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Turnover Rates at Regulatory Phosphorylation Sites on Myosin II in Endothelial Cells

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Abstract Assembly and motor activity of non-muscle myosin II can be regulated by phosphorylation. Because myosin II-containing structures undergo continuous assembly, disassembly, and remodeling in living cells, especially during cell migration, myosin II should undergo frequent phosphorylation and dephosphorylation. This study examines the turnover of phosphate on myosin II in stationary and migrating endothelial cells. Cultured bovine aortic endothelial cells were metabolically labeled with ^{32}P-phosphate, and the incorporation of phosphate into myosin II was assessed by quantitative phosphor imaging of electrophoretic gels of myosin II immunoadsorbed from cell lysates. Likewise, phosphate turnover was measured upon chasing the ^{32}P with unlabeled phosphate. Phosphate incorporated very slowly into heavy chains, taking >8 h to plateau, and turned over at $\leq 12.7\%$ per hour. Regulatory light chains became completely labeled in ≤ 4 h, and turnover occurred at two rates: 49% turned over at 20% per hour, the remainder at 67% per hour. Peptide mapping showed light chain phosphorylation at serine 19 and threonine 18, but phosphate turnover was the same in mono- and diphosphorylated lights chains, indicating that rates are not different at the two sites. When cells were stimulated to migrate by wounding a confluent monolayer, the rate of light chain dephosphorylation increased and the rate of phosphate incorporation decreased causing a net 10% dephosphorylation of light chains. This process persisted during migration and returned to baseline when the wound was closed. There was no effect on heavy chain phosphates. Light chain dephosphorylation may facilitate migration by mobilizing myosin II during cytoskeletal remodeling. J. Cell. Biochem. 75:629–639, 1999.

Key words: myosin light chain; cell motility; cytoskeleton; assembly; wound healing; angiogenesis

Phosphorylation of myosin II from smooth-muscle and non-muscle cells regulates the assembly of myosin II into filaments and its activity as an actin filament motor in vitro [reviewed by Tan et al., 1992]. For vertebrate myosin II, this regulation occurs primarily via phosphorylation of specific sites on the regulatory light chains (RLCs) [Tan et al., 1992; Trybus, 1991b]. In vivo, phosphorylation of the RLCs is associated with a variety of cytoskeletal functions. In smooth muscle, phosphorylation of the RLCs of myosin II is necessary and sufficient for muscle contraction [Itoh et al., 1989]. In non-muscle cells, phosphorylation of RLCs can drive contraction of the cytoskeleton in several different permeabilized cell models [Cande and Ezzell, 1986; Ehrlich et al., 1991; Holzapfel et al., 1983; Masuda et al., 1984; Stark et al., 1991; Wysolmerski and Lagunoff, 1990], and dephosphorylation of RLCs causes dissolution of stress fibers and cell rounding [Fernandez et al., 1990, 1991; Lamb et al., 1988, 1990]. During cytokinesis, specific phosphorylations on RLCs are associated with accumulation of myosin II in the cleavage furrow [Matsumura et al., 1998; Satterwhite et al., 1992; Yamakita et al., 1994; DeBiasio et al., 1996], and during polarized locomotion large spatial differences exist between phosphorylated and unphosphorylated RLCs along the axes of migration [DeBiasio et al., 1996; Matsumura et al., 1998; Post et al., 1995]. These observations suggest that RLC phosphorylation plays an important role in directing myosin II behavior during shape changes and cell locomotion.

Abbreviations used: RLC, regulatory light chain of myosin II; BAEC, bovine aortic endothelial cell.

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Phosphorylation of myosin II has been studied extensively in endothelial cells, where RLC phosphorylation has been implicated in regulating transendothelial permeability. RLC phosphorylation is associated with increased paracellular leakiness, presumably resulting from the activation of myosin-based contractile activity that pulls apart cell-cell contacts [Garcia et al., 1995; Goeckeler and Wysolmerski, 1995; Moy et al., 1993; Sheldon et al., 1993; Verin et al., 1998; Wysolmerski and Lagunoff, 1990]. We have recently shown that RLC phosphorylation coincides with assembly of myosin II into the endothelial cytoskeleton (Kolega and Kumar, 1999), indicating that RLC phosphorylation can affect cytoskeletal organization at a very fundamental level. In addition, because asymmetries in myosin II assembly correlate with the direction of cell locomotion [Kolega, 1997; Kolega and Taylor, 1993; DeBiasio et al., 1996; Matsumura et al., 1998; Post et al., 1995], and because disruption of myosin II causes depolarization of cell locomotion [Höner et al., 1988; Wessels and Soll, 1990; Wessels et al., 1988], RLC phosphorylation may also play an important role in directing the orientation of the locomotive machinery of an endothelial cell during wound-healing and angiogenesis.

Despite the wide-ranging interest in myosin phosphorylation as a regulatory signal in nonmuscle, relatively little is known about the dynamics of myosin phosphorylation and dephosphorylation in vivo. Most studies have focused on net levels of phosphorylation, with minimal concern for how rapidly phosphates cycle on and off of myosin II. However, in nonmuscle cells, the actin-myosin cytoskeleton is extremely dynamic. In addition to the ongoing cytoskeletal rearrangements associated with force generation, shape change, and cell motility, actin and myosin filaments continuously undergo assembly and disassembly, even in stationary cells [Giuliano and Taylor, 1990]. Consequently, it is important to examine the turnover of potential regulatory phosphates if we are to understand how steady-state levels of phosphorylation are controlled. Toward this end, the present study describes the dynamics of myosin II phosphorylation in resting endothelial cells and how these dynamics change when the cells are stimulated to undergo migration.

MATERIALS AND METHODS

Cells and Reagents

Bovine aortic endothelial cells (BAECs) were cultured from an early-passage primary culture provided by the laboratory of Dr. Scott Diamond (State University of New York at Buffalo, now at University of Pennsylvania). The cells were confirmed as endothelial cells by staining for Factor VIII and by specific uptake of low-density lipoproteins. They were grown and maintained in Dulbecco's modified Eagle's medium (DMEM)(Life Technologies, Grand Island, NY) supplemented with 10% fetal calf serum (FCS) (Atlanta Biological, Atlanta, GA). All experiments were conducted on cells that were between their 12th and 18th passage after isolation.

Radiolabeling of Cells

For most experiments, cells were radiolabeled with both ^{35}S-methionine/cysteine and ^{32}P-orthophosphate, using a procedure based on that of Goeckeler and Wysolmerski [1995]. Cells were labeled for 24 h in low-methionine medium (methionine-free medium to which 5% complete medium and 5% FCS were added) containing 150 μCi/ml Expres^{35}S-tag protein labeling mix (a mixture of methionine and cysteine from NEN Life Science Products, Boston, MA). The cells were then washed twice with low-phosphate medium (phosphate-free medium to which 5% complete medium and 5% FCS were added) and immediately labeled in low-phosphate medium containing 100 μCi/ml ^{32}P-phosphoric acid (ICN Pharmaceuticals, Costa Mesa, CA). Because the turnover of myosin II is very slow (<5% per hour; unpublished observations), this sequential labeling method can be used with relatively little loss of the initial ^{35}S-methionine/cysteine label. Labeling of cells with a single radionuclide was performed using the same media and nuclide concentrations.

Determination of Phosphorylation Levels

The relative levels of phosphorylation on myosin II were quantified by two methods. To monitor phosphate turnover, the one-dimensional method of Chrzanowska-Wodnicka and Burridge [1996] was used. Cells were radiolabeled with both ^{35}S-methionine/cysteine and ^{32}P-orthophosphate; then myosin II was immunoadsorbed from cell lysates and the heavy and light

chains separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 12% or 20% gels. Gels were dried and exposed to phosphor screens (Molecular Imager, Bio-Rad Laboratories, Hercules, CA) along with a set of spots of pure ^{35}S-methionine/cysteine and ^{32}P-phosphoric acid standards. Gels were exposed twice. One exposure gave an image in which the spot intensities were the sum of ^{35}S and ^{32}P emissions. A second exposure was then made through four sheets of Reynolds heavy-duty aluminum foil, which absorbed >99% of the ^{35}S emissions but was penetrated by ^{32}P emissions. The attenuation of the ^{32}P signal during the second exposure was determined by measuring the intensities of the ^{32}P-phosphoric acid standard spots, and this was used to calculate the contribution of ^{32}P emissions to the signal in the first exposure. This contribution was subtracted from the signal in the second exposure to obtain the ^{35}S signal:

$$^{35}\text{S signal} = E_u - (P_u/P_f \times E_f)$$

where E_u is the intensity from the unfiltered exposure, E_f is the intensity from the foil-filtered exposure, P_u is the intensity of the ^{32}P standard spots during the unfiltered exposure, and P_f is the intensity of the 32P standard spots during the filtered exposure.

To determine absolute levels of phosphorylation, 2-D electrophoresis was used as described by Ludowyke et al. [1989]. Myosin II was immunoadsorbed from cells radiolabeled with ^{35}S-methionine/cysteine and the light chains separated in the first dimension by isoelectric focusing over a pH range of 4.5–5.4, and in the second dimension by SDS-PAGE on a 20% acrylamide gel. Spots were visualized by exposing dried gels to phosphor screens, and the amount of light chain in each spot was measured by integrating the ^{35}S signal on the phosphor image. Identification of the phosphorylated light chains was confirmed by comparing the spot pattern with that of cells labeled with ^{32}P-phosphate.

All measurements of radioactivity were made by using a Bio-Rad Molecular Imager to image the phosphor screens and Molecular Analyst software to integrate the signal associated with each spot or band in the images.

Identification of Phosphorylation Sites

The phosphorylation sites were determined by one-dimensional isoelectric focusing of tryptic digests as described by Daniel and Sellers [1992]. Identities of the bands were assigned by comparing the band pattern with digests of bovine platelet myosin II phosphorylated in vitro with ATP-γ^{32}P (ICN Pharmaceuticals), using myosin light chain kinase (a generous gift of Dr. A. Edelman, SUNY-Buffalo) or protein kinase C (Calbiochem, San Diego, CA).

RESULTS

Phosphate Turnover on Myosin II in BAECs

The rate at which phosphates are incorporated into myosin II in vivo was determined by incubating cells with radiolabeled phosphate and examining the incorporation of label into myosin II heavy and light chains. BAECs were first labeled overnight with ^{35}S-cysteine/methionine to label total protein, so that the amount of myosin II recovered from the cells in each experiment could be determined precisely. The cells were then incubated in medium containing ^{32}P-phosphate and either lysed after various periods of labeling, or subjected first to a cold chase with unlabeled phosphate. Myosin II was then immunoadsorbed from the lysates, the heavy and light chains separated by SDS-PAGE, and the amount of phosphate associated with each of the peptides determined by phosphor imaging as described under Methods.

Figure 1 shows that ^{32}P-phosphate was incorporated into the RLCs more rapidly than into the heavy chains of myosin II. RLC phosphorylation reached maximal levels within ~4 h, indicating that the label had access to the entire pool of phosphorylated RLCs. In other words, virtually all the phosphates on RLCs turned over during this period. By contrast, heavy chain phosphorylation had not plateaued even after 8 h had elapsed.

The rate at which phosphate turned over was determined by measuring how fast ^{32}P-phosphate was chased from myosin II by unlabeled phosphate. The results of a typical "cold chase" experiment are shown in Figure 2. Phosphates on the heavy chains turned over very slowly (Fig. 2A). The data could be fit to a linear equation ($r = 0.98$) with a slope of 12.7% per hour. This represents a maximum turnover rate, as only 53% of the label had turned over at the end of the longest period measured. In any case, the basal rate of dephosphorylation of the heavy chains was very slow.

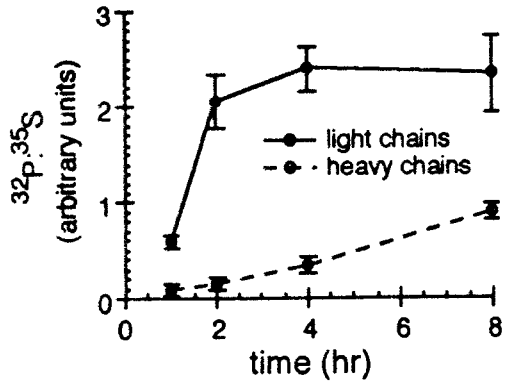

Fig. 1. Incorporation of ^{32}P-phosphate into myosin II in bovine aortic endothelial cells (BAECs). BAECs were radiolabeled with ^{35}S-methionine/cysteine and then incubated in the presence of ^{32}P-phosphoric acid for the indicated times. After lysing cells and immunoadsorbing their myosin II, the amount of ^{32}P associated with myosin heavy chain (solid circles) and light chains (open circles) was determined by phosphor imaging of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Cells from triplicate dishes were used for each data point; error bars indicate 1 SD from the mean. The amount of ^{32}P per light chain reached a plateau within 2–4 h, whereas ^{32}P continued to be incorporated into the heavy chains for at least 8 h. Note that ratios are based on uncallibrated radiation counts and so are not the actual molar ratio between the two isotopes.

Turnover of the phosphates on the RLCs was more complex. The time course of phosphate turnover indicated the presence of at least two different populations of RLC phosphate (Fig. 2B). The steep slope of the curve indicates rapid turnover of a large fraction of the phosphate during the first hour of the cold chase. However, a second population turned over much more slowly, as demonstrated by the persistence of label after 4 h of chasing. Isotope levels over the later timepoints ($\geq$2 h) could be fit to a straight line with a high confidence level (R = 0.98) and the amount labeled approached 0% at the end of the observation period, suggesting that this period represented the turnover of a single population of phosphates. Extrapolating back to t = 0, this population constituted 49% of the RLC phosphates, and complete turnover took 5.0 h (giving a turnover rate of 20% per hour within this "slow" population). The remaining 51% of the label on RLCs must have turned over during the first 2 h, and the excess over the "slow" turnover gives its turnover rate during this period. Because the early timepoints ($\leq$1 h) fit a straight line (R = 0.99), there was apparently only a single "fast" rate. From the curve fits in Figure 2 B, the "fast" label was calculated to be completely released after 1.5 h, giving a turnover rate of 67% per hour for this population.

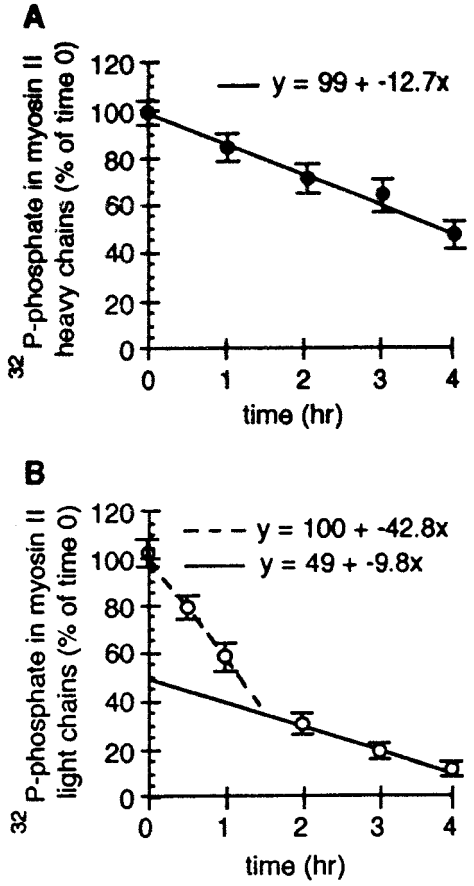

Fig. 2. Turnover of ^{32}P-phosphate on myosin II in bovine aortic endothelial cells (BAECs). BAECs were double-labeled with ^{32}P-phosphate and ^{35}S-methionine/cysteine, then incubated in medium with unlabeled phosphate for the indicated times. The amount of ^{32}P-phosphate still associated with heavy chains **(A)** or light chains **(B)** was determined by immunoadsorption of myosin II and phosphor imaging of SDS-PAGE gels. Measurements were made on myosin from triplicate dishes for each time, and the data shown is from one representative experiment. Curve fits are by linear regression with R values of $<$0.98. B: The dotted line represents a line fitted to the data for times of $<$1.5 h; the solid line was fitted for times of $>$.5 h.

"Fast" and "Slow" Turnover Reflect Different Populations of Myosin II, Not Different Phosphorylation Sites

RLCs in bovine microcapillary endothelial cells [Kolega and Kumar, 1999] and in human umbilical vein endothelial cells [Goeckeler and Wysolmerski, 1995] have been shown to be phosphorylated at both serine 19 and threonine 18, and to a much lesser degree at serine 1 or threonine 9, or both. To determine where the RLCs were phosphorylated in BAECs, RLCs were isolated from cells after 4 h in ^{32}P-phosphate, digested with trypsin, and the phosphorylated peptides mapped by isoelectric focusing

and autoradiography. As shown in Figure 3, RLCs were phosphorylated almost exclusively at serine 19 and threonine 18.

Fast and slow turnover could represent a difference in turnover at serine 19 versus threonine 18. Alternatively, serine 19 and threonine 18 could turn over together, but at different rates in two different populations of myosin II molecules. These alternatives can be distinguished by comparing phosphate turnover on mono- and diphosphorylated RLCs. Because phosphorylation at threonine 18 alone has not been observed either in vitro or in vivo, the monophosphorylated RLCs presumably represents phosphorylation at serine 19. If serine 19 turns over more rapidly than threonine 18, label will incorporate more rapidly in the monophosphorylated form and will persist longer in the diphosphorylated population when label is chased. Conversely, if threonine 19 turns over more rapidly, label would exchange more rapidly on the diphosphorylated RLCs than in the monophosphorylated population. Exchange of label would occur at the same rate in mono- and diphosphorylated RLCs only if the two phosphorylation sites are processed at similar rates.

In order to compare phosphate turnover on mono- and diphosphorylated RLCs, cells were pulse-labeled with ^{32}P-phosphate, myosin II immunoadsorbed from cell lysates, and the various phosphorylated species resolved by two-dimensional gel electrophoresis. The amount of label associated with each spot was quantified by phosphor imaging as described under Materials and Methods, and the results are shown in Figure 4. After 1.5 h in ^{32}P-phosphate, when the rapidly exchanging phosphates should be almost completely labeled and only 30% of the slowly exchanging phosphate would be labeled, the ratio of label to protein in the diphosphorylated spot is twice that of the monophosphorylated spot (2.01 ± 0.09; n = 3). Continued labeling for an additional 1.5 h did not increase the relative labeling of mono- versus diphosphorylated RLCs (mono:di = 1.89 ± 0.22; n = 3), even though additional label would have incorporated at slowly exchanging sites. Thus, rapidly exchanging and slowly exchanging phosphates must be equally distributed between mono- and diphosphorylated RLCs. Furthermore, both mono- and diphosphorylated RLCs were still labeled even after a 2-h cold chase, during which all the "fast" phosphate should exchange, leaving label only at the "slow" sites. Therefore, there must be slowly exchanging phosphates at both serine 19 and threonine 18. In addition, because the ratio of label in mono- and diphosphorylated RLCs was not significantly different from the pre-chase ratio (mono:di = 1.91 ± 0.12; n = 3), the phosphates at serine 19 must exchange at very similar rates to those at threonine 18.

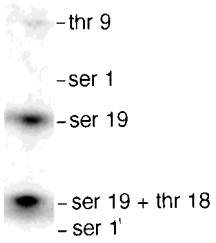

Fig. 3. Peptide mapping of phosphorylation sites on bovine aortic endothelial cell (BAEC) myosin II. Trypsin digests were performed on myosin II immunoadsorbed from BAECs that were metabolically labeled with ^{32}P-phosphate in vivo. The fragments were separated on isoelectric focusing gels, and the positions of the phosphorylated fragments were visualized by phosphor imaging. The positions of standard phosphorylated fragments are indicated on the right. Almost all of the phosphate label was found in fragments phosphorylated at serine 19 alone or at both serine 19 and threonine 18. A faint signal was sometimes also detected at the threonine 9 fragment.

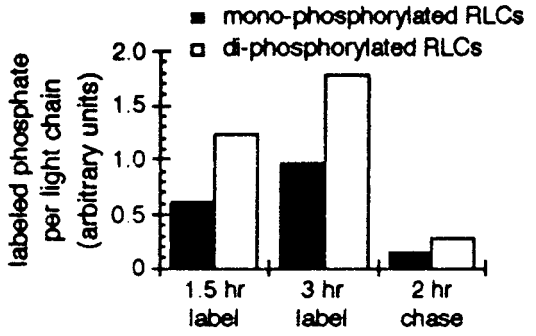

Fig. 4. Phosphate incorporation and turnover in mono- versus diphosphorylated regulatory light chains (RLCs). Bovine aortic endothelial cells (BAECs) were labeled with ^{35}S-methionine/cysteine for 24 h, followed by ^{32}P-phosphate for either 1.5 h, 3 h, or for 3 h followed by 2 h in phosphate without ^{32}P (= 2-h chase). The amounts of ^{32}P incorporated into mono- and diphosphorylated RLCs were then determined by phosphor imaging of two-dimensional electrophoretic gels, and the amount of RLC associated with each spot determined from the ^{35}S signal. Labeled phosphate was incorporated into both mono- and diphosphorylated RLCs, with twice as much label entering the diphosphorylated RLCs. Note that this proportionality remained constant after "fast" sites were labeled (1.5 h), after all RLCs were labeled (3 h), and after "fast" sites should have turned over leaving only "slow" sites labeled (2 h chase).

Increased Phosphate Turnover During Wound Healing

Phosphorylation of RLCs is associated with assembly of myosin II in vitro and in vivo. Because the actin-myosin II cytoskeleton is continuously assembled and disassembled when cells migrate, rapidly moving cells should have higher turnover rates than those of stationary cells. To compare migrating and stationary cells, BAECs were grown until they formed confluent monolayers. This provided a large population of cells that are not migrating. Migratory cells were then obtained by subjecting the monolayer to two orthogonal passes of a fine metal comb, scraping away cells in a grid-shaped pattern (Fig. 5). Most cells were then at the edge of a freshly denuded surface, where they immediately began to migrate into the newly available free space. Because cells began to move within seconds after scrape-wounding and typically translocated more than a full cell diameter in an hour, we focused on turnover during the first 1–2 h after wounding. There was no detectable change in the turnover of phosphates on myosin II heavy chains in response to wounding (Fig. 6A), but phosphates on the RLC turned over more rapidly in wounded cultures than in unwounded controls (Fig. 6B). The exact rates varied from experiment to experiment, which may reflect differences in passage number and the accompanying variability in growth and migration rates. Nonetheless, in six separate trials an increase in the rate of turnover was observed in every case (Fig. 7), with a mean increase of 42.8 ± 19.1%.

In order to determine whether increased phosphate turnover reflected an overall increase in the rate of cycling of myosin II through the phosphorylation-dephosphorylation cycle, the rate of phosphate incorporation was also examined. When the incorporation of ^{32}P-phosphate into myosin II in scrape-wounded monolayers was compared with unwounded monolayers, no significant difference between the rates of phosphorylation of the heavy chains was found at any time over the first 8 h after wounding (Fig. 8A). By contrast, the rate of at which labeled phosphate was incorporated into the light chains was diminished during the first 2 h after wounding (Fig. 8B). Once again, the absolute rates varied among experiments, but a decrease in the rate of incorporation was consistently observed (Fig. 9), with a mean decrease of 46.8 ± 6.2% (n = 4). Our data do not give the rate beyond the initial 2-h period, because labeling of the light chains in unwounded cells saturated at 2–4 h. However, most of the cells have become fully motile within the first 1–2 h after wounding (Fig. 5).

RLC Phosphorylation Decreases During Wound Healing

Because the rate of phosphorylation of RLCs decreased while the rate of dephosphorylation increased, there must be a net decrease in phosphorylation of RLC during wound healing. This was confirmed by two-dimensional gel electrophoresis. Myosin II was immunoadsorbed from lysates of confluent monolayers of BAECs at various times before or after scrape wounding. Phosphorylated and unphosphorylated RCLs were separated by two-dimensional gel electrophoresis and the relative amounts of each form determined by quantitative image analysis of the gels. The results are shown in Figure 10.

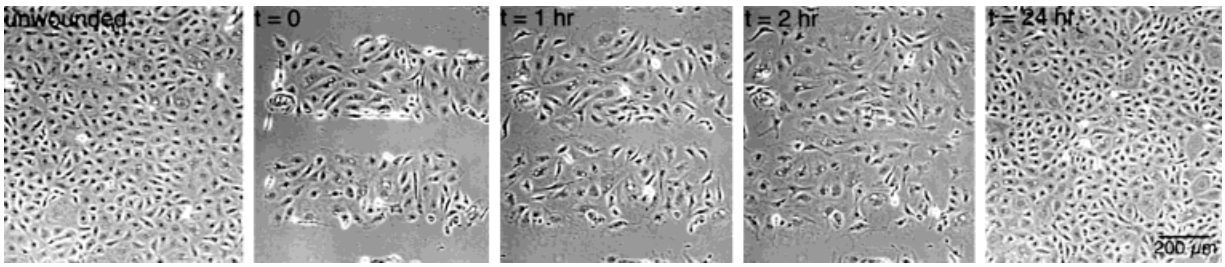

Fig. 5. Wounding of bovine aortic endothelial cell (BAEC) monolayers. A confluent monolayer of BAECs (unwounded) was scraped in the horizontal and vertical directions with a fine metal comb to remove cells in a grid-like pattern, leaving narrow square or rectangular islands of cells (t = 0 h). Cells at the edges of the islands immediately extended broad lamellipodia from their free edges and began migrating onto the newly denuded substratum, so that by t = 1 h, many cells had already translocated onto the wound surface. By 2 h after wounding, all the cells at the margins of the islands had migrated onto the wound surface, opening up empty space between cells at the center of the island, so that all cells could migrate. 24 h after wounding, cell spreading and replication had filled the wound space so that the culture was a confluent monolayer again.

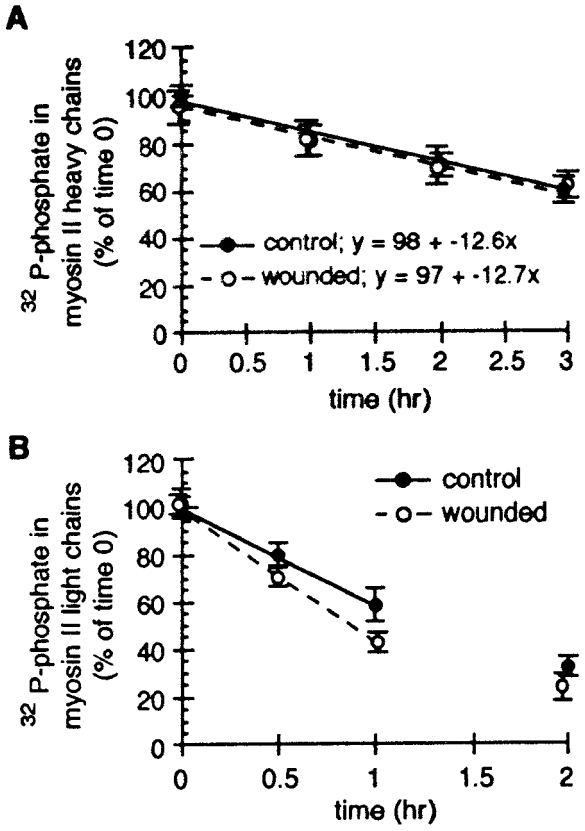

Fig. 6. Phosphate turnover in wounded bovine aortic endothelial cell (BAEC) monolayers. Confluent monolayers of BAECs were double-labeled with ^{32}P-phosphate and ^{35}S-methionine/cysteine, then scrape-wounded as in Fig. 5. The cultures were then immediately changed into medium with unlabeled phosphate, and the amount of ^{32}P-phosphate that was still associated with myosin heavy chains **(A)** or light chains **(B)** was determined in duplicate wounded and unwounded cultures at the indicated times after wounding. Each point represents the average of measurements from three dishes; bars represent one standard deviation from the mean. A: There was no significant difference between the amount of labeled phosphate on myosin heavy chains in wounded cultures (open circles) versus unwounded controls (solid circles) nor in the turnover rates. Equations for the two lines were obtained by a linear regression fit, with R values of 0.97 for the wound data and 0.98 for controls. B: On regulatory light chains (RLCs), ^{32}P-phosphate turned over more rapidly in wounded cultures (open circles) than in controls (solid circles). Slopes for lines that were fitted to the data for the first 1 h are given in Fig. 7 for a series of six replicate experiments.

Within 1 h of wounding, there was a small but significant decrease in both mono- and diphosphorylated RLCs, with a corresponding increase in the amount of completely unphosphorylated light chains. RLCs were further dephosphorylated at 2 h after wounding, and this reduced level of RLC phosphorylation persisted as cells continued to migrate 4 h after wounding. By 24 h

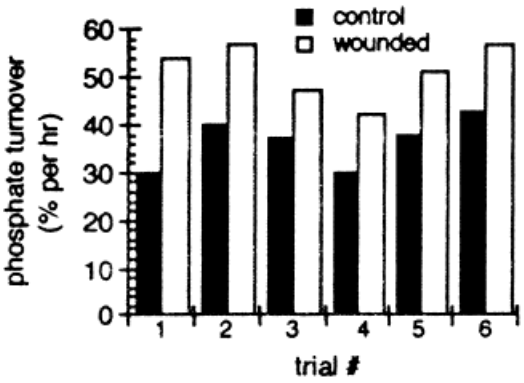

Fig. 7. Increased turnover of phosphate on regulatory light chains (RLCs) in wounded monolayers. The rate of phosphate turnover on RLCs was determined from plots of ^{32}P-phosphate content versus time in cold-chase experiments, as shown in Fig. 6B. Turnover rates were higher in wounded cultures in all six experiments.

after wounding, when the monolayer was reestablished and migration stopped, RLC phosphorylation had returned to pre-wounding levels.

DISCUSSION

Constitutive Phosphorylation and Dephosphorylation of Myosin II in Resting Endothelial Cells

In the absence of chemical or mechanical stimulation, confluent monolayers of endothelial cells display very little locomotive or contractile activity. Yet the present study demonstrates that myosin II is continuously phosphorylated and dephosphorylated, even in this quiescent state. In particular, a population of myosin II exists in which the phosphate on the RLCs is completely turned over in 1.5 h. What is the function of this energy-expensive activity? Giuliano and Taylor [1990] demonstrated that myosin-containing cytoskeletal structures are very dynamic even in cell that are not migrating, observing that stress fibers are continuously assembled in the periphery of serum-starved cells, transported centripetally, and disassembled in the perinuclear cytoplasm. Such behavior appears to be a fundamental constitutive activity in most non-muscle cells [Bray and White, 1988], and similar dynamics are observed in endothelial cells in culture [Kolega, 1997] (J. Kolega, unpublished observations). Because RLC phosphorylation correlates with myosin II assembly, and dephosphorylation with its disassembly, the cycle of phosphorylation and dephosphorylation of RLCs may be

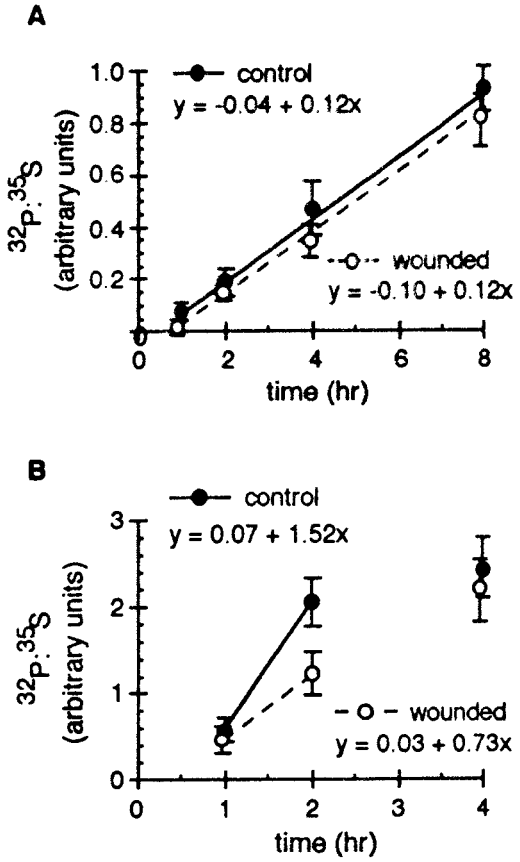

Fig. 8. Incorporation of ^{32}P-phosphate into myosin II in wounded monolayers. Confluent monolayers were labeled for 24 h with ^{35}S-methionine/cysteine and then wounded. Immediately after wounding, ^{32}P-phosphate was added to the medium, and the amount incorporated into myosin II was determined at the indicated times. Each point represents the average of measurements from three dishes; bars represent one standard deviation from the mean. **A:** Myosin II heavy chains incorporated ^{32}P-phosphate at the same rate in wounded (open circles) and unwounded (solid circles) cultures. R values for the linear curve fits were 0.99 for both wounded and control cultures. **B:** Between 1 and 2 h after wounding, the amount of ^{32}P-phosphate in RLCs in wounded cultures (open circles) fell significantly behind the unwounded controls (solid circles). Some of the difference appears to be made up by 4 h after wounding, but recall that phosphate incorporation normally reaches a plateau between 3 and 4 h (Fig. 1). Slopes for lines fitted to the data for the first 2 h are given in Fig. 9 for a series of four replicate experiments.

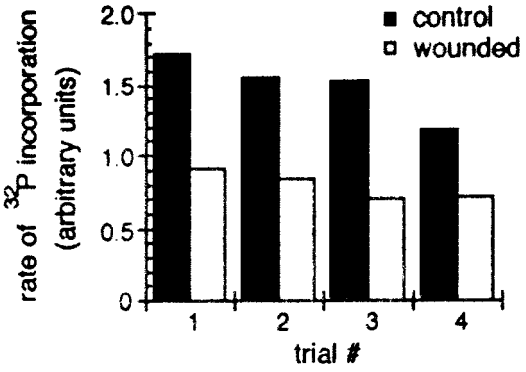

Fig. 9. Decreased rates of phosphate incorporation into regulatory light chains (RLCs) in wounded cultures. The rate of phosphate incorporation into RLCs was determined from plots of ^{32}P-phosphate incorporation versus time, as shown in Fig. 6B. Incorporation rates were significantly lower in wounded cultures in all four experiments.

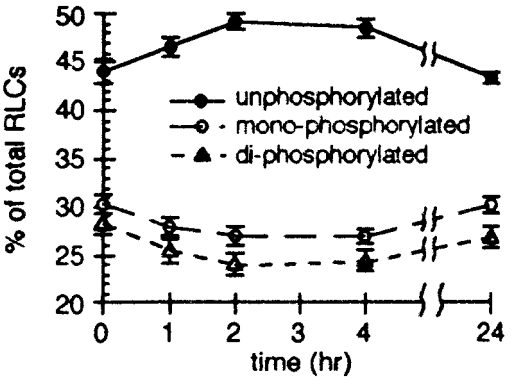

Fig. 10. Regulatory light chain phosphorylation in wounded monolayers. Confluent monolayers of bovine aortic endothelial cells (BAECs) were labeled with ^{35}S-methionine for 24 h and then wounded. At various times after wounding, cells in three identical dishes were lysed, myosin II was immunoadsorbed from the lysates, and the unphosphorylated, mono- and diphosphorylated forms of the RLCs separated by two-dimensional gel electrophoresis. The fraction of the total RLC radiolabel associated with each spot was determined by quantitative phosphor imaging. Each point on the graph represents the mean of three determinations; error bars = 1 SD. Unphosphorylated RLCs (solid circles) increased from 48% to 54% during the first 2 h after wounding and remained at this new level through 4 h. Note that there was a decrease in both monophosphorylated (open circles) and diphosphorylated (triangles) light chains at the same time, and that the mono- and diphosphorylated RLCs decreased in close parallel. The proportions of all three forms of RLCs returned to the original, pre-wound levels 24 h after wounding.

necessary to redistribute myosin II as the cytoskeleton is continuously assembled and disassembled. Based on the rate of fiber movement reported by Giuliano and Taylor and the diameter of their cells, it takes 1–5 h for a complete cycle of fiber assembly and disassembly in serum-deprived fibroblasts, which is notably similar to the turnover time for RLC phosphorylation in BAECs.

Myosin II heavy chains also undergo constitutive phosphorylation and dephosphorylation, but at a much slower rate than the RLCs. In fact, the observation periods used in the present study were not long enough to determine how long it takes for complete turnover of the

heavy chain phosphate. However, the short-term data indicate that it would take at least 8 h, even in migrating cells. Thus, heavy chain phosphorylation is unlikely to play a role in constitutive cytoskeletal assembly/disassembly nor in cytoskeletal remodeling during cell migration. Heavy chain phosphorylation could play a role in myosin redistribution during cytokinesis as in *Dictyostelium* [Sabry et al., 1997], as this is a relatively infrequent event that occurs over a longer time scale (the doubling time for these BAECs is 18–24 h). It would be worthwhile to examine turnover rates at different phases of the cell cycle in a synchronized population of cells.

What Is the Difference Between RLCs With "Fast" Versus "Slow" Phosphate Turnover?

There are two possible explanations for "fast" and "slow" turnover of RLC phosphates: (1) phosphates at serine 19 and threonine 18 turn over at different rates, and (2) different populations of myosin II are phosphorylated/dephosphorylated at different rates. The constant ratio of label in mono- versus diphosphorylated RLCs during both phosphate incorporation and turnover suggests that serine 19 and threonine 18 are processed coordinately at the same rates. This is not surprising, because myosin light chain kinase will phosphorylate both sites in vitro, and both sites can be dephosphorylated by type I protein phosphatase. However, in smooth muscle cells, phosphorylated serine 19 and phosphorylated threonine 18 have different spatial distributions and can be differentially affected by kinase and phosphatase inhibitors [Sakurada et al., 1998]. It is not known whether similar effects can be produced in BAECs or whether this reflects a difference between smooth and non-muscle cells in the regulation of RLC phosphorylation. In any case, in unperturbed BAEC monolayers, the different turnover rates must be attributed to different populations of myosin II, rather than differences between sites within the RLC.

"Fast" and "slow" RLCs are probably present in different locations in the cytoplasm. For example, myosin II associated with very dynamic structures such as lamellipodia may undergo much more frequent assembly and disassembly than myosin at the core of a stress fiber beneath the center of the cell. What determines the rate of phosphorylation/dephosphorylation at any given site in the cell is unknown. Because both myosin light chain kinase [DeLanerolle et al., 1981; Guerriero et al., 1981] and myosin phosphatase [Hirano et al., 1999; Inagaki et al., 1997; Murata et al., 1997] colocalize with myosin II in the cytoskeleton, spatial heterogeneity in enzyme distribution is probably not responsible. It is more likely that local activation or inhibition of kinase and phosphatase activities determines the rate. The increased rate of dephosphorylation during wound healing would then represent an activation of phosphatases in or around more stable myosin structures (with slowly cycling phosphates), thereby increasing the proportion of RLCs that are being rapidly dephosphorylated and consequently increasing the overall turnover rate.

Dephosphorylation of RLCs During Cell Migration

When quiescent BAECs were permitted to migrate by wounding a confluent monolayer, net dephosphorylation of RLCs was observed. The change was small, leading to complete dephosphorylation of only an additional 5–10% of the RLCs, but closely mirrored the locomotive behavior of the cells. That is, phosphorylation decreased as cells began to migrate, remained low for hours while BAECs continued to move, and returned to original levels when the monolayer reached confluence again and migration stopped. Because RLC phosphorylation correlates with assembly of myosin II into filaments in vitro [Trybus, 1991a] and into the insoluble cytoskeleton in vivo (Kolega and Kumar, 1999), dephosphorylation during migration implies that the pool of soluble myosin II increases. This additional mobilization of myosin II may reflect, or even facilitate, remodeling of the cytoskeleton as cells move.

Factors that induce a contractile response in endothelia, such as histamine, typically cause an increase in RLC phosphorylation [Garcia et al., 1995; Moy et al., 1993; Sheldon et al., 1993; Wysolmerski and Lagunoff, 1991]. RLC phosphorylation also activates myosin contractility in vitro. Thus, migrating BAECs are apparently less contractile than their quiescent counterparts, even though cytoplasmic contraction is a necessary part of amoeboid locomotion. This implies that turning on myosin II contractility does not activate movement; that is, cells do not require additional myosin II-based force-generating capacity to pull them forward or to push out protrusions. Disassembly of static

structures and redistribution of the motor components may be more important limiting events. For example, myosin II with phosphorylated RLCs may crosslink actin filaments in static bundles, analogous to the "latch-bridge" state hypothesized in smooth muscle. Dephosphorylation would then permit sliding-filament contraction, relaxation and expansion of the bundle, and/or translocation of actin and myosin II for assembly into new sites in the cytoplasm.

Control of RLC Phosphorylation/Dephosphorylation Rates

The rate at which phosphate cycles on and off RLCs can be dictated by the activities of myosin light chain kinases and phosphatases. Until recently, myosin light chain kinase was considered the main enzyme responsible for phosphorylating RLCs at serine 19/threonine 18, but it is now recognized that rho-associated kinases may also play a significant role. The rho kinase, p160(ROCK), phosphorylates RLCs in vitro [Amano et al., 1996], and inhibitors of rho activation will block phosphorylation of RLCs in endothelial cells in response to thrombin [Vouret-Craviari et al., 1998; Essler et al., 1998] or transforming growth factor-a [Wojciak-Stothard et al., 1998]. Interestingly, rho-kinase also phosphorylates the myosin-binding subunit of protein phosphatase-1 and inhibits dephosphorylation of RLCs [Hartshorne et al., 1998; Essler et al., 1998]. Thus, downregulation of the rho signaling pathway could produce the simultaneous depression of RLC phosphorylation and augmentation of RLC dephosphorylation that occurs in BAECs during wound healing.

After the initial changes in phosphorylation rates (1–2 h after wounding), a new equilibrium is reached, because net phosphorylation does not change between 2 and 4 h after wounding (Fig. 10). An important question that remains is whether the new steady-state cycle of phosphorylation/dephosphorylation runs faster in moving cells than in the quiescent monolayer. Future studies will examine steady-state on-off rates at later times in wound healing and will also determine the effects of chemotactic and angiogenic stimuli to determine if acceleration of the cycle might be a mechanism for speeding up wound healing and angiogenesis in vivo.

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REFERENCES

Amano M, Itoh M, Kimura K, Fukata Y, Chihara K, Nakano T, Matsuura Y, Kaibuchi K. 1996. Phosphorylation and activation of myosin by Rho-associated kinase (Rho-kinase). J Biol Chem 271:20246–20249.

Bray D, White JG. 1988. Cortical flow in animal cells. Science 239:883–888.

Cande WZ, Ezzell RM. 1986. Evidence for regulation of lamellipodial and tail contraction of glycerinated chicken embryonic fibroblasts by myosin light chain kinase. Cell Motil Cytoskeleton 6:640–648.

Chrzanowska-Wodnicka M, Burridge K. 1996. Rho-stimulated contractility drives the formation of stress fibers and focal adhesions. J Cell Biol 133:1403–1415.

Daniel JL, Sellers JR. 1992. Purification and characterization of platelet myosin. Methods Enzymol 215:78–88.

DeBiasio RL, LaRocca GM, Post PL, Taylor DL. 1996. Myosin II transport, organization, and phosphorylation: evidence for cortical flow/solation-contraction coupling during cytokinesis and cell locomotion. Mol Biol Cell 7:1259–1282.

DeLanerolle P, Adelstein RS, Feramisco JR, Burridge K. 1981. Characterization of antibodies to smooth muscle myosin kinase and their use in localizing myosin kinase in nonmuscle cells. Proc Natl Acad Sci USA 78:4738–4742.

Ehrlich HP, Rockwell WB, Cornwell TL, Rajaratnam JBM. 1991. Demonstration of a direct role for myosin light chain kinase in fibroblast-populated collagen lattice contraction. J Cell Physiol 146:1–7.

Essler M, Amano M, Kruse HJ, Kaibuchi K, Weber PC, Aepfelbacher M. 1998. Thrombin inactivates myosin light chain phosphatase via Rho and its target Rho kinase in human endothelial cells. J Biol Chem 273:21867–21874.

Fernandez A, Brautigan DL, Mumby M, Lamb NJC. 1990. Protein phosphatase type-1, not type 2-A, modulates actin microfilament integrity and myosin light chain phosphorylation in living nonmuscle cells. J Cell Biol 111:103–112.

Fernandez A, Mery J, Vandromme M, Bassett M, Cavadore J-C, Lamb NJC. 1991. Effective intracellular inhibition of the cAMP-dependent protein kinase by microinjection of a modified form of the specific inhibitor peptide PKI in living fibroblasts. Exp Cell Res 195:468–477.

Garcia JGN, Davis HW, Patterson CE. 1995. Regulation of endothelial cell gap formation and barrier dysfunction: role of myosin light chain phosphorylation. J Cell Physiol 163:510–522.

Giuliano KA, Taylor DL. 1990. Formation, transport, contraction, and disassembly of stress fibers in fibroblasts. Cell Motil Cytoskeleton 16:14–21.

Goeckeler ZM, Wysolmerski RB. 1995. Myosin light chain kinase-regulated endothelial cell contraction: the relationship between isometric tension, actin polymerization, and myosin phosphorylation. J Cell Biol 130:613–627.

Guerriero VJ, Rowley DR, Means AR. 1981. Production and characterization of an antibody to myosin light chain kinase and intracellular localization of the enzyme. Cell 27:449–458.

Hartshorne DJ, Ito M, Erdödi F. 1998. Myosin light chain phosphatase: subunit composition, interactions and regulation. J Muscle Res Cell Motil 19:325–341.

Hirano M, Niiro N, Hirano K, Nishimura J, Hartshorne DJ, Kanaide H. 1999. Expression, subcellular localization, and cloning of the 130-kDa regulatory subunit of myosin phosphatase in porcine aortic endothelial cells. Biochem Biophys Res Commun 254:490–496.

Holzapfel G, Wehland J, Weber K. 1983. Calcium control of actin-myosin based contraction in triton models of mouse 3T3 fibroblasts is mediated by the myosin light chain kinase (MLCK)-calmodulin complex. Exp Cell Res 148: 117–126.

Höner B, Citi S, Kendrick-Jones J, Jockusch BM. 1988. Modulation of cellular morphology and locomotory activity by antibodies against myosin. J Cell Biol 107:2181–2189.

Inagaki N, Nishizawa M, Inagaki M. 1997. Myosin binding subunit of smooth muscle myosin phosphatase at the cell-cell adhesion sites in MDCK cells. Biochem Biophys Res Commun 230:552.

Itoh T, Ikebe M, Kargacin GJ, Hartshorne DJ, Kemp BE, Fay FS. 1989. Effects of modulators of myosin light-chain activity in single smooth muscle cells. Nature 338:164–167.

Kolega J. 1997. Asymmetry in the distribution of free versus cytoskeletal myosin II in locomoting microcapillary endothelial cells. Exp Cell Res 231:66–82.

Kolega J, Kumar K. 1999. Regulatory light chain phosphorylation and the assembly of myosin II into the cytoskeleton of microcapillary endothelial cells. Cell Motil Cytoskeleton 43:255–268.

Kolega J, Taylor DL. 1993. Gradients in the concentration and assembly of myosin II in living fibroblasts during locomotion and fiber transport. Mol Biol Cell 4:819–836.

Lamb NJC, Fernandez A, Conte MA, Adelstein R, Glass DB, Welch WJ, Feramisco JR. 1988. Regulation of actin microfilament integrity in living nonmuscle cells by cAMP-dependent protein kinase and the myosin light chain kinase. J Cell Biol 106:1955–1971.

Lamb NC, Fernandez A, Watrin A, Labbe J-C, Cavadore J-C. 1990. Microinjection of p34cdc2 kinase induces marked changes in cell shape, cytoskeletal organization, and chromatin structure in mammalian fibroblasts. Cell 60:151–165.

Ludowyke RI, Peleg I, Beaven MA, Adelstein RS. 1989. Antigen-induced secretion of histamine and the phosphorylation of myosin by protein kinase C in rat basophilic leukemia cells. J Biol Chem 264:12492–12501.

Masuda H, Owaribe K, Hiyashi H, Hatano S. 1984. Ca^{2+}-dependent contraction of human lung fibroblasts treated with triton X-100: a role of Ca^{2+}-calmodulin-dependent phosphorylation of myosin 20,000-dalton light chain. Cell Motil Cytoskeleton 4:315–331.

Matsumura F, Ono S, Yamakita Y, Totsukawa G, Yamashiro S. 1998. Specific localization of serine 19 phosphorylated myosin II during cell locomotion and mitosis of culture cells. J Cell Biol 140:119–129.

Moy AB, Shasby SS, Scott BD, Shasby DM. 1993. The effect of histamine and cyclic adenosine monophosphate on myosin light chain phosphorylation in human umbilical vein endothelial cells. J Clin Invest 92:1198–1206.

Murata K, Hirano K, Vill-Moruzzi E, Hartshorne DJ, Brautigan DL. 1997. Differential localization of myosin and myosin phosphatase subunits in smooth muscle cells and migrating fibroblasts. Mol Biol Cell 8:663–673.

Post PL, DeBiasio RL, Taylor DL. 1995. A fluorescent protein biosensor of myosin II regulatory light chain phosphorylation reports a gradient of phosphorylated myosin II in migrating cells. Mol Biol Cell 6:1755–1768.

Sabry JH, Moores SL, Ryan S, Zang J-H, Spudich JA. 1997. Myosin heavy chain phosphorylation site regulate myosin localization during cytokinesis in living cells. Mol Biol Cell 8:2605–2615.

Sakurada K, Seto M, Sasaki Y. 1998. Dynamics of myosin light chain phosphorylation at Ser^{19} and Thr^{18}/Ser^{19} in smooth muscle cells in culture. Am J Physiol 274:C1563–C1572.

Satterwhite LL, Lohka MJ, Wilson KL, Scherson TY, Cisek LJ, Corden JL, Pollard TD. 1992. Phosphorylation of myosin-II regulatory light chain by cyclin-$p34^{cdc2}$: a mechanism for the timing of cytokinesis. J Cell Biol 118:595–605.

Sheldon R, Moy A, Lindsley K, Shasby S, Shasby DM. 1993. Role of myosin light-chain phosphorylation in endothelial cell retraction. Am J Physiol 265:L606–L612.

Stark F, Golla R, Nachmias VT. 1991. Formation and contraction of a microfilamentous shell in saponin-permeabilized platelets. J Cell Biol 112:903–913.

Tan JL, Ravid S, Spudich JA. 1992. Control of nonmuscle myosins by phosphorylation. Annu Rev Biochem 61:721–759.

Trybus KM. 1991a. Assembly of cytoplasmic and smooth muscle myosins. Curr Opin Cell Biol 3:105–111.

Trybus KM. 1991b. Regulation of smooth muscle myosin. Cell Motil Cytoskeleton 18:81–85.

Verin AD, Cooke C, Herenyiova M, Patterson CE, Garcia JG. 1998. Role of Ca^{2+}/calmodulin-dependent phosphatase 2B in thrombin-induced endothelial cell contractile responses. Am J Physiol 275:L788–L799.

Vouret-Craviari V, Boquet P, Pouyssegur J, Obberghen-Schilling EV. 1998. Regulation of the actin cytoskeleton by thrombin in human endothelial cells: role of Rho proteins in endothelial barrier function. Mol Biol Cell 9:2639–2653.

Wessels D, Soll DR. 1990. Myosin II heavy chain null mutant of *Dictyostelium* exhibits defective intracellular particle movement. J Cell Biol 111:1137–1148.

Wessels D, Soll, DR, Knecht,D, Loomis WF, DeLozanne A, Spudich J. 1988. Cell motility and chemotaxis in *Dictyostelium* amebae lacking myosin heavy chain. Dev Biol 128:164–177.

Wojciak-Stothard B, Entwistle A, Garg R, Ridley AJ. 1998. Regulation of TNF-alpha-induced reorganization of the actin cytoskeleton and cell-cell junctions by Rho, Rac, and Cdc42 in human endothelial cells. J Cell Physiol 176:150–165.

Wysolmerski RB, Lagunoff D. 1990. Involvement of myosin light-chain kinase in endothelial cell retraction. Proc Natl Acad Sci USA 87:16–20.

Wysolmerski RB, Lagunoff D. 1991. Regulation of permeabilized endothelial cell retraction by myosin phosphorylation. Am J Physiol 261:C32–C40.

Yamakita Y, Yamahiro S, Matsumura F. 1994. In vivo phosphorylation of regulatory light chain of myosin II during mitosis of cultured cells. J Cell Biol 124:129–137.