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Myosin isoform determines the conformational dynamics and cooperativity of actin filaments in the strongly bound actomyosin complex

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SUMMARY

We have used transient phosphorescence anisotropy (TPA) to detect the microsecond rotational dynamics of erythrosin iodoacetamide (ErIA)-labeled actin strongly bound to single-headed fragments of muscle myosin (muscle S1) and non-muscle myosin V (MV). The conformational dynamics of actin filaments in solution are markedly influenced by the isoform of bound myosin. Both myosins increase the final anisotropy of actin at sub-stoichiometric binding densities, indicating long-range, non-nearest neighbor cooperative restriction of filament rotational dynamics amplitude, but the cooperative unit is larger with MV than muscle S1. Both myosin isoforms also cooperatively affect the actin filament rotational correlation time, but with opposite effects; muscle S1 decreases rates of intrafilament torsional motion, while binding of MV increases the rates of motion. The cooperative effects on the rates of intrafilament motions correlate with the kinetics of myosin binding to actin filaments such that MV binds more rapidly, and muscle myosin more slowly, to partially decorated filaments than to bare filaments. The two isoforms also differ in their effects on the phosphorescence lifetime of the actin-bound ErIA; while muscle S1 increases the lifetime, suggesting decreased aqueous exposure of the probe, MV does not induce a significant change. We conclude that the dynamics and structure of actin in the strongly bound actomyosin complex is determined by the isoform of the bound myosin, in a manner likely to accommodate the diverse functional roles of actomyosin in muscle and non-muscle cells.

Keywords

actin; dynamics; phosphorescence; non-muscle myosin; allostery

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INTRODUCTION

Elucidation of the structure of the actomyosin complex is critical for understanding the mechanism of contractility. Crystallographic and electron microscopy studies performed on isolated muscle and non-muscle myosin isoforms have detected structural changes in myosin light chain domains and actin-binding regions in response to binding of nucleotides and nucleotide analogs, indicating that all myosins undergo structural transitions during the force-generating actomyosin ATPase cycle ^{1; 2; 3; 4; 5; 6; 7}. Structures of strongly bound actomyosin complexes, obtained by fitting crystal structures of muscle actin and the myosin head into a three-dimensional reconstruction from electron micrographs of the complex, showed remarkable similarities at the interface between actin and various myosin isoforms, such as skeletal and smooth muscle S1 ^{6; 8}, myosin V ⁵ and myosin VI ⁹, supporting a common structural basis for the mechanism of actomyosin function.

On the other hand, biochemical studies showed that the extent of actin-activated ATPase, as well as the *in vitro* sliding speed of actin filaments, strongly depends on the interacting myosin isoform ^{10; 11; 12; 13}. Kinetic studies indicate that these biochemical differences probably reflect differences in the rate limiting step of the actomyosin ATPase for different myosin isoforms. For example, the rate-limiting step for acto-MV ATPase is ADP release ^{14; 15}, while for acto-muscle S1 ATPase, the rate limiting step is a combination of ATP hydrolysis and phosphate (P_i) release ¹⁶. Consequently, MV spends most of its actomyosin ATPase cycle strongly bound to actin ¹⁴, which contributes to processive motility, while non-processive muscle S1 spends most of the ATPase cycle in the low affinity, weakly bound state and enters a strongly bound state after ATP hydrolysis and P_i release. Thus, the molecular mechanism of interaction between actin and various myosin isoforms is fine-tuned to the isoform-specific functions and properties of myosin. The goal of the present study is to determine whether the mechanism of this interaction involves isoform-specific changes in the structural dynamics of actin filaments.

We have used transient phosphorescence anisotropy (TPA) to determine the dynamics of actin in complex with the strongly-bound, nucleotide-free state of muscle S1 or a truncated, single-headed nonmuscle myosin V construct (MV). Our previous studies have established that the dynamics of actin on the microsecond time scale, as detected by TPA, is significantly affected by a variety of actin-binding proteins such as skeletal muscle myosin, gelsolin, cofilin or dystrophin ^{17; 18; 19; 20; 21; 22}. The dynamics and cooperative interactions depend on the bound protein, indicating that actin dynamics are determined by protein-specific interactions at the binding interface. The present results show that the conformational dynamics of actin filaments, and therefore the binding interface, also depend on the type of interacting myosin.

RESULTS

The effect of muscle S1 and MV on TPA of actin

Time courses of TPA decays (Figure 1) measured in KMg50 buffer and the absence of nucleotide show that binding of saturating amounts of muscle S1 and MV to ErIA-actin results in a substantial increase in the final anisotropy, indicating that both myosins decrease the amplitude of actin's rotational microsecond dynamics. The effects of the two myosin isoforms on actin dynamics were analyzed in more detail by performing measurements over a wide range of myosin concentrations (from 0.025 μ M to 0.6 μ M). Under these conditions, myosin binds actin (0.5 μ M) stoichiometrically to saturation, since the K_d is in the range of 0.004–0.03 μ M for nucleotide-free muscle S1 and MV ^{18; 23}. The interaction of these myosins with actin filaments is not significantly affected by ErIA-labeling of actin ¹⁸ (Supplementary Information).

Changes in actin dynamics resulting from an increased fraction of bound muscle S1 and MV were first analyzed according to the effects on the final anisotropy (Figure 2), defined as the average anisotropy over a 400–500 μ s time window, which is the most sensitive and direct indicator of changes in the amplitude of actin's microsecond rotational dynamics¹⁹. Fitting the changes in the final anisotropy to a one-dimensional linear lattice model (Eq. 1) shows that changes induced by the two myosins display long-range, non-nearest neighbor cooperativity. However, the extent of cooperativity, defined as the number of filament subunits (cooperative unit, N) affected by a single bound myosin, is much higher for MV ($N = 12.61 \pm 0.74$) than for muscle S1 ($N = 4.33 \pm 0.56$).

Experiments performed in the presence of ADP and absence of Mg^{2+} or in the presence of MgADP did not detect significant effect of the nucleotide on anisotropy decays of actin in the same range of bound MV concentrations (data not shown). This observation indicates that TPA does not distinguish between the documented effects of ADP- and nucleotide-free MV²³; 24.

The effects of muscle S1 and MV on actin are further compared using model-independent analysis of TPA decays (Eq. 2; Eq. 3) which yielded two more parameters of actin dynamics: the initial anisotropy and the average rotational correlation times. The initial anisotropy reflects amplitudes of sub-microsecond motions (immobilized erythrosin phosphorescent probe has an initial anisotropy of 0.205). The binding of either myosin increases the initial anisotropy of actin, indicating decreased amplitudes of these motions, and in this case both isoforms have similar effects (data not shown). Both myosin isoforms also affect the average correlation time $\langle \phi \rangle$ indicating changes in the rates of intrafilament rotational motions (Figure 3), but the changes are in the opposite directions. While binding of MV decreases the value of $\langle \phi \rangle$, binding of muscle S1 increases $\langle \phi \rangle$. These data indicate that binding of MV significantly increases the rates of intrafilament motions, while binding of muscle S1 decreases the rates. In both cases, changes in $\langle \phi \rangle$ are cooperative, but the fit to the linear lattice model showed that the extent of cooperative unit (N) depends on the myosin isoform. As observed for the final anisotropy (Figure 2), myosin V is more cooperative: $N = 5.94 \pm 2.13$ than muscle S1: $N = 1.95 \pm 0.52$. These values of N are different from those obtained by analysis of the final anisotropy (Figure 2), indicating uncoupling between molecular changes associated with amplitudes and rates of intrafilament motions.

The effect of muscle S1 and MV on the phosphorescence intensity of actin

The analysis of phosphorescence intensity decays of actin in the presence of increasing fraction of bound muscle S1 and MV show that these myosins have substantially different effects on the average phosphorescence lifetime $\langle \tau \rangle$ of actin (Figure 4). Binding of MV induces small - up to 13% - and non-cooperative changes in $\langle \tau \rangle$ of actin (Figure 4), while muscle S1 increases $\langle \tau \rangle$ by about 61% and the increase is cooperative, with N of 2.06 ± 0.17 . Since $\langle \tau \rangle$ is very sensitive to the quenching action of oxygen in solution, detected differences indicate that each myosin induces different, isoform-specific local structural changes in the environment of the probe conjugated to the C-terminus of actin.

The effect of myosin binding to actin on binding rate

The TPA results indicate that MV binding to actin filaments induces structural changes in the actin in a cooperative manner, affecting between ~6 and 13 (Figure 2 and Figure 3) actin subunits, while muscle S1 shows less cooperative changes in the actin filament. The functional effect of these structural changes on the actin filament was evaluated by measuring the rate of myosin binding to pyrene actin filaments partially decorated with varying amounts of either MV or muscle S1. The observed rate constant of binding for MV to pyrene actin increases as a function of the pre-bound MV binding density, from 62 s^{-1} for bare filaments ($k_+ = 62$

$\mu\text{M}^{-1} \text{s}^{-1}$) to a maximal value of $\sim 66 \text{s}^{-1}$ ($k_+ = 66 \mu\text{M}^{-1} \text{s}^{-1}$) when filaments are decorated at $\sim 10\%$ with pre-bound MV (Figure 5). The relative increase in the rate constant is small ($\sim 5\text{--}10\%$), but statistically significant (ANOVA, $p < 0.02$). Fitting the data to the linear lattice model indicates that the accelerated binding has a cooperativity of $N = 15.1 \pm 5.2$ subunits, in close agreement with the cooperative unit associated with intrafilament motions (Figure 3), suggesting that the acceleration of binding for subsequent MV molecules occurs because vacant filament sites have adopted the more dynamic MV-bound conformation.

In the presence of ADP, the effect of pre-bound MV on the observed binding rate constant is more pronounced than in the absence of nucleotide (Figure 5), suggesting filament dynamics are greater though they were not detected by TPA, perhaps because they occur on a slower time scale. The increase in the mole fraction of the pre-bound MV increased the rate constant by a statistically significant 30% (ANOVA, $p < 0.001$), from $\sim 40 \text{s}^{-1}$ to $\sim 50 \text{s}^{-1}$ under our experimental conditions, with $N = 3.4 \pm 1.0$.

In contrast, muscle S1 displays negative cooperativity in binding actin filaments, as reported²⁵. The observed rate constant for muscle S1 binding to pyrene actin decreases as a function of the pre-bound muscle S1 binding density (Figure 5), and the maximum effect observed at pre-bound density of $>80\%$ is a $\sim 30\%$ decrease in the observed rate constant for binding. Pre-bound muscle S1 shows less cooperativity than MV in effect on binding rate to pyrene actin with $N = 2.2 \pm 0.3$, in agreement with the TPA results. The observation of opposing effects on binding rate constants based on the myosin isoform pre-bound to actin in experiments performed in parallel eliminates the possibility that the observed effects are due to experimental artifacts and indicates that the conformational dynamics of intrafilament motions is linked to myosin binding.

Acceleration of the observed MV binding rate constant can arise from an increase in either or both of the association or dissociation rate constant. We favor an interpretation in which the more rapid observed rate constant results from a more rapid association rate constant of myosin V, since an increase in the dissociation rate constant by several orders of magnitude is required to account for the magnitude of change observed. Thus, both MV-induced and muscle S1-induced changes in actin dynamics and structure are associated with isoform-specific effects on the rate of myosin binding to vacant sites on the filament.

Comparison of the proposed regions of strong interaction of muscle S1 and MV isoforms with actin

Structure alignment of muscle S1 (PDB: 2MYS:A) and MV (PDB: 1OE9) showed 36.3% sequence homology between the two myosins, indicating substantial differences in their primary structure. We focused specifically on differences within the sequences of the proposed regions of interaction with actin: loop2, lower 50K domain, “cardiomyopathy loop”, and the secondary binding site for the neighboring actin monomer^{8, 27} (Figure 6). The most prominent difference was found in the length of loop 2, which is much longer in MV (52 residues) than in muscle S1 (35 residues). Furthermore, all three regions of the primary actin binding site (loop 2, lower 50K domain and the cardiomyopathy loop) show myosin-dependent differences in overall charge and amino acid composition. The secondary actin binding site shows difference in amino acid composition, while its length (25 residues in MV and 29 residues in muscle S1 and), as well as charge (+4 in MV and +4.2 in muscle S1) are similar. Thus, cumulative effects of differences in the regions of interaction with actin could provide major contribution to the differences in the effects of these two myosins on actin dynamics and their binding kinetics.

DISCUSSION

Actin rotational dynamics in the strongly bound complex

The principal finding of this study is that the dynamics and structure of actin in the actomyosin complex substantially depend on the kind of bound myosin isoform, muscle S1 or non-muscle MV. Cooperative increase in the final anisotropy of actin with an increasing fraction of bound myosin (Figure 2) shows that while each myosin restricted amplitudes of motions of several protomers in an actin filament, bound MV allosterically affects a significantly longer segments of the filament than muscle S1. Each myosin also affects rates of intrafilament motions as indicated by changes in the rotational correlation time, but while MV increased the rates, muscle myosin had the opposite effect, decreasing the rates (Figure 3). It is also noticeable that the extent of cooperativity for $\langle\phi\rangle$ is different than that for final anisotropy (Figure 2), indicating isoform-specific uncoupling between molecular changes associated with amplitudes and rates of intrafilament motions. That is, the increased rates of motion do not simply arise from higher amplitude fluctuations. Rather, they suggest isoform-specific changes in the intrafilament flexibility of actin²². This interpretation suggests that actin is more flexible in complex with MV than with muscle S1. This increased torsional flexibility likely contributes to the more rapid myosin binding kinetics (Figure 5).

The isoform-dependent effects on actin dynamics cannot be due to differential effects of actin-bound ErIA or phalloidin on interaction with each myosin. Our previous study found that ErIA label does not affect strong binding affinity of muscle S1¹⁸, and that phalloidin, while necessary to stabilize labeled actin from depolymerization and denaturation²⁶, does not affect functional interaction with myosin^{19; 27}. Furthermore, phalloidin-stabilized actin has been used in experiments demonstrating significant differences in the kinetics of acto-muscle S1 and acto-MV¹⁴ as well as in the processivity of MV and muscle myosin in the *in vitro* motility assay^{28; 29; 30}.

While our study was performed using skeletal muscle actin, we cannot exclude possibility that isoform-specific effects of myosins on actin dynamics will be different for non-muscle actin, which is natural partner of MV but not of muscle myosin. However, this possibility does not affect validity of our approach: our results provide structural complement to previously detected differences in function and kinetics of acto-MV and acto-muscle myosin, which were detected using skeletal muscle actin, e.g.¹⁴. In addition, the effects of actin isoform on myosin V enzymology were minimal³¹.

The observed reduction in correlation time and increased final anisotropy of actin with MV qualitatively resembles changes previously detected upon binding of the actin binding proteins, dystrophin and utrophin, which were proposed to result in an increased resistance of filaments to breaking by the contractile force of muscle²². By analogy, a decreased correlation time and increased final anisotropy of actin upon binding of MV could be involved in the mechanism of stabilizing intracellular actin during processive transport, which is much more dynamic in non-muscle cells than in the well-organized contractile apparatus of skeletal muscle.

Different effects of MV and muscle myosin could also represent a molecular adaptation of MV for transport along a filament, where a recessively moving myosin has to accommodate 36 nm steps, corresponding to actin's pseudo-helical repeat of myosin-binding regions. The number of protomers cooperatively affected by one head (Figure 2) is comparable with 11 – 15 protomers separating two heads of actin-bound MV³² suggesting that conformational changes in actin induced by one bound head could be transferred along the filament and affect the binding site for another head. This possibility is supported by the results of kinetic experiments (Figure 5 and Figure 6). The rate of MV binding was accelerated by the presence of the pre-bound MV suggesting that myosin-induced changes in actin structure and dynamics provide

a functional adaptation, affecting binding of MV to the next site during processive motility. Regulated binding of other myosin isoforms via myosin-induced structural changes in an actin filament is supported by electron microscopy and optical trap studies^{33; 34}.

Myosin-induced local changes in actin

Isoform-specific effects of myosin on the structure of actin protomers were observed in electron microscopy studies, in which 3D-reconstructions of the complexes of smooth and skeletal muscle myosins with actin revealed myosin-specific structural changes in subdomain 1 as well as in the structurally coupled subdomain 2⁶. Our current analysis of the lifetime of the actin-bound ErIA probe indicates that at least the C-terminus in subdomain 1 is differently affected by muscle S1 than by MV. A cooperative increase in the ErIA lifetime upon binding of muscle S1 indicated changes in the environment of the probe resulting in increased protection from the quenching effect of oxygen; however the effect of MV on ErIA lifetime was much less pronounced indicating significantly smaller structural changes (Figure 4). Thus, MV presumably affects the environment of Cys374, but in different manner than muscle S1. Since the changes in the state of the C-terminus of actin are associated with changes in the intermonomer contacts and structure of actin filament³⁵, possible different effects of the two myosin isoforms on this region can provide one of the explanations of different effects on the dynamics of bound actin. It is possible that the myosin-linked dissociation of stabilizing actin filament-associated ions contributes to the enhanced filament dynamics, as reported for cofilin³⁶.

Structural transitions in myosin during binding to actin

The alignment of crystal structures of muscle S1 and MV showed that despite overall similarity of the structures, the structures are not identical and isoform-specific differences between nucleotide-free myosins in solution exist, particularly in the extent of actin-binding cleft closure³. The state of the actin-binding cleft in free myosin may be crucial to the mechanism of interaction with actin, since it has been predicted that the cleft has to close to create the rigor complex with actin^{8; 37}, presumably by movement of subdomains in myosin head^{3; 6}. This prediction is supported by 3D reconstruction of cryo-electron micrographs of actomyosins, where actin-bound muscle S1 as well as MV show a closed actin-binding cleft^{5; 6}.

Isoform-specific conformational changes in muscle S1 and MV associated with actin binding are further supported by kinetic studies: while temperature-dependent binding of muscle S1 indicated different structural states before and after binding to actin³⁸, lack of temperature dependence for nucleotide-free MV binding to actin indicated that binding is limited by encounter (i.e. conformational changes after encounter are more rapid) and/or the structural state of MV catalytic domain in solution is nearly equivalent to that after binding to actin¹⁴.

We propose that isoform-specific effects on actin dynamics and structure (Figure 2–Figure 4) are associated with isoform-specific structural transitions in myosins during binding to actin. The proposed coupling of structural transitions in myosin with actin dynamics is supported by our previous TPA studies. We have detected coupling between the order-disorder transition in muscle S1 and transition in the dynamics of the interacting actin during strong-to-weak transition in the actomyosin ATPase cycle¹⁹. Furthermore, perturbation on myosin structure by oxidative modifications affected the extent of strong-to-weak transition in actin indicating that the dynamics of actin interacting with myosin depends on the structural state of myosin^{19; 26}.

Structural differences between muscle S1 and MV

(i) heavy chain—Isoform-specific structural transitions in myosin during binding to actin are probably associated with overall low (36.3%) sequence homology between the heavy

chains of the two isoforms, particularly with differences in the amino acid sequences in the proposed actin-binding regions of muscle S1 and MV (Figure 7). Most prominent differences are in loop 2, which is disordered in unbound myosin but becomes ordered in strongly bound complex^{5; 6}. The length of the loop and structural changes accompanying binding to actin were proposed as essential for the mechanism of processive movement of MV, which is not observed for muscle S1⁵. It has been suggested that net positive charge of this loop, which is about twice higher than that of muscle S1, allows MV to bind actin with high affinity, preventing its diffusion from actin and increasing processivity⁵. Crucial role of the structure of loop 2 in interaction with actin is further supported by mutational studies of Dictyostelium myosin, which have shown that charge, length and amino acid sequence affect myosin's affinity to actin¹¹. Mutational studies on Dictyostelium myosin also found that formation of actomyosin complex is substantially affected by site-specific mutations in other regions of the primary binding site, lower 50K domain and cardiomyopathy loop^{39; 40}. This suggests that differences in these regions of muscle S1 and MV (Figure 7) provide further contribution to different structural effects of the two myosin isoforms on actin.

(ii) light chains—Myosin isoforms used in this study have differences not only in the sequences of heavy chains, but also in the type of the essential light chains: muscle S1 has two isoforms of the essential light chains while MV has been coexpressed with one human essential light chain^{14; 31}. Possible role of the type of light chain in the structure of actomyosin complex is suggested by their pronounced functional effects, observed for muscle myosin. Biochemical and physiological studies showed that light chain isoforms affect calcium regulation of fiber shortening⁴¹, the extent of actin-activated ATPase, and the speed of actin filament sliding in the *in vitro* motility assay^{42; 43; 44}. Electron microscopy showed that the A1 essential chain of muscle S1 interacts with the C-terminus of actin⁴⁵ in the absence and presence of ATP⁴⁶. In the case of MV, the kind of light chain isoform was shown to affect kinetic properties of MV³¹, however its role in the structure of the actomyosin complex has not been determined.

Summary of results

We report that non-muscle MV, similar to muscle myosin S1, restricts the microsecond dynamics of strongly bound actin. However, specific parameters of actin dynamics including final anisotropy, rates of intrafilament motions as well as the environment of the actin C-terminus, are dependent on the kind of bound myosin isoform. Partial saturation of filament with myosin V allosterically affects vacant filament subunits, putting them in a "myosin bound" conformation. These non-nearest neighbor effects on filament dynamics increase the association of subsequent myosin V molecules since incoming myosin molecules can bind actin without having to introduce this change. It has been proposed that actin filaments assume multiple conformations⁴⁷ and structural changes induced by various binding proteins, including different myosin isoforms, likely reflect a shift in this conformational equilibrium towards one most suitable for interaction. We propose that such conformational flexibility of actin allows adjustment of the structure of its binding interface to the structure and function of specific myosin isoforms for optimal performance of the actomyosin complex in a variety of intracellular structures.

MATERIALS AND METHODS

Protein preparations

Skeletal muscle actin was prepared from rabbit skeletal muscle as previously described¹⁹, by extracting acetone powder in cold water, polymerizing with 30 mM KCl for 1 hr at room temperature, and centrifuging for 30 min at 350,000×g. The pellet was suspended in G-Ca buffer: 5 mM Tris pH 7.5, 0.5 mM ATP, 0.2 mM CaCl₂; pH values of all buffers are reported for 25° C.

Muscle myosin S1 (referred to as muscle S1) was obtained by α -chymotryptic digestion of rabbit skeletal muscle myosin, as described previously¹⁹.

The cDNA for chicken myosin V was truncated at the Arg792 codon, generating a construct consisting of the motor domain and the first IQ motif. The human essential light chain (LC-1sa) was subcloned into pVL1393 for baculovirus expression and co-expressed with myosin V-1IQ. Expression and purification of myosin V (referred to as MV) was performed as described previously¹⁴.

Purified myosins were stored in 50 mM KCl 0.2 mM CaCl₂, 10 mM imidazole pH 7.0, 1 mM DTT, 50% glycerol at -20°C.

Labeling of actin at Cys 374 with ErIA (AnaSpec) was performed as described previously¹⁹. Actin (48 μ M) was polymerized with 50 mM KCl in 20 mM Tris pH 7.5, ErIA, freshly dissolved in DMF, was added at a concentration of 480 μ M and the sample was incubated 2 hrs incubation at 25 °C. Labeling was stopped by adding 10 mM DTT, actin was ultracentrifuged 30 min at 350,000 $\times$ g, pellets were suspended in G-Ca-buffer, clarified by 10 min centrifugation at 300,000 $\times$ g, and actin was polymerized for 30 min at 25°C by adding 50 mM KCl or 2 mM MgCl₂. After ultracentrifugation for 1 hr at 200000 $\times$ g, pellets were suspended in K50 (50 mM KCl, 10 mM imidazole pH 7.0, 0.2 mM CaCl₂) or KMg50 buffer (50 mM KCl, 2 mM MgCl₂, 10 mM imidazole pH 7.0, 0.2 mM CaCl₂) containing 0.2 mM ATP, and the labeled F-actin was immediately stabilized against depolymerization and denaturation by adding 1 molar equivalent of phalloidin. The stabilization of actin by phalloidin was critical, since multiple control experiments showed that at pH >7.0 phalloidin-free ErIA-actin filaments are unstable and show time-dependent depolymerization and/or denaturation. The extent of labeling (mol dye/mol actin), determined by measuring absorbance of labeled actin at 538 nm and assuming molar extinction coefficient of 83000, was 0.83 ± 0.13 (mean $\pm$ SD, n=11)

The concentration of myosin's S1 and of labeled actin was measured by the Bradford protein assay⁴⁸ (BioRad) using BSA or unlabeled actin of known concentration, respectively, as a standard.

TPA experiments

Phalloidin-stabilized ErIA-F-actin was diluted in KMg50 buffer to 0.5 μ M, and acto-S1 complexes were formed by adding 0.025 – 0.6 μ M S1, as indicated in the text. To remove residual ATP from actin, all samples prior to measurement were incubated for 20 min with 0.5 unit/ml apyrase. To prevent photobleaching of the dye during TPA measurement, oxygen was removed from the sample by 5 min incubation with glucose oxidase (55 μ g/ml), catalase (36 μ g/ml), and glucose (45 μ g/ml)¹⁹.

Phosphorescence was measured at 25°C as described previously²¹ with slight modification of the TPA spectrometer. Actin-bound ErIA was excited with a vertically polarized 1.2-ns pulse from a FDSS 532-150 laser (CryLas) at 532 nm, operating at a repetition rate of 100 Hz. Phosphorescence emission was selected by a 670 nm glass cut-off filter (Corion), detected by a photomultiplier (R928, Hamamatsu), and digitized by a transient digitizer (CompuScope 14100, GaGe) at a time resolution of 1 μ s/channel. The time-resolved phosphorescence anisotropy decay was calculated as $r(t) = [I_v(t) - GI_h(t)]/[I_v(t) + 2GI_h(t)]$, where $I_v(t)$ and $I_h(t)$ are vertically and horizontally polarized components of the emission signal, detected at 90° with a single detector and a Polaroid sheet polarizer that alternated between the two orientations every 500 laser pulses. G is an instrumental correction factor, determined by performing the measurement with horizontally polarized excitation, for which the corrected anisotropy value is set to zero. The time-dependent anisotropy decays of free actin and its

complexes with myosin S1 were obtained by recording 30 cycles of 1000 pulses (500 in each orientation of the polarizer).

TPA Data analysis

Final anisotropy was defined as the average value of r in the time window from 400 to 500 μ s, which has been shown previously to provide the most sensitive and precise measurement of actin's microsecond rotational dynamics¹⁷. The effects of strongly bound S1 on the final anisotropy of actin was analyzed in terms of the linear lattice model as in our previous work^{17; 18}, assuming that perturbation of one protomer in an actin filament affects a segment containing N protomers:

$$r = r_{\max} - (r_{\max} - r_{\text{actin}})(1 - x)^N \quad \text{Eq. 1}$$

where r_{actin} and $r_{\max}$ are the limiting values of anisotropy at 0 and infinite concentrations S1, and $x = [\text{S1}]/[\text{actin}]$. The adjustable parameters in the least-squares fit were $r_{\max}$ and N .

Other parameters of TPA decay (initial anisotropy r_0 and correlation times ϕ_1 and ϕ_2) were determined by fitting the decay to the double exponential and a constant r_{∞} :

$$r(t) = r_1 \exp(-t/\phi_1) + r_2 \exp(-t/\phi_2) + r_{\infty} \quad \text{Eq. 2}$$

This method of analysis was established previously^{17; 18; 49} and was validated by comparing residuals and χ^2 for fits: fits of the current data were optimal for $n=2$ with residuals not exceeding 2% of the maximum anisotropy. For model-independent comparison of the effects of used myosin isoforms, the component lifetimes and amplitudes of the decay were used to calculate a single average correlation time $\langle\phi\rangle$:

$$\langle\phi\rangle = (\phi_1 r_1 + \phi_2 r_2) / (r_1 + r_2) \quad \text{Eq. 3}$$

The phosphorescence intensity decays of actin-bound ErIA $I(t) = (I_v(t) + 2 * GI_h(t)) / 3$ were best fitted to three exponential terms:

$$I(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + a_3 \exp(-t/\tau_3) \quad \text{Eq. 4}$$

The amplitudes a_i and the triplet excited lifetimes τ_i were used to calculate a single average lifetime τ :

$$I(t) = (a_1 \tau_1 + a_2 \tau_2 + a_3 \tau_3) / (a_1 + a_2 + a_3) \quad \text{Eq. 5}$$

Transient kinetic fluorescence measurements

Transient kinetic measurements of MV binding to pyrene actin filaments was performed in KMg50 buffer (50 mM KCl, 2 mM MgCl₂, 1 mM EGTA, 1 mM DTT, and 10 mM imidazole, pH 7.0) using an Applied Photophysics SX.18MV-R stopped flow apparatus thermostated at 25 $\pm$ 0.1°C. Actin was labeled with pyrene iodoacetamide as described previously¹⁴. 100 nM pyrene actin stabilized with equimolar amount of phalloidin was preincubated with increasing concentrations of MV up to 1.2 mol MV/mol actin in the presence of 0.1 U/mL apyrase (no nucleotide) or 20 μ M MgADP (+ADP). After addition of 1 μ M MV, the time course of fluorescence quenching of pyrene actin ($\lambda_{\text{ex}} = 366$ nm) was monitored at 90° through a 400-

nm, long-pass colored glass filter. Typically two to four time courses were averaged then fitted to a sum of exponential terms using OriginPro 8.0. Errors are reported as standard errors of the fit. Data in Figure 5 is fit to the linear lattice model presented in Eq. 1, with r_{actin} and r_{max} representing observed rate constants at 0 and infinite concentrations of S1, respectively.

Steady state ATPase

The actin-activated steady state ATPase of myosin was determined using the NADH-coupled assay^{14; 15}.

Statistical analysis of data

Each result is reported as mean $\pm$ SEM, unless indicated otherwise. The statistical significance of differences was evaluated by one-way ANOVA, with Tukey post-hoc tests performed for multiple comparisons. Differences were considered significant for $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

The effect of muscle S1 and MV on TPA decays of actin in the absence of nucleotide. S1/A=1.2, KMg50, 0.5 U/ml apyrase. Smooth lines represent fits to the sum of two exponential terms (Eq. 2).

Fig. 2.
The effect of increasing fraction of strongly bound muscle S1 and MV on the final anisotropy of actin.

Fig. 3.
The effect of increasing fraction of strongly bound muscle S1 and MV on the average correlation time $\langle \phi \rangle$ of actin.

Fig. 4.

The effect of strongly bound muscle S1 and MV on the average phosphorescence lifetime of actin-conjugated ErIA. The $\langle\tau\rangle$ in the presence of muscle S1 is fitted to the linear lattice model Eq. 1, and $\langle\tau\rangle$ in the presence of MV is represented by the linear fit for visualization.

Fig. 5.

The effect of the pre-bound S1 binding density on the relative k_{on} of myosin binding to pyrene actin for muscle S1 (squares) or MV in the absence of nucleotide (filled circles) or MV in the presence of 20 μM MgADP (empty circles). Data points represent fits to averages of 2–4 individual time courses at indicated pre-bound S1 density. The concentration dependence is fitted to the linear lattice model (Eq. 1). Uncertainties represent errors in the fit.

Loop2

MV	F594-L645	FQDEEKAI SPTSATPSGRVPLSRT	
MS1	F624-L658	FATYGGEAEGGGGKKGGKKKGSSF	
MV		PVKPAKARPGQTSKEHKKT VGHQFRNSL	(+6.2)
MS1		-----QTVSALFRENL	(+3.0)

Lower 50K domain

MV	L499-L533	LIEAKMGVLDLLDEECKMPKGSDDTWAQKLYNTHL	(-2.9)
MS1	L524-L560	LIEXPMGIFSI LEEECMF PKATDTSFXNXLYDEHL	(-5.9)

Cardiomyopathy loop

MV	M372-I392	MAHWL--CHRKLATATET YIKPI	(+2.1)
MS1	L399-G417	LLKALCYPRVG VGN---EAVTXG	(+1.0)

Secondary actin binding site

MV	N534-V357	N-KCALFEKPRL---SNKAFI IKHFADKV	(+4.0)
MS1	G561-V589	GKSNNFQKPKPAKGKAEAHFSLVHYAGTV	(+4.2)

Fig. 6.

Structural alignment of the proposed regions of interaction of muscle S1 (MS1) and MV with actin. Numbers in parentheses show charges of the peptides calculated using Innovagen's Peptide Property Calculator program.