The Light Chain-binding Domain of the Smooth Muscle Myosin Heavy Chain Is Not the Only Determinant of Regulation*

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Interactions between the dephosphorylated regulatory light chains (RLCs) of smooth muscle myosin are involved in maintaining the enzymatically "off" state. Expressed chimeric smooth muscle heavy meromyosins containing skeletal muscle myosin heavy chain (HC) sequences were used to assess the relative importance of the light chain-binding domain (or "neck") to regulation. Surprisingly, regulation remained intact with a skeletal RLC-binding site. A chimera with the entire α-helical neck composed of skeletal HC sequence showed 2-fold regulation of motility and nearly 5-fold regulation of actin-activated ATPase activity. Complete activation of the dephosphorylated state (i.e. complete loss of regulation) occurred when skeletal HC sequence extended from the head/rod junction to the SH1-SH2 helix. Smooth muscle-specific sequences near the motor domain may therefore position the regulatory domain in a way that optimizes RLC-rod-head interactions, thus enabling a completely off state when the RLC is dephosphorylated. Conversely, a chimera that joins the motor domain from unconventional myosin V to the smooth muscle myosin neck and rod showed only 2-fold regulation. The presence of the smooth muscle light chain-binding region and rod is therefore not sufficient to confer complete phosphorylation-dependent regulation upon all motor domains of the myosin family.

It is well established from biochemical studies that the activity and motor properties of smooth muscle myosin are controlled by regulatory light chain (RLC) phosphorylation. The low activity in the dephosphorylated state is increased several hundredfold upon Ser-19 phosphorylation (1). Skeletal muscle myosin RLC, when exchanged into smooth muscle myosin, had the unexpected effect of putting the molecule permanently into an "off" state even when phosphorylated (3). Conversely, skeletal muscle myosin with a smooth muscle RLC does not acquire phosphorylation-dependent regulation of activity, but remains active irrespective of RLC phosphorylation (4).

A chimeric HMM consisting of a skeletal motor domain and the neck and rod from smooth muscle myosin was recently shown to decrease its actin-activated ATPase activity from 4.3 to 0.5 s⁻¹ upon dephosphorylation (5). This result suggested that the presence of the smooth muscle LC-binding region conferred the motor domain with the ability to inhibit phosphate release. The motility of the phosphorylated chimera, however, was unexpectedly low (0.12 μm/s), and thus, this construct also has some inexplicable features. These data were interpreted as supporting the idea that the regulatory domain is the sole determinant of regulation.

It was previously shown, however, that mutations in the motor domain of smooth muscle HMM can result in the loss of the inhibited state, despite the fact that its regulatory domain and rod are intact. When the sequence of the "actin-binding loop," which bridges the 50- and 20-kDa tryptic domains of the myosin head, was mutated to that found in skeletal muscle myosin, the chimera was equally active when dephosphorylated or phosphorylated, with actin-activated ATPase values at least as high as that seen in the wild-type progenitor (6). The major conclusion from this study was that sequences in the motor domain are capable of precluding regulation, despite the presence of a native neck and rod region.

When considering the nature of the regulatory interactions in smooth muscle, it is important to remember that the presence of an α-helical coiled-coil rod is also necessary for regulation (7). The myosin II source is unimportant, as long as the length of the rod is minimally the length of the myosin head. The charge distribution of the rod, which is a conserved feature among myosin II rods, is likely to be important in the interactions that the rod contributes to stabilizing the off state.

Here, we assess the properties of two types of chimeric HMMs to test the hypothesis that the light chain-binding domain (attached to a myosin rod) is the sole or at least a major determinant of regulation. The first type of chimera contains predominantly smooth muscle myosin sequence and increasing

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¶ The abbreviations used are: RLC, regulatory light chain; HMM, heavy meromyosin; LC, myosin light chain; HC, myosin heavy chain; ELC, myosin essential light chain.
amplification of skeletal sequence, starting from the invariant proline and extending through the neck region into the motor domain. The complementary chimera joins the motor domain of unconventional myosin V with the regulatory domain and rod from smooth muscle myosin. When the sequence of the regulatory domain is derived from an unregulated myosin, the chimeric smooth muscle HMM is only partially regulated. The presence of a smooth muscle regulatory domain and rod is not sufficient, however, to confer complete regulation upon every heterologous myosin motor domain. Regulation is therefore not determined by any one region of the structure, but instead depends on multiple weak interactions throughout the molecule.

MATERIALS AND METHODS

Construction of Heavy Chain cDNAs—The backbone for the constructs was a smooth muscle heavy meromyosin composed of amino acids 1–1175. The constructs were engineered to contain the FLAG epitope (DYKDDDDK) at the C terminus of the rod for purification purposes. The chimeric constructs were generated by interchanging smooth and skeletal sequence into which compatible restriction sites either existed or were engineered. Accordingly, XhoI sites were introduced into nucleotides 2131–2136 and 2350–2355 of the smooth muscle myosin heavy chain (8) and the comparable regions of the skeletal heavy chain (9). A BstEI site exists at nucleotides 2556–2562 of the smooth muscle heavy chain, and a BstEI site was engineered into the comparable region of the skeletal heavy chain. Chimeras were then made by interchanging either XhoI-BstEI fragments (for the Sk 787–852 and Sk 719–852 chimeras) or XhoI-XhoI fragments (for the Sk 719–787 chimera).

For the chimeras that introduced skeletal sequence between amino acids 805 and 848, flanking silent mutations were engineered in the smooth muscle heavy chain cDNA to create unique restriction sites. The method of Kunkel (10) was followed for mutagenesis. The introduction of silent restriction sites in the smooth sequence flanking the codons for amino acids 805–848 allowed introduction of skeletal sequence via the synthesis and ligation of double-stranded oligonucleotide “linkers.” Point mutations in the RLC-binding site (Q839P and Q839P/W841M) were also introduced by mutagenesis following the method of Kunkel (10).

The chimera with the murine myosin V motor domain (11) and the smooth muscle neck and rod was produced by introducing a XhoI site into the myosin V sequence at the point of interchange (coding for amino acids LE at positions 758 and 759 of myosin V). A XhoI site had been previously introduced at the LE sequence of the comparable region of the smooth muscle sequence (amino acids 784–785) for the construction of the Sk 787–852 chimera described above. The sequence at the joining region for this chimera was QVAYLEERD. The sequence QVAY was derived from myosin V; LE is common to both myosins; and EERD starts the smooth muscle sequence.

Expression of Mutant HMMs—Recombinant baculovirus was isolated by conventional protocols (12). Sf9 cells in suspension culture were cocultivated with viral particles containing the HMM HC construct and a virus expressing both the smooth muscle regulatory and essential light chains. The cells were harvested at 65–75 h, and the recombinant HMM molecules were purified either by actin binding and release by MgATP (13) or on an anti-FLAG affinity column (International Biotechnologies Inc.). The expressed HMM was phosphorylated by addition of Ca++, calmodulin, and myosin light chain kinase and dephosphorylated by addition of protein phosphatase 1-M (gift from T. Haystead). Immunoblots of glycerol gels verified RLC phosphorylation (13).

Biochemical Analysis of Expressed Constructs—Actin-activated ATPase assays were performed in 10 mM imidazole, pH 7, 8 mM KCl, 4 mM MgCl2, 1 mM EGTA, and 2 mM MgATP at 37 °C. The reaction was stopped with SDS at six time points per actin concentration, and inorganic phosphate was determined colorimetrically (14). The concentration of active heads was determined by NH4+ ATPase activity relative to a myosin standard (25 mM Tris, pH 7.5 at 37 °C, 0.4 M NH4Cl, 2 mM EDTA, 0.2 mM sucrose, 1 mM diothreitol, and 1 mg/ml bovine serum albumin). The motility assay was performed at 30 °C in 25 mM imidazole, pH 7.5, 25 mM KCl, 4 mM MgCl2, 1 mM EGTA, and 0.5% methylcellulose using monoclonal antibody S2.1 or S2.2 for attachment (15).

RESULTS

A Chimeric HMM with a Skeletal Muscle Myosin Neck Is Partially Regulated—A series of chimeric constructs were designed to determine if the source of the neck region completely determines whether or not the activity of a myosin motor can be regulated by RLC phosphorylation. The first class of chimeric HMMs that were expressed in the baculovirus/insect cell system contained the motor domain from regulated smooth muscle myosin and part or all of the regulatory domain from unregulated skeletal muscle myosin. Fig. 1 shows the sequence comparison of the neck regions of smooth and skeletal muscle myosins. In the 73 residues from Gly-779 to Gln-852 (smooth muscle myosin heavy chain numbering), only 31 amino acids are identical. Thus, there are many isoform-specific sequences in the regulatory domain of myosin that could potentially determine whether or not a myosin motor is regulated.

The first chimera that was analyzed (Sk 787–852) contained all smooth muscle HC sequence (amino acids 1–1175), except for amino acids 787–852, which were derived from the skeletal HC sequence. The amino acid sequence LEE (amino acids 784–786) is conserved between these two isoforms and a number of other species, and this sequence was used as the point of interchange. Thus, both the ELC- and RLC-binding sites in this chimera, as well as a limited amount of additional sequence closer to the motor, were derived from an unregulated myosin. Fig. 2 shows the location of the skeletal HC sequences in the three-dimensional structure of the myosin head. Smooth muscle LCs bind well to the skeletal heavy chain sequence (3), and so this chimera was expressed with either smooth or skeletal muscle LCs since either RLC isoform can be phosphorylated.

With smooth LCs, the dephosphorylated skeletal neck chimera (Sk 787–852) moved actin at half the rate of the phosphorylated chimera (Fig. 3B and Table I), and thus, a significant degree of regulation was lost. The average rate of motility in the phosphorylated state was 1.31 ± 0.19 μm/s, compared with 1.12 ± 0.15 μm/s for the wild-type HMM control, showing that the chimeric construct did not compromise the molecule’s motor properties. The average actin-activated ATPase activity of the dephosphorylated chimera was one-fourth of that in the phosphorylated state (0.41 s–1 versus 1.95 s–1, n = 3) (Fig. 4A and Table I). Thus, the ratio of activation (dephosphorylated/ phosphorylated) is somewhat different when determined by ATPase activity and by motility. This discrepancy is in part due to the fact that the ATPase activity of the phosphorylated chimera is lower than that obtained with wild-type HMM (Table I), yet this increased ATPase activity did not result in a correspondingly higher rate of motility.

With skeletal LCs, the dephosphorylated chimera (Sk 787–852) moved actin at half of the rate of the phosphorylated construct. The average rate of motility obtained with phosphorylated skeletal RLC was 0.60 ± 0.10 μm/s (Fig. 3B and Table
II), lower than that of control wild-type HMM. It should be noted, however, that wild-type HMM with skeletal LCs did not move actin at all in a motility assay (Fig. 3A), a result that was previously reported (3). Thus, the incorporation of skeletal HC sequence in the neck actually overrides the inhibition imposed by the skeletal RLC when bound to the smooth muscle myosin neck. The actin-activated ATPase activity of the construct with skeletal LCs was lower than that obtained with smooth LCs, qualitatively consistent with the reduced rate of motility observed when skeletal LCs were bound to this chimera (Fig. 4 and Table II).

The construct described above retained 3 smooth muscle-specific residues at the beginning of the α-helix (residues 780–783; see Fig. 1). To rule out the possibility that these residues that interact with the motor domain might be the critical determinant for completely activating the dephosphorylated species, the properties of the Sk 781–852 chimera were also determined. They were, however, not significantly different from those of the Sk 787–852 chimera (Fig. 4A, compare circles and triangles; and Table I).

**Chimeras Containing Skeletal HC Sequence in Part of the Neck**—To identify which region of the heavy chain in the neck was responsible for the partial loss of regulation, several other mutants were engineered. The expectation was that the sequence constituting the RLC-binding site would be most important in giving rise to activation of the dephosphorylated state. We therefore first focused on a region of the RLC-binding site that has a distinct bend in it near a sequence that is WQWW (amino acids 838–841) in smooth and scallop myosins and WPWM in skeletal muscle myosin. Mutants with either a single amino acid mutation (Q839P) or a double point mutation (Q839P/W841M), however, had actin-activated ATPase activity and motility that were indistinguishable from those of wild-type HMM (data not shown).

Larger pieces of skeletal sequence were next engineered into smooth muscle HMM. One chimera (Sk 827–849) corresponds to skeletal sequence only under the RLC-binding site, and two others (Sk 815–849 and Sk 806–849) contained increasing amounts of skeletal sequence in the region to which the ELC binds (see Figs. 1 and 2A). The chimera Sk 827–849 resembles wild-type HMM in its motility properties (Fig. 3C), whereas there is a modest activation of the dephosphorylated state in the Sk 815–849 and Sk 806–849 constructs (Table I). The first chimera to show a high level of motility and activity was the Sk 827–849. The construct described above retained 3 smooth muscle-specific residues at the beginning of the α-helix (residues 780–783; see Fig. 1). To rule out the possibility that these residues that interact with the motor domain might be the critical determinant for completely activating the dephosphorylated species, the properties of the Sk 781–852 chimera were also determined. They were, however, not significantly different from those of the Sk 787–852 chimera (Fig. 4A, compare circles and triangles; and Table I).

**Regulation of Smooth Muscle Myosin**
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Table I
Actin-activated ATPase activity of chimeric HMMs with smooth muscle LCs

| Chimeric HC constructs | Actin-activated ATPase activity | Motility |
|-----------------------|--------------------------------|---------|
|                       | Dephos. RLC | Phos. RLC | Ratio (D/P) | Dephos. RLC | Phos. RLC | Ratio (D/P) |
| Wild-type             | 0.04       | 1.21 (2)  | 0.03       | 0           | 1.12 ± 0.15 (4) | 0       |
| Sk 827–849            | 0.06       | 0.81 (4)  | 0.07       | 0           | 0.58 ± 0.07 (2) | 0       |
| Sk 815–849            | 0.13       | 0.64 (2)  | 0.20       | 0.14 ± 0.03 | 0.71 ± 0.09 (2) | 0.20    |
| Sk 806–849            | 0.18       | 0.89 (3)  | 0.26       | 0.17 ± 0.04 | 0.71 ± 0.13 (2) | 0.24    |
| Sk 787–852            | 0.41       | 1.95 (3)  | 0.21       | 0.73 ± 0.18 | 1.31 ± 0.19 (4) | 0.56    |
| Sk 781–852            | 0.63       | 2.22 (2)  | 0.28       | 0.92 ± 0.16 | 1.13 ± 0.17 (1) | 0.73    |
| Sk 719–852            | 1.57       | 2.20 (2)  | 0.71       | 0.72 ± 0.80 | 0.84 ± 0.13 (1) | 0.86    |
| Sk 719–787            | 0.45       | 1.12 (2)  | 0.40       | ND          | 0.21 ± 0.04 (2) |         |

*Expressed chimeric HMM constructs contain all smooth muscle myosin heavy chain sequence except for the amino acids indicated, which were derived from the chicken fast skeletal heavy chain sequence. For example, Sk 827–849 has skeletal heavy chain sequence inserted into the smooth muscle backbone from amino acids 827 to 849, with the remainder of the HMM being composed of smooth muscle heavy chain sequence (amino acids 1–1175).

Actin-activated ATPase rates were obtained at five time points in duplicate at 40 μM actin. Activity in the absence of actin was subtracted from the reported values. 40 μM actin gives ATPase values that are close to V_max and assures good linearity of phosphate release as a function of time because the relatively low viscosity allows good mixing.

Dephos., dephosphorylated; Phos., phosphorylated; D/P, dephosphorylated/phosphorylated; ND, not determined.

For each preparation, the means ± S.D. of 15–30 actin filaments are reported.

Figure 4
Actin-activated ATPase activity of chimeric HMMs as a function of actin concentration with phosphorylated (closed symbols) and dephosphorylated (open symbols) RLCs. Both LCs are from smooth muscle, except in C. The chimeric HMMs contain skeletal HC sequence in the neck (Sk 787–852 (circles) and Sk 781–852 (triangles)) (A), in the neck and converter (Sk 719–852) (B), in the neck (Sk 787–852) but with skeletal LCs (sk LCs) (C), and in the converter region only (Sk 719–787) (D).

787–852 construct (described above), in which essentially the entire α-helix has been mutated to skeletal sequence.

Complete Loss of Regulation Requires Skeletal Sequence in the "Converter" Region of the Motor Domain and Neck—Changing the entire sequence of the neck region to that found in skeletal muscle myosin significantly activated the dephosphorylated state, but not to the level of the phosphorylated chimera. We therefore made a chimera that consisted of even more skeletal sequence, from the conserved SH1-SH2 helix of the motor domain through the invariant proline that marks the head/rod junction. This chimera has the entire light chain-binding α-helix as well as the domain that has been referred to as the converter region, composed of skeletal sequence (Fig. 2C).

This chimera, unlike the others described, showed complete loss of regulation. It displayed similar activity in the dephosphorylated and phosphorylated states, both with respect to motility (Fig. 3D) and actin-activated ATPase activity (Fig. 4B). Moreover, the results obtained with smooth and skeletal LCs were much more similar than in any of the other chimeras. Thus, sequences in both the motor domain and the neck region are required to fully abolish the regulatory capabilities of smooth muscle HMM.

To test the possibility that the skeletal sequences in the motor domain were primarily responsible for the loss of regulation, we expressed a chimera (Sk 719–787) that contained skeletal sequence only in the motor domain, from the SH1-SH2 helix to the top of the α-helix to which the light chains bind (i.e. the converter domain) (Fig. 2D). This chimera was more regulated than Sk 719–852 (Fig. 4D and Table I), implying that complete loss of regulation was due to a cumulative effect of changes to both the motor and regulatory domains. Despite the high ATPase activity of this chimera (Fig. 4D), there was a low rate of motility in the phosphorylated state (Table I).

The Smooth Muscle Regulatory Domain Does Not Confer Regulation upon the Motor Domain of Unconventional Myosin V—The second type of chimera that was engineered consisted of the motor domain from unconventional myosin V joined to the neck and rod from smooth muscle HMM. Myosin V is quite divergent from myosin II, and thus, this chimera is a stringent test of whether the neck region is the sole determinant of regulation. The activity of native myosin V is regulated by calcium binding to calmodulin, which is bound to six IQ motifs in the neck region (16). There is also a specific light chain bound to one of the IQ motifs, although at present its position relative to the motor domain has not been established. The strategy used to design the chimera HMM (myosin V motor domain and smooth neck and rod) was similar to that used to generate the Sk 787–852 chimera (described above), namely the conserved LE sequence (amino acids 784 and 785; see Fig. 1) was used as the point of interchange. Four independent preparations of this chimera showed the following ratios of actin-activated ATPase activities in the dephosphorylated/phosphorylated state: 0.67, 0.54, 0.70, and 0.53 (Table III).
TABLE II

Actin-activated ATPase activity of chimeric HMMs with skeletal muscle LCs

| Preparation No. | Actin-activated ATPase activity | Motility | Actin-activated ATPase activity | Motility |
|-----------------|---------------------------------|----------|---------------------------------|----------|
|                 | Dephos. RLC                      | Phos. RLC | Ratio (D/P)                      | dephos. RLC | Phos. RLC | Ratio (D/P) |
|                 | µmol/min/mg                      | µmol/min/mg |                                   | µmol/min/mg | µmol/min/mg | µmol/min/mg |
| 1               | 1.54                             | 2.29      | 0.67                            | 1.54      | 2.29      | 0.67       |
| 2               | 0.60                             | 1.10      | 0.54                            | 0.60      | 1.10      | 0.54       |
| 3               | 0.49                             | 0.71      | 0.70                            | 0.49      | 0.71      | 0.70       |
| 4               | 0.64                             | 1.20      | 0.53                            | 0.64      | 1.20      | 0.53       |

a Dephos., dephosphorylated; Phos., phosphorylated; D/P, dephosphorylated/phosphorylated.

chimaera therefore consistently showed less than a 2-fold degree of regulation, establishing that the presence of the smooth muscle myosin neck and LCs is not sufficient to confer this motor with the ability to completely inhibit phosphate release.

DISCUSSION

Smooth muscle myosin’s ability to inhibit phosphate release requires multiple interactions to take place, which creates a degree of regulation that is unique in its completeness. At the same time, the sheer number of critical interactions makes the determinants of the off state difficult to define, while easy to perturb. Altering any one of these interactions causes varying degrees of activation of the dephosphorylated state, and thus, a continuum in the observed degree of regulation can be attained. The adjacent head is important for complete inhibition of the dephosphorylated state since dephosphorylated single-headed myosin has two-thirds the activity of phosphorylated single-headed myosin (17). The rod also plays a role since two heads that were dimerized by addition of a “leucine zipper” coiled-coil, but that contained little or no native rod sequence, were also active when phosphorylated (7). Both head-head and head-rod interactions are therefore major elements involved in regulation.

The nature of the head-head interactions has not been fully established, but is thought to include interactions between dephosphorylated RLCs on adjacent heads that are abolished upon phosphorylation. Thus, it was expected that a chimera with skeletal HC sequence in the neck region would be active when dephosphorylated since this would presumably perturb RLC-RLC interactions. While this prediction was fulfilled, several aspects of this study were unexpected. One surprising feature was that regulation was essentially unperturbed if only the RLC-binding site was mutated to the skeletal sequence (Sk 827–849). There are numerous sequence differences between smooth and skeletal myosins in this region of the HC (Fig. 1), and based on sequence comparisons alone, one might have predicted that these differences were sufficient to account for whether or not a particular myosin is regulated. The results obtained here, however, imply that the smooth RLC binds similarly to the skeletal and smooth muscle IQ motif sequences, at least with respect to positioning the two lobes of the RLC adequately for regulation. When the entire LC-binding α-helix was changed to skeletal sequence, a degree of regulation still remained. For dephosphorylated HMM to have the same actin-activated ATPase activity and motility as phosphorylated HMM, both the neck region and the converter had to be mutated to skeletal sequence. Changing the converter sequence alone resulted in more activation of ATPase activity than changing the neck sequence alone, but neither chimera produced full activation of the dephosphorylated state.

If RLC-RLC interactions play a major role in maintaining the inhibited state, why do sequences nearer the motor domain have a larger activating effect? One way to interpret these results is that sequences near the motor domain dictate the orientation of the neck as it emerges from the motor domain, which ultimately affects the way RLCs on adjacent heads will interact with each other. A comparison of the crystallographic structure of the neck regions from regulated scallop myosin (18) and unregulated skeletal myosin S1 (2) shows differences in the angle of the HC at the ELC/RLC interface as well as in the RLC-binding site (see Fig. 5 in Ref. 19). Thus, small changes at the top of the α-helix might be propagated to larger changes in orientation of the RLC-binding site. In support of this idea, a chimeric smooth muscle myosin HMM that contained the HC neck sequence from regulated scallop myosin showed a high degree of regulation by phosphorylation. Thus, incorporation of any foreign sequence in the neck does not necessarily activate the dephosphorylated state. Instead, some feature of the neck that is common to regulated myosins, but that is not conserved in the skeletal muscle myosin neck, determines whether or not a motor with the potential to be regulated does in fact exhibit control of phosphate release.

The results described above are consistent with the idea that the neck is involved in interactions necessary to obtain an inhibited state. A more stringent test of whether the regulatory domain is the sole determinant of regulation is to analyze the properties of a chimera with the smooth muscle neck and rod attached to a heterologous motor domain. Using this strategy, it was reported that a chimera containing the motor domain from skeletal muscle myosin and the neck and rod from smooth muscle HMM conferred a 9-fold degree of regulation upon this previously unregulated motor (5). Here, the properties of a similar construct containing the motor domain from an unconventional myosin V and the neck and rod from smooth muscle HMM were analyzed. We found less than a 2-fold degree of regulation, suggesting that the smooth muscle neck cannot confer complete regulation upon all motor domains of the myosin superfamily. The inability of the smooth muscle neck to regulate myosin V is consistent with our previous observation.

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that sequences in the motor domain can preclude regulation, even when the construct has a completely native neck and rod derived from smooth muscle myosin (6).

The emerging picture of smooth muscle myosin regulation is that the light chain-binding domain of the myosin HC is only one of the elements necessary for complete regulation. The perturbations that activate dephosphorylated myosin to the greatest extent are as follows: mutations in the actin-binding loop (6), mutations in the converter region of the motor domain (this work), removal of one of the myosin heads (17), and removal of the native myosin rod sequence while retaining a dimeric structure with a leucine zipper (7). Conversely, substitution of the skeletal RLC for the smooth RLC completely locks the system off (3). Thus, creation of an inactive state likely begins with interactions between the two native dephosphorylated RLCs, which are then stabilized by interactions between the myosin rod and both heads. The nature of the motor domain is important for regulation since we show here that not all motor domains can be regulated. This study also suggests that, in some way (requiring sequence extending from the RLC-binding site through the converter domain), the positioning of the regulatory domain of smooth muscle myosin relative to the motor domain is critical and has been optimized to facilitate these RLC-rod-head interactions, thus enabling a completely off state in the absence of RLC phosphorylation.

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