Coupling of ATPase Activity and Motility in Smooth Muscle Myosin Is Mediated by the Regulatory Light Chain

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Abstract. Smooth muscle myosin acts as a molecular motor only if the regulatory light chain (RLC) is phosphorylated. This subunit can be removed from myosin by a novel method involving the use of trifluoperazine. The motility of RLC-deficient myosin is very slow, but native properties are restored when RLC is rebound. Truncating 6 residues from the COOH terminus of the RLC had no effect on phosphorylated myosin's motor properties, while removal of the last 12 residues reduced velocity by ≈30%. Very slow movement was observed once 26 residues were deleted, or with myosin containing only the COOH-terminal RLC domain. These two mutants thus mimicked the behavior of RLC-deficient myosin, with the important difference that the mutant myosins were monodisperse when assayed by sedimentation velocity and electron microscopy. The decreased motility therefore cannot be caused by aggregation. A common feature of RLC-deficient myosin and the mutant myosins that moved actin slowly was an increased myosin ATPase compared with dephosphorylated myosin, and a lower actin-activated ATPase than obtained with phosphorylated myosin. These results suggest that the COOH-terminal portion of an intact RLC is involved in interactions that regulate myosin's "on-off" switch, both in terms of completely inhibiting and completely activating the molecule.

DEPHOSPHORYLATED smooth muscle myosin has a low actin-activated ATPase activity and is unable to move actin in a motility assay. Phosphorylation of Ser 19 of the regulatory light chain (RLC)[1] activates both enzymatic activity and motility, producing a molecule that acts as a molecular motor (for review see Sellers, 1991). In both states, there is a tight coupling between enzymatic activity and movement. Exchange of mutant chimeric LCs into myosin further showed that the minimal requirements for smooth muscle myosin to move actin were a phosphorylatable serine and the native COOH-terminal half of the LC. Thus, myosin with phosphorylated N-skeletal/C-smooth RLC moved actin and had a high actin-activated ATPase, while myosin containing a phosphorylated N-smooth/C-skeletal chimeric RLC was locked in the "off" state (Trybus and Chatman, 1993).

An important question raised by these results is whether specific interactions involving the LC and the HC are necessary to generate movement, or whether RLC removal will produce a constitutively active myosin. Similar questions have been addressed in thick filament regulated scallop myosin, whose RLCs are weakly bound and can be readily removed. Such "desensitized" scallop myofibrils have high enzymatic activity both in the presence and absence of calcium, leading to the conclusion that the LC acts as a repressor, and that calcium binding relieves this inhibition (for review see Szent-Györgyi and Chantler, 1986). Despite this high ATPase, movement of RLC-deficient scallop myosin-coated beads on *Nitella* actin cables was reduced 10–20-fold. RLC-deficient myosin aggregates near the head/rod junction with other molecules, however, and thus no firm conclusions could be drawn regarding the role of the LC in coupling activity to movement (Vale et al., 1984).

In contrast, removal of the homologous RLC from the thin filament regulated skeletal muscle myosin decreased velocity only 2.5-fold, from 8.5 μm/s to 3.1 μm/s, with little change in actin-activated activity (Lowey et al., 1993). A more striking change was obtained upon removal of the essential LC (ELC), which abuts the globular domain of the head that contains the ATP and actin-binding sites. Motility was decreased sixfold without a corresponding decrease in actin-activated ATPase activity; moreover, this myosin remained monodisperse (Lowey et al., 1993). These results are consistent with the hypothesis that the LCs amplify a small structural change in the globular domain of the head that occurs upon product release (Rayment et al., 1993), thereby allowing skeletal myosin to move actin at rates characteristic of fast muscles.

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1. *Abbreviations used in this paper*: LC, light chain; RLC, regulatory light chain; ELC, essential light chain; WT, wild-type light chain.

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Here we show that RLC removal from smooth muscle myosin decreases both its actin-activated activity and its ability to move actin, compared with phosphorylated myosin. Similar results were obtained with a completely monomeric myosin that contained a phosphorylated RLC lacking only the last 26 COOH-terminal amino acids. Both myosins also had an increased myosin ATPase activity relative to dephosphorylated myosin. An intact dephosphorylated RLC thus causes myosin's enzymatic activity to be completely suppressed, while specific interactions involving phosphorylated Ser 19 and portions of the COOH-terminal half of the RLC release this inhibition in the presence of actin, insuring that high actin-activated activity is coupled to fast motility only upon phosphorylation.

Materials and Methods

Protein Preparation

Dephosphorylated smooth muscle myosin was prepared from turkey gizzards (Sellers et al., 1981). Smooth muscle myosin LC kinase was isolated essentially according to Adelstein and Klee (1981). Actin was purified from chicken pectoralis acetone powder by the method of Pardee and Spudich (1982) and stored as F-actin at 4°C. Turkey gizzard tropomyosin was prepared from an ethanol-acetone gizzard powder (Smillie, 1982). Protein concentrations were determined at 280 nm using the following extinction coefficients (1 mg/ml): myosin, 0.5; actin, 1.1; tropomyosin, 0.29; wild-type RLC 0.22. The concentration of the deletion mutants, and NH_2- and COOH-terminal domains was determined by the Bradford method (1976) using myosin as a standard. Myosin or LCs were thiophosphorylated by addition of smooth muscle myosin LC kinase, calmodulin (Pharmacia LKB Biotechnology, Piscataway, NJ), calcium, and MgATP-gamma-S (Boehringer-Mannheim Corp., Indianapolis, IN) (Trybus and Lowey, 1984). Glycerol/acrylamide gels were used to monitor the state of phosphorylation (Perrie and Perry, 1970). Using this gel system, all mutant LCs were shown to be completely thiophosphorylated before incorporation into myosin because they migrated as a single band of faster mobility than the corresponding unphosphorylated LC (data not shown; examples in Trybus and Lowey, 1984).

Construction of Mutant LCs

Wild-type LC in the expression vector pT7-7 was expressed with an NH_2-terminal tag of 4 residues (ARIL) in *E. coli* BL21 (DE3) as previously described (Trybus and Chatman, 1993). Deletion mutants of this construct, lacking the last 6, 12, or 26 residues, were engineered by introducing a stop codon followed by a new BamHI restriction site by PCR mutagenesis. The EcoRI/BamHI fragment was cloned into pT7-7 and expressed in the same way as the wild-type construct. The resultant clones were sequenced around the site of mutagenesis. The 34 COOH-terminal residues of the smooth muscle RLC (residues 138-171), and the amino acid at which the three deletion mutations stop are indicated:

VDEMYREA(▲26)PIDKKGNFNYVEFT(▲12)RILKHG(▲6)AKDKDD.

Although smooth and skeletal RLCs are 53% identical overall, only 8 of the last 34 residues are the same (common residues are shown in boldtype). For comparison, the last 34 residues of the chicken skeletal RLC (residues 134–167) are:

IKNMWAAFPPDVAGNVDYKNICYVITHGEDKEGE (Reinach and Fischman, 1985).

The truncated NH_2-terminal domain (residues 1–84) was engineered by introducing a stop codon by mutagenesis in M13mp19 (Amersham Corp., mutagenesis kit). Construction of the COOH-terminal domain (residues 89–171) was previously described (Trybus and Chatman, 1993).

RLC Removal and Readdition of Mutant LCs

Regulatory LCs were removed from smooth muscle myosin by a modification of the method used to remove troponin-C from skeletal myosin fibers (Putkey et al., 1991). Myosin (5 mg/ml) in high salt buffer (20 mM imidazole pH 6.8, 0.4 M NaCl, 5 mM EDTA, 2 mM EGTA, 3 mM NaN_3) was incubated with 1.5 mM ATP, 10 mM DTT, 5 mM trifluoperazine (TFP), 0.05% Triton X-100 for 1 h at 0°C. FPLC gel filtration (Superose 6, 1 × 30 cm, 0.5 ml/min) was used to separate myosin from free LCs (same buffer as in incubation, but with 0.5 mM TFP, 0.5 mM ATP, 1 mM DTT). After removal of TFP, mutant or WT LCs (10 μM) were added to LC-deficient myosin (10 mM imidazole, pH 6.8, 0.4 M KCl, 6 mM $MgCl_2$, 2 mM EGTA, 1 mM DTT). If necessary, unbound LCs were removed from myosin filaments by centrifugation.

In Vitro Motility Assay

The motility assay was performed as previously described (Trybus and Chatman, 1993). Myosin heads that cannot dissociate from actin in the presence of MgATP were removed by centrifugation in the presence of actin and MgATP just before the assay to prevent slowing of movement by these non-functional heads. Motility assays were sometimes performed without removal of excess LCs by binding monomeric myosin via a monoclonal antibody that was adsorbed to a nitrocellulose coverslip (Ab S2.2; Trybus and Henry, 1989). This procedure prevents LCs from competing with myosin for binding to the nitrocellulose, and also minimizes unfavorable interactions of the myosin head with the substratum. When excess LCs were removed, myosin monomers were usually directly adsorbed onto the nitrocellulose. Both methods gave similar results.

Antibody-bound LC deficient-myosin could be reconstituted with phosphorylated WT LC in the flow cell by incubation with 5 μM LC (10 mM imidazole, pH 7.5, 0.4 M KCl, 4 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.5 mg/ml BSA) for 90 min at 4°C in a humid chamber.

The percent of rapidly moving actin filaments was quantitated by marking the initial position of all the filaments in the field, advancing the videotape by ~20 s, and counting those filaments whose position was unchanged. At least 100 filaments were counted in each case. Velocities obtained for 15 or more filaments are reported as means and standard deviations of the means. In some instances (e.g., Fig. 2), extended observation times were not used, and thus no movement could not be distinguished from slow movement (<0.1 μm/s). Quantitation of these slow rates requires extended observation periods (>1 min) for sufficient movement to occur.

ATPase Assays

ATPase assays were performed in 20 mM imidazole pH 7.5, 70 mM KCl, 4 mM $MgCl_2$, 1 mM EGTA, 2 mM MgATP at 37°C, with 10 μM actin, 2.5 μM tropomyosin, and 20–50 μg/ml myosin. Reactions were stopped at 3–4 times with SDS, and inorganic phosphate determined colorimetrically (Taussky and Shorr, 1953).

Chymotryptic Digestion

Myosin (0.3 mg/ml in 5 mM imidazole pH 7.0, 10 mM NaCl, 1 mM DTT, 1 mM NaN_3, with either 2 mM $MgCl_2$ or 2 mM EDTA) was digested with 20 μg/ml chymotrypsin at room temperature for 15 min, and stopped with 1 mM phenylmethylsulphonyl fluoride. Soluble and precipitated materials were separated by centrifugation at 23 psi (Beckman airfuge) for 15 min.

Characterization of the Physical State of the Molecule

Sedimentation velocity measurements were carried out at 20°C in a Beckman Model E analytical ultracentrifuge, using the Schlieren optical system. Metal-shadowed images were obtained by diluting myosin into 0.5 M ammonium acetate, 66% glycerol and rotary shadowing with platinum (Trybus and Lowey, 1984). Images were observed on a Philips EM301 electron microscope operated at 60 kV.

Results

Enzymatic and Motor Properties of RLC-Deficient Myosin

Smooth muscle myosin containing only heavy chain and the 17-kd ELC was prepared by incubating myosin with trifluoperazine, a phenothiazine that also binds to the homologous proteins calmodulin and troponin-C (Massom et al., 1990). (Fig. 1 *A*, lane 2). A similar approach was used to

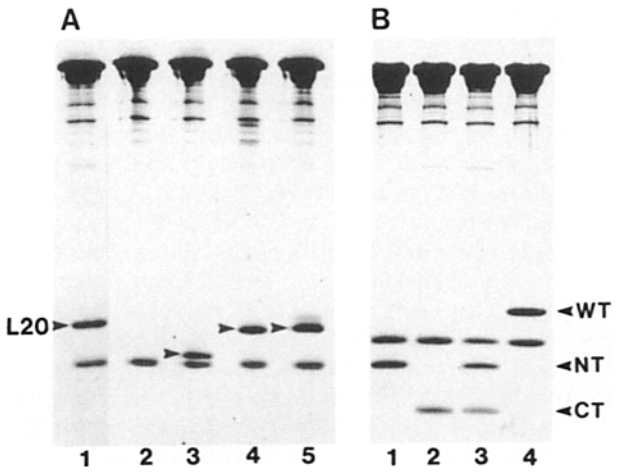

Figure 1. RLC-deficient myosin reconstituted with mutant LCs. (*A*) 15% SDS-gel showing gizzard myosin (lane *1*), RLC-deficient myosin (lane *2*), and myosin reconstituted with LC▲26 (lane *3*), LC▲12 (lane *4*), and WT RLC (lane *5*). Arrowheads point to incorporated RLC. The ELC is the fastest migrating band in each lane. (*B*) Myosin reconstituted with the NH_2-terminal domain (*NT*, lane *1*), the COOH-terminal domain (*CT*, lane 2), both halves of the LC (*NT* + *CT*, lane *3*), or WT RLC (lane *4*). The unmarked low molecular weight band is the ELC.

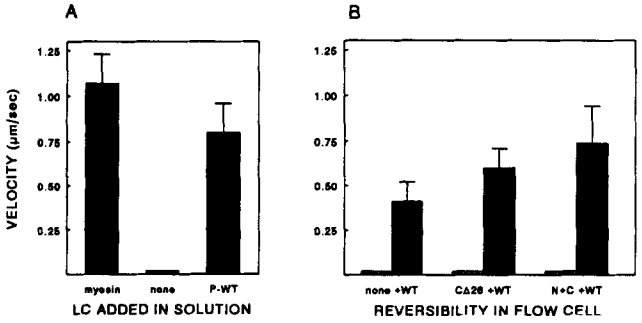

Figure 2. Reversibility of RLC removal. (*A*) Velocity of actin movement in a motility assay by phosphorylated myosin, myosin from which the RLC has been removed, and RLC-deficient myosin to which phosphorylated WT RLC was re-added in solution. In this experiment, no movement was not distinguished from velocities <0.1 μm/s. (*B*) Phosphorylated WT RLC was added to non-motile myosins within the flow cell. The bound myosin contained either no RLC, phosphorylated C▲26, or the phosphorylated NH_2-terminal and COOH-terminal domains. Upon incubation with phosphorylated WT RLC, movement was restored to varying degrees. Velocity of control myosin (1.07 ± 0.16 μm/s) is shown in *A*. In both *A* and *B*, myosin was attached to the coverslip via an anti-rod antibody.

remove troponin-C from skeletal fibers (Putkey et al., 1991). Binding of this drug to the RLC appears to weaken its affinity for the heavy chain. Antibody-affinity columns can also be used to remove RLC, but the small amounts of material obtained by this method precluded an extensive characterization of RLC-deficient myosin (Trybus and Lowey, 1988). The method described here allows larger amounts of RLC-deficient smooth muscle myosin to be readily prepared for the first time. If the RLC represses a constitutively active molecule, then its removal should produce myosin that moves actin and has a high actin-activated ATPase activity. Alternatively, interactions specifically involving the phosphorylated LC might be required for smooth muscle myosin to express activity and movement.

When tested by a motility assay, RLC deficient-myosin moved actin filaments at very slow rates (0.095 ± 0.035 μm/s, n = 21). This rate of movement was indistinguishable from no motility during the short observation times typically used to quantify movement by phosphorylated smooth muscle myosin (~30 s per field). Rates did not increase if the myosin was captured via an antibody bound to the nitrocellulose, instead of being directly adhered to a nitrocellulose-coated coverslip. Incubation of RLC-deficient myosin with phosphorylated LC in solution restored rapid motility: 72% of the actin filaments moved at 75% the rate (0.80 ± 0.16 μm/s) of untreated, control myosin (1.07 ± 0.16 μm/s, >95% movement) (Fig. 2 *A*). Thus, despite the tendency of RLC-deficient myosin to aggregate with other molecules as shown both from metal-shadowed images and by sedimentation velocity (Fig. 3, *A* and *C*; see also Trybus and Lowey, 1988),

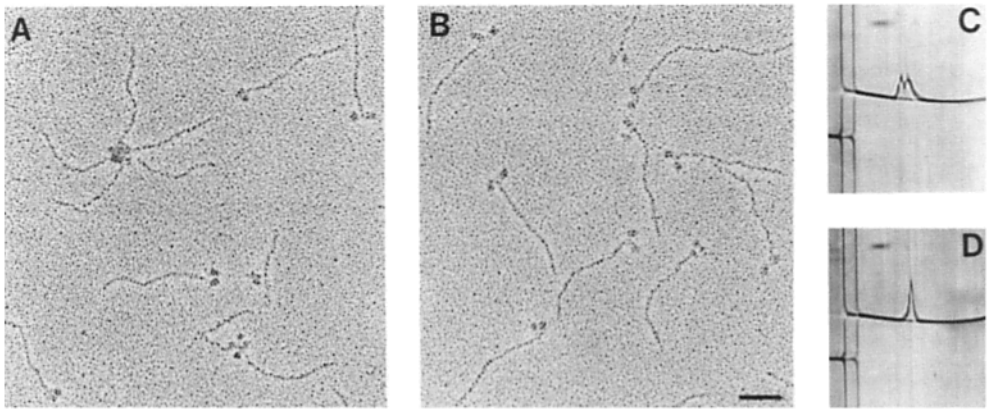

Figure 3. Physical characterization of RLC-deficient and reconstituted myosins. (*A* and *C*) Metal shadowed RLC-deficient myosin shows a mixture of monomers and small oligomers that aggregate near the head/rod junction. Consistent with this observation, the sedimentation velocity pattern of RLC-deficient myosin (*C*) shows a slower monomeric boundary and a faster sedimenting boundary due to small oligomers. (*B* and *D*) Myosin reconstituted with the COOH-terminal half of the RLC restores the monomeric state, as can be seen both by electron microscopy and by the single symmetrical sedimenting boundary (*D*). Monomers were also obtained upon addition of any of the COOH-terminal deletion mutants or WT RLC.

the myosin can be reversibly reconstituted into a rapid motor. The possibility that this aggregation could explain the slow motility was ruled out by the results obtained with a deletion mutant (see next section), where the monomeric state of myosin was preserved, but motility was still greatly inhibited. Moreover, metal-shadowed images showed that there was sufficient monomeric RLC-deficient myosin (37%, $N = 197$) to support movement at full velocity, even if the oligomers are non-functional.

A striking feature of the activity of RLC-deficient myosin was its increased rate of ATP turnover in the absence of actin: the rate was twofold higher than myosin reconstituted with a dephosphorylated WT or truncated LC (C▲6, see below), and 11-fold higher than untreated dephosphorylated myosin (Table I). Since some of the increased rate of the reconstituted control myosin is likely due to a small fraction of myosin with no RLC, the myosin ATPase activity is at least doubled by RLC removal. Actin further increased the activity of RLC-deficient myosin less than twofold, giving rise to a small actin-activated ATPase relative to that obtained with native or control phosphorylated myosin.

COOH-terminal Deletion Mutants and Severed Domains Identify Interactions Required for Movement

The results obtained with RLC-deficient myosin suggested that some part of the RLC is needed to convert myosin into a rapid molecular motor. Previous work showed that the minimal requirements for movement are a phosphorylation site and a native COOH-terminal half of the RLC (Trybus and Chatman, 1993). Only 8 of the last 34 residues of the smooth and skeletal myosin LC are identical, thus this divergent region was chosen as our initial target for deletion mutagenesis (see Materials and Methods for a comparison of the sequences).

Three COOH-terminal deletion mutants, lacking the last 6, 12, or 26 residues of the RLC, were expressed, fully phosphorylated, and bound to RLC-deficient myosin (Fig. 1 *A*). The expressed NH_2-terminal (residues 1–84) and COOH-terminal halves (residues 89–171) were also bound to myosin to test whether the two domains must be connected for proper function (Fig. 1 *B*). It was previously shown that the COOH-terminal domain alone competes poorly in an exchange reaction with the native RLC (Trybus and Chatman, 1993). It was therefore essential to remove the endogenous RLC from myosin before incorporating the weaker binding deletion mutants (e.g., C▲26) and the domains, since extremely high concentrations of the weaker binding mutants would be needed to compete effectively with the stronger binding native RLC in an exchange reaction.

Removal of 6 residues from the COOH terminus of the LC had no effect on motility. Removal of 12 residues (essentially all of the H helix, Rayment et al., 1993) caused a small decrease in velocity, while removal of 26 residues (containing the H helix and the non-functional fourth EF hand) essentially abolished motility (Fig. 4, *solid bars*). Complete removal of the LC thus had the same effect as removing the last 26 residues. Similarly, addition of the COOH-terminal domain or the severed NH_2- and COOH-terminal domains drastically reduced myosin's motor properties. An important difference between RLC deficient-myosin and myosin containing any of the deletion mutants was that these hybrid myosins were completely monomeric as determined both by electron microscopy and sedimentation velocity. The COOH-terminal domain alone was sufficient to keep myosin monomeric (Fig. 3, *B* and *D*). Aggregation therefore cannot explain these molecules' inability to move actin rapidly. Since both the COOH-terminal domain and C▲26 restore the monomeric state, the "sticky patch" that causes LC-deficient molecules to aggregate involves a region of the heavy chain in the neck that interacts with portions of the COOH-terminal half of the RLC (approximately residues Q817-L833 by homology with skeletal subfragment-1).

The enzymatic activity of the monomeric myosins that moved actin slowly (C▲26 and the severed NH_2- and COOH-terminal domains) resembled that obtained with RLC-deficient myosin (Table I). The myosin ATPase activity was at least twofold higher than myosin reconstituted with dephosphorylated C▲6. Actin increased this rate to a much lesser extent than that obtained with native or control phosphorylated myosin, giving rise to a low actin-activated ATPase activity, which was similar regardless of the state of phosphorylation of the added mutant RLC (Table I). The hybrid myosins that moved actin slowly thus had "activation ratios" less than 1 (actin-activated rate divided by the myosin ATPase rate; Fig. 4, *shaded bars*).

Table I. ATPase Activity of Myosin Containing Mutant RLCs

	Myosin	WT	▲6	▲12	▲26	N-term	N +C	C-term	-LC
DEPHOS									
− actin	.02 ± .02	.10 ± .01	.11 ± .04	.18	.25 ± .03	.27	.39	.27 ± .06	.22 ± .05
+ actin	.05 ± .02	.15 ± .04	.12 ± .05	.31	.32 ± .01	.43	.51	.43 ± .08	.34 ± .04
rate (s^{-1})	.03 ± .02 (8)	.05 ± .03 (4)	.02 ± .02 (4)	.13 (2)	.08 ± .08 (4)	.16 (1)	.12 (1)	.16 ± .05 (4)	.12 ± .02 (5)
PHOS									
− actin	.09 ± .03	.10 ± .02	.16 ± .04	.18	.26 ± .03	.27	.29		
+ actin	.68 ± .22	.53 ± .05	.80 ± .18	.45	.37 ± .08	.48	.46		
rate (s^{-1})	.58 ± .21 (7)	.43 ± .07 (3)	.64 ± .18 (4)	.27 (2)	.11 ± .08 (4)	.21 (1)	.17 (2)		

The ATPase activity of myosin containing dephosphorylated or phosphorylated WT or mutant RLCs was measured in the presence or absence of 10 μM actin, 2.5 μM tropomyosin. Rates were obtained from four time points with each preparation. The mean and standard deviation of the mean from independent preparations is indicated, with the number of preparations indicated in parentheses. For myosins that supported motility only at very slow rates, phosphorylation did not significantly increase activity. The mutants ▲6, ▲12, and ▲26 indicate the number of residues deleted from the COOH-terminus of the RLC. NH_2-term, NH_2-terminal RLC domain; COOH-term, COOH-terminal RLC domain; N + C, NH_2-terminal and COOH-terminal domains.

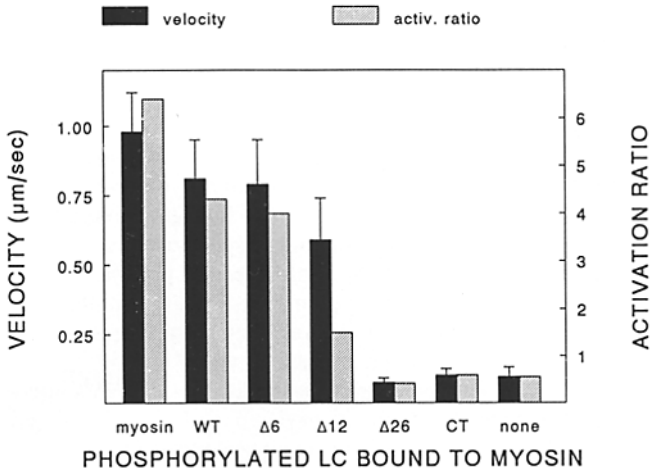

Figure 4. Motility and activity of myosin with mutant phosphorylated RLCs. The velocity of actin movement is shown for hybrid myosins containing the indicated phosphorylated WT or mutant RLCs (*solid bars*). CT indicates the COOH-terminal domain alone. Values are means and standard deviations of the mean for at least 10 filaments. The motility of control phosphorylated myosin is shown for comparison. The shaded bars are the activation ratio obtained from ATPase measurements (see Table I). The activation ratio is the average actin-activated ATPase rate divided by the average myosin ATPase rate for the phosphorylated species, except when there is no phosphorylation site, i.e., with the COOH-terminal domain or no RLC.

Reconstitution of Immobilized Myosin with Phosphorylated WT RLC

Incubation of immobilized LC-deficient myosin with phosphorylated LC in the flow cell restored myosin's ability to move actin rapidly: 79% of the actin filaments moved at a rate of 0.41 ± 0.11 μm/s (Fig. 2 *B*). Reversibility was further enhanced if the immobilized myosin contained either C▲26 or the two severed halves of the LC (Fig. 2 *B*). Both of these mutants compete poorly with native RLC in an exchange reaction, and thus should be displaced by the WT RLC (Trybus and Chatman, 1993). The higher velocities observed when the phosphorylated WT RLC displaced the mutant LCs is most likely due to the ability of the mutant LCs to maintain myosin in a monomeric state (Fig. 3, *B* and *D*). Because rapid movement was restored after incubation with phosphorylated RLC in the flow cell, it is unlikely that the poor motility of the RLC-deficient myosin was due to an irreversible, unfavorable interaction of myosin heads with the antibody bed to which the myosin was bound.

Proteolytic Digestion as a Probe of Structural Changes in the Head

Limited digestion with α-chymotrypsin was used to probe for structural changes in the head that might accompany the observed changes in enzymatic activity. At high ionic strength, chymotryptic digestion of myosin containing either WT RLC, C▲26, or no RLC yielded heavy meromyosin. In low ionic strength buffer in the presence of $MgCl_2$, however, the cleavage pattern of myosin with WT RLC differed from that obtained with myosin containing C▲26 or no RLC (Fig. 5). Based on solubility and immunoblots of the cleaved material, it was deduced that the primary cleavage obtained with myosin containing WT RLC was within the head, resulting in 140 kd (rod + 20 kD) and 70 kd (NH_2-terminal head) fragments that remain noncovalently attached in benign solvents. In contrast, the myosins that only supported slow movement were cleaved near the head/rod junction, generating soluble head fragments and an insoluble rod. These differences in cleavage patterns were abolished in the absence of divalent cations.

Discussion

Myosin's ability to move actin rapidly could be abolished by as small a change as deleting the last 26 residues of the RLC. Severing the connection between the two halves of the LC, or complete removal of the RLC, likewise decreased motility to very low rates. The NH_2- and COOH-terminal halves also lack four residues in the connecting region between the two domains (FLTM, 85–88), which may contribute to altered contacts with the heavy chain.

The mutant myosins' impaired motor properties share a common mechanistic basis: ATP turnover in the absence of actin is increased relative to dephosphorylated myosin, and the further enhancement observed when actin is added is insensitive to the state of phosphorylation, and small compared to that obtained with phosphorylated myosin. A region of the COOH terminus of the intact dephosphorylated LC is thus needed to completely repress myosin's MgATPase activity, and to allow the phosphorylated serine to form specific new interactions which fully turn on the molecule for rapid movement and high actin-activated activity.

Comparison with Other Myosins

RLC removal from scallop myosin abolishes calcium sensitivity of the actomyosin ATPase activity. Changing the last 11 residues of the scallop RLC to 15 random amino acids (Goodwin et al., 1990), or deletion of 12 or more residues from the COOH terminus of the smooth muscle RLC (Rowe

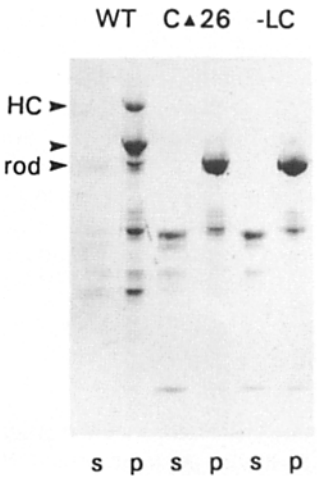

Figure 5. Chymotryptic digestion identifies LC-dependent structural changes in the myosin head. Myosin containing WT LC, C▲26, or no RLC (-LC) was digested with α-chymotrypsin at low ionic strength in the presence of $MgCl_2$. Soluble fragments (S) were separated from insoluble ones (P) by centrifugation. Myosin with WT LC is cleaved internally within the head (*arrowhead* points to the ≈140-kd band consisting of the ≈120-kd rod and the 20-kD portion of the head). Myosin containing no LC or C▲26 is cleaved at the head/rod junction (*arrowhead*, *rod*). *HC*, intact heavy chain, 200 kd. 12.5% SDS-gel.

and Kendrick-Jones, 1993), also resulted in high activity of scallop actomyosin both in the presence and absence of calcium. The high calcium-insensitive ATP turnover of desensitized scallop actomyosin was mainly due to an enhanced MgATPase activity (Szent-Györgyi, A. G., personal communication), as we observed here with LC-deficient smooth muscle myosin (Table I). RLC-deficient scallop myosin adhered to beads also showed greatly impaired movement on *Nitella* actin cables, but since RLC removal causes molecules to aggregate, no firm conclusions regarding the role of the LC in motility could be drawn (Vale et al., 1984). Here we show by sedimentation velocity and electron microscopy that aggregation cannot account for the poor motility of smooth muscle myosin with the C▲26 RLC (or the severed domains), and that completely monomeric species were still unable to move actin filaments rapidly. Thus, in smooth muscle myosin, an intact RLC is clearly necessary to obtain high actin-activated ATPase activity coupled to rapid movement.

Removal of calmodulin from the single-headed brush border myosin I resulted in strikingly similar behavior to removal of the RLCs from smooth muscle myosin. Partial depletion of bound calmodulin enhanced MgATPase activity from 0.047 sec^{-1} to 0.403 sec^{-1}, and this rate was increased only slightly in the presence of actin (Collins et al., 1990). This species was unable to translocate actin filaments, but addition of calmodulin restored motility and actin-activated ATPase activity, suggesting that in this motor "light chains" also mediate coupling of ATPase activity to movement.

Skeletal myosin without its RLC moved at approximately one-third the rate of myosin with both classes of LCs, and showed little decrease in actin-activated ATPase activity (Lowey et al., 1993). Thus in thin-filament regulated myosins, the RLC modulates the speed at which actin is translocated rather than acting as an on-off switch. In fact, reconstitution of skeletal heavy chain with both the regulatory and the essential LCs from smooth muscle myosin decreased motility only slightly (from 8.4 μm/s with skeletal LCs to 7.6 μm/s with smooth LCs). The LCs appear to play primarily a structural role in stabilizing the heavy chain, which depends on overall conformation rather than on a particular sequence (Lowey et al., 1993). In contrast, smooth muscle myosin was completely "off" when a phosphorylated RLC from skeletal myosin was incorporated, suggesting that activation requires a particular RLC sequence in thick filament regulated myosins (Trybus and Chatman, 1993).

The properties of a recombinant *Dictyostelium* myosin lacking the regulatory light chain-binding site, and hence also the RLC, were only somewhat different from wild type myosin: motility was decreased twofold, while the actin-activated ATPase was increased by twofold (Uyeda and Spudich, 1993). Thus, even though light chain phosphorylation can regulate this myosin's activity in vitro, the effects of RLC removal do not mimic that observed with smooth muscle myosin. A major difference between the two studies is the concomitant removal of a portion of the heavy chain from the *Dictyostelium* myosin.

What Does Structural Information Tell Us about Regulation?

It has been suggested that phosphorylation dependent regulation in a number of enzymes may share a common structural basis; three dimensional information obtained from unrelated enzymes such as glycogen phosphorylase in the phosphorylated and dephosphorylated states may therefore be relevant to changes that occur with the RLC (Sprang et al., 1988). The NH_2 terminus of glycogen phosphorylase contains a cluster of basic residues that are required for kinase recognition, but also causes this segment to be disordered. Upon phosphorylation, the positive charge in this region is reduced, and the NH_2 terminus folds into an ordered helical structure. Phosphorylation thus triggers an active conformation of this region of the molecule: the phosphorylated serine forms ion-pair interactions with two arginines, and displaces the COOH-terminal residues from the sites they bound to in the dephosphorylated state.

Similarly, a key issue here is to understand Ser 19's interactions in both the dephosphorylated and phosphorylated states. The recent three dimensional structure of the skeletal myosin head shows no electron density for the first 19 residues of the RLC, including Ser 14 which is the residue phosphorylated in this isoform (Rayment et al., 1993). This segment of the molecule is probably disordered in the dephosphorylated state, consistent with proteolytic studies which show that the NH_2 terminus of the dephosphorylated RLC is readily cleaved by proteolytic enzymes (Jakes et al., 1976). Phosphorylation protects the LC from digestion, thus partial neutralization of the positive charge in the highly basic NH_2 terminus of the LC probably favors a more ordered structure. The neutralization of charge alone, however, is not sufficient to obtain activation of smooth muscle myosin. Replacement of both Arg13 and Arg16 with alanines does not activate myosin without phosphorylation, and replacement of both Thr18 and Ser19 with either glutamic acid or aspartic acid only partially activates motility (Sweeney et al., 1994).

Based on both the deletion mutations and results obtained with chimeric LCs, the target for interaction with the phosphorylatable Ser may reside in the COOH terminus of the LC, which is structurally feasible given the observed proximity of the amino and carboxyl terminal residues. The COOH-terminal half of the LC also contains a strong binding site for the heavy chain (Trybus and Chatman, 1993), consistent with the observation from the crystal structure that helices in the COOH-terminal half have changed their position to grip the myosin heavy chain more tightly, while the conformation of the NH_2-terminal half of the LC is essentially identical to that of free calmodulin (Rayment et al., 1993).

The α-helical 20-kd heavy chain fragment to which the LCs are bound extends near the ATP and actin-binding sites, so it is possible that phosphorylation-induced perturbations to the LC can be transmitted via the heavy chain to the globular domain of the head. The COOH-terminal half of the RLC also closely abuts the ELC, and thus this subunit may be involved in the pathway to the active site. Based on homology with the scallop regulatory domain (Xie et al., 1994), the 26 residues deleted from the COOH terminus of the RLC would not directly contact the ELC. Nonetheless, their removal could alter the interaction of adjacent residues in domain 3 of the RLC that do make contacts with the ELC. In scallop myosin, the regulatory calcium-binding site that controls activity is in fact formed by an EF-hand in domain I of the ELC, and stabilized by interactions involving residues both from domain III of the RLC and the heavy chain (Xie et al., 1994). Although these exact interactions have obviously not been conserved in smooth muscle myosin, an on-

off switch based on alterations of the interactions at the interface between the LCs may be a common theme for thick filament regulated myosins.

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