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Kinetic and Motor Functions Mediated by Distinct Regions of the Regulatory Light Chain of Smooth Muscle Myosin^{1,2}

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Abstract

To understand the importance of selected regions of the regulatory light chain (RLC) for phosphorylation-dependent regulation of smooth muscle myosin (SMM), we expressed three heavy meromyosins (HMMs) containing the following RLC mutants; K12E in a critical region of the phosphorylation domain, GTDP⁹⁵⁻⁹⁸/AAAA in the central hinge, and R160C a putative binding residue for phosphorylated S19. Single-turnover actin-activated Mg²⁺-ATPase ($V_{\max}$ and K_{atpase}) and in vitro actin sliding velocities were examined for both unphosphorylated (up-) and phosphorylated (p-) states. Turnover rates for the upstate (0.007–0.030 s⁻¹) and velocities (no motion) for all constructs were not significantly different from the up-wild type (WT) indicating that they were completely turned off. The apparent binding constants for actin in the presence of ATP (K_{atpase}) were too weak to measure as expected for fully regulated constructs. For p-HMM containing GTDP/AAAA, we found that both ATPase and motility were normal. The data suggest that the native sequence in the central hinge between the two lobes of the RLC is not required for turning the HMM off and on both kinetically and mechanically. For p-HMM containing R160C, all parameters were normal, suggesting that R160C is not involved in coordination of the phosphorylated S19. For p-HMM containing K12E, the $V_{\max}$ was 64% and actin sliding velocity was ~50% of WT, suggesting that K12 is an important residue for the ability to sense or to promote the conformational changes required for kinetic and mechanical activation.

Keywords

Myosin regulatory light chain phosphorylation; myosin light chain kinase; smooth muscle myosin; actin-activated ATPase activity; smooth muscle; regulation; in vitro motility

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²Abbreviations: RLC or smRLC, regulatory light chain from smooth muscle; skRLC from skeletal muscle myosin; HMM, heavy meromyosin; up-HMM, unphosphorylated HMM; p-HMM, phosphorylated or thiophosphorylated HMM; SMM, smooth muscle myosin; HC, heavy chain; ELC, essential light chain; S1, subfragment 1 of myosin; S2, subfragment 2 of myosin; WT, wild type; ATPγS, adenosine 5'-O-(thiotriphosphate); MLCK, myosin light chain kinase; Mant-ATP, 2-(3)-O-(N-methylanthraniloyl)-ATP.

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1. Introduction

The actin-activated MgATPase and motor properties of SMM and its C-terminally truncated subfragment, HMM, are activated by phosphorylation of the RLC at Ser19 by the Ca^{2+} /calmodulin-dependent myosin light chain kinase [1]. The RLC is a calmodulin-like structure that binds largely through hydrophobic interactions with the heavy chain helix. The N-lobe of the RLC (excluding the N-terminal domain see below) binds to a prominent bend in the heavy chain at the head-tail junction and the C-lobe binds further toward the heavy chain N-terminus (toward the motor domain). Phosphorylation of the RLC on each of the two head domains is sufficient to activate the ATPase by ~ 1000 fold [2,3] and to enable the molecule to move actin filaments, whereas the unphosphorylated state is inactivated in both respects. Since the RLC is located at the head-rod junction [4], it is unlikely that its phosphorylation site, which remains crystallographically undetermined, can interact directly with the ATP or actin binding sites more than ~ 10 nm away. The mechanism by which phosphorylation of S19 accelerates the rate of phosphate release from the active site [3], thus activating the ATPase, remains unknown.

The RLC has been the focus of numerous previous mutagenesis studies related to phosphorylation-dependent regulation of SMM [5-11]. Several unifying themes have emerged. Coupling of the ATPase to movement is mediated by the RLC [9]. The ATPase activity of RLC-deficient SMM is much higher than unmodified up-SMM. However, RLC-deficient SMM cannot move actin in an in vitro motility assay. With 26 residues truncated from the C-terminus of the RLC, the molecule behaves exactly like the LC deficient myosin regardless of the state of phosphorylation [9]. Without these residues, the dephosphorylated form of the protein has a high ATPase activity both in the absence and presence of actin. Therefore, the C-terminal lobe of the RLC is involved in interactions that regulate myosin's on-off switch, both in terms of inhibition and activation. Although the C-terminal domain is clearly critical for regulation of the ATPase and motor function, there have been no site-directed mutagenesis studies addressing the role of specific residues. Many of the studies were done with deletions, which severely weaken the binding of the RLC to the heavy chain, raising the possibility for global changes in structure.

The extreme N-terminal region of the RLC containing the phosphorylated Ser-19 is critical for regulation [5,7]. Sequential deletion of N-terminal residues 1-16 from this phosphorylation domain incrementally increases the unphosphorylated and decreases the phosphorylated actin-activated ATPase activities, whereas activities remain relatively low in the absence of actin. This behavior might be expected if the entire region provides stabilization energy toward the switched-off and switched-on states, respectively. The phosphorylation domain can sense the nucleotide state of the active site but only in the context of a regulated construct, i.e. double-headed HMM, but not in the unregulated S1 head construct [12]. Unfortunately, no electron density has been observed for the first 24 amino acids of the RLC in any of the myosin X-ray structures. A region of the domain, $^4\text{RAKAKTTKKRPQR}^{16}$, can be cross-linked to Cys-108 in the C-terminal lobe of the other head in the up-state, but not in the p-state [13]. These residues lie within a region that probably constitutes an independent flexible domain [14,15]. It is possible that the phosphorylated Ser-19 coordinates with a residue(s) in this domain but this has not been determined. Although this important domain has been extensively studied, nothing is known about its effects upon the motor function.

Previous work has shown that another important functional region of the RLC is the central linker between the N- and C-lobes. It has been proposed that a putative hinge region between the helices (D and E) that connects the two lobes of the RLC, Gly⁹⁵-Pro⁹⁸, plays a significant role in phosphorylation-dependent regulation [16]. However, certain questions remain unanswered because most mutants were again deletions. Indeed the effect of these deletions

on the motor properties were similar to the above-mentioned RLC-deficient SMM, that is the mechanochemical coupling of the ATPase to motility was broken. This raises the possibility that the phenotype was due to improper binding of the RLC to the HC, and not to the specific importance of the hinge itself. The one mutation that was a substitution gave motility and ATPase characteristics that are difficult to reconcile considering the known parameters that control actin sliding velocity (see Discussion).

The present study represents initial work to address the above-mentioned deficiencies and uncertainties in our understanding of the role of the C-terminal, N-terminal, and central hinge domains of the RLC with respect to regulation by phosphorylation. Although prior studies in this area represent founding and significant work, many questions remain because of factors that make study-to-study comparisons ambiguous. For example, studies were done with different protein preparations. Those include full length tissue-purified SMM, tissue purified HMM and expressed HMM. ATPase assays were performed under different conditions and some work was completed prior to the common use of the in vitro motility assay to assess motor function. Importantly, ATPase assay results were often reported for only one actin concentration that was well below the $V_{\max}$ raising concerns over K_m (K_{ATPase}) effects. Usually, the steady-state method of assay was used which can overestimate the ATPase activity of the inhibited state [3]. In earlier reports, the heat-mediated [6] or trifluoroperazine-mediated [9] RLC exchange procedures were used to reconstitute mutant RLC with the heavy chain/ELC, which often resulted in mixtures of molecules with different RLC subunits. Also, many mutants severely weakened the binding affinity of the RLC for the heavy chain. These two latter problems often dictated that ATPase activities be adjusted by calculation. More recent work addressing other portions of the SMM molecule, for example the rod and motor domains, have successfully expressed HMM-like constructs using the baculovirus expression system [17-19], thus avoiding many of the aforementioned problems. We have used that approach here. Also, we have used the single-turnover method of ATPase assay and determined the $V_{\max}$ and K_{ATPase} for each construct, in addition to assessing motor function by the in vitro motility assay.

We have expressed selected smooth muscle HMM constructs containing RLC mutations in the 3 critical domains discussed above, some of which had been previously investigated, that we felt merited further study for reasons outlined above. K12 in the N-terminal phosphorylation domain is of interest due to recent modeling work showing that this residue lies in a critical location within the phosphorylation domain that undergoes a large conformational change upon phosphorylation [14,15]. The GTDP/AAAA mutation in the central hinge is of interest due to the above-mentioned unusual properties, allowing for robust motility with low ATPase. R160 in the C-terminal lobe is of interest due to previous work suggesting that it may coordinate the negative charges of phosphorylated Ser-19. The motor properties and actin-activated Mg^{2+} -ATPase activity of the mutants were evaluated by motility and transient kinetic single turnover of Mant-ATP, respectively. We found that the actin sliding velocity and ATPase kinetics of HMM with the R160C and GTDP/AAAA mutations on the RLC are similar to WT in both up- and p-states, suggesting that the putative phospho-binding residue, Arg¹⁶⁰, is not important for phosphate coordination, and the Gly⁹⁵-Pro⁹⁸ native sequence constituting the hinge region between the N- and C-lobes of the RLC is not required for phosphorylation-dependent regulation of SMM. In addition, we show that in the p-state a K12E mutation modestly inhibits the actin-activated ATPase and the actin-sliding motor activity, suggesting that the negative charge may destabilize but not prevent the proposed phosphorylation-dependent ordering of the phosphorylation domain.

2. Materials and Methods

2.1. Reagents

Restriction enzymes, calmodulin, Mant-ATP, and ATP γ S were purchased from New England Biolabs (MA), Sigma, Molecular Probes (Eugene, OR), and Roche Molecular Biochemicals, respectively. Actin was purified from frozen rabbit skeletal muscle acetone powder [20]. MLCK was prepared from frozen chicken gizzard smooth muscle [21], except that Superdex 200 (GE Healthcare) was used for gel filtration and Super Q (Tosohass) was used for ion exchange.

2.2. Constructs of HMM heavy chain, RLC, and ELC

Chicken gizzard SMM heavy chain DNA coding for residues 1-1106 (CAA29793 following correction P10587) contains 6 histidine amino acid residues (His₆) at the C-terminus for protein purification. The RLC DNA (CAA29684) was cloned into the pFastBac vector [22]. The RLC R160C construct was obtained using a site directed mutagenesis kit (Stratagene, CA). RLC mutants K12E and GTDP/AAAA were obtained by multi-PCR. The RLC was subcloned into the pFastBac vector (Invitrogen, CA) with Bam H1/E.coR1 at 5'- or 3'-sites, respectively. The original chicken gizzard ELC DNA (AAA48978) was in the PAcC4 vector [19]. The ELC DNA was obtained by PCR and subcloned into the pFastBac vector with E.coR1/Hind III at 5'- or 3'-sites, respectively. The sequences of all of the constructs were confirmed by sequencing.

2.3. Expression and purification of HMM

All constructs were expressed in a baculovirus system by co-infecting Sf9 cells (Invitrogen) with three separate recombinant baculoviruses; WT heavy chain, WT or mutant RLC, and WT ELC. Infected Sf9 insect cells were incubated at 28°C in flasks (SARSTEDT, 175cm²) for 3 days. The following steps were performed at 4°C. The cells were collected by centrifugation at 1500 rpm for 5 min and either frozen or processed immediately. The cell pellets were resuspended in lysis buffer (0.3 M NaCl, 0.2 mM EGTA, 5 mM MgCl₂, 5 mM ATP, 0.05% Triton X-100, 0.2 mM PMSF, 10 µg/ml leupeptin, 0.1% 2-mercaptoethanol, 30 mM Tris-HCl, pH 7.5) and sonicated for 30 sec. The lysed cells were centrifuged at 18900 g for 20 min, and the supernatant was rotated with Ni-NTA Agarose (Qiagen) in conical tubes for 1 hr. The resin suspension was collected by centrifugation at less than 100 g for 5 min and the beads were washed with wash buffer containing 0.3 M NaCl, 0.1 mM EGTA, 5 mM MgCl₂, 25 mM imidazole, 1 mM DTT, 1 µg/ml leupeptin, and 50 mM Tris-HCl pH 7.5. After washing, the beads were loaded onto a column (BioRad, 0.8 × 4 cm) and the proteins were eluted by gravity with elution buffer containing 0.2 M imidazole, 50 mM NaCl, 0.1 mM EGTA, 5 mM MgCl₂, 1 mM DTT, and 1 µg/ml leupeptin and 10 mM MOPS, pH 7.0. ATP was removed by a Hi-trap desalting column (GE Healthcare) equilibrated in 50 mM NaCl, 0.1 mM EGTA, 5 mM MgCl₂, 1 mM DTT, 1 µg/ml leupeptin and 10 mM MOPS, pH 7.0. The protein concentration was determined using the extinction coefficient (1 mg/ml; 280 nm): HMM, 0.62. All the protein samples were used within two weeks and were always kept on ice.

2.4. Actin-activated single turnover of Mant-ATP

HMMs were assayed at 25 °C as described [2,23] to accurately measure the low ATPase activities of the unphosphorylated state. The excitation wavelength was 365 nm, and the excitation bandwidth was 1.8 nm. Emitted light was collected through a filter (KV299, Schott in the presence of actin and KV289 in the absence of actin). The experiments were done with a Hi-tech stopped-flow spectrophotometer with two syringes and the mixing ratio was 1:1. All concentrations stated in the figures are after mixing. The first syringe contained various concentrations of F-actin in 50 mM NaCl, 0.2 M ATP, 1 mM MgCl₂, 1 mM DTT and 10 mM

MOPS, pH 7.0. MantATP (1 μ M final) and $MgCl_2$ (2 μ M final) were rapidly mixed by hand with 1 μ M HMM heads in HMM buffer (50 mM NaCl, 0.1 mM EGTA, 1 mM DTT, and 10 mM MOPS, pH 7.0) in a 9 well plate and the mixture was loaded into the second syringe. Shots were taken after maximal nucleotide binding was reached (indicated by maximal fluorescence, measured in an independent experiment, not shown). Data was analyzed with Kinetic Studio software (Hi-tech).

2.5. In Vitro Motility Assays

In vitro motility assays were performed using a Nikon TE2000 epifluorescence microscope and a Roper Cascade 512B (Princeton Instruments, Trenton, NJ) camera. F-actin was labeled by TRITC- phalloidin (Sigma) immediately prior to use. HMM mutants were mixed with an equimolar amount of unlabeled F-actin (2mM ATP in HMM buffer), and clarified at 321,000 g for 20 min at 4°C to eliminate HMM heads that irreversibly bound to F-actin. The supernatants were collected for the motility assay. The procedure [24] was performed at room temperature and modified as follows. HMM (0.5 mg/ml) was applied to a nitrocellulose-coated glass flow cell and incubated for 1 min. The flow cells were washed with HMM buffer twice, treated with 0.5% BSA twice in actin buffer (50 mM KCl, 40 mM HEPES, 6 mM $MgCl_2$, pH 7.4) for 1 min, and 10 nM TRITC-actin twice for 1 min. The flow cells were washed with 40 μ l of actin buffer twice followed by 40 μ l of motility buffer containing 25mM KCl, 20mM HEPES (pH 7.0), 3mM $MgCl_2$, 10mM DTT, 2mM ATP, 0.5% methylcellulose, and oxygen scavenger containing 29% glucose, 281 unit/ml glucose oxidase and 4,207 unit/ml catalase prior to observing motion and recording videos. At least 2 flow cells were analyzed. For each flow cell, 3-6 movies were recorded for 30 sec, each at different slide fields. Data obtained from these 2 slides $\times$ 3-6 fields/slide constituted one ($n = 1$) experiment. Moving filament velocities were manually collected and the mean velocity and standard deviation was calculated from 4-8 moving filaments per field (minimum of 24-96 total filaments). Not all filaments moved, but better movement was obtained at higher myosin concentrations, lower ionic strengths, and without imidazole buffer. There were no statistical differences between the mutants with regard to number of moving filaments (data not shown). K12E differed from the other constructs only in the velocities of the moving filaments. Movement did not appear to be improved by eliminating HMM heads that irreversibly bound to F-actin (described above). Attempts to use an anti-histag antibody to attach the HMM to the surface gave poor motion. For each determination, tissue-purified skeletal myosin or phosphorylated SMM are also assayed to ensure that all reagents were viable. All mutants were prepared independently at least twice ($n=2$) and the average and standard deviation in Table 1 were calculated from these two determinations. We used Simple PCI tracking software (Compix, Sewickley, PA) to obtain actin-sliding velocities. Objects were defined by applying an exclusionary area threshold to minimize background noise. Intersect filters were applied to exclude intersecting filaments.

2.6 Phosphorylation of HMM

HMM was thiophosphorylated in 10 mM MOPS, pH 7.0, 50 mM NaCl, 5 mM $CaCl_2$, 0.2 mM ATP, 0.05 mM EGTA, 5 μ g/ml MLCK, 5 μ g/ml CaM, 4 mM $MgCl_2$ and 1 mM $ATP\gamma S$ on ice overnight. RLC phosphorylation levels we assayed using urea gel electrophoresis [12].

3. Results

HMMs were expressed in Sf9 cells by co-infection with viruses to express residues 1-1106 of the heavy chain with a C-terminal His-tag, wild type ELC, and WT-, K12E-, GTDP/AAAA- or R160C-RLC. Figure 1 shows a homology model of the regulatory domain of SMM indicating the mutated residues on the RLC. The expressed HMMs were purified by Ni-NTA chromatography in a single step. All HMM constructs showed similar ratios of subunits as determined by staining on an SDS gel (Fig. 2A). These data are consistent with previous work

on SMM [25] and HMM [2] purified from tissue, showing that there is normal binding of all RLC mutants to the heavy chain. All experiments were performed at least twice from independently expressed and purified batches of protein.

HMM was thiophosphorylated using ATP γ S in the presence of MLCK/Ca²⁺-CaM, under conditions in which only S19 of the RLC is phosphorylated [26,27]. The Coomassie-stained urea-PAGE gel in Fig. 2B shows that all samples showed prominent gel shifts upon phosphorylation as expected [12,28], consistent with essentially complete phosphorylation.

To evaluate the effects of the RLC mutations on phosphorylation-dependent regulation we measured the actin-activated single-turnover rate of MantATP in both the up- (Figure 4A) and p-states (Figure 3 and 4B) as a function of actin concentration. Actin stimulates myosin's turnover rate by inducing structural changes that are associated with the transition from a weak actin binding state to a strong actin binding state. During this process, inorganic phosphate (Pi) is released from the myosin active site.

The observed transients in the up-state fit well to a single exponential. The data for all the up-HMM constructs were not significantly different and showed that they were completely inhibited. Data are very similar to that for tissue-purified up-HMM and up-SMM [2,3,12,27, 29]. It is not possible to calculate a $V_{\max}$ (rate at saturating actin) and K_{atpase} (the actin concentration at half-maximal ATPase) from these data, as the actin binding is too weak as expected for a well-regulated (turned off) preparation, consistent with the presence of few unregulated heads. The motility data (Table 1) show no movement for all constructs in the up-state, also consistent with fully turned off behavior. Therefore, the data in Figure 4A and Table 1 suggest that the up-WT construct is fully turned off and that the RLC mutations do not significantly alter the turnover kinetics or motility in the up-state.

The observed transients for the p-states for all mutants fit well to a double exponential model as found previously for tissue-purified HMM [2] using a different nucleotide, formycin triphosphate. Figure 3 shows the dependence upon actin concentration of the two rates for p-WT-HMM. All mutant p-HMM samples behaved in a similar manner with respect to the presence of two rates (Table 1). Figure 4B shows the weighted average of the two rates. The $V_{\max}$ and K_{atpase} for the fit to the weighted average, to the fast phase only, and to the slow phase only, are in Table 1. As expected, the $V_{\max}$ for the p-WT-HMM was elevated about 130 fold over the up-state, consistent with prior studies [2,3]. The K_{atpase} of the expressed p-WT-HMM ($32 \pm 21 \mu\text{M}$) is consistent with the value measured for native HMM ($38 \mu\text{M}$; [30]). The ATPase parameters and motility function of the p-R160C and p-GTDP/AAAA RLC mutations were not significantly different from p-WT.

The only mutant with behavior significantly different from WT was K12E. $V_{\max}$ for the p-HMM with the K12E mutation was 64% of p-WT-HMM and the actin-gliding velocity was 50% of p-WT. These data suggest that this mutant is compromised in the ability to undergo the weak actin binding (Acto-myosin-ADP.Pi) to strong (Acto-myosin-ADP + Pi) actin binding transition [31] by about 50%. In general, the ATPase and the motility data suggest that the R160C and GTDP/AAAA mutations had little effect whereas the K12E mutation caused the molecule to partially lose the ability to sense or to promote the conformational changes required for kinetic and mechanical activation.

4. Discussion

4.1 General observations

For all mutants and WT HMM, we observed a transient in the MantATP turnovers that fit to a double exponential model. Both extracted rates were dependent upon actin concentration,

and the dependence fit the Michaelis-Menton equation. At this time we do not know the molecular basis for the double exponential decay of the Mant-ADP signal. The assay monitors the release of Mant-ADP from the active site. Active site bound Mant-ADP has a higher fluorescence than free Mant-ADP. Since it is known that Pi release is the rate-limiting step in the ATPase cycle, our assay will report Pi release rates. We considered that one of the two heads was not binding to actin or was binding extremely weakly, but this does not appear to be consistent with the data. The slower rate indeed saturates at higher actin concentrations with similar K_{ATPase} to the fast rate (Fig. 3). In addition we see double exponentials for the single headed construct, S1 (data not shown). The behavior is not attributed to the 2'- and 3'-isomers of MantATP because we see similar behavior for formycin triphosphate, which is not a mixture of isomers [2]. Further kinetic studies will be required to determine the basis for the observations.

4.2. R160C

The results from two previous studies have given clues that R160 might be an important residue to stabilize the on-state. This prediction was made with suggestive but not conclusive data, giving us reason to do a definitive experiment. In the first study [8], a sequence VEFTR¹⁶⁰ILK from the smRLC was replaced with the sequence, KNICY¹⁶⁰VIT from the skRLC (CEX2 in the publication), after noting the lack of sequence conservation in this region. The authors found that the p-mutant was not activated by actin, as measured by a steady-state ATPase assay. The same phenotype was observed for a N-lobe_{smooth}/C-lobe_{skeletal} construct [6], consistent with the idea that the C-lobe is critical for activation by phosphorylation. Amongst the residues substituted, R160 to Y was the least conservative. In a second study, the authors attempted to delineate the structural elements within the C-lobe that are absent in the skRLC and are necessary for regulation [10]. They replaced 6 residues that are conserved in regulated myosin RLCs, but not in nonregulated RLCs, among them was R160, in the background of the skRLC. They found that there was a partial return of regulation, i.e. the inhibited activity of the mutated phosphorylated skRLC attached to the smooth HC, was increased. However, further mutagenesis to determine which of the 6 residues to be the most important was not reported. The authors noted that the positive charge on R often coordinates to the negative charge on phosphate groups. As part of our ongoing studies to detect conformational changes in myosin related to phosphorylation-dependent regulation, we decided that it was important to test whether or not the positive charge on R160 was required for activation of the myosin ATPase and motility. We mutated the R to a C to be used in future studies as an attachment site for photocross-linkers, should the residue prove to be required.

Our results clearly demonstrate that the positive charge of R160 in the H-helix of the C-terminal lobe is not important to either mechanical or kinetic activation upon phosphorylation. If it were playing such a role, we would have expected the ATPase and motility of the p-R160C mutant to be diminished. Instead, we found that these characteristics of the mutant were essentially identical to the WT construct. In the model in Figure 1, R160 interacts with Y832 on the HC and E156 (H-bonding) on the RLC. Our results suggest that these interactions do not appear to be critical for regulation. The fact that R160 is occupied through these interactions might explain why it is not available to coordinate the phosphorylated S19. Our finding suggests that the loss of actin-activated ATPase of the V EFT¹⁶⁰ILK to KNICY¹⁶⁰VIT mutant [8] was possibly due to: i) residues other than R160, or ii) a change in the global structure of the C-lobe of the RLC, consistent with the fact that the 8 residue mutation weakened the binding affinity of the RLC to the heavy chain, or iii) that fact that their study mutated R160 to a Y instead of a C (our work). With regard to the study reporting the simultaneous effects of 6 residues [10], we are in the process of mutating the other 5 residues one at a time to determine which are most important, if any.

4.3. GTDP/AAAA

It has been proposed that the hinge region between the helices (D and E) that connects the two lobes of the RLC, Gly⁹⁵-Pro⁹⁸, plays a significant role in phosphorylation-dependent regulation [16]. The p-state of SMM with an RLC containing a deletion of the 4 hinge residues (p-ΔGTD) showed no measurable actin motility in an in vitro motility assay, in contrast to the normal motion observed for the p-state of the WT [16]. The intrinsic motor of ΔGTD was not compromised because the S1 construct had normal motion. The motility of the up-state for ΔGTD was normal, that is zero. The actin-activated ATPase activity of up-ΔGTD was elevated about 3-fold above WT and the p-ΔGTD was inhibited by about a factor of 2.4 relative to WT. Therefore this hinge deletion caused a commonly-observed phenotype for the ATPase activity of RLC mutations in that the up-state is more active and the p-state is less active than the WT. However, the mechanochemical coupling of the ATPase to motility was broken. In this regard, ΔGTD behaves similarly to RLC-deficient SMM [9] and SMM with 26 residues truncated from the C-terminus of the RLC (weakly binds to the HC). This raises the question of whether or not the ΔGTD truncated RLC binds normally to the heavy chain. If not, abnormal HC binding could explain the mutant phenotype, rather than the specific importance of the hinge itself.

In further work, the same authors [16] lessened the potential problem of HC binding and prepared a 4 amino acid substituted RLC (GTD/AAAA), which had a unique phenotype not previously observed. Even though the actin-activated ATPase in the up-state was almost completely normal (that is low), the sliding velocity of actin filaments was activated in the up-state, to about 1/3 of the p-state (0.23 vs. 0.64 μm/s). This suggested that the inhibitory effect of the up-RLC on the motor activity but not the ATPase activity is diminished by mutation at the hinge. The implications of this finding are that δ/r would have to be increased, since actin sliding velocity = $(\delta/r)ATPase$, where δ = working stroke distance and r = duty ratio (the fraction of time that myosin is strongly bound to actin during the ATPase cycle) [32]. We assume that δ could not be increased without changing the lever arm length [33]. Using the above equation and the values reported, r for up-GTD/AAAA HMM would have to be ~10 fold lower than r for p-WT HMM or p-GTD/AAAA and therefore would require 1/ r or 10-fold more active heads to be in proximity of a single actin filament to allow for the observed continuous movement [32]. Because a 10-fold decrease in duty ratio would require roughly a 10-fold increase in myosin surface density to maintain optimal motility, it is unlikely that up-GTD/AAAA HMM would support motility.

Due to the apparent disagreement between the above predictions and the prior work [16], we prepared HMM with the GTD/AAAA mutation in the RLC to double check the result, as a first step to further explore the basis of the finding. Our data show that the GTD/AAAA mutation has no significant effect upon the ATPase kinetics (either up-state or p-state), in agreement with the original finding [16]. However, in contrast to the previous report, our construct did not support motility in the unphosphorylated state, in agreement with the above calculations. The up-state was completely turned off kinetically and mechanically and the p-state was fully activated in both respects. Therefore, our data suggests that the native sequence of the hinge is not required for normal phosphorylation-dependent regulation. Should further studies support our conclusion this result will have important implications on the structural mechanism of regulation.

4.4. K12E

We chose to investigate the K12E RLC mutant because of recent modeling work [15] showing that this residue lies in a critical location within the phosphorylation domain that undergoes a large conformational change upon phosphorylation [12,14]. In the unphosphorylated state, residues 12-15 appear to be most stable as a random coil, whereas upon phosphorylation the

peptide adopts a helical conformation. A previous report [7] found that deletion of the NH₂-terminal 12 residues from the smRLC diminished the actin-activated ATPase in the phosphorylated state, but not as much as for the respective K12E mutant. Therefore, it appeared that the positive charge of the K residue was important, and changing the positive charge to a negative charge gave a more severe phenotype of only 20% of wild type. However, the effect of the mutation on the actin motility was not measured. Our results showed that the K12E mutation had a modest but significant effect on the $V_{\max}$ of the actin-activated ATPase of the p-state (64% of WT) and the effect on ATPase was mirrored in the actin sliding velocity (48% of WT). There was no effect on the up-state with regard to either parameter. The fact that the K12E mutation affected only the p-state (not the up-state) is in accord with the idea that the residue lays in a disordered environment in the up-state which orders to a helix in the p-state. The removed positive charge replaced with a negative charge may destabilize the proposed helix.

4.5 Conclusions

In summary, our findings suggest that i) the positive charge of R160 on the RLC is not required for activation of the ATPase or motility and is therefore not an important residue for coordination of phosphorylated S19, ii) the native sequence of the hinge region between the two lobes of the RLC is not required for normal phosphorylation-dependent regulation with regard to both inactivation and activation, and iii) K12 likely contributes stability to the helical conformation of the phosphorylation domain in the p-state and mutation to an E destabilizes such helical conformation.

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Fig. 1. Ribbon structure of the RLC bound to the heavy chain

The hinge amino acids, GTDP, and R160 are indicated. K12 is within the region of the phosphorylation domain that is not crystallographically resolved. Model is from [13].

Fig. 2.

A. SDS-PAGE of expressed HMMs. Electrophoresis was in 4-20% polyacrylamide gels (Invitrogen). Lane 1, molecular weight standards; Lane 2, wild type HMM; lane 3, HMM containing K12E RLC; lane 4, HMM containing GTDP/AAAA RLC; lane 5, HMM containing R160C RLC. The RLC/ELC ratios for all mutants were similar to the WT (within 10%). **B. Analysis of RLC phosphorylation levels using urea gel electrophoresis** [12]. WT, wild type; Up, unphosphorylated, P, phosphorylated.

Fig. 3. Effect of actin on MantATP single turnover rates for WT p-HMM

HMM was phosphorylated as described in Methods. The final conditions in the single turnover assay were (after mixing) 0.5 μ M HMM heads and indicated actin in 10 mM MOPS (pH 7.0), 50 mM NaCl, 0.1 mM EGTA, 1 mM DTT, 0.5 μ M MantATP, 100 μ M ATP, 0.5 mM MgCl_2 . Data was fit to a double exponential model (fast rate, solid circles; slow rate, open circles). The solid lines indicate the fit to the data using the Michaelis-Menton equation (see *footnote b*, Table 1). The V_{max} and K_{atpase} obtained from the fits are tabulated in Table 1.

Fig. 4. Actin-activated single turnover of MantATP

HMMs were assayed at 25 °C as described in Fig. 2. A, in up-state. B, in p-state. Data are the weighted average of the two rates (see *Footnote b*, Table 1). Values are the average of replicate determinations from two independently expressed and purified preparations. The range of the two values never exceeded $\pm 10\%$ from the average value. For B, the fit to the WT and K12E data are shown as solid lines.

Table 1
Summary of ATPase and actin sliding velocity data for HMM constructs

RLC Mutant	State	$k_{\text{turnover w/o actin}}^a$ (sec ⁻¹)	V_{max}^b (sec ⁻¹)	K_{atpase}^b (μM)	Actin Gliding Velocity ^c (μm sec ⁻¹)
WT	Up	0.015 ± 0.006	d	d	NOM ^e
	P	0.033 ± 0.001	2.66 ± 0.37	57 ± 16	0.25 ± 0.04
			3.51 ± 0.18 (F)	19 ± 3 (F)	
R160C			0.49 ± 0.03 (S)	13 ± 3 (S)	
	Up	0.033 ± 0.001	-	-	NOM
	P	0.049 ± 0.011	2.37 ± 0.22	47 ± 10	0.25 ± 0.05
K12E			3.47 ± 0.43 (F)	31 ± 10 (F)	
	Up	0.012	0.21 ^f ± 0.03 (S)	13 ± 6 (S)	
	P	0.031 ± 0.014	-	-	NOM
GTPD/AAAA			1.70 ^f ± 0.16	83 ± 14	0.12 ± 0.03 ^f
			1.92 ^f ± 0.25 (F)	36 ± 12 (F)	
			0.29 ± 0.14 (S)	55 ± 56 (S)	
	Up	0.019 ± 0.003	-	-	NOM
	P	0.040 ± 0.002	2.72 ± 0.25	69 ± 12	0.25 ± 0.03
			3.76 ± 0.43 (F)	60 ± 14 (F)	
			0.48 ± 0.05 (S)	38 ± 9 (S)	

^aSingle turnover of MantATP in the absence of actin from Figure 4A. Values represent the average and ± 1/2 range of replicate determinations from two different protein preparations. Replicate shots for one preparation varied by less than 0.003 sec⁻¹. Four preparations were examined for the WT.

^bObtained by fitting the data in Figures 4A and 4B to the Michaelis-Menton equation, $V = (V_{\text{max}}[\text{actin}] / (K_{\text{atpase}} + [\text{actin}] + C)$. C = the rate in the absence of actin. V_{max} is the maximal turnover rate and K_{atpase} is the concentration of actin at half-maximal activity corresponding to the dissociation constant of HMM for actin in the weakly bound state. Uncertainties represent the 95% confidence intervals of the fit. See ^{footnote a} for replicate information. The first value represents the weighted average of the fits = (rate 1) (fractional amplitude 1) + (rate 2) (fractional amplitude 2). Fractional amplitudes 1 and 2 add to 1. (F), fast phase; (S), Slow phase. Total amplitudes were similar for all samples and 60-80% of the total amplitude was attributed to the fast phase at 100 μM actin.

^cMean value ± standard deviation from two independent preparations (n=2). See Methods for details.

^d V_{max} and K_{atpase} could not be accurately determined due to weak binding to actin in the presence of ATP. See Figure 3A.

^eNOM = No observed motility.

^fSignificantly different from the corresponding value for the WT (Student's t-test) with p less than or equal to 0.05. All other values had 0.25 > p > 0.1.