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Myosin II Phosphorylation and the Dynamics of Stress Fibers in Serum-Deprived and Stimulated Fibroblasts

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The actin-based cytomatrix generates stress fibers containing a host of proteins including actin and myosin II and whose dynamics are easily observable in living cells. We developed a dual-radioisotope-based assay of myosin II phosphorylation and applied it to serum-deprived fibroblasts treated with agents that modified the dynamic distribution of stress fibers and/or altered the phosphorylation state of myosin II. Serum-stimulation induced an immediate and sustained increase in the level of myosin II heavy chain (MHC) and 20-kDa light chain (LC20) phosphorylation over the same time course that it caused stress fiber contraction. Cytochalasin D, shown to cause stress fiber fragmentation and contraction, had little effect on myosin II phosphorylation. Okadaic acid, a protein phosphatase inhibitor, induced a delayed but massive cell shortening preceded by a large increase in MHC and LC20 phosphorylation. Staurosporine, a kinase inhibitor known to effect dissolution but not contraction of stress fibers, immediately caused an increase in MHC and LC20 phosphorylation followed within minutes by the dephosphorylation of LC20 to a level below that of untreated cells. We therefore propose that the contractility of the actin-based cytomatrix is regulated by both modulating the activity of molecular motors such as myosin II and by altering the gel structure in such a manner as to either resist or yield to the tension applied by the motors.

INTRODUCTION

Lewis and Lewis (1924) described the dynamics of the earliest tissue culture cells and concluded that their rate of migration was inversely related to the consistency of the semifluid cytoplasmic gel. They believed that temporary cytoplasmic structures termed longitudinal striae or fibrillae appeared along the lines of tension where a contractile substance had coagulated. Furthermore, the authors proposed that if these structures were produced by tension, then their disappearance was a result of altered tension or relaxation. Using a light and electron microscopic approach, Buckley and Porter (1967) further described longitudinal striae, stress fibers as we now know them, in living cells as phase dark linear structures whose width ranges from 0.15 to 1.0 μm. These fibers have been shown to contain bundles of actin filaments (Ishikawa *et al.*, 1969; Sanger and Sanger, 1980). The cortical cytoplasm contains a rich meshwork of these filaments not resolvable at the light microscopic level (Buckley and Porter, 1967; Hartwig and Shevlin, 1986).

Stress fibers are most prominent in well-attached, nonlocomoting cells and their appearance is attributed to the development of tension between cytoplasmic actin and the site of substrate adhesion (Burridge, 1981). Actin, myosin II, and a host of other proteins are distributed in a pseudosarcomeric arrangement along the length of stress fibers (Sanger *et al.*, 1984; Amato and Taylor, 1986; Langanger *et al.*, 1986; Mittal *et al.*, 1987) and potentially act as part of a contractile complex to generate cytoplasmic tension. Stress fibers, therefore, are specialized, highly ordered, cytoskeletal structures whose properties are probably very similar to the underlying, less ordered, cytoplasmic meshwork from which they arise.

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We take advantage of the fact that stress fibers are easily resolved in the light microscope and use them as tools within living cells to study the dynamics of the actin-based cytomatrix (Giuliano and Taylor, 1990) and to probe regulation and dynamics of contraction (Kolega *et al.*, 1991). The model system of choice for these studies is the serum-deprived fibroblast. This flattened, stationary cell exhibits an extensive array of dynamic stress fibers that form constitutively at the cytoplasm-membrane interface and are slowly transported toward the perinuclear region where they disappear. Furthermore, serum-stimulation causes many stress fibers to contract (Giuliano and Taylor, 1990).

The role that 20-kDa light-chain phosphorylation plays in the regulation of myosin II filament formation, actin-activated Mg^{2+}-ATPase activity, and contractility in conjunction with actin has been extensively studied in smooth muscle (Sellers and Adelstein, 1987; Itoh *et al.*, 1989; Trybus, 1991; Sellers, 1991) and, although not as completely, in nonmuscle cells (Bayley and Rees, 1986; Kawamoto *et al.*, 1989; Ludowyke *et al.*, 1989) and permeabilized cell models (Holzapfel *et al.*, 1983; Masuda *et al.*, 1984; Cande and Ezzell, 1986; Wysolmerski and Lagunoff, 1991). The physiological role of myosin II heavy-chain phosphorylation in smooth and nonmuscle systems is unclear, but data accumulate describing the site of phosphorylation and the type of kinase involved (Kawamoto and Adelstein, 1988; Kamm *et al.*, 1989; Kawamoto *et al.*, 1989; Ludowyke *et al.*, 1989; Sellers, 1991). Conti *et al.* (1991) pointed out a unique property of human platelet myosin II heavy chains by showing that the single site phosphorylated by protein kinase C is absent in vertebrate smooth muscle myosin II heavy chains. Kinetic changes in the phosphorylation of heavy and 20-kDa light chains have been shown to parallel each other and are correlated with histamine release in rat basophilic leukemia cells (Ludowyke *et al.*, 1989).

Recent work supports the notion that a minimal level of myosin II 20-kDa light-chain phosphorylation is required to maintain stress fiber integrity (Lamb *et al.*, 1988; Fernandez *et al.*, 1990). Cell permeant inhibitors of myosin light-chain kinase induce the dissolution of stress fibers in serum-deprived cells (Kolega, Giuliano, and Taylor, unpublished data). The loss of phosphate from the 20-kDa light chains of myosin II is accompanied by the rapid splaying of stress fibers into their constituent filaments. We also presented evidence suggesting that the dynamic structure of the actin-based cytomatrix is an important regulator of stress fiber integrity and movement, thus supporting the main tenet of the solation-contraction coupling hypothesis (Janson *et al.*, 1991; Kolega *et al.*, 1991).

The potential importance of myosin II phosphorylation to the maintenance of both the actin-based cytomatrix and contractility encouraged us to measure the phosphorylation state of myosin II in the serum-deprived cell model system under conditions where we have detailed information concerning the dynamic distribution of stress fibers. We developed and report on here a novel dual-radioisotope–based assay of temporal changes in the phosphorylation state of cellular myosin II heavy chains and 20-kDa light chains. Our data suggest that variations in the phosphorylation level of myosin II and the gel state of the actin-based cytoskeleton act in concert to regulate cytoplasmic structure and contractility.

MATERIALS AND METHODS

Materials

A polyclonal antibody raised against human platelet myosin II (BT-561) was obtained from Biomedical Technologies, Inc. (Stoughton, MA) and was used undiluted. We purchased TRAN^{35}S-LABEL, a mixture consisting of 70% ^{35}S-L-methionine, 15% ^{35}S-L-cysteine, and various nonlabeled amino acids (^{35}S-amino acids), specific activity 1000–1200 Ci/mmole and carrier-free ^{32}P-orthophosphate from ICN Biomedicals (Irvine, CA). Okadaic acid and the protein kinase inhibitor KT5926 were from Calbiochem (La Jolla, CA). Protein A immobilized on Sepharose 4B (P-9424), cytochalasin D, Sephadex G-25, and staurosporine were obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals were reagent grade.

Cell Culture

Swiss 3T3 fibroblasts (ATCC No. CCL92; passage 121–128) were cultured in Dulbecco's modified Eagle's medium (DMEM) plus 10% calf serum at 37°C in a humidified, 5% CO_2 atmosphere. Petri dishes (60 mm) were seeded with 1×10^5 cells in DMEM plus 10% calf serum. For myosin II phosphorylation assays the culture medium was changed the next day to 2 ml of modified essential medium containing no phosphate salts or L-methionine plus 0.2% calf serum. At this time we added either 100 μCi ^{32}P-orthophosphate or 100 μCi ^{35}S-amino acids or a mixture of both to each culture dish. Cells incubated this way for 48 h were subconfluent and considered serum deprived. The 2-d labeling period was required for uniform and reproducible results because we found the rate of incorporation of radioisotopes by serum-deprived cells to be very low. Cells were treated with serum or drugs in the presence of radioisotopes for the time periods indicated in the figure legends. For experiments requiring microinjection of the fluorescent analogue of myosin II, cells were cultured as described by Giuliano and Taylor (1990).

Microscopy

Cells microinjected with the acetamido-tetramethylrhodamine analog of smooth muscle myosin II (AR-myosin II; DeBiasio *et al.*, 1988) were allowed to recover for 1.5 h before an experiment. Cells were observed with the multimode microscope (Giuliano and Taylor, 1990; Giuliano *et al.*, 1990) using a Plan 100/1.25 objective and a sampling frequency of 2.5 min. The results presented here represent only a few images from multiframe time-lapse movie loops.

Immunoprecipitation of Myosin II

Immunoprecipitation of myosin II from untreated and serum or drug-treated radiolabeled cells was based on the method of Ludowyke *et al.* (1989). After treatment, Petri dishes were drained of medium and, without rinsing, the cells scraped with 2 washes of 100 μl each of ice-cold immunoprecipitation buffer (25 mM tris(hydroxymethyl)-aminomethane [Tris], pH 8.1, 1% (wt/vol) NP-40, 100 mM sodium

pyrophosphate, 100 mM NaF, 250 mM NaCl, 10 mM ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid [EGTA], 5 mM EDTA, 1 mM dithiothreitol, 10 μg/ml leupeptin, 1 μM aprotinin and pepstatin, and 1 mM phenylmethylsulfonylfluoride). We centrifuged the samples for 12 min at 10 000 × *g* and 4°C to pellet nuclei and insoluble material, carefully removed the supernatant, and incubated it for 2 h at 4°C with 20 μl of anti-myosin II. The entire mixture was then incubated with 100 μl of a 50% slurry of protein A immobilized on Sepharose 4B for 1 h at 4°C. We washed the beads twice with 750 μl ice-cold immunoprecipitation buffer and then once with 750 μl ice-cold water. Immunoadsorbed proteins were then solubilized and subjected to electrophoresis. This immunoprecipitation method results in the recovery of 70–90% of the myosin II heavy chain from whole rat basophilic leukemia cells (Ludowyke *et al.*, 1989).

One-Dimensional Gel Electrophoresis

Proteins adsorbed to protein A beads were solubilized by boiling for 1 min in 100 μl of a buffer containing 60 mM Tris, pH 8.8, 2% (wt/vol) sodium dodecyl sulfate (SDS), 10% (wt/vol) glycerol, 5 mM dithiothreitol, 0.005% (wt/vol) bromophenol blue, and 25 μg smooth muscle myosin II. We separated the proteins using the acrylamide slab gel system described by Pollard (1982). The diallyltartardiamide-cross-linked gels resolved both the heavy and light chains of myosin II. After electrophoresis, gels were stained and prepared for either fluorography or liquid scintillation counting. For liquid scintillation counting, the bands corresponding to the heavy and light chains of smooth muscle myosin II were cut out of the gel and solubilized by continuous mixing in 1 ml of 2% (wt/vol) sodium m-periodate solution for 1 h at room temperature (Springer, 1991). Ten milliliters of BioSafe II (Research Products International Corp., Mt. Prospect, IL) was added to the samples before liquid scintillation counting.

Two-dimensional Gel Electrophoresis

Proteins adsorbed to protein A beads were solubilized for 1 h at room temperature in 100 μl of a buffer containing 50 mM Tris, pH 8.5, 8 M urea, 2% (wt/vol) NP-40, 5 mM dithiothreitol, 25 μg of smooth muscle myosin II, and 0.005% (wt/vol) methyl red. The samples were loaded onto cylindrical isoelectric focusing gels (2.5 × 110 mm) containing 5%T 2.7%C acrylamide-bisacrylamide, 9 M urea, 2% (wt/vol) NP-40, 0.4% (vol/vol) 3–10 ampholyte, 1.6% (vol/vol) 4–6 ampholyte, 60 mM 3-[*N*-morpholino]propane-sulfonic acid, 0.05% (wt/vol) ammonium persulfate, and 0.07% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine. We electrophoresed the gels for 18–20 h at 0.1 W per gel tube using 0.085% phosphoric acid as the anolyte and 20 mM NaOH as the catholyte. This system provides enhanced separation of acidic proteins like the light chains of myosin II (Tindall, 1986). After electrofocusing, the gels were incubated for 30 min in 20% (vol/vol) acetic acid followed by 3 more 30 min incubations in 47.5% (vol/vol) ethanol. Gels were then stored in 15% (vol/vol) ethanol at 4°C for up to 1 mo. We incubated the isoelectric focusing gels for 30 min in buffer containing 125 mM Tris, pH 6.8, 25% (wt/vol) glycerol, 0.5% (wt/vol) SDS, 5 mM dithiothreitol, and 0.005% (wt/vol) bromophenol blue before second-dimension electrophoresis on 12.5%T 2.7%C acrylamide-bisacrylamide gels (Giuliano, 1991). After electrophoresis, gels were stained with Coomassie blue R-250, soaked in Fluoro-Hance autoradiography enhancer (Research Products International Corp.), dried, and subjected to fluorography with X-ray film.

Phosphopeptide Mapping of the 20-kDa Light Chain

Myosin II immunoprecipitates from untreated and serum or drug-treated ^{32}P-labeled cells were subjected to one-dimensional SDS-gel electrophoresis as described above. The 20-kDa light chain was cut from the gel, homogenized in 300 μl water using a disposable pestle (Kontes, Vineland, NJ), quickly frozen in liquid nitrogen, and lyophilized. The resulting powder was rehydrated with 500 μl of 50 mM ammonium bicarbonate solution, pH 8.5, containing 5 μg sequencing grade trypsin (Promega V511A; Madison, WI) and incubated at 37°C for 20–24 h. The solution was briefly centrifuged and the supernatant plus two 900-μl water washes of the remaining gel pieces were combined and lyophilized. The resulting peptides were solubilized in the urea buffer described above for isoelectric focusing and loaded onto an isoelectric focusing slab gel containing 10%T 3.75%C acrylamide-diallyltartardiamide, 8 M urea, 2% (wt/vol) NP-40, 0.7% (vol/vol) 3–10 ampholyte, 1.3% (vol/vol) 4–6 ampholyte, 20 mM 3-[*N*-morpholino]propane-sulfonic acid, 0.05% (wt/vol) ammonium persulfate, and 0.07% (vol/vol) *N,N,N',N'*-tetramethylethylenediamine. We electrophoresed the gel for 2 h at 10 W using 0.085% phosphoric acid (4°C) as the anolyte and 20 mM NaOH as the catholyte. After electrofocusing, the gel was dried and subjected to fluorography with X-ray film. This one-dimensional isoelectric focusing system resolved the trypsin-generated phosphopeptides of the 20-kDa light chains into species with isoelectric points ranging from 3.6 to 7.5 (Nakabayashi *et al.*, 1991).

Preparation of Quenched ^{35}S and ^{32}P Standard Solutions

We used the specific activity data from the radioisotope manufacturer to prepare a set of 10 standard solutions containing 1.69 × 10^6 dpm ^{35}S-amino acids or 5.53 × 10^5 dpm ^{32}P-orthophosphate in 10 ml BioSafe II (Research Products International Corp.). Nine samples from each set were quenched with 10–90 μl of nitromethane. A quench curve for each isotope was constructed with a Beckman (Beckman Instruments, Fullerton, CA) model LS 5801 liquid scintillation counter using the external quench monitor, H* method (Horrocks, 1964).

Preparation and Separation of ^{32}P-Labeled Phosphoproteins from Whole Cell Extracts

Serum-deprived cells were labeled with ^{32}P-orthophosphate as described above, subjected to serum and drug treatments, and harvested by scraping with 150 μl of buffer containing 120 mM Tris, pH 8.8, 2% (wt/vol) SDS, 20% (vol/vol) glycerol, 10 mM dithiothreitol, and 0.005% (wt/vol) bromophenol blue. The whole cell extract was placed into a Sephadex G-25-based sample microconcentrator (Saul and Don, 1984) and eluted with an additional 100 μl of cell scraping buffer that was diluted twofold. The microconcentrator significantly reduced the viscosity of whole cell extracts, probably by shearing the DNA, making them easier to handle. We boiled the protein samples for 1 min and loaded 2 × 10^5 dpm into each well of an SDS-gel. After electrophoresis, the gels were stained and subjected to autoradiography.

RESULTS

Immunoprecipitation of Myosin II from Swiss 3T3 Cells

Figure 1 shows a representative fluorogram of a one-dimensional SDS-gel containing the radiolabeled proteins immunoprecipitated from permeabilized, serum-deprived Swiss 3T3 fibroblasts using a polyclonal antiserum to nonmuscle myosin II. Proteins comigrating with the heavy and light chains of smooth muscle myosin II and skeletal muscle actin were the prominent species found in the ^{35}S-labeled immunoprecipitate. The major proteins in the ^{32}P-labeled immunoprecipitate were a high molecular weight band and a closely spaced doublet that comigrated with the heavy and 20-kDa light chains of smooth muscle myosin II, respectively.

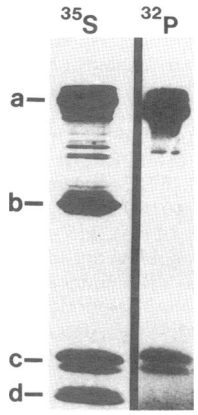

Figure 1. Immunoprecipitation of myosin II from Swiss 3T3 fibroblasts: one-dimensional SDS-PAGE analysis. Proteins immunoprecipitated from cells labeled with either ^{35}S-amino acids or ^{32}P-orthophosphate were solubilized and subjected to one-dimensional SDS-PAGE. A fluorogram of the gel is presented to demonstrate the specificity of the antibody. Denoted are the migration positions of smooth muscle myosin heavy chains (a), 20-kDa (c), and 17-kDa (d) light chains, and the migration position of rabbit skeletal muscle actin (b).

The two-dimensional gel analysis of the immunoprecipitated proteins revealed a heterogeneous distribution of the nonmuscle myosin II light chains (Figure 2). The ^{35}S-labeled immunoprecipitate consisted of a mixture of species migrating near the positions of the unphosphorylated and phosphorylated 20-kDa light chains of smooth muscle myosin II and at least four species of lower molecular weight proteins of which only one comigrated with the 17-kDa light chains of smooth muscle myosin II.

Liquid Scintillation Counting of Dual-Labeled Samples

The average particle energies of the β-radiation emitted from ^{35}S and ^{32}P are different enough to permit quantitation of each in a gel slice containing both isotopes using a liquid scintillation counter. We separated the signal produced by each isotope and corrected for the amount of signal quench present in each sample by first dividing the liquid scintillation counting window into upper and lower parts and measuring the counting efficiency of standard solutions of each isotope in both windows under varying conditions of signal quench (Figure 3). The activity of ^{32}P in a mixed isotope gel slice was determined directly from the quench-corrected counts in the upper window because the counting efficiency of ^{35}S in this window was zero. The contribution of ^{32}P to the sum of the quench-corrected ^{32}P and ^{35}S activities in the lower window was then subtracted to yield the activity of ^{35}S in the lower window. We calculated the specific activity of a mixed isotope protein sample by dividing the activity (in dpm) of ^{32}P by the activity of ^{35}S. This ratio, when determined for both the heavy and 20-kDa light chains of myosin II immunoprecipitated from untreated serum-deprived cells, fell within the range 4.00×10^{-3} to 9.00×10^{-3}. To make comparison of data from separate experiments easier, we normalized the specific activity values obtained from

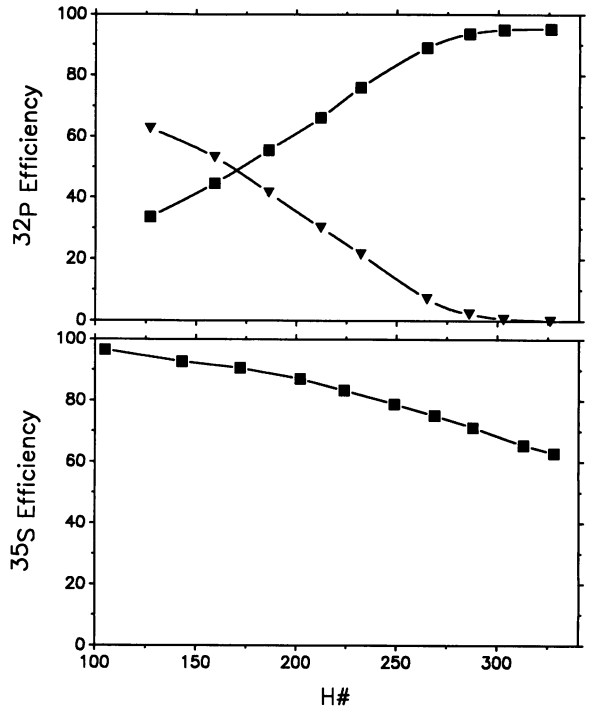

Figure 3. Liquid scintillation signal separation and quench correction of samples containing a mixture of ^{35}S-amino acids and ^{32}P-orthophosphate. We prepared a set of quenched ^{35}S and ^{32}P standards as described in MATERIALS AND METHODS and assayed them in a liquid scintillation counter using a lower window setting of 0–670 (■) and an upper window setting of 670–1000 (▲). Counting efficiencies are plotted as a function of H*, the quench detection system used by the instrument, for ^{32}P (top) or ^{35}S (bottom). The counting efficiency for ^{35}S in the upper window was zero in all cases whereas the counting efficiency for ^{32}P was greatest in this window under conditions of minimal quench. The H* values for solubilized gel slices fell within the range 130–150.

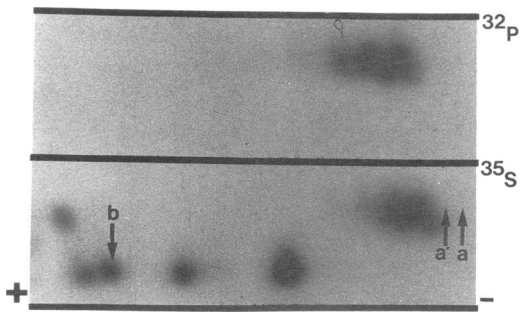

Figure 2. Immunoprecipitation of myosin II from Swiss 3T3 fibroblasts: two-dimensional PAGE analysis. Proteins immunoprecipitated from cells labeled with either ^{32}P-orthophosphate (upper gel) or ^{35}S-amino acids (lower gel) radioisotopes were solubilized and subjected to two-dimensional PAGE. Fluorograms of gel regions containing the myosin light chains are presented with the acidic (+) and basic (−) ends of the isoelectric focusing gel denoted. The migration positions of the unphosphorylated (a) and phosphorylated (a') 20-kDa light chains and 17-kDa light chains (b) of smooth muscle myosin II are denoted and were used to align the two gels.

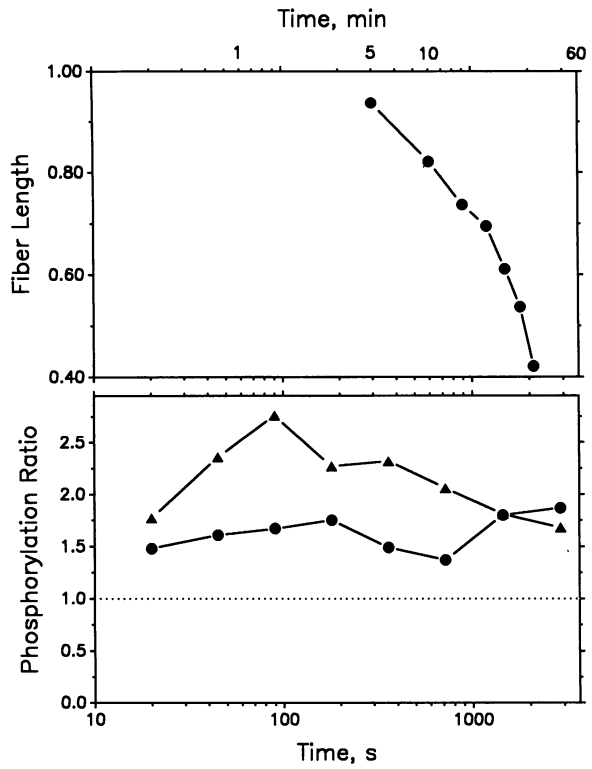

Figure 4. Serum modulates the phosphorylation ratio of myosin II and stress-fiber shortening in serum-deprived fibroblasts. We treated dual-isotopically labeled serum-deprived fibroblasts with 10% calf serum for various times. Top: The fractional length of a fluorescently labeled stress fiber (Giuliano and Taylor, 1990) continually decreased over the same time course that serum induced an increase in the phosphorylation ratio of myosin II. Bottom: The phosphorylation ratio of the myosin II heavy chains (●) and 20-kDa light chains (▲) at various times after treatment. The data are a representative set of five separate experiments.

treated cells to the average value of the specific activity obtained from untreated cells during the same experiment and called this the phosphorylation ratio.

Serum-Stimulated Cell Model

The addition of serum to serum-deprived cells causes an immediate and sustained cytoskeletal reorganization wherein many stress fibers shorten in such a manner as to suggest a contraction (Giuliano and Taylor, 1990). Figure 4 (top) shows the kinetics of shortening for a single fiber. Shortening begins immediately upon serum-stimulation and continues for >1 h, but kinetic data are collected every 5 min to minimize cellular photodamage and fluorophore photobleaching (Giuliano and Taylor, 1990). Serum treatment resulted in a rapid and sustained increase in the phosphorylation ratio of the heavy and 20-kDa light chains of myosin II (Figure 4, bottom). The phosphorylation ratio of the 20-kDa light chains rose within seconds to a level three times that of the initial value followed by a slow decay during the next 45 min.

Time Course of Myosin II Phosphorylation by Other Effectors of Cytoskeletal Structure

Cytochalasin D. Cytochalasin D treatment of serum-deprived cells causes a solation of the cytoplasmic gel structure resulting in the contraction of stress fibers in serum-deprived cells (Kolega *et al.*, 1991). Figure 5 shows that cytochalasin D caused a relatively small variation in the phosphorylation ratio of both the heavy or 20-kDa light chains of myosin II over the time course that the same dose of the drug causes stress-fiber contraction.

The data in Figure 5 also describe the accuracy with which we measure the phosphorylation ratio during an experiment. Phosphorylation ratio values between 0.75 and 1.2 are characteristic of the data scatter within all of the experiments described here and are considered to represent insignificant changes in myosin II phosphorylation.

Okadaic Acid. Okadaic acid, a potent tumor-promoting protein phosphatase inhibitor, caused the phosphorylation ratio of the heavy and 20-kDa light chains of myosin II to immediately increase to a level similar to that induced by serum treatment (Figure 6, top). In contrast to serum treatment, the early elevation of the phosphorylation ratio did not persist, but fell to a level < 75% that of pretreatment levels within 15 min. After the drop, there was an abrupt transient increase in the phosphorylation ratio of both the heavy and 20-kDa light chains of myosin II. The second phosphorylation ratio peak was 2.5–3 times that induced by serum treat-

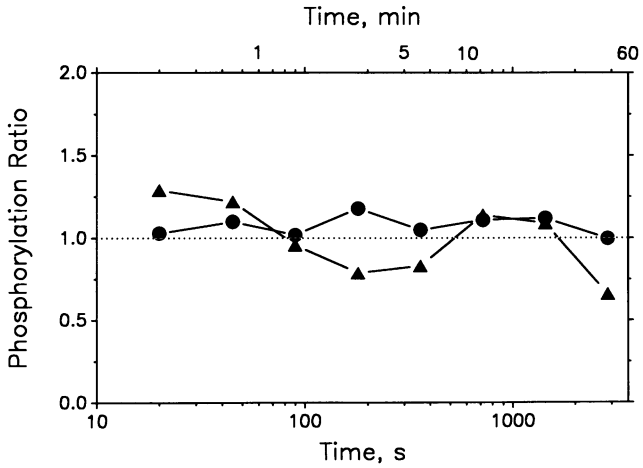

Figure 5. Cytochalasin D has little effect on the phosphorylation ratio of myosin II. We treated dual-isotopically labeled serum-deprived fibroblasts with 0.5 μM cytochalasin D and measured the phosphorylation ratio values of the myosin II heavy chains (●) and 20-kDa light chains (▲) at various times after treatment.

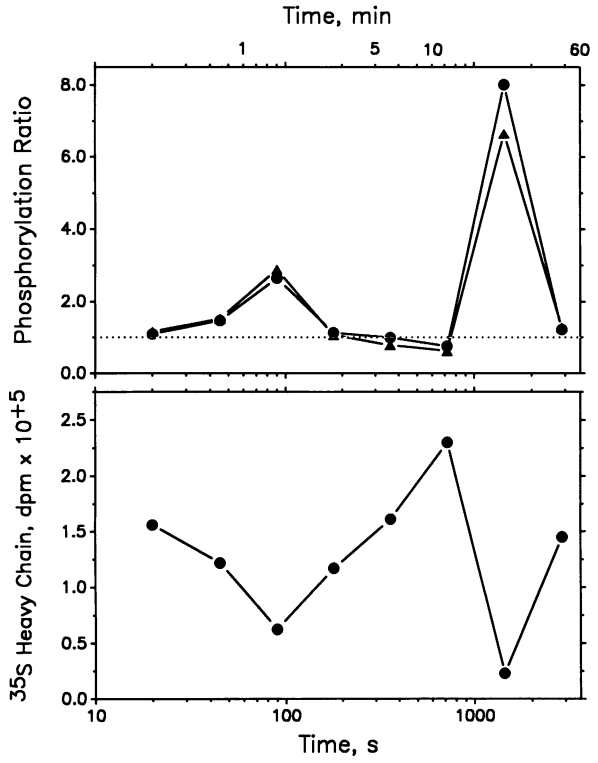

Figure 6. Okadaic acid modulates the phosphorylation ratio and recovery of myosin II from permeabilized cells. Top: We treated dual-isotopically labeled serum-deprived fibroblasts with 0.4 μM okadaic acid for various times and measured the phosphorylation ratio of myosin heavy chains (●) and 20-kDa light chains (▲). Bottom: The amount of ^{35}S radioactivity associated with the myosin heavy chains immunoprecipitated from the cellular soluble fraction at various times after drug treatment. At time zero there were $1.84 \times 10^{+5}$ dpm ^{35}S associated with the solubilized myosin heavy chains.

ment. We also present data correlating the magnitude of the phosphorylation ratio of myosin II heavy chains and the total amount of ^{35}S radioactivity associated with the myosin II heavy chains (Figure 6, bottom). Treatment of serum-deprived cells with okadaic acid for 24 min resulted in a 10-fold decrease in the amount of ^{35}S-labeled myosin II heavy chain extracted and immunoprecipitated from the cells. In contrast, serum-induced myosin phosphorylation (Figure 4) resulted in no significant change in the amount of myosin II heavy and light chains immunoprecipitated from the cells.

The delayed phosphorylation of myosin II caused by okadaic acid correlated with the cell morphological changes induced by the drug. Figure 7 shows a time series of fluorescence images taken of a serum-deprived cell microinjected with the tetramethylrhodamine analogue of smooth muscle myosin II before treatment with okadaic acid. Okadaic acid caused a subtle reorganization of the cytoskeleton and the rounding of cell edges during the first 30 min after treatment began (Figure 7, A–C). A dramatic shortening of stress fibers and retraction of the cell edges then ensued, followed within minutes by the entire cell contracting into a sphere (Figure 7D). Cells remained in this state until they lifted off of the culture dish several hours later.

Staurosporine. When serum-deprived cells are treated with the cell-permeant protein kinase inhibitor staurosporine, cellular protrusive activity increases, stress fibers dissolve, and a distinct loss of phosphate from the 20-kDa light chains of myosin II occurs (Kolega, Giuliano, and Taylor, unpublished data). Figure 8 shows that the drug differentially modulated the phosphorylation ratio of myosin II heavy and 20-kDa light chains in serum-deprived fibroblasts. Within seconds, the phosphorylation ratio of the 20-kDa light chain increased to a level two times that of untreated cells followed by a rapid decrease to a sustained value half that of untreated cells. In contrast, the phosphorylation ratio of the myosin II heavy chains immediately increased to a level 1.4 times that of untreated cells but remained relatively unchanged for the duration of the experiment. The dephosphorylation of the 20-kDa light chains observed here is temporally correlated with the dispersal and disappearance of stress fibers in staurosporine-treated serum-deprived cells (Kolega, Giuliano, and Taylor, unpublished data). These cells show an increased amount of ruffling activity but the loss of stress fiber structure does not result in cell shortening.

Effect of Serum and Drug-Treatment on Cellular Protein Phosphorylation

To test the effects that serum and drug treatments have on cellular phosphoproteins, we electrophoresed ^{32}P-labeled whole cell extracts on SDS-gels (Figure 9). The distribution of phosphoproteins changed little as a result of any one of the treatments, underscoring the need for a sensitive and specific assay of protein phosphorylation. The data also indicate that substantial cytoskeletal rearrangements need not be accompanied by large changes in protein phosphorylation.

Effect of Serum and Drug-Treatment on the Site of 20-kDa Light-Chain Phosphorylation

We determined the site of 20-kDa light-chain phosphorylation 30 min after treating serum-deprived cells with either serum, okadaic acid, or staurosporine because these agents had near maximal effect on the phosphorylation ratio of the 20-kDa light chain after this time (Figures 4, 6, and 8). Trypsin cleavage of the ^{32}P-labeled mouse nonmuscle 20-kDa light chain generated one acidic phosphopeptide in both treated and untreated cells (Figure 10). Furthermore, this phosphopeptide had the same isoelectric point as a phosphopeptide generated from the 20-kDa light chain of

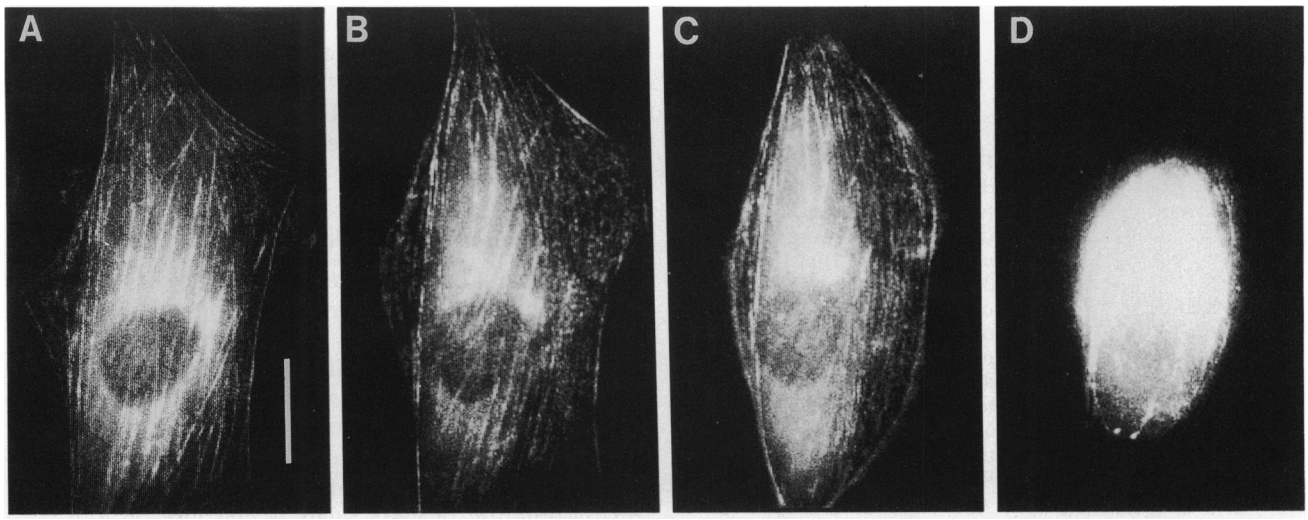

Figure 7. Okadaic-acid–induced changes in cell morphology and cytoskeletal structure. Serum-deprived fibroblasts were microinjected with AR-myosin II and perfused with medium containing 0.4 μM okadaic acid. We obtained fluorescence images every 2.5 min for 1 h after drug treatment. Image A was taken immediately before drug treatment while the rest of the images were obtained post-treatment at various times: B, 15 min; C, 30 min; and D, 60 min. Bar, 10 μm.

smooth muscle myosin II phosphorylated with myosin light-chain kinase.

DISCUSSION

Determination of Intracellular Myosin II Phosphorylation Level

The most sensitive technique for measuring the phosphorylation level of myosin II in nonmuscle cells is based on the incorporation of a single-isotope, ^{32}P (Bayley and Rees, 1986; Ludowyke *et al.*, 1989). The phosphorylation level of myosin II is determined by radioactive phosphate incorporation into living cells, immunoprecipitation and separation of the myosin II heavy and light chains by SDS-polyacrylamide gel electrophoresis (PAGE), and quantitative densitometry of protein gel autoradiograms. We modified the method by first metabolically labeling cellular proteins with ^{35}S-amino acids and ^{32}P-orthophosphate followed by immunoprecipitation and separation of the myosin II as described by Ludowyke *et al.* (1989). We determined the specific activity or "phosphorylation ratio" of each myosin II subunit through liquid scintillation counting of solubilized gel slices. One advantage of the double-isotope method is that the phosphorylation ratio was determined without autoradiography and quantitative densitometry, a process taking days or even weeks. The most important advantage of the method is its precision. We measured the amount of phosphate incorporated per unit protein thus permitting detection of small changes in phosphorylation level. Using the single-isotope method, Kolega, Giuliano, and Taylor (unpublished data) show that 20 nM staurosporine greatly reduces the amount of phosphate associated with the myosin II 20-kDa light chains, a result we confirmed here in a set of kinetic experiments.

The distribution of myosin II 20-kDa light chains on two-dimensional gels was heterogeneous regarding isoelectric point and molecular weight. Likewise, the 20-kDa light chains of human platelet myosin II consist of two major isoforms present in approximately equal amounts, but differing in their molecular weights and

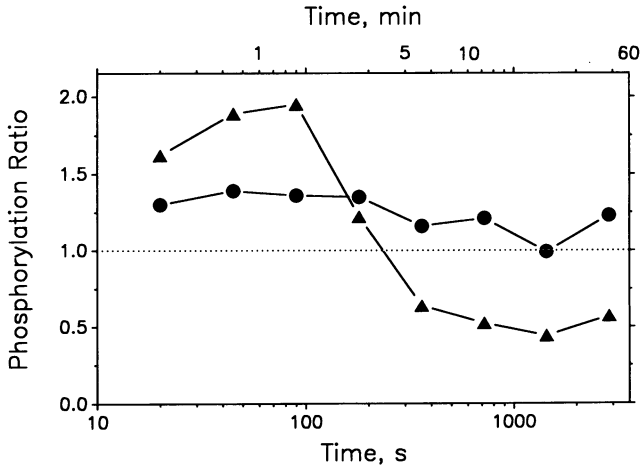

Figure 8. Staurosporine-induced changes in the phosphorylation ratio of myosin II extracted and immunoprecipitated from permeabilized serum-deprived cells. We treated dual-isotopically labeled fibroblasts with 20 nM staurosporine for various times and determined the phosphorylation ratio of the myosin II heavy chain (●) and 20-kDa light chain (▲).

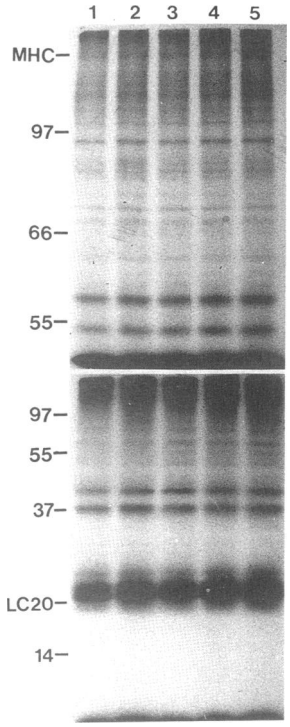

Figure 9. Effect of serum and drug-treatment on cellular protein phosphorylation. Cultures of ^{32}P-labeled cells were either left untreated (lane 1) or treated for 30 min in the presence of 10% calf serum (lane 2), 0.4 μM okadaic acid (lane 3), 20 nM staurosporine (lane 4), or 2 μM KT5926, a protein kinase inhibitor (lane 5) before the preparation and separation of whole cell extracts on SDS-gels (see MATERIALS AND METHODS). The upper and lower gels were 7.5%T 2.7%C acrylamide-bisacrylamide and 12.5%T 2.7%C acrylamide-bisacrylamide, respectively. Protein markers (in kDa) are listed to the left of the gels. The migration position of smooth muscle myosin II heavy chain (MHC) and 20-kDa light chain is also denoted.

isoelectric points (Kawamoto *et al.*, 1989). We found that all but one of the ^{35}S-labeled 20-kDa mouse nonmuscle light-chain spots comigrated with a ^{32}P-labeled spot, suggesting that no more than 50% of the 20-kDa light chains were phosphorylated in serum-deprived cells.

The heavy and 20-kDa light chains of smooth and nonmuscle myosin II can be phosphorylated at several sites with distinct kinases producing different effects on function (Kawamoto and Adelstein, 1988; Conti *et al.*, 1991; Sellers, 1991; Trybus, 1991). In serum-deprived, serum-stimulated, and drug-treated fibroblasts we detected a single phosphopeptide from the 20-kDa light chain. This peptide comigrated with a peptide phosphorylated in vitro with myosin light-chain kinase, suggesting that the activity of this enzyme is coupled to the actin-based cytoskeletal dynamics we observe.

Using high concentrations of myosin light chain kinase inhibitors, we routinely drive the phosphorylation level of the 20-kDa light chains to a level where we can no longer detect incorporation of ^{32}P-orthophosphate (Kolega, Giuliano, and Taylor, unpublished data). We report here that okadaic acid induced a several-fold increase in the phosphorylation ratio of the myosin II heavy and 20-kDa light chains. Taken together these data imply that physiologically relevant phosphorylation ratio values vary between those being very close to zero and those approaching 10 with the value of serum-deprived cells normalized to 1.

We are developing a fluorescent indicator that will ideally yield real-time high-resolution temporal and spatial information about the phosphorylation state of the myosin II 20-kDa light chains in living cells (Hahn *et al.*, 1993). The data presented here will serve as a reference point during the development of this probe.

Serum-Stimulated Cell Model

Serum-stimulation initiates a cascade of molecular events that we are only beginning to understand (McNeil and Taylor, 1987). Among other events, growth factors initiate an intracellular Ca^{2+} transient (Tucker and Fay, 1990; Byron *et al.*, 1992; Hahn *et al.*, 1993) and an increase in phosphoinositide metabolism (Stossel, 1989) that most likely lead to a reduction in the actin-based gel structure of cytoplasm by activating actin-modulating proteins such as gelsolin, profilin, and α-actinin. Using the smooth muscle paradigm, we would also predict that an increase in intracellular Ca^{2+} would result in an increased level of 20-kDa light-chain phosphorylation and thus a more potent myosin II motor (Sellers and Adelstein, 1987; Kamm and Stull, 1989). Indeed, an increase in the phosphorylation level of 20-kDa light chains at a single time point after serum stimulation has been observed (Bockus and Stiles, 1984). We have extended these studies to include several more time points and conclude that serum causes an immediate and sustained increase in the phosphorylation level of both the heavy and 20-kDa light chains. Unlike smooth muscle cell contraction, the isotonic shortening of stress fibers and condensation of cytoplasmic material in nonmuscle cells can occur without whole cell shortening (Giuliano and Taylor, 1990). The manner by which serum induces stress fiber shortening is therefore

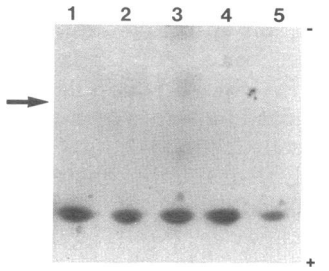

Figure 10. Fluorogram of one-dimensional isoelectric focusing peptide maps of tryptic digests of ^{32}P-labeled 20-kDa light chains. Smooth muscle myosin II was phosphorylated in vitro with myosin light-chain kinase and the isolated 20-kDa light chain was subjected to tryptic digestion and peptide mapping as described in MATERIALS AND METHODS (lane 1). Peptide maps of mouse nonmuscle 20-kDa light chains isolated from serum-deprived 3T3 fibroblasts (lane 2) or cells treated for 30 min with 10% calf serum (lane 3), 0.4 μM okadaic acid (lane 4), or 20 nM staurosporine (lane 5) are also presented. The acidic (+) and basic (−) ends of the gel are denoted. The arrow indicates the migration position of the undigested 20-kDa light chain.

consistent with a combination of the reduction in cytoplasmic gel structure and an increase in myosin II motor activity.

Drug Effects on Cytoskeletal Structure

Micromolar cytochalasin D induces a cessation of stress-fiber formation and transport and causes immediate stress-fiber fragmentation and contraction (Kolega *et al.*, 1991). Cytochalasin D most likely acts by partially solating the highly cross-linked actin gel in the stress fiber, thus reducing the force required to contract the fiber (Janson *et al.*, 1991; Kolega *et al.*, 1991). The work presented here supports this hypothesis because the drug induced no significant change in the level of myosin II heavy or 20-kDa light-chain phosphorylation. The level of myosin II phosphorylation found in serum-deprived cells generated some isometric tension that was converted into an isotonic contraction of stress fibers by merely solating a portion of the highly cross-linked actin gel that constitutes the stress fibers. Therefore, the mechanism whereby serum induces stress-fiber shortening appears to be more complicated than that of cytochalasin D.

Okadaic acid induces the phosphorylation of myosin II light chain in the nuclear/cytoskeletal fraction but not in the cytoplasmic fraction of human fibroblasts (Guy *et al.*, 1992). Our data indicated that the drug caused myosin II to associate with the detergent-insoluble cytoskeleton, suggesting that a large increase in phosphorylation level caused the protein to partition into a distinct phase of the cytoplasm.

The cellular response to okadaic acid gives us a glimpse of how tightly regulated the level of myosin II phosphorylation in a serum-deprived cell is. The drug, acting most likely by inhibiting the phosphatase of which myosin II is a substrate, caused an immediate increase in heavy and 20-kDa light-chain phosphorylation that was quickly reversed by the cell with some overcompensation to below pretreatment levels. This response is similar to the response serum-deprived cells have to a continuous alkaline loading of their cytoplasm. The cells immediately begin to buffer the intracellular pH and sometimes overshoot and maintain an intracellular pH lower than the prealkalinization value (Giuliano and Gillies, 1987).

While okadaic acid failed to induce an immediate large increase in myosin II phosphorylation, its continued presence of okadaic acid eventually caused an increase in the phosphorylation of the heavy and 20-kDa light chains to a level several-fold higher than that induced by serum. This resulted in a massive retraction of the entire cell even though the phosphorylation ratio eventually returned to the pretreatment value. Cell edge retraction without a rapid respreading was seldom seen in serum-stimulated cells where, coincidentally, the phosphorylation ratio also decays over time, suggesting that okadaic acid increased myosin II motor strength and thus cytoplasmic tension to a point where it overcame the resisting strength of the actin meshwork. The violent retraction of the entire cell implies that the attachment sites between the actin-based cytoplasmic gel and other key structural components of the cell were maintained throughout the cytoplasmic contraction. Continuous treatment with okadaic acid may have eventually led to the inhibition of the kinases that phosphorylate myosin II, thus causing a net loss of phosphate from the protein. In spite of the dephosphorylation, we never observed stress-fiber reformation or cellular respreading in the continued presence of the drug. However, the contraction induced by okadaic acid does not involve the concomitant decrease in actin-gel structure that occurs after treatment with serum or cytochalasin D. The recycling of actin-based gel components may be necessary to reform cytoplasmic structure. This is consistent with the solation contraction coupling hypothesis.

Furthermore, the stress-fiber dynamics induced by okadaic acid in the serum-deprived fibroblast are difficult to compare with the reported effects the drug has on smooth muscle. Okadaic acid can elicit contraction of intact and skinned smooth muscle fibers (reviewed by Hartshorne *et al.*, 1989) but Tansey *et al.* (1990) treat strips of smooth muscle with a 7.5-fold higher dose of okadaic acid than we used to treat fibroblasts and find that it fails to increase the phosphorylation of the myosin II 20-kDa light chain or induce contraction. We are now investigating the role 20-kDa light-chain phosphorylation plays in stress fiber dynamics by specifically modulating myosin light-chain kinase activity in living cells with antibodies and inhibitory-pseudosubstrates.

Staurosporine was the only agent we studied that gave a differential response with heavy and 20-kDa light-chain phosphorylation. The drug caused an immediate and sustained slight increase in heavy-chain phosphorylation but a very different response concerning the phosphorylation of the 20-kDa light chain. The drug ultimately caused a decrease in 20-kDa light-chain phosphorylation. Staurosporine also causes the splaying and disappearance of stress fibers without contraction (Kolega, Giuliano, and Taylor, unpublished data), suggesting that the dephosphorylation of the 20-kDa light chain measured here is sufficient to both weaken the forces required to stabilize actin filament bundles as well as to decrease the contractile force of the myosin II. In contrast to serum-stimulation (Giuliano and Taylor, 1990) and cytochalasin D treatment (Kolega *et al.*, 1991), staurosporine-induced solation of stress fibers is not coupled to contraction because the motor strength is decreased simultaneously.

Implications for the Molecular Dynamics of Cytoplasm in Serum-Deprived Cells

In serum-deprived cells, stress fibers are part of a structured cytoplasmic gel that resists the contractile force exerted by myosin II (Kolega *et al.*, 1991). The drugs we used to treat serum-deprived cells act with partial specificity for those enzymes responsible for myosin II phosphorylation yet we cannot detect serum or drug-induced changes in the phosphorylation level of proteins on one-dimensional SDS-gels of whole cell extracts. However, actin-binding proteins are also candidates for drug-induced modification because these proteins have been implicated in actin-linked events that control the dynamic structure of cytoplasm (Taylor and Fechheimer, 1982; Stossel *et al.*, 1985; Pollard and Cooper, 1986; Hartwig and Kwiatkowski, 1991; Kolega *et al.*, 1991). Other potential phosphoproteins that serve cytoskeletal or regulatory roles must also be examined.

The observed cyclical formation, transport, and disappearance of stress fibers in serum-deprived cells (Giuliano and Taylor, 1990) is therefore consistent with a model wherein the phosphorylation level of myosin II near the cytoplasm-membrane interface would have to be above a critical threshold for the protein to express both some motor activity and actin-cross-linking capability because stress-fiber formation is primarily initiated here in serum-deprived cells. Areas near the plasma membrane would most likely provide a substrate for actin filament assembly because this is where amphiphilic phosphoinositides are likely to localize. These metabolites weaken the actin-monomer–sequestering ability of several proteins including profilin (Lassing and Lindberg, 1988; Goldschmidt-Clermont *et al.*, 1991), destrin and cofilin (Yonezawa *et al.*, 1990), and gelsolin (Janmey and Stossel, 1989). In contrast, we would predict the phosphorylation level of myosin II in the perinuclear region to be below some threshold, thus causing stress-fiber dispersal without contraction (Kolega, Giuliano, and Taylor, unpublished data). The free calcium level remains below 100 nM in serum-deprived (quiescent) fibroblasts (McNeil *et al.*, 1985; Tucker and Fay, 1990), suggesting that if calcium-independent actin-binding proteins, including actin severing and depolymerizing proteins (Pollard and Cooper, 1986; Giuliano *et al.*, 1988; Yonezawa *et al.*, 1990), were maximally active in this region of the cell during the loss of stress fiber structure, they would produce subunits that diffuse back to the cell periphery. This model essentially describes a supramolecular form of treadmilling where subunits of the actin-based cytomatrix are preferentially lost from the cytoplasmic gel in the perinuclear region, diffuse throughout the cytoplasm, and are preferentially added back to the cytoplasmic gel, sometimes in the form of stress fibers, at the cytoplasm-membrane interface. Testing this hypothesis will require a detailed temporal and spatial map of the phosphorylation state of myosin II and the activity of key actin-filament-modulating proteins in serum-deprived cells.

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