Regulation of Asymmetric Smooth Muscle Myosin II Molecules*

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The emerging view of smooth/nonmuscle myosin regulation suggests that the attainment of the completely inhibited state requires numerous weak interactions between components of the two heads and the myosin rod. To further examine the nature of the structural requirements for regulation, we engineered smooth muscle heavy meromyosin molecules that contained one complete head and truncations of the second head. These truncations eliminated the motor domain but retained two, one, or no light chains. All constructs contained 37 heptads of rod sequence. None of the truncated constructs displayed complete regulation of both ATPase and motility, reinforcing the idea that interactions between motor domains are necessary for complete regulation. Surprisingly, the rate of ADP release was slowed by regulatory light chain dephosphorylation of the truncated construct that contained all four light chains and one motor domain. These data suggest that there is a second step (ADP release) in the smooth muscle myosin-actin-activated ATPase cycle that is modulated by regulatory light chain phosphorylation. This may be part of the mechanism underlying “latch” in smooth muscle.

The regulation of vertebrate smooth and nonmuscle myosin II is based on reversible phosphorylation of one of the two myosin light chains, referred to as the regulatory light chain (RLC)1 (1–3). The mechanism by which inhibition of activity is obtained is thought to involve numerous interactions within the molecule, including interactions between the two heads (4) and between the heads and the myosin rod (5). Within the heads, the nature of the RLC itself is important, as substitution of the skeletal RLC for the smooth RLC completely locks the system “off” (6). The nature of the motor domain is important for regulation, because we have shown that not all motor domains can be regulated simply by attachment of the smooth muscle neck and light chains (2). In addition, we have suggested that smooth muscle-specific sequences near the motor domain may contribute to the proper positioning of the light chains that is critical to facilitate the RLC-rod-head interactions necessary for achieving a completely off state in the absence of RLC phosphorylation (2). Thus, creation of the fully inhibited state of smooth muscle myosin likely begins with interactions that are facilitated by the two native dephosphorylated RLCs, which are then stabilized by interactions between the myosin rod and both heads.

The observation that proteolytically prepared single-headed myosin does not achieve the enzymatically off state in the absence of RLC phosphorylation established that head-head interactions are needed for regulation (4). However, this study did not address the issue of which part of the head is involved in the interactions. A number of lines of evidence have led to the proposal that there must be interactions between the two light chain binding domains of the heads (3, 7). If these are the most important interactions for regulation, then it might be possible to preserve regulation by eliminating one of the two smooth muscle myosin motor domains, while retaining one or both of the light chain binding domains of that head, as well as the complete second head. In contrast, the recently published structure of the smooth muscle HMM regulatory complex derived from two-dimensional crystals reveals interactions between the motor domains but not between the two light chain binding domains (8). To ascertain whether interactions between the light chain binding domains can confer a high degree of regulation, we constructed a series of asymmetric HMMs and assessed the degree of regulation conferred on these constructs by RLC phosphorylation.

MATERIALS AND METHODS

Construction, Expression, and Purification of Proteins—Fig. 1 is a schematic diagram of the asymmetric HMM constructs that were created. In each case, the expression involved co-expression of two different heavy chain constructs, derived from the chicken gizzard smooth muscle myosin cDNA (9). The first construct, which was co-expressed in all cases, began at amino acid 1 and ended at amino acid 1112, without a FLAG tag appended to the end. The other heavy chain constructs deleted the motor domain but preserved the coding sequence for both light chain binding sequences (designed to create an asymmetric HMM with a single intact head and an essential light chain and regulatory light chain in place of the second head, designated as SH(ER), i.e. single-headed HMM with binding sites for the RLC and the RLC, the regulatory light chain only (to create SHIR), i.e. single-headed HMM with binding sites for the RLC), or deleted the entire head (used to create SH, i.e. single-headed HMM). The amino acid position that followed the start codon of each of the N-terminal truncations was as follows: Arg788 for SH(ER), Phe812 for SHIR, and Pro649 for SH (complete removal of second head). Following the codon for amino acid 1112 of each of the three N-terminal truncated heavy chains, a FLAG tag was inserted before the stop. Thus the following three different HMM species resulted from co-expression: (a) a two-headed homodimer without a FLAG tag; (b) a homodimer containing no motor domains (i.e. no actin binding) but with two FLAG tags; and (c) the desired asymmetric heterodimer, containing one complete head capable of binding actin, and one heavy chain with a truncated head and FLAG tag at the C terminus. Thus sequential purification involving actin binding followed by binding to an anti-FLAG affinity column could be used to purify the asymmetric species.

The HMM-like constructs were used to produce recombinant baculoviruses, as described previously (10). Sf9 insect cells were co-infected with recombinant virus containing chicken smooth ELC (LC1) and RLC cDNAs and with recombinant viruses coding for the truncated
myosin heavy chains.

Three days after infection, Sf9 cells were lysed in a high ionic strength buffer containing ATP, dithiothreitol, Nonidet P-40, and protease inhibitors. This was followed by low-speed centrifugation, ammonium sulfate fractionation, and overnight dialysis at 4 °C in the presence of F-actin, and hexokinase and glucose to remove ATP. Dialysis was followed by centrifugation at 500,000 x g for 20 min, which pelleted the F-actin-HMM complexes. The pellet was washed and then resuspended in a buffer containing 1 mM MgATP to dissociate the HMM. The F-actin was pelleted by centrifugation, and the supernatant was removed and passed through an anti-FLAG epitope antibody affinity column. Only HMM that bound and released from actin and that contained a C-terminal FLAG epitope bound to the column. The HMM was eluted via FLAG peptide competition.

Actin was purified from rabbit back leg and skeletal muscles, following the procedure of Spudich and Watt (11). All protein concentrations were determined using the Bradford (12) method of assay. Purity of all myosin and actin preparations was confirmed on 10% discontinuous SDS polyacrylamide gels (13).

**Single-headed Myosin—** Single-headed myosin was prepared by limited papain digestion of chicken gizzard myosin prepared from tissue, as described in Tyska et al. (14). Data from such a preparation is included for comparison to the expressed single-headed HMM, SH.

**RLC Phosphorylation—** Myosin was incubated with 2 μg calmodulin, 100 μM ATP, and 0.15 μM skeletal muscle myosin light chain kinase in the presence of 1 mM MgATP for 30 min. This achieved high levels of regulatory light chain phosphorylation (>90%). Levels of HMM phosphorylation for each preparation were determined using glycerol/urea gels (15).

**ATPase Assays of Phosphorylated HMM-like Fragments—** The actin-activated ATPase assays involved the measurement of the rate of radioactive P32γ generation from γ-phosphate-labeled ATP, by the method of Pollard (16). The conditions of the assay were 2 mM MgCl2, 10 mM MOPS (pH 7.0), 0.1 mM EGTA, 1 mM ATP at 25 °C. Vmax and KATPase were determined from Lineweaver-Burk plots. The actin concentration used was over the range of 0–150 μM. Assays for determination of the potassium EDTA-ATPase activity followed the methodology of Pollard (16).

**Metal Shadowing—** Rotary-shadowed platinum images were obtained in 0.5 mM ammonium acetate, 66% glycerol and observed with a Philips EM301 electron microscope operated at 60 kV (17).

**In Vitro Motility Assay—** The motility assay was performed at 30 °C in 25 mM imidazole, pH 7.5, 25 mM KCl, 4 mM MgCl2, 1 mM EGTA, 0.5% methanolcellulose, using monoclonal antibody S2.2 for attachment of the HMM (18). For single-headed myosin, the conditions were 30 °C in 25 mM imidazole, pH 7.5, 60 mM KCl, 4 mM MgCl2, 1 mM EGTA, 0.5% methanolcellulose, with the myosin directly attached to the nitrocellulose.

**Rate of ADP Dissociation from acto-HMM—** Acto-HMM or acto-SH(ER) was mixed with 50 or 100 μM ADP in 10 mM imidazole, pH 7.0, 1 mM dithiothreitol, 1 mM EGTA, and 1 mM MgCl2 at 25 °C. The actin concentration prior to mixing was 5 μM, and the SH(ER) concentration was 2 μM. For experiments with HMM, the actin concentration was increased to 10 μM, and the HMM concentration was 1 μM. The acto-HMM-ADP (acto-SH(ER)-ADP) was rapidly mixed with a 2 mM MgATP using stopped-flow techniques (10). The rate of dissociation of acto-HMM by ATP as measured by light scattering was assumed to be rate-limited by the rate of ADP dissociation from the active site.

**RESULTS AND DISCUSSION**

Three asymmetric variants of HMM, shown diagrammatically in Fig. 1, were produced by expression using the baculovirus/insect cell expression system. Each construct contains one intact heavy chain and one truncated heavy chain. No construct has two motor domains. Depending on the nature of the truncated heavy chain (see “Materials and Methods” for details), there is either no light chain binding domain, a truncated light chain binding domain that binds only the RLC, or a light chain binding domain that binds both ELC and RLC. These three constructs were referred to as SH (single-headed HMM), SH(R) (single-headed HMM with an RLC binding site), or SH(ER) (single-headed HMM with an ELC and RLC binding site), respectively.

The gel shown of SH(ER) (Fig. 2A, right lane) illustrates that the expression and purification scheme described under “Materials and Methods” resulted in the production of an asymmetric HMM, with one intact and one truncated heavy chain, which differ in molecular weight by the expected amount. Rotary shadowing confirmed that the asymmetric constructs contained only one intact myosin head.

The potassium EDTA-ATPase revealed that all constructs had the same extent of intrinsic ATPase activity (Table I). The actin-activated ATPase activities revealed that none of the asymmetric constructs were fully regulated by RLC phosphorylation. As reported in Table I, the dephosphorylated species all had significant activity at actin concentrations between 5 and 50 μM actin. At 50 μM actin, SH(ER) shows approximately a 2-fold increase in activity as a consequence of RLC phosphorylation. However, the KATPase values for all of the single-headed constructs were significantly elevated compared with the two-headed construct and precluded determination of the Vmax values for the actin-activated ATPase activity. Thus we only can conclude that there is not complete regulation of any of the asymmetric constructs as assessed by ATPase activity but cannot determine what, if any, degree of regulation is maintained at high actin concentrations. This same increase in KATPase was seen for a single-headed construct of Dictyostelium myosin II (the full-length myosin equivalent of our SH construct) (19), which prevented an accurate determination of the ATPase Vmax of the single-headed myosin in that study. Those authors suggested that this was indicative of cooperativity between the myosin heads that is lost in the one-headed construct.

In vitro motility provides another way to quantify the degree
TABLE I
Kinetic parameters for HMM-like constructs with and without RLC phosphorylation

| HMM-like construct (± RLC phosphorylation) | K(EDTA) (μM) | Actin-activated ATPase (50 μM actin) | Rate of ADP release from actomyosin |
|-------------------------------------------|-------------|-----------------------------------|----------------------------------|
|                                            |             | s^−1 per head | s^−1 per head | s^−1|
| HMM − RLC phos.                           | 1.9 ± 0.3   | 0.01            | 80 ± 4         |
| HMM + RLC phos.                           | 2.0 ± 0.4   | 1.28 ± 0.12     | 82 ± 6         |
| SH(ER) − RLC phos.                       | 1.8 ± 0.4   | 0.34 ± 0.08     | 29 ± 6^6       |
| SH(ER) + RLC phos.                       | 1.9 ± 0.5   | 0.70 ± 0.07     | 84 ± 8         |
| SH(R) − RLC phos.                        | 1.5 ± 0.4   | 0.45 ± 0.06     | ND              |
| SH(R) + RLC phos.                        | 1.7 ± 0.5   | 0.50 ± 0.09     | ND              |
| SH − RLC phos.                           | 1.5 ± 0.6   | 0.57 ± 0.08     | ND              |
| SH + RLC phos.                           | 1.5 ± 0.7   | 0.65 ± 0.14     | ND              |

^a Derived from slower rate of a two-exponential fit (see Fig. 4).

of regulation that is maintained in each of our asymmetric constructs. The in vitro motility values summarized in Fig. 3 reveal that only SH(ER) did not support actin filament movement in the absence of RLC phosphorylation. The average in vitro motility value of the phosphorylated SH(ER) was approximately 30–40% of that obtained with double-headed HMM. The lowered motility of the single-headed species was also observed with single-headed myosin that was prepared by proteolysis of tissue-purified myosin. Phosphorylated single-headed myosin moved actin at ~40% the rate of the phosphorylated double-headed myosin (0.49 ± 0.13 μm/sec versus 1.30 ± 0.16 μm/sec for the control; note that these data were obtained at slightly higher salt conditions where movement is faster; see “Materials and Methods”). This is also consistent with previous studies of single-headed, full-length myosin II species that have shown a rate of 50 Dictyostelium myosin II (Ref. 19) and 30% (smooth muscle myosin II; Ref. 4) decrease in in vitro motility. Furthermore, the single-headed Dictyostelium myosin II results in severely impaired in vivo function (20). The fact that the single-headed asymmetric molecules display reduced in vitro motility suggests that either the rod provides residual inhibition in the absence of a complete second head or that cooperative interactions between the heads are necessary for maximal motility. A decreased motility for single-headed myosin is not seen for skeletal muscle myosin (21), suggesting that it may be a property of regulated myosins.

How can one reconcile the apparent differences in the degree of regulation obtained with ATPase assays versus motility assays in the case of SH(ER)? First, it is more likely that the lowered actin-activated ATPase activity and motility of all of the single-headed constructs represent a partial inhibition because of head-rod interactions, rather than a loss of cooperativity. This assertion is based on the fact that smooth muscle myosin S1 has the same actin-activated ATPase activity (per head) and nearly the same motility as HMM. Thus it is likely that partial residual inhibition of actin-activated ATPase activity and motility exists in the single-headed constructs with and without phosphorylation. What is the physical meaning of this partial regulation? Partial regulation of the asymmetric mutants may result from either simply slowing the rate of the weak to strong transition on actin, or, more likely, it may result from shifting the equilibrium of an off state and an on state transition that precedes the weak to strong transition on actin. That is to say that complete regulation of intact smooth muscle myosin is achieved by trapping 100% of the molecules in an off state in the absence of phosphorylation (preventing transition 1 in Scheme 1), and RLC phosphorylation shifts the equilibrium to 100% in the on state. Thus the activity (per head) is identical between S1 and phosphorylated HMM. In the current study, and likely in other instances where structural modifications have resulted in partially regulated molecules, partial regulation may consist of creating an equilibrium distribution between a completely off and fully on state in which both states are significantly populated, rather than modulating the rate of the weak to strong transition and Pi release.

Applying this logic to the current study, one derives insight from the work of Warshaw et al. (22), in which motility was determined as a function of the ratio of phosphorylated (on) to dephosphorylated (off) smooth muscle myosin. For motility to be stopped, approximately 85% of the myosin had to be dephosphorylated. Such a mixture would generate an ATPase activity of 15% of fully phosphorylated HMM, which may help explain our observation; no motility of dephosphorylated HMM(ER) but with an actin-activated ATPase activity that was 25% of phosphorylated HMM. Additional slowing of dephosphorylated SH(ER) motility may come from a reduction in the rate of ADP release; see below. Thus with SH(ER), the apparent dichotomy in the regulation of ATPase activity versus motility might be explained by 25% of the heads being on at any given time in the absence of RLC phosphorylation and the instantaneous value increasing to 55% (based on the actin-activated ATPase activity) with RLC phosphorylation.

An equilibrium distribution between off and on molecules could be the underlying mechanism of the partial regulation by RLC phosphorylation that is normally seen in a variety of myosin II molecules, such as Dictyostelium myosin II (19, 20). Furthermore, this model may underlie the partial regulation that has been reported for a variety of mutations in smooth muscle myosin, including truncations in the rod (5) and more recently, phosphorylation of only one head of intact HMM (23).

In the case of both Dictyostelium myosin II and smooth muscle HMM with one phosphorylated RLC, the molecules appeared to be more regulated when assayed by motility assays than by actin-activated ATPase activity. In the case of the smooth muscle HMM with one phosphorylated RLC, the molecules appeared to be more regulated when assayed by motility assays than by actin-activated ATPase activity. In the case of the smooth muscle HMM with one phosphorylated RLC, single-turnover studies were attempted to clarify the nature of the partial regulation (23). However, the results of those measurements may not be meaningful if the single RLC-phosphorylated species has a significantly higher K_{ATPase} than the doubly phosphorylated species, as is the case for our one-headed versus two-headed constructs. (High actin concentrations cannot be used in single-turnover experiments because of interference from light scattering.)

This study demonstrates that full regulation of both ATPase activity and motility can only be obtained with two intact heads, suggesting a role for motor domain interactions in stabilizing the inhibited conformation. This conclusion agrees with recent structural evidence obtained from two-dimensional crystals of smooth muscle HMM, which directly shows interactions between the two motor domains of HMM in the dephos-
to explain the lack of motility of the dephosphorylated SH(ER), although a 3-fold reduction in ADP release rate is not sufficient either a phosphorylated or dephosphorylated state (Table I). PHosphorylation state of SH(ER) is depicted in Fig. 4. Whereas an alteration of the rate of ADP release as a function of the phosphorylation state of SH(ER) is proposed to happen in smooth muscle during a phenomenon termed “latch” (26–30). Latch occurs in some smooth muscles under conditions of low levels of myosin RLC phosphorylation. It is manifested by slowly cycling cross-bridges that support high force isometric contractions but with greatly reduced shortening velocity when the tissue is unloaded.

Based on this reasoning, we measured the rate of ADP release from both HMM and SH(ER) bound to actin, with and without RLC phosphorylation. Surprisingly, we saw more than a 3-fold decrease in the rate of ADP release from 84 ± 8 s⁻¹ when SH(ER) was phosphorylated to 29 ± 6 s⁻¹ in the dephosphorylated state. A representative record showing that there is an alteration of the rate of ADP release as a function of the phosphorylation state of SH(ER) is depicted in Fig. 4. Whereas the dissociation from actin of the phosphorylated SH(ER) is best fit by a single exponential, the dephosphorylated species gave rise to two exponential processes, 247 and 28 s⁻¹. The amplitude of the fast process was approximately 35% at 50 μM ADP and fell to approximately 20% in the presence of 100 μM ADP. The fast process represents heads without ADP bound that are rapidly dissociated by ATP. The slower rate represents ADP dissociation, which must occur before ATP can bind and dissociate the heads from actin. Thus, the dephosphorylated SH(ER) displays a slower ADP release and a lower affinity for ADP (because the heads are not saturated with ADP at 50–100 μM ADP). The rate achieved when SH(ER) is phosphorylated was the same as the rate seen for the two-headed HMM in either a phosphorylated or dephosphorylated state (Table I). Although a 3-fold reduction in ADP release rate is not sufficient to explain the lack of motility of the dephosphorylated SH(ER), it certainly will contribute to a slowing of motility. Perhaps the lack of motility in the absence of RLC phosphorylation is because of a combination of drag contributed by heads that are in an off state and heads that are slow to release ADP.

This modulation of the rate of ADP release via RLC phosphorylation has a number of important implications for the regulation and function of smooth muscle myosin. First, it suggests that the presumably weak interactions involving the light chain domains in the absence of RLC phosphorylation are sufficiently strong to prevent the transition from a state that releases ADP with a reduced rate to a state where the ADP off rate is accelerated. This possibly could correspond to the structural transition between an ADP-bound position of the power-stroke to the nucleotide-free position that has been seen using cryo-electron microscopy (31). If such a movement is necessary to release ADP, or at least to accelerate ADP release, it provides a highly strain-dependent ADP release step for smooth muscle myosin. Such a mechanism has been proposed (31–33) and would be well suited for smooth muscle, in which economic maintenance of force is a specialized feature of the tissue. By slowing ADP release under an imposed load, the duty cycle would be increased and the economy of force production enhanced (i.e. latch).

Why would the phosphorylation-dependent slowing of ADP release be manifested in the asymmetric construct but not in the native double-headed construct? We have proposed that the coiled-coil immediately following the heads of smooth muscle myosin is sufficiently weak that it could melt when both heads are bound to actin (5), and it may be necessary for this to occur for both heads of HMM to bind to actin strongly. If both heads were bound strongly, then interactions involving the light chain domains would likely be broken, regardless of whether the RLCs are phosphorylated. Thus the rate of ADP release would be independent of the state of phosphorylation. Rosenfeld et al. (3) noted that the rate of ADP release from dephosphorylated HMM is slower than that of phosphorylated HMM if one begins the reaction with HMM-ADP not bound to actin. Under those conditions, the two heads may initially have in-
Interactions between the heads involving the light chain domains that must be broken before both heads can attach to actin and release ADP.

We are proposing that RLC phosphorylation modulates at least two separate steps in the actin-smooth HMM kinetic cycle. The absence of RLC phosphorylation prevents the weak interactions between the heads involving the light chain domains that must be broken before both heads can attach to actin and release ADP. Thus under conditions where one myosin head is attached to actin and the second head is detached, the interactions involving the dephosphorylated light chain domains of the two heads may slow ADP release. Although the slowing of the weak to strong transition would simply reduce the number of strongly attached cross-bridges, slowing the ADP release rate would increase the amount of time a cross-bridge spends in the force-generating states (i.e. increase the duty cycle). This would increase the economy of force generation and could contribute to the phenomenon of latch.

In conclusion, this study provides further evidence that attainment of the complete off state of smooth muscle myosin involves interactions between two intact heads, consistent with the recent structure from two-dimensional crystals (8). Furthermore, interactions involving the light chain domains can confer modulation of the ADP release step, thus prolonging the strongly attached state in the absence of RLC phosphorylation. Whether the interactions that achieve this slowing of ADP release are a subset of interactions used to achieve the off state or whether they involve a different set of interactions between the light chain domains themselves is unknown.

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