Phosphorylation-dependent Regulation Is Absent in a Nonmuscle Heavy Meromyosin Construct with One Complete Head and One Head Lacking the Motor Domain*

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To understand the domain requirements of phosphorylation-dependent regulation, we prepared three recombinant constructs of nonmuscle heavy meromyosin IIB containing 1) two complete heads, 2) one complete head and one head lacking the motor domain, and 3) one complete head and one head lacking both motor and regulatory domains. Steady-state ATPase measurements showed that phosphorylation did not alter the affinity for actin by more than a factor of 2 for any construct. Phosphorylation increased V_{max} by a factor of 10 for construct 1 and 1.5–3 for construct 2 but had no effect for construct 3. Single turnover measurements, a better measure of slow rates inherent to unphosphorylated regulated myosins, showed that the single-headed construct 2, like construct 3 retains less than 1% of the regulatory properties of the double-headed construct 1 (300-fold activation). Therefore, a complete head cannot be down-regulated by a regulatory domain (without the motor domain) on the partner head. Two motor domains are required for regulation. This result is predicted by a structural model (Wendt, T., Taylor, D., Messier, T., Trybus, K. M., and Taylor, K. A. (1999) J. Cell Biol. 147, 1385–1390) showing interaction between the motor domains for unphosphorylated smooth muscle myosin, if motor-motor interaction is the basis for down-regulation.

The conventional class II myosins are hexameric proteins consisting of two heavy chains and two pairs of light chains. The carboxyl-terminal half of the two heavy chains dimerize via an α-helical coiled-coil to form a long rod through which myosin can self-assemble to form filaments. The amino-terminal half of the two heavy chains forms the two separate motor (catalytic) domains, which bind actin and hydrolyze ATP. Each is connected to the rod via a neck region that contains the binding site for the two light chains. The class of light chains that binds nearest to the motor domain is termed the essential light chain (ELC) whereas the class that binds nearest to the coiled-coil rod is termed the regulatory light chain (RLC). One ELC and one RLC together with the portion of the heavy chain to which they bind are commonly referred to as the “regulatory domain.” The motor domain and the regulatory domain together form the soluble myosin “head” termed subfragment-1 (S1). Truncation of the coiled-coil rod of myosin produces a soluble two-headed structure termed heavy meromyosin (HMM).

The actin-activated MgATPase activity and motor properties of smooth muscle and nonmuscle myosins are regulated by phosphorylation of the RLC (1–3). The unphosphorylated forms of these regulated myosins have low ATPase activity and are unable to move actin filaments, whereas the phosphorylated forms are activated in both respects. S1 is active in both unphosphorylated and phosphorylated states (4–6), whereas HMM is well regulated by phosphorylation (4, 7). Single-headed myosin, which lacks the motor domain and regulatory domain of one head, is not regulated by phosphorylation (8, 9). Similarly, recombinantly expressed fragments that were sufficiently truncated from the carboxyl terminus to destabilize the coiled-coil, and thus the two-headed structure, were also not regulated by phosphorylation (10–12). These data suggest that the presence of two heads is critical for down-regulation, suggesting that head-head interaction is an important feature of the unphosphorylated state. However, these studies do not elucidate which domain interactions are critical for regulation.

Recent analysis of two-dimensional crystals of smooth muscle HMM suggests a structural mechanism for phosphorylation-dependent regulation (13, 14). Reconstructions of images of unphosphorylated HMM showed an asymmetrical structure with an interaction between the actin-binding region of the motor domain of one head and the converter region of the motor domain of the partner head. This interaction between the motor domains was not evident in phosphorylated HMM. It is not known whether this motor-motor domain interaction is a requirement for down-regulated kinetic properties, but if it is, a prediction of the mechanism is that a myosin lacking one full head would not be regulated, which is what has been observed. However, a more constrained prediction is that a myosin with one full head and a partner head lacking just the motor domain, but retaining the regulatory domain would also not be regulated.

Recently, three studies (5, 15, 16) have tested this prediction using various asymmetric recombinant fragments of smooth muscle HMM that contained an intact head partnered with a head that was truncated from the amino terminus. All three studies produced at least one asymmetric HMM construct whose MgATPase activity was increased 2–3-fold by RLC phosphorylation, and two of the studies found that some of these molecules required phosphorylation for the movement of actin filaments in an in vitro motility assay. However, the interpre-
Experimental Procedures

Construc ts—PCR using the chicken nonmuscle IIB heavy chain cDNA (GenBank accession number Q02015) as a template was carried out to make various amino-terminal-truncated species shown in Fig. 1A. The Motor-ER-S2 (or MHC II-B1 HMM-like) fragment was subcloned into pBlueBAC II baculotransfer vector described in detail by Pato et al. (22). Using this vector as a template, an ER-S2-DNA fragment was made using a 5′-end PCR primer upstream to the RLC binding site (amino acid residue 795) plus a BamHI restriction site and ATG start codon. The 3′-end PCR primer at the end of the HMM-like fragment (amino acid residue 1242) included a FLAG epitope, a TAG stop codon, and an EcoRI restriction site. Similarly, the S2 DNA fragment was made using a PCR primer downstream to the ELC binding site (amino acid residue 854) plus a BamHI restriction site and ATG start codon together with the same 3′-end PCR primer used for the ER-S2 fragment.

Preparation of Recombinant Proteins—Baculoviruses were produced in a manner similar to that described in Pato et al. (22). Recombinant proteins are diagrammed in Fig. 1B. HMM was made and purified according to Pato et al. (22). The SH(ER) protein was made by co-infecting insect cells with the virus containing the Motor-ER-S2 construct, the ER-S2-DNA construct, and the RLC/ELC construct (22). The SH protein was made by co-infecting insect cells with the viruses containing the S1-ER-S2 construct, the S2 construct, and the RLC/ELC construct. The proteins containing only one motor domain were purified from the cell extract by FLAG affinity chromatography followed by actin selection to isolate the proteins containing motor domains. A detailed procedure is described in Wang et al. (23).

The concentrations of all samples were determined by the Bradford assay (Bio-Rad) using smooth muscle HMM as a standard. The concentration of some preparations of HMM was measured using d_{1.43} = 0.63 mg cm/ml (kindly determined by Dr. Walter Stafford, Boston Biomedical Research Institute). The molecular masses in daltons of the various constructs were: HMM, 362,000; SH, 272,000; SH, 228,000.

Preparation of Other Proteins—F-actin was prepared from rabbit muscle according to the method of Spudich and Watt (24), myosin light chain kinase was from turkey gizzard (25), and calmodulin was prepared as described (26).

Electron Microscopy—Images were collected on a Hitachi 600 electron microscope operated at 75 kV. Specimens were prepared at 22 mg/ml protein in 66% glycerol, 0.5 mM KCL, 20 mM Tris, pH 8.2, 10 mM DTT, 1 mM EGTA.

Steady-state Actin-activated MgATPase Activity—Actin-activated MgATPase activities of the various HMM fragments were measured using the method of Pollard and Korn (27) in a buffer containing 10 mM MOPS (pH 7.0), 2 mM MgCl₂, 1 mM ATP, 0.1 mM EGTA at 35 °C or in 50 mM NaCl, 10 mM MOPS (pH 7.0), 2 mM MgCl₂, 1 mM ATP, 0.1 mM EGTA at 25 °C in order to better mimic the conditions of the single turnover experiments. The actin concentration was varied according to the HMM being measured. HMM was either used as purified (unphosphorylated) or prephosphorylated in a similar buffer to the above, but containing additionally 0.2 mM CaCl₂, 10 mM calmodulin, and 2 nM myosin light chain kinase (MLCK), for 20 min at 25 °C prior to initiating the assay.

Double-mixing Single Turnover of FTP in the Presence of Actin—This protocol is a modification of our previous method (17). Assays were performed at 25 °C in a temperature-controlled stopped-flow fluorometer (Hi-Tech SF 61-DX-2) equipped with a 150-watt Hg-Xe lamp and an electronic anti-bleaching shutter designed to avoid photolysis over prolonged time courses. The excitation wavelength was 313 nm, and the emission bandwidth was 4 nm. Emitted light was collected through a LG 370 filter (Corning). The experiment was done with 2 syringes. The first syringe contained actin at twice the final concentration, 200 µM ATP, 10 mM MOPS (pH 7.0), 50 mM NaCl, 0.1 mM EGTA, 0.8 mM MgCl₂, and 1 mM DTT. The second syringe contained 0.6–0.8 µM active sites in 10 mM MOPS (pH 7.0), 50 mM NaCl, 0.1 mM EGTA, 1 mM DTT. Immediately prior to data collection, MgFTP (FTP equimolar to active sites, MgCl₂ equal to two times the FTP concentration) was added to the second syringe. Fifty microliters were repetitively shot (approximately every min) from both syringes. These initial shots were not analyzed per se but were used only to observe when the initial fluorescence reached a maximum (indicating maximal binding of FTP to HMM, ~3 min after FTP addition). The maximal binding time was also measured in independent experiments (as data shown). As the complete turnover was then collected from one additional shot. The decrease in fluorescence after mixing reflects the off-rate of MgFDP, which is most likely limited by phosphate release. Between 500 and 1000 data points were collected depending upon the total acquisition time. One hundred and twenty-eight points were collected in the first 2.56 s after which the shutter was activated. The shutter open time for each data point collected thereafter was 12 ms.

A “no turnover” control experiment similar to that previously described (17) was performed to ensure that the change in fluorescence observed was because of the binding and release of FTP/FDP from the active site, and to confirm that photolysis was not appreciable under the conditions described above. The result (not shown) showed that the fluorescence changes observed in the turnover experiment were due to binding and release of FTP/FDP from the active site.

All turnover experiments reported here were completed within a week of preparing the protein, and samples were stored on ice. Older samples and samples frozen in liquid nitrogen tended to exhibit more complex kinetics, with an additional rapid phase appearing with significant amplitude.

RESULTS

An HMM-like fragment of nonmuscle myosin IIB (Fig. 1B, HMM) was expressed in Sf9 cells by coinfection with a virus producing residues 1–1242 of the heavy chain (Fig. 1A, top, Motor-ER-S2) and another virus producing both the RLC and ELC (not shown). To express different asymmetric HMM-like molecules two additional heavy chain constructs were made (Fig. 1A, middle and bottom). ER-S2 started at codon 795, which deletes the motor domain, but retains the binding sites for both ELC and RLC. S2 started at codon 854, which encodes just the S2 (subfragment-2) region. Both of these latter two constructs contain a FLAG epitope at their carboxyl terminus. The expression of asymmetric HMM molecules, which have both the RLC and ELC bound to the motorless head (Fig. 1B, SH/ER)) was achieved by co-expressing heavy chain constructs Motor-ER-S2 and ER-S2 (Fig. 1A) along with an RLC/ELC encoding construct (not shown). This molecule will be referred to as SH/ER) as previously suggested (16). The expression of a single-headed protein (Fig. 1B, SH) was achieved by co-expressing heavy chain constructs motor-ER-S2 and S2 along with the construct expressing the RLC/ELC. SH/ER) can be activity purified by a combination of FLAG affinity chromatography, which eliminates HMM, and the ability to bind to actin in an ATP-dependent manner, which eliminates (ER), theimerized fraction containing no motor domains. A similar purification strategy is used to produce SH, which has light chains only on the head with the motor domain. The latter molecule is similar to single-headed myosins or single-headed...
HMM made using proteolytic methods. We attempted to produce SH(R) with only the regulatory light chain binding site on the truncated heavy chain, but this heavy chain underwent proteolysis in the RLC binding region such that a portion of the molecules would not be expected to bind the RLC. Therefore we did not further characterize SH(R).

SDS gels of the three molecules used in this study are shown in Fig. 2. In the case of the SH(ER), a heavy chain with an \( M_r \) of 144,000 corresponding to Motor-ER-S2 along with a lower molecular weight heavy chain (\( M_r \) of 57,000) corresponding to ER-S2 can be seen.

Three methods were used to characterize the native molecules. Nondenaturing gel electrophoresis (Fig. 3) of two independent preparations showed that HMM migrated as a single species that co-migrated with proteolytic smooth muscle HMM (prepared as described in Ref. 17). This suggests that the expressed HMM was fully assembled. SH(ER), SH, and (ER) migrated as single species with mobilities consistent with their relative \( M_r \). More directly, rotary-shadowed images (Fig. 4) of HMM and SH(ER) revealed that the molecules showed the appropriate shape. For comparison an image of (ER), the molecule bearing two regulatory domains but lacking both motor domains is also shown. The (ER) molecules have a smaller globular shape as expected. Finally, all constructs were native in that myosin light chain kinase could fully phosphorylate the RLC (Fig. 5).

Table I shows a summary of the steady-state actin-activated MgATPase maximal rate (\( V_{\text{max}} \)) and apparent actin affinity in the presence of ATP (\( K_{\text{ATPase}} \)) for the three constructs at 35 °C and under slightly different ionic conditions at 25 °C. After purification, all constructs were completely unphosphorylated, and treatment with myosin light chain kinase resulted in full phosphorylation (as in Fig. 5). The steady-state actin-activated MgATPase activity of the double-headed HMM was increased 9.6-fold at 25 °C and 10.6-fold at 35 °C by phosphorylation of the RLC. Most of the regulation is at the level of \( V_{\text{max}} \) as opposed to \( K_{\text{ATPase}} \) where less than a 2-fold effect is observed.

The steady-state \( V_{\text{max}} \) of SH(ER) is activated 1.7-fold (25 °C) and 3.0-fold (35 °C) by phosphorylation. In contrast, neither the \( V_{\text{max}} \) nor the \( K_{\text{ATPase}} \) of SH is greatly affected by phosphorylation. Interestingly, the \( V_{\text{max}} \) of phosphorylated SH(ER) and phosphorylated SH was about 2-fold less than phosphorylated HMM (Table I). These data for the SH(ER) and SH are similar in magnitude to the activation of homologous constructs derived from gizzard smooth muscle myosin (5, 15, 16).

To properly quantify the extent of regulation, it is necessary to accurately measure the very slow rate of the unphosphorylated proteins. It has been well documented that steady-state assays are a poor method for determining the true activity of properly regulated unphosphorylated myosins (17, 19, 21). This is because the activity of unregulated molecules is so much higher than that of the regulated ones that even a small fraction of unregulated molecules in a mixture of predominately regulated molecules can mask their low activity. The steady-state assay is sufficient to properly measure the rates of the phosphorylated myosins because their activities are high and small amounts of damaged or otherwise unregulated molecules will not significantly alter the measurement. A better method for examining regulation is to use single turnover assays where each of the HMM molecules in the preparation bind and turnover a single ATP molecule (17, 19, 21). Data for a single...
Specimens were prepared as in Ref. 8 at 22°C performed on a Joel JEM 1000 electron microscope operated at 80 kV. Gels were prepared as previously described (34). RLC plus sign is phosphorylated. pRLC is the phosphorylated RLC indicated below the respective lane; minus sign is unphosphorylated, SH muscle myosin; SM HMM (proteolytic); SH(ER) (motorless) are nonmuscle HMM IIB constructs as defined in Fig. 1.

![Fig. 3. Nondenaturing gels of constructs.](image)

Gels (10 cm × 8 cm; 0.5 mm thick) were run in the presence of sodium pyrophosphate according to Ref. 33. SMS1, tissue purified proteolytic smooth muscle myosin S1; SMHMM, tissue purified proteolytic smooth muscle myosin HMM; SMM, tissue purified smooth muscle myosin; HMM, SH(ER), SH and ER (motorless) are nonmuscle HMM IIB constructs as defined in Fig. 1.

![Fig. 4. Rotary shadowing of constructs.](image)

Electron microscopy was performed on a Joel JEM 1000 electron microscope operated at 80 kV. Specimens were prepared as in Ref. 8 at 22°C at 66% glycerol, 0.5 M KCl, 20 mM Tris, pH 8.2, 10 mM DTT, 1 mM EGTA. Representative images of HMM (a), SH(ER) (b) and (ER) (c; construct without a motor domain on either head). Bar is 50 nm.

![Fig. 5. Urea gels of constructs showing phosphorylation of the RLC.](image)

Gels were prepared as previously described (34). SMM, smooth muscle myosin; SM HMM, smooth muscle HMM (proteolytic); SH(ER) and SH, see the legend to Fig. 1. The state of phosphorylation is indicated below the respective lane; minus sign is unphosphorylated, plus sign is phosphorylated. pRLC is the phosphorylated RLC.

population of kinetically identical molecules will give a single exponential decay whereas data for a mixed population of two kinetically nonidentical molecules will better fit a biexponential decay. Therefore, unlike the steady-state technique, the single turnover technique can identify different populations of molecules within a sample.

Single turnover measurements require the use of a fluorescent ATP derivative to follow the rate of nucleotide release, which is likely to be limited by P, release in this case. We used FTP, which decreases in fluorescence upon release from the active site, and performed the measurements at 25°C in the presence of 50 mM NaCl. Table II summarizes these single turnover data in the absence of actin and at 60 μM actin for all constructs. Fig. 6A shows a representative single turnover trace for unphosphorylated HMM at 40 μM actin and its fit to a single exponential model (0.00084 s⁻¹). Under these conditions the steady-state rate was much faster at 0.016 s⁻¹. This indicates that the preparation must be a mixture of slowly cycling molecules (>95%) detected by the single turnover experiment and a smaller portion (<5%) of rapidly cycling material (~0.3 s⁻¹). Close examination of the early portions of the traces reveals a faster phase with a small (~5% or less) amplitude. However, attempts to fit these data to a double exponential yielded a spurious fit. A single exponential model was sufficient to fit traces for unphosphorylated HMM at all actin concentrations tested (Fig. 7A). A Michaelis Menten fit (not shown) to the data in Fig. 7A gave V_max = 0.00071 s⁻¹ and K_MATP = 15 ± 6 μM. The single turnover V_max = 0.00071 s⁻¹ is in marked contrast to the steady-state V_max = 0.022 s⁻¹ determined at 25°C and demonstrates that the true degree of regulation is much greater than the ~10-fold determined by the steady-state methods. The true degree of regulation can be estimated by dividing the steady-state V_max for phosphorylated HMM at 25°C (0.21 s⁻¹) by the single turnover V_max = 0.00071 s⁻¹ for unphosphorylated HMM which yields a 300-fold activation. It is clear that the extent of regulation calculated using the single turnover value for the unphosphorylated HMM (300-fold) is much greater than that estimated from steady-state methods (10-fold). This greater degree of regulation measured by the single turnover approach allows one to more carefully assess the significance of intermediate levels of regulation that could potentially be attributed to partially regulated molecules or mixtures of unregulated and regulated molecules.

FTP single turnovers for phosphorylated HMM were best fit to two exponentials (e.g., Fig. 6B; 40 μM actin; Fig. 7A) despite the fact that samples were fully phosphorylated. Two exponentials were also observed for phosphorylated smooth HMM (17). The calculated V_max (1.5 s⁻¹; Table II) was higher than the respective steady-state V_max for ATP (0.212 s⁻¹; Table I). This discrepancy was not evident for the SH(ER) or the SH constructs (compare Tables I and II), nor was it for proteolytically prepared phosphorylated smooth muscle HMM (17) or for scallop myosin (19). Thus while in most cases the two nucleotides (ATP and FTP) have similar turnover kinetics, this is not the case for phosphorylated NMIIB HMM. We obtain the same steady-state rate using an NADH-linked assay (data not shown), which rapidly converts ADP back into ATP, as we obtain from the radioactive method used to generate the data in Table I. Therefore, we have ruled out ADP inhibition of the steady-state rate of HMM (which would not occur in the transient experiments because ADP does not build up during the course of the experiment). Although other explanations are possible, the conclusions of our study remain the same.

Single turnover analyses with unphosphorylated SH(ER) yielded a turnover rate of 0.0004 s⁻¹ in the absence of actin and 0.033 s⁻¹ in the presence of 60 μM actin (see Table II). Data at all actin concentrations (Figs. 6C and 7B) were well fit by a single rate constant, and no evidence for a much slower phase (which would represent a well regulated fraction) was detected. It was not possible to obtain a V_max as good mixing at high actin concentrations is difficult in the stopped-flow spectrophotometer. In the presence of 60 μM actin, the single turnover rate (0.033 s⁻¹) was comparable with the steady-state rate for the unphosphorylated SH(ER) under the same conditions (0.038
s$^{-1}$). These values in the absence of phosphorylation can be compared with the phosphorylated steady-state value of 0.085 s$^{-1}$ at 60 μM actin ($V_{\text{max}} = 0.10$ s$^{-1}$) or the single turnover rate of 0.081 s$^{-1}$ at 60 μM actin, revealing that the SH(ER) molecule is not well regulated by phosphorylation regardless of the assay method. At this actin concentration the rate for SH(ER) is only increased 2-fold by phosphorylation, compared with 300-fold for HMM. Therefore, if we use HMM as the fully regulated example (100% regulated), SH(ER) is less than 1% regulated.

We have used truncated constructs of nonmuscle myosin IIB to address the question of whether regulation of smooth muscle myosin requires two intact heads, or whether an asymmetric HMM containing one intact head and just a regulatory domain on the other head could adopt the off position. Three previous studies have utilized a similar strategy to determine the nature of this regulation for smooth muscle myosin constructs. Interestingly all three of the smooth muscle myosin studies produced rather similar steady-state data to ours in terms of relative rates of the constructs. The actin-activated MgATPase activity of HMM was activated more than 10-fold by phosphorylation while the MgATPase activity of SH was hardly affected. The latter finding is in agreement with studies of proteolytically produced single-headed smooth muscle myosin (8, 9). In addition, all three studies produced SH(ER) or SH(R) (construct without an ELC on the truncated head). Two interesting findings were consistently found in each of these studies. The steady-state actin-activated MgATPase activities were activated 2–3-fold by phosphorylation, while the magnitude of the rates of the phosphorylated species were significantly lower than that of the phosphorylated double-headed HMM. Two of the three smooth muscle HMM studies (5, 15), and the present study of nonmuscle HMM IIB measured the kinetic constants, $V_{\text{max}}$ and $K_{\text{ATPase}}$, for the asymmetric constructs while the third examined the rates at a single actin concentration (16). The former two studies showed that the depression in the rate for the phosphorylated truncated constructs persisted even after extrapolation to infinite actin. Sweeney et al. (16) suggested that the depression of activity observed with the asymmetric constructs could be attributed to residual inhibition due to head interaction with the rod. However, this proposed mech-

### Table I

**Summary of steady-state actin-activated MgATPase data**

| Construct | Temp | RLC | $V_{\text{max}}$ | $K_{\text{ATPase}}$ | Fold activation | N$^d$ |
|-----------|------|-----|----------------|-------------------|----------------|------|
| HMM       | 25°C | P   | 0.21 ± 0.07$^d$ | 15 ± 14           | 9.6            | 5    |
|           |      | deP | 0.022 ± 0.006  | 21 ± 13           | 2              |      |
| SH(ER)    | 25°C | P   | 0.103 ± 0.009  | 33 ± 30           | 1.7            | 4    |
|           |      | deP | 0.06 ± 0.02    | 39 ± 33           | 3              |      |
| SH        | 25°C | P   | 0.094 ± 0.006  | 33 ± 4            | 10.6           | 4    |
|           |      | deP | 0.034 ± 0.005  | 10 ± 5            | 2              |      |
| SH(ER)    | 35°C | P   | 0.19 ± 0.02    | 25 ± 7            | 3              |      |
|           |      | deP | 0.07 ± 0.01    | 64 ± 24           | 3              |      |
| SH        | 35°C | P   | 0.15           | 57               | 1.1            | 1    |
|           |      | deP | 0.14           | 42               | 1              |      |

$^a$ $V_{\text{max}}$ is the maximal rate of ATP hydrolysis under steady-state conditions.

$^b$ $K_{\text{ATPase}}$ is the apparent affinity of the construct for actin in the presence of ATP.

$^c$ Fold activation is the ratio, $V_{\text{max}}$ phosphorylated/$V_{\text{max}}$ dephosphorylated.

$^d$ N, number of determinations on different protein preparations.

$^e$ P indicates phosphorylated and deP indicates without phosphorylation.

$^f$ Data were fit to the Michaelis Menten equation, rate = $(\text{actin})[V_{\text{max}}/K_{\text{ATPase}} + \text{actin}]$. Values are the mean ± S.D.

### Table II

**Summary of FTP single-turnover data**

The two data sets (from Fig. 7) from independent samples of each construct were combined and fit. For SH(ER) and SH and the slow phase of phosphorylated HMM, data were fit to an equation for a line. For unphosphorylated HMM and the fast phase of phosphorylated HMM, data were fit to the Michaelis Menten equation, rate = $(\text{actin})[V_{\text{max}}/K_{\text{ATPase}} + \text{actin}]$. From these fits, the $V_{\text{max}}$ and rates at 60 μM actin are reported (see Fig. 7).

| Construct | No actin | 60 μM actin | $V_{\text{max}}$ | No actin | 60 μM actin | $V_{\text{max}}$ |
|-----------|----------|-------------|----------------|----------|-------------|----------------|
| HMM       | 0.00035  | 0.00091     | 0.00071        | 0.00090  | 1.1$^b$     | 1.5$^c$        |
| SH(ER)    | 0.00040  | 0.033       | n.d.$^a$       | 0.00095  | 0.081       | n.d.           |
| SH        | 0.00034  | 0.066       | n.d.           | 0.0022   | 0.058       | n.d.           |

$^a$ n.d., not determined.

$^b$ The weighted average of the two phases from a biexponential fit; 80% amplitude at 1.8 s$^{-1}$.

$^c$ The weighted average of the two phases from a biexponential fit; 80% amplitude at 1.3 s$^{-1}$ and 20% amplitude at $-0.3$ s$^{-1}$. 

We conclude that the MgATPase activity of SH(ER) is unregulated and therefore reject the concept of partial regulation for this construct. Furthermore, as the turnover data could be fit to a single exponential model (Fig. 6C), there is no evidence for a slow equilibrium (interconverting at slower than or equal to the rate-limiting step) between two populations in which one is fully turned off and the other is fully turned on.

The single turnover rate for unphosphorylated SH increased from a value of 0.00034 s$^{-1}$ in the absence of actin to 0.066 s$^{-1}$ in the presence of 60 μM actin (comparable with the value of 0.067 s$^{-1}$ determined in the steady-state at 60 μM actin). Under the same conditions, the single turnover rate (0.058 s$^{-1}$) was comparable with the steady-state rate for phosphorylated SH (0.09 s$^{-1}$). Like the SH(ER), single turnover data for both the unphosphorylated (Fig. 6D) and the phosphorylated (data not shown) SH were best fit to a single exponential function. Therefore, single turnover results with SH showed no evidence for regulation, similar to the steady-state results.

**DISCUSSION**

We have used truncated constructs of nonmuscle myosin IIB to address the question of whether regulation of smooth muscle
amplitude at 0.016 s⁻¹ and 15% of amplitude at 0.025 s⁻¹; C, single exponential fit for unphosphorylated SH(R) at 36 μM actin; 0.925 ± 0.001 s⁻¹. D, single exponential fit for unphosphorylated SH at 40 μM actin; 0.050 ± 0.001 s⁻¹.

amplitude may not be correct because S1 (without any rod) also has a lower Vₘₐₓ than phosphorylated HMM (4–6, 28).

Li et al. (15) and Sweeney et al. (16) additionally measured the rate of in vitro motility of actin filaments by their various constructs. Li et al. (15) found that unphosphorylated SH(R) did not move actin filaments, whereas the phosphorylated species moved filaments at a rate about one-third of that of phosphorylated double-headed HMM. Sweeney et al. (16) found that while SH and SH(R) moved actin filaments in a phosphorylation-independent manner, the movement of actin filaments by SH(ER) was regulated by phosphorylation, in that the unphosphorylated species did not support movement of actin filaments.

The interpretations applied to these data differed dramatically in the three studies. Li et al. (15) and Konishi et al. (5) emphasized that the activation seen by phosphorylation of SH(ER) or SH(R), compared with the lack of activation of SH, implied the importance of RLC-RLC interaction for regulation. This was strongly reinforced in Li et al. (15) by the apparent regulation observed with SH(R) in the in vitro motility studies and by the fact that the actin-activated MgATPase activities of unphosphorylated HMM and unphosphorylated SH(R) were similar. In contrast, Sweeney et al. (16) pointed out that true regulation should be larger than suppression to one-half of the phosphorylated values and suggested that interactions between the two heads of the same molecule were essential to obtain the off-state. However, their in vitro motility data with SH(ER) was counter to this claim. To explain this, they suggested that there was an equilibrium between a fully off-state and fully on-state that was shifted by phosphorylation. Myosin molecules in the off-state such as unphosphorylated smooth or nonmuscle HMM, which are themselves incapable of moving actin filaments, have been shown to be able to exert a drag on activated cycling myosins in this same type of assay (29, 30). Thus, Sweeney et al. (16) rationalized that the completely turned off molecules in their preparation would act to prevent the movement of the fully on molecules in the same preparation and thus slow the observed sliding velocity. The presence of an equilibrium between completely off and fully on molecules implies that a significant portion of the molecules in this prep are capable of at least transiently adopting a fully regulated conformation without the presence of two motor domains. While these authors generally support the Wendt et al. model (13, 14) for smooth muscle myosin regulation, it is not clear how they reconcile the existence of an off configuration of the single-headed SH(ER), which they propose to exist in their equilibrium mixture.

The steady-state data using nonmuscle myosin IIB HMM-like constructs in the present study is similar in principle to that obtained by the smooth muscle studies. The steady-state MgATPase activity of double-headed HMM is activated approximately 10-fold by phosphorylation in the presence of actin (Table I). The steady-state MgATPase of the SH construct was not greatly affected by phosphorylation, whereas that of SH(ER) was activated 2–3-fold by phosphorylation (Table I). In addition, the Vₘₐₓ of phosphorylated SH and SH(ER) was slower than double-headed phosphorylated HMM. The rate of in vitro motility was not measured, because the phosphorylated nonmuscle myosin IIB HMM moves very slowly (0.1 μm/s) and any reduction in this rate would be difficult to quantify and interpret. In addition, the motility assay is an inherently poor method of assessing the degree of regulation of myosin. For example, a small fraction of active molecules can bring about movement in an otherwise well regulated unphosphorylated preparation (17). Alternatively, a small fraction of inactive, rigor-like heads can attenuate or stop the movement of a population of active cycling myosins (31).

Several studies have shown that steady-state methods typically underestimate the true degree of regulation of myosin (17, 19, 21). A small percentage of unregulated myosins with an activity equal to that of the active species can disproportionately raise the level of activity observed under the off, i.e. unphosphorylated conditions. For example, the steady-state MgATPase activity of unphosphorylated smooth muscle HMM demonstrates a hyperbolic dependence on the actin concentration with a Vₘₐₓ of 0.07 s⁻¹ head⁻¹ compared with a Vₘₐₓ of 1.8 s⁻¹ head⁻¹ when phosphorylated, giving a fold increase of about 25 (6). The single turnover rate on the same unphosphorylated HMM preparation was 0.002 s⁻¹ in the absence of actin, and this rate is not further activated by actin (21). Thus, single turnover experiments reveal that unphosphorylated smooth muscle HMM is essentially perfectly regulated by actin and that the true activation by phosphorylation is close to 1000 (1.8/0.002). These results were confirmed with a different proteolytic preparation of smooth HMM (17). It follows that the increase in activity of unphosphorylated HMM observed with increasing actin concentration in steady-state measurements actually represents titration of an unregulated portion of the preparation.

Using a single turnover approach we show that the unphosphorylated double-headed HMM does have a significantly lower Vₘₐₓ (0.00071 s⁻¹) than is seen in the steady-state (0.022 s⁻¹). In contrast, single turnover experiments with unphosphorylated SH demonstrates that the MgATPase activity is increased by increasing actin concentrations (Fig. 7C) and is quite similar to steady-state measurements made under the same conditions. Furthermore, this rate is not affected by RLC phosphorylation in either the single turnover or steady-state experiments. This implies that SH is truly unregulated and
that the steady-state measurements accurately reflect this. The important question is whether the 2–3-fold activation of the MgATPase activity of SH(ER) observed in the steady-state measurements represents regulation or merely a minor modulation of the activity. If the reason for the appreciable activity observed with unphosphorylated SH(ER) is due to a mixture of completely off and fully on molecules as suggested by Sweeney et al. (16), the single turnover data should be best fit by two disparate rates of exponential decay. If on the other hand, phosphorylation of this species merely doubles or triples the rate of the rate-limiting step of an otherwise active unphosphorylated species, then only a single exponential decay should be observed. Our data clearly show that the single turnover transients of both unphosphorylated (Fig. 6C) and phosphorylated SH(ER) (data not shown) were well fitted by single exponentials and that the rates agreed well with those determined by steady-state methods. This strongly implies that the MgATPase activity of SH(ER) is not well regulated by phosphorylation and that the unphosphorylated species cannot adopt the off-state. Furthermore, the fact that the data were fitted by a single exponential function demonstrates that, at least with the nonmuscle myosin IIB SH(ER) construct, there is not a slow (equal to or slower than rate-limiting) equilibrium between a completely off-state and a fully on-state as implied by Sweeney et al. (16) for the smooth muscle construct. 

Konishi et al. (5) and Li et al. (15) interpreted their steady-state ATPase data to conclude that the minimum requirement for regulation was two RLC molecules. Both observed that the steady-state MgATPase rates of the unphosphorylated asymmetric HMM (bearing at least at RLC on the truncated heads) were essentially the same as the rate of the unphosphorylated HMM. They reasoned that the regulation for asymmetric HMM was not as large as for HMM due to lower MgATPase rates for phosphorylated asymmetric HMMs versus phosphorylated HMM. Our results show, and other studies with unphosphorylated smooth muscle HMM (17, 21) and myosin (32) confirm, that this argument is flawed because steady-state measurements greatly overestimate (by a factor of ~30 in our hands) the true turnover rate of unphosphorylated HMM. On the other hand, based on our results, it is likely that their steady-state measurements of the asymmetric HMMs do actually reflect the true turnover rate, and it is probable that these rates are, in fact, considerably higher than the true turnover rate for unphosphorylated HMM. Based on this logic, one could conclude that their constructs were, like ours, only slightly modulated by phosphorylation and that true regulation requires the presence of two intact motor domains.

It is nonetheless interesting that phosphorylation does increase the MgATPase activity of the asymmetric HMM containing two RLCs by up to a factor of three while the activity of S1 is found to be completely independent of phosphorylation (4, 5). This would suggest that there might be an interaction specific to the asymmetric HMMs even though it may not be of great physiological relevance.

Therefore, in contrast to three previous studies using smooth muscle myosin constructs, we show that two motor domains are absolutely required to obtain the fully off-state of nonmuscle myosin IIB. The presence of two regulatory domains in a single-headed HMM is not sufficient to obtain the off-state. A recent structural model for smooth muscle myosin regulation proposed by Wendt et al. (13, 14) shows the actin-binding surface of an unphosphorylated head interacting asymmetrically with the converter portion of the motor domain of its partner head. Such a head-head interaction could not be obtained in the absence of two motor domains. Therefore, our results support this structural model, if such a motor-motor domain interaction is the molecular basis for down-regulation.

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FIG. 7. FTP single turnover rates for constructs versus actin concentration. For each construct and respective state of phosphorylation, data for two independent preparations are plotted with similar symbols. A, HMM construct. Data for thiophosphorylated HMM were fit to a double exponential model (fast rate, square symbols; slow rate, solid circles). Data for the fast phase were fit (not plotted) to the Michaelis Menten equation giving \(k_{\text{on}} = 1.8 \pm 0.2 \, \text{sec}^{-1}\) and \(K_{\text{ATP}} = 33 \pm 15 \, \mu M\). Data for the slow phase was not fitted and represented ~15–20% of the amplitude at >10 \(\mu M\) actin. Values from fits to a single exponential model are plotted for unphosphorylated HMM (triangular symbols). A Michaelis Menten fit (not plotted) gave \(k_{\text{on}} = 0.00071 \, \text{sec}^{-1}\) and \(K_{\text{ATP}} = 15 \pm 6 \, \mu M\). B, SH(ER) construct. Values from fits to a single exponential model are plotted for unphosphorylated SH(ER) (triangular symbols) and phosphorylated SH(ER) (square symbols). C, SH construct. Values from fits to a single exponential model are plotted for unphosphorylated SH (triangular symbols) and phosphorylated SH (square symbols). The final conditions for the turnovers were 25 °C in 10 mM MOPS (pH 7.0), 50 mM NaCl, 0.1 mM EGTA, 1 mM DTT, 0.6–0.8 \(\mu M\) active sites, 0.6–0.8 \(\mu M\) FTP, indicated actin, 100 \(\mu M\) ATP, 0.4 mM MgCl₂.

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Phosphorylation-dependent Regulation Is Absent in a Nonmuscle Heavy Meromyosin Construct with One Complete Head and One Head Lacking the Motor Domain

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