Evaluation of the symmetric model for myosin-linked regulation: effect of site-directed mutations in the regulatory light chain on scallop myosin

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Regulatory myosins are controlled through mechanisms intrinsic to their structures and can alternate between activated and inhibited states. However, the structural difference between these two states is unclear. Scallop (*Pecten maximus*) striated adductor myosin is activated directly by calcium. It has been proposed that the two heads of scallop myosin are symmetrically arranged and interact through their regulatory light chains [Offer and Knight (1996) *J. Mol. Biol.* **256**, 407–416], the interface being strengthened in the inhibited state. By contrast, vertebrate smooth-muscle myosin is activated by phosphorylation. Its structure in the inhibited state has been determined from two-dimensional crystalline arrays [Wendt, Taylor, Trybus and Taylor (2001) *Proc. Natl. Acad. Sci. U.S.A.* **98**, 4361–4366] and is asymmetric, requiring no interaction between regulatory light chains. Using site-directed mutagenesis of the scallop regulatory light chain, we have tested the symmetric model for scallop adductor muscle myosin. Specifically, we have made myosin hybrid molecules from scallop

(*P. maximus*) myosin, in which the normal regulatory light chains have been replaced by expressed light chains containing mutations in three residues proposed to participate in the interaction between regulatory light chains. The mutations were R126A (Arg¹²⁶ → Ala), K130A and E131A; made singly, in pairs or all three together, these mutations were designed to eliminate hydrogen bonding or salt linkages between heads, which are key features of this model. Functional assays to address the competence of these hybrid myosins to bind calcium specifically, to exhibit a calcium-regulated myofibrillar Mg-ATPase and to display calcium-dependent actin sliding were performed. We conclude that the symmetrical model does not describe the inhibited state of scallop regulatory myosin and that an asymmetric structure is a plausible alternative.

Key words: actin sliding assay, calcium-specific binding, muscle regulation, regulatory myosin.

INTRODUCTION

Myosin-linked regulation is observed in both muscle and non-muscle cells of vertebrates and invertebrates. Here, the interaction between actin and myosin can be regulated by conformational events on the myosin molecule alone [1–4]. Two forms of regulatory myosin have been found in muscle. In vertebrates, myosin-linked mechanisms control the contractile activity of smooth- and non-muscle cells [5], although forms of actin-based regulation may play a complementary role [6]. Myosin regulatory light chains (R-LCs) become phosphorylated by myosin light-chain kinase, which has, in turn, been activated by calcium-calmodulin [3,7]. These actions result in myosin activation and muscle contraction, provided that myosin-binding sites on actin have been made available – often through mechanisms also responsive to calcium-calmodulin [3,6]. By contrast, the type of regulatory myosin found in many invertebrate striated-muscle cells becomes activated directly through the binding of calcium to calcium-specific sites, one on each myosin essential light chain (E-LC) [8,9]; thin-filament-linked regulation is not required, although it may operate in a complementary manner in some muscles [10,11]. Detailed structural analysis of scallop (*Pecten maximus*) striated adductor muscle myosin-regulatory domain indicates that calcium binding probably affects the pivotal hydrogen bonding arrangement linking each E-LC (Phe²⁰ and Arg²⁴) to the R-LC (Gly¹¹⁷) on the same head, leading to activation [12,13].

Co-operative mechanisms are responsible for myosin activation in both phosphorylation- [14,15] and calcium-dependent [16–19] motors. The inhibited state is maintained through interactions

between the two heads of the bipartite myosin molecule. This state is of considerable interest and, for many years, has been surmised to require the close proximity of the light-chain-binding regions (regulatory domains), located on the neck regions of the molecule, to the head–tail junction [myosin heavy chains (HCs)] [1]. Suggestions about the nature of this interaction arose when Offer and Knight [20] tackled the problem of how the two myosin heads might be joined to the coiled-coil tail and, hence, how they might interact. They noted that, in the crystal structure of the scallop-regulatory domain, the C-terminal α -helix extends up to the invariant proline residue that starts the myosin coiled-coil tail. They suggested that the C-terminal α -helix might continue into a strand of the coiled-coil without interruption. This strategy yielded a model of the head–tail junction of a two-headed myosin molecule with the two heads symmetrically arranged (Figure 1, upper panel). In this symmetrical model, the two heads contact one another through the R-LCs, providing a basis for understanding the head–head interactions, which was consistent with both fluorescence resonance energy transfer [21] and cross-linking [22] results. Particular attention was placed on one type of contact, which appears to be invariant in molluscan striated myosins. This included three residues in the N-terminal half of one R-LC (Ala⁴², Glu⁴⁵ and Gln⁴⁶) lying close to three residues in the C-terminal half of the other R-LC (Arg¹²⁶, Lys¹³⁰ and Glu¹³¹). The implication was that there might be a salt link between Glu⁴⁵ and Lys¹³⁰ as well as contacts between the other opposing residues. It was suggested that the structural differences between inhibited and activated states might be subtle, but that the interface might be further strengthened in the inhibited state.

Abbreviations used: E-LC, myosin essential light chain; HC, myosin heavy chain; R-LC, myosin regulatory light chain.

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Figure 1 Different models of scallop and smooth-muscle myosins

Upper panel: RasMol diagrams depicting the symmetrical model of scallop myosin as described in [20]. The views are of the N-terminal region of the coiled-coil as it joins the regulatory domain, seen from the side and from the top; the motor domain is not shown. The pair of backbone HCs are coloured in shades of blue; the pair of essential light chains are coloured in shades of green; the pair of regulatory light chains are shown in red and yellow. The critical residues that are proposed to participate in contacts between R-LCs are indicated. Lower panel: RasMol diagram depicting the asymmetrical model of smooth-muscle myosin (code 1B84, Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, Piscataway, NJ, U.S.A.) adapted to illustrate the location of the critical residues depicted for scallop myosin. The residues in the R-LCs that are proposed to contact each other in the Offer and Knight model [20] (upper panel) are indicated; note that these sets of residues, on each of the regulatory light chains, are far apart from each other in this model. The pair of backbone HCs are coloured in shades of blue; the pair of essential light chains are coloured in shades of green; the pair of regulatory light chains are shown in red and yellow.

More recently, an alternative model has been suggested for the inhibited state of a regulatory myosin based on results obtained from electron microscopy of smooth-muscle myosin in the 'off-state' within lipid monolayers [23,24]. This model is asymmetric and shows no points of contact between adjacent R-LCs (Figure 1, lower panel). Here, the contacts between the heads are between the actin-binding surface of one head and the converter domain of the second head. Any suggestion that this distinctive arrangement is artifactual, arising from the stresses placed on individual smooth-muscle myosin molecules when ordered within a lipid monolayer, is countered by recent observations of a similar configuration using single-particle analysis of negatively stained molecules [25]. One possible explanation of the different structures observed for these two regulatory myosins could be that, whereas the smooth-muscle myosin structure was obtained by image analysis of real data, the scallop myosin structure was determined indirectly by modelling. An alternative possibility is that

Figure 2 Urea-gel electrophoresis of scallop hybrid myofibrils that have incorporated mutant regulatory light chains

Lanes A–J, intact, desensitized or recombined hybrid myofibrils that have incorporated expressed normal or mutant regulatory light chains. Lane A, intact *Pecten maximus* myofibrils; lane B, desensitized myofibrils with the R-LC removed; lane C, desensitized myofibrils with bound R126A R-LC; lane D, desensitized myofibrils with bound K130A R-LC; lane E, desensitized myofibrils with bound E131A R-LC; lane F, desensitized myofibrils with bound expressed R-LC without point mutations; lane G, desensitized myofibrils with bound R126A/K130A/E131A R-LC; lane H, desensitized myofibrils with bound R126A/K130A R-LC; lane I, desensitized myofibrils with bound R126A/E131A R-LC; lane J, desensitized myofibrils with bound K130A/E131A R-LC. The mobility of the mutant R-LCs on the urea gel changed according to the charge alterations as compared with the unmodified R-LC. Lanes C (R126A), D (K130A) and G (R126A/K130A/E131A) have lost a positive charge overall, and have increased mobility. Lane H (R126A/K130A) has lost two positive charges and so runs with an even greater mobility. Lane E (E131A) has lost a negative charge and so has decreased mobility. The rest of the R-LCs show no overall charge change and therefore run alongside the unmodified R-LC. Gains or losses of charge are indicated. Gels were stained with Coomassie Blue. Loadings were approx. 25 µg/well.

both structures are correct: the structure required for inhibition of a calcium-regulated myosin is necessarily different from the structure required to maintain a phosphorylatory myosin in the 'off-state'.

In this paper, we present results from experiments designed to discriminate between these two models. We have taken advantage of the detailed information provided by the Offer and Knight model [20] to perform site-directed mutagenesis of the R-LCs with respect to each of the three residues in the C-terminal half, which participate in the head–head contact of their model. Using pure hybrid scallop myosin molecules [26] made from such mutated R-LCs, we have tested the functional importance of these residues by assessing their ability to maintain calcium sensitivity of regulation when incorporated into hybrid myosins. We have done this by analysing the ability of such hybrid myosins to support calcium-specific binding, calcium-regulated actin-activated Mg-ATPase activity and calcium-dependent *in vitro* motility.

EXPERIMENTAL

Preparation of proteins, EDTA desensitization and hybridization

Myofibrils and myosin from scallop (*P. maximus*) striated adductor muscles were prepared by standard procedures [27,28,34]. EDTA desensitization is the term given to the removal of R-LCs from scallop myofibrils or myosin, accomplished through metal-ion chelation and leading to the reversible loss of calcium sensitivity [28]. In the present study, it was performed using methods described previously [27,28], with only minor changes. Scallop myofibrils at 4 mg/ml were warmed to 30 °C in (–)Mg buffer (40 mM NaCl/5 mM P_i/0.1 mM EGTA/3 mM NaN₃, pH 7.0) and desensitized by the addition of 10 mM EDTA for 5 min, leading to the removal of R-LC (see Figure 2, lane B). The desensitized myofibrils were centrifuged, washed with (–)Mg buffer and then

Table 1 Oligonucleotides used for site-directed mutagenesis

List of oligonucleotides used in the present study to facilitate site-directed mutagenesis of the scallop (*Pecten maximus*) striated adductor muscle R-LC. Residues that were altered from the native sequence are underlined.

Mutant R-LC	Oligonucleotide sequence
R126A R-LC	5'-CAAAGATGAGGTGGCCATGACATTCAAGGAAGCCCC-3'
K130A R-LC	5'-GTGAGGATGACATTGCGGAGGCCCTGTGGAAGG-3'
E131A R-LC	5'-GACATTCAAGGCAGCCCCCTGTGG-3'
R126A/K130A R-LC	5'-CAAAGATGAGGTGGCCATGACATTGCGGAAGCCCCCTGTGGAAGG-3'
R126A/E131A R-LC	5'-CAAAGATGAGGTGGCCATGACATTCAAGGCAGCCCCCTGTGGAAGG-3'
K130A/E131A R-LC	5'-CAAAGATGAGGTGAGGATGACATTGCGGAGGCCCTGTGGAAGG-3'
R126A/K130A/E131A R-LC	5'-CAAAGATGAGGTGGCCATGACATTGCGGAGGCCCTGTGGAAGG-3'

pelleted again before re-suspension in (+)Mg buffer (40 mM NaCl/1 mM $MgCl_2$ /5 mM P_i /3 mM NaN_3 , pH 7.0). Expressed forms of mutant and unmodified scallop R-LCs were hybridized with desensitized scallop myosin using techniques based on previous methods [26]. Desensitized myofibrils (5 mg) were incubated overnight with 1 mg of purified R-LC at 4 °C with gentle rocking in 1.5 ml of (+)Mg buffer. The tubes were microfuged for 1 min at 4 °C to spin down the myofibrils and the pellet was re-suspended in (+)Mg buffer. Scallop myosin was treated in a similar way but desensitization was performed at 25 °C. The success of R-LC exchange was monitored by urea-gel electrophoresis. Actin was prepared by standard methods [29] from actin acetone powder.

Site-directed mutagenesis of scallop R-LCs

Mutant R-LCs were constructed by site-directed mutagenesis of R-LC cDNA using the Promega GeneEditor site-directed mutagenesis system *in vitro*. This involved synthesis of mutant oligonucleotides (MWG Biotech; Table 1) designed to anneal to the R-LC expression vector R-LCpET21a described previously [30]. The resulting mutants were checked by restriction digestion and sequencing before expression in BL21(DE3) pLysS *Escherichia coli*. Charged residues were changed to alanine residues, so as to disrupt potential ionic or hydrogen-bonded interactions.

R-LC expression

Cultures (500 ml) of the R-LC expression vectors in BL21(DE3) pLysS *E. coli* were grown at 37 °C until an A_{600} of 1 was reached. The cultures were then induced by the addition of 0.4 mM IPTG for 4–6 h. In every case, most of the expressed R-LC was found to be in the insoluble inclusion body fraction of the bacteria. These inclusion bodies were isolated from the bacterial pellet by disrupting the cells in 20 ml of 20 mM Tris/HCl (pH 8.0), with two 10 s bursts in a Polytron at 4 °C. The insoluble fraction was then pelleted and the disruption was repeated twice with 20 ml of isolation buffer [2 M urea, 20 mM Tris/HCl, 0.5 M NaCl and 2% (v/v) Triton X-100, pH 8.0]. Inclusion bodies were frozen at –20 °C overnight. R-LCs were solubilized by stirring at room temperature (20–22 °C) for 1 h with solubilization buffer (6 M guanidinium chloride/20 mM Tris/HCl/0.5 M NaCl/10 mM imidazole, pH 8.0). The remaining insoluble fraction was removed by centrifugation and the supernatant dialysed against two changes of PBS for 16 h at 4 °C. This solution was clarified by centrifugation and filtered through a 0.45 μ m filter before purification on an ion-exchange column (Q Sepharose fast flow; Amersham Biosciences). The running buffer was 25 mM NaCl/3 mM $MgCl_2$ /0.2 mM $CaCl_2$ /5% (v/v) poly(ethylene glycol)/5 mM NaP_i /3 mM azide, pH 7.0. The

fractions were eluted against an increasing NaCl gradient to 0.5 M. The purity of each R-LC was analysed by SDS/PAGE; if judged to be >90% pure, the R-LCs were frozen at –20 °C for storage. If the purity was <90%, R-LCs were further purified by gel filtration on a HiPrep 16/60 Sephacryl S-100 high-resolution column with 150 mM NaCl/5 mM NaP_i /2 mM $MgCl_2$ /10 μ M $CaCl_2$ /3 mM azide, pH 7.0.

ATPase measurements

Myofibrillar Mg-ATPase measurements were made using a colorimetric assay, with minor modifications being made to techniques described previously [16]. Myofibrils (1.2 mg) were placed in a 1.5 ml reaction volume with ATPase buffer (20 mM NaCl/0.2 mM EGTA/3 mM $MgCl_2$ /20 mM Tris, pH 7.5). For each experiment, duplicate tubes were prepared with and without 0.5 mM $CaCl_2$. The reaction was started at room temperature (20–22 °C) with the addition of 2 mM ATP. At various time points, 0.25 ml aliquots of the reaction solution were removed and the reaction stopped by acid precipitation; the amount of P_i was determined colorimetrically [27]. After application of the colour reagent, the insoluble material was pelleted and the A_{700} of the resulting supernatant was measured 2 min later. The absorbance value was compared with that obtained from a phosphate standard curve and the amount of phosphate released was estimated.

Measurement of the *in vitro* motility activity

The ability of the mutant light chains to support actin motility was examined using an *in vitro* motility assay [31]. Soluble myosin (1–3 mg/ml) was added in high-salt buffer [0.3 M KCl/0.1 mM EGTA/4 mM $MgCl_2$ /0.2 mM $CaCl_2$ /25 mM Hepes (pH 7.5); 140 mM 2-mercaptoethanol was added just before use] to a flow cell constructed from a glass slide and a nitrocellulose-coated coverslip, separate chambers having been created by tracks of grease [32]. Excess myosin was removed by washing with the high-salt buffer after 1 min. The buffer was then washed with a low-salt buffer (25 mM KCl/0.1 mM EGTA/4 mM $MgCl_2$ /0.2 mM $CaCl_2$ /25 mM Hepes, pH 7.5/140 mM 2-mercaptoethanol at 25 °C). Rhodamine-phalloidin-labelled actin was introduced to the flow cell at approx. 1 nM concentration in low-salt buffer and immediately washed again with the low-salt buffer. The ATP mix was then added (low-salt buffer with 0.1 mg/ml glucose oxidase, 23 μ g/ml catalase, 2.5 mg/ml glucose, 1 mg/ml BSA and 1 mM ATP). To study the effect of calcium on actin motility, the concentration of $CaCl_2$ in the ATP mixture was varied between 0 and 0.2 mM. Note that there was no need in these measurements to add sonicated actin for the purpose of removing 'dead-heads' [32]: scallop myosin and hybrids showed >90% actin movement in all fields in the presence of calcium.

Table 2 Myofibrillar Mg-ATPase and calcium-specific binding results of hybrid scallop myofibrils possessing expressed normal and mutant regulatory light chains

Values of the R-LC/E-LC ratio are presented for mutant R-LCs. Values for the total (mutant plus residual) R-LC are given in parentheses. Because the mutant and native R-LCs in samples indicated by an asterisk cannot be resolved by urea-gel electrophoresis, the amount of mutated R-LC was estimated by subtracting the mean value of residual native R-LC (0.10), obtained from those samples where the bands could be resolved. Mg-ATPase rates, in the absence or presence of calcium, are expressed in $\text{nmol P}_i \cdot (\text{mg myofibril})^{-1} \cdot \text{min}^{-1}$. The quotient, $-\text{Ca}^{2+}$ rate/ $+\text{Ca}^{2+}$ rate, provides an indication of the degree of restoration of regulation; as with all hybrid results, the mutant rates should be compared with those from hybrids possessing the expressed, unmodified R-LC. Calcium-specific binding results are expressed in mol Ca^{2+} bound/mol myosin. Data from independent determinations on different preparations (n) have been pooled. Results are presented as means $\pm$ S.D.

Hybrid	Ratio of mutant R-LC/E-LC (Ratio of total R-LC/E-LC)	ATPase rate ($-\text{Ca}^{2+}$)	ATPase rate ($+\text{Ca}^{2+}$)	ATPase rate ratio ($-\text{Ca}^{2+}/+\text{Ca}^{2+}$)	Calcium-specific binding (mol Ca^{2+} /mol myosin)
Intact	1.02 ± 0.25 $n = 10$	9.9 ± 4.9 $n = 23$	142.8 ± 51.4 $n = 23$	0.07	2.13 ± 0.20 $n = 16$
Desensitized	0.13 ± 0.12 $n = 10$	104.1 ± 40.9 $n = 23$	107.5 ± 42.9 $n = 23$	0.97	0.00 $n = 8$
Resensitized R-LC	0.91^* (1.01 ± 0.31) $n = 6$	41.0 ± 12.2 $n = 11$	144.3 ± 13.8 $n = 11$	0.29	2.03 ± 0.12 $n = 8$
R126A R-LC	0.94 ± 0.17 (0.99 ± 0.15) $n = 6$	35.4 ± 13.2 $n = 11$	136.5 ± 26.2 $n = 11$	0.26	1.91 ± 0.14 $n = 8$
K130A R-LC	0.98 ± 0.19 (1.01 ± 0.22) $n = 6$	38.8 ± 15.4 $n = 11$	132.8 ± 21.5 $n = 11$	0.29	2.02 ± 0.12 $n = 8$
E131A R-LC	0.78 ± 0.11 (0.90 ± 0.31) $n = 5$	77.4 ± 17.2 $n = 9$	133.4 ± 22.4 $n = 9$	0.58	1.87 ± 0.15 $n = 8$
R126A/K130A R-LC	0.73 ± 0.08 (0.90 ± 0.40) $n = 4$	60.5 ± 10.7 $n = 10$	123.8 ± 21.6 $n = 10$	0.49	1.75 ± 0.12 $n = 8$
R126A/E131A R-LC	0.82^* (0.92 ± 0.11) $n = 4$	46.5 ± 34.4 $n = 10$	141.2 ± 31.7 $n = 10$	0.33	1.95 ± 0.15 $n = 8$
K130A/E131A R-LC	0.84^* (0.94 ± 0.32) $n = 4$	46.4 ± 11.7 $n = 10$	129.4 ± 22.2 $n = 10$	0.36	2.07 ± 0.12 $n = 8$
R126A/K130A/E131A R-LC	0.87 ± 0.26 (0.97 ± 0.35) $n = 4$	54.8 ± 13.4 $n = 10$	128.9 ± 29.2 $n = 10$	0.43	1.73 ± 0.15 $n = 8$

The movement of the actin filaments was recorded on videotape and velocity data were obtained from a computer using a tracking program (Retrac 1.8) designed for this purpose (courtesy of Dr Nick Carter, <http://mc11.mcrl.ac.uk/Retrac/index.html>).

Calcium binding

Calcium binding was determined using a ^{45}Ca -EGTA buffering system, based on methods described previously [27,28]. Weighed myofibrillar pellets [33] were dissolved in Folin–Lowry buffer before scintillation counting and protein determination. The free calcium concentration used, calculated by an iterative procedure [28], was 2.1×10^{-6} M.

Other

Protein concentrations were determined either spectrophotometrically (for myosin, $E_{1\%,1\text{ cm},280\text{ nm}} = 5.3$ [34]) or with the Bradford protein assay kit from Bio-Rad Laboratories, using BSA as reference protein. Urea/acrylamide gel and SDS/acrylamide gel electrophoresis experiments were performed as described previously [28]. The relative amounts of protein in different gel bands were determined through quantitative densitometry (model GS-

690 imaging densitometer; Bio-Rad Laboratories) of Fast Green- or Coomassie Blue-stained urea gels.

RESULTS

Creation of the mutant R-LCs and hybrid myosins

Putative contact between the R-LCs of the two heads was tested by introducing point mutations in the R-LC at each of the three charged C-terminal residues suggested previously [20] to be potential participants in the contact. These mutations were introduced singly, in pairs or all three together. These amino acids were changed to alanine residues so as to disrupt potential ionic interactions and hydrogen bonds (for details see the Experimental section). Nucleotide sequences of all mutated transcripts were verified. Expression of all seven mutant R-LCs was achieved (yields of 10–20 mg/l bacterial broth). The characteristic migration pattern of the expressed, purified mutant R-LCs as seen by urea-gel electrophoresis of hybrid myofibrils was determined (Figure 2). Quantitative analysis of the R-LC/E-LC ratios (Table 2) indicated that, in most recombination experiments, complete rebinding of mutant R-LCs was achieved. However, in the E131A ($\text{Glu}^{131} \rightarrow \text{Ala}$) and R126A/K130A R-LC mutant hybrids, only 70–80% rebinding was achieved.

Figure 3 Restoration of myofibrillar Mg-ATPase regulation by re-addition of expressed normal and mutant regulatory light chains to desensitized scallop myofibrils

Histogram showing the restoration of regulation by expressed normal and mutant R-LCs, as estimated through measurement of the calcium sensitivity of the myofibrillar Mg-ATPase. Columns, labelled in order from left to right, depict myofibrils that have been desensitized (DS) or resensitized with expressed normal R-LC, single-mutant R-LCs (R126A, K130A, E131A), double-mutant R-LCs (R126A/K130A, R126A/E131A, K130A/E131A) and triple-mutant R-LC (R126A/K130A/E131A). Regulation was defined as $\{1 - [\text{ATPase}(-\text{Ca}^{2+})/\text{ATPase}(+\text{Ca}^{2+})]\} \times 100$; for ease of comparison, all values have been normalized such that the expressed, unmodified R-LC hybrid displays a value set at 100%.

Regulation of the myofibrillar Mg-ATPase of the R-LC mutant hybrid myosins

The combined myofibrillar Mg-ATPase and calcium-binding results of myosin hybrids, made from each of the mutant R-LCs, are shown in Table 2 and Figure 3. In all cases, with the possible exceptions of the E131A and R126A/K130A hybrids noted above, calcium-specific binding and calcium-sensitive actin-activated Mg-ATPase have been substantially restored to the levels attained by the unmodified, expressed R-LC control hybrid, a sample of which was always included in all experiments. Calcium-specific binding broadly corresponds to the amount of rebound R-LC. For the E131A and R126A/K130A hybrids, myofibrillar Mg-ATPase rates are somewhat less calcium-sensitive than the unmodified control (ratios of 0.50–0.60 as compared with 0.30 respectively). Because the recombination ratio (R-LC/E-LC) is lower in these hybrids when compared with the other mutants, it is probable that the lack of complete rebinding is the source of the lowered calcium sensitivity.

Regulated *in vitro* motility activity of the R-LC mutant hybrid myosins

The results of actin sliding velocity measurements for each of the R-LC mutant hybrids are displayed both as velocity histograms (Figure 4) and in tabular form (Table 3), indicating the mean velocities and standard deviations. In the absence of calcium, no filament movement was detected except in desensitized myosin, the mean velocity of which was relatively low (approx. one-seventh of activated intact myosin rate) and similar in magnitude to its rate in the presence of calcium (Table 3). In the presence of calcium, mean velocities of actin sliding propelled by the recombinant myosins were in the range 1.28–1.79 $\mu\text{m/s}$, except for the E131A hybrid that had a mean rate of 0.81 $\mu\text{m/s}$, significantly lower than the others. As in the case of the ATPase assays, it is probable that this lower rate is partly due to the impaired uptake of this light chain by desensitized scallop myosin. Intact myosin shows the greatest spread of velocities in comparison with the recombinant forms (Figure 4). All recombinant myosins show comparable velocity spreads, with means significantly greater than those obtained for desensitized scallop myosin, further indicating (in addition to an absence of measurable velocity in

the absence of calcium) that calcium sensitivity of the myosin hybrids has been restored.

DISCUSSION

The mechanism by which a regulatory myosin maintains the 'off-state' remains unknown, despite being of considerable therapeutic potential as well as of intrinsic interest. Whereas myosin from scallop striated adductor muscles and other invertebrates is activated directly by calcium binding and does not require the intervention of additional enzymes [1,4], vertebrate smooth-muscle myosin requires kinase-dependent phosphorylation of a specific residue, Ser¹⁹, on its R-LC to become activated [3]. In the case of scallop myosin, calcium-specific binding takes place at a specific site located on the essential light chain [8,12] where it initiates the switch that turns the myosin molecule on by a co-operative mechanism [16–19]. Phosphorylation of smooth-muscle myosin on the R-LC also occurs through a co-operative mechanism [14,15]. Regulatory–regulatory light chain interactions have been suggested to underlie the maintenance of the 'off-state' and facilitate the co-operative mechanism in both scallop striated adductor muscles [22,28] and vertebrate smooth muscles [15,35]. Despite these different trigger mechanisms, it is probable that the subsequent conformational events that control the rate of hydrolysis through actin activation are similar in both types of regulatory myosin. It was intriguing, therefore, when very different structures for the inhibited molecule were proposed for the two cases. The symmetric structure, which involved specific R-LC–R-LC interactions (Figure 1, upper panel) as proposed for scallop myosin [20], contrasts dramatically with an asymmetric structure that does not require any interaction between R-LCs on opposing heads (Figure 1, lower panel), as proposed for smooth-muscle myosin [23–25].

The experiments described in the present study were designed to assess whether or not the three charged C-terminal residues Arg¹²⁶, Lys¹³⁰ and Glu¹³¹, proposed in the scallop myosin model to participate in binding adjacent R-LCs together through interaction with Gln⁴⁶, Ala⁴² and Glu⁴⁵ [20], were indeed important for regulation. We have successfully engineered three single mutants, three double mutants and one triple mutant by replacing Lys¹³⁰ and/or Arg¹²⁶ and/or Glu¹³¹ by alanine residues and have recombined these mutant R-LCs with desensitized scallop myosins to create hybrid molecules (Figure 2). In all cases, the functional control for comparative purposes was the hybrid molecule created by re-addition of an expressed, unmodified R-LC, having a sequence identical with that found in *P. maximus* R-LC [30], to desensitized scallop myosin. Calcium-specific binding (Table 2) and calcium sensitivity of the actin-activated Mg-ATPase (Table 2; Figure 3) were substantially restored to desensitized myosin by all mutant R-LCs created for the present study, and the exact degree to which control values were attained depended on the amount of rebound R-LC. In this regard, the E131A single-mutant R-LCs and the R126A/K130A double-mutant R-LCs failed to achieve reproducible stoichiometric rebinding (Table 2); a 25–30% deficiency in R-LC complement is detrimental to the restoration of calcium sensitivity because resensitization begins to occur only when the second mole equivalent of R-LC is rebound [28]. Interestingly, the triple mutant was capable of restoring calcium-specific binding and calcium sensitivity to values closer to those of the controls (Tables 1 and 2; Figure 2), suggesting that the conformation required for facile rebinding was impaired by the E131A mutation or by both mutations in R126A/K130A, yet restored in the triple mutant and in the other mutations.

Figure 4 Frequency distribution of actin sliding velocities as propelled by immobilized scallop hybrid myosins possessing expressed normal and mutant R-LCs

Actin filament sliding velocities were measured using a motility assay *in vitro* in the presence of 0.1 mM $\text{Ca}^{2+}_{\text{free}}$. Each point represents the rate of movement of one actin filament. This rate was derived from a data set obtained from 20 consecutive frames, captured at 1.25 s intervals, yielding 19 separate estimates of the rate that were averaged to give a single value. The numerical values of the velocities analysed in each frame set were placed in bins (bin width of 0.1 $\mu\text{m/s}$) giving rise to a frequency distribution. Histograms are shown for intact myosin (I), desensitized myosin (DS), R-LC (hybrid myosin with expressed normal scallop R-LC) and for mutant R-LC hybrid myosins possessing single (R126A, K130E, E131A), double (R126A/K130E, R126A/E131A, K130E/E131A) and triple (R126A/K130E/E131A) mutations in their R-LCs.

Measurement of the sliding velocity imparted to actin filaments by our immobilized recombinant myosins further indicates that mutation of residues Arg¹²⁶, Lys¹³⁰ and Glu¹³¹ does not abolish regulation. Only desensitized scallop myosin exhibits any movement at all in the absence of calcium (Table 3). This rate, of the order of 0.44 $\mu\text{m/s}$, is similar to its velocity in the presence of calcium (0.49 $\mu\text{m/s}$) and is approx. 7-fold less than the

rate of movement of intact myosin in the presence of calcium (3.08 $\mu\text{m/s}$). These results, obtained from desensitized and intact myosins of the striated adductor muscles of *P. maximus*, are comparable with analogous rates obtained previously by myosins prepared from *Argopecten irradians* and examined for movement on *Nitella* actin filaments [36]. In the presence of calcium, all recombinant myosin hybrids exhibit sliding velocities that are

Table 3 Mean values of the actin sliding velocities as propelled by immobilized scallop hybrid myosins possessing expressed normal and mutant R-LCs

Mean values of the actin sliding velocities as determined from the frequency distribution of velocities given in Figure 4. Results are expressed in $\mu\text{m/s}$, in the presence and absence of calcium, and include standard deviations and the number of separate observations made.

Hybrid	Mean velocity ($\mu\text{m/s}$)	
	+ Ca^{2+}	– Ca^{2+}
Intact	$3.08 \pm 1.53, n = 527$	0
Desensitized	$0.49 \pm 0.31, n = 605$	$0.44 \pm 0.28, n = 256$
R-LC (resensitized)	$1.28 \pm 0.72, n = 244$	0
R-LC R126A	$1.66 \pm 1.22, n = 312$	0
R-LC K130A	$1.45 \pm 0.88, n = 620$	0
R-LC E131A	$0.81 \pm 0.62, n = 423$	0
R-LC R126A/K130A	$1.56 \pm 0.93, n = 716$	0
R-LC R126A/E131A	$1.38 \pm 0.92, n = 281$	0
R-LC K130A/E131A	$1.41 \pm 0.85, n = 158$	0
R-LC R126A/K130A/E131A	$1.79 \pm 1.13, n = 128$	0

significantly higher than desensitized myosin (Figure 4; Table 3). In this regard, actin translocation brought about by desensitized myosin is still significant, implying that cross-bridge cycling occurs. Based on earlier motility studies *in vitro*, in which pairwise mixtures of myosins were used to distinguish between the inhibitory effects caused by slower cycling heads as opposed to non-cycling heads [37], we suggest that the lower sliding velocities produced by desensitized scallop myosin are due to a lower rate of detachment of heads from the actin filament. Interestingly, the triple mutant shows the highest rate, whereas the unmodified, expressed R-LC gives the lowest rate of the group with one exception. This exception, the E131A R-LC mutant, nevertheless shows a rate significantly higher than that of desensitized myosin; as noted above, the lower than expected rate probably correlates with its reduced capacity for full re-uptake of the modified R-LC (Table 2). The fact that all mutant hybrid myosins except one show actin sliding rates similar to, or higher than, the recombined unmodified hybrid indicates a complete restoration of regulation.

The sliding velocities achieved by intact scallop myosin are distributed over a very broad range in comparison with any of the recombinant forms (Figure 4). This is likely to be due to the fact that all hybrids used in the present study are formed by recombination of light chain with desensitized myosin; residual desensitized molecules will inevitably remain within this population. Such a situation exerts a more noticeable effect on sliding within an *in vitro* motility assay than it would in an ATPase assay. Measurement of the latter will yield an average rate in direct proportion to the population of molecules present. However, the effect of inhibitory molecules will act disproportionately in a motility assay; the overall measured actin translocation velocity will be lower if active, yet slower, desensitized myosin molecules perturb the action of the uninhibited fraction. For example, a 50% decrease in velocity of actin sliding was obtained with a mixture of slow (platelet) and fast (skeletal muscle) myosins when only 8% of this mixture comprised the slow component [37]. Consequently, the range of velocities measured for our scallop hybrid myosins will cluster towards values lower than those of intact myosin, as was also observed previously with *A. irradians* myosin in a *Nitella* actin motility assay [36]. It may be noted that it can be difficult to demonstrate regulation, even with well-regulated molecules, using the actin sliding assay [38]; consequently, our

evidence for the fact that mutant light chains can restore regulation using this assay passes a stringent test, further indicating that our conclusions are robust.

In summary, functional analyses of mutant scallop R-LC hybrid myosins, in which R-LC residues Arg¹²⁶, Lys¹³⁰ and Glu¹³¹ have been mutated to alanine residues, singly or in combination, do not support the proposed symmetrical structure for the 'off-state' of scallop striated adductor muscle myosin (Figure 1, upper panel) [20]. Although our results suggest that the symmetric model does not explain the inhibited state, additional work would be necessary to attest to the validity of the asymmetric model with respect to scallop myosin. The R-LC mutants of our study do not address this point because R-LC–R-LC interactions are not present in the proposed asymmetric structure (Figure 1, lower panel) [24]. Nevertheless, it remains a possibility that the activated 'on-state' has a structure resembling the symmetric model, at least for some portion of the actomyosin duty cycle. Kinetic studies on scallop myosin fragments have provided results that are consistent with an asymmetric inhibited structure. In the absence of calcium, only one ADP molecule binds to two-headed heavy meromyosin fragments at physiological ADP concentration [18]; furthermore, the second head of these fragments is bound more tightly to actin than is the equivalent head in skeletal-muscle myosin [19]. Consequently, the logistical beauty inherent in the symmetric structure, which provided a coherent argument for maintenance of the 'off-state' and the origins of co-operativity, must now be discarded. Ways to examine the putative asymmetric structure of scallop myosin in the 'off-state' and ways to correlate this structure with its regulatory function must be devised.

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