A Fluorescent Protein Biosensor of Myosin II Regulatory Light Chain Phosphorylation Reports a Gradient of Phosphorylated Myosin II in Migrating Cells

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Phosphorylation of the regulatory light chain by myosin light chain kinase (MLCK) regulates the motor activity of smooth muscle and nonmuscle myosin II. We have designed reagents to detect this phosphorylation event in living cells. A new fluorescent protein biosensor of myosin II regulatory light chain phosphorylation ($^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$) is described here. The biosensor depends upon energy transfer from fluorescein-labeled regulatory light chains to rhodamine-labeled essential and/or heavy chains. The energy transfer ratio increases by up to 26% when the regulatory light chain is phosphorylated by MLCK. The majority of the change in energy transfer is from regulatory light chain phosphorylation by MLCK (versus phosphorylation by protein kinase C). Folding/unfolding, filament assembly, and actin binding do not have a large effect on the energy transfer ratio. $^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$ has been microinjected into living cells, where it incorporates into stress fibers and transverse fibers. Treatment of fibroblasts containing $^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$ with the kinase inhibitor staurosporine produced a lower ratio of rhodamine/fluorescein emission, which corresponds to a lower level of myosin II regulatory light chain phosphorylation. Locomoting fibroblasts containing $^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$ showed a gradient of myosin II phosphorylation that was lowest near the leading edge and highest in the tail region of these cells, which correlates with previously observed gradients of free calcium and calmodulin activation. Maximal myosin II motor force in the tail may contribute to help cells maintain their polarized shape, retract the tail as the cell moves forward, and deliver disassembled subunits to the leading edge for incorporation into new fibers.

INTRODUCTION

In vitro, the activity of smooth muscle and mammalian nonmuscle myosin II is controlled by phosphorylation of the regulatory light chain. Phosphorylation by myosin light chain kinase (MLCK),¹ at serine 19 and threonine 18, induces a conformational change

from a folded, 10S monomer, to an extended, 6S molecule that can assemble into filaments; the actin-activated Mg^{2+} ATPase activity of the phosphorylated myosin II is increased as well (Sellers and Adelstein, 1987; Sellers, 1991; Trybus, 1991b). Phosphorylation by protein kinase C (PKC), at threonine 9 and serine 1/serine 2, inhibits the actin-activated MgATPase of myosin II that was previously phosphorylated at serine 19. In addition, phosphorylation of unphospho-

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¹ Abbreviations used: FRET, fluorescence resonance energy transfer; $^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$, fluorescein-labeled regulatory light chain exchanged myosin II; $^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$, fluorescein-labeled regulatory light chain exchanged rhodamine-labeled myosin II;

MLCK, myosin light chain kinase; PKC, protein kinase C; $^{\text{R}}\text{myosin II}$, rhodamine-labeled myosin II.

rylated myosin II by PKC reduces the rate of phosphorylation by MLCK (Nishikawa *et al.*, 1984).

In vivo, however, myosin II knock-out studies in *Dictyostelium discoideum* demonstrated that some form of cell locomotion could continue in the absence of myosin II. In addition, a type of cytokinesis, traction-mediated cytofission, could occur in adherent motile cells (DeLozanne and Spudich, 1987; Knecht and Loomis, 1987; Manstein *et al.*, 1989), but cytokinesis could not occur in cells in suspension (Knecht and Loomis, 1987). Anti-myosin II antibodies block cytokinesis when injected into dividing cells (Mabuchi and Okuno, 1977; Kiehart *et al.*, 1982), but do not block the locomotion of fibroblasts, although abnormal shapes are induced (Höner *et al.*, 1988). Yet surprisingly, *Dictyostelium* cells lacking residues phosphorylated by MLCK, which regulate motor activity (Ostrow *et al.*, 1994), or those phosphorylated by a myosin II heavy chain kinase, which regulate filament assembly (Engelhoff *et al.*, 1993), are essentially normal. A major unanswered question remains about the role of myosin II and its regulation in fundamental cell functions including locomotion and cytokinesis.

Biochemical measurements of the level of myosin II regulatory light chain phosphorylation of cell populations have provided valuable information about the intracellular role of myosin II regulatory light chain phosphorylation (Bayley and Reese, 1986; Lamb *et al.*, 1988; Itoh *et al.*, 1989; Giuliano *et al.*, 1992), including during mitosis (Yamakita *et al.*, 1994). However, these methods require the extraction of myosin II from many cells and phosphorylation levels may change during this purification. Furthermore, these studies determine the average level of phosphorylation of the entire cell population and not cell-to-cell variations and temporal/spatial changes within the same cell.

To help understand the contribution of myosin II to nonmuscle cell motility and the significance of its regulation by light chain phosphorylation, we are designing reagents, fluorescent protein biosensors, to monitor this and associated events in individual, living cells (Hahn *et al.*, 1993; Post *et al.*, 1994; Giuliano *et al.*, 1995). Fluorescent protein biosensors are fluorescently labeled, functional proteins that report protein activities (such as conformational changes, binding of ligands, or covalent modifications) via a fluorescence spectroscopic change (Hahn *et al.*, 1992, 1993; Giuliano *et al.*, 1995). Detection methods to monitor protein activity include the site-specific placement of solvent-sensitive fluorophores (Hahn *et al.*, 1990, 1992; Richieri *et al.*, 1992; Brune *et al.*, 1994; Post *et al.*, 1994), introduction of fluorescence resonance energy transfer fluorophores (FRET) (Taylor *et al.*, 1981; Herman and Fernandez, 1982; Adams *et al.*, 1991), or labeling with fluorescent dyes to monitor changes in anisotropy (Carraway and Cerione, 1993; Gough and Taylor, 1993).

Previously, we used a molecular genetic approach to engineer a single cysteine adjacent to serine 19 on the myosin II regulatory light chain, which was subsequently labeled with a polarity-sensitive fluorescent dye (Post *et al.*, 1994). This reagent has been valuable for in vitro studies but severe quenching limited the value of this reagent in live cell studies. Here we describe a new fluorescent protein biosensor of myosin II regulatory light chain phosphorylation. It uses a different labeling approach in that it employs native myosin II and depends upon energy transfer between fluorescein-labeled regulatory light chains and rhodamine-labeled essential and/or heavy chains. This biosensor has overcome the in vivo spectroscopy limitations of the previously described acrylodan reagent (Post *et al.*, 1994) and has enabled visualization of phosphorylated myosin II in polarized, migrating cells undergoing wound healing. We have used this new biosensor to define a part of the mechanism of cell locomotion.

MATERIALS AND METHODS

Protein Preparation

Turkey gizzard myosin II was purified as described previously (DeBiasio *et al.*, 1988). Turkey gizzard myosin II regulatory light chains were purified as described (Post *et al.*, 1994). Actin, MLCK, and bovine-brain calmodulin were purified according to the methods of Spudich and Watt (1971), Ngai *et al.* (1984), and Hahn *et al.* (1990), respectively. Purified light chains were labeled with 5-iodoacetamidofluorescein (Molecular Probes, Eugene, OR) according to the procedure described by Post *et al.* (1994) for acrylodan. Rhodamine-myosin II was prepared with tetramethylrhodamine-5-(and-6)-iodoacetamide (Molecular Probes) as described (DeBiasio *et al.*, 1988). The dye-to-protein ratio of rhodamine-labeled myosin II and fluorescein-labeled regulatory light chain varied from 4.0–6.0 and 0.8–1.1, respectively. Fluorescein-labeled regulatory light chains were exchanged into rhodamine-myosin II as detailed by Post *et al.* (1994) with minor modifications. Before dialysis into the high salt exchange buffer, rhodamine-myosin II was extracted by addition of 0.2 mM ATP and 100 mM NaCl to the rhodamine-myosin II storage buffer and then centrifuged at $75,000 \times g$ for 5 min. The supernatant was removed and dialyzed into high salt exchange buffer for the exchange.

³⁵S-RLC-myosin II was chemically cross-linked according to the method of Kolega and Taylor (1993) except that myosin II was labeled first with rhodamine and fluorescein as described above and then cross-linked with 1-3-(3-dimethylaminopropyl)carbodiimide (Pierce, Rockford, IL).

Detection of Energy Transfer Measured in the Fluorometer

Fluorescence emission scans were recorded on a SPEX Fluorolog-2 system (Edison, NJ) corrected for the intensity of the exciting light and the sensitivity of the detection system. Slit bandpass widths were 2 nm, scans were collected over 1-nm increments, and integration times were for 1 s (unless otherwise indicated). Samples were excited at 480 nm and emission was scanned from 500–650 nm. The "energy transfer ratio" is the ratio of the emission at 578 nm (acceptor) divided by the emission at 520 nm (donor).

Efficiency of energy transfer was determined by donor quenching according to the method of Wang and Taylor (1981). Emission scans were taken in the fluorometer of equal concentrations of donor-

labeled myosin II (F RLC-myosin II), acceptor-labeled myosin II (R myosin II), donor- and acceptor-labeled myosin II (F RLC- R myosin II), and unlabeled myosin II. Efficiency (E) was calculated by $E = 1 - (I_2 - I_3)/(I_1 - I_4)$, where I_1 is the emission intensity of the donor alone, I_2 is donor- and acceptor-labeled myosin II, I_3 is acceptor-labeled myosin II, and I_4 is unlabeled myosin II. To simulate intracellular concentrations (10% labeled myosin II, 90% unlabeled myosin II) during in vitro experiments (further explained in RESULTS), the in vitro phosphorylation studies below were all performed with a 1:10 dilution of labeled myosin II with unlabeled myosin II excess of unlabeled gizzard myosin II.

Phosphorylation Assays

F RLC- R myosin II was scanned in phosphorylation buffer (15 mM Tris, pH 7.5, at room temperature, 175 mM KCl, 5 mM $MgCl_2$, 0.1 mM EGTA, 1 mM $MgATP$, 1 mM dithiothreitol [DTT]) in the fluorometer, to keep myosin II folded (10S) when unphosphorylated and extended (6S) when phosphorylated by MLCK. Myosin II was phosphorylated with the addition of MLCK (to 1 μ M), calmodulin (to 1 μ M), and $CaCl_2$ (to 1 mM).

Kinase Specificity Assays

To achieve maximal phosphate incorporation, light chains were phosphorylated first and then exchanged onto myosin II heavy chains. Fluorescein-labeled regulatory light chains (2 mg/ml) were phosphorylated with MLCK for 45 min at room temperature in 10 mM MOPS (pH 7.0), 1 mM $CaCl_2$, 1 mM ATP, 1 mM $MgCl_2$, 1 mM DTT, 0.8 μ M MLCK, 0.8 μ M calmodulin, 1 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.2 mM phenylmethylsulfonyl fluoride, and 3 mM NaN_3 . Fluorescein labeled regulatory light chains (2 mg/ml) were phosphorylated with PKC for 2 h at 30°C in 20 mM Tris (pH 7.5), 5 mM $MgCl_2$, 0.4 mM $CaCl_2$, 15 mM KCl, 1 mM ATP, 50 μ g/ml phosphatidyl serine (Avanti Polar Lipids, Alabaster, AL), 8 mg/ml dioleoyl glycerol (Avanti Polar Lipids), 5 μ g/ml PKC (Calbiochem, La Jolla, CA), 1 mM sodium pyrophosphate, 50 mM sodium fluoride, 0.2 mM phenylmethylsulfonyl fluoride, and 3 mM NaN_3 . MLCK-phosphorylated fluorescein light chains were then phosphorylated by PKC. Extent of phosphorylation was verified by urea/glycerol gel electrophoresis (Trybus and Lowey, 1984) and phosphorylated fluorescein light chains were exchanged into rhodamine-labeled myosin II. Phosphorylated light chains were kept in the phosphatase inhibitors throughout the exchange procedure and experiments. Urea/glycerol gel electrophoresis showed that phosphorylation levels had remained the same after experiments were completed. Light chains were phosphorylated at at least one site by MLCK, at least two sites by PKC, and at least three sites when phosphorylated by MLCK and PKC.

Myosin II Filament Disassembly Assays

These assays were based on those described by Hahn *et al.* (1993). As in the other spectroscopy assays, F RLC- R myosin II was first diluted with unlabeled myosin II to approximate its intracellular mixture with endogenous protein after microinjection. For these assays, the dilution was 1:1 of labeled myosin II:unlabeled myosin II (rather than 1:10) to optimize the signal/noise during the scans. Clarified unlabeled myosin II plus clarified biosensor (0.74 μ M total concentration) was scanned in the fluorometer in filament assembly buffer (150 mM KCl, 10 mM $MgCl_2$, 1 mM EGTA, 0.1 mM DTT, 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid [HEPES], pH 7.5, at room temperature). Samples were diluted with filament assembly buffer (to 0.21 μ M, 0.10 μ M, and 0.02 μ M) and scanned after each dilution.

ATP Binding Assays

F RLC- R myosin II, diluted 1:10 with unlabeled myosin II to make a final concentration of 1 μ M myosin II, was scanned in filament

assembly buffer (6S conformation). ATP was added to make a final concentration of 5 mM, samples were centrifuged at $75,000 \times g$ for 5 min to remove remaining filaments, and supernatants were scanned again (10S conformation).

Actin Binding Assays

Actin binding assays were performed in 0.1-mm pathlength rectangular glass capillary tubes (or "microslides") on a Multimode microscope (Giuliano *et al.*, 1990). To ensure optimal conditions for actin binding to phosphorylated myosin II, the buffer for these assays and the protein concentrations are those routinely used to measure the actin-activated Mg^{2+} ATPase activity of myosin II in our laboratory (Sellers *et al.*, 1981). Unphosphorylated or phosphorylated F RLC- R myosin II (0.3 mg/ml) was diluted into ATPase buffer (30 mM KCl, 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 15 mM Tris, pH 7.5, at room temperature) and drawn into a capillary tube. The capillary was sealed and attached to a glass slide with VALAP (a mixture of equal parts of Vaseline, lanolin, and paraffin). Images were acquired through a 1.25NA 63 $\times$ Plan NEOFLUAR objective (Carl Zeiss, Thornwood, NY) with a 0.1 ND filter. Ratio images were collected with a fluorescein excitation filter (485 nm with 22-nm bandpass) and either a fluorescein emission filter (530 nm with a 30-nm bandpass) and 510-nm longpass dichroic or a rhodamine emission filter (590 nm with a 35-nm bandpass) and 572-nm longpass dichroic. To eliminate artifacts from photobleaching, the order of emission collection was alternated. G-actin was diluted into ATPase buffer and polymerized. For scans using actin, phosphorylated F RLC- R myosin II (0.3 mg/ml) and 0.3 mg/ml actin were mixed and quickly drawn into a capillary tube. The mean pixel intensity of all background-subtracted images was calculated with BDS-Image (now Oncor-Image, Gaithersburg, MD) and ratios were made of the background-subtracted rhodamine emission divided by the background-subtracted fluorescein emission.

Cell Culture and Microinjection

Swiss mouse 3T3 fibroblast cells (American Type Culture Collection, Rockville, MD), passage 118–128, were grown on 40-mm round, glass coverslips, and wounded as described by DeBiasio *et al.* (1988). Briefly, cells were grown to confluency and then wounded with a razor blade to create a straight wound edge. Medium was changed to fresh DMEM (without phenol red) plus 10% calf serum and incubated for 2–3 h before microinjection.

Live Swiss mouse 3T3 cells were microinjected as described (Amato *et al.*, 1983) with clarified F RLC- R myosin II (with or without cascade blue 10-kDa dextran; Molecular Probes) in myosin injection buffer (2 mM HEPES, 100 mM NaCl, 0.2 mM $MgATP$, 0.1 mM EGTA, 1 mM DTT, pH 7.5, at room temperature). Medium was changed to fresh DMEM (without phenol red) plus 10% calf serum. Cells were allowed to recover for approximately 30 min before an experiment.

Microscopy and Image Analysis

Cellular experiments were performed in a sealed chamber on a temperature-controlled stage of the Multimode microscope as described (Kolega *et al.*, 1993). Cells were imaged with a 1.25 NA 63 $\times$ Plan NEOFLUAR objective (Carl Zeiss) with fluorescein and rhodamine filter sets (described above). Cascade blue fluorescence was collected with a cascade blue filter set (380-nm excitation filter with a 22-nm bandpass, 405-nm longpass dichroic, and 450-nm emission filter with a 50-nm bandpass). A 576 $\times$ 384 Thompson chip-cooled CCD camera (Photometrics, Tucson, AZ) was used to acquire all images. Healthy cells were selected, monitored, and focused with video-enhanced contrast differential interference contrast microscopy to avoid excessive illumination of the fluorescent dyes.

For staurosporine experiments, serum-deprived cells (maintained in 0.2% serum for 48 h before experiments) were imaged as described above. Cells were then perfused with 20 nM staurosporine

in DMEM plus 0.2% calf serum for 3 min and allowed to recover for 15 min before image acquisition.

To avoid photobleaching artifacts from dual fluorescein excitation required for energy transfer imaging, fluorescein emission was collected via excitation of the fluorescein dye and rhodamine emission was collected via direct excitation of the rhodamine dye. Changes in energy transfer efficiency were detected by dividing the phosphorylation-insensitive rhodamine emission image by the fluorescein (phosphorylation sensitive, or donor quenched) emission image to produce a ratio image (DeBiasio *et al.*, 1988). Although this ratio image is not the true "energy transfer ratio," it is an indicator of energy transfer and is referred to simply as the "ratio" or "ratio image." Ratios generated *in vitro* via direct excitation of both fluorophores gave the same ratio value as the true energy transfer ratio. Because both the donor and acceptor dyes are on the same protein, the rhodamine image normalizes the fluorescein image to differences in cell pathlength, accessible cell volume, and local protein concentration, while the fluorescein image shows phosphorylation-dependent donor-quenching. Ratio images were generated with BDS-Image (now Oncor-Image), each fluorescein and rhodamine image was background subtracted, superimposed, and masked to the region of interest. The pixel value of each processed rhodamine image was divided by the corresponding pixel value of each fluorescein image, producing the ratio image. The resulting ratio image was pseudocolored, creating a map of the spatial distribution of phosphorylated myosin II. The ratio image of fluorescein images divided by cascade blue dextran images is a map of the spatial distribution of total myosin II relative to the volume indicator. Average ratio values of entire cells or regions of cells were quantified with Oncor-Image or NIH-Image, respectively. We focused on single time points in this study to avoid necessary corrections for photobleaching in time-lapse studies. The absolute ratios varied between preparations of the biosensor because the extent of energy transfer is a function of the exchange efficiency; however, all comparative experiments used the same batch of biosensor.

RESULTS

Biosensor Design

This phosphorylation biosensor employs FRET between fluorescein-labeled regulatory light chains (F RLC) and rhodamine-labeled myosin II heavy chains and/or essential light chains (R myosin II). To prepare the biosensor, smooth muscle myosin II was first labeled with tetramethylrhodamine-iodoacetamide in a low salt buffer in the presence of ATP (DeBiasio *et al.*, 1988). This labeling results in covalent binding of six rhodamine dye molecules to myosin II, while maintaining a majority of the myosin's native activity (DeBiasio *et al.*, 1988; Hahn *et al.*, 1993). Dye is bound to the 17-kDa essential light chains and to the heavy chains. One labeling site on the heavy chain is in the 70-kDa amino-terminal portion of the S1 region and the other is in the S2 region, as previously determined by papain digestion (Hahn *et al.*, 1993). Regulatory light chains remain unlabeled.

Second, purified turkey-gizzard regulatory light chains were labeled with iodoacetamidofluorescein on the single endogenous cysteine (position 108). These labeled light chains were subsequently incorporated into rhodamine-labeled myosin II via a myosin II regulatory light chain exchange procedure described in MATERIALS AND METHODS, which does not alter

the biological activity of the myosin II (Morita *et al.*, 1991). The doubly labeled myosin II is called F RLC- R myosin II.

Fluorescein-labeled Regulatory Light Chains Transfer Energy to Rhodamine-labeled Myosin II

F RLC- R myosin II in phosphate buffer was excited at 480 nm with a band width of 2 nm. Emission from the sample revealed energy transfer from the fluorescein regulatory light chains to the rhodamine-myosin II (see Figure 1A). To assay the extent of energy transfer, emission was collected from equal concentrations of F RLC-myosin II, R myosin II, and F RLC- R myosin II. Figure 1A shows all scans on the same axis. Energy transfer causes a large quenching of the donor fluorescence and sensitized emission of the acceptor fluorescence. The efficiency of energy transfer was determined to be 59%, derived by the method of donor quenching (Wang and Taylor, 1981). Energy transfer is probably occurring between the regulatory light chains and the essential light chains, although some transfer to the heavy chain labeling sites is also possible (see DISCUSSION). Therefore, distance changes between the energy donors and acceptors could not be determined.

Figure 1. Fluorescein-labeled regulatory light chains transfer energy to rhodamine myosin II and change efficiency with phosphorylation by MLCK. All emission scans shown were collected in the fluorometer through excitation at 480 nm with a 2-nm band width. (A) Emission was collected from equal concentrations of F RLC- R myosin II (—), donor-labeled myosin II (---), or acceptor-labeled myosin II (.....) with excitation at 480 nm. Energy transfer produces a large quenching of fluorescein fluorescence and sensitized emission of rhodamine. For scans in panels B, C, and D, — represents unphosphorylated F RLC- R myosin II and - - - represents phosphorylated F RLC- R myosin II. (B) Phosphorylation of F RLC- R myosin II by MLCK produces up to a 26% increase in the energy transfer ratio (emission at 578 nm/520 nm). (C) Phosphorylation of donor-labeled myosin II (F RLC-myosin II) causes only a small (4.3%) quenching. (D) Phosphorylation of acceptor-labeled myosin II (R myosin II) produces a small (6.6%) quenching.

Phosphorylation of $FRLC-R$ myosin II by MLCK Causes a Change in the Energy Transfer Ratio

$FRLC-R$ myosin II has been used in living cells to observe changes in the level of myosin II phosphorylation by MLCK. Inside the cell, however, the reagent mixes with the endogenous pool of unlabeled myosin II. As a rule, for cellular experiments, the concentration of the fluorescent analogue or biosensor is kept at $\leq 10\%$ of the endogenous concentration of the protein of interest (Taylor *et al.*, 1986). To simulate intracellular concentrations (10% labeled myosin II, 90% unlabeled myosin II) during in vitro experiments, the in vitro phosphorylation studies below were all performed with a 10-fold molar excess of unlabeled gizzard myosin II.

Phosphorylation of $FRLC-R$ myosin II by MLCK produced up to a 26% change in the energy transfer ratio from 1.15 (unphosphorylated) to 1.45 (phosphorylated) (see Figure 1B). (The ratios varied from a phosphorylation-dependent ratio increase of 17–26% between preparations of the reagent. This was likely due to minor variations in the dye-to-protein ratio of the rhodamine-labeled myosin II). Urea/glycerol gel electrophoresis demonstrated that phosphorylated $FRLC-R$ myosin II samples were 100% phosphorylated and unphosphorylated samples remained 100% unphosphorylated. Phosphorylation of $FRLC$ -myosin II as above produced only a small fluorescence emission quenching of 4.3% (see Figure 1C). Similarly, phosphorylation of R myosin II results in an emission quenching of 6.6% (see Figure 1D), which explains the lower than expected sensitized fluorescence (see Figure 1B).

Phosphorylation by MLCK Induces the Majority of the Change in the Energy Transfer Ratio

To test the specificity of the ratio change for phosphorylation by MLCK, regulatory light chains of $FRLC-R$ myosin II were phosphorylated with MLCK, PKC, or both kinases. Phosphorylation of regulatory light chains of $FRLC-R$ myosin II by PKC caused only a small increase in the energy transfer ratio (3.4%) from unphosphorylated $FRLC-R$ myosin II (Figure 2). Phosphorylation of the regulatory light chain by MLCK and PKC gave a ratio value intermediate to that of PKC or MLCK phosphorylated biosensor (Figure 2).

Because phosphorylation of smooth muscle and nonmuscle myosin II induces filament formation in vitro, the effects of filament formation on the energy transfer ratio were examined in the fluorometer. Unphosphorylated $FRLC-R$ myosin II filaments were co-assembled with unlabeled myosin II in filament assembly buffer (as described in MATERIALS AND METHODS). Energy transfer ratios were compared above and below the critical concentration of unphosphorylated myosin II. The energy transfer ratio of

Figure 2. The majority of the energy transfer ratio change is from phosphorylation by MLCK. The energy transfer ratios of $FRLC-R$ myosin II phosphorylated at different kinase sites were compared. Shown are energy transfer ratios of $FRLC-R$ myosin II phosphorylated with MLCK, PKC, both kinases, or left unphosphorylated. PKC phosphorylation does not cause a large change in energy transfer ratio from unphosphorylated $FRLC-R$ myosin II. $FRLC-R$ myosin II containing light chains phosphorylated at both kinase sites had an energy transfer ratio intermediate to that phosphorylated by PKC or MLCK alone. Error bars represent SEM.

nonfilamentous biosensor below the critical concentration was 4% lower than that above the critical concentration, suggesting that assembly of $FRLC-R$ myosin II may be responsible for only a small part of the total change in ratio observed with phosphorylation of the biosensor (see Figure 3A).

Under physiological conditions, phosphorylation of myosin II induces a change from a folded (10S) conformation to an extended (6S) conformation that as-

Figure 3. Characterization of the phosphorylation-induced energy transfer ratio change. (A) Filament assembly assays. Unphosphorylated $FRLC-R$ myosin II was diluted 1:1 with unlabeled unphosphorylated myosin II (to avoid possible inter-protein dye quenching specific to in vitro experiments) to total concentrations of 0.74 μ M, 0.21 μ M, 0.1 μ M, and 0.02 μ M and assayed above and below the critical concentration of 0.1 μ M (Kendrick-Jones *et al.*, 1987). (B) Folded (10S) vs extended (6S) myosin II assays. Unphosphorylated $FRLC-R$ myosin II was assayed in filament assembly buffer with ATP (10S conformation) or without ATP (6S conformation). Unfolding of the labeled myosin II caused a 7.3% decrease in the energy transfer ratio. However, ATP binding may also cause some or most of this change. Error bars represent SEM.

Table 1. Actin binding to ^FRLC-^Rmyosin II

Myosin	Ratio
Unphosphorylated myosin	1.44 ± 0.02 SD
Phosphorylated myosin	1.73 ± 0.05 SD
Phosphorylated myosin + actin	1.79 ± 0.11 SD

Actin binding does not produce a large change in the energy transfer ratio. The energy transfer ratio of unphosphorylated ^FRLC-^Rmyosin II, phosphorylated ^FRLC-^Rmyosin II, or phosphorylated ^FRLC-^Rmyosin II (0.3 mg/ml) plus actin (0.3 mg/ml) in ATPase buffer was measured in flat capillary tubes under the Multimode microscope. Note that actin binding caused only a 3.5% change in the energy transfer ratio.

sembles into filaments. Therefore, the 10S-6S conformational change was tested to determine whether it was responsible for some component of the phosphorylation-dependent change in the energy transfer ratio. The energy transfer ratio of 6S unphosphorylated ^FRLC-^Rmyosin II was determined before and after addition of ATP to induce formation of the 10S conformation. Unfolding to the 6S conformation caused a 7.3% decrease in the energy transfer ratio (see Figure 3B), however, effects of folding and effects of ATP-binding to myosin II on the ratio could not be separated. Because ^FRLC-^Rmyosin II phosphorylation was always assayed in the presence of ATP, ATP binding was not a component of the change reported here (in Figure 1B). If unfolding causes a decrease in the energy transfer ratio, then this may suppress the increase of the energy transfer ratio observed with phosphorylation (and subsequent unfolding) of ^FRLC-^Rmyosin II.

Binding of Actin to ^FRLC-^Rmyosin II Has a Minor Effect on the Energy Transfer Ratio

It was also important for interpretation of cellular images to determine whether actin-binding would cause a large change in the energy transfer ratio of ^FRLC-^Rmyosin II. Experiments to assay this were performed in flat capillary tubes with the Multimode microscope (Giuliano *et al.*, 1990; Farkas *et al.*, 1993). To maximize fluorescence emission, ^FRLC-^Rmyosin II was not diluted with unlabeled myosin II in these experiments. Phosphorylated ^FRLC-^Rmyosin II was scanned in the fluorometer with or without F-actin. Unphosphorylated ^FRLC-^Rmyosin II was also imaged in capillary tubes to compare changes in FRET efficiency between unphosphorylated and phosphorylated myosin II detected under the microscope versus the fluorometer. Images were taken of various regions along the capillaries by exciting with a fluorescein excitation filter and then collecting fluorescein and rhodamine emission. The average pixel value was ob-

tained from each image and ratios were generated by dividing the rhodamine emission value by the fluorescein emission value. Results are presented in Table 1. Phosphorylation caused a 20% increase in the ratio of ^FRLC-^Rmyosin II, which is comparable to the change observed in the fluorometer (Figure 1B) in spite of the differences in the way ratios were performed (see MATERIALS AND METHODS). Addition of actin to phosphorylated ^FRLC-^Rmyosin II caused only a 3.5% increase in the energy transfer ratio of ^FRLC-^Rmyosin II.

Cells Containing ^FRLC-^Rmyosin II Treated with a Kinase Inhibitor Have a Lower Ratio than Untreated Cells

Giuliano *et al.* (1992) found that Swiss 3T3 fibroblasts treated with 20 nM staurosporine inhibited both PKC and MLCK phosphorylation of the regulatory light chain. These investigators found that staurosporine treatment caused an immediate rise in the incorporation of phosphate and then a decrease (90 s after treatment) to a phosphorylation level that was half that of untreated cells (and sustained for at least 1 h). Using the conditions of Giuliano *et al.* (1992), this experiment was repeated in individual living cells containing ^FRLC-^Rmyosin II as a test of intracellular function of the phosphorylation biosensor. Data was collected during the time determined by Giuliano *et al.* (1992) where RLC phosphate incorporation was at lowest levels. The average ratio (rhodamine emission/fluorescein emission) of untreated cells was 2.034 ± 0.038 SEM (Figure 4). Cells were perfused with 20 nM staurosporine and then the treated cells were imaged. To avoid photobleaching artifacts, cells on the same coverslip were imaged only one time, either as untreated or as inhibitor treated. The average ratio of staurosporine-treated cells was 1.853 ± 0.057 SEM. Thus, staurosporine treatment produced a statistically significant ratio decrease to a *p* value of <0.01 (as determined by a *t* test), which is expected from a lower level of myosin II regulatory light chain phosphorylation. This change correlates well with the spectroscopic change measured in solution studies (Figure 1B).

Polarized, Migrating Cells Show a Gradient of Activated Myosin II that Is Greatest in the Tail Region

To look at the spatial distribution of myosin II regulatory light chain phosphorylation, a wound healing model system was used to generate polarized, migrating cells. ^FRLC-^Rmyosin II incorporated into the transverse fibers of these cells in a periodic and punctate manner, as previously described for the myosin II fluorescent analogue (DeBiasio *et al.*, 1988; Kolega and Taylor, 1993).

Figure 4. Cells treated with the kinase inhibitor staurosporine have a lower energy transfer ratio than untreated cells. Live cells microinjected with $^{\text{F}}\text{RLC-R}$ myosin II were imaged before ("untreated cells," $n = 18$) or after ("treated cells," $n = 14$) perfusion with 20 nM staurosporine under the Multimode microscope. The average ratio of the rhodamine/fluorescein emission was determined for each cell. Treatment with the kinase inhibitor caused approximately a 9% decrease ($p < 0.01$) in the energy transfer ratio, which suggests a lower level of myosin II regulatory light chain phosphorylation. A biochemical analysis of cells under the same conditions confirmed that staurosporine lowers the level of myosin II regulatory light chain phosphorylation (Giuliano *et al.*, 1992). Error bars represent SEM.

Figure 5 depicts ratio images of four cells along a wound that contain either $^{\text{F}}\text{RLC-R}$ myosin II or control reagents. The average ratio values (rhodamine emission/fluorescein emission) of the front-, middle-, and tail-third regions of each group of cells analyzed were quantitated and results are graphed in Figure 6. To facilitate cell-to-cell comparisons, the ratio value of each section of these cells was normalized to that of the middle region (making the middle ratio 1.0 in every cell).

Figure 5A shows the pseudocolored ratio image of a typical polarized, migrating cell (moving in the direction of the arrow), microinjected with $^{\text{F}}\text{RLC-R}$ myosin II. These cells show a gradient of myosin II phosphorylation that is lowest in the leading edge and highest in the tail. The average ratio values for the front, middle, and tail sections quantitated for the 55 polarized cells analyzed containing the phosphorylation biosensor is shown in Figure 6A. This distribution of phosphorylated myosin II is consistent with the gradient of free calcium and activated calmodulin previously observed in wound healing cells (Hahn *et al.*, 1992; Gough and Taylor, 1993).

Cells along or near the edge of the wound area that have not yet begun to migrate remain unpolarized, characterized by having the nucleus near the center of

the cell and no primary leading edge. Figure 5B is a ratio image of a typical unpolarized cell microinjected with $^{\text{F}}\text{RLC-R}$ myosin II. Unpolarized cells do not have a particular pattern of distribution of phosphorylated myosin II. Figure 6B shows the average ratio value in the "front," "middle," and "tail" third (chosen relative to the wound edge) of each of the unpolarized cells examined.

Polarized, Migrating Cells Containing Control Reagents Exhibit No Gradient

To determine whether the gradient of myosin II phosphorylation was caused by a faster rate of photobleaching of fluorescein than rhodamine or via a non-phosphorylation-induced quenching of fluorescein in the tail region, wound healing cells were injected with two control reagents: a mixture of fluorescein- and rhodamine-labeled 70-kDa dextrans or chemically cross-linked $^{\text{F}}\text{RLC-R}$ myosin II. The ratio image of a typical dextran-injected cell remained relatively uniform and did not exhibit a gradient (see Figure 5C). The average ratio in the front, middle, and tail of the 28 cells investigated is quantified in Figure 6C.

$^{\text{F}}\text{RLC-R}$ myosin II was chemically cross-linked in the folded 10S conformation (Kolega and Taylor, 1993). The biosensor phosphorylation experiment shown in Figure 1 was repeated for the cross-linked phosphorylation biosensor. There was essentially no change in the ratio of the cross-linked phosphorylation biosensor ($1.2 \pm 6.5\%$ SD) in six experiments performed. In vivo, there was no incorporation of the cross-linked phosphorylation biosensor into stress fibers and its distribution was similar to that of a dextran (Kolega and Taylor, 1993). The ratio image of a polarized, migrating cell containing cross-linked $^{\text{F}}\text{RLC-R}$ myosin II is shown in Figure 5D and ratios quantified according to cell region for all 34 cells analyzed are shown in Figure 6D. A slight gradient can be detected but it is much smaller than that observed in experimental cells (compare Figure 6, C and D vs. A). It is possible that some phosphorylation of the cross-linked $^{\text{F}}\text{RLC-R}$ myosin II occurs and this alters the ratio slightly. The ratio change in the experimental cells (Figure 6A) is significantly different from that of the controls ($p < 0.005$, as determined by a *t* test).

Phosphorylation of Myosin II versus Localization of Myosin II

Because the phosphorylation biosensor is both a biologically active fluorescent analogue of myosin II as well as a biosensor of its activity, it can be used to define both the relative concentration, as well as phosphorylation of myosin II. Therefore, a volume indicator (cascade blue 10-kDa dextran) was coinjected into wound healing cells with $^{\text{F}}\text{RLC-R}$ myosin II and either the rhodamine or fluorescein images were divided by

Figure 5. Polarized, migrating cells have a gradient distribution of activated myosin II. Wound healing cells (3–4 h postwounding) were microinjected with $^{\text{F}}\text{RLC}^{\text{R}}$ -myosin II and imaged on the Multimode microscope. Ratio images were generated by dividing the rhodamine image by the fluorescein image, as described in MATERIALS AND METHODS. Images are pseudocolored such that warmer (red) colors represent higher ratio values (= greater myosin II regulatory light chain phosphorylation) and cooler (blue) colors represent lower ratio values (lower myosin II regulatory light chain phosphorylation). The distribution of phosphorylated myosin II is shown as follows: (A) a polarized, migrating cell microinjected with the phosphorylation biosensor, (B) an unpolarized cell along the wound edge microinjected with $^{\text{F}}\text{RLC}^{\text{R}}$ -myosin II, (C) a polarized, migrating cell microinjected with rhodamine- and fluorescein-labeled 70-kDa dextrans, and (D) a polarized, migrating cell microinjected with chemically cross-linked (inactivated) $^{\text{F}}\text{RLC}^{\text{R}}$ -myosin II. Note that only panel A shows a gradient pattern of distribution of the ratio. The arrows indicate the direction of migration of each cell. The “N” denotes the nucleus of each cell. Bar, 10 μm .

the cascade blue images. Figure 7A shows a map of the activated myosin II in this migrating fibroblast cell

(rhodamine image/fluorescein image) and Figure 7B shows a map of the relative concentration of myosin II

Figure 6. Quantitation of the distribution of activated myosin II. Average ratios were quantitated in the front, middle, and tail thirds of all cells studied. Average ratio values for each region of cells analyzed were normalized to the middle region, making the middle ratio equal to 1.0. Each graph shows the average ratio value in these regions of all cells evaluated in the experimental and control groups of Figure 5. (A) Polarized migrating cells microinjected with $^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$ ($n = 55$); (B) unpolarized cells microinjected with $^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$ ($n = 6$); (C) polarized, migrating cells microinjected with rhodamine- and fluorescein-labeled 70-kDa dextrans ($n = 28$); and (D) polarized, migrating cells microinjected with chemically cross-linked (inactivated) $^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$ ($n = 34$). Error bars represent standard deviation of the mean. Note that panel A shows the strongest gradient.

in the same cell (fluorescein image/cascade blue image). Myosin II concentration, relative to the volume indicator, is maximal in the anterior lamellum and the very end of the tail (Figure 7B; Kolega and Taylor, 1993). These are regions of maximum membrane surface area to cell volume ratio (Kolega and Taylor, 1993). Myosin II is maximally activated in the rear half of the cell (Figure 7A). This suggests that regulatory events other than regulatory light chain phosphorylation at serine 19 may be controlling myosin II assembly and that maximal contraction of the cell is occurring in the tail, where calcium and calmodulin activation are also at their greatest levels.

DISCUSSION

Present Biosensor Design

This fluorescent protein biosensor of myosin II regulatory light chain phosphorylation is a reporter of both

the endogenous myosin II localization and activity. The biosensor depends on FRET between fluorescein-labeled regulatory light chains and rhodamine-labeled essential and/or heavy chains. This biosensor has overcome the in vivo spectroscopy limitations of the previously described acrylodan reagent (Post *et al.*, 1994).

FRET has been used with fluorescence microscopy to study protein dynamics (Herman and Fernandez, 1982; Adams *et al.*, 1991). There are several design considerations for constructing FRET-based fluorescent protein biosensors for use in vivo. Donor and acceptor molecules should be positioned (or capable of moving) within 1.0–7.0 nm of each other, however, protein labeling can be difficult within this restricted area. Donor and acceptor fluorophores must have good spectral overlap, yet filters should be chosen that allow good excitation of donor, with little excitation of acceptor. The light source should provide optimal excitation of the donor. Perhaps the biggest problem with applying FRET in vivo is dilution of donor- and acceptor-labeled macromolecules with the endogenous (unlabeled) macromolecules.

An advantage of the labeling approach chosen here for the phosphorylation biosensor is that both dyes of the energy transfer pair are on one protein, which eliminates problems of diffusion of donor and acceptor apart, upon mixing with endogenous protein. Because the myosin II light chains are not covalently bound to the heavy chains, it is feasible that some exchange of labeled light chains with endogenous, unlabeled light chains can occur. However, given the nonphysiological conditions required for in vitro exchange of regulatory light chains (10 mM EDTA, 0.6 M NaCl, heated to 42°C for 30 min) and the time required for incorporation of microinjected fluorescently labeled regulatory light chains into stress fibers of live cells (from minutes to ca. 2 h; Post, unpublished observations), it is unlikely that a significant number of biosensor molecules exchanged the fluorescein-labeled regulatory light chain with endogenous light chains.

The change in FRET efficiency after regulatory light chain phosphorylation may occur from a change in distance between fluorescein on cysteine 108 of the regulatory light chain and rhodamine on cysteines of the essential light chain and/or rhodamine on the heavy chain cysteines of the S2 rod and/or the N-terminal 70-kDa portion of S1 (Hahn *et al.*, 1993). The C-terminal domain of the regulatory light chain is necessary for regulation of the actin-activated ATPase of smooth muscle and scallop myosin II (Rowe and Kendrick-Jones, 1992; Trybus and Chatman, 1993; Ikebe *et al.*, 1994; Jancso and Szent-Györgyi, 1994; Trybus *et al.*, 1994). Thus, phosphorylation of the N-terminal domain of the regulatory light chain at the head/rod junction must transmit a signal from the

Figure 7. Myosin II phosphorylation vs. myosin II localization. Wound healing cells were coinjected with $^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$ and a volume indicator (cascade blue 10-kDa dextran). Ratio images shown are as follows: (A) the ratio of the rhodamine emission divided by fluorescein emission and (B) the ratio of the fluorescein emission divided by cascade blue emission (division of rhodamine emission by cascade blue emission gave the same result). As in Figure 5, these ratio images have been pseudocolored such that warmer (red) colors represent higher levels of myosin II phosphorylation (A) or myosin II distribution relative to cell volume (B) and cooler, blue colors represent lower levels. The "N" denotes the nucleus of each cell. Bar, 8 μm .

regulatory light chain, down the heavy chain to the ATP and actin binding sites of myosin II. This signal may be mediated by the C-terminal domain of the regulatory light chain via contacts with the heavy chain in this region (Ikebe *et al.*, 1994).

Also nearby the C-terminal region of the regulatory light chain is the N-terminal domain of the essential light chain, which abuts (skeletal muscle myosin II; Rayment *et al.*, 1993) or actually contacts, via hydrogen bonds, (scallop myosin II; Xie *et al.*, 1994) the regulatory light chain. One can speculate that movement of the C-terminal domain of the regulatory light chain caused by N-terminal phosphorylation would change the energy transfer efficiency to nearby regions of the heavy chain and/or essential light chain. Further use of mutants where specific dye locations can be controlled will optimize reagent design and construction.

Biosensor Characterization

Previous studies have shown that neither labeling myosin II with rhodamine according to the protocol used here nor exchanging a fluorescently labeled regulatory

light chain affects the major biological activity of myosin II. The rhodamine-myosin II analogue retains the ability to polymerize and to shift from a folded (10S) to extended (6S) conformation. The rates of K^+EDTA ATPase activity and of regulatory light chain phosphorylation do not differ significantly from those of native myosin II; the analogue retains phosphorylation-dependent activation of the actin-activated MgATPase, with activity remaining 60–100% that of unlabeled myosin II (Hahn *et al.*, 1993). Both $^{\text{F}}\text{RLC-}^{\text{R}}\text{myosin II}$ and rhodamine-myosin II incorporated in a periodic and punctate manner into stress fibers (DeBiasio *et al.*, 1988; Kolega and Taylor, 1993). Gizzard myosin II exchanged with other fluorescently labeled regulatory light chains has also been biochemically characterized (Morita *et al.*, 1991; Post *et al.*, 1994). This exchanged myosin II has biochemical properties very similar to that of untreated myosin II; most importantly, the exchanged myosin II retains phosphorylation-dependent activation of the actin-activated MgATPase activity. Assays of kinase specificity (Figure 2), filament assembly (Figure 3A), folding/

unfolding (Figure 3B), and actin binding (Table 1) of the phosphorylation biosensor described here suggest that the change in energy transfer efficiency observed here is largely from a conformational change that occurs between the regulatory light chain and the rest of the labeled myosin II after MLCK phosphorylation.

Intracellular Distribution of Phosphorylated Myosin II

The gradient of phosphorylated myosin II in wound healing cells corresponds to gradients of free calcium and activated calmodulin previously observed in wound healing fibroblasts (Hahn *et al.*, 1992; Gough and Taylor, 1993) as well as calcium gradients in polarized eosinophils (Brundage *et al.*, 1991) and free living amoeba (Taylor *et al.*, 1980a). In all these amoeboid cells, free calcium is low in the leading edge and high in the tail. Therefore, a calcium-calmodulin-dependent phosphorylation of the regulatory light chain of myosin II can explain the results. The gradient of phosphorylated myosin II runs opposite to gradients previously observed of actin assembly (Giuliano and Taylor, 1994) and myosin II assembly (Kolega and Taylor, 1993) relative to cell volume in wound healing fibroblasts. Both actin and myosin II incorporate into transverse fibers, which form at the base of lamellipodia and are then transported toward the perinuclear region of migrating cells. As these fibers transport rearward, they contract and disassemble (Conrad *et al.*, 1993; Kolega and Taylor, 1993; Giuliano and Taylor, 1994). Actin shows maximal assembly in the peripheral cortex at the cytoplasm/membrane interface at the leading edge of lamellipodia, while myosin II shows maximal assembly in the transverse fibers at the base of lamellipodia. Both proteins exhibit a decrease in assembly in the noncortical cytoplasm of the perinuclear region, based on photobleaching results (Kolega and Taylor, 1993; Giuliano and Taylor, 1994). Three-dimensional imaging reveals fibers of both proteins in the cell cortex, suggesting a cortical contraction coupled to a solation at the site of maximal contraction (Janson and Taylor, 1993; Kolega and Taylor, 1993; Giuliano and Taylor, 1994).

The fiber dynamics observed and the distribution of calcium, activated calmodulin, and phosphorylated myosin II support the role of solation-contraction coupling in the locomotion of these cells (Taylor *et al.*, 1980a,b; Taylor and Fechheimer, 1982; Janson *et al.*, 1991; Janson and Taylor, 1993; Kolega *et al.*, 1993; Kolega and Taylor, 1993). Here, a solation of the actin-gel structure in the tail cortex would allow for maximal contractility in the presence of a maximally activated motor (Janson and Taylor, 1993). As transverse fibers, as well as the less structured cortex, approach the region of elevated calcium and activated calmodulin, calcium-activated actin severing proteins (such as

Figure 8. Model of the role of phosphorylation of myosin II in locomoting fibroblasts. Calcium (Hahn *et al.*, 1992), activated calmodulin (Gough and Taylor, 1993; Hahn *et al.*, 1993), and phosphorylated myosin II are at highest concentrations in the tail of locomoting fibroblasts and at lowest concentrations toward the leading edge. Myosin II is maximally assembled at the base of lamellipodia (Kolega and Taylor, 1993), while actin is maximally assembled at the leading edge (Wang, 1985; DeBiasio *et al.*, 1988; Giuliano and Taylor, 1994). Actin/myosin II transverse fibers are transported rearward (Conrad *et al.*, 1993), contracting as they enter regions of elevated calcium, activated calmodulin, and (presumably) activated MLCK. Solation of the actin gel from increased activity of some actin-severing proteins (gelsolin) and reduced affinity of some actin-bundling proteins (α -actinin) in the tail, where calcium levels are elevated, in combination with the increased activity of the phosphorylated myosin II motor, may allow for maximum contraction in this region (designated by arrows). These events may help locomoting cells to maintain their polarized shape, retract their tails as lamellipodia move forward, and generate a positive hydrostatic pressure in the tail to help deliver disassembled fiber subunits to the leading edge for incorporation into new fibers.

gelsolin) and the lowered affinity of some actin-binding proteins (such as α -actinin) weaken the gel strength, while calcium/calmodulin-activated MLCK could phosphorylate the regulatory light chain, thus increasing the strength of the motor protein (Figure 8). The cortical-tail contractions do not result in a dense concentration of actin and myosin II. Hence the self-destruct mechanism, which is a central part of the solation-contraction coupling hypothesis, can generate subunits of actin and myosin II for delivery to the front of the cell (Janson and Taylor, 1993; Kolega and Taylor, 1993).

A phosphorylation biosensor that is phosphorylated at both the PKC and MLCK sites demonstrates a 10% ratio increase (Figure 2). It is possible that at least some of the myosin II in either the tail or the leading lamella is phosphorylated at both of the kinase sites. The effects of phosphorylation at both of these sites in vivo are uncertain. Several groups have demonstrated that phosphorylation at the PKC and MLCK sites of myosin II inhibits the actin-activated Mg^{2+} ATPase

activity because it increases the K_m of the myosin II for actin, with little effect on the maximum rate of the actin-activated Mg^{2+} ATPase activity (Nishikawa *et al.*, 1984; Bengur *et al.*, 1987; Ikebe *et al.*, 1987; Ikebe and Reardon, 1990). However, Okagaki *et al.* (1991) demonstrated that gizzard myosin II phosphorylated at the MLCK and PKC sites moved actin filaments in an in vitro motility assay at the same velocity as myosin phosphorylated only at the MLCK sites. These authors propose that the sliding velocity is specified by the V_{max} of the actin-activated Mg^{2+} ATPase and not the affinity for actin. However, the solation-contraction hypothesis predicts that the site of maximal activation must also involve the beginning of inactivation of myosin II to allow 10S myosin II to be transported to the front of the cell after contraction (Janson and Taylor, 1993; Kolega and Taylor, 1993).

What Drives Myosin II Filament Assembly at the Base of Lamellipodia?

If myosin II regulatory light chains are maximally phosphorylated by MLCK in the tail of locomoting fibroblasts, yet the myosin II is maximally assembled into filaments at the base of lamellipodia, then what drives myosin II filament assembly in transverse fibers at the base of lamellipodia (DeBiasio *et al.*, 1988; Fisher *et al.*, 1988; Conrad *et al.*, 1993; Kolega and Taylor, 1993)? The prevailing dogma, from in vitro studies, states that smooth muscle and nonmuscle myosin II regulatory light chain phosphorylation by a calcium-calmodulin-regulated MLCK induces filament assembly (Suzuki *et al.*, 1978; Trybus, 1991a). However, it has been demonstrated that the free calcium concentration and calmodulin activation are minimal at the leading edge of migrating cells (Hahn *et al.*, 1993). In addition, myosin II "assembles" into semi-sarcomeric arrays in stress fibers that constitutively form, transport, and disassemble in serum-deprived fibroblasts where the free calcium ion concentration is low (ca. 10^{-8} M) (Giuliano and Taylor, 1990, 1994; Kolega and Taylor, 1993). Therefore, free calcium and calcium/calmodulin-based regulation does not appear to be necessary for some forms of myosin II assembly, while it is important for contraction.

Separate mechanisms may be involved in myosin II assembly and full activation of the motor. Perhaps a currently undefined calcium-independent phosphorylation might induce assembly and/or local high concentrations of actin or myosin II-binding proteins could help drive assembly of unphosphorylated or minimally phosphorylated myosin II. For instance, cellular titin has been found to be localized to stress fibers in fibroblast cells and evidence suggests that it helps to organize myosin II bipolar filaments in vivo (Eilertsen *et al.*, 1994). Furthermore, in vitro studies demonstrated that actin can mediate filament assem-

bly of *Dictyostelium* (Mahajan *et al.*, 1989) and gizzard myosin II (Applegate and Pardee, 1992). The local actin filament concentration is very high in the base of lamellipodia where myosin II appears to assemble into transverse fibers (DeBiasio *et al.*, 1988; Fisher *et al.*, 1988; Giuliano and Taylor, 1994). The potential of separate mechanisms controlling myosin II assembly and maximal motor activity must be explored.

Future Phosphorylation Biosensors

Although the in vivo phosphorylation biosensor described here is markedly improved over the previously described acrylodan biosensor in that it can now be used to map the in vivo dynamics of myosin II regulatory light chain phosphorylation (Post *et al.*, 1994), there can be important improvements. For instance, the rapid rate of photobleaching of fluorescein relative to that of rhodamine produces artifacts in ratio images of the two fluorophores when fluorescein is excited multiple times, requiring extensive corrections of images. For this reason, the present study limited the excitation of the donor and sacrificed temporal analyses. Efforts are underway to design a more photostable sulfhydryl-reactive donor to rhodamine through modification of cyanine and other dyes (Ernst *et al.*, 1989). Also, the phosphorylation-dependent change in energy transfer efficiency is not large (Figure 1B), limiting intracellular calibrations of actual levels of regulatory light chain phosphorylation. Future phosphorylation biosensors will use information in the recently published crystal structure of myosin II (Rayment *et al.*, 1993; Xie *et al.*, 1994) and combine the approaches of strategically placing fluorophores through genetic engineering of cysteine residues with the sensitivity of fluorescence resonance energy transfer to target protein regions predicted to undergo large conformational changes. The recent success of expressing enzymatically active heavy meromyosin in a baculovirus system may now allow molecular genetic manipulation of cysteines on the essential light chains and heavy chains (Trybus, 1994). The goal is to produce a highly sensitive phosphorylation biosensor of myosin II that can be used in conjunction with other cytoskeletal probes and biosensors to examine the temporal and spatial dynamics of motor mechanisms generated during cell locomotion and division throughout the entire cell cycle.

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