Behavior of a Fluorescent Analogue of Calmodulin in Living 3T3 Cells

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ABSTRACT We have prepared and partially characterized a lissamine-rhodamine B fluorescent analogue of calmodulin, LRB-CM. The analogue had a dye/protein ratio of ~1.0 and contained no free dye or contaminating labeled proteins. LRB-CM was indistinguishable from native calmodulin upon SDS PAGE and in assays of phosphodiesterase and myosin light chain kinase. The emission spectrum of LRB-CM was insensitive to changes in pH, ionic strength, and temperature over the physiological range, but the apparent quantum yield was influenced somewhat by divalent cation concentration. LRB-CM injected into living Swiss 3T3 fibroblasts became associated with nitrobenzoxadiazole-phallacidin staining stress fibers in some interphase cells. LRB-CM and acetamidofluorescein-labeled actin co-injected into the same cell both became associated with fibers in some cells, but in most cases association of the two analogues with fibers was mutually exclusive. This suggests that calmodulin may differ from actin in the timing of incorporation into stress fibers or that we have distinguished distinct populations of stress fibers. We were able to detect no direct interaction of LRB-CM with actin by fluorescence photobleaching recovery (FRAP) of aqueous solutions. Interaction of LRB-CM with myosin light chain kinase also was not detected by FRAP. This suggests that the mean lifetime of the calmodulin-myosin light chain kinase complex is too short to affect the diffusion coefficient of calmodulin. We examined various fluorescent derivatives of proteins and dextrans as suitable control molecules for quantitative fluorescent analogue cytochemistry in living cells. Fluorescein isothiocyanate-dextran-were found to be preferable to all the proteins tested, since their mobilities in cytoplasm were inversely dependent on molecular size and there was no evidence of binding to intracellular components. In contrast, FRAP of LRB-CM in the cytoplasm of living 3T3 cells suggested that the analogue interacts with intracellular components with a range of affinities. The mobility of LRB-CM in the cytoplasm was sensitive to treatment of the cells with trifluoperazine, which suggests that at least some of the intracellular binding sites are specific for calmodulin in the calcium-bound form. FRAP of LRB-CM in the nuclei of living 3T3 cells indicated that the analogue was highly mobile within the nucleus but entered the nucleus from the cytoplasm much more slowly than fluorescein isothiocyanate-dextran of comparable molecular size and much more slowly than predicted from its mobility in cytoplasm.

Various biochemical evidence has led to speculation that the calcium-dependent regulator protein, calmodulin, is a fundamental regulator of cellular motility (Means et al., 1982; Kakiuchi and Sobue, 1983). For example, calmodulin activates the smooth muscle regulatory protein, myosin light chain kinase (MLCK)1 (Dobrowska et al., 1977; Adelstein and Klee, 1981). It also enhances the calcium sensitivity of microtubules in vitro (Marcum et al., 1978) and has been implicated in the regulation of microtubule disassembly in extracted cell models (Schliwa et al., 1981; Deery et al., 1984). Similar experiments with extracted cell models have implied after photobleaching; LRB-CM, lissamine-rhodamine B derivative of calmodulin; MLCK, myosin light chain kinase; NBD, nitrobenzoxadiazole; TFP, trifluoperazine.
cated calmodulin in the control of actin and myosin based contractility in human lung fibroblasts as well (Masuda et al., 1984). A recent report also suggests that calmodulin can modify indirectly the polymerization of actin in vitro (Piazza and Wallace, 1985). In fact, calmodulin reportedly binds to a number of actin-binding proteins and to microtubule-associated proteins (Rebhun et al., 1980; Sobue et al., 1981; Glenney et al., 1982; Lee and Wolff, 1984; for review see Kakuchi and Sobue, 1983). However, the distribution and activity of calmodulin in intact cells is not yet known in detail.

Immunocytochemical studies have reproducibly localized calmodulin in the mitotic apparatus and intercellular bridge during cell division (Andersen et al., 1978; Welsh et al., 1978; Welsh et al., 1979; DeMey et al., 1980; Willingham et al., 1983), but the distribution of calmodulin in interphase cells is less well characterized. Welsh et al. (1978) reported anti-calmodulin antibodies associated with stress fibers in interphase BALB/c 3T3 cells. Deery et al. (1984) recently have shown that anti-calmodulin antibodies bind to cytoplasmic microtubules in extracted Swiss 3T3 cells under certain conditions. However, Willingham et al. (1983) could not find any correspondence between the localization of anti-calmodulin antibodies and the distribution of cytoskeletal elements in interphase Swiss 3T3 cells. Recent studies of living cells microinjected with fluorescent analogues of calmodulin or with underivatized calmodulin have confirmed the localization of calmodulin in the mitotic apparatus and intercellular bridge, but again provided contradictory results about its interaction with cytoskeletal elements in interphase cells (Hamaguchi and Iwasa, 1980; Keith et al., 1983; Zavortink et al., 1983). Zavortink et al. (1983) injected tracer amounts of tetramethylrhodamine-labeled calmodulin into both PK1 and 3T3 cells and observed no specific localization of the analogue during interphase. Keith et al. (1983) injected underivatized calmodulin along with 4 mM CaCl2 into gerbil fibrobra and 3T3 cells in a 15-100-fold excess of total cellular calmodulin and found evidence that both microfilaments and microtubules near the injection site depolymerized. Their controls indicated this was a specific effect of Ca-calmodulin and not a result of the occurrence of damage to the cell during microinjection. Although this latter study suggests an interaction between calmodulin and the cytoskeleton of living interphase cells, the excessive intracellular concentrations of calmodulin and calcium attained in these experiments makes it difficult to assess the physiological relevance of the results.

We have synthesized and partially characterized a functional lissamine-rhodamine B derivative of calmodulin (LRB-CM). We microinjected it into living interphase Swiss 3T3 cells and found that, in contrast to the results of Zavortink et al. (1983), our analogue apparently was associated with stress fibers in some cells. In this report we present evidence that calmodulin is localized on a subset of actin-containing fibers in interphase 3T3 cells. We also report the results of fluorescence photobleaching studies in which the mobility of calmodulin was compared with the mobility of various control molecules within the cytoplasm and nucleus of interphase 3T3 cells.

MATERIALS AND METHODS

Preparation of LRB-CM: Calmodulin was isolated and purified from bovine brain by the method of Burgess et al. (1980). The identity of calmodulin was established by its ability to activate CAMP phosphodiesterase, by differential mobility upon SDS PAGE in the presence and absence of Ca2+, by its ultraviolet absorption spectrum, and by its amino acid composition. Purified calmodulin was lyophilized and stored at -70°C until use. For labeling, lyophilized calmodulin was dissolved in 10 mM sodium carbonate buffer, pH 9.6, 1 mM EGTA, at a concentration of 5-10 mg/ml in a small vial containing a stirrer. A threefold molar excess of lissamine-rhodamine B sulfonyl chloride on celite (Molecular Probes Inc., Junction City, OR) was added to the protein solution in five aliquots, over a period of 50 min, with constant stirring at room temperature. The solution was stirred for 15 min more after the last addition. The contents of the vial were centrifuged for 10 min at 10,000 g to remove the insoluble celite, and the protein solution was desalted on Sephadex G-25 (Sigma Chemical Co., St. Louis, MO) in 10 mM Tris-CI, pH 7.5, 0.1 M NaCl, 1 mM ß-mercaptoethanol, 1 mM EDTA (buffer A) to remove free dye. The void volume from the column was loaded directly onto a 1.5 × 60 cm column of DE-52 (Whatman Chemical Separation Inc., Clifton, NJ) equilibrated in buffer A. The column was washed with three column volumes of buffer A to wash out residual dye and then eluted with a 200-ml linear gradient of 0.15-0.6 M NaCl in buffer A, and 0.5-mL fractions were collected. Fractions containing LRB-CM were diazylized against buffer A and concentrated by chromatography on DE-52. After determination of dye/protein ratio and specific activity, LRB-CM was diazylized against injection buffer (2 mM K+-PI1ES, pH 7.0, 0.05 mM MgCl2) and stored frozen at -70°C.

Determination of Dye/Protein: The concentration of dye in LRB-CM was estimated using a molar extinction coefficient at 550 nm for the LRB-protein of 1.3 × 104 M −1 cm−1. A recent report also suggests that calmodulin can be determined by the method of Lowy et al. (1951) or Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA) using unlabeled calmodulin standards. The concentration of unlabeled calmodulin standards was determined spectroscopically using ε562 = 2.2 (Dedman et al., 1977).

Phosphodiesterase Assay: The ability of calmodulin to stimulate the hydrolysis of CAMP by phosphodiesterase was assayed spectrophotometrically according to the method of Kessin et al. (1979). Activator-deficient bovine heart phosphodiesterase (P-0520) and adenosine deaminase (A-9626) were purchased from Sigma Chemical Co. Alkaline phosphatase was purchased from Boehringer-Mannheim Biochemicals, Indianapolis, IN (Catalogue No. 108138).

Phosphodiesterase activity was assayed at 37°C as a function of varying concentration of calmodulin in the presence of 10 mM CaCl2. A Gilford model 250 spectrophotometer (Gilford Instrument Laboratories, Oberlin, OH) was used to monitor the decrease in OD 265 as adenosine is converted to inosine.

Spectroscopy: Corrected fluorescence spectra of LRB-CM were obtained using a Spex Fluorolog (Spex Industries, Inc., Metuchen, NJ) at a half band width of 10 nm. Except where noted, spectra were obtained in 10 mM Tris buffer, 0.1 M NaCl, pH 7.5. For some experiments pH and ionic strength were varied independently by adjusting the concentrations of HCl and NaCl, using buffer tables constructed according to Perrin and Dempsey (1974). The pH and conductivity of the buffers were verified by measurement.

SDS PAGE: SDS PAGE of LRB-CM was carried out in 12% gels in the presence of 1-mercaptoethanol. The Ca2+ concentration of each sample was adjusted by addition of CaCl2 or EGTA to a final concentration of 5 mM.

Labeling of Other Proteins: Acetamidofluorescein actin (AF-actin) was labeled according to the method of Wang and Taylor (1980) and either used directly after preparation or stored frozen in liquid nitrogen until use. Alpha-lactalbumin, ovalbumin, bovine serum albumin (BSA), and IgG were labeled as previously described (Wang and Taylor, 1979).

Cell Culture: Swiss 3T3 cells were grown in Dulbecco's modified Eagle's medium with 10% calf serum (Gibco Laboratories Inc., Grand Island, NY) and 1% penicillin-streptomycin (Gibco Laboratories Inc.) added. Cell cultures were maintained at 37°C in a humidified 5% CO2 atmosphere. For microinjection, cells were subcultured onto chamber slides (Lab-Tek Div., Miles Laboratories, Inc., Naperville, IL) or plain glass slides at least 24 h before use.

Microinjection and Fluorescence Microscopy: Labeled proteins and dextrans were microinjected into 3T3 cells as previously described (Amato et al., 1983). The volume of LRB-CM solution injected was estimated to be ≤10% of the cell (Diacumakos, 1973). Since the concentration of LRB-CM in this solution was 1-4 mg/ml, we estimate that the equivalent of <1% of total cellular calmodulin was injected (Chaffoulas et al., 1982).

To control for path length and accessible volume artifacts, LRB-CM was sometimes co-injected with fluorescent isothiocyanate (FITC)-dextran (4.057 mol wt; Sigma Chemical Co.). This size dextran was chosen because its radius of gyration (~23 Å) (Benoit, 1948) is comparable to the Stokes radius of calmodulin (21 Å; see Klee and Vanaman, 1982). The concentrations of the two macromolecular species were adjusted so that the fluorescence intensities of the two fluorophores were nearly equal, resulting in a final concentration of...
dextran in the injection solution of ~1 mg/ml. For co-injection of LRB-CM and AF-actin, the final concentrations of both proteins in the injection buffer were 0.05 mg/ml. Fluorescence microscopy was performed as previously described (Amano et al., 1983; Luby-Phelps et al., 1984).

Nitrobenzoxadiazole (NBD)-Phallacidin Staining of Stress Fibers: Cells to be stained with NBD-phallacidin were fixed for 15 min in 3.7% formaldehyde in phosphate-buffered saline (PBS), then rinsed well with PBS, and finally extracted with acetone at −20°C for 5 min. After the cells had been allowed to air dry, NBD-phallacidin (Molecular Probes Inc.) was applied to each slide in 100 μl PBS (4 U/slide), and the slides were incubated for 30 min at 37°C. The slides were then rinsed well with PBS and viewed immediately.

Anti-tubulin Immunocytochemistry: After a brief rinse in PBS at 37°C, cells were fixed for 1 h at room temperature in 3% formaldehyde in 80 mM PIPES, pH 7.3, 1.0 mM EGTA, 0.5 mM MgCl₂. The slides were rinsed three times in PBS at room temperature, and the cells were then extracted with −20°C acetone for 3 min and allowed to air dry for 5 min. The cells were incubated in a mouse monoclonal anti-β-tubulin antibody (Walsh, 1984) for 1 h at 37°C, rinsed well with PBS, and incubated in fluorescein-labeled goat anti-mouse IgG (20 μg/ml; Cappel Laboratories, Cochranville, PA) for 40 min at 37°C. After further rinsing in PBS, the slides were viewed immediately or transferred to 100% toulene to dehydrate them before mounting in Flo-Texx (Lerner Laboratories, New Haven, CT) under a No. 1 coverslip.

Fluorescence Recovery after Photobleaching (FRAP): Fluorescence photobleaching was performed using an argon-ion laser (Spectra-Physics Inc., Mountain View, CA) focused through a Zeiss Universal microscope equipped with epifluorescence optics. The laser was operated at 190 mW. Rhodamine was bleached with the 514 nm line, fluorescein with the 488 nm line. The duration of the photobleaching pulses ranged from 1.8 ms to 1 s, depending on spot size and fluorescence intensity of the specimen. The laser beam was attenuated 3,600 times for monitoring FRAP. FRAP was monitored either by video, or by a photometer input to a chart recorder. To determine the width parameter, w, of the quasi-Gaussian laser spot, a fluorescent spot was defined spatial intervals to generate a representation of the beam intensity profile. The resulting curves were fit to a Gaussian equation by least squares and w was determined by calculation of the position on the diameter of the spot where intensity falls to 1/e². FRAP curves recorded from solution and from cells were analyzed by the method of Yguerabide et al. (1982), and diffusion coefficients (mobilities) were calculated from the equation \( D = \frac{3}{2} \cdot \frac{w^2}{4t} \). Percent mobile fractions were calculated according to Axelrod et al. (1976). For FRAP of aqueous solutions, samples were contained in 0.1-mm flat capillaries (Vitro Dynamics Inc., Rockaway, NJ). FRAP measurements on living cells were made while the cells were maintained at a constant temperature of 37°C, and calculated values for mobility were corrected to 22°C.

RESULTS

Characterization of LRB-CM

CHROMATOGRAPHY OF LRB-CM ON DE52: When the void volume of the G-25 gel filtration step in the preparation of LRB-CM was chromatographed on DE52, three absorbance peaks at 230 nm were resolved (Fig. 1). The first contained no rhodamine and comprised unlabeled calmodulin and another strongly absorbing component presumed to be EDTA. Both the second and third fractions were labeled with rhodamine. The dye/protein ratio of the first labeled fraction was ~1:1, and the dye/protein ratio of the second labeled fraction was often as high as 4:1 or 5:1. The first labeled fraction was monitored by absorbance at 550 nm. The first of the two rhodamine-containing fractions, designated LRB-CM (arrowhead) had a dye/protein ratio of 1 and was able to activate phosphodiesterase and MLCK. --, OD 230 nm. - ...., OD 550 nm... The second labeled fraction was often as high as 4:1 or 5:1. The first labeled fraction was found to be fully as active as unlabeled calmodulin in assays of phosphodiesterase (nanomoles cAMP hydrolyzed per minute per microgram PDE). Open circles, native calmodulin; triangles, LRB-CM + 5 mM EGTA. The slides were then rinsed well with PBS and viewed immediately. The second labeled fraction exhibited a reduced ability to activate this enzyme. The first labeled fraction, designated LRB-CM, was also able to activate MLCK (Dr. Jimmy Collins, personal communication), and was the fraction chosen for all further experiments.

SDS PAGE OF LRB-CM: When analyzed by SDS PAGE in 12% gels (Fig. 1 b), LRB-CM ran as a single band in the presence of EGTA, and exhibited the differential mobility in fractions 1 and 3. Figure 1 (a) Chromatography of labeled calmodulin on DE52. The void volume from G-25 filtration of the reaction mixture was loaded onto a 1.5 x 60 cm column of DE52 and then eluted with a 0.15-0.6 M linear salt gradient (see Materials and Methods). Elution of protein was monitored by absorbance at 230 nm. Elution of rhodamine-containing fractions was monitored by absorbance at 530 nm. The first of the two rhodamine-containing fractions, designated LRB-CM (arrowhead) had a dye/protein ratio of 1 and was able to activate phosphodiesterase and MLCK. --, OD 230 nm. ...., OD 550 nm... [NaCl] beginning at start of gradient (0.15 M). (b) SDS PAGE of LRB-CM. Electrophoresis of LRB-CM was carried out in 12% polyacrylamide in the presence and absence of free Ca²⁺. Gels were stained with Coomassie Blue. Lane 1, LRB-CM in 5 mM CaCl₂. Lane 2, LRB-CM in 5 mM EGTA. Lane 3, molecular weight standards. LRB-CM was indistinguishable from native calmodulin by Coomassie Blue staining.

the presence and absence of Ca²⁺ characteristic of active calmodulin. No free dye or contaminating labeled proteins were observed in the LRB-CM preparation, based on both fluorescence and absorption analysis.

FLUORESCENCE SPECTROSCOPY OF LRB-CM: The fluorescence spectrum of LRB-CM was found to have a single
excitation peak at 550 nm and a single emission peak at 603 nm. The fluorescence spectrum of LRB-CM was found to be insensitive to the concentration of LRB-CM over the range from 10–100 μM, to temperatures between 22 and 37°C, to changes in ionic strength over the range 0 to 0.2, and to changes in pH over the range of 6.8 to 8.0. However, the apparent quantum yield of the labeled calmodulin was somewhat sensitive to calcium concentration (Fig. 3a). LRB-CM exhibited ~30% greater fluorescence emission in the presence of 5 mM CaCl₂ than in the presence of 5 mM EGTA. Since lissamine-rhodamine B tested in the same buffer did not show any difference in fluorescence emission in the presence of CaCl₂ or EGTA, the increase apparently was not an effect of Ca²⁺ directly on the fluorophore (Fig. 3b). Substitution of 5 mM MgCl₂ for CaCl₂ decreased the amplitude of emission to below the level observed in the presence of 5 mM EGTA (Fig. 3). This may represent the interaction of Mg²⁺ with Ca²⁺ binding sites, since it has been shown that at high concentrations Mg²⁺ appears to compete with Ca²⁺ for some sites and can induce a change in conformation of calmodulin (Klee and Vanaman, 1982; Ogawa and Tanokura, 1984). We have not ruled out a possible effect of EGTA on LRB-CM fluorescence, but in view of the known effects of Ca²⁺ binding on the conformation of calmodulin, it seems very likely that the increase in fluorescence emission of LRB-CM in the presence of calcium can be attributed to changes in the environment of the fluorescent moiety as a result of calcium binding to calmodulin. The magnitude of the difference in fluorescence emission in the presence of 5 mM CaCl₂ vs. 5 mM EGTA was not affected by changes in temperature, pH, and ionic strength. Similar effects of CaCl₂ concentration and MgCl₂ concentration were observed when spectra were obtained in injection buffer or in 50 mM 2-(N-morpholino)propane-sulfonic acid, 0.2M KCl, pH 7.3. Including saturating amounts of phosphodiesterase had no effect on LRB-CM spectra obtained in the presence or absence of CaCl₂, which suggests the fluorescence of the analogue is not influenced by binding to this target enzyme.

** Localization of LRB-CM in Living 3T3 Cells **

In a typical experiment, a mixture of LRB-CM and 4,057 mol wt FITC-dextran was injected by microneedle into the cytoplasm of 50–100 interphase cells, and the subsequent distributions of the two fluorescent probes were monitored for up to 48 h. In all, ~5,000 cells were studied. Immediately after microinjection, both LRB-CM and FITC-dextran diffused throughout the cytoplasm of the cell and eventually entered the nucleus. Both analogues appeared to be excluded from the nucleolus. In cells that had passed into mitosis at some time after microinjection, LRB-CM was found at the poles of the forming spindle at late prophase, in the half-spindles during metaphase and anaphase, and near the proximal ends of the midbody during late telophase, whereas FITC-dextran showed no such localization (e.g., Fig. 4, c–f). This distribution of LRB-CM during cell division is consistent with the localization of calmodulin reported by previous investigators who have used other fluorescent analogues of calmodulin or immunofluorescence techniques in their studies (Hamaguchi and Iwasa, 1980; Zavortink et al., 1983; Andersen et al., 1978; Welsh et al., 1978; Willingham et al., 1983).

In interphase cells, LRB-CM showed a variable distribution. In some cells, the analogue exhibited a diffuse distribution indistinguishable from the distribution of FITC-dextran in the same cell. In others it formed a striking pattern of fine, rectilinear, fluorescent fibers, clearly distinct from the diffuse distribution of FITC-dextran (Fig. 4a). There was no apparent periodicity of fluorescence along the long axis of these fibers. In any experiment, the percentage of cells that exhibited fibrous localization of LRB-CM varied from 0 to 50%. This variability was not a function of the passage number of the culture and was not affected by lowering the level of serum in the medium to promote cell spreading. Similar heterogeneity was observed whether the cells were cultured in newborn or fetal calf serum. The localization of LRB-CM in fluorescent fibers appeared independent of cell morphology and size except that cells with an obvious motile morphology (broad leading ruffle and long, thin tail) never exhibited fluorescent fibers. The appearance of fluorescent fibers correlated loosely with time elapsed after microinjection in that fluorescent fibers were not positively identified before 1 h after injection and that fluorescent fibers were always observed in some percentage of cells 18–24 h after injection. By 48 h after microinjection, the fluorescence of LRB-CM in the cells had been reduced to a barely detectable level, presumably as a result of cell division and, perhaps, autophagocytosis.

** LRB-CM Co-localized with Actin-containing Fibers **

The fibrous pattern of LRB-CM fluorescence described above resembles the distribution of actin-containing "stress"
FIGURE 4 Distribution of LRB-CM in living 3T3 cells. 4,057 mol wt FITC-dextran was co-injected into the same cells as a control for path length and accessible volume. (a) An example of LRB-CM associated with stress fibers (arrowheads) in many interphase cells. The distribution of FITC-dextran in the same cell is shown in b. (c) LRB-CM appeared at the poles of the forming spindle in cells entering mitosis. (d) The distribution of FITC-dextran in the same cell. (e) LRB-CM was localized in the region of the midbody in the intercellular bridge of daughter cells undergoing cytokinesis (The arrow points to the midbody region). The distribution of FITC-dextran in the same cells is shown in f. Bar, 5 μm. × 3,000.
fibers as seen in various fibroblastic cells by immunofluorescence (e.g., Lazarides and Weber, 1974) and by fluorescent analogue cytochemistry (Kreis et al., 1979; Wehland and Weber, 1980; Kreis et al., 1982; Glacy, 1983; Luby-Phelps et al., 1984, Wang, 1984). To determine whether LRB-CM was in fact localized on actin-containing fibers, cells that had been injected with LRB-CM alone (no dextran) were recorded, then fixed, extracted, and stained with NBD-phallacidin to detect F-actin. When an LRB-CM image was compared with the NBD-phallacidin image from the same cell, it could be seen that fibers containing LRB-CM corresponded closely to fibers stained by NBD-phallacidin (Fig. 5 a). In contrast, when a similar procedure was carried out using anti-tubulin antibody to localize microtubules in the cell, the localization of LRB-CM bore no obvious relation to the localization of cytoplasmic microtubules in the same cell (data not shown).

Association of Calmodulin with Stress Fibers Is Independent of Actin Incorporation

When LRB-CM and AF-actin were co-injected into the same cells and then viewed 4–6 h later, both the calmodulin analogue and the actin analogue exhibited a fibrous localization in some cells. However, in the great majority of cases, the incorporation of actin and calmodulin into fibers appeared to be mutually exclusive in any one cell. In observations of ~500–1,000 cells, many cells exhibited actin fibers but showed a more uniform distribution of calmodulin (Fig. 6). Fewer cells exhibited calmodulin associated with fibers and showed a uniform distribution of actin (Fig. 7). Very few cells (<1%) exhibited co-linear localization of actin and calmodulin on stress fibers (Fig. 8). In general, AF-actin became associated with fibers much more rapidly than LRB-CM (minutes vs. hours) but was also eliminated from the cell much more rapidly, virtually disappearing by ~24 h after injection. The progressive accumulation of fluorescein-containing vesicles near the nucleus after injection of AF-actin suggests that the analogue is eliminated through autophagocytosis. In contrast, whereas LRB-CM became associated with fibers relatively slowly (hours), it persisted in the cell for up to 48 h, and accumulation within vesicles was not usually observed.

**FRAP of LRB-CM**

Table I reports the diffusion coefficients (mobilities) calcu-
FIGURE 7 Co-injection of LRB-CM and AF-actin. In some cells, such as this one, LRB-CM is associated with stress fibers (a), whereas AF-actin does not appear to be associated with fibers (b). Bar, 10 μm. × 1,400.

FIGURE 8 Co-injection of LRB-CM (a) and AF-actin (b). In this very large cell LRB-CM and AF-actin appear to co-localize on some fibers. The incidence of co-localization was ≤1%, which suggests that incorporation of actin and calmodulin into stress fibers may be spatially and/or temporally distinct events. Bar, 5 μm. × 1,600.

TABLE I. FRAP of Dextrans and Proteins in Aqueous Solution and in 3T3 Cell Cytoplasm

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Mol wt</th>
<th>Daq × 10^6</th>
<th>Mcyto × 10^7</th>
<th>% mobile (cyto)</th>
</tr>
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<tr>
<td>FITC-dextran</td>
<td>20,000</td>
<td>1.01 ± 0.20</td>
<td>1.8 ± 0.5 (4)</td>
<td>100</td>
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<tr>
<td>FITC-dextran</td>
<td>39,000</td>
<td>0.70 ± 0.03</td>
<td>1.3 ± 0.4 (4)</td>
<td>100</td>
</tr>
<tr>
<td>FITC-dextran</td>
<td>65,500</td>
<td>0.52 ± 0.05</td>
<td>1.0 ± 0.23 (11)</td>
<td>100</td>
</tr>
<tr>
<td>FITC-lactalbumin</td>
<td>16,700</td>
<td>1.02 ± 0.11</td>
<td>0.69 ± 0.36 (2)</td>
<td>42 ± 4.9*</td>
</tr>
<tr>
<td>FITC-ovalbumin</td>
<td>43,000</td>
<td>0.69 ± 0.27</td>
<td>0.59 ± 0.23 (8)</td>
<td>78 ± 4.6*</td>
</tr>
<tr>
<td>FITC-BSA</td>
<td>66,000</td>
<td>0.67 ± 0.02</td>
<td>0.68 ± 0.56 (8)</td>
<td>77 ± 15.6*</td>
</tr>
<tr>
<td>FITC-IgG</td>
<td>150,000</td>
<td>0.46 ± 0.05</td>
<td>0.67 ± 0.18 (2)</td>
<td>54 ± 24.4*</td>
</tr>
<tr>
<td>LRB-CM</td>
<td>16,700</td>
<td>1.02 ± 0.13</td>
<td>0.40 ± 0.15 (12) (fast)</td>
<td>81 ± 5.9</td>
</tr>
<tr>
<td>AF-actin</td>
<td>42,000</td>
<td>0.72 ± 0.35*</td>
<td>0.34 ± 0.05 (13)</td>
<td>54 ± 8.6</td>
</tr>
</tbody>
</table>

Daq, aqueous diffusion coefficient (cm²/s). Mcyto, cytoplasmic mobility (cm²/s) as defined in Materials and Methods and Discussion. % mobile, percentage of initial fluorescence recovered during time course of experiment (see Materials and Methods). FRAP data are presented as mean ± SEM. Numbers in parentheses are the number of observations used in calculating the mean. Cytoplasmic mobilities were measured while cells were maintained at 37°C and were corrected to 22°C. Observed values were 1.4 times higher.

* FRAP of these proteins yields visible persistent spots, indicating significant interaction with cellular components.

† From Lanni and Ware, 1984.
lated from fluorescence photobleaching recovery curves of LRB-CM and various other proteins and dextrans, both in aqueous solution and in the cytoplasm of living interphase 3T3 cells. As expected, both the dextrans and the proteins exhibited an inverse relation between their diffusion coefficients in aqueous solution and their molecular weights. The observed diffusion coefficients of all the proteins in aqueous solution agreed well with previously reported values.

FRAP of Reconstituted Stress Fiber Components: Since LRB-CM co-localized with actin-containing fibers in living 3T3 cells, we investigated the effect of F-actin on its aqueous diffusion coefficient as a first step toward reconstituting stress fibers with fluorescent analogues (Table II). LRB-CM (final concentration, 20 μM) was mixed with G-actin (final concentration, 50 μM) in actin buffer A (Wang and Taylor, 1980), and polymerization was initiated by the addition of KCl to a final concentration of 100 mM. After from 30 min to 1 h of incubation at room temperature, no interaction of LRB-CM with F-actin could be detected: the diffusion coefficient of LRB-CM under these conditions was within experimental error of the diffusion coefficient of LRB-CM alone. Furthermore, the presence of saturating amounts of MLCK in this mixture also had no effect on the aqueous diffusion coefficient of LRB-CM. In contrast, NBD-phallacidin exhibited a dramatic decrease in its aqueous diffusion coefficient in the presence of 50 μM F-actin (Table II), demonstrating that high affinity interactions could have been detected under our experimental conditions.

FRAP of Living Cells: The average mobilities of labeled proteins and dextrans in the cytoplasm of 3T3 cells were determined by FRAP using bleached spots 5–7 μm in diameter. In agreement with the results of Wojcieszyn et al. (1981) and others (Wang et al., 1982; Kreis et al., 1982), all extrinsic control proteins tested exhibited very similar mobilities within the cytoplasm of 3T3 cells. It is interesting that all of the extrinsic control proteins tested also showed an immobile component and that persistent bleached spots remained visible minutes after photobleaching. In contrast, the various molecular weight dextrans exhibited mobilities that were graduated inversely with molecular weight, and there was no evidence of immobile components.

Because LRB-CM exhibited a complex FRAP curve that could not be fit to fewer than three diffusive terms (Fig. 9), we report in Table I only the mobilities of the fastest and the slowest components of the recovery. The mobility of the fastest component of LRB-CM is slightly slower than the mobility of all extrinsic control proteins tested. The mobilities of the fast and slow components did not differ significantly between cells in which LRB-CM was localized on fibers and cells in which LRB-CM showed no clear localization. In addition to the slow moving component, an average of 15–20% of LRB-CM fluorescence failed to recover in the time frame of our experiments, which suggests the presence of even less mobile components in the calmodulin pool. The average size of this relatively immobile class of LRB-CM was the same whether or not LRB-CM was localized on fibers, although it did increase from an average of 5% immediately after injection to a final value of 15% after ~18 h.

**TABLE II. FRAP of Partially Reconstituted Stress Fibers**

<table>
<thead>
<tr>
<th>Molecule(s)</th>
<th>Daq x 10^6</th>
<th>% mobile (aq)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRB-CM + F-actin</td>
<td>1.1 ± 0.46</td>
<td>100</td>
</tr>
<tr>
<td>LRB-CM, F-actin, MLCK</td>
<td>1.1 ± 0.35</td>
<td>100</td>
</tr>
<tr>
<td>NBD-phallacidin</td>
<td>3.0 ± 0.73</td>
<td>100</td>
</tr>
<tr>
<td>NBD-phallacidin + F-actin</td>
<td>10</td>
<td>100</td>
</tr>
</tbody>
</table>

FRAP of partially reconstituted stress fibers. Aqueous diffusion coefficients (Daq) were determined for LRB-CM in the presence of F-actin plus or minus MLCK to see whether the association of LRB-CM with stress fibers in interphase cells could be explained simply by binding of LRB-CM to actin directly, or indirectly through association with MLCK. No interactions were detected as, in both cases, the aqueous diffusion coefficient of LRB-CM was within experimental error of the diffusion coefficient of LRB-CM alone. This means that even the interaction of LRB-CM with MLCK was not detected, which suggests that the lifetime of the calmodulin–MLCK complex is shorter than the half-time of recovery of LRB-CM from photobleaching. FRAP data for the actin-binding fluorescent peptide, NBD-phallacidin, are included in this Table to demonstrate that FRAP can be used to study high affinity interactions between molecules. 90% of the population of NBD-phallacidin molecules are essentially immobilized in the presence of F-actin. Mean values are given ± SEM. % mobile, percentage of initial fluorescence recovered during time course of experiment (see Materials and Methods).

**FIGURE 9 FRAP of LRB-CM in living interphase 3T3 cells. This is an example of the complex photobleaching recovery curves exhibited by LRB-CM in the cytoplasm of 3T3 cells. These curves could not be fit to fewer than three components. The rapid rise at the beginning of the curve indicates a relatively fast moving component, and the slowly increasing tail indicates the presence of slower components. In addition, our analysis indicates the presence of a relatively immobile fraction (~20%) which did not exhibit significant recovery during the time of these experiments. This type of recovery curve probably reflects interaction of LRB-CM with various intracellular targets with a range of affinities for calmodulin, F^* fluorescence before bleaching, F^* fluorescence immediately after bleaching (calculated from curve fitting routine). Δ, change of chart recorder speed from 10 to 1 mm/s.**

**EFFECT OF TRIFLUOPERAZINE (TFP) ON CYTOPHASIC LRB-CM:** The effect of 10 μM TFP (Sigma Chemical Co.) on the localization and mobility of LRB-CM in the cytoplasm of 3T3 cells was tested. The effect of the drug on the localization and mobility of AF-actin was used as a control. Cells that exhibited LRB-CM localization on fibers showed a marked change in LRB-CM localization over the course of a 10-min exposure to TFP. Although the pattern of fluorescence was still roughly linear, it appeared as broad stripes that were indistinguishable from the path length facts that are sometimes seen when extrinsic "control" proteins are injected into well spread cells (Luby-Phelps et al., 1984). At this time, the localization of AF-actin on fibers was unaffected by the drug. The mobility of the fastest moving components of the recovering species of both LRB-CM and AF-actin was slightly increased after 10 min in the presence
TABLE III. Effect of 10 μM TFP on the Mobility of LRB-CM and AF-actin in Cells

<table>
<thead>
<tr>
<th>Molecule</th>
<th>Mcyto (fast) × 10⁶</th>
<th>% mobile</th>
</tr>
</thead>
<tbody>
<tr>
<td>LRB-CM</td>
<td>4.0 ± 1.5 (12)</td>
<td>81 ± 5.9</td>
</tr>
<tr>
<td>LRB-CM + TFP</td>
<td>4.8 ± 1.2 (4)</td>
<td>95 ± 2.0</td>
</tr>
<tr>
<td>AF-actin</td>
<td>3.4 ± 0.5 (13)</td>
<td>54 ± 8.6</td>
</tr>
<tr>
<td>AF-actin + TFP</td>
<td>4.8 ± 0.3 (5)</td>
<td>65 ± 15.0</td>
</tr>
</tbody>
</table>

Effect of TFP on the mobility of LRB-CM and AF-actin in living 3T3 cells. Cyanosoluble mobilities were determined before and after treatment of cells with 10 μM TFP for 10 min. TFP had no effect on the distribution of AF-actin and little effect on its cytosolic mobility. In contrast, TFP somewhat altered the distribution of LRB-CM (see Results) and decreased the size of the immobile fraction to nearly zero. Mean values for mobility are presented as the mean ± SEM. Numbers in parentheses are the number of observations used to calculate the mean.

LUB-Phelps et al. (1981) and Taylor et al. (1984) have stressed the importance of characterizing fluorescent analogues before using them as probes of cellular activity. By using LRB-CM and their fluorescent analogue of calmodulin and stated that it was excluded from the nucleus. One possible reason that our results differ from those of Zavortink et al. is that our choice of fluorophore, reactive group, and reaction conditions differs from theirs and could certainly influence the site of labeling and thus the behavior of the fluorescent product.

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The results of experiments in which LRB-CM and AF-actin were co-injected into the same cell suggest that there may exist in Swiss 3T3 cells at least two types of actin-containing fibers whose composition is regulated independently. Although LRB-CM co-localized with fibers that could be stained with NBD-phallacidin, these were not necessarily fibers that could incorporate AF-actin. In most co-injected cells, incorporation of LRB-CM and AF-actin appeared to be mutually exclusive. These results suggest that actin and calmodulin differ in the timing of incorporation into stress fibers and/or that we have distinguished populations of stress fibers of differing biochemical composition. The idea of subsets of stress fibers that are regulated independently has also been advanced to account for variation in the tropomyosin content of stress fibers (Lazarides, 1976; Matsumura et al., 1983; Lin et al., 1984) and the occurrence of tropomyosin isoforms in various non-muscle cell types (for review, see Payne and Rudnick, 1984).

FRAP of Partially Reconstituted Stress Fibers

FRAP of fluorescent analogues in aqueous solution can be used to detect the interaction between the labeled species and other proteins in a mixture, and to determine binding constants for these interactions (e.g., Lanni and Ware, 1984). Analysis of the interactions possible under controlled conditions in vitro will be important as the basis for understanding the molecular interactions of molecules in living cells. A test experiment showed that we could detect the high affinity interaction between NBD-phallacidin and F-actin by this method. In contrast, although LRB-CM associated with actin-containing stress fibers in living cells, we detected no direct interaction of LRB-CM and AF-actin in the nucleus.

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interaction between LRB-CM and actin. This is in agreement with previous studies by other methods in which no direct interaction of calmodulin and actin was detected (Howe et al., 1980; Sobue et al., 1982a; Piazza and Wallace, 1985). Inclusion of saturating amounts of MLCK in the mixture also had no detectable effect on the mobility of LRB-CM. Calmodulin and MLCK form a complex with a Stokes radius of 79 Å (Klee and Vanaman, 1982). This complex was not detected in our experiments, which suggests that the dissociation rate of calmodulin and MLCK is faster than the half-time of recovery of LRB-CM from photobleaching. (If dissociation of calmodulin from MLCK is dependent only on the rate of dissociation of calcium from calmodulin, this could be as fast as 0.095 s⁻¹ [Malencik et al., 1981].) This might account for the observation that MLCK does not appear to mediate binding of LRB-CM to F-actin, despite reports that MLCK binds to actin (Dabrowska et al., 1982; Sobue et al., 1982a).

Alternatively, according to the flip-flop mechanism proposed by Sobue et al. (1982a, b), MLCK may not bind to actin when it is bound to calmodulin.

**FRAP of LRB-CM in Living Cells**

Although the mobilities reported for calmodulin and other molecules within the cytoplasm of 3T3 cells were calculated from the equation for diffusion, these values should not be taken as true diffusion coefficients. The mobility of macromolecules within the cell may reflect a combination of factors, such as bulk cytoplasmic flow and interaction with cellular components, as well as diffusion of the unbound species. Because this is especially true in the case of proteins such as calmodulin and actin, which are suspected to bind to specific sites within the cell, the values in Table I are always referred to as mobilities in this report and are best interpreted relative to one another.

**NUCLEUS:** The fact that nuclear fluorescence of LRB-CM could be depleted selectively by extensive photobleaching within the nucleus suggests that entry of LRB-CM into the nucleus is constrained in some way. Given the mobility of LRB-CM in the cytoplasm (4.0 × 10⁻⁴ cm² s⁻¹), and treating the depleted nucleus as a bleached spot of average diameter 15 μm, if the nuclear envelope were freely permeable to LRB-CM, the half-time of recovery would be ∼5 s. In fact, the half-time for re-equilibration of LRB-CM fluorescence between the cytoplasm and the nucleus is several minutes, whereas the recovery of the fluorescence of a FITC-dextran of comparable size is much faster. These data raise the possibility that calmodulin in the cytoplasm exists as a complex with another protein(s) with a combined particle diameter of about the diameter of the nuclear pore (see Paine and Horowitz, 1980 for discussion of nucleocytoplasmic exchange). If so, this complex apparently is insensitive to TFP, since treatment with TFP did not affect the rate of entry of LRB-CM into the nucleus.

**CYTOPLASM:** LRB-CM within the cytoplasm of 3T3 cells exhibited a complex FRAP curve, just as would be expected of a protein such as calmodulin, which probably has an array of intracellular binding sites of differing affinities. The fastest component exhibited a mobility four times slower than a FITC-dextran of comparable size, and therefore probably does not represent unbound calmodulin. It could represent calmodulin either transiently associated with rapidly exchanging targets and/or associated with relatively mobile targets (for discussions of FRAP complicated by transient binding, see Koppel, 1981 and Elson and Reidler, 1979). Since the upper limit of detection of mobile species by FRAP is determined by spot size and dead time of the instrument, we have not ruled out the possibility that higher mobility components were not resolved in our experiments. The slower mobility components appear to represent binding of LRB-CM to specific, exchangeable, but low mobility calmodulin targets, since these species are abolished during treatment of the cells with the anti-calmodulin drug, TFP. In contrast, the FRAP curve of AF-actin within 3T3 cells could be resolved into two predominant components, one highly mobile and one highly immobile. Based on previous FRAP studies of AF-actin in aqueous solution (Lanni et al., 1981; Lanni and Ware, 1984) these two components may represent mobile monomers or small oligomers of actin, and polymerized or immobilized actin, respectively. If so, then ∼50–60% of the actin in interphase 3T3 cells exists as "soluble," unpolymerized actin. This value is similar to estimates of G-actin reported by other investigators for a variety of cell types (e.g., Blikstad et al., 1978; Kreis et al., 1982).

LRB-CM and AF-actin were >15 times less mobile in 3T3 cells than in aqueous solution. In contrast, the three FITC-dextrans were on the average only about four times less mobile in 3T3 cells (using uncorrected values of mobility obtained at 37°C) than in aqueous solution. The mobility of FITC-dextrans both in aqueous solution and in the cytoplasm of 3T3 cells was inversely dependent on molecular size, as predicted from diffusion theory. The results of regression analysis of these preliminary data suggested that the diffusion coefficient of FITC-dextrans in aqueous solution scales with the square root of molecular weight, as would be expected for long chain polymers that diffuse as random coils. There was no evidence from analysis of FRAP curves or visual inspection of bleached cells that FITC-dextrans bind with high affinity to any intracellular component. Therefore, FITC-dextrans may reasonably be regarded as indicators of that part of the effective cytoplasmic viscosity that is due to the true viscosity of the solvent water and to the effect of macromolecular obstructions to free diffusion. This corresponds to a solvent viscosity of ∼4 centipoise—not too different from values determined in other cells by other techniques (Wojcieszyn et al., 1982; Lepock et al., 1983; Mastro et al., 1984; Jacobson and Wojcieszyn, 1984).

All proteins, regardless of molecular size, move considerably more slowly than these dextrans within 3T3 cells. Unlike those of the dextrans, the intracellular mobilities of all extrinsic “control” proteins tested did not appear to depend on molecular size. Previous investigators have noted qualitatively similar results and have postulated that these proteins may be bound to intracellular components (Wojcieszyn et al., 1981; Wang et al., 1982; Gershon et al., 1982; Mastro et al., 1984; Jacobson and Wojcieszyn, 1984). In this study, apparent binding of these proteins to intracellular components was detected: as an “immobile” component in analysis of FRAP curves and as a persistent bleached spot by visual inspection of bleached cells. Clearly, dextrans are better control molecules than those we have used, as they do not appear to have any inherent affinity for intracellular components.
use of a very small spot size (radius 1.5 μm). Alternatively, the discrepancy may lie in some basic difference between primary cells and tissue culture cells.

Since FITC-dextran appears to be faithful indicators of cytoplasmic viscosity, they can be used to probe the microstructure of cytoplasm. More specifically, they may be useful in determining the pore size of the cytoplasmic gel. This parameter must be determined in order to understand fully the relative contributions of intramolecular interactions vs. cytoplasmic structure to mobilities determined by FRAP. Furthermore, FITC-dextran can be used to test the sol-gel contraction-coagulation hypothesis of cell motility (see Taylor and Fechheimer, 1982, for review), and also to detect possible changes in cytoplasmic viscosity under various experimental conditions.

Amino acid analysis was performed for us by the Amino Acid Analysis Facility at the Carnegie-Mellon University, Pittsburgh, PA. The continued help of the scientists and engineers of Hamamatsu Photonics, K. K. is gratefully acknowledged. Their interest in this field has made possible rapid development in the use of imaging systems and quantitative microscopy in the biomedical sciences. Antibulin antibody was the generous gift of Dr. Charles J. Walsh, University of Pittsburgh. We benefited from the helpful suggestions of Drs. Claude Klee, Thomas Vanaman, and Anthony Means. Dr. Klee also kindly provided us with a sample of her fluorescent analogue of calmodulin for comparison. We are grateful to Dr. Jimmy Collins and his associates at the University of Pittsburgh for testing the ability of LRB-CM to activate MLC. We thank Drs. Philip Amato, Gary Bright, Lauren Ernst, Marcus Fechheimer, Jeanne Heiple, Paul McNeil, Joel Swanson, Lerson Tanasugarn, and Alan Wagner for much helpful discussion and advice; Jacob Franke for his assistance in working out the phosphodiesterase assay for calmodulin; and Mark Rockwell, Nancy O’Connor, Keiko Ozaki, and Andrew Miranker for technical support.

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