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The C-terminal helix in subdomain 4 of the regulatory light chain is essential for myosin regulation

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In vertebrate smooth/non-muscle myosins, phosphorylation of the regulatory light chains by a specific calmodulin-activated kinase controls both myosin head interaction with actin and assembly of the myosin into filaments. Previous studies have shown that the C-terminal domain of the regulatory light chain is crucial for the regulation of these myosin functions. To further dissect the role of this region of the light chain in myosin regulation, a series of chicken smooth muscle myosin regulatory light chain mutants has been constructed with successive C-terminal deletions. These mutants were synthesized in *Escherichia coli* and analysed by their ability to restore Ca^{2+} regulation to scallop myosin that had been stripped of its native regulatory light chains ('desensitized'). The results show that regulatory light chain mutants with deletions in the C-terminal helix in subdomain 4 were able to reform the regulatory Ca^{2+} binding site on the scallop myosin head, but had lost the ability to suppress scallop myosin filament assembly and interaction with actin in the absence of Ca^{2+}. Further deletions in the C-terminal domain led to a gradual loss of ability to restore the regulatory Ca^{2+} binding site. Thus, the regions in the C-terminal half of the regulatory light chain responsible for myosin regulation can be identified.

Key words: actomyosin interaction/EF-hand subdomains/inhibition/myosin light chains/regulation

Introduction

In vertebrate smooth/non-muscle myosins (myosin IIs) one regulatory light chain (RLC) and one essential light chain (ELC) are associated with the neck region of each myosin head and are involved in regulating contractile activity. In these myosins, 'motor' activity is controlled by phosphorylation of the RLCs by a Ca^{2+}–calmodulin-dependent myosin light chain kinase. *In vitro* studies have provided compelling evidence that RLC phosphorylation regulates vertebrate smooth/non-muscle myosin filament assembly, as well as myosin interaction with actin. For example, under physiological conditions unphosphorylated myosin filaments disassemble in the presence of MgATP into monomers with folded tails and a sedimentation coefficient of 10S (Onishi and Wakabayashi, 1982; Trybus *et al.*, 1982; Craig *et al.*, 1983). RLC phosphorylation, by unfolding the 10S monomers into transient 'assembly-competent' 6S intermediates, promotes filament assembly. In the folded (10S) state the myosin is enzymatically inactive, i.e. the products of ATP hydrolysis are effectively 'trapped' at the active site and are released very slowly (Cross *et al.*, 1986, 1988). Thus it is believed that in cells there may exist a cytoplasmic pool of inert 10S monomers that can be recruited into enzymatically active filaments when the RLCs are phosphorylated and assembled with actin to form a contractile apparatus (e.g. in the contractile ring during cytokinesis). The recent genetic studies of Karess *et al.* (1991) have provided direct proof that the RLC is required for cytokinesis in *Drosophila*.

At present only limited structural information is available on the myosin light chains. On the basis of sequence analysis they are homologous to members of the EF-hand family of Ca^{2+} binding proteins such as calmodulin and troponin-C (Kretsinger, 1980). The crystal structures of calmodulin and troponin have been solved (reviewed by Strynadka and James, 1989) and they have served as models for predicting light chain structure. The characteristic feature of these proteins is that they are composed of four EF-hand subdomains, each consisting of a 12 residue Ca^{2+} binding loop flanked by α-helices of 10–13 residues. The subdomains are designated 1–4 and the helices A–H sequentially from the N- to the C-terminus. In all the RLCs, three of the Ca^{2+} binding loops have deletions and non-conservative substitutions that would render them incompetent to bind Ca^{2+}. Only the first subdomain has retained all the residues necessary to form a competent high affinity divalent metal binding site (Collins, 1976; Kendrick-Jones and Jakes, 1976). This site binds Ca^{2+}/Mg^{2+} non-selectively and has a structural role (Reinach *et al.*, 1986; Goodwin *et al.*, 1990). It is distinct from the regulatory Ca^{2+}-specific site, unique to molluscan myosins, which is believed to be located on the ELC (Kwon *et al.*, 1990, 1992). The recent elucidation of the crystal structure of a vertebrate skeletal muscle myosin head and associated light chains (Rayment *et al.*, 1993) will greatly increase our understanding of the structure of the light chains and how they interact with the myosin head.

Data from mutagenesis studies are beginning to reveal information on the interactions between the RLC and the myosin head which are important for function. By constructing chimeras with subdomains swapped between gizzard and skeletal muscle myosin RLCs, it has been shown previously (Rowe and Kendrick-Jones, 1992) that subdomain 3 appears to be the part of the RLC that interacts with the scallop heavy chain–ELC complex to reform the specific Ca^{2+} binding site and thus regulate myosin function. These observations are consistent with those of Trybus and Chatman (1993) who also constructed chimeric mutants and demonstrated that the identity of the C-terminal domain dictates the ability of the RLC to mediate phosphorylation-dependent control of smooth muscle myosin motor function. Furthermore, Goodwin *et al.* (1990) showed that in scallop

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myosin substitution of the last 11 residues at the C-terminus of the RLC with an unrelated sequence led to a loss of regulation, but specific Ca^{2+} binding was retained. Therefore, interactions between the C-terminal subdomains of the RLC and the heavy chain appear to be crucial for myosin regulation.

In this paper a series of vertebrate smooth muscle myosin RLC mutants with successive deletions of three amino acids from the C-terminus have been constructed to explore further the role of this region of the RLC in myosin regulation. The regulatory properties of these RLC mutants were assessed by their ability to restore Ca^{2+} sensitivity to desensitized scallop myosin (Chantler and Szent-Györgyi, 1980). Scallop myosin is the only myosin where we can reversibly remove the RLCs and has thus been used as a model system to test the structural and functional properties of RLCs/mutants in many previous studies (Kendrick-Jones *et al.*, 1991). The obvious question is how appropriate is the scallop myosin system for testing the functional capability of these smooth muscle myosin RLC mutants? Several observations have suggested that in molluscan and vertebrate smooth/non-muscle myosins the regulatory systems mediated by the RLCs operate by similar basic mechanisms, although the regulatory signals differ. For instance, smooth/non-muscle myosin RLCs are able to mediate Ca^{2+} or phosphorylation-dependent regulation of scallop myosin function (Sellers *et al.*, 1980; Kendrick-Jones *et al.*, 1982). Both types of myosin are able to adopt a folded monomer (10S) conformation, where binding of the tail to the neck region of the myosin heads traps the products of ATP hydrolysis at the active site (Cross *et al.*, 1988; Ankrett *et al.*, 1991). In both systems, subfragment 1 (a single myosin head or motor domain) is unregulated, whereas heavy meromyosin (two heads attached to a portion of rod) is regulated, implying a crucial role for the second head domain or the subfragment 2 portion of the rod in regulation (Stafford *et al.*, 1979; Seidel, 1980). The underlying similarity between these regulatory systems suggests that the results obtained with RLC-desensitized scallop myosin hybrids are relevant to the situation in vertebrate smooth/non-muscle myosins where the regulatory signal is RLC phosphorylation. So using the desensitized scallop myosin system, it was found that C-terminal deletions of three to six amino acids had little effect on the ability of the gizzard RLC to restore Ca^{2+}-dependent regulation to the actomyosin MgATPase. Mutants with further deletions of nine to 15 amino acids were able to interact with the scallop heavy chain–ELC complex and fully restore Ca^{2+} binding, but were unable to suppress the actomyosin MgATPase in the absence of Ca^{2+}. RLCs with further deletions (up to 39 residues, i.e. the entire subdomain 4) still bound to scallop myosin but were non-functional. These findings indicate that the inhibitory and Ca^{2+} regulatory capabilities (operation of the on/off switch) of the gizzard RLC can be uncoupled and demonstrate that the C-terminal helix in the fourth subdomain of the RLC is essential for 'switching off' the myosin (inhibition) in the absence of Ca^{2+}.

Results

Construction and expression of the RLCs

The truncated gizzard RLCs were synthesized in *Escherichia coli* using the pMW172 vector (Way *et al.*, 1990). They were isolated in amounts ranging from 5 to 50 mg per litre of bacterial culture and could be purified to at least 95% purity (as judged by SDS–PAGE). Nine mutants were synthesized (Figure 1): eight with successive deletions of three residues from the C-terminus (denoted Cd3–Cd24) and one lacking 39 residues (Cd39). In Cd3 and Cd6, residues were removed from the highly charged acidic C-terminus of the RLC, while in Cd9–Cd15 the predicted C-terminal helix (helix H) was also truncated. In the mutants with further deletions (Cd18–Cd24) residues were also removed from the defunct Ca^{2+} binding loop, and in Cd39 the whole of the fourth subdomain was deleted. The deduced N-terminal amino acid sequences of the wild-type expressed RLC, and Cd3–Cd24 were identical to the N-terminal sequence of the native RLC purified from chicken gizzard tissue (Pearson *et al.*, 1984), except for the presence of an additional glycine residue at the N-terminus. This extra residue, which resulted from the subcloning procedure used to construct these mutants, was not present at the N-terminus of Cd39. All the expressed RLCs lacked the acetyl blocking group present on the N-terminal amino acid of the native gizzard RLC.

Binding of the RLCs to desensitized scallop myofibrils

To check whether the RLC mutants refolded into a conformation similar to that of the native protein, their binding affinities to desensitized scallop muscle myofibrils (DMFs) were measured. In the presence of Mg^{2+}, all native RLCs bind to scallop DMFs with such high affinity that it is impossible, using conventional binding assay techniques, to determine their true binding constants. For instance, they bind to scallop DMFs in a 1:1 molar ratio of RLCs to myosin heads when added at this equimolar ratio, even in the 10–100 nM range. Therefore, to compare the relative affinities of the RLC mutants for scallop DMFs the following procedure was employed: RLC–DMF hybrids (prepared by adding the RLCs to DMFs at a slight molar excess of RLCs over myosin heads) were washed with a Mg^{2+}-containing buffer at temperatures of 4, 12 and 25°C and analysed by PAGE to determine whether the RLCs remained bound to the myosin heads at the expected 1:1 molar ratio. Gel densitometric analysis revealed that, after washing at 4 and 12°C, all the expressed RLCs remained bound to scallop DMFs at a molar ratio of about 0.7 mol RLCs or more per mol of myosin heads (see Figure 2). After washing at 25°C the band intensity of Cd39 was reduced to a value of 0.4 mol RLC per mol of myosin heads, whereas the band intensities of Cd3–Cd12 were similar to those observed at the lower temperatures and there was only a slight reduction in Cd15–Cd24 ($<10\%$) (data not shown). Thus, Cd39 (but not Cd3–Cd24) was partially released from DMFs by washing at 25°C because of its decreased affinity for myosin heads.

Competitive scallop DMF binding assays were also used to estimate the relative myosin binding affinities of the RLC mutants. In these assays scallop DMFs were added to a mixture of two different RLCs in competition (at a molar ratio of 1 mol of each RLC per mol of myosin heads). The resulting RLC–DMF hybrids were washed extensively to remove any non-specifically bound RLCs and analysed by PAGE to determine the proportion of each RLC bound to the scallop DMFs. Previously it was shown that different RLCs compete for the same site on desensitized myosin

Fig. 1. Amino acid sequences of the wild-type (wt) gizzard RLC and C-terminal deletion mutants. Shown at the top is a schematic representation of the RLC with subdomains 1−4 stippled. The complete primary sequence of the fourth subdomain (Met129−Asp171) is shown and the positions of the ligating residues (X, Y, Z, −Y, −X, −Z) of the defunct Ca^{2+} binding loop and predicted α-helices G and H are indicated. The C-terminal sequences of the deletion mutants are also shown.

heads and do not bind to native scallop myosin (Messer and Kendrick-Jones, 1991). When bound to scallop DMFs in competition with the wild-type gizzard RLC, Cd3−Cd12 represented at least 40% of the total amount of RLCs that were associated with the myosin heads (Table I). Further deletions caused a progressive reduction in the proportion of each RLC mutant bound to scallop DMFs relative to the wild-type gizzard RLC (from 35% for Cd15 to only 5% for Cd39). Therefore in these mutants, sites on the RLC that are concerned with binding to the scallop heavy chain−ELC complex were either removed or conformationally altered such that the affinities of the RLCs for scallop DMFs were reduced.

Regulation of scallop myosin interaction with actin by the RLC mutants

The regulatory capabilities of the RLC mutants were assessed by measuring their abilities to restore Ca^{2+}-dependent function to scallop DMFs. The Ca^{2+} binding affinities of the RLC−DMF hybrids were measured at 2.1 μM Ca^{2+} in the presence of 2 mM Mg^{2+}. Under these conditions, scallop myofibrils bind 2 mol of Ca^{2+} per mol of myosin at the regulatory Ca^{2+}-specific sites located on the scallop heavy chain−ELC complex (Chantler and Szent-Györgyi, 1980; Kwon *et al.*, 1990). The Ca^{2+} binding affinities and steady-state actomyosin MgATPase activities of the RLC−DMF hybrids were determined at 12°C to ensure that a significant amount of Cd39 remained bound to the scallop DMFs during the course of these assays (see above).

The results of the Ca^{2+} binding and actomyosin MgATPase assays are shown in Table II. As expected, the scallop DMF control sample had a low Ca^{2+} binding affinity and its actomyosin MgATPase was elevated in the absence and presence of Ca^{2+} (Szent-Györgyi *et al.*, 1973). Rebinding the scallop or gizzard RLCs restored both high affinity Ca^{2+} binding and Ca^{2+} regulation, although the wild-type gizzard RLC−DMF hybrid had a reduced affinity for Ca^{2+} compared with the scallop RLC−DMF hybrid as previously observed (Sellers *et al.*, 1980). It was found that Cd3 and Cd6 restored Ca^{2+} sensitivity to DMFs, like the wild-type gizzard RLC, indicating that deletions of up to six residues from the C-terminus had relatively little effect on the regulatory capability of the RLC. Further deletions of nine to 15 residues disrupted the inhibitory function of the gizzard RLC without affecting its ability to restore Ca^{2+} binding. Therefore, the region deleted in these mutants (corresponding to the predicted C-terminal helix H of subdomain 4) is essential for the inhibitory function of the gizzard RLC. The mutants with deletions of 18−39 residues, although remaining bound to scallop DMFs during the course of these assays at a molar ratio of about 0.7 mol of RLC per mol of myosin heads (Figure 2), exhibited a progressively decreased ability to restore Ca^{2+} binding. Cd39 appeared to be completely non-functional. Because the

binding affinities of Cd18–Cd39 for scallop DMFs were also reduced (Table I), it is likely that the loss of their abilities to restore Ca^{2+} sensitivity to actin–myosin interactions are caused by either the gross disruption of their tertiary structures or the deletion of residues involved in stabilizing the conformation of the C-terminal domain of the RLC.

Regulation of scallop myosin solubility mediated by the RLCs

Under roughly physiological conditions (0.1–0.2 M NaCl in the presence of millimolar MgATP and at pH 7.0), Ca^{2+} can control scallop myosin filament assembly by regulating the equilibrium between folded 10S monomers, transient extended 6S monomers and filaments (Ankrett *et al.*, 1991). At >0.2 M NaCl the formation of 6S monomers and filaments is favoured, whereas <0.1 M NaCl filaments predominate (irrespective of Ca^{2+} levels). In a previous study to monitor Ca^{2+} regulation of filament assembly (Rowe and Kendrick-Jones, 1992), a sedimentation assay was used to measure the solubility of RLC-desensitized scallop myosin hybrids as a function of the NaCl concentration in the presence or absence of Ca^{2+} ($\pm Ca^{2+}$). Here, the same assay has been used to compare the abilities of the wild-type gizzard RLC and Cd15 to mediate Ca^{2+} regulation of scallop myosin filament assembly. At 0.1–0.2 M NaCl the solubility of native scallop myosin (at 0.5 mg/ml) was Ca^{2+}-dependent (Figure 3A) because it exists largely as the soluble 10S monomer in the absence of Ca^{2+} and as insoluble filaments in the presence of Ca^{2+} (Ankrett *et al.*, 1991). On removal of the RLCs, the resulting desensitized scallop myosin (DMY) was almost totally filamentous $\pm Ca^{2+}$, i.e. Ca^{2+} regulation of filament

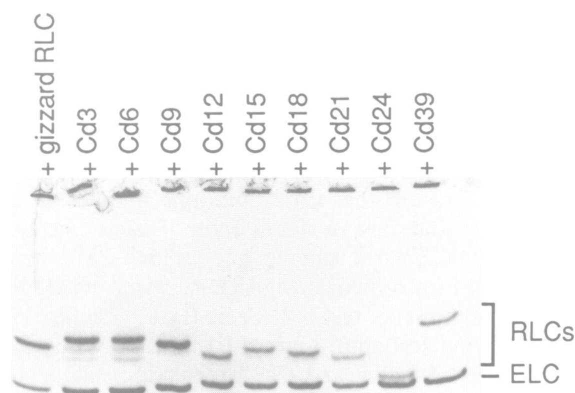

Fig. 2. Binding of the RLCs to DMFs analysed by urea–glycerol (10% polyacrylamide gel) PAGE. The RLCs were incubated overnight with DMFs at a 1.1:1 molar ratio (RLC:myosin heads). The resulting RLC–DMF hybrids were washed twice at 12°C with 50 volumes of a 50 mM NaCl, 4 mM $MgCl_2$, pH 7.0 buffer solution and samples of the final pellets were removed for electrophoresis. Note, on this gel the RLCs are separated on the basis of their charge and the DMF preparation (not shown) was completely devoid of its endogenous RLC (which would run just ahead of the gizzard RLC on these gels). The scallop ELC is not released by the 'desensitization' procedure and therefore serves as an internal control for quantitating the amount of each mutant RLC bound to the DMY. An ~1:1 molar ratio of mutant RLC to scallop ELC indicates maximum binding. The molar ratios (RLC:ELC) as measured by gel densitometry were as follows: wild-type gizzard RLC (1.21), Cd3 (0.97), Cd6 (0.90), Cd9 (1.09), Cd12 (0.78), Cd15 (0.77), Cd18 (0.80), Cd21 (0.68), Cd24 (0.72) and Cd39 (0.68). The minor bands running just ahead of Cd3 and Cd6 are RLC degradation products.

Table I. Competitive binding of two different RLCs to desensitized myofibrils

RLC mixes added	Band intensity of each RLC bound (scallop ELC = 1.0)	% Each RLC bound
gizzard RLC alone	1.55	–
gizzard RLC	0.90	57
Cd3	0.69	43
gizzard RLC	0.93	57
Cd6	0.70	43
gizzard RLC	1.00	58
Cd9	0.72	42
gizzard RLC	1.17	60
Cd12	0.78	40
gizzard RLC	1.08	65
Cd15	0.58	35
gizzard RLC	1.37	70
Cd18	0.59	30
gizzard RLC	1.39	75
Cd21	0.47	25
gizzard RLC	0.79	87
Cd24	0.12	13
gizzard RLC	1.41	95
Cd39	0.07	5

Table II. The ability of the RLCs to restore high affinity Ca^{2+} binding and Ca^{2+} regulation to desensitized scallop myofibrils

Desensitized myofibrils	Ca^{2+} binding at 2.1 μM Ca^{2+} (mol Ca^{2+}/mol myosin)	Actomyosin MgATPase (μmol H^+/min/mg)	
		$-Ca^{2+}$	$+Ca^{2+}$
control	0.56	0.12	0.12
+ scallop RLC	2.17	0.02	0.16
+ gizzard RLC	1.25	0.05	0.14
+ Cd3	1.17	0.04	0.13
+ Cd6	1.20	0.04	0.12
+ Cd9	1.29	0.06	0.11
+ Cd12	1.45	0.12	0.13
+ Cd15	1.45	0.10	0.12
+ Cd18	1.07	0.12	0.13
+ Cd21	0.87	0.11	0.13
+ Cd24	1.02	0.10	0.13
+ Cd39	0.57	0.13	0.12

A typical experiment using one DMF preparation is shown. This experiment was repeated three times with fresh preparations and all the above observations were confirmed, although the actual ATPase rates and Ca^{2+} binding values varied slightly between the preparations. Both sets of assays were carried out at 12°C to minimize the release of the mutant RLCs from the DMF–RLC hybrids. The amounts of each of the RLC mutants remaining bound at the end of the assays were measured and were similar to the values given in Figure 2, i.e. all the mutant RLCs, with the exception of Cd39, remained bound to the scallop DMFs at a molar ratio of at least 0.7 (the ratio for Cd39 was 0.6).

assembly was lost. The gizzard RLC was able to restore Ca^{2+}-dependence to the solubility of DMY because the gizzard RLC–DMY hybrid was partially soluble in the absence of Ca^{2+} and insoluble in the presence of Ca^{2+}. Examining the solubility curves of the Cd15–DMY hybrid (Figure 3B) revealed that this hybrid was almost totally insoluble $\pm Ca^{2+}$. In other words, Cd15 had lost the ability to promote the formation of 10S monomers in the absence of Ca^{2+}. Therefore, the C-terminal helix of the gizzard RLC is essential for inhibiting both myosin filament assembly and interaction with actin, i.e. for maintaining the myosin head in an inhibitory conformation.

Exchange of the RLCs into vertebrate smooth muscle myosin

Although it is not possible to selectively remove the RLCs from gizzard myosin, partial exchange of added RLCs into this myosin can be achieved under specific conditions (Morita *et al.*, 1991). When present in a large molar excess over myosin (20-fold molar excess), added wild-type gizzard RLCs can be incorporated into smooth muscle myosin in high levels (Rowe and Kendrick-Jones, 1992; Trybus and Chatman, 1993). Therefore, we explored whether it would be possible similarly to exchange the gizzard RLC C-terminal deletion mutants to study their effect on phosphorylation-dependent regulation of their parent myosin. It was found that the levels of exchange of Cd3 and Cd6 were at least 90%, implying that their affinities for the smooth muscle myosin were comparable with that of the native gizzard RLC. Further deletions of residues from the C-terminus, however, decreased the myosin binding affinity of the gizzard RLC; e.g. Cd9 was incorporated to a level of ~40% and no incorporation was detected for Cd12–Cd39.

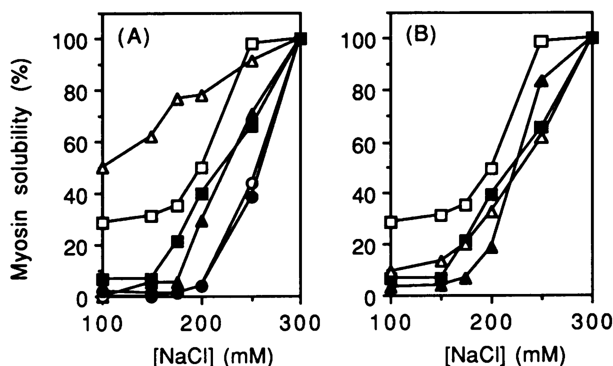

Fig. 3. Ca^{2+} regulation of the assembly of RLC-desensitized scallop myosin hybrids (RLC–DMY) into filaments. The filament assembly properties of the hybrids in the presence and absence of Ca^{2+} were determined by their solubility at 0.1–0.3 M NaCl (open symbols, in the absence of Ca^{2+}; closed symbols, in the presence of Ca^{2+}). **(A)** (△, ▲) native scallop myosin; (○, ●) desensitized myosin (DMY); (□, ■) gizzard RLC–DMY. **(B)** (□, ■) gizzard RLC–DMY; (△, ▲) Cd15–DMY. The RLCs were incubated overnight with desensitized scallop myosin at 0.5 mg/ml in a 0.5 M NaCl, 4 mM $MgCl_2$ (pH 7.0) buffer solution, then dialysed against a 0.1 M NaCl (pH 7.2) buffer solution in the presence of 2 mM MgATP and either 1 mM EGTA ($-Ca^{2+}$) or 0.1 mM $CaCl_2$ ($+Ca^{2+}$). The NaCl concentration was adjusted to 0.1–0.3 M to allow the myosin to equilibrate between soluble monomers and filaments. The samples were centrifuged to sediment myosin filaments and the initial solutions and supernatants run on SDS–PAGE. The amount of soluble myosin in the supernatants was determined by densitometry of the SDS–PAGE gels. Note, at 0.3 M NaCl all the myosins were 100% soluble.

After RLC exchange, the effects of phosphorylation by Ca^{2+}–calmodulin-dependent myosin light chain kinase on the actin-activated MgATPase activities of the gizzard myosins were measured. All the myosins, irrespective of whether they were exchanged with the wild-type gizzard RLC or Cd3–Cd9, could be fully phosphorylated and they exhibited actomyosin MgATPase activities that were activated by phosphorylation to the same extent. Unfortunately, because of the lower myosin binding affinities of Cd12–Cd39, we were unable to exchange any of these deletion mutants into smooth muscle myosin and thus we were unable to assess their effects on the actin-activated MgATPase of this myosin. These results suggest that the loss of inhibitory function of mutants Cd12–Cd39 when reconstituted with desensitized scallop myosin may be caused by the disruption of their correct tertiary structures.

Discussion

To explore the role of the C-terminus of the RLC in the regulation of vertebrate smooth muscle myosin, site-directed mutagenesis was employed to construct a series of gizzard RLC mutants each with successive C-terminal deletions of three amino acids. They were analysed using the desensitized scallop myosin test system. Our results show that the structural and functional properties of mutants Cd3 and Cd6 are similar to those of the wild-type gizzard RLC. For example, when added to scallop DMFs they bind with comparable affinity (Table I) and restore both Ca^{2+} binding and Ca^{2+} regulation to the DMFs in a quantitatively similar manner to the wild-type RLC (Table II). The high levels of exchange of these RLC mutants into intact chicken smooth muscle myosin (>90%) provide further strong evidence that their myosin binding affinities are comparable with that of the native RLC. Onishi *et al.* (1992) showed that in papain-digested smooth muscle myosin subfragment 1, the three aspartate residues at the C-terminus of the RLC (Asp168, Asp170 and Asp171; see Figure 1) can be crosslinked using a zero-length crosslinker to a lysine on the heavy chain (Lys841; Yanagisawa *et al.*, 1987) that lies very close to the C-terminal end of the myosin head. Our results with Cd3 and Cd6 indicate that if there are specific interactions between these residues in the heavy and light chains, as Onishi *et al.* (1992) suggested, then they are not necessary for either tight binding of the RLC to the heavy chain or inhibition/regulation of myosin interaction with actin.

Deletions in the predicted helix H of subdomain 4 (mutants Cd12 and Cd15) (see Figure 1) abolished the ability of the gizzard RLC to suppress the actomyosin MgATPase of scallop DMFs in the absence of Ca^{2+}, but did not affect its ability to restore Ca^{2+} binding (Table II). Therefore, this region is essential for the inhibitory behaviour of the gizzard RLC. The most plausible explanation for these results is that deletions in helix H alter the structure of the C-terminal domain of the RLC such that it is no longer able to attain an inhibitory conformation in the absence of Ca^{2+}. The close correlation between the loss of inhibitory capability of the deletion mutants (Table II) and the reduction in their heavy chain binding affinities (as assessed by the levels of RLC exchange into smooth muscle myosin) support this explanation. Further support is provided by the previous protein engineering studies of Reinach *et al.* (1986) and Goodwin *et al.* (1990) where they showed that RLCs with

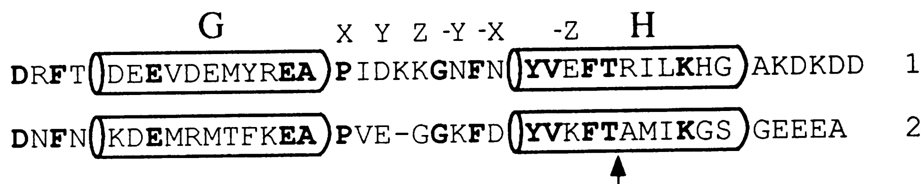

Fig. 4. Alignment of the C-terminal subdomain 4 sequences of the gizzard (1) and scallop (2) RLCs. The positions of helices G and H and the ligating residues in the defunct Ca^{2+} binding loop of subdomain 4 are shown. Identical residues in both sequences are shown in bold type. Goodwin *et al.* (1990) reported that a scallop RLC mutant, where the last 11 residues of the C-terminus were substituted with a sequence of 15 unrelated amino acids, restored Ca^{2+} binding but not Ca^{2+} regulation to DMFs. The position of the substitution in the scallop RLC sequence corresponds exactly to the region deleted in our gizzard RLC mutant, Cd12 (as indicated by the arrow).

point mutations in the Ca^{2+} binding loop in subdomain 1 were unable to regulate scallop myosin function in the absence of Ca^{2+}. Thus the ability to 'switch off' the myosin head (maintain an inhibited state) must require a specific conformation of the RLC involving a number of precise interactions between its N- and C-terminal subdomains.

Although, at present, only limited information is available on the 3-D structure of the light chains, the high degree of sequence homology with EF-hands (subdomains) in parvalbumin, calmodulin and troponin-C (whose structures are known) provides useful models for predicting possible interactions between the different subdomains in the RLCs. The pattern of hydrophobic contacts identified in the crystal structures of troponin and calmodulin (Strynadka and James, 1989) is well conserved in the gizzard RLC and so one may predict that in this RLC, helix H forms a hydrophobic interface with helix E of subdomain 3, as well as with helix G in subdomain 4. The hydrophobic interactions stabilizing helices E and H involve conserved Phe residues (Phe106 and Phe158 in the gizzard RLC). Thus on the basis of this structural data it becomes clear why deletions in helix H destabilize subdomain 3, a region of the RLC known to play a crucial role in Ca^{2+} regulation (Rowe and Kendrick-Jones, 1992). Interestingly Goodwin *et al.* (1990) showed that substitution of the last 11 residues at the C-terminus of scallop RLC with an unrelated sequence of 15 amino acids yielded a mutant that was able to restore Ca^{2+} binding but not Ca^{2+} regulation to scallop DMFs. These results indicate that disruption of helix H, without deletion of the residues involved in hydrophobic interactions with neighbouring helices, is sufficient to abolish Ca^{2+} regulation. Because the substitution in the scallop RLC mutant begins at the same position in helix H as the deletion in our gizzard RLC mutant Cd12 (Figure 4) it is not surprising that both types of mutants have similar properties, i.e. in both cases Ca^{2+} regulation was lost.

The gizzard RLC mutants with deletions of 18–39 residues exhibited a progressively decreased ability to restore Ca^{2+} binding, as well as being unable to regulate the actomyosin MgATPase of DMFs (Table II). Although these RLC mutants remained bound to myosin heads during the course of the assays, their heavy chain binding affinities were also reduced. In the reported structures of the EF-hand proteins each domain is stabilized by interactions between the intimately-associated subdomains (EF-hands), i.e. subdomains 1 with 2 and 3 with 4 (reviewed by Strynadka and James, 1989). Therefore in those RLC mutants with the largest deletions, such as Cd39 where all subdomain 4 has been deleted, the loss of all regulatory functions must be caused by the severe disruption in the structure of the C-terminal domain of the molecule.

In summary, by employing a site-directed mutagenesis approach it has been shown that helix H in subdomain 4 of the smooth muscle myosin RLC is essential for the inhibition of myosin function under resting conditions. Structural data from related EF-hand proteins (Strynadka and James, 1989) predict that this helix is crucial for stabilizing the structure of the RLC so that an 'OFF' (inhibitory) conformation can be achieved. In vertebrate smooth/non-muscle myosins, phosphorylation at the N-terminus of the RLCs must somehow overcome this inhibited state so that the myosin is able to assemble into stable filaments and interact with actin to produce the force necessary to drive cellular contractile events such as cytokinesis and smooth muscle contraction. So far, biochemical and molecular biological studies have provided the most revealing insights into this regulatory switch and now that the 3-D structure of the myosin head has just been revealed (Rayment *et al.*, 1993), they should begin to uncover the fine details of the mechanisms involved.

Materials and methods

Materials

Analytical grade reagents, used throughout, were obtained from BDH Chemicals Ltd (Poole, UK), Bethesda Research Laboratories Inc. (Gaithersburg, MD) and Sigma Chemical Co. Ltd (Poole, UK). Enzymes, supplied by New England Biolabs (Beverly, MA) and Promega Corporation (Madison, WI) were used according to the manufacturer's standard assay conditions. Live scallops (*Pecten maximus*) were supplied by the Marine Biological Station, Millport, UK. Desensitized scallop myofibrils and myosin were prepared from the striated adductor muscle, as described by Chantler and Szent-Györgyi (1980) and Ankrett *et al.* (1991), respectively. Chicken gizzards were obtained from G.W.Padley Ltd, Bury St. Edmunds, UK and myosin was prepared from them as described (Kendrick-Jones *et al.*, 1983). Native scallop and gizzard RLCs were prepared as outlined in Kendrick-Jones *et al.* (1976). Chicken skeletal muscle actin, bovine brain calmodulin and chicken gizzard myosin light chain kinase were prepared as described in Kendrick-Jones *et al.* (1983). Radiochemicals were supplied by Amersham International (Amersham, UK). Oligonucleotides were synthesized on an Applied Biosystems 380B automated synthesizer (Applied Biosystems Inc., Foster City, CA) by Jan Fogg and Terry Smith (Laboratory of Molecular Biology, Cambridge, UK).

Construction, expression and purification of the RLCs

Unless otherwise stated, all DNA manipulations were performed as described by Sambrook *et al.* (1989). M13 site-directed mutagenesis was carried out by the method of Kunkel *et al.* (1987) using the *dut⁻ ung⁻ E.coli* strain CJ236 which incorporates uracil in place of thymine. The mutants were selected by DNA sequencing. The gizzard RLC clone, plasmid CGX2 (Messer and Kendrick-Jones, 1991), was digested with *Bam*HI and *Eco*RI and the DNA fragment containing the gizzard RLC coding sequence subcloned into M13mp19. Site-directed mutagenesis was performed using an oligonucleotide 5′ AGGGCCCCCAAGGGATCCAGCAAACGT to obtain a clone (M13CGX2BamHI) containing a *Bam*HI site at the start of the gizzard RLC coding sequence. To construct the mutants Cd3–Cd24, mutagenesis was performed using M13CGX2BamHI as template DNA and oligonucleotide primers (36 oligonucleotides long) designed to generate the

appropriate C-terminal deletion. For example, the oligonucleotide 5′ GGC-AACTTCAACTATGTGTAGAGCTGAGAGCCGCCC was used to construct Cd15. Mutant clones were digested with *Bam*HI and *Eco*RI and cDNA fragments containing the RLC coding sequences ligated into the expression vector, pMW172 (Way *et al.*, 1990). Cd39 was generated by PCR using the wild-type gizzard RLC–pMW172 construct (Rowe and Kendrick-Jones, 1992) as template DNA and the following oligonucleotide primers: 5′ ATCGATCGAGTCGACCATATGTCCAGCAAACGTGCC-AAAGCC and 5′ AGCCATCGAGAATTCATCGATCTCCCATGGTGG-TCAGCAGTT. The primers were designed to insert *Sal*I and *Nde*I sites at the 5′ end of the RLC coding sequence and a stop codon (TGA) and *Eco*RI site at the end of the third subdomain. The PCR product was digested with *Sal*I and *Eco*RI and ligated into M13mp18. A clone of the correct full-length sequence was digested with *Nde*I and *Eco*RI and ligated into pMW172.

The RLC–pMW172 vectors were expressed in *E.coli* BL21(DE3) cells (Studier and Moffat, 1986). Transformed colonies were inoculated into 2 × TY medium containing 100 μg/ml ampicillin, grown overnight at 34°C then induced with 0.6 mM IPTG and grown for a further 2 h. The cells were harvested by centrifugation and resuspended in 25% sucrose, 2 mM EDTA, 2 mM EGTA, 50 mM Tris–HCl, pH 8.0, plus a battery of protease inhibitors (Citi and Kendrick-Jones, 1986). The cells were lysed with 3% trichloroacetic acid to inactivate bacterial proteases and the precipitated proteins pelleted. The pellet was resuspended in Tris–HCl, pH 7.5. and dialysed against 0.5 mM $MgCl_2$, 0.5 mM DTT, 50 mM Tris–HCl, pH 7.5. Solid urea was added to 6 M final concentration and the insoluble material removed by centrifugation. The RLCs were purified further by chromatography on DEAE–cellulose ion exchange and Sephacryl S200 gel filtration columns as described previously (Rowe and Kendrick-Jones, 1992).

Analysis of the RLCs

Competitive scallop DMF binding assays were performed as follows: scallop DMFs were added to a mixture of two different RLCs at a molar ratio of 1 mol of each RLC per mol of myosin heads (at a final concentration of 1 mg/ml DMFs) in wash buffer (0.1 M NaCl, 4 mM $MgCl_2$, 1 mM DTT, 10 mM Na phosphate, pH 7.0). After incubation overnight at 4°C with gentle stirring the non-specifically bound RLCs were removed by washing three times with 50 volumes of the above 0.1 M NaCl buffer. The final myofibril pellets were analysed by urea–glycerol PAGE and the amounts of the RLC mutants and scallop ELC bound to the myosin heads measured by gel densitometry. A ~1:1 ratio of RLC to scallop ELC indicates full binding.

The RLC–DMF or RLC–DMY hybrids were prepared by adding the RLCs to scallop DMF or DMY at a molar ratio of 1.1:1 (mol RLC:myosin heads) in wash buffer (at a final concentration of 1 mg/ml DMF or DMY) and incubating overnight at 4°C. The myofibrils or myosin were then washed twice with a 50-fold excess of wash buffer containing 50 mM NaCl. Their actomyosin MgATPase activities were measured by monitoring proton liberation in a pH stat at pH 7.8 and 12°C (to ensure that the mutant RLCs remain bound) in 10 ml of 30 mM KCl, 5 mM $MgCl_2$, 1.5 mM ATP and either 0.2 mM EGTA ($-Ca^{2+}$) or 0.2 mM EGTA and 0.22 mM $CaCl_2$ ($+Ca^{2+}$) using 1 mg of myofibrils and 0.5 mg of rabbit skeletal muscle actin filaments per assay. Each ATPase assay was repeated twice $\pm Ca^{2+}$ and a third time if the rates differed by >10%. The calcium binding affinities of the RLC–DMF hybrids were measured in triplicate using a $^{45}Ca^{2+}$–EGTA buffer system with free [Ca^{2+}] at 2.1 μM and using the binding assay described by Chantler and Szent-Györgyi (1980). To calibrate the assay system Ca^{2+} binding measurements, on the wild-type, smooth and scallop RLC–DMF hybrids and DMF control at free [Ca^{2+}] from 10^{-5} to 10^{-7} M, were carried out. Myosin filament assembly in the absence and presence of Ca^{2+} was monitored using a sedimentation assay to measure the solubility of the RLC–DMY hybrids as a function of the NaCl concentration as described previously (Rowe and Kendrick-Jones, 1992). The myosin hybrids were centrifuged at 100 000 *g* for 20 min in a Beckman TL-100 ultracentrifuge (Beckman Instruments Inc., Fullerton, CA) to sediment the filamentous myosin, and the supernatants were analysed by SDS–PAGE. The amount of soluble myosin in each supernatant was determined by gel densitometry of the stained PAGE gels using a Molecular Dynamics model 300A computing densitometer (Molecular Dynamics, Sunnyvale, CA).

Exchange of the RLCs into smooth muscle myosin

The RLCs were incorporated into chicken gizzard myosin under similar conditions to those described by Morita *et al.* (1991). The myosin (1 mg/ml) was incubated in the presence of a 20-fold molar excess of added RLCs in 0.5 M NaCl, 30 mM Tris–HCl, pH 7.5, 10 mM EDTA, 10 mM ATP, 10 mM DTT at 42°C for 30 min. After exchange, the myosins were reprecipitated by dialysis against 30 mM NaCl, 5 mM $MgCl_2$, 1 mM DTT, 10 mM imidazole, pH 6.5. The reprecipitated myosins were washed twice with 50 volumes of the above dialysis buffer to remove the unincorporated RLCs then resolubilized in 0.6 M NaCl, 2 mM $MgCl_2$, 1 mM DTT, 10 mM imidazole, pH 7.0, centrifuged at 200 000 *g* for 1 h and filtered through a 0.2 μm filter (Minisart-Sartorius AG, Göttingen, Germany). Samples of the clarified myosins were run on 20% polyacrylamide SDS gels which enabled the deletion mutants to be resolved from the native RLC and thus permitted the levels of RLC incorporation to be quantified by densitometry. The myosins were phosphorylated by incubating them at 25°C for 10 min in a solution containing purified chicken gizzard myosin light chain kinase (0.01 mg/ml), purified calmodulin (0.02 mg/ml), 0.2 mM $CaCl_2$, 2 mM MgATP, 0.1 M NaCl, 1 mM DTT, 10 mM Tris–HCl, pH 7.5. The actin-activated MgATPase activities of the smooth muscle myosin were measured at 37°C [with a 4:1 (w/w) excess of rabbit actin over myosin] by the isotopic [^{32}P]phosphate assay (Seals *et al.*, 1978).

Gel electrophoresis

SDS–PAGE (mini 5–20% acrylamide gradient gels) and urea–glycerol (10% polyacrylamide, 40% glycerol) gels were run as described [Matsudaira and Burgess (1978) and Perrie and Perry (1970), respectively].

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