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Fluorescent Analogues of Myosin II for Tracking the Behavior of Different Myosin Isoforms in Living Cells

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Abstract Fluorescently labeled smooth muscle myosin II is often used to study myosin II dynamics in non-muscle cells. In order to provide more specific tools for tracking non-muscle myosin II in living cytoplasm, fluorescent analogues of non-muscle myosin IIA and IIB were prepared and characterized. In addition, smooth and non-muscle myosin II were labeled with both cy5 and rhodamine so that comparative, dynamic studies may be performed. Non-muscle myosin IIA was purified from bovine platelets, non-muscle myosin IIB from bovine brain, and smooth muscle myosin II from turkey gizzards. After being fluorescently labeled with tetramethylrhodamine-5-iodoacetamide or with a succinimidyl ester of cy5, they retained the following properties: (1) reversible assembly into thick filaments, (2) actin-activatable MgATPase, (3) phosphorylation by myosin light chain kinase, (4) increased MgATPase upon light-chain phosphorylation, (5) interconversion between 6S and 10S conformations, and (6) distribution into endogenous myosin II-containing structures when microinjected into cultured cells. These fluorescent analogues can be used to visualize isoform-specific dynamics of myosin II in living cells. J. Cell. Biochem. 68:389–401, 1998.

Key words: cytoskeleton; cell motility; intracellular dynamics; stress fibers; heavy chain

Myosin II, or "conventional" myosin, is the likely contractile motor in a variety of dynamic cytoskeletal structures in non-muscle cells, including stress fibers [Burridge, 1981], the cytokinetic contractile ring [DeLozanne and Spudich, 1987; Knecht and Loomis, 1987], and peripheral bands that circumscribe the apices of cells in endothelial and epithelial sheets [Bement and Capco, 1991; Martin and Lewis, 1992]. In order to understand the function of myosin II-containing structures in cytoplasmic shape and movement, it is useful to be able to see how structures form, contract, and disperse at particular locations, and how they are interconverted as a cell migrates or divides. An extremely powerful tool for doing this is fluorescent-analogue cytochemistry (i.e., the attachment of fluorescent dyes to cellular constituents), allowing them to be tracked in the cytoplasm of living cells [Wang, 1989]. Fluorescent-analogue cytochemistry has elucidated the dynamic behavior of myosin II in vertebrate non-muscle cells, revealing cyclical formation, intracellular movement, and dissolution of myosin II structures [DeBiasio et al., 1988; Giuliano and Taylor, 1990; McKenna et al., 1989; Sanger et al., 1989; Verkhovsky et al., 1995], regional differences in the state of assembly and contractile activity of myosin II [Kolega et al., 1991; Kolega and Taylor, 1993; Verkhovsky et al., 1995], and even spatial and temporal variation in phosphorylation of myosin II's regulatory light chains [Post et al., 1995].

Because myosin II is not very abundant in non-muscle tissues, fluorescent analogues derived from smooth muscle myosin II have typically been used to monitor myosin II dynamics in non-muscle cells. This heterologous combination has been justified by the similar enzymatic and assembly properties of vertebrate smooth and non-muscle myosin II in vitro. Unlike that of skeletal or cardiac muscle, the ATPase activity of purified smooth or non-muscle myosin II

Abbreviations: MHC-A, isoform A of myosin II heavy chain; MHC-B, isoform B of myosin II heavy chain; BMEC, bovine microcapillary endothelial cell.

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is regulated by phosphorylation of the 20,000-kD regulatory light chains [Sellers, 1991; Tan et al., 1992; Trybus and Lowey, 1987]. In addition, filaments of smooth or non-muscle myosin II are more plastic in vitro, disassembling in the presence of ATP in physiological salt concentrations. For both smooth and non-muscle myosin II this plasticity is also regulated by phosphorylation of the regulatory light chains [Sellers, 1991; Tan et al., 1992; Trybus and Lowey, 1987]. However, smooth and non-muscle myosin II do have different amino acid sequences, and are expressed in very different populations of cells. Furthermore, there are at least two major isoforms of non-muscle myosin II, designated myosin IIA and myosin IIB, which have distinct subcellular distributions in neurons [Cheng et al., 1992; Miller et al., 1992; Rochlin et al., 1995], *Xenopus* blastomeres and cultured fibroblasts[Kelley et al., 1996], and cultured human melanoma and carcinoma cells [Maupin et al., 1994]. Consequently, it is important to distinguish behavior of specific populations of myosin II. Fluorescent analogues described in the present study will permit the examination of the behavior of either non-muscle myosin IIA or non-muscle myosin IIB in living cells. In addition, two different fluorophores have been used to label non-muscle and smooth muscle myosin II, which will make it possible to monitor the distribution of different forms simultaneously in the same cell.

MATERIALS AND METHODS

Proteins

Smooth muscle myosin II was isolated from frozen turkey gizzards (Pel-Freeze Biologicals, Rogers, AK) using the procedures described by DeBiasio et al. [1988] for chicken gizzard myosin II. Platelet myosin II was isolated from bovine blood obtained from a local slaughterhouse: platelets were purified by the method of Ikebe and Reardon [1990], and myosin II was then isolated by the method of Daniel and Sellers [1992]. Bovine brain myosin II was isolated from frozen, stripped brains (Pel-Freeze Biologicals, Rogers, AR) using essentially the same procedure as Li et al. [1994], except that MOPS was used in place of imidazole as the buffering agent in all solutions, and a narrower ammonium sulfate cut was taken (i.e., myosin was collected with proteins precipitating between 40-60% saturation, rather than 35-65%). Actin was purified from rabbit skeletal muscle as described by Pardee and Spudich [1982]. Myosin light chain kinase from chicken gizzard was provided by A. Edelman (SUNY, Buffalo, NY).

Fluorescent Analogues

Myosin II was labeled with tetramethylrhodamine as described by DeBiasio et al. [1988]. Briefly, purified myosin II was solubilized in 10 mM HEPES, 50 mM KCl, 2 mM ATP, 2 mM $MgCl_2$, 0.2 mM EGTA, pH 7.0, at a concentration of 12–15 mg/ml. A 10-fold molar excess of tetramethylrhodamine-5-iodoacetamide (Molecular Probes, Eugene, OR) was then added and the mixture incubated 4 h on ice. The labeling reaction was stopped by addition of dithiothreitol to 2 mM, and labeled protein was separated from unbound dye by dialyzing to remove ATP, then precipitating myosin II with 10 mM $MgCl_2$. Labeling with cy5 was similar except: a 20-fold molar excess of cy5-monofunctional NHS-ester (FluoroLink monofunctional dye; Amersham Life Sciences, Arlington Heights, IL) was used, the reaction time was 1 hr on ice, and labeling was terminated by the addition of 20 mM lysine. The number of bound dye molecules per myosin II was 4–6 for rhodamine- and 1–2 for cy5-labeled analogues. The analogues were stored on ice as thick filaments suspended in 2 mM HEPES, pH 7.5, 0.1 mM EGTA, 1 mM DTT, and were used within 1 week of purification and labeling.

Determination of 6S and 10S Conformation

The 6S and 10S conformations of myosin II were distinguished by size-exclusion chromatography as described by Trybus et al. [1982]: A 2×100 cm column of Sepharose CL-4B was equilibrated in 5 mM MgCl, 1 mM EGTA, 20 mM potassium phosphate, pH 7.5, containing either 0.15 M KCl and 5 mM ATP (to induce the 10S conformation) or 0.6 M KCl without ATP (for the 6S conformation). Aliquots of purified myosin II were suspended and chromatographed in the corresponding buffer. Elution of myosin II was monitored by determining protein concentration in the eluted fractions (by absorbance at 280 nm or by Bradford assay) or, for fluorescent analogues, measuring fluorescence at appropriate emission and excitation wavelengths using an Aminco-Bowman Series 2 spectrofluorometer.

ATPase Assay

The ATPase activities of myosin II and its analogues were determined using a procedure based on that of Ikebe and Reardon [1990]. Myosin II was incubated at 25°C in 60 mM KCl, 30 mM Tris, pH 7.5, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, in the presence or absence of 0.8 mg/ml actin. At 5-15-min time intervals, the reaction was stopped by the addition of 3% sodium dodecyl sulfate (SDS) and 30 mM EDTA. The amount of phosphate released was then determined colorimetrically, and the rate of the ATPase reaction measured from the linear portion of the time course. For determining the ATPase of phosphorylated myosin, myosin was first phosphorylated with myosin light chain kinase as previously described [Hahn et al., 1993]. Phosphorylation of the light chains was confirmed by two-dimensional gel electrophoresis.

Cells

Microcapillary endothelial cells isolated from bovine adrenal gland (BMECs) [Furie et al., 1984] were provided by Dr. Martha Furie (SUNY, Stony Brook, NY). The cells were cultured in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum (FBS) with 10 mM HEPES, pH 7.4, and were used between the 14th and 19th passage after their original isolation. Cos fibroblasts were from American Type Cell Culture (Gaithersburg, MD), and were cultured in the same medium used for BMECs. Culture medium was purchased from Life Technologies (Grand Island, NY) and FBS serum from Atlanta Biological (Norcross, GA).

Microinjection

Cells were microinjected as described by Amato et al. [1983]. The concentration of myosin II in the solution that was injected varied with each preparation, but the maximum was 1–2 mg/ml. The volume that was injected was estimated to be 2–5% of the volume of the cell, so that the final intracellular concentration was $\leq$0.1 mg/ml. This corresponds to $<$2% of the endogenous myosin II, assuming a concentration of myosin II in endothelial cells of approximately 8 mg/ml [Schnittler et al., 1990]. Injected analogues became distributed throughout the accessible volume of the cytoplasm in $\leq$15 min and were allowed to equilibrate in the cytoplasm for $\geq$2 hr before image acquisition.

Fluorescence Imaging

Living cells were imaged in a simple viewing chamber described by Radice and Keller [1977], consisting of a slide with a 15-mm-diameter hole and coverglasses affixed over the hole with a ring of vacuum grease. The temperature of the cells was maintained at 37°C (as measured by a wire thermistor placed inside the chamber) by an electrical heating tape wrapped around the microscope objective. Fluorescence microscopy was performed through a Zeiss Axiovert 135 microscope with a 100 W mercury arc lamp for excitation illumination, using a 100× Plan-NEOFLUAR oil-immersion objective. Fluorescence images were acquired by using Oncor-Image software (Oncor, Gaithersburg, MD) to sum 32 frames of the video output from a Paultek CCD camera equipped with a Gen II-MCP intensifier (Paultek Systems, Nevada City, CA). The pixel density in the final digitized images was 4.2 pixels/μm.

Quantitative Immunoblotting

Quantitative immunoblotting was performed as previously described Kolega [1997]. Briefly, samples were spotted on nitrocellulose paper, stained with antibodies, and the amount of staining quantified by densitometry of the stained paper. Along with each sample, known amounts of purified myosin standards were spotted on the same paper, so that the amount of a particular antigen in each sample could determined by comparison with the standards.

Antibodies against specific isoforms of the myosin II heavy chain were generously provided by R. Adelstein (National Institutes of Health [NIH]). These antibodies were raised against synthetic peptides corresponding to the unique C-termini of MHC-A, (isoform A of myosin II heavy chain) and MHC-B (isoform B of myosin II heavy chain), and do not recognize other myosin II heavy chains [Maupin et al., 1994; Phillips et al., 1995]. A polyclonal antibody against smooth and non-muscle myosin II was obtained from Biomedical Technologies Inc. (Stoughton, MA). The secondary antibody for western blotting was an affinity-purified, peroxidase-conjugated goat antibody against rabbit IgG heavy and light chains (Kirkegaard and Perry Laboratories, Gaithersburg, MD).

Ratio Images

Image manipulation and calculations were performed using NIH-Image or Oncor-Image software. Ratio images were generated as previously described Kolega and Taylor [1993], based on the methods of Bright et al. [1987], DeBiasio et al. [1988;], and Tanasugarn et al. [1984]. Before ratios were calculated, background images were subtracted from each image, and the image intensities normalized by multiplying each image by an appropriate constant, so that the integrated intensity over the entire, background-subtracted image was equal for all images.

RESULTS

Cy5-Labeled Myosin II

The ultimate goal of this endeavor was to produce fluorescent analogues that could be used to compare the behavior of different myosins within the same cell. Therefore, it was important to be able to label myosin II with different, spectrally separable fluorophores. Because rhodamine-labeled analogues of smooth muscle myosin II are routinely used to study myosin dynamics in living cells, we first set out to make a complementary fluorescent analogue of smooth muscle myosin II. Fluorescein is frequently used with rhodamine in paired fluorescence, and fluorescein-labeled skeletal muscle myosin II has been prepared by Johnson et al. [1988]. However, fluorescein is prone to photobleaching and its fluorescence is sensitive to local pH. Both of these qualities make it difficult to use quantitatively in living cells. Therefore, we labeled smooth muscle myosin II with the more stable, pH-insensitive dye, cy5 [Southwick et al., 1990]. For comparison, we also prepared a rhodamine-labeled analogue, which was characterized and described previously [Hahn et al., 1993].

Smooth muscle myosin II isolated from turkey gizzard was labeled with cy5 as described under Materials and Methods. The biological function of the cy5-labeled analogue was tested in vitro using several criteria. First, it was put through two cycles of assembly and disassembly to make certain that it was capable of reversible assembly into thick filaments: Upon dialysis into 50 mM KCl, 10 mM $MgCl_2$, ≥90% of the analogue could be sedimented by centrifugation for 15 min at 100,000*g*. The sedimented material consisted of filamentous bundles, as determined by electron microscopy (not shown). The sedimented, cy5-labeled, smooth muscle myosin II was then resuspended in a high-salt buffer containing 300 mM KCl without Mg^{++}, and clarified by centrifugation, with ≥90% remaining in the supernatant. The high-salt-soluble cy5-myosin II was once again assembled and sedimented, and then resuspended in physiological salt (100 mM KCl). Under these conditions, 90% of myosin II remained insoluble. However, the same material could be solubilized in 100 mM KCl by the addition of 2 mM MgATP. Thus, the cy5-labeled platelet myosin II reversibly assembled into filaments that were salt- and ATP-sensitive, as are native smooth muscle myosin II [Suzuki et al., 1978] and previously described rhodamine-labeled analogues [DeBiasio et al., 1988; McKenna et al., 1989].

The cy5-labeled form of smooth muscle myosin II also underwent normal folding and unfolding. When solubilized by ATP in physiological salt concentrations, unphosphorylated nonmuscle or smooth muscle myosin II exists as a compact, folded monomer. In the presence of high salt, the monomer unfolds into an extended rod. The folded and unfolded conformations (designated the 10S and 6S forms, respectively, because of their sedimentation rates) can be distinguished by a shift in mobility on Sepharose CL-4B chromatography [Trybus et al., 1982]. Figure 1a shows that unlabeled, cy5-labeled, and rhodamine-labeled gizzard myosin II all migrated more rapidly through Sepharose CL-4B in high salt. Thus, both the unlabeled myosin II and its analogues assumed a less compact conformation in high salt, consistent with a 10S→6S unfolding.

Cy5-labeled gizzard myosin II remained active as an ATPase. The MgATPase activity of cy5-labeled gizzard myosin II was measured under several different conditions (Fig. 1b). Like the MgATPase of native gizzard myosin II and rhodamine-labeled gizzard myosin II, this activity was stimulated by the presence of F-actin and was modulated by phosphorylation: The 20-kD regulatory light chains of the analogue could be phosphorylated by myosin light chain kinase, as demonstrated by the incorporation of ^{32}P-phosphate and a concomitant shift in isoelectric point on 2-dimensional electrophoretic gels (not shown). Phosphorylation by myosin light chain kinase increased both the MgATPase and the extent of its activation by F-actin, and the

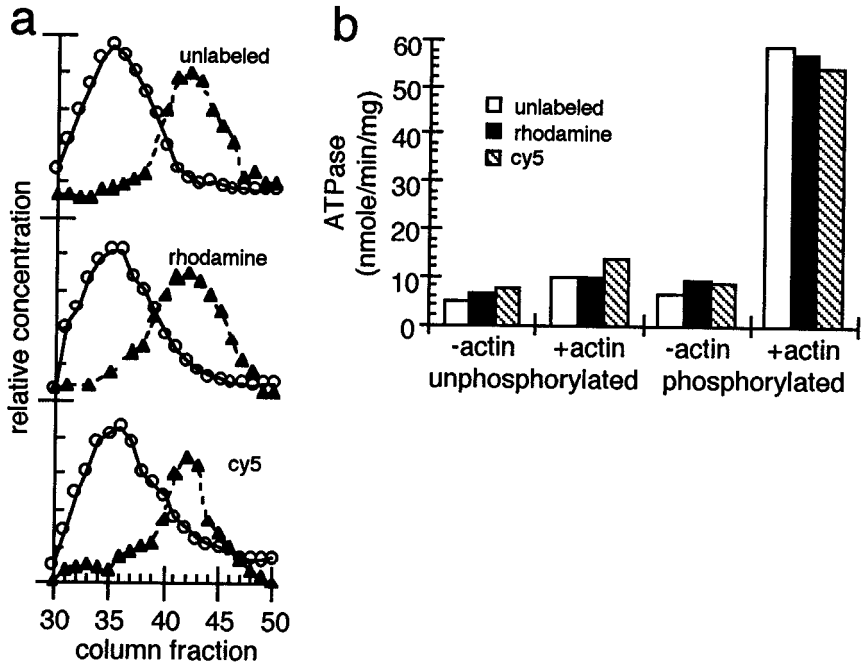

Fig. 1. Biochemical characterization of smooth muscle analogues. **a:** 6S↔10S transition. Fluorescent analogues of myosin II derived from turkey gizzards were chromatographed over Sepharose CL-4B as described in Methods. The portions of the elution profiles that contain myosin II are illustrated here. Open circles, the elution profile in the presence of 0.6 M KCl to induce unfolding to the 6S conformation; solid triangles, elution in 0.15 M KCl and 5 mM ATP to induce the folded, 10S form. Elution profiles are shown for unlabeled gizzard myosin II and for its rhodamine- and cy5-labeled analogues. In each case, myosin II migrated as a single peak in the presence of high salt. In low salt containing ATP, unlabeled myosin II and both analogues migrated more slowly, indicating that all three assume a more compact conformation under these conditions. **b:** MgATPase activities. The MgATPase of myosin II which had unphosphorylated 20-kD light chains, or which had been phosphorylated by myosin light chain kinase, was determined in the presence and absence of 0.8 mg/ml actin as described in Methods. MgATPase activities for unlabeled (empty bars), rhodamine-labeled (solid bars) and cy5-labeled gizzard myosin II (hatched bars) are shown. In the absence of actin, MgATPase was low for all three forms whether or not the light chains were phosphorylated. However, the MgATPase of the phosphorylated myosins was higher than the unphosphorylated forms. In all three cases, actin stimulated ATPase of the unphosphorylated myosin II only slightly, but dramatically increased the ATPase activity of phosphorylated myosin II.

levels of these activities were comparable between labeled and unlabeled myosin II (Fig. 1b).

To test the function of cy5-labeled gizzard myosin II in vivo, it was microinjected into cultured endothelial cells. The analogue distributed through most of the cytoplasm, and was excluded from the nucleus (Fig. 2a). Much of it incorporated into stress fibers, where it frequently displayed a periodic, punctate distribution, as has been observed for rhodamine-labeled gizzard myosin II microinjected in fibroblasts [Giuliano and Taylor, 1990; McKenna et al., 1989]. This distribution was identical to that of rhodamine-labeled gizzard myosin II injected into the same cell (Fig. 2b,c).

Fluorescent Analogues of Non-muscle Myosin IIA

Fluorescent analogues of non-muscle myosin IIA were prepared by labeling myosin II purified from bovine platelets. Platelets contain only the A isoform of non-muscle myosin II heavy chain (MHC-A) [Murakami et al., 1991]. The presence of MHC-A and the absence of the B isoform (MHC-B) in our preparations were confirmed by Western blotting using antibodies that distinguish the two isoforms [Maupin et al., 1994; Phillips, et al., 1995]. As shown in Figure 3, antibody against MHC-A recognized bovine platelet myosin II and its rhodamine- and cy5-labeled analogues, whereas neither the unlabeled myosin II nor the analogues were recognized by an antibody directed against MHC-B. Neither isoform was detected in myosin II isolated from smooth muscle.

Platelet myosin IIA was labeled with cy5 and with rhodamine as described for gizzard myosin II, and the biological function tested using the same criteria. Both analogues assembled

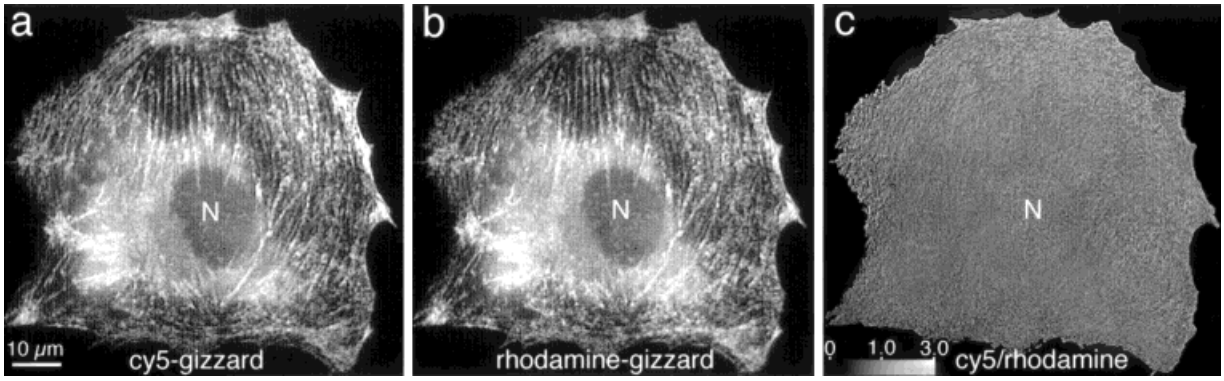

Fig. 2. Distribution of fluorescently labeled, smooth muscle myosin II in cytoplasm. BMECs were microinjected with a mixture of cy5-labeled gizzard myosin II and rhodamine-labeled gizzard myosin II, and allowed to recover for 4 h. Separate fluorescence images were then acquired through cy5 and rhodamine filter sets. **a:** Distribution of cy5-labeled gizzard myosin II. Note that the nucleus (N) stands out as dark (fluorescence-excluding) structure against a background of diffuse fluorescence in the perinuclear cytoplasm. Most of the fluorescent myosin is located in large linear structures (stress fibers) or in a pattern of small spots that is particularly predominant in the thin, peripheral cytoplasm. **b:** Rhodamine-labeled myosin II. The distribution of the rhodamine-labeled analogue is virtually indistinguishable from that of the cy5-labeled myosin II in (a). **c:** Ratio image comparing a and b. The cy5 image was divided by the rhodamine image as described in Methods to create an image in which the pixel intensity represents the ratio of the intensities in the original images. The image is displayed with the gray levels depicting different ratio values, as indicated by the scale bar. The uniformity of the image, with most of the gray values around 1.0, indicates that there is very little difference between the two images.

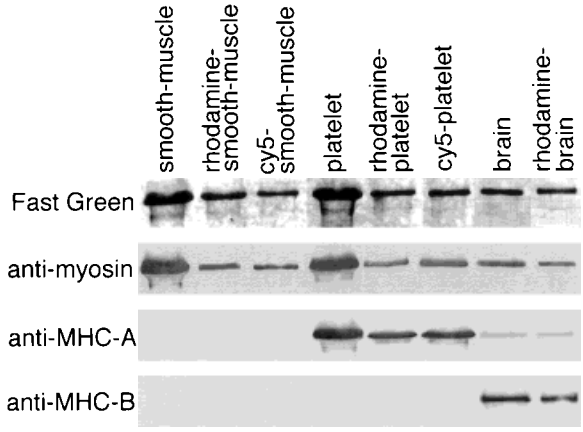

Fig. 3. Isotype specificity of fluorescent analogues. Purified gizzard, platelet, and smooth muscle myosin II, along with their fluorescent analogues, were run on 8.5% polyacrylamide gels and transferred to nitrocellulose paper. Four identical blots were stained with (1) Fast Green (to reveal total protein), (2) an antibody against smooth and non-muscle myosin II, (3) an antibody specific for MHC-A, or (4) an antibody against MHC-B. The portion containing the myosin heavy chains is shown for each blot. No antibody staining was detected elsewhere on the blots. Although similar amounts of total myosin were loaded in the lanes containing rhodamine-platelet, cy5-platelet, brain, and rhodamine-brain myosin II, they stained very differently for MHC-A and MHC-B. Platelet analogues stained strongly for MHC-A and not at all for MHC-B, while myosin II from brain stained strongly for MHC-B but only very weakly for MHC-A.

into thick filaments when dialyzed into 50 mM KCl, 10 mM $MgCl_2$, and were solubilized in either 300 mM KCl or in 100 mM KCl plus 2 mM MgATP. This behavior was also observed for unlabeled platelet myosin IIA, and is consistent with previously reported assembly properties of non-muscle myosin II from platelets [Ikebe, 1989], thymus [Citi and Kendrick-Jones, 1986; Scholey et al., 1980], and intestinal brush border [Citi and Kendrick-Jones, 1986].

The rhodamine-labeled form of platelet myosin IIA also underwent normal 6S↔10S conformational change (Figure 4a), and had actin-activatable and phosphorylation-stimulated ATPase activity (Fig. 4b), as previously reported for native platelet myosin II [Adelstein and Conti, 1975]. The actin-activated ATPase activity of phosphorylated platelet myosin IIA in our preparations ranged from 60–80 nmol/mg/min, which is within the range of previously published values [Adelstein and Conti, 1975; Ikebe, 1989].

When microinjected into BMECs, the rhodamine-labeled platelet myosin IIA behaved as the smooth muscle analogues: it distributed through most of the cell, was excluded from the nucleus, localized heavily in stress fibers, and frequently displayed a periodic, punctate distribution (Fig. 5a). This is very similar to the

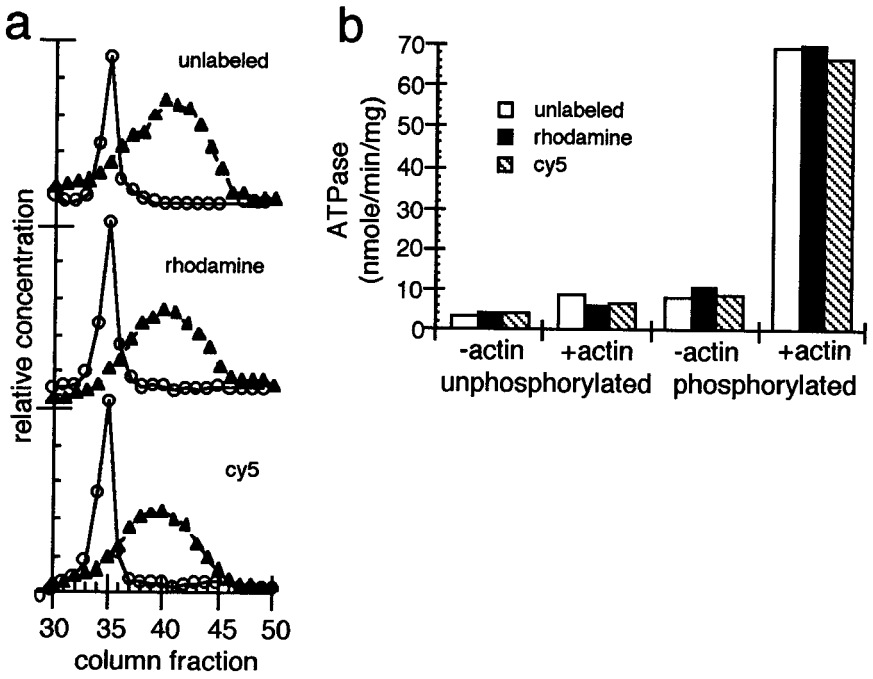

Fig. 4. Characterization of platelet myosin II and its analogues. **a:** 6S↔10S conformational change. Platelet myosin II was chromatographed over Sepharose CL-4B as in Fig. 1. Unlabeled platelet myosin II and its rhodamine- and cy5-labeled analogues each migrated as a single, sharp peak in 0.6 M KCl (open circles), and all three migrated more slowly in 0.15 M KCl plus ATP (solid triangles). Thus, both the analogues and unlabeled myosin are less compact in high salt, consistent with unfolding from a 10S to 6S conformation. **b:** MgATPase activities. The MgATPase activities of unlabeled (open bars), rhodamine-labeled (solid bars) and cy5-labeled platelet myosin II (hatched bars) were determined as in Fig. 1. Actin mildly stimulated MgATPase of unphosphorylated platelet myosin II and dramatically stimulated MgATPase of phosphorylated platelet myosin II. Phosphorylation alone, in the absence of actin, elevated MgATPase levels only slightly. Similar activities were observed for the rhodamine- and cy5-labeled forms of platelet myosin II in each of the four conditions.

distribution of endogenous myosin II revealed by immunofluorescence (Figure 5b). Like the gizzard-myosin analogues, both cy5- and rhodamine-labeled platelet myosin II displayed essentially identical distributions (Fig. 6).

Rhodamine-Labeled Non-Muscle Myosin IIB

Fluorescent analogues of non-muscle myosin IIB were prepared by labeling myosin II purified from bovine brain. Because of the large quantities of reagents and effort consumed and the very small amounts of myosin II obtained in brain preparations, only rhodamine-labeled analogues were characterized. In brain, the heavy chains of myosin II are mostly of the B isoform (MHC-B) [Murakami and Elzinga, 1992; Murakami et al., 1991]. However, western blotting also showed small amounts of MHC-A in our preparations (Fig. 3). To estimate how much MHC-A was present, western blots were performed on known amounts of brain myosin II alongside a series of lanes containing known amounts of platelet myosin II (to provide a standard curve for MHC-A). The blots were stained with antibody against MHC-A, and the staining quantified by densitometry as previously described [Kolega, 1997]. These blots indicated that the A isoform constituted 10-15% of the myosin II in our purified brain myosin II and in the analogues prepared from it.

Brain myosin II was labeled and characterized as described above for platelet and gizzard myosin II. Compared to gizzard or platelet myosin II, brain myosin II and its analogues were less soluble in low salt in the presence of ATP, with 50–60% of the myosin sedimenting when centrifuged for 20 min at 10,000*g*. This is probably due to the tendency of brain myosin II to form minifilaments under these conditions [Matsumura et al., 1989]. Nonetheless, the soluble portion still underwent a conformational change when placed in high salt, as indicated by size-exclusion chromatography (Fig. 7a). Labeled myosin II behaved in close parallel

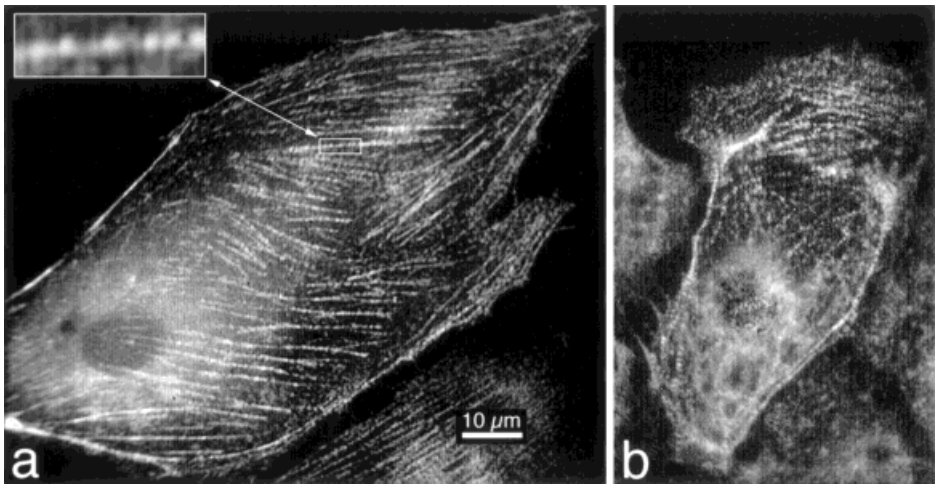

Fig. 5. Distribution of myosin IIA in cultured endothelial cells. **a:** Distribution of microinjected platelet myosin II in the cytoplasm of living cells. Rhodamine-labeled platelet myosin II was microinjected into BMECs and allowed to equilibrate in the cytoplasm for 4 h. Fluorescence images of the living (unfixed) cells were then acquired. The rhodamine-labeled myosin II incorporated into an extensive array of stress fibers, very often displaying a periodic, bead-like distribution along the length of the fibers (e.g., magnified fiber, *inset*). Diffuse cytoplasmic distribution was also observed, particularly in the perinuclear region. **b:** Distribution of endogenous myosin IIA in BMECs. BMECs were fixed and stained with antibody against MHC-A. Note that the distribution of the fluorescent analogue (a) is consistent with the pattern of organization displayed by the endogenous myosin IIA; i.e., many stress fibers, some diffuse perinuclear distribution, and punctate arrays along stress fibers and in peripheral cytoplasm.

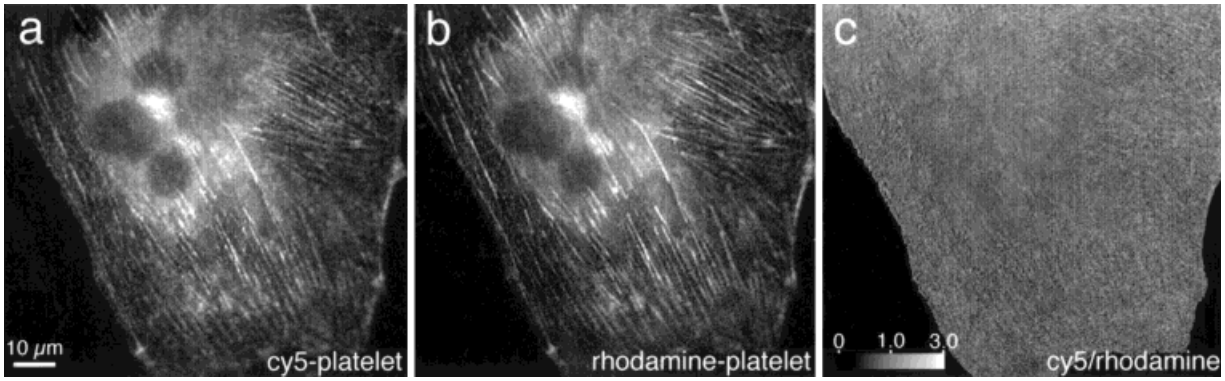

Fig. 6. Co-distribution of cy5- and rhodamine-labeled platelet myosin II. BMECs were microinjected with **(a)** cy5-labeled platelet myosin II and **(b)** rhodamine-labeled platelet myosin II. Fluorescence images of the living cells were acquired 4 h after injection. Both analogues distribute into the same regions and structures in the cell. **c:** Ratio image obtained by dividing the cy5 image by the rhodamine image. The relationship between pixel intensity and the ratio value is indicated by the shaded bar. The nearly uniform gray levels in the cell indicate that there is very little difference in the distribution of the two analogues. Scale bar = 10 μm.

with its unlabeled counterpart (Fig. 7a, compare upper and lower panels).

The ATPase activity of rhodamine-labeled brain myosin II also matched the activity of unlabeled brain myosin II, and this activity was stimulated by actin and by phosphorylation of the 20-kD regulatory light chains (Fig. 7b). These activities were lower than those of gizzard and platelet myosin II, as has been previously reported by others [Hobbs and Frederiksen, 1980; Malik et al., 1983; Matsumura et al., 1988].

Finally, to test the function of the rhodamine-labeled brain myosin II in cytoplasm, the analogue was injected into cos fibroblasts. Cos cells contain the B isoform of the myosin II heavy chain, but little or no MHC-A, as shown by Western blotting of cos cell extracts (Fig. 8) and by immunofluorescence staining of cultured cells (Fig. 9a,b). The endogenous MHC-B was found

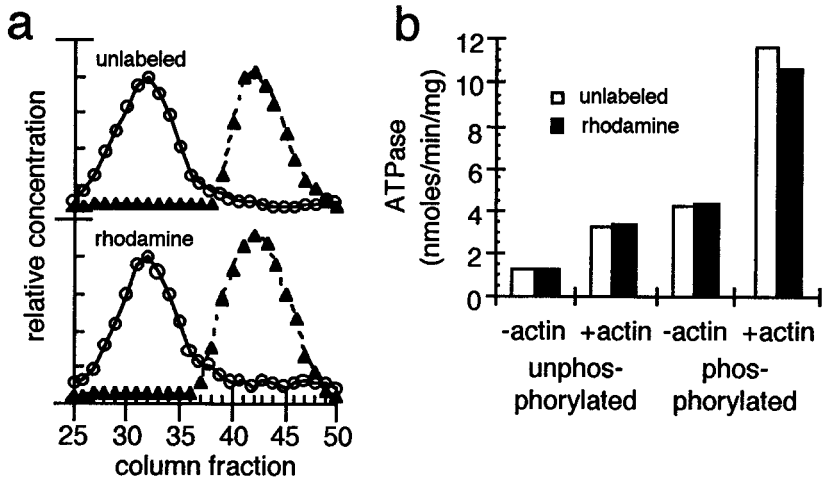

Fig. 7. Characterization of brain myosin II and rhodamine-labeled brain myosin II. **a:** 6S↔10S conformational change. Brain myosin II was chromatographed over Sepharose CL-4B as in Fig. 1. Unlabeled and rhodamine-labeled brain myosin II both migrated more rapidly in 0.6 M KCl (open circles) than in 0.15 M KCl plus ATP (solid triangles), indicating that they are less compact in high salt. The locations of the peaks are consistent with a 6S conformation in high salt and a 10S conformation in low salt with ATP. **b:** ATPase activities. The ATPase activities of unlabeled (open bars), rhodamine-labeled (solid bars) brain myosin II were determined as in Fig. 1. The ATPase of unphosphorylated myosin II was stimulated by the presence of actin, and this stimulation was increased by phosphorylation of the myosin light chains. ATPase activities for rhodamine-labeled and unlabeled brain myosin II were similar in each of the four conditions.

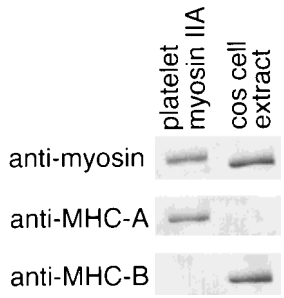

Fig. 8. Isoforms of non-muscle myosin II in cos cells. Identical lanes of a Western blot of platelet myosin IIA and whole-cell extracts of cos cells were stained for total myosin II, MHC-A, or MHC-B, using the same antibodies as in Fig. 3. Myosin II in the cos cell extracts was recognized by the antibody against MHC-B, but no MHC-A was detected.

mostly in stress fibers, where it was frequently arranged in periodic spots or striations. Some diffusely distributed MHC-B was also observed, especially in the perinuclear cytoplasm. When rhodamine-labeled brain myosin II was microinjected into cos cells, it distributed with precisely these same characteristics (Fig. 9c).

DISCUSSION

The fluorescent analogues described above provide three important new capabilities. First, a well-characterized, functional myosin II that is labeled with cy5 provides an alternative to existing, rhodamine-labeled analogues. This extends the range of fluorescent probes that may be visualized simultaneously with myosin II in living cells. Second, analogues of myosin II from bovine platelet and brain provide the first fluorescent indicators for non-muscle myosin II in higher vertebrates. Third, these analogues permit examination of the dynamic behavior of specific isoforms of non-muscle myosin II.

Cy5-Labeled Analogues of Myosin II

Fluorescent analogues of myosin II that have been previously described [Hahn et al., 1993; McKenna et al., 1989; Sanger, 1989; Johnson, 1988] were labeled with iodoacetamides of rhodamine or fluorescein, which react with sulfhydryl groups. Cy5-labeled analogues were prepared in the present study using an ester of cy5 that reacts with lysines, rather than sulfhydryls. Despite the different labeling chemistry, cy5-labeled analogues maintained function as well as the rhodamine analogues. This may be attributed to three factors: First, the final labeled analogue was selected for functionality by a cycle of assembly and disassembly in two different conditions. Second, the labeling conditions used should maintain myosin II in a folded 10S conformation, protecting the ATP-binding site [Cross et al., 1986], as well as sites critical to 6S-10S folding. Third, because of the brightness of cy5 and the sensitivity of intensified

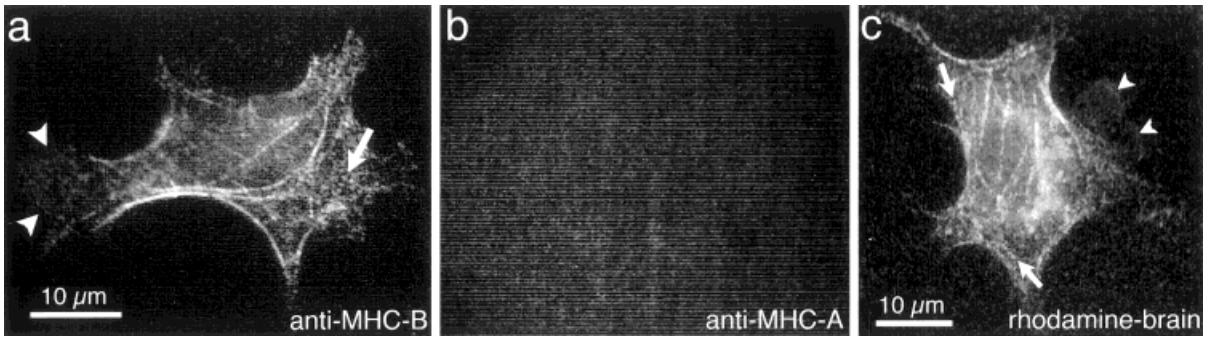

Fig. 9. Distribution of myosin IIB and brain myosin II in cultured fibroblasts. **a,b:** Cos fibroblasts were fixed, then stained by direct immunofluorescence with antibodies against MHC-B and MHC-A. a: MHC-B was localized mostly in stress fibers and punctate arrays scattered through the cytoplasm (arrows). Some weak, diffuse staining was seen in lamellipodia (arrowheads). b: Cos cells stained with antibodies against MHC-A: no staining was observed. **c:** Cos cells were microinjected with rhodamine-labeled brain myosin II, allowed to recover for 4 h, then imaged by fluorescence microscopy. Like endogenous myosin IIB (a), the microinjected brain myosin II concentrated in stress fibers and linear, punctate arrays (arrows), and small amounts were diffusely distributed in lamellipodia (arrowheads). Scale bars = 10 μm.

CCD cameras in the red wavelengths, the labeling stoichiometry could be kept very low (1-2 fluorophores per myosin), minimizing the likelihood that the bound dye would interfere with a critical site on the myosin II.

The availability of cy5-labeled analogues of myosin II will permit visualization of myosin II simultaneously with other fluorescent indicators of cytoskeletal components and regulators, such as rhodamine-labeled actin, α-actinin or profilin. Even where fluorescein-labeled alternatives are available, cy5-labeled analogues are particularly valuable when studying dynamic processes because they permit the use of cy5-rhodamine pairs instead of fluorescein-rhodamine combinations. Because cy5 and rhodamine are both much more resistant to photobleaching than fluorescein, this permits more images to be acquired of a single, changing cell. Because its fluorescence is relatively insensitive to pH, cy5 also minimizes artifacts arising from intracellular pH changes.

Cy5-labeled myosin II will also allow comparisons of the intracellular dynamics of different forms of myosin II. Borrione et al. [1990] reported that some endothelial cells in culture transiently express both smooth and non-muscle myosin II, and that isoform-specific antibodies give different staining patterns by immunofluorescence. The functional significance of this is entirely unknown, but an examination of the dynamic behavior of smooth and non-muscle II in such cells could reveal how their roles may differ. Although analogues of smooth and non-muscle myosin II distributed similarly in BMECs in the present study, there may be subtle differences revealed by co-localization of isoforms in the same cell, or there may be differences in the speed of intracellular movements of the two types of myosin II. It will be interesting to see if either form is preferentially incorporated into the contractile ring during cytokinesis, or if transient differences in localization occur during the contraction of endothelia that occurs in response to thrombin and various other stimuli. It should be noted that the similarity between the distribution of platelet and smooth muscle myosin II is in stark contrast to what is observed when smooth and skeletal muscle myosins are injected into fibroblasts [Johnson et al., 1988]. Skeletal muscle myosin II fails to distribute into many structures and tends to form filamentous aggregates of a morphology not ordinarily observed in fibroblasts.

Fluorescent Analogues of Platelet Myosin II

As was observed for gizzard myosin II, labeling of platelet myosin II with rhodamine or cy5 did not alter its function in a broad range of in vitro assays. Nor did it appear to disturb its ability to function in vivo, as rhodamine- and cy5-labeled platelet myosin II become correctly distributed when reintroduced into the cytoplasm of living cells. Thus, these fluorescent analogues should be useful tools for studying the dynamics of non-muscle myosin II in cells. The fluorescent analogues of platelet myosin II have two main distinctions from the smooth

muscle analogues used in past studies [DeBiasio et al., 1988; Sanger et al., 1989; Giuliano and Taylor, 1990; Kolega et al., 1991; Kolega and Taylor, 1993; McKenna et al., 1989; Post et al., 1995; Verkhovsky et al., 1995]. First, they can potentially reveal behavior that is unique for non-muscle myosin II. Although the differences detected in vitro between smooth and non-muscle myosin II are small, they may translate into larger effects in the cytoplasm. Or myosin II behavior in the cell may depend on properties that have simply not been assayed for in vitro. Second, the heavy chains of platelet myosin II are all of the A isoform [Murakami et al., 1991], so fluorescently labeled platelet myosin II can be used to discern isoform-specific behavior. Most vertebrate non-muscle tissues contain mixtures of MHC-A and MHC-B, but the functional significance of this is not known. The ability to examine the dynamic behavior of myosin IIA versus myosin IIB, may provide an answer to this question (see below).

Brain Myosin II

Brain myosin II was also labeled without disruption of its enzymatic, assembly, or solubility properties. Fluorescently labeled brain myosin II offers an excellent complement to fluorescently-labeled platelet myosin II, because it consists primarily of non-muscle myosin IIB. It does contain a small amount of myosin IIA, so the distribution revealed by this analogue must be interpreted with care. However, assuming that rhodamine reacts with myosin IIA and IIB with equal efficiency during labeling, 85-90% of the fluorescence signal should be attributable to myosin IIB. This should provide a good picture of the gross distribution and dynamics of myosin IIB in living cells. In fact, the distribution off the analogue was found to be in excellent agreement with the distribution of MHC-B revealed by immunofluorescence (Fig. 9).

Fluorescently labeled brain myosin II will be most useful in probing the respective roles of myosin IIA and IIB in non-muscle cells. Kelley et al. [1996] recently showed that actin filaments are moved faster by non-muscle myosin IIA than by non-muscle myosin IIB, suggesting that myosin IIA and IIB could have different dynamics in the cell. Although in situ dynamics have not been observed directly, differences between the distribution of MHC-A and MHC-B have been observed. In cultured neurons, myosin IIB is preferentially associated with the active leading edges of the growth cone [Rochlin et al., 1995], whereas, in non-neuronal cells, myosin IIA is usually biased toward the cells' edges [Maupin et al., 1994]. And, in mitotic cells, myosin IIA, but not IIB, localizes to the mitotic spindle [Kelley et al., 1996]. Comparison of the dynamic behavior of myosin IIA with myosin IIB may clarify how and why these differences arise. For example, are the distinct distributions of these two non-muscle isoforms due to intrinsic properties of the two proteins, or to spatially separated expression, as has been observed for some actin isoforms [Hoock et al., 1991]? When do myosin IIA and IIB separate during formation of a growth cone [Rochlin et al., 1995]? Are myosin IIA and IIB distributed differently in the mitotic spindle [Kelley et al., 1996] because myosin IIA selectively moves into the mitotic apparatus, because myosin II B selectively moves out, or both?

Other Fluorescent Analogues of Non-Muscle Myosin II

Moores et al. [1996] have genetically engineered a fluorescent, non-muscle myosin II by fusing the sequence encoding green fluorescent protein to the gene for the heavy chain of myosin II in Dictyostelium. This permits visualization of myosin II in entire populations of amoeba without having to microinject each cell. A similar approach will no doubt eventually be used to express fluorescent myosins in vertebrate cells, and will be an extremely powerful and useful reagent. Nonetheless, direct chemical labeling of myosin II still provides a very useful compliment to the genetic approach. The greatest advantage of "chemical" over "genetic" fluorescent analogues is that they can be immediately introduced, at virtually any concentration, into any cell that can be microinjected. This makes it possible to observe behavior of the myosin II in a wide variety of conditions, and before significant compensatory gene expression is likely to occur. Furthermore, the wide choice of fluorophores available for labeling makes it possible to observe multiple analogues in the same cell. This permits comparisons and correlations between different myosins or between myosin and other cytoskeletal proteins. Finally, green fluorescent protein is very large (M_r = 27,000–30,000) compared to most fluorescent dyes, and thus poses a much greater danger of sterically hindering intermolecular interactions. This is of particular concern in the highly organized,

tightly packed structures that make up the cytoskeleton.

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