Gradients in the Concentration and Assembly of Myosin II in Living Fibroblasts During Locomotion and Fiber Transport

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Assembly and motor activity of myosin II affect shape, contractility, and locomotion of nonmuscle cells. We used fluorescent analogues and imaging techniques to elucidate the state of assembly and three-dimensional distribution of myosin II in living Swiss 3T3 fibroblasts. An analogue of myosin II that was covalently cross-linked in the 10S conformation and unable to assemble served as an indicator of the cytoplasmic volume accessible to 10S myosin II. Ratio-imaging of an analogue that can undergo 10S $\rightarrow$ 6S conversion versus the volume indicator revealed localized concentration of assembly-competent myosin II. In stationary serum-deprived cells and in cells locomoting at the edge of a wound, it was most concentrated in the peripheral cytoplasm, where fibers containing myosin II assemble, and least concentrated in the perinuclear cytoplasm, where they disassemble. Furthermore, fluorescence photobleaching recovery showed myosin II to be less mobile in the periphery than in perinuclear cytoplasm. These results indicate a gradient in the assembly of myosin II. Three-dimensional microscopy of living cells revealed that fibers containing myosin II were localized in the cortical cytoplasm, whereas myosin II was diffusely distributed in the deeper cytoplasm, suggesting that myosin II is assembled preferentially near the cell surface. Localized protein phosphorylation may play a role, because a kinase inhibitor, staurosporine, abolished the gradient of myosin II assembly.

INTRODUCTION

In muscle, actin and myosin II form the force-generating machinery that drives contraction. Nonmuscle myosin II also forms contractile structures, and there is considerable evidence that myosin II-based contractility affects movement and shape change in nonmuscle cells. During cytokinesis, myosin II is the motor in the contracting array of actin-myosin II that divides the cell. In many cultured cells, actin and myosin II organize into contractile stress fibers. Myosin II also appears to localize, albeit with less clearly organized structure, in the contractile tails of locomoting cells such as amoebae, fibroblasts, and leukocytes, whereas myosin I shows a slight bias toward the leading edge (Fukui *et al.*, 1989; Conrad *et al.*, 1993). Furthermore, although cells that have been made myosin II-deficient by genetic engineering or antibody injection are still capable of locomotion, they display distorted locomotive morphologies (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Höner

et al., 1988), reduced rates of translocation (Wessels *et al.*, 1988), and reduced speed and directionality of intracellular particle movement (Wessels and Soll, 1990). This suggests possible roles for myosin II in polarizing cell shape, maintaining directional locomotion, maximizing the rate of locomotion, and/or facilitating the delivery of components of the contractile cytoskeleton to the leading edge (Spudich, 1989; Wessels and Soll, 1990; Conrad *et al.*, 1993).

In all these instances, structures containing actin and myosin II must be assembled and disassembled at specific locations in the cell at the appropriate times. To understand the regulation of these events and the consequences for cell shape and motility, we have been studying the distribution and dynamics of actin and myosin II in Swiss 3T3 fibroblasts. These cells display two types of behavior that are particularly useful models of cytoskeletal dynamics. The first occurs when 3T3 fibroblasts are made quiescent by serum-deprivation. In this condition, the cells stop translocating and develop an extensive array

of dynamic stress fibers. The stress fibers form constitutively at the periphery of the cell, transport centripetally through the cytoplasm, and appear to disassemble in the perinuclear region (Giuliano and Taylor, 1990). In addition, the fibers can be induced to contract by stimulating cells with complete serum or purified growth factors (Giuliano and Taylor, 1990) or by solating the actin-based cytoplasmic gel with cytochalasin (Kolega *et al.*, 1991). Thus, serum-deprived fibroblasts provide an excellent venue for examining assembly, transport, contraction, and disassembly of actin-myosin II structures in a stationary cell that exhibits little shape change. The second behavior we have investigated is polarized locomotion. Fibroblasts cease locomotion when grown to confluence but will immediately resume movement when the confluent sheet is disrupted. By "wounding" a monolayer of cells, one obtains a large number of cells that undergo uniformly polarized and relatively synchronized locomotion (DeBiasio *et al.*, 1988; Fisher *et al.*, 1988; Conrad *et al.*, 1989). In the course of wound healing, fibroblasts reorganize actin and myosin II, displaying discrete regions (and timing) of assembly, transport, contraction, and disassembly of supramolecular actin-myosin II structures.

It is not known how the assembly of structures containing myosin II is controlled in the living cell. Extensive studies of myosin II assembly in vitro (see recent reviews by Sellers, 1991; Trybus, 1991; Tan *et al.*, 1992) have shown that monomeric myosin has two forms: the unfolded, or 6S, configuration and the more compact, or 10S, configuration. In filaments, myosin II is unfolded, and it is believed that control of the 10S $\rightarrow$ 6S transition plays an important role in regulating the assembly of smooth-muscle and nonmuscle myosin II. Treatments that cause these myosins to assemble, such as phosphorylation of the 20-kDa regulatory light chains, cause the myosin II to unfold, and myosin II that has been cross-linked in the 10S configuration does not assemble (Trybus and Lowey, 1984). We expect that assembly and disassembly of the cytoskeleton releases myosin II subunits in vivo and predict that unassembled myosin II diffuses through the cytoplasm in the 10S monomeric form.

In cells, the restructuring of actin-myosin II assemblies is complicated by their contractility. When the actin-based cytoplasmic gel is partially solated by cytochalasin without increasing myosin II phosphorylation, stress fibers contract (Kolega *et al.*, 1991; Janson *et al.*, 1991; Giuliano *et al.*, 1992). This suggests that regulating the gel structure of the actin-based cytoskeleton can modulate contractility. On the other hand, stress fiber contraction is also regulated through the contractile motor, for example, via phosphorylation of the regulatory light chains of myosin II (Giuliano *et al.*, 1992). These and other studies (Janson *et al.*, 1992) have demonstrated that contraction in nonmuscle cells is under dual regulation: a balance between motor force and gel structure (the solation-contraction coupling hypothesis) (reviewed

by Taylor and Fechheimer, 1982). As a consequence, there appear to be at least two distinct pathways for restructuring actin-myosin II assemblies. One pathway does not involve contraction but a disassembly of fibers and possibly disassembly of the constituent actin and myosin II filaments. Examples of this process include the constitutive formation, transport and disassembly of stress fibers in serum-deprived fibroblasts (Giuliano and Taylor, 1990), and the disassembly of stress fibers in the transition between interphase and prophase during the cell cycle (Sanger *et al.*, 1989). A second pathway involves disassembly of actin and myosin II fibers in conjunction with contraction of the actin-myosin II structure. Examples of this process include cytokinesis (Kitanishi-Yumura and Fukui, 1989; Sanger *et al.*, 1989), serum and growth factor stimulation of quiescent cells (Giuliano and Taylor, 1990; Giuliano *et al.*, 1992), shape changes during wound healing by fibroblasts (DeBiasio *et al.*, 1988; Fisher *et al.*, 1988; Conrad *et al.*, 1993), and tail retraction during amoeboid movement (Taylor and Condeelis, 1979).

Fluorescent analogue cytochemistry makes it possible to examine the dynamics of proteins in living cells (reviewed by Taylor *et al.*, 1986; Wang, 1989). To monitor myosin II in nonmuscle cells, analogues have been prepared using myosin II from smooth muscle, because it is readily obtained in large quantities. In vitro, myosin II from skeletal, smooth and nonmuscle will coassemble to yield functional filaments (Pollard, 1975; Citi *et al.*, 1987). Smooth muscle myosin II has been compared with nonmuscle myosin II from a wide range of tissues and species, and, among vertebrates, they are extremely similar. They have identical solubility properties and enzymatic activities, undergo the same conformational transitions, have their assembly and ATPase regulated via phosphorylation by myosin light chain kinase, and have highly conserved sequences around the phosphorylation sites on their regulatory light chains (see reviews by Sellers, 1991; Trybus, 1991; Tan *et al.*, 1992). Although some differences have been observed in phosphorylation of smooth and nonmuscle myosin II by protein kinase C in vitro (Conti *et al.*, 1990; Ikebe and Reardon, 1990), when fluorescent analogs of chicken gizzard smooth muscle myosin II are injected into 3T3 mouse fibroblasts, they display the identical distribution as the endogenous, nonmuscle myosin II revealed by immunofluorescence localization (McKenna *et al.*, 1989; Kolega *et al.*, 1993).

Here we use fluorescent analogues of smooth muscle myosin II to examine the state of assembly of myosin II in living fibroblasts. Fluorescence photobleaching recovery (FPR)¹ and ratio imaging reveal the molecular

¹ Abbreviations used: AF-10S myosin II, iodoacetamidofluorescein analogue of cross-linked 10S myosin II; AR-myosin II, acetamidotramethylrhodamine analogue of native myosin II; FPR, fluorescence photobleach recovery; VEC-DIC, video-enhanced contrast, differential-interference contrast.

dynamics and local concentration of myosin II, which, in turn, indicate where myosin II is assembled and disassembled in the cytoplasm. We also define the distribution of myosin II in three dimensions in living fibroblasts. We report spatial gradients in the assembly and relative concentration of myosin II both in the serum-deprived state and during polarized locomotion. These gradients identify particular regions of the cell where important events regulating myosin II behavior must occur.

MATERIALS AND METHODS

Reagents

Culture media and calf serum were purchased from GIBCO/BRL (Grand Island, NY), 5-iodoacetamidofluorescein from Molecular Probes (Eugene, OR), ethyl 1-3-(3-dimethylaminopropyl)carbodiimide from Pierce (Rockford, IL), and staurosporine from Calbiochem (LaJolla, CA). All other chemicals were from Sigma Chemical (St. Louis, MO) unless otherwise indicated. Ultrafiltration was performed using Centrprep and Centricon filters (Amicon, Beverly, MA) with a molecular weight cutoff of 30 kDa.

The acetamido-tetramethylrhodamine analogue of chicken gizzard smooth muscle myosin II (AR-myosin II) was prepared as described by DeBiasio *et al.* (1988). The AR-myosin II analogue is indistinguishable from native myosin II in vitro in terms of its solubility in physiological salt solutions, actin-activated ATPase, phosphorylation by myosin light chain kinase, and ability to assemble into filaments and undergo 6S $\leftrightarrow$ 10S conformational changes (Hahn *et al.*, 1993).

Fluoresceinated Cross-linked 10S Myosin II

Smooth muscle myosin II was isolated from chicken gizzard as described by Sellers *et al.* (1991) with minor variations. Purified myosin II was cross-linked in the 10S configuration as described by Trybus *et al.* (1982) but using ethyl 1-3-(3-dimethylaminopropyl)carbodiimide as the cross-linker (Trybus and Lowey, 1988). Briefly, myosin II was dialyzed into 0.15 M KCl, 5 mM MgCl₂, 5 mM ATP, 1 mM ethylene glycol-bis(β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), 20 mM potassium phosphate, pH 7.0 and clarified by centrifugation for 10 min at $10\,000 \times g$. Myosin II concentration was adjusted to 2 mg/ml, and 21 mM 1-3-(3-dimethylaminopropyl)carbodiimide was added from a freshly prepared 10X aqueous stock solution to a final volume of 9 ml. After 3 h at 4°C, the reaction was stopped by adding glycine to 0.1 M, and the myosin II was concentrated to 16 mg/ml by ultrafiltration and dialyzed into 0.6 M KCl, 5 mM MgCl₂, 1 mM EGTA, 20 mM potassium phosphate, pH 7.5. The cross-linked 10S myosin was separated from 6S myosin by chromatography over a 2×100 cm Sepharose CL-4B column. Fractions eluting at the location of 10S myosin II were pooled and concentrated to 6 mg/ml by ultrafiltration for subsequent labeling. The locations of the 10S and 6S myosin II peaks were previously determined by chromatography of native myosin II in 5 mM MgCl₂, 1 mM EGTA, 20 mM potassium phosphate, pH 7.5 containing 0.15 M KCl and 5 mM ATP or 0.6 M KCl without ATP, respectively (Figure 1).

Monomeric cross-linked 10S myosin II was fluorescently labeled by adding a 20-fold molar excess of 5'-iodoacetamidofluorescein from a freshly prepared 1 mg/ml stock solution in dimethyl formamide. The reaction was stopped after 18 h at 4°C by addition of 2 mM dithiothreitol, and the labeled protein was separated from free dye by chromatography on a 1×5 cm Sephadex G50-50 column. The molar ratio of dye to protein in the fluoresceinated, cross-linked 10S myosin II was 0.2. The final product was dialyzed into 150 mM KCl, 2 mM N-2-hydroxyethylpiperazine-N'-ethanesulfonic acid (HEPES), pH 7.0, 1 mM dithiothreitol, and concentrated to 8 mg/ml by ultra-

filtration. Small aliquots were stored at -80°C and thawed immediately before use.

Cells

Swiss 3T3 murine fibroblasts (CCL92) from American Type Culture Collection (Rockville, MD) were used between passages 118 and 128. Cells were maintained in Dulbecco's minimal essential medium (DME) supplemented with 10% calf serum. For wound-healing experiments, cells were grown to confluence and used within 24 h of reaching confluence. The monolayer was wounded by scraping away cells with a razor blade as described by DeBiasio *et al.* (1988). Experiments with serum-deprived cells were carried out at low cell densities, such that there were few contacts between individual cells. The cells were serum-deprived by reducing serum to 0.2% for 48 h (Giuliano and Taylor, 1990).

Microinjection

Cells were microinjected with fluorescent analogues as described by Amato *et al.* (1983). The analogues were injected in the unphosphorylated state and diffused throughout the accessible volume of the cell in <15 min. Needle concentrations of both analogues were 1–2 mg/ml, and the injected volume was 2–5% of the cell volume. Thus, the final intracellular concentration of each analog was ≤ 0.1 mg/ml or $\leq 10\%$ of the endogenous myosin II, assuming a concentration of 1 mg/ml in fibroblast cytoplasm (Clark and Spudich, 1977; Höner *et al.*, 1988). Because the properties of myosin II copolymers are dominated by the species at highest concentration (Pollard, 1975; Citi *et al.*, 1987), the 10-fold excess of endogenous myosin II over the fluorescent analogue further minimizes any effects that might arise from the use of a heterologous myosin II analogue.

FPR

Measurements of FPR were made by the Gaussian spot method of Axelrod *et al.* (1976) as described by Pagliaro and Taylor (1988). Diffusion coefficients were determined by fitting recovery curves using the method of Yguerabide *et al.* (1982), and percentage mobile fraction was calculated as described by Axelrod *et al.* (1976). Measurements in cells were made using a $100\times$ Zeiss Plan oil-immersion objective (Thornwood, NY) and spot radii of 1 to 3 μm . It was important to use a small spot size so that the characteristic recovery time for diffusion, τ_D , was much faster than recovery because of flow, τ_f . Assuming a flow rate of 20 $\mu\text{m}/\text{min}$ (retrograde fiber transport occurs at an average rate of 15 $\mu\text{m}/\text{min}$ in wound-healing cells and only 0.17 $\mu\text{m}/\text{min}$ in serum-deprived fibroblasts) (Fisher *et al.*, 1988; Giuliano and Taylor, 1990), $\tau_f/\tau_D = 6$ for a 10^{-8} cm²/sec diffusion coefficient and a 2 μm spot. When $\tau_f/\tau_D > 2$, the contributions of flow are not distinguishable above diffusion when fitting recovery curves by the method of Yguerabide *et al.* (1982). Temperature was maintained at 37°C with an air curtain regulated by feedback from a thermistor placed adjacent to the field of view on the specimen. Bleaching times of 1–5 ms were used to give 15–30% bleaching.

Image Acquisition

Fluorescence microscopy and image acquisition were performed on a BDS Multimode microscope (Biological Detection Systems, Pittsburgh, PA) (Giuliano *et al.*, 1990; Taylor *et al.*, 1992; Farkas *et al.*, 1993). All images for analysis were acquired using a cooled CCD camera (model CH250, Photometrics, Tucson, AZ) as 12-bit, 512×384 pixel arrays. Cells were prepared for observation as described previously (Giuliano *et al.*, 1990; Kolega *et al.*, 1991) except during perfusion experiments, when they were observed in an open chamber to facilitate exchange of media. In open chamber experiments, cells were transferred to DME buffered with 25 mM HEPES, pH 7.4, without bicarbonate, immediately before observation. For short periods (<3 h) cell behavior under these conditions was indistinguishable from that observed in

bicarbonate-buffered DME in closed chambers. All images were acquired using a 1.3 NA 100X Plan-NEOFLUAR objective (Zeiss).

Image Processing

Images were processed using BDS-Image software (Biological Detection Systems) on Macintosh personal computers (Apple Computers, Cupertino, CA).

"Optical sectioning" was performed by acquiring a through focus series of 20–30 images for each cell. The highest and lowest image planes were chosen so as to include at least one or two planes above and below the full thickness of the cell, with a z-axis spacing of 0.23 μm between image planes. Out-of-plane fluorescence was then removed using the nearest-neighbor deconvolution method (Agard, 1984). Three-dimensional (3-D) reconstructions and stereo-images were made from the deconvolved image sets using BDS-Render (Biological Detection Systems).

Ratio images were generated by division of registered, background-subtracted images as previously described (Tanasugarn *et al.*, 1984; DeBiasio *et al.*, 1988; Bright *et al.*, 1989). All images that were used for ratios had maximum intensities that fell within the linear range of the cooled CCD camera. The numerator for our ratio measurements was AR-myosin II fluorescence, representing the distribution of total myosin II in the cytoplasm and including both assembled and un-assembled myosin II species. The denominator was the fluorescence of AF-10S myosin II, which does not assemble. It should be noted that this denominator is proportional to the volume accessible to 10S myosin II not the volume of the cell. Consequently, the ratio is not a measure of the absolute concentration (myosin II per unit volume of cytoplasm) but of the relative concentration of the assembly-competent myosin II (in all its forms) compared to its unassembled counterpart (i.e., myosin II per unit volume of cytoplasm accessible to myosin II). Within the volume accessible to myosin II, the ratio is always nonzero, as both analogues are present. The ratio will be zero only where there is no detectable AR-myosin II. Where AR-myosin II is present, the ratio will be at a minimum where there is no local enrichment of the assembly-competent AR-myosin II, i.e., where there is no assembly of myosin II. We will operationally define the regions with the highest ratio of assembly-competent myosin II analogue to assembly-incompetent myosin II analogue as being the regions of highest assembly of myosin II.

The images used as illustrations in this paper were linearly scaled in order to display the full dynamic range of the data but were not otherwise processed. To obtain black-and-white photomicrographs, digitized images were photographed with conventional 35 mm film using a Slidewriter Film Recorder and Conductor software (Agfa Matrix Division, Orangeburg, NY). Color images were printed directly using a Kodak SV6510 video printer (Eastman Kodak, Rochester, NY).

RESULTS

Fluorescently Labeled Chemically Cross-Linked 10S Myosin II Is a Volume Reference for Myosin II Assembly and Binding

In both serum-deprived and locomoting fibroblasts, fibers containing myosin II appear at the edge of the cell, move centripetally, and apparently disassemble in the perinuclear region. Localized assembly and disassembly would be expected to create gradients in myosin II concentration within the cell. Unassembled unbound 10S myosin II would distribute uniformly in the accessible space, whereas assembly and/or local binding would enrich myosin II at the sites of assembly. Although a two-dimensional image of AR-myosin II provides a good map of myosin II distribution, the intensity in this image does not indicate the relative concentration of myosin

II. To obtain a measure of relative myosin II concentration, one must compensate for variations in cell thickness and for partial exclusion of myosin II from some regions of the cell (Tanasugarn *et al.*, 1984; DeBiasio *et al.*, 1988; Bright *et al.*, 1989; Luby-Phelps *et al.*, 1986). Dividing the AR-myosin II image by an image representing the volume accessible to 10S myosin II corrects for both of these factors (Tanasugarn *et al.*, 1984).

An ideal volume indicator for myosin II is the folded, 10S form of myosin II itself. Therefore, we prepared an analogue of myosin II that was covalently cross-linked in the folded 10S configuration and labeled with iodoacetamidofluorescein (see MATERIALS AND METHODS). To demonstrate that the analogue was cross-linked, we assayed its configuration by size ex-

Figure 1. Size-exclusion chromatography of native and AF-10S myosin II. Chicken gizzard smooth muscle myosin II and AF-10S myosin II were chromatographed separately over CL-4B Sepharose under conditions that favor either the 10S or 6S conformation. (a) In low salt in the presence of ATP (0.15 M KCl, 5 mM ATP, 5 mM MgCl_2 , 1 mM EGTA, 20 mM potassium phosphate, pH 7.5), conditions that cause myosin to fold in the 10S conformation, both native myosin II (solid line) and its cross-linked fluorescent analogue, AF-10S myosin II (dashed line), migrate at the same location. (b) In high salt without ATP (0.6 M KCl, 5 mM MgCl_2 , 1 mM EGTA, 20 mM potassium phosphate, pH 7.5), which causes native myosin II to unfold to the less compact 6S conformation, native myosin II migrates more rapidly. AF-10S myosin II, however, continues to migrate at the location of 10S myosin II.

Figure 2. Ratio-imaging of myosin II concentration in serum-deprived fibroblasts. Serum-deprived fibroblasts were microinjected with a mixture of AR-myosin II and AF-10S myosin II and the analogues allowed to equilibrate in the cytoplasm for ≥ 2 h. Fluorescence images of AR-myosin II (a) and AF-10S myosin II (b) were acquired in rapid succession (< 6 s apart), and then ratio images (c-f) were generated by dividing the AR-myosin II image by the AF-10S myosin II image. a-c show the AR-myosin II, AF-10S myosin II, and ratio images, respectively, from the same cell, and d-f show additional examples of ratio images of serum-deprived cells. AR-myosin II incorporates into stress fibers, whereas AF-10S myosin II does not. The images a and b have been printed so that the distribution of the fluorescent analogues in the thin peripheral cytoplasm is visible. Consequently, the perinuclear fluorescence appears overexposed. In both images, the most intense fluorescence is in the cytoplasm surrounding the nucleus (N), where the cell is thickest. The ratio images (c-f) were linearly scaled with the highest ratio value displayed as white and zero displayed as black. Note that the ratio is truly zero only where there is no AR-myosin II present in image (a). In contrast to the images of the fluorescent analogues, the intensity of the ratio image (c) is very low in the perinuclear region. The highest intensities are observed in the stress fibers in the periphery of the cell. In addition, many more stress fibers are visible, and they stand out more sharply against the interfiber cytoplasm. Bar, 10 μ m.

clusion chromatography. As shown in Figure 1, the fluoresceinated cross-linked myosin II (hereafter referred to as AF-10S myosin II) eluted in the same volume as native myosin II and AR-myosin II when chromatographed in 0.15 M KCl/5 mM ATP, which keeps myosin II in the folded 10S configuration. When chromatographed in 0.6 M KCl, native myosin II and AR-myosin II unfolded to the more rapidly eluting 6S configuration, but all the AF-10S myosin II continued to elute at the location of 10S myosin II. In addition, AF-10S myosin II remained soluble in 0.15 M KCl in the absence of ATP, conditions under which native myosin II and AR-

myosin II unfold and form sedimentable filaments. Thus, AF-10S myosin II appeared to be cross-linked in the 10S form and consequently unable to unfold and assemble into filaments.

When AF-10S myosin II was microinjected into living cells, it became diffusely distributed throughout the cytoplasm (Figure 2b). Like AR-myosin II, it was excluded from the nucleus and from small, presumably newly formed, protrusions (DeBiasio *et al.*, 1988). But unlike AR-myosin II, it did not become incorporated into fibers. We confirmed that the microinjected AF-10S myosin II remained free by FPR. AF-10S myosin II would be pre-

dicted to exhibit a single diffusion coefficient and 100% mobility, because it should not unfold to the 6S species, assemble into filaments, and/or bind to actin. When AF-10S myosin II was photobleached, we observed complete recovery of fluorescence in spots bleached in either the perinuclear or peripheral cytoplasm (Table 1). This indicated that the AF-10S myosin II remained soluble throughout the cytoplasm. In addition, the recovery curves could be described by single exponential curves with very good fit ($\chi^2 < 0.005$), implying a single mobile species. A slight difference was observed between the diffusion coefficients in the perinuclear and peripheral cytoplasm. This may reflect local variations in the structure of the cytoplasmic matrix that restrict diffusion of very large molecules (Luby-Phelps *et al.*, 1986, 1987; Luby-Phelps and Taylor, 1988).

Myosin II Exhibits a Gradient in Concentration that Is Highest at the Periphery and Lowest in the Perinuclear Region in Serum-deprived Cells

Because AF-10S myosin II is the same size as myosin II in its folded configuration, it distributes in the volume accessible to 10S myosin II. The assembly-competent AR-myosin II distributes in this same space but can also assemble into structures containing myosin II. Therefore, the ratio of AR-myosin II:AF-10S myosin II is a measure of how much the assembly-competent myosin II is concentrated in any particular region of the cell relative to myosin II that does not assemble. If myosin II assembly is heterogeneous within the cell, the ratio will be higher where myosin II has been localized or stabilized by assembly and lower where myosin II is unassembled. This provides a means for observing spatial variation in the state of assembly across the cell.

Figure 2 illustrates ratio imaging of AR-myosin II:AF-10S myosin II for a typical serum-deprived fibroblast. In the AR-myosin II image (Figure 2a), the highest intensities were associated with stress fibers in the pe-

riphery of the cell and with the diffuse perinuclear fluorescence. Identical distribution is observed when endogenous myosin II is localized by immunofluorescence (Kolega *et al.*, 1993; see also Conrad *et al.*, 1989, 1993; McKenna *et al.*, 1989). The volume indicator, AF-10S myosin II, was also most intense in the perinuclear region (Figure 2b), reflecting the greater thickness of the cell in this region. When the AR-myosin II image was divided by the AF-10S myosin II image, the perinuclear region consistently showed the lowest intensity in the ratio image. This indicates that the strong perinuclear fluorescence in the AR-myosin II image was because of cell thickness and not to specific localization of AR-myosin II in this region. In contrast, the intensity of the ratio image was highest in the stress fibers in the peripheral cytoplasm. In other words, in terms of the volume accessible to myosin II, there was much more AR-myosin II in stress fibers than in an equal accessible volume of perinuclear cytoplasm. To obtain an indication of the degree of enrichment of assembly-competent myosin II in stress fibers relative to the perinuclear cytoplasm, we compared the ratio intensity measured on the brightest stress fibers with the intensity measured in the perinuclear cytoplasm for 34 different cells. The former was higher than the latter by 2.8 ± 1.0 fold (mean $\pm$ SD). The ratio intensity decreased in the space between stress fibers in the peripheral cytoplasm but was not as low as in the perinuclear cytoplasm (Figure 2c). This suggests that the sparse, isotropic, actin-based gel between fibers contains some assembled myosin II but less than that of the dense, actin-filament bundles in stress fibers. Figure 2, d-f shows additional examples of serum-deprived fibroblasts after ratio imaging of AR-myosin II:AF-10S myosin II.

Myosin II Is More Assembled in the Cell Periphery than in the Perinuclear Region

Myosin II molecules can become concentrated in particular regions of the cytoplasm if they assemble into

Table 1. Mobility of Myosin II Analogues in 3T3 Fibroblasts

	% mobile ¹		Diffusion coefficient ¹ ($\times 10^{-9}$ cm ² /s)	
	Perinuclear	Edge	Perinuclear	Edge
AF-10S myosin II, serum-deprived cells	100 $\pm$ 12 (42)	99 $\pm$ 11 (42)	8.0 $\pm$ 2.2 (42)	6.5 $\pm$ 2.2 (42)
AR-myosin II, serum-deprived cells	97 $\pm$ 10 (30)	65 $\pm$ 24 (30)	9.4 $\pm$ 2.7 (30)	4.9 $\pm$ 2.4 (30)
AR-myosin II, serum-deprived cells, 20 nM staurosporine	97 $\pm$ 14 (35)	92 $\pm$ 14 (35)	9.9 $\pm$ 2.7 (35)	8.1 $\pm$ 2.9 (35)
AR-myosin II, wound-healing cells	96 $\pm$ 14 (43)	54 $\pm$ 30 (43)	13.2 $\pm$ 4.3 (43)	6.8 $\pm$ 3.8 (40)

¹ Mean $\pm$ SD; number of observations is given in parentheses.

Figure 3. Myosin II mobility in serum-deprived, staurosporine-treated, and wound-healing fibroblasts. AR-myosin II was microinjected into serum-deprived fibroblasts and allowed to equilibrate for ≥ 2 h. The size of the mobile fraction of myosin II was then determined by FPR as described in MATERIALS AND METHODS. Two spots were measured in each cell as indicated in d: one positioned within $5 \mu\text{m}$ of the nucleus ("perinuclear"), the other within $5 \mu\text{m}$ of the edge of the cell ("periphery"). The two measurements were made on the same side of the cell, and the order of photobleaching (i.e., whether the edge or perinuclear spot was measured first) was alternated with each cell. All measurements were made at 37°C . a displays the distribution of the percentage of mobile myosin II observed in serum-deprived fibroblasts in a typical experiment. A smaller percentage of myosin II is mobile, and the size of the mobile fraction is much more variable near the edge of the cell. (b) Serum-deprived fibroblasts were treated with 20 nM staurosporine for 20 min before measuring myosin II mobility. FPR measurements were then made over a period of 20 min in the continued presence of staurosporine. Myosin II mobility near the cell edges is shifted toward 100%, and the variability in the percentage mobile fraction is decreased. (c) Myosin II mobility was measured in migrating cells at the margin of a wounded monolayer ~ 6 h after wounding. FPR measurements were made in the perinuclear cytoplasm and near the leading edge of the cell as indicated in d. As in serum-deprived cells, more myosin II is mobile in the perinuclear region. The size of the mobile fraction is both smaller and more variable near the leading edge of the cell.

filaments and/or bind to localized cellular components such as actin filaments. Either myosin II assembly or binding to actin filaments would lead to an enrichment of assembly-competent myosin II over the assembly-incompetent AF-10S myosin II. On the basis of the concentration gradient revealed by ratio imaging, we predicted that myosin II in the perinuclear region would be less assembled than in the peripheral cytoplasm. The more diffuse distribution of myosin II in perinuclear cytoplasm suggested that it was no longer organized in large fibers. However, myosin II could remain assembled in very fine filaments that are not resolved by fluorescence imaging. In vitro, actin and myosin II can form isotropic gels, the structure of which is not resolvable in the standard light microscope (Janson *et al.*, 1991), and electron microscopy suggests that some fraction of

the cytoplasmic actin is in just such a fine filamentous gel (Buckley and Porter, 1967; Small and Celis, 1978; Heuser and Kirschner, 1980). To distinguish between reorganization of myosin II into finer filaments and actual disassembly of myosin II, we examined the mobility of myosin II by fluorescence photobleaching recovery.

As shown in Figure 3 and Table 1, myosin II in the perinuclear cytoplasm was virtually 100% mobile. This indicated that the majority of the diffuse myosin II was not assembled into any large structures such as fibers or gels. The diffusion coefficient provides an estimate of the maximum size of the mobile myosin II structures. Upon comparing the diffusion coefficient in the perinuclear region for AR-myosin II with that of AF-10S myosin II, we found no significant difference (Table 1). Because AF-10S myosin II cannot assemble, this sug-

gested that the mobile fraction of AR-myosin II in the perinuclear cytoplasm consisted predominantly of 10S monomers, although it could include small filaments no larger than a few myosin II molecules.

Myosin II mobility was quite different in the peripheral cytoplasm. An average of only 65% of AR-myosin II was mobile. The size of the mobile fraction in the peripheral cytoplasm was much more variable than in the perinuclear cytoplasm, ranging from 10 to 100%. This may be because of greater spatial heterogeneity in myosin II structure in the periphery of the cell. Ratio images showed that the relative concentration of myosin II was also variable in the peripheral cytoplasm and could change sharply over very small distances. If localized concentration of myosin II was because of assembly, this would be consistent with the highly variable mobile fraction in the peripheral cytoplasm.

The diffusion coefficient for the mobile fraction of AR-myosin II was significantly lower in the peripheral cytoplasm than in the perinuclear cytoplasm (Table 1). This may be partly because of differences in the cytoplasmic matrix as noted above for AF-10S myosin II. However, the diffusion coefficient for AR-myosin II in the peripheral cytoplasm was also much lower than that of AF-10S myosin II in the same region. This could be explained if AR-myosin II unfolds to the larger, more slowly diffusing 6S configuration in the cell periphery. Alternatively, it might undergo transient binding to other molecules or structures, such as actin filaments, slowing its diffusion relative to AF-10S myosin II.

Staurosporine Disrupts the Gradients of Myosin II Assembly and Concentration

When 3T3 fibroblasts are treated with the kinase inhibitor, staurosporine, they lose their stress fibers, actin becomes diffusely distributed in the cytoplasm (Hedberg *et al.*, 1990), and the regulatory light chains of myosin II become dephosphorylated (Giuliano *et al.* 1992). We expected that this disruption of the actin cytoskeleton might be accompanied by disassembly of myosin II, and thereby provide a test of our measurements of assembly and relative concentration. Treatment of serum-deprived fibroblasts for 30 min with 20 nM staurosporine caused a loss of myosin II-containing stress fibers and the dispersal of myosin II to a uniform distribution throughout most of the cytoplasm (Figure 4, a and b). FPR measurements demonstrated that in the presence of 20 nM staurosporine the mobility of myosin II in the periphery of the cell increased to >90% and was no longer significantly different from the mobility of myosin II in the perinuclear cytoplasm (Table 1). When we examined relative myosin II concentration in staurosporine-treated cells by ratio imaging, we found that the spatial heterogeneity was almost completely lost (Figure 4, c–f). The relative concentration of myosin II became uniform throughout most of the cytoplasm, al-

though a slightly higher concentration in the periphery of the cell persisted. We have previously shown that this treatment of Swiss 3T3 fibroblasts causes dephosphorylation of the endogenous myosin II (Giuliano *et al.*, 1992). Interestingly, a small, but reproducible, difference also persisted between the diffusion coefficient measured by FPR in the peripheral cytoplasm and that in the perinuclear cytoplasm. Thus, myosin II may still undergo some transient binding or assembly in the most peripheral cytoplasm in the presence of staurosporine.

Myosin II Is More Assembled Near the Leading Edge of Migrating Cells and Less Assembled in the Perinuclear Region

As fibroblasts begin to migrate at the edge of a wound, actin becomes enriched in extending lamellipodia and assembles into numerous transverse fibers beginning at the base of lamellipodia in the anterior portion of the cell (DeBiasio *et al.*, 1988; Conrad *et al.*, 1989, 1993). Myosin II is initially excluded from newly formed lamellipodia but is found at the base of lamellipodia (DeBiasio *et al.*, 1988). Therefore, we expected that myosin II would be assembled into the transverse fibers beginning at the base of lamellipodia in migrating cells. In addition, myosin II would be expected to be disassembled in the perinuclear region, where despite the retrograde movement of transverse fibers from the anterior cytoplasm (Conrad *et al.*, 1993), fibers are less numerous. Populations of uniformly polarized, locomoting fibroblasts were generated by wounding confluent monolayers of cells (DeBiasio *et al.*, 1988). Cells at the edge of the wound were microinjected with AR-myosin II and AF-10S myosin II and images acquired at various times after wounding.

By 5–7 h after wounding, most cells were elongated perpendicular to the wound edge and undergoing lateral contraction. Large lamellar protrusions extended into the wound surface from the front of the cell, and the nucleus remained in the rear. This corresponds to the "lamellar contraction phase" defined by Conrad *et al.* (1993). Ratio imaging indicated that assembly-competent myosin II was heavily concentrated in the transverse fibers in the anterior half of the cell and was lowest in the perinuclear cytoplasm (Figure 5). The steepness of this concentration gradient was more pronounced in migrating cells than in serum-deprived cells, as the ratio in anterior stress fibers was higher than that in the perinuclear cytoplasm by 5.0 ± 2.2 -fold (mean $\pm$ SD, $n = 20$). The mobility of myosin II was also quite different in these two regions. Myosin II was >90% mobile in the perinuclear cytoplasm, whereas only one-half of the myosin II was mobile in the leading edge (Figure 3 and Table 1).

Fibroblasts become polarized during the lamellar contraction phase of wound-healing primarily by extension of their leading edges with little, if any, trans-

Figure 4. Disruption of stress fibers and concentration gradients by staurosporine. Serum-deprived fibroblasts were microinjected with a mixture of AR-myosin II and AF-10S myosin II. a and b depict the AR-myosin II distribution in a typical cell immediately before (a) and 30 min after (b) perfusion of 20 nM staurosporine. Numerous stress fibers are visible in the peripheral cytoplasm before treatment (a), but stress fibers are completely disrupted and myosin II becomes diffusely distributed in the presence of staurosporine (b). The cell also displays a dramatic increase in protrusive activity resulting in its spreading toward the top of the field. c and d show the ratio-images (AR-myosin II divided by AF-10S myosin II) for a and b, respectively. The two images are presented with the same scaling, contrast, and exposures. A steep gradient between very bright stress fibers and very dark perinuclear cytoplasm can be seen in c, whereas the ratio is a more uniform gray after staurosporine treatment (d). Note the loss of fibrous structures, as well as the increased ratio in the perinuclear region. e and f are ratio images of a cell that underwent a similar loss of gradient and stress fibers but with less extensive changes in cell shape. Bar, 10 μ m.

Figure 5. Ratio-imaging of myosin II concentration in wound-healing fibroblasts. Fibroblasts migrating at the edge of a wound (a–d) or located within an unwounded monolayer (e and f) were microinjected with a mixture of AR-myosin II and AF-10S myosin II, and the analogues allowed to equilibrate in the cytoplasm for ≥ 2 h. Between 5 and 7 h after wounding, fluorescence images of AR-myosin II (a) and AF-10S myosin II (b) were acquired in rapid succession (< 6 s apart), then ratio images (c–f) were generated by dividing the AR-myosin II image by the AF-10S myosin II image. a–c show the AR-myosin II, AF-10S myosin II, and ratio images, respectively, from the same cell. As for serum-deprived cells, fibrous structures are visible in the AR-myosin II image (a) but not with AF-10S myosin II (b), and both analogues display the most intense fluorescence in the cytoplasm around the nuclei, where the cell is thickest. The ratio image (c) reveals a strong gradient in the relative concentration of myosin II between the perinuclear region and the leading edge. This gradient is highly polarized in the direction of cell migration (the cells in a–d are all migrating toward the left of the field). d–f compares the ratio images of wound-healing cells (d) with that of cells in an unwounded monolayer (e and f). The latter display little structure, and there is no directionality to the heterogeneities that are observed. The ratio images are all presented with the same scaling, contrast, and exposures. Bar, 10 μ m.

location of the cell body. To observe actual migration, we also examined cells at later stages after wounding. Ratio images of 43 cells at the wound edge were acquired between 8 and 10 h after wounding, and the locomotive behavior of these cells was recorded by time-lapse imaging using video-enhanced contrast, differential-interference contrast (VEC-DIC) optics. Of those cells, 35 displayed continuous movement at rates of ≥ 0.5 μ m/min. Locomoting cells displayed a strikingly consistent pattern of myosin II concentration in the cytoplasm between the nucleus and the leading edge, which is illustrated in Figure 6. The relative concentration of assembly-competent myosin II was always lowest in the broad lamellipodium at the leading edge of the cell. The lamellipodium was distinct from more proximal cytoplasm by its extreme uniformity under

VEC-DIC, the absence of intracellular organelles and granules, and the presence of ruffling and surface undulations that could be seen in time-lapse sequences. At the base of the lamellipodium, the assembly-competent myosin II was markedly enriched, even though the highly structured transverse fibers observed during the lamellar contraction phase were no longer present. Overall, the general pattern of myosin II assembly remained very similar between these two different stages of wound healing. Assembly-competent myosin II was highest at the base of the lamellipodia and decreased toward the perinuclear regions of the cell. This gradient is clearly evident in a pseudocolor display of the ratio image (Figure 7).

In the tail of the cell, the relative concentration of assembly-competent myosin II did not display a simple

Figure 6. Relative concentration of myosin II in a translocating fibroblast. Fibroblasts migrating at the edge of a wound were imaged at 10 min intervals using DIC optics (a) between 9 and 10 h after wounding. The time-lapse series showed that this cell was migrating from left to right at a rate of $0.8 \mu\text{m}/\text{min}$. Fluorescence images of AR-myosin II and AF-10S myosin II were acquired immediately after the DIC image in a. b shows the ratio image of AR-myosin II:AF-10S myosin II, reflecting the relative concentration of myosin II. Note the broad hyaline lamellipodium at the front of the cell indicated by the brackets in a. Arrows in (b) mark the leading edge of the lamellipodium; arrows mark the base of the lamellipodium and the characteristic band of high myosin II concentration. The quantitative variations in myosin II concentration can be seen more clearly in the pseudocolor representation in Figure 7. Bar, $10 \mu\text{m}$.

pattern. It was high in the rear of some cells but not in others. Myosin II concentration and assembly may vary in the rear of the cell depending on the stage of the tail retraction.

3-D Imaging of Living Fibroblasts Demonstrates that Stress Fibers Are Localized in the Cortical Cytoplasm

Both FPR and ratio-imaging indicated that myosin II was less assembled in the perinuclear cytoplasm than in the peripheral regions of the cell. However, the quantities measured by FPR and ratio-imaging do not reveal variations in myosin II organization in the z axis. They can only represent the average state of assembly through the full thickness of the cell. Therefore, living cells containing AR-myosin II were optically sectioned as described in MATERIALS AND METHODS to obtain a map of myosin II distribution in three dimensions. Serial sections through 22 serum-deprived cells were examined, and sections from a representative series are illustrated in Figure 8, b–d. It should be noted that it is difficult to illustrate 3-D distributions in static, two-dimensional (2-D) images. Our determinations were aided considerably by examining each through-focus series as a movie loop and by rendering it as a rotating 3-D reconstruction. A stereo-pair (Figure 8e) allows a view of the 3-D organization. With these visual aids, the 3-D distribution of myosin II was determined to be as

follows: Fibers containing myosin II were confined to the cortical cytoplasm. In the most peripheral regions of the cytoplasm, the cell is so thin that individual fibers appose both the dorsal and ventral surfaces. In the perinuclear cytoplasm, fibers were still localized in the cortex but did not exhibit a consistent pattern of association with one surface or the other. Although it was more common for fibers to be found at the dorsal surface of the cell in the perinuclear region, many cells had fibers only along the ventral surface or displayed both dorsal and ventral fibers. In the noncortical perinuclear cytoplasm, myosin II was diffuse and amorphously distributed (Figure 8c). Thus, although the bulk of the myosin II was unassembled in the perinuclear cytoplasm, some myosin II could remain assembled in a thin layer near the membrane.

Optical sectioning and stereo-rendering revealed that fibers were also localized in the cortical cytoplasm of migrating cells (Figure 9). In cells exhibiting the wound-healing response, fibers in the anterior lamellar regions of the cytoplasm (where the cell was very flat) were associated with both the dorsal and ventral surfaces, as in serum-deprived cells. In the more posterior cytoplasm, transverse fibers were associated with either the dorsal or ventral surface of the cell, whereas myosin II in the deeper perinuclear cytoplasm was distributed diffusely (Figure 9c). Many cells contained fibers in the posterior cytoplasm that were parallel to the direction of movement. These longitudinal fibers were also lo-

Figure 7. Gradients in the relative concentration of myosin II in a translocating fibroblast. The ratio image in Figure 6b is shown here in pseudocolor with warm colors representing high ratios and cool colors representing low ratios. The color scale is linear with arbitrary units of ratio intensity. The lowest relative concentration of myosin II (blue colors) is found in the lamellipodium; the highest relative concentration (red colors) is found in a circumferential band at the base of the lamellipodium. Note the gradual decrease in relative concentration from the base of the lamellipodium to the perinuclear region. Bar, 10 μ m.

calized in the cortex and could be found extending either above or below the nucleus (Figure 9, b and d).

DISCUSSION

Myosin II Is Localized by Assembly

The relative concentration of assembly-competent myosin II in various regions of the cytoplasm can be observed by ratio imaging. Although the cytoplasm is broadly accessible to the myosin II molecule (as indicated by the distribution of the assembly-incompetent analogue, AF-10S myosin II), assembly-competent myosin II becomes specifically enriched in some regions. Our interpretation is that this is driven by myosin II assembling into filaments and/or binding to actin-based structures. Thus, the relative concentration of myosin II is highest in stress fibers, because myosin II molecules are assembled into filaments. Meanwhile, the relative concentration is lowest in the perinuclear cytoplasm, where fibers disassemble. FPR measurements in both serum-deprived cells and cells locomoting at the edge of a wound confirm that myosin II is mostly unassembled in the perinuclear region, whereas there is a large immobile fraction in the peripheral cytoplasm where myosin II is most concentrated. Furthermore, when fibers containing myosin II are disassembled by treating cells with staurosporine, a treatment known to cause dephosphorylation of myosin II (Giuliano *et al.*, 1992), the gradient in the relative concentration of myosin II is drastically diminished. Thus, by revealing localized

enrichment of assembly-competent myosin II, ratio-imaging provides an excellent indicator of differential assembly in living cells.

Myosin II Undergoes 10S $\leftrightarrow$ 6S Transitions In Vivo

The detection of an immobile fraction by FPR indicates that a significant portion of myosin II is stably associated with very large structures, as would be expected if it were assembled and interacting with the actin-based gel. The diffusion coefficient of the remaining mobile portion provides some indication of the size of the diffusing molecules. In the perinuclear cytoplasm of serum-deprived cells, the diffusion coefficient of AR-myosin II is not significantly different from the cross-linked 10S analogue. Unfolding of myosin II from a 10S to 6S configuration would be expected to decrease the diffusion coefficient by 40%, or more if the unfolded dimensions approach or exceed the pore size of the cytoplasmic matrix. Likewise polymerization of 6S myosin II into filaments would decrease the diffusion coefficient even further. Thus, our observations are consistent with a significant fraction of myosin II being unassembled, 10S monomer in the perinuclear cytoplasm.

In the periphery of both locomoting and serum-deprived cells, the diffusion coefficient of AR-myosin II is about one-half that of the perinuclear cytoplasm. This is partly because of local differences in the cytoplasmic matrix (Luby-Phelps *et al.*, 1986, 1987; Luby-Phelps and Taylor, 1988), because diffusion of AF-10S myosin II is also slower. However, the diffusion coefficient of AR-

Figure 8. Three-dimensional distribution of myosin II in serum-deprived fibroblasts. Serum-deprived Swiss 3T3 fibroblasts were microinjected with AR-myosin II and a through-focus series of fluorescence images acquired. a shows the distribution of AR-myosin II as visualized by conventional fluorescence microscopy. The focal plane is very close to the substratum ($<0.5 \mu\text{m}$ above it), where the thin peripheral cytoplasm is optimally visualized. The cell displays an extensive network of stress fibers in the peripheral cytoplasm. In the much thicker perinuclear cytoplasm, AR-myosin II fluorescence is more complicated, and individual fibers are more difficult to distinguish. b shows the image from a after deconvolution to remove out-of-focus fluorescence. The perinuclear fluorescence is dramatically reduced. Fibers that are now clearly resolved in the perinuclear region lie in the same plane as the peripheral fibers. Some of these fibers (e.g., arrow) extend beneath the nucleus. The image in c is from a plane $1.15 \mu\text{m}$ above that in b. Most of the perinuclear myosin II is amorphously distributed. Peripheral fibers are no longer visible. Fibers that slant upward (eventually extending over the nucleus in d) can be seen in tangential section (arrow). d is near the top of the cell, $2.76 \mu\text{m}$ above a and shows that the fiber marked in (c) arches over the nucleus. This can be seen more clearly in the stereo-pair shown in e. e is a stereo-pair obtained by 3-D rendering of the full set of through-focus images after they have been deconvolved. The z axis has been stretched by a factor of ten to enhance the 3-D structure. Fibers are confined to the cortical cytoplasm, whereas the deeper perinuclear cytoplasm contains only diffusely distributed myosin II. Bar, $10 \mu\text{m}$.

Figure 9. Three-dimensional distribution of myosin II in wound-healing fibroblasts. Wound-healing fibroblasts were microinjected with AR-myosin II and a through-focus series of fluorescence images acquired 6 h after wounding. a shows the distribution of AR-myosin as visualized by conventional fluorescence microscopy. The focal plane is within $0.5\ \mu\text{m}$ of the substratum where the leading edge is optimally visualized. The direction of migration is toward the top of the field. Numerous closely spaced transverse fibers (oriented perpendicular to the direction of migration) fill the anterior portion of the cell. These are more clearly delineated in deconvolved images (b). Fibers are less numerous around the nucleus and tend to lie parallel to the direction of migration. b–d are images from the through-focus series after deconvolution. The image in b is the same plane as shown in a, c is from a plane $1.38\ \mu\text{m}$ above that in b, and d is near the top of the cell, $2.76\ \mu\text{m}$ above a. (b) The flat anterior portion of the cell contains a nearly continuous array of transverse fibers with myosin II distributed in a semisarcomeric pattern (arrows mark the ends of one such fiber). A section through the deeper perinuclear cytoplasm (c) shows little fiber structure; most of the perinuclear myosin II is diffusely distributed. The optical section in d passes tangentially through two large fibers (arrows) lying above the nucleus in the dorsal, cortical cytoplasm. Bar, $10\ \mu\text{m}$.

myosin II is still $\sim 25\%$ lower than for AF-10S myosin II. This may reflect unfolding of 10S myosin into the 6S form and/or transient binding of AR-myosin II. It is interesting to note that diffusion of inert spheres becomes extremely hindered when the hydrodynamic radius exceeds $\sim 16\ \text{nm}$, presumably because of limiting pore size of the cytoplasmic matrix (Luby-Phelps *et al.*, 1986, 1987; Luby-Phelps and Taylor, 1988). Because this is very close to the dimensions of folded, monomeric myosin II (Trybus *et al.*, 1982), unfolded or multimeric myosin II may diffuse poorly through the cytoplasmic matrix. Folding to the 10S configuration may facilitate

delivery of myosin II to sites of assembly by improving its transport properties. This might also explain the very low AR-myosin II:AF-10S myosin II ratio observed in lamellipodia (see also DeBiasio *et al.*, 1988).

Fibers Disassemble and Contract in Locomoting Cells, but Disassembly Occurs Without Contraction in Serum-deprived Fibroblasts

In fibroblasts locomoting at the edge of a wound, transverse fibers undergo both disassembly and contraction (Conrad *et al.*, 1993) in what appears to be an example

of solation-contraction coupling (reviewed by Taylor and Fechheimer, 1982). Hahn *et al.* (1992b) recently demonstrated that gradients of free Ca^{2+} concentration and of calmodulin activation are associated with contraction of transverse fibers in wound-healing fibroblasts. The free Ca^{2+} concentration and the activation of calmodulin is highest toward the posterior of locomoting cells as has been observed in amoeba (Taylor *et al.*, 1980) and neutrophils (Brundage *et al.*, 1991). Elevated free Ca^{2+} should solate the actin-based component of the actin-myosin II gel by activating actin-capping and -severing proteins such as gelsolin (Yin and Stossel, 1979) and by inhibiting the cross-linking activity of some actin-binding proteins (e.g., nonmuscle α -actinin) (Burridge and Feramisco, 1981). Furthermore, elevated free Ca^{2+} and Ca^{2+} binding to calmodulin should activate myosin II light chain kinase, which phosphorylates the 20-kDa regulatory light chains of myosin II and thereby activates myosin II motor activity (recently reviewed by Sellers, 1991; Trybus, 1991; Tan *et al.*, 1992). Therefore, the loss of fiber structure appears to involve a self-destruct contraction in which calcium activates the contractile motor and decreases the resistance of the load on the motor, as described by the solation-contraction hypothesis (Taylor and Fechheimer *et al.*, 1982; Kolega *et al.*, 1991; Janson *et al.*, 1992).

The situation is quite different during the disassembly of stress fibers in serum-deprived cells, where constitutive disassembly occurs without a rise in free Ca^{2+} or contraction (Giuliano and Taylor, 1990; Hahn *et al.*, 1993). The >90% mobility of myosin II in the perinuclear region of serum-deprived cells indicates that the dispersal of fibers in this region is not just a splaying apart of bundled filaments; it does indeed involve disassembly of at least the myosin II. In fact, release of myosin II may be an early step in the disassembly of stress fibers to avoid solation-induced contraction, because solation of the actin-myosin II-based gel can also induce contraction of stress fibers, even in the absence of additional activation of myosin II motor activity (Janson *et al.*, 1991; Kolega *et al.*, 1991; Giuliano *et al.*, 1992). Experiments are currently underway to determine where and when disassembly of actin occurs.

It is not known what regulates myosin II assembly and disassembly in serum-deprived fibroblasts. Free Ca^{2+} and pH are both known to be low (c. 50–100 nM and 6.8–7.2, respectively) and spatially uniform (MacNeil and Taylor, 1987; Bright *et al.*, 1989; Byron and Villerreal, 1989; Tucker and Fay, 1990; Hahn *et al.*, 1993). Thus, myosin II undergoes constitutive assembly and disassembly without changing either free Ca^{2+} or pH. Because the regulatory light chains of myosin II are partially phosphorylated in serum-deprived cells (Giuliano *et al.*, 1992), Ca^{2+} /calmodulin-dependent myosin light chain kinase may have a low calcium-independent level in cells, or light chain phosphorylation may have another regulatory mechanism under these conditions.

Regulating dephosphorylation could play an important role. Serum-deprived cells may exhibit a gradient of myosin II phosphorylation, highest at the periphery and lowest in the perinuclear region but still below the level in stimulated cells (Giuliano *et al.*, 1992). Interestingly, myosin II assembly at the periphery of serum-deprived cells and at the base of the lamellipodium in migrating cells occurs at low free Ca^{2+} (Hahn *et al.*, 1992), suggesting Ca^{2+} -independent regulation of assembly in these regions. The possible role of phosphorylation of the myosin II heavy chains must also be explored, along with other sites of phosphorylation on the regulatory light chains. Multiple mechanisms for regulating myosin II assembly may allow the activation of assembly and motor activity to be uncoupled.

Myosin II Gradients Are Parallel to Fiber Motion

The concentration, mobility, and structural organization of myosin II all suggest a higher degree of myosin II assembly at the distal edges of the cell and lower assembly in the perinuclear region. The gradient in assembly of myosin II coincides with the motion of actin-myosin II structures. That is, in serum-deprived cells, stress fibers assemble constitutively at the periphery of the cell, move centripetally, and disassemble in the perinuclear cytoplasm (Giuliano and Taylor, 1990). Likewise, in wound-healing fibroblasts, assembly of fibers containing actin and myosin II occurs at or near the leading edge with continuous centripetal transport of the assembled structures (DeBiasio *et al.*, 1988; Fisher *et al.*, 1988; Conrad *et al.*, 1993). McKenna *et al.* (1989) have also observed constitutive assembly and centripetal movement of bead-like structures containing myosin II in the leading edge of fibroblasts. Presumably, the disassembly of myosin II structures in the perinuclear cytoplasm releases material that is recycled for the assembly of cytoskeleton occurring at the membrane-cytoplasm interface (Dunn, 1980; Bray and White, 1988). This proposed model for myosin II assembly and dynamics is summarized in Figure 10.

Myosin II is absent from initial protrusions (DeBiasio *et al.*, 1988); it is relatively unassembled in rapidly moving lamellipodia (Figures 6 and 7), and it is not necessary for protrusive activity (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987). What then is the function of myosin II assembly in the peripheral cytoplasm, particularly at the base of lamellipodia? Dunn (1980) proposed that continuous contraction of the cytoskeleton after its assembly at the edge of the cell could generate force for the forward translocation of the cell body. For continuous centripetal contraction to occur, the contractile motor (e.g., myosin II) must be incorporated at the beginning of the contraction in the cell periphery and released at the end of the contraction. An integration of the cortical flow concepts (Bray and White, 1988) and the solation-contraction coupling hypothesis (Tay-

Figure 10. Proposed pattern of myosin II assembly and disassembly in situ. This schematic cross-section of a migrating fibroblast illustrates the spatial bias in assembly of myosin II in a locomoting cell. Folded 10S monomers of myosin II ($\circ$) (assembled myosin II, $\bullet$) diffuse throughout the cytoplasm. Although initially excluded from newly formed protrusions at the leading edge of the cell, myosin II eventually enters the lamellipodium but does not assemble, despite extensive assembly of actin filaments in this region. Myosin II becomes incorporated into the cytoskeleton at the base of the lamellipodium (a), where transverse fibers are first formed (represented here as aggregates of assembled myosin II but also containing bundled actin filaments). The cell is very flat in the peripheral cytoplasm, and transverse fibers can be associated with the dorsal, ventral, or both surfaces of the cell. Myosin II may also assemble into the less ordered cortical meshwork of microfilaments, which for clarity is not illustrated here. Fibers move toward the nucleus, carrying assembled myosin II away from the leading edge. It is likely that some assembly and disassembly continues to occur throughout the lifetime of the fiber ($\leftrightarrow$). In the thicker regions of the cell, fibers remain associated with either the dorsal or ventral surface. In the perinuclear region, which lies near the posterior of a locomoting cell, myosin II disassembles (b), creating a large pool of monomers, which diffuse and/or are transported back toward sites of assembly in the more peripheral cytoplasm.

lor *et al.*, 1980; Taylor and Fehcheimer, 1982) could explain the process.

Myosin II-null mutants of *Dictyostelium* move more slowly than wild-type (Wessels *et al.*, 1988; Wessels and Soll, 1990). Perhaps they are less able to exert strong forces for pulling the cell body forward after extending a protrusion. Or they may not deliver disassembled subunits efficiently from the rear to the front of the cell. Myosin II-deficient cells also move in more erratic paths than wild-type strains and display increased randomness in the location of expansion zones (Wessels *et al.*, 1988; Wessels and Soll, 1990), suggesting that myosin II is involved in polarizing movement. This could occur if myosin II-based contractility were to limit expansion of the leading edge (Spudich, 1989). Assembly-driven expansion of the gel has been proposed as a mechanism for protrusive activity and centripetal flow (Bray and White, 1988; Stossel, 1990), and lateral contraction of the gel would limit expansion. In fibroblasts at the edge of a wound, fibers in the anterior part of the cell are oriented perpendicular to the direction of movement, such that sliding-filament type contractility would decrease lateral expansion of the cell. This might also serve to enhance the continued protrusion of the cell into the wound space, facilitate transport into protrusions by increasing hydrostatic pressure, and separate actively migrating cells from their neighbors. The band of myosin II assembly at the base of the lamellipodium in

rapidly translocating cells may have a similar function. It is noteworthy that myosin II-deficient *Dictyostelium* (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987) and fibroblasts that have been injected with antibodies against myosin II (Höner *et al.*, 1988) both display abnormally broad regions of protrusive activity at their leading edges. We are currently examining the dynamics of myosin II assembly during the complete process of amoeboid locomotion. For example, how does myosin II assembly vary as the direction and rate of protrusive activity change? Does myosin II assemble in the posterior part of the cell for tail retraction? Is the forward flow of cytoplasm, as indicated by the movement of cytoplasmic organelles, correlated with the state of myosin II assembly?

A Cell Surface-associated Signal Could Generate Myosin II Assembly Gradients

In a 2-D projection of the cell, gradients in the assembly and relative concentration of myosin II extend over large distances, spanning the cytoplasm from the very edge of the cell to the nucleus. However, considered in three-dimensions, a much shorter gradient that lies normal to the cell surface could produce this distribution. If myosin II were preferentially assembled near the cell surface, even a thin cortical layer of high concentration/assembly could fill the peripheral cytoplasm, where the cell is only a few hundred nanometers thick. In contrast, in the much thicker perinuclear region, where the cytoplasm is 2–3 μm deep, most of the cytoplasm could still contain unassembled myosin II. The strict cortical localization of actin-based stress fibers suggests that assembly might indeed occur preferentially near the cell surface.

What controls the localized assembly and disassembly of myosin II? As noted above, in serum-deprived fibroblasts some regulator other than intracellular pH or free Ca^{2+} must be invoked. The disruption of concentration and assembly gradients by staurosporine implicate protein kinase(s) in maintaining myosin II gradients. Staurosporine does not cause massive disassembly of the actin cytoskeleton, as an extensive network of actin filaments remains even after complete disruption of stress fibers (Hedberg *et al.*, 1990; Kolega *et al.*, 1993). We suspect that inhibition of the phosphorylation of myosin II itself may be involved. Microinjection of peptide and antibody inhibitors of myosin light chain kinase also causes loss of stress fibers (Lamb *et al.*, 1988), and phosphorylation of the 20-kDa regulatory light chains is known to affect myosin II assembly and contractile activity (see reviews by Sellers, 1991; Trybus, 1991; Tan *et al.*, 1992). We have recently shown that myosin II regulatory light chains are rapidly dephosphorylated in the presence of staurosporine (Giuliano *et al.*, 1992) and that dephosphorylation and stress fiber disassembly show parallel dose-response curves (Kolega *et al.*, 1993).

In any case, it is not yet known where in the cell phosphorylation and dephosphorylation of any cytoskeletal proteins occur. For example, are myosin II light chains phosphorylated in the cell periphery and dephosphorylated in the perinuclear cytoplasm? Might heavy chain phosphorylation or phosphorylation of other actin-binding proteins, such as caldesmon, be involved? A new class of fluorescent analogues, protein-based optical biosensors (Hahn *et al.*, 1992, 1993), should help answer these questions.

These observations are just the first step in obtaining a spatial map of cytoskeletal assembly and the dynamics of assembly. Understanding the function of the cell's locomotive machinery will require knowledge of the local concentrations and states of assembly of basic structural elements, such as actin, and of other motor proteins such as myosin I. Ratio-imaging, FPR, photoactivation of fluorescence, and imaging rotational diffusion via fluorescence anisotropy (Gough and Taylor, 1993) can generate such a map, which will help pinpoint where key structural transitions take place and where the first line of cytoskeletal regulation must occur.

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