Phosphorylation on Threonine-18 of the Regulatory Light Chain Dissociates the ATPase and Motor Properties of Smooth Muscle Myosin II

ARTICLE *in* BIOCHEMISTRY · NOVEMBER 1995

Impact Factor: 3.02 · DOI: 10.1021/bi00039a012 · Source: PubMed

4 AUTHORS, INCLUDING:

Anne R Bresnick
Albert Einstein College of Medicine
77 PUBLICATIONS **3,427** CITATIONS
SEE PROFILE

Otto Baumann
Universität Potsdam
78 PUBLICATIONS **1,529** CITATIONS
SEE PROFILE

Phosphorylation on Threonine-18 of the Regulatory Light Chain Dissociates the ATPase and Motor Properties of Smooth Muscle Myosin II

Anne R. Bresnick, Vicki L. Wolff-Long, Otto Baumann, and Thomas D. Pollard

Biochemistry, **1995**, 34 (39), 12576-12583• DOI: 10.1021/bi00039a012 • Publication Date (Web): 01 May 2002

Phosphorylation on Threonine-18 of the Regulatory Light Chain Dissociates the ATPase and Motor Properties of Smooth Muscle Myosin II[†,‡]

Anne R. Bresnick,[§] Vicki L. Wolff-Long,[§] Otto Baumann,[∥] and Thomas D. Pollard*[,§]

Department of Cell Biology and Anatomy, Johns Hopkins School of Medicine, Baltimore, Maryland 21205, and Institut fur Zoologie, Universitat Regensburg, D-93040 Regensburg, Germany

Received June 9, 1995; Revised Manuscript Received August 7, 1995[⊗]

ABSTRACT: We cloned the full-length cDNA for the cytoplasmic myosin II regulatory light chain (RLC) from a stage 1−2 *Xenopus* oocyte library. The *Xenopus* RLC is 94% identical to the chicken smooth muscle myosin RLC. All of the protein kinase C and myosin light chain kinase phosphorylation sites are conserved. Using trifluoperazine [Trybus, K. M., Waller, G. S., & Chatman, T. A. (1994) *J. Cell Biol. 124,* 963−969], we removed the RLC of smooth muscle myosin and replaced it with recombinant *Xenopus* RLCs. The wild-type *Xenopus* RLC substitutes for the gizzard RLC in actin-activated ATPase and *in vitro* motility assays. We made alanine substitutions of the two residues phosphorylated by myosin light chain kinase, Ser-19 and Thr-18. All of the myosin hybrids, regardless of their mutations or phosphorylation, have similar K^+EDTA ATPase activities. As expected, the T18A, S19A hybrid had no actin-activated ATPase, whereas the T18A hybrid phosphorylated on Ser-19 had an actin-activated ATPase similar to that of wild-type hybrids phosphorylated only on Ser-19. The actin-activated ATPase of myosin phosphorylated only on Thr-18 is approximately 15-fold lower than that of myosin phosphorylated on Ser-19. Phosphorylation of either Ser-19 or Thr-18 permits the formation of filaments. Remarkably, in the gliding filament assay, myosin phosphorylated only on Thr-18 moves actin filaments at velocities similar to myosin phosphorylated on Ser-19 or both Thr-18 and Ser-19.

Phosphorylation of the regulatory light chain (RLC)[1] of myosin II initiates the contraction of vertebrate smooth muscle (Somlyo & Somlyo, 1994) and is presumed to play a similar role in the functions of myosin in nonmuscle cells. For example, light chain phosphorylation of cytoplasmic myosin has been implicated in cytokinesis, receptor capping, and cell locomotion (Yamakita et al., 1994; Berlot et al., 1985, 1987; Bourguignon et al., 1981). RLC phosphorylation primarily occurs on Ser-19 and is catalyzed by the Ca^{2+}/calmodulin-dependent myosin light chain kinase (MLCK). RLC phosphorylation promotes myosin polymerization, enhances the actin-activated ATPase of myosin, and is essential for the movement of actin filaments by myosin *in vitro* (Sellers et al., 1985; Ikebe & Hartshorne, 1985).

In general, it is believed that phosphorylation at Ser-19 is sufficient to support the ATPase and motor functions of myosin. *In vitro*, high concentrations of MLCK phosphorylate Thr-18 in addition to Ser-19 (Ikebe et al., 1986). Although phosphorylation on both Ser-19 and Thr-18 of the RLC increases the actin-activated ATPase of myosin, additional phosphorylation on Thr-18 has no effect on the velocity of myosin-coated beads moving along actin cables (Umemoto et al., 1989). It is unknown if phosphorylation on Thr-18 supplements phosphorylation on Ser-19 *in vivo*, or if this secondary phosphorylation site is physiologically irrelevant.

The RLC is also a substrate for protein kinase C (PKC) and $p34^{cdc2}$ kinase which phosphorylate three residues on the N-terminus of the RLC, Ser-1, Ser-2, and Thr-9 (Nishikawa et al., 1984; Satterwhite et al., 1992). Myosin phosphorylated by both PKC and MLCK has a lower actin-activated Mg^{2+} ATPase than myosin phosphorylated by MLCK alone, due to lower a affinity for actin. Phosphorylation by PKC also inhibits phosphorylation on Ser-19 of the RLC by MLCK (Nishikawa et al., 1984; Ikebe et al., 1986).

To clarify the role of Thr-18 phosphorylation in the regulation of myosin, we made alanine mutations at the MLCK sites of the *Xenopus* RLC and reconstituted the recombinant RLCs with the heavy chain of chicken smooth muscle myosin. We used the *Xenopus* RLC of cytoplasmic myosin to correlate *in vitro* biochemistry with ongoing *in vivo* experiments. The *Xenopus* wild-type RLC substitutes for the gizzard RLC. Phosphorylation only on Thr-18 allows filament formation; however, the actin-activated ATPase of this myosin is 15-fold lower than the ATPase of myosin phosphorylated on Ser-19 or both Thr-18 and Ser-19. Interestingly, phosphorylation at Thr-18 supports the movement of actin filaments at velocities comparable to those for myosin phosphorylated on Ser-19.

MATERIALS AND METHODS

Cloning of the Xenopus Regulatory Light Chain cDNA. A myosin II RLC cDNA was cloned from a stage 1−2 *Xenopus* oocyte lambda ZAP cDNA library (kindly provided

[†] This work was supported by grants from the American Cancer Society (A.R.B.) and the Human Frontiers Science Program, by NIH grant GM26132, and by Deutsche Forschungsgemeinschaft Ba1284/3-1 (O.B.).

[‡] The nucleotide sequence presented in this paper has been submitted to GenBank under Accession Number U31118.

[§] Johns Hopkins School of Medicine.

[∥] Universitat Regensburg.

[⊗] Abstract published in *Advance ACS Abstracts,* September 15, 1995.

[1] Abbreviations: RLC, regulatory light chain; XRLC, *Xenopus* regulatory light chain; MLCK, myosin light chain kinase; PKC, protein kinase C; TFP, trifluoperazine.

0006-2960/95/0434-12576$09.00/0

by Alan Wolffe) using the rat aortic smooth muscle RLC cDNA as the probe (kindly provided by Mark Taubman). The nucleotide sequence was determined on both strands of DNA by the dideoxynucleotide termination method (Sanger et al., 1977).

Construction of the XRLC Expression Vector. Using PCR mutagenesis, an *Nde*I site was made at the start methionine of the XRLC cDNA. An *Nde*I fragment of the XRLC was subcloned into the *Nde*I site of the T7 expression vector pMW172 (Way et al., 1990). Correct orientation of the XRLC was confirmed by sequencing.

Bacterial Expression and Purification of the XRLC. BL21 (DE3) cells were transformed with pMW172-XRLC. A colony was grown overnight at 37 °C in 50 mL of LB medium containing 100 μg/mL ampicillin. A 1:100 dilution of the overnight culture was used to inoculate 5 × 600 mL of enriched medium (2% bactotryptone, 1% yeast extract, 0.5% NaCl, 2% glycerol, 50 mM KPO_4) containing 100 μg/mL ampicillin. The cultures were grown at 37 °C to an OD_{600} of 0.8 and then induced with 1 mM IPTG for 5 h. The cells were harvested at 15000*g* for 10 min. The cell pellet was resuspended in 60 mL of 50 mM Tris, pH 7.5, 2 mM DTT, 1 mM EDTA, and 8 M urea and sonicated for 6 × 10 s on ice. The lysate was centrifuged at 100000*g* for 30 min, and the supernatant was dialyzed for 36 h against 4.0 L of buffer P (20 mM imidazole, pH 7.0, 0.5 mM DTT, 1 mM EDTA, 0.02% NaN_3) with two buffer changes. Solid NaCl was added to the dialyzed supernatant to 4 M with stirring on ice and centrifuged at 100000*g* for 30 min. The clarified supernatant was applied to a Phenyl Sepharose (Pharmacia) column (2.5 × 25 cm) equilibrated in buffer P containing 4 M NaCl. The column was washed with buffer P containing 4 M NaCl and developed with a 4−0 M NaCl linear gradient, followed by a second buffer P wash. Fractions containing the XRLC were pooled and dialyzed against 4.0 L of buffer D (20 mM Tris, pH 8.0, 0.2 mM DTT, 1 mM EDTA, 20 mM NaCl, 0.02% NaN_3). The dialyzed pool was applied to a Macro-Prep Q (Bio-Rad) column (2.5 × 27 cm) equilibrated in buffer D. The column was washed with buffer D and developed with a 20−300 mM NaCl linear gradient. Fractions containing the XRLC were pooled and applied to a hydroxylapatite (Bio-Rad) column (2.5 × 12 cm) equilibrated in buffer H (1 mM potassium phosphate, pH 6.0, 0.1 mM DTT, 1 mM EDTA, 0.02% NaN_3). The column was washed with buffer H and developed with a 0−0.4 M potassium phosphate linear gradient. The average protein yield was 12 mg per liter of cells. The purified protein was dialyzed into 2 M urea, 5 mM KPO_4, pH 7.0, and 0.1 mM DTT and concentrated in an Amicon filtration unit using a PM10 membrane. DTT was added to 1 mM, and the concentrated XRLCs were stored at −80 °C.

Construction of Mutant XRLCs. Three oligonucleotides, 5′-TCAACGCGCAGCATCCAATG-3′, 5′-ACGCGCAACAGCCAATGTAT-3′, and 5′-TCAACGCGCAGCAGCCAATGTAT-3′, were used to mutate Thr-18 to Ala, Ser-19 to Ala, and both Thr-18 and Ser-19 to Ala, respectively. Site-directed mutagenesis was performed using the Bio-Rad Muta-Gene Phagemid *in vitro* Mutagenesis kit. The cDNA sequences of mutant XRLCs were confirmed by sequencing the coding region of the DNA. The mutant XRLCs were subcloned into pMW172, expressed, and purified as described for the wild-type XRLC.

Protein Purification. Unphosphorylated smooth muscle myosin was prepared from chicken gizzards as described (Sellers et al., 1981). Rabbit skeletal muscle actin was prepared from acetone powder by the method of Spudich and Watt (1971) and further purified by gel filtration on Sephadex G150 to remove low levels of contaminating ATPase activity. Chicken gizzard tropomyosin was purified as a by-product of the myosin purification using two rounds of acid precipitation and solubilization followed by chromatography on hydroxylapatite (Smillie, 1982). Chicken gizzard myosin light chain kinase (MLCK) was purified as described by Conti and Adelstein (1991). Bovine calmodulin was purchased from Calbiochem. Native gelsolin and recombinant gelsolin were kindly provided by Paul Janmey and Michael Way, respectively.

Phosphorylation of Recombinant XRLCs. Recombinant XRLCs (2−4 mg/mL) were phosphorylated in 20 mM Tris-HCl, pH 7.5, 15 mM KCl, 5 mM $MgCl_2$, 0.4 mM $CaCl_2$, 2 mM DTT, 0.02% NaN_3, 1 mM ATP, 1 μM MLCK, and 1.2 μM calmodulin at 30 °C for 45 min. To phosphorylate wild-type XRLC at both Thr-18 and Ser-19, 1.5 μM MLCK and 1.5 μM calmodulin were used. The extent of phosphorylation was monitored using glycerol polyacrylamide gels according to Perrie and Perry (1970).

Denuding and Reconstitution of Myosin. Myosin (1.5−3 mg/mL) in 20 mM imidazole, pH 7.0, 0.5 M NaCl, 0.02% NaN_3, 1 mM DTT, 5 mM EDTA, 5 mM CDTA, and 2 mM EGTA was incubated with 10 mM DTT, 1.5 mM ATP, 0.05% Triton X-100, and 1.5−3 mM trifluoperazine (TFP, Sigma) for 30 min at 0 °C. Free RLCs and myosin were separated on a S300 column (2.5 × 12.5 cm) equilibrated in the same buffer as the incubation except with 1 mM DTT, 0.5 mM ATP, and 1 mM TFP. Denuded myosin was passed through a Sephadex G25 (coarse) spin column, equilibrated in 20 mM imidazole, pH 7.0, 0.5 M NaCl, 0.02% NaN_3, 2 mM $MgCl_2$, and 0.5 mM DTT, to remove free TFP. Denuded myosin was incubated with an excess of recombinant XRLCs, 5 mM $MgCl_2$, and 5 mM DTT at 0 °C for 60 min. Reconstituted myosin was dialyzed overnight against 5 mM PIPES, pH 6.5, 20 mM NaCl, 1 mM DTT, 5 mM $MgCl_2$, and 0.02% NaN_3. Unbound XRLCs were removed by four cycles of centrifugation at 5000*g* for 10 min. The final myosin pellet was resuspended in a small volume of 10 mM imidazole, 0.55 M NaCl, 2 mM DTT, and 0.02% NaN_3 and incubated on ice for 90 min. The myosin was clarified by centrifugation at 240000*g* for 25 min. Typically, the final yield of reconstituted myosin was 20−25% of the starting material.

ATPase Measurements. Mg^{2+} ATPase assays were performed at 22 °C using an ATP-regenerating system (Catterall & Pederson, 1971). Gel-filtered G-actin was polymerized in the presence of a 500:1 molar ratio of actin to gelsolin and preincubated with tropomyosin at a 4:1 molar ratio. Myosin hybrids (10−20 μg/mL) were incubated with F-actin (0−30 μM) in 20 mM Tris, pH 7.5, 18−28 mM KCl, 5 mM $MgCl_2$, 0.2 mM EGTA, 1 mM DTT, 2 mM Mg^{2+}ATP, 0.5 mM phosphoenolpyruvate (Sigma), 0.25 mM NADH (Boehringer Mannheim, $\epsilon = 6.2$ OD_{340}/mM), 5 units of lactate dehydrogenase (Sigma), and 2 units of pyruvate kinase (Sigma) per 500 μL volume. The rate of oxidation of NADH to NAD was measured by absorbance at 340 nm. The dependence of the ATPase rate on the concentration of actin

```
CTCATTTAATCTCAGCCTGCAGAGCAGACTACTCACTACCCTCTCTCCCTCCCACACGAA    60
                          •         *         *         *

TAACCCACCGCCAAGATGTCCAGCAAAAGAGCAAAGACCAAGACCACCAAAAAGCGCCCT   120
                M  S  S  K  R  A  K  T  K  T  T  K  K  R  P     15
   *         *         *         *         •         •

CAACGCGCAACATCCAATGTATTCGCCATGTTTGACCAGTCCCAGATCCAGGAGTTCAAG   180
 Q  R  A  T  S  N  V  F  A  M  F  D  Q  S  Q  I  Q  E  F  K     35
   *         *         *         •         •         •

GAGGCGTTTAACATGATCGATCAGAACCGTGACGGCTTTATTGATAAAGAAGATCTGCAT   240
 E  A  F  N  M  I  D  Q  N  R  D  G  F  I  D  K  E  D  L  H     55
   •         *         •         •         •         •

GACATGCTTGCCTCTCTTGGGAAAAACCCTACAGACGAGTATTTAGATGCCATGATGAAT   300
 D  M  L  A  S  L  G  K  N  P  T  D  E  Y  L  D  A  M  M  N     75
   *         *         *         *         *         *

GAAGCCCCCGGCCCTATCAACTTCACCATGTTCCTTACCATGTTTGGTGAGAAGCTGAAT   360
 E  A  P  G  P  I  N  F  T  M  F  L  T  M  F  G  E  K  L  N     95
   •         *         *         •         •         •

GGGACCGACCCTGAGGATGTCATTAGGAACGCATTTGCATGCTTTGATGAAGAAGCAATA   420
 G  T  D  P  E  D  V  I  R  N  A  F  A  C  F  D  E  E  A  I    115
   *         *         •         *         *         *

GGTTTTATCCAGGAGGACAATCTGAGGGAATTGCTGACAACAATGGGTGACCGATTTACA   480
 G  F  I  Q  E  D  N  L  R  E  L  L  T  T  M  G  D  R  F  T    135
   *         *         •         *         •         *

GATGAGGACGTAGATGAGCTTTTCAGAGAGGCCCCAATCGACAAGAAGGGCAATTTCAAT   540
 D  E  D  V  D  E  L  F  R  E  A  P  I  D  K  K  G  N  F  N    155
   *         *         *         •         •         •

TACAACGAATTCACCCGCATCTTGAAACATGGAGCTAAAGACAAGGATGACTAAGCTACA   600
 Y  N  E  F  T  R  I  L  K  H  G  A  K  D  K  D  D  *          172
AGTGCCACTGCAACAAGAACTGCACTGCTCTCTGCCATTCTCATGTCTGTCTTCAACTCA   660
TTGCATGCATATAGCTCTAGATCTTTTCTTTTCTGTGTATTTATGGATCTGTACCAGGCC   720
TTTTTGGTACATGGGCACATACTGTATGTAACCAAACTGGAAAACAAAAAGTGCAATGTT   780
AACAAGCAGTACACAGCAGGTCTTTTCTGGGGACTTAAGCTGGATTTTTTGCAATTTTGT   840
AATAAAATTTGGAATTTAAATTAACTGTCAGTTGAAACTTTTGTCCGAATGCTCTGGCAT   900
ATCCATGGAATATTACAGATGCACTGAAACACAGTTTTTGTTGCCGTGAGCTACACACAC   960
AATTTTTTTTTTATATGACTGTGTGTTTGTGTGTATACACACAGTCATATGAAAATGTTG  1020
GGAATATATAACATGCCTTATGGAAACAGTAGTATTTCAGCAGTGACATAAAGTTTATTG  1080
GATTAACAGAAAATATACAATATGCATCATAACAAAATTAGACAGGTGAATAAATTTGGG  1140
CACCCCAACAGAGATATTACATCAATACTTAGTTGAGCCTCCTTTTGCAAATGTAACAGC  1200
CTCTAGACTCC                                                    1211
```

```
Xen RLC    MSSKRAKTKTTKKRPQRATSNVFAMFDQSQIQEFKEAFNMIDQNRDGFID
                    |
ChG RLC    MSSKRAKAKTTKKRPQRATSNVFAMFDQSQIQEFKEAFNMIDQNRDGFID

Xen RLC    KEDLHDMLASLGKNPTDEYLDAMMNEAPGPINFTMFLTMFGEKLNGTDPE
                     |                ||  |
ChG RLC    KEDLHDMLASMGKNPTDEYLEGMMSEAPGPINFTMFLTMFGEKLNGTDPE

Xen RLC    DVIRNAFACFDEEAIGFIQEDNLRELLTTMGDRFTDEDVDELFREAPIDK
                           |   |  |                  |   ||
ChG RLC    DVIRNAFACFDEEASGFIHEDHLRELLTTMGDRFTDEEVDEMYREAPIDK

Xen RLC    KGNFNYNEFTRILKHGAKDKDD
                    |
ChG RLC    KGNFNYVEFTRILKHGAKDKDD
```

FIGURE 1: (A, top) Amino acid and nucleotide sequence of the *Xenopus* RLC of cytoplasmic myosin. (B, bottom) Alignment of translated amino acid sequences of the *Xenopus* and gizzard RLCs. Bold indicates the phosphorylation sites. The *Xenopus* RLC is 94% identical to the gizzard RLC.

was fit to an equation for a hyperbola using the computer program Regression.

K^+EDTA ATPase assays were performed with minor modifications of the method of Geladopoulos et al. (1991). Myosin (20−40 μg/mL) was incubated at 22 °C in 0.5 M KCl, 10 mM imidazole, pH 7.0, 2 mM EDTA, 0.02% NaN_3, 1 mM DTT, and 1.5 mM K^+ATP. Inorganic phosphate was quantitated in a microtiter plate by quenching 10 μL aliquot time points in 200 μL of MGAMPVST [0.023% malachite green, 1.6% ammmonium molybdate in 6 N HCl, 0.32% poly(vinyl alcohol) and 0.034% sterox (Bacharach)]. After 1 min, 50 μL of 34% sodium citrate was added to stop complex production. Samples were incubated for 1−1.5 h to obtain full color development prior to reading at a wavelength of 600 nm.

In Vitro Motility Assay. The gliding actin filament assay was performed at 24−26 °C essentially as described by Kron et al. (1991) and Sellers et al. (1993). For some experiments myosin hybrids were attached to the nitrocellulose with the smooth muscle myosin monoclonal antibody S2.2 (Horowitz & Trybus, 1992), kindly provided by Kathy Trybus. Filament velocities were quantitated by marking the centroid position of the filament at 5 s intervals for a total of 20 s. Under conditions in which the filament velocity was slow, the velocity was measured for ≥1 min. For each experimental condition, the velocities of 20 or more filaments are presented as the means and the standard deviations of the means.

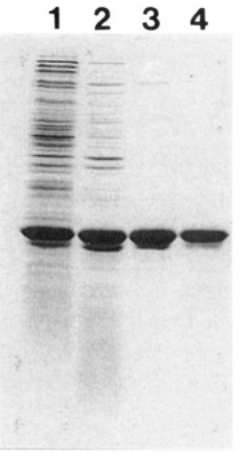

FIGURE 2: Bacterial expression and purification of the wild-type XRLC. A 14% SDS−polyacrylamide gel electrophoresis stained with Coomassie Blue showing (lane 1) the whole cell lysate, (lane 2) Phenyl Sepharose pool, (lane 3) Macro-Prep Q pool, and (lane 4) hydroxylapatite pool.

RESULTS

Cloning of the Xenopus cytoplasmic regulatory light chain. The myosin II regulatory light chain cDNA from a stage 1−2 oocyte *Xenopus* library consisted of a 76 nucleotide 5′-untranslated region, a 519 nucleotide open reading frame, and a 617 nucleotide 3′-untranslated region (Figure 1A). The predicted amino acid sequence of the XRLC is 94% identical to the sequence of chicken smooth muscle RLC (Messers & Kendrick-Jones, 1988) (Figure 1B). In particular, all three PKC phosphorylation sites (Ser-1, Ser-2, Thr-9) and both MLCK phosphorylation sites (Thr-18, Ser-19) are conserved. Of the 12 amino acid differences between the *Xenopus* and chicken gizzard RLCs, eight substitutions are conservative. We constructed mutants of the *Xenopus* RLC in which Thr-18 and Ser-19 were individually or both replaced with alanine. These mutations allowed us to assess the effect of Thr-18 phosphorylation on the activity of myosin.

Bacterial Expression and Purification of XRLCs. We purified bacterially expressed *Xenopus* RLC by sequential chromatography on Phenyl Sepharose, anion-exchange, and hydroxylapatite columns (Figure 2). This method yielded approximately 12 mg of homogenous XRLC per liter of culture. Purification of the *Xenopus* RLC by anion-exchange chromatography of solubilized inclusion bodies (Sweeney et al., 1994; Trybus et al., 1994; Kamisoyama et al., 1994) yielded only small amounts of impure *Xenopus* RLC.

Phosphorylation of Recombinant XRLCs. Myosin light chain kinase (MLCK) quantitatively phosphorylated acceptor sites on all of the recombinant mutant XRLCs (Figure 3). Thus wild-type had two phosphates (lane 2), the T18A and

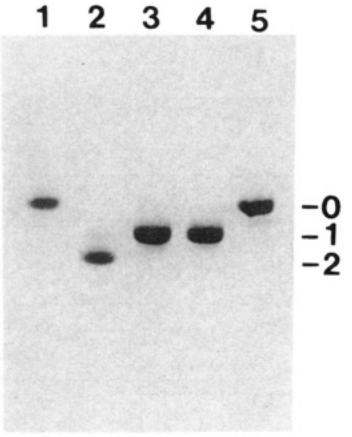

FIGURE 3: Recombinant XRLCs phosphorylated with chicken gizzard MLCK. Phosphorylation was analyzed using glycerol PAGE stained with Coomassie Blue. (Lane 1) Unphosphorylated wt XRLC; (lane 2) wt XRLC phosphorylated at Thr-18 and Ser-19; (lane 3) T18A XRLC phosphorylated at Ser-19; (lane 4) S19A XRLC phosphorylated at Thr-18; and (lane 5) unphosphorylated T18A,S19A XRLC. Marks indicate the mobility of light chains with zero, one, or two phosphates.

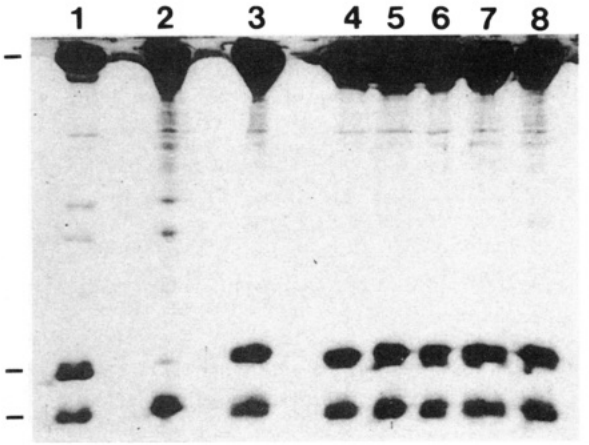

FIGURE 4: Reconstitution of denuded myosin with recombinant XRLCs. Myosin analyzed by gel electrophoresis in SDS and stained with Coomassie Blue. (Lane 1) Native gizzard myosin; (lane 2) myosin denuded of RLC; (lane 3) myosin hybrid with wt XRLC phosphorylated on Ser-19; (lane 4) myosin hybrid with unphosphorylated wt XRLC; (lane 5) myosin hybrid with T18A,S19A XRLC; (lane 6) myosin hybrid with wt XRLC phosphorylated on Thr-18 and Ser-19; (lane 7) myosin hybrid with unphosphorylated S19A XRLC; and (lane 8) myosin hybrid with S19A XRLC phosphorylated on Thr-18.

S19A single mutants each had one phosphate (lanes 3 and 4), and the T18A,S19A double mutant had no phosphates (lane 5).

Reconstitution of Smooth Muscle Myosin with XRLCs. Gizzard myosin, denuded of its endogenous RLC by the method of Trybus et al. (1994), was reconstituted with phosphorylated recombinant XRLCs. The addition of trifluoperazine caused >98% of the endogenous RLC to be dissociated from the heavy chain of myosin (Figure 4, lane 2). The extent of reconstitution of the gizzard myosin heavy chain with the recombinant XRLCs was the same for all of the mutant light chains (Figure 4).

ATPase Activity of XRLC/Myosin Hybrids. Regardless of their mutations or phosphorylation, most of the myosin hybrids had K^+EDTA ATPase activities approximately 2-fold higher than that of native myosin (Table 1). Even myosin hybrids in which the XRLC was exchanged onto gizzard

Table 1: K^+EDTA ATPase of XRLC/Gizzard MII Hybrids[a]

	activity [nmol of P_i of (nmol of head)$^{-1}$ s^{-1}]	
gizzard MII-0P[b]	0.79 ± 0.3	(6)
gizzard MII-2P[c]	1.5 ± 0.4	(3)
wt XRLC/MII-0P	2.0 ± 1.0	(4)
wt XRLC/MII-1P	1.8 ± 0.6	(11)
wt XRLC/MII-2P	1.9 ± 0.5	(8)
T18A XRLC/MII-1P	1.5 ± 0.3	(10)
S19A XRLC/MII-0P	0.9	(1)
S19A XRLC/MII-1P	1.7 ± 0.6	(13)
T18AS19A XRLC/MII-0P	0.95	(1)

[a] The mean and standard deviation of the mean from independent preparations is indicated with the number of preparations shown in parentheses. [b] Native gizzard myosin, unphosphorylated. [c] Gizzard myosin heavy chain reconstituted with diphosphorylated gizzard RLC.

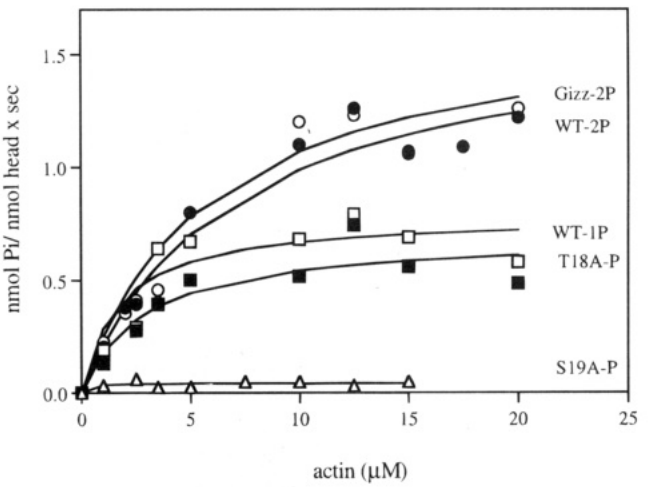

FIGURE 5: Actin-activated Mg^{2+} ATPase activities of myosin hybrids in 20 mM Tris, pH 7.5, 18−28 mM KCl, 5 mM $MgCl_2$, 0.2 mM EGTA, 1 mM DTT, 2 mM ATP, and 0−30 μM actin at 22 °C. The low rates of hydrolysis by actin alone and myosin alone are subtracted. (○) Gizzard RLC phosphorylated on Thr-18 and Ser-19; (●) wt XRLC phosphorylated on Thr-18 and Ser-19; (□) wt XRLC phosphorylated on Ser-19; (■) T18A XRLC phosphorylated on Ser-19; (△) S19A XRLC phosphorylated on Thr-18. Symbols represent averages of two or three observed data sets.

myosin by the method of Morita et al. (1991) had a higher K^+EDTA ATPase (data not shown). Thus removal of the gizzard RLC and reconstitution with recombinant RLCs is not deleterious to the ATPase of myosin.

Gizzard myosin reconstituted with either gizzard or *Xenopus* RLCs phosphorylated on both Thr-18 and Ser-19 had a maximum actin-activated ATPase activity of approximately 1.7 s^{-1} (Figure 5 and Table 2). Myosin hybrids containing RLCs phosphorylated only on Ser-19 had an actin-activated ATPase activity approximately one-half of those hybrids containing RLCs phosphorylated on both Ser-19 and Thr-18 (Figure 5 and Table 2). These results confirm that phosphorylation on Thr-18 enhances the actin-activated ATPase of myosin phosphorylated on Ser-19 of the RLC (Ikebe & Hartshorne, 1985; Ikebe et al., 1986). Myosin reconstituted with the T18A, S19A double mutant had no detectable actin-activated ATPase activity even if treated with MLCK.

In 25 mM KCl and 30 μM actin filaments, myosin phosphorylated on Thr-18 had an actin-activated ATPase of 0.05 s^{-1} as compared to 0.74 s^{-1} for myosin phosphorylated on Ser-19 alone, and 1.63 s^{-1} for myosin phosphorylated on both Ser-19 and Thr-18. Kamisoyama et al. (1994)

Table 2: Actin-Activated ATPase Activity of XRLC/Gizzard MII Hybrids[a]

	V_{max} [nmol of P_i (nmol head)$^{-1}$ s^{-1}]	
gizzard RLC/MII-2P	1.75	(2)
wt XRLC/MII-2P	1.63 ± 0.49	(3)
wt XRLC/MII-1P	0.82 ± 0.33	(4)
T18A XRLC/MII-1P	0.66	(2)
S19A XRLC/MII-1P	0.05 ± 0.025	(3)
T18AS19A XRLC/MII-0P	0	(2)

[a] The mean and standard deviation of the mean from independent preparations is indicated with the number of preparations shown in parentheses. ATPase values for myosin hybrids phosphorylated on Thr-18 (S19A XRLC/MII-1P) are only for those preparations which supported motility similar to that of myosin hybrids phosphorylated on Ser-19. The ATPase activity in the absence of actin is subtracted.

determined actin-activated ATPase values of 0.08, 0.18, and 0.41 s^{-1} for smooth muscle myosin phosphorylated on Thr-18 alone, Ser-19 alone, or both Ser-19 and Thr-18. Their conditions were 65 mM KCl and 48 μM actin. In our ATPase assay using KCl concentrations of 65−80 mM, the maximum actin-activated ATPase rates were 0.01, 0.21, and 0.38 s^{-1} for myosin phosphorylated on Thr-18 alone, Ser-19 alone, or both Thr-18 and Ser-19. The S19-P and S19-P, T18-P values are similar to those of Kamisoyama et al. (1994), although in our hands the actin-activated ATPase for myosin phosphorylated only on Thr-18 was 15-fold lower than myosin phosphorylated on Ser-19 at both low and high KCl concentrations.

In the buffer used for the ATPase assays, more than 90% of the myosin containing XRLCs phosphorylated on either Ser-19 or Thr-18 pelleted when centrifuged at 250000*g* for 20 min (data not shown). This suggests that phosphorylation on Thr-18 stabilizes myosin in the 6S extended conformation, allowing polymerization, and confirms observations by Kamisoyama et al. (1994) that smooth muscle myosin phosphorylated only on Thr-18 favors the 6S conformation and forms filaments.

Motor Properties of XRLC/Myosin Hybrids. We assessed the movement of actin filaments by myosin hybrids in an *in vitro* motility assay. Myosin hybrids containing XRLCs phosphorylated on both Thr-18 and Ser-19 or Ser-19 alone moved actin filaments at a velocity of 0.81 ± 0.10 μm s^{-1} (Figure 6). Myosin reconstituted with the T18A, S19A double mutant did not move actin filaments. Although the myosin hybrid containing the XRLC phosphorylated only on Thr-18 had a very low actin-activated ATPase, it moved actin filaments at velocities similar to those for myosin phosphorylated at Ser-19 (0.65 ± 0.13 μm s^{-1}).

To test whether low concentrations of active myosin are capable of producing maximum actin filament motility, we diluted active myosin with either buffer or inactive myosin. Monomeric smooth muscle myosin phosphorylated on Ser-19 moves actin filaments at the maximum velocity down to a concentration of 10 μg/mL (data not shown). This is a lower threshold than filamentous phosphorylated smooth muscle myosin with which movement becomes discontinuous at concentrations below 62 μg/mL (Warshaw et al., 1990). However, dilution of wild-type hybrids phosphorylated on Ser-19 with either unphosphorylated wild-type hybrids or unphosphorylatable T18A, S19A hybrids slowed the gliding motility such that mixtures consisting of myosin with 12.5% WT-1P and 87.5% WT-0P or T18A, S19A moved actin

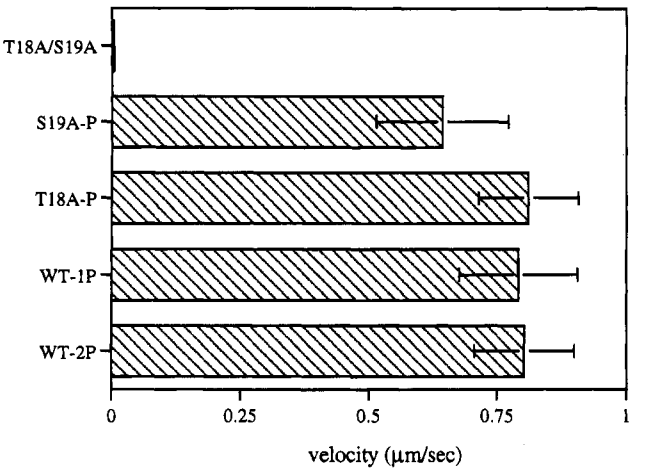

FIGURE 6: *In vitro* motility of myosin hybrids. The velocity of actin filament motility was assayed for myosin hybrids. Values are the means and standard deviations of the mean for a minimum of 20 filaments for two preparations of T18A, S19A, 18 preparations of S19A-P, eight preparations of T18A-P, 13 preparations of WT-1P, and eight preparations of WT-2P. Myosin hybrids phosphorylated on Thr-18 and Ser-19, or Ser-19 alone, and Thr-18 alone move actin filaments at the similar velocities.

filaments at only 0.128 μm s^{-1} (Figure 7A). The movement of the actin filaments was discontinuous in mixtures containing ≤25% of the WT-1P myosin. The velocity of actin filament motility was constant over a wide range of concentrations of the phosphorylated S19A hybrid (Figure 7B).

All preparations of myosin hybrids containing XRLCs phosphorylated on Ser-19 moved actin filaments at constant velocity with little variation over time (Figure 8A,B). Although myosin phosphorylated on Thr-18 moves actin filaments at velocities comparable to myosin phosphorylated on Ser-19, the velocity and quality of actin filament motility of the 18 different preparations was more variable than other myosin hybrids. Myosin reconstituted with the XRLC phosphorylated only on Thr-18 produced three types of actin filament motility: (1) in some preparations of the S19A-P myosin the actin filaments moved persistently, but with greater variation in their velocity (Figure 8C); (2) in some preparations of this hybrid the actin filaments moved consistently at a slow velocity (0.201 μm s^{-1}) (Figure 8D); and (3) in other preparations of the S19A-P hybrid the actin filaments moved intermittently (Figure 8D). One of these patterns of actin filament motility predominated in each hybrid preparation. All preparations of myosin hybrids phosphorylated on Thr-18 had similar K^+EDTA and actin-activated ATPase activities.

Tethering of the myosin to the nitrocellulose via an antibody did not improve the performance of myosin containing RLC phosphorylated on Thr-18. Instead the actin filament velocity was lower when the myosin was attached directly to the coverslip with a smooth muscle myosin monoclonal antibody (Figure 9A). The addition of free RLCs phosphorylated on Ser-19 to myosin hybrids phosphorylated on Thr-18 failed to increase the velocity of actin filament motility (Figure 9B).

DISCUSSION

Myosin II phosphorylated on Thr-18 of the RLC has low actin-activated ATPase activity but moves actin filaments

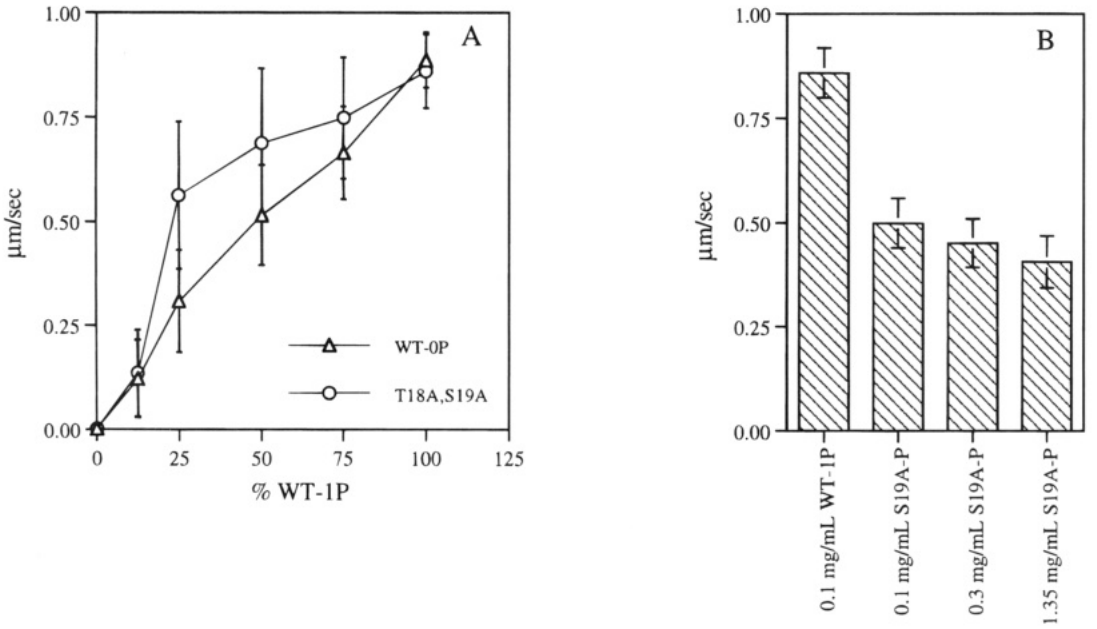

FIGURE 7: (A) Velocity of actin filament motility by mixtures of myosin hybrid containing wt XRLC phosphorylated on Ser-19 and myosin hybrid containing unphosphorylated wt XRLC (△) or myosin hybrid containing wt XRLC phosphorylated on Ser-19 and myosin hybrid containing unphosphorylated T18A, S19A XRLC (○). (B) Assessment of hybrid concentration on the velocity of actin movement. Myosin hybrid with wt XRLC phosphorylated on Ser-19 at 0.1 mg/mL; myosin hybrid with S19A XRLC phosphorylated on Thr-18 at 0.1, 0.3, and 1.35 mg/mL.

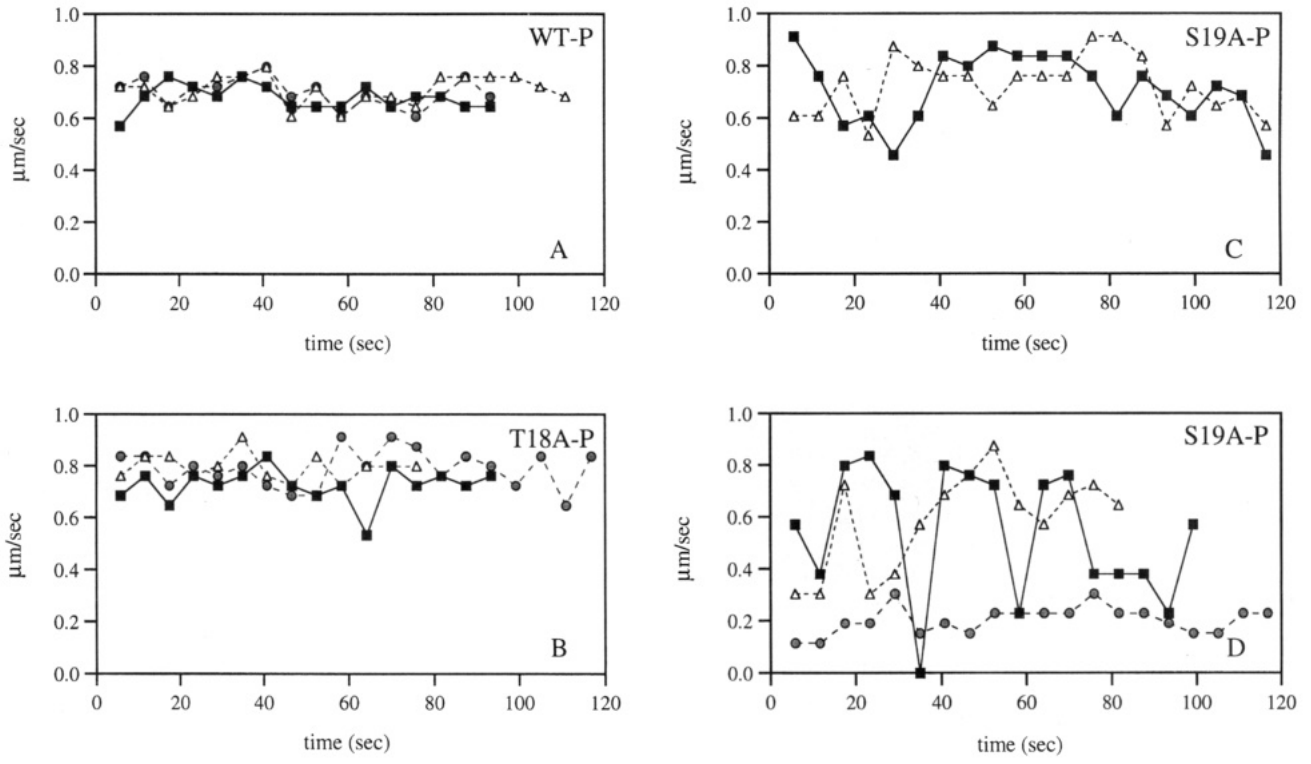

FIGURE 8: Motility of individual actin filaments by myosin hybrids. (A and B) hybrids phosphorylated on Ser-19; (C and D) different preparations of myosin hybrids phosphorylated on Thr-18 produce more variable actin filament motility.

in an *in vitro* motility assay nearly as well as myosin phosphorylated on Ser-19 which has a 15-fold higher ATPase activity. The actin filaments moved with velocities that were 79% of the velocities obtained for myosins phosphorylated on Ser-19, or both Thr-18 and Ser-19, and an average of 90% of the filaments in the field moved. The average velocity determined for the S19A-P hybrid (Figure 6) includes filaments that moved at the same velocities observed for wild-type phosphorylated hybrids as well as filaments which moved discontinuously. Although all preparations of the S19A-P myosin had the same K^+EDTA and actin-activated ATPase activities, this hybrid was variable in the gliding filament assay. It is possible that direct binding of the S19A-P myosin to the nitrocellulose impairs its motor properties, causing variability in the movement of actin filaments. However, attachment of the S19A-P myosin to the coverslip via an antibody did not improve the actin filament motility. The absence or poor binding of the S19A-P RLC to the myosin heavy chain could also contribute to the variability in movement observed with the S19A-P

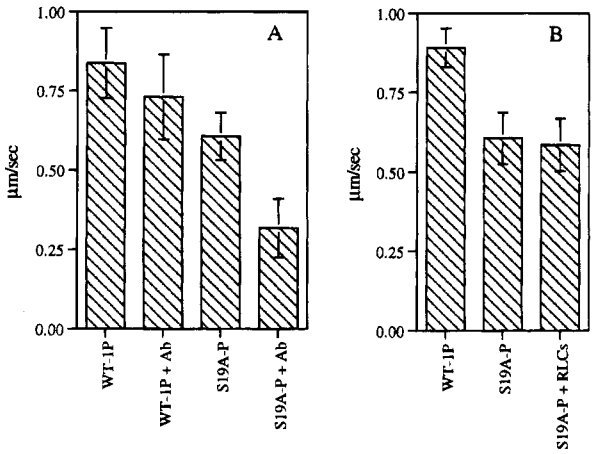

FIGURE 9: (A) Velocity of actin filaments by myosin hybrids tethered by an antibody. Myosin hybrid phosphorylated on Ser-19, or attached by the S2.2 antibody; myosin hybrid phosphorylated on Thr-18, or attached by the S2.2 antibody. (B) Effect of free phosphorylated XRLCs on the myosin phosphorylated on Thr-18. Myosin hybrid phosphorylated on Ser-19; myosin hybrid phosphorylated on Thr-18; myosin hybrid phosphorylated on Thr-18 incubated with free wt XRLC phosphorylated on Ser-19.

myosin. The addition of an excess of free S19-P RLCs did not alter the actin filament motility, indicating that the S19A-P RLC binds the myosin heavy chain as well as the S19-P RLC. Although we have been unable to determine why the S19A-P myosin is variable in the *in vitro* motility assay, clearly this hybrid can move actin filaments at velocities similar to those observed for myosin phosphorylated on Ser-19.

One interpretation is that most of the myosin phosphorylated on Thr-18 is inactive, accounting for the low actin-activated ATPase, and that a small fraction of active heads moves the actin filaments in the gliding filament assay. However, several lines of evidence suggest this explanation is incorrect. The K^+EDTA ATPase of myosin hybrids phosphorylated on Thr-18 is comparable to the ATPase observed for other myosin hybrids, thus indicating that all of the heads are active. In a pelleting assay, myosin phosphorylated on Thr-18 forms filaments as well as myosin phosphorylated on Ser-19, suggesting the S19A-P hybrid favors the 6S extended conformation. Thus it is unlikely that the low ATPase of the S19A-P myosin results from the trapping of ADP and Pi in the folded 10S conformation (Cross et al., 1988). If the ATPase measured for the S19A-P myosin were due to a mixture of fully active heads (like myosin phosphorylated on Ser-19) and inactive heads (with zero activity), only 6−8% of the heads would be active. Motility assays with mixtures of WT-1P (active heads) and either WT-0P or T18A, S19A hybrids (inactive, nonrigor heads) indicate that a fraction of less than 12% active heads does not support the movement of actin filaments (Figure 7A). Therefore 7% active heads could not move actin filaments at 79% the velocity of wild-type motility. These observations suggest that most of the heads in the S19A-P myosin preparations are active and participate in the movement of actin filaments.

It is surprising that myosin with a low actin-activated ATPase supports the motility of actin filaments. One explanation is that the S19A-P myosin has a low affinity for actin and requires significantly higher actin concentrations for activation than those needed for myosin phosphorylated on Ser-19. The local concentration of actin on the surface of the myosin-coated surface may be much higher than the 20 μM limit that we could test in solution, resulting in a high ATPase and the observed motility. The methycellulose in the buffer used for the gliding filament assay decreases the lateral diffusion of actin filaments (Umemoto & Sellers, 1990), thus creating a local high concentration of actin.

This is not an isolated example of the steady state actin-activated ATPase activity not being correlated with the velocity of actin filaments in the gliding filament assay. Myosin phosphorylated on both Thr-18 and Ser-19 has a 2-fold higher actin-activated ATPase than myosin phosphorylated only on Ser-19, yet both move actin filaments at the same velocities (Figure 6). Smooth muscle and platelet myosin have similar actin-activated ATPase activities (Sellers et al., 1981), but smooth muscle myosin moves actin filaments 1.5 times faster that platelet myosin (Umemoto & Sellers, 1990). Recent experimental evidence suggests that a low actin-activated ATPase can support the motility of actin filaments *in vivo*. *Dictyostelium* myosin containing a mutant RLC in which the MLCK phosphorylated serine was substituted with alanine has a maximum actin-activated ATPase of 0.09 s^{-1}. Expression of this mutant RLC in *Dictyostelium* RLC^- null cells rescues defects in cytokinesis and development as well as the wild-type RLC (Ostrow et al., 1994).

Phosphorylation at Ser-19 of the RLC of smooth muscle myosin stabilizes the myosin in the 6S extended conformation and enhances its actin-activated ATPase (Suzuki et al., 1978; Sellers et al., 1981; Craig et al., 1983). Although phosphorylation at Thr-18 occurs *in vivo* (Colburn et al., 1988; Haeberle et al., 1988), this phosphorylation only occurs in conjunction with phosphorylation at Ser-19 in the diphosphorylated RLC. The contribution of phosphorylation at Thr-18 to the physiological activity of myosin remains unknown.

The N-terminus of the RLC contains several positively charged amino acids. The reduction in net charge which occurs as a result of phosphorylation of the RLC is believed to regulate the transition from the 10S folded monomer to the 6S extended conformation. The replacement of Thr-18 and Ser-19 with negatively charged amino acids unfolds smooth muscle myosin to the 6S extended conformation; however, these mutations fail to activate the ATPase of myosin (Kamisoyama et al., 1994) or promote the motility of actin filaments (Sweeney et al., 1994). These observations suggest that although electrostatic interactions regulate the conformation of myosin, the regulation of its ATPase and motile activities specifically requires phosphorylation.

The pK_as of phosphoserine (Vogel & Bridger, 1982) and related functional groups (Dawson et al., 1986) are reported to be in the range of 6.1−6.6. Therefore it is likely that at neutral pH the phosphate moieties on Thr-18 and Ser-19 will be nearly dianionic. Incorporation of a dianionic phosphate at Ser-19 is sufficient to activate the ATPase of myosin and promote the movement of actin filaments. Our results demonstrate that the incorporation of a dianionic phosphate at Thr-18 cannot significantly activate the ATPase of myosin. However, phosphorylation at Thr-18 combined with an additional negative charge at position 19 via substitution of Ser-19 with either an aspartic or glutamic acid activates the ATPase (Kamisoyama et al., 1994) and motile (Sweeney et al., 1994) activities of myosin to levels observed for myosin phosphorylated on Ser-19. These observations suggest that

electrostatic interactions not only regulate the 10S to 6S transition of myosin but also are involved in the regulation of its ATPase activity.

ACKNOWLEDGMENT

We thank Dr. Douglas Murphy for the use of his microscope and image processing equipment, Dr. Kathy Trybus for the S2.2 antibody to the chicken smooth muscle myosin heavy chain, Dr. Paul Janmey for native gelsolin, Dr. Michael Way for recombinant gelsolin, Dr. Jim Sellers for his advice regarding motility assays, and Drs. Steven Almo and Lee Sweeney for helpful discussions.

REFERENCES

Berlot, C. H., Spudich, J. A., & Devreotes, P. N. (1985) *Cell 43*, 307−314.
Berlot, C. H., Devreotes, P. N., & Spudich, J. A. (1987) *J. Biol. Chem. 262*, 3918−3926.
Bourguignon, L. Y. W., Nagpal, M. L., & Hsing, Y.-C. (1981) *J. Cell Biol. 91*, 889−894.
Catterall, W. A., & Pederson, P. L. (1971) *J. Biol. Chem. 246*, 4987−4994.
Colburn, J. C., Michnoff, C. H., Hsu, L.-C., Slaughter, C. A., Kamm, K. E., & Stull, J. C. (1988) *J. Biol. Chem. 263*, 19166−19173.
Conti, M. A., & Adelstein, R. S. (1991) *Methods Enzymol. 196*, 34−47.
Craig, R., Smith, R., & Kendrick-Jones, J. (1983) *Nature 302*, 436−439.
Cross, R. A., Jackson, A. P. Citi, S., Kendrick-Jones, J., & Bagshaw, C. R. (1988) *J. Mol. Biol. 203*, 173−181.
Dawson, R. M. C., Elliott, D. C., Elliott, W. H., & Jones, K. M. (1986) *Data for Biochemical Research*, pp 62−63 and 70−71, Clarendon, Oxford.
Geladopoulos, T. P., Sotiroudis, T. G., & Evangelopoulos, A. E. (1991) *Anal. Biochem. 192*, 112−116.
Haeberle, J. R., Sutton, T. A., & Trockman, B. A. (1988) *J. Biol. Chem. 263*, 4424−4429.
Horowitz, A., & Trybus, K. M. (1992) *J. Biol. Chem. 267*, 26091−26096.
Ikebe, M., & Hartshorne, D. J. (1985) *J. Biol. Chem. 260*, 10027−10031.
Ikebe, M., Hartshorne, D. J., & Elzinga, M. (1986) *J. Biol. Chem. 261*, 36−39.
Kamisoyama, H., Araki, Y., & Ikebe, M. (1994) *Biochemistry 33*, 840−847.
Kron, S. J., Toyoshima, Y. Y., Uyeda, T. Q. P., & Spudich, J. A. (1991) *Methods Enzymol. 196*, 399−416.
Messer, N. G., & Kendrick-Jones, J. (1988) *FEBS Lett. 234*, 49−52.
Morita, J., Takashi, R., & Ikebe, M. (1991) *Biochemistry 30*, 9539−9545.
Nishikawa, M., Sellers, J. R., Adelstein, R. S., & Hidaka, H. (1984) *J. Biol. Chem. 259*, 8808−8814.
Ostrow, B. D., Chen, P., & Chisholm, R. L. (1994) *J. Cell Biol. 127*, 1945−1955.
Perrie, W. T., & Perry, S. V. (1970) *Biochem. J. 119*, 31−38.
Sanger, F., Nicklen, S.,, & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5464.
Satterwhite, L. L., Lohka, M. J., Wilson, K. L., Scherson, T. Y., Cisek, L. J., Corden, J. L., & Pollard, T. D. (1992) *J. Cell Biol. 118*, 595−605.
Sellers, J. R., Pato, M. D., & Adelstein, R. S. (1981) *J. Biol. Chem. 256*, 13137−13142.
Sellers, J. R., Spudich, J. A., & Sheetz, M. P. (1985) *J. Cell Biol. 101*, 1897−1902.
Sellers, J. R., Cuda, G., Wang, F., & Homsher, E. (1993) *Methods Cell Biol. 39*, 24−49.
Smillie, L. B. (1982) *Methods Enzymol. 85*, 234−241.
Somlyo, A. P., & Somlyo, A. V. (1994) *Nature 372*, 231−236.
Spudich, J. A., & Watt, S. (1971) *J. Biol. Chem. 246*, 4866−4871.
Suzuki, H., Onishi, H., Takahashi, K., & Watanabe, S. (1978) *J. Biochem. (Tokyo) 84*, 1529−1542.
Sweeney, H. L., Yang, Z., Zhi, G., Stull, J. T., & Trybus, K. M. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1490−1494.
Trybus, K. M., Waller, G. S., & Chatman, T. A. (1994) *J. Cell Biol. 124*, 963−969.
Umemoto, S., & Sellers, J. R. (1990) *J. Biol. Chem. 265*, 14864−14869.
Umemoto, S., Bengur, A. R., & Sellers, J. R. (1989) *J. Biol. Chem. 264*, 1431−1436.
Vogel, H. J., & Bridger, W. A. (1982) *Biochemistry 21*, 5825−5831.
Way, M., Pope, B., Gooch, J., Hawkins, M., & Weeds, A. G. (1990) *EMBO J. 9*, 4103−4109.
Warshaw, D. M., Desrosiers, J. M., Work, S. S., & Trybus, K. M. (1990) *J. Cell Biol. 111*, 453−463.
Yamakita, Y., Yamashiro, S., & Matsumura, F. (1994) *J. Cell Biol. 124*, 126−137.

BI951295+