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Isolation of the regulatory domain of scallop myosin: Role of the essential light chain in calcium binding

(muscle/calcium regulation/renaturation)

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ABSTRACT **The regulatory domain of scallop myosin, consisting of a regulatory light chain (R-LC), an essential light chain (E-LC), and a portion of heavy chain, occupies the neck region of myosin. This domain is directly involved in the regulation of molluscan muscle contraction, which is triggered by direct Ca^{2+} binding to myosin. We have isolated a soluble functional complex (regulatory complex) comprised of R-LC, E-LC, and a 10-kDa heavy chain fragment in a 1:1:1 stoichiometry by clostripain digestion of the myosin head (papain subfragment 1). N termini of the heavy chain fragments were either leucine-812 or valine-817. The isolated complex retained the specific Ca^{2+}-binding site and bound Ca^{2+} with a similar affinity and selectivity as myosin. The individual components of the regulatory complex were isolated after complete denaturation with guanidine hydrochloride. The regulatory complex was reconstituted from isolated light chains and the heavy chain fragment. The renatured complex regained Ca^{2+} binding quantitatively. To elucidate the function of the E-LC in Ca^{2+} binding, we constructed hybrid regulatory complexes. The hybrid complexes reconstituted with molluscan E-LC and R-LC regained the specific Ca^{2+}-binding site, whereas the hybrid complex formed with rabbit skeletal E-LC [alkali LC 2 (A2-LC)] and scallop R-LC did not. The results demonstrate that E-LCs from myosins regulated by direct Ca^{2+} binding are required for the specific Ca^{2+} binding in the molluscan muscle.**

Contraction of molluscan muscle is triggered by direct Ca^{2+} binding to myosin (1). Molluscan myosin is a regulated molecule that has a specific Ca^{2+}-binding site on the globular head region [subfragment 1 (S1)]. In the absence of Ca^{2+}, the interaction between actin and myosin is suppressed by the regulatory light chain (R-LC), a subunit of myosin, and the muscle remains in the relaxed state. The binding of Ca^{2+} to the specific Ca^{2+}-binding site of S1 releases the inhibition, resulting in muscle contraction.

Each myosin head contains two different subunits: a R-LC and an essential light chain (E-LC). Both types of light chains (LCs) belong to a family of Ca^{2+}-binding proteins such as parvalbumin and troponin C (2, 3) and are involved in Ca^{2+} regulation. The functional role of the R-LC has been well established since the R-LCs of scallop myosin can be removed and replaced without loss of contractile activity. When R-LCs are removed from scallop myosin, the specific Ca^{2+}-binding site and Ca^{2+} sensitivity of the actin-activated ATPase are lost—i.e., the actin-activated ATPase in the absence of Ca^{2+} is no longer inhibited (4, 5). Correspondingly, Ca^{2+} sensitivity of tension generation requires R-LCs in bundles of skinned fibers (6). R-LCs isolated from scallop or other myosins can rebind to desensitized myosin. These hybrids regain the specific Ca^{2+}-binding site and Ca^{2+} sensitivity only when the source of the LC is a regulated myosin (7, 8). Therefore, R-LCs of regulated and unregulated myosins differ functionally, although all R-LCs have a common attachment site(s) to the heavy chain.

Although R-LC is required for the specific Ca^{2+} binding, this subunit does not contain the specific Ca^{2+}-binding site. Primary sequence analysis indicates that R-LCs have only one potential divalent cation-binding site. However, this site is a nonspecific divalent cation-binding site that is present in the R-LCs of regulated and unregulated myosins (9). Site-directed mutagenesis, which destroys the potential divalent cation-binding site of scallop R-LC, does not abolish the specific Ca^{2+}-binding site when this subunit of scallop myosin is replaced by the mutant R-LC, although Ca^{2+} sensitivity is not restored (10).

E-LCs are also implicated in Ca^{2+} regulation because antibodies specific to the E-LC can desensitize scallop myosin (11). One possible function of the E-LCs could be to provide the specific Ca^{2+}-binding site. The E-LCs of molluscan myosins contain a potential divalent cation-binding site. This site is not present in the E-LCs of those myosins that are not regulated by direct Ca^{2+} binding to myosin (12). Since isolated E-LCs do not bind Ca^{2+} selectively, the contribution of the E-LC to specific Ca^{2+} binding should be evaluated on scallop myosins hybridized with foreign E-LCs. If the E-LC is a necessary constituent of the specific Ca^{2+}-binding site, only the E-LCs from Ca^{2+}-binding myosins could be able to form such a site; hybrids formed with E-LCs of unregulated myosins would not be able to bind Ca^{2+}. Because of the difficulty of replacing scallop E-LCs with foreign E-LCs without denaturation of scallop myosin, it has not been possible so far to test directly the function of these subunits in specific Ca^{2+} binding.

Both types of LCs are located close together in the neck region of myosin (13, 14) and form a Ca^{2+}-binding regulatory region of the molecule together with a portion of the heavy chain. This region was first visualized by electron microscopy of a proteolytic fragment of scallop myosin produced by digestion with a protease from *Pseudomonas aeruginosa* (15). The fragment consists of the complete rod heavy chain joined to a C-terminal head domain associated with the R-LC and E-LC. When scallop papain S1 is extensively digested by trypsin, a heavy chain fragment of around 14 kDa associated with E-LC and R-LC is formed and this complex (called here the regulatory complex) is resistant to further digestion (16). As in scallop myosin, the binding of R-LC to the complex requires the presence of divalent cations (16). The intrinsic tryptophan fluorescence change of the regulatory complex

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Abbreviations: LC, light chain; E-LC, essential LC; R-LC, regulatory LC; S1, subfragment 1; A2-LC, alkali LC 2.

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induced by Ca^{2+} suggests that the complex can bind Ca^{2+} (17).

We show here that when isolated, the regulatory complex quantitatively retains its specific Ca^{2+}-binding site. Taking advantage of the structural simplicity of the isolated complex, we have developed a method to reconstitute the functional regulatory domain from isolated LCs and heavy chain fragment. It is possible, therefore, to examine the function of the E-LCs in specific Ca^{2+} binding by constructing hybrid regulatory complexes. We find that a hybrid regulatory complex containing both types of the molluscan *Mercenaria mercenaria* myosin LCs binds Ca^{2+}, whereas another hybrid containing rabbit skeletal E-LC [alkali LC 2 (A2-LC)] and scallop R-LC does not.

MATERIALS AND METHODS

Chemicals. $^{45}Ca^{2+}$ solution was purchased from DuPont/NEN. Clostripain was purchased from Sigma and papain was from Boehringer Mannheim.

Protein Preparation. Scallop myosin and papain S1 were prepared according to Stafford *et al.* (18). Scallop and *Mercenaria mercenaria* LCs were prepared as described by Kendrick-Jones *et al.* (19); rabbit skeletal A2-LC was prepared as described by Wagner and Weeds (20).

Preparation of the Regulatory Domain. Scallop papain S1 (10–20 mg/ml) in digestion buffer (20 mM NaCl/3 mM $MgCl_2$/0.1 mM EGTA/2 mM $CaCl_2$/0.5 mM dithiothreitol/5 mM sodium P_i, pH 7.0) was digested at 20°C for 45 min with 1:100 (wt/wt) activated clostripain. EGTA and iodoacetic acid were added to 10 mM and 5 mM, respectively, to terminate the reaction. After bringing the dithiothreitol concentration to 10 mM, insoluble proteins were removed by centrifugation at 48,000 × *g* for 30 min. The supernatant was applied to a Sephadex G-75 exclusion column (32 × 800 mm) equilibrated in 10 mM NaCl/3 mM $MgCl_2$/0.1 mM EGTA/0.2 mM $CaCl_2$/1 mM NaN_3/5 mM sodium P_i, pH 7.0. The fractions of the second peak containing mostly regulatory complex were pooled and applied to a Fast-Q (Pharmacia) ion-exchange column (8 × 200 mm) equilibrated in 25 mM NaCl/3 mM $MgCl_2$/0.2 mM $CaCl_2$/5% PEG 400/5 mM sodium P_i, pH 7.0. The regulatory complex eluted as a single peak with a NaCl gradient of 25 mM to 1 M. The regulatory complex was concentrated by ultrafiltration (Omegacell 30k, Pharmacia; Centricon 30, Amicon).

Preparation of Heavy Chain Fragment. Heavy chain fragment was isolated by reverse-phase HPLC (Hi-pore RP-318; Bio-Rad) after complete denaturation with 6 M guanidine hydrochloride. Since the heavy chain fragment aggregates in the absence of denaturing agent, it was stored in acetonitrile. N-terminal sequences of two heavy chain fragments were determined using a microsequencing technique (21).

Ca^{2+}-Binding Assay. Ca^{2+} binding of the regulatory complex was measured using a gel filtration method adapted from Hummel and Dreyer (22). Forty-five microliters of regulatory fragment (15–20 mg/ml) equilibrated with pCa buffer (20 mM NaCl/2 mM $MgCl_2$/30 mM Hepes, pH 7.6) was mixed with 5 μl of pCa buffer containing 1.1 mM $^{45}Ca^{2+}$ (2 μCi/ml; 1 Ci = 37 GBq) and sufficient EGTA to bring pCa to the desired value. The protein was applied to a Sephadex G-10 column (3 × 400 mm) equilibrated with corresponding pCa buffers containing 0.11 mM $^{45}Ca^{2+}$ (0.2 μCi/ml) at room temperature. Fractions of five drops were collected in preweighed scintillation vials. Ten microliters of each sample was taken out for measurement of protein concentration by the Bradford method. The samples were immediately weighed and assessed for radioactivity. Excess Ca^{2+} due to binding was followed by a dip in Ca^{2+} concentration as reported by Hummel and Dreyer (22).

Ca^{2+} binding of myosin was measured according to Chantler *et al.* (23) except that the included volume was determined by weighing the centrifuged pellet and imidazole was replaced by Hepes buffer.

Renaturation of the Regulatory Domain. Both types of LCs were completely reduced at 20°C for 30 min in 6 M guanidine hydrochloride/10 mM dithiothreitol/10 mM Hepes, pH 7.9. After bringing the urea concentration to 6 M with solid urea, heavy chain fragment (0.5–1.0 mg/ml) was dialyzed overnight at 4°C against renaturation solution (40 mM NaCl/1 mM $MgCl_2$/0.1 mM EGTA/0.2 mM dithiothreitol/10 mM Hepes, pH 7.0) containing 6 M urea. Aggregated protein was removed by centrifugation. The heavy chain fragment was mixed with a stoichiometric amount of E-LC and dialyzed against renaturation solution containing 0.5 M urea for >5 hr at 4°C. A stoichiometric amount of R-LC was added and the regulatory complex was renatured by dialysis overnight at 4°C against renaturation solution. After removing aggregated proteins by centrifugation, the renatured regulatory complex was isolated by Fast-Q ion-exchange chromatography and gel-exclusion chromatography as described above. In protection experiments, the renatured regulatory complex or free R-LC (2 mg/ml) was digested with 1:50 (wt/wt) clostripain in digestion buffer at 20°C for 10–30 min.

RESULTS

Isolation of the Regulatory Domain. The regulatory complex was prepared by extensive digestion of scallop papain S1 with clostripain (Fig. 1). The advantage of using clostripain over trypsin was that the regulatory complex was completely protected from clostripain digestion, so that a relatively homogeneous population of the complex could be obtained in high yield after extensive proteolysis. A major fraction of digested fragments became insoluble after clostripain digestion and was readily separated by centrifugation from the

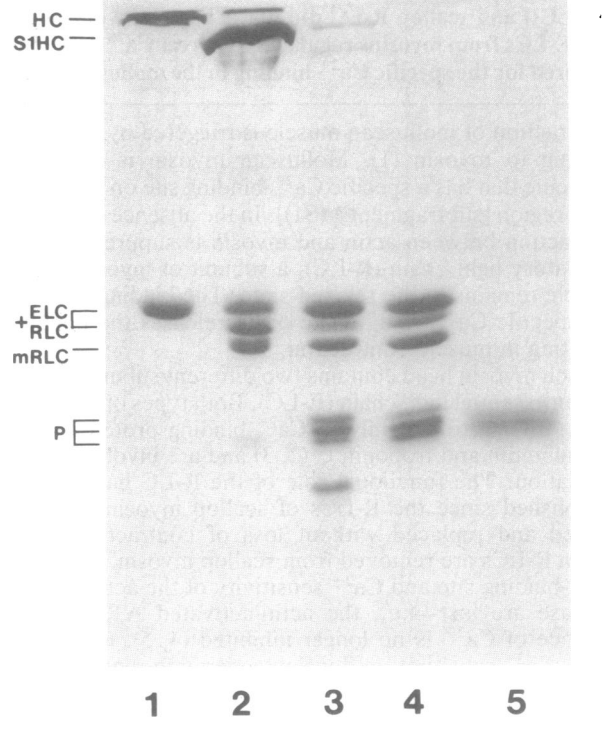

FIG. 1. Isolation of the regulatory domain: SDS/PAGE of myosin (lane 1), papain S1 (lane 2), the supernatant of whole digestion after centrifugation (lane 3), the regulatory complex (lane 4), and the heavy chain fragments (lane 5). HC, myosin heavy chain; S1HC, S1 heavy chain; mRLC, modified R-LC; P, heavy chain fragment.

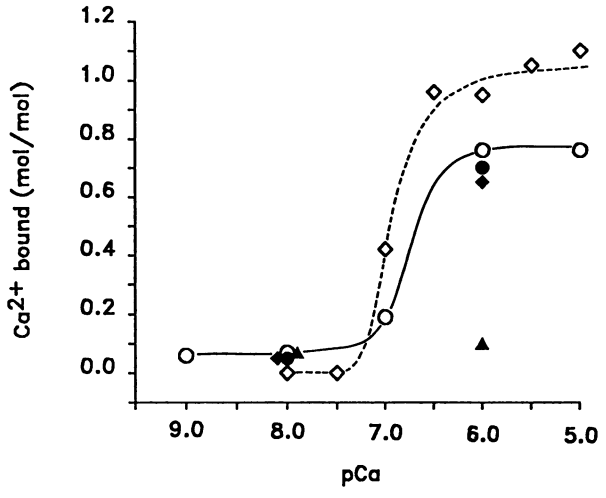

FIG. 2. Ca^{2+} binding by the regulatory domain. ◇, Myosin; ○, the regulatory complex; ●, the renatured regulatory complex; ◆, the renatured hybrid regulatory complex with *Mercenaria* E-LC and R-LC; ▲, the renatured hybrid regulatory complex with rabbit skeletal A2-LC and scallop R-LC. The unit of Ca^{2+} binding by myosin is mol/mol of myosin head.

soluble regulatory complex. The yield of the purified regulatory complex was nearly one-third of the theoretical maximum. The complex could be concentrated by ultrafiltration to 20 mg/ml or more without aggregation. The complex contained an intact E-LC and a R-LC that was clipped during papain digestion (mR-LC). It appeared that three major heavy chain fragments were formed, each weighing around 10 kDa. N-terminal sequences of two of the heavy chain fragments have been determined and localized as ^{812}Leu-Gly-Thr-Thr-Lys-Val-Phe-Phe-Lys-Ala-Gly-Val-Leu-Gly-Asn-Leu-Glu-Glu and ^{817}Val-Phe-Phe-Lys-Ala-Gly-Val-Leu-Gly-Asn-Leu-Glu-Glu based on homology with other myosin sequences (24). Therefore, the binding sites for both LCs fall within Val-817 and the C terminus of scallop S1. This finding was in general agreement with the study by Mitchell *et al.* (25), which localized the binding sites of R-LC within Gly-780 and the C terminus of S1 in rabbit skeletal myosin.

Ca^{2+} Binding by the Regulatory Domain. The isolated complex bound Ca^{2+} with a similar specificity and affinity as myosin (Fig. 2). Ca^{2+} was bound with 0.76 mol/mol stoichiometry and Ca^{2+} binding was saturated at about pCa 6.0 in the presence of 2 mM $MgCl_2$ at pH 7.6. Thus, the regulatory domain retained much of the intact Ca^{2+}-binding site even after extensive digestion. Control studies with parvalbumin showed a binding of 1.8 mol of Ca^{2+} per mol of protein in the presence of 2 mM $MgCl_2$ saturating at pCa 6.0. Bovine serum albumin did not bind Ca^{2+}.

Renaturation of the Regulatory Domain. The regulatory complex contained stoichiometric amounts of R-LC, E-LC, and the heavy chain fragment. The heavy chain fragment could be isolated from the LCs by the use of 6 M guanidine hydrochloride. The heavy chain fragment was dialyzed against 6 M urea. The regulatory complex was reconstituted from stoichiometric amounts of isolated R-LC, E-LC, and the heavy chain fragment by stepwise removal of urea. Without LCs, the heavy chain fragment aggregated during renaturation; in contrast, aggregation was prevented by the binding of LCs. The renatured regulatory complex was isolated from unbound components by Fast-Q ion-exchange chromatography. The renatured complex eluted at the same position as the native one and contained heavy chain fragment and both types of LCs in a 1:1:1 stoichiometry as estimated by SDS and urea gel electrophoresis (Fig. 3). Further evidence that the renatured complex regained its original conformation was its resistance to degradation by clostripain under conditions in which free R-LC was completely digested (Fig. 3*a*). The specific Ca^{2+}-binding site was also restored by renaturation: Ca^{2+} was bound in a 0.7 mol/mol stoichiometry at pCa 6.0, with only negligible binding at pCa 8.0 (Fig. 2).

Construction of Hybrid Regulatory Domain. To study the contribution of E-LCs to specific Ca^{2+} binding, we have constructed two hybrid regulatory complexes, one with *Mercenaria mercenaria* E-LC and R-LC and the other with rabbit skeletal E-LC (A2-LC) and scallop R-LC. Both of the renatured hybrids contained stoichiometric amounts of E-LC, R-LC, and scallop heavy chain fragment (Fig. 3). The hybrid regulatory complex with *Mercenaria* E-LC and R-LCs bound Ca^{2+} stoichiometrically at pCa 6.0 but not at pCa 8.0. In contrast, the hybrid complex formed from rabbit skeletal A2-LC and scallop R-LC did not bind Ca^{2+} either at pCa 6.0 or at pCa 8.0 (Fig. 2). Since *Mercenaria* myosin is Ca^{2+} regulated and rabbit skeletal myosin is not, we conclude that E-LCs from myosin regulated by direct Ca^{2+} binding are necessary to constitute a specific Ca^{2+}-binding site. These results also suggest that the binding domain for the heavy

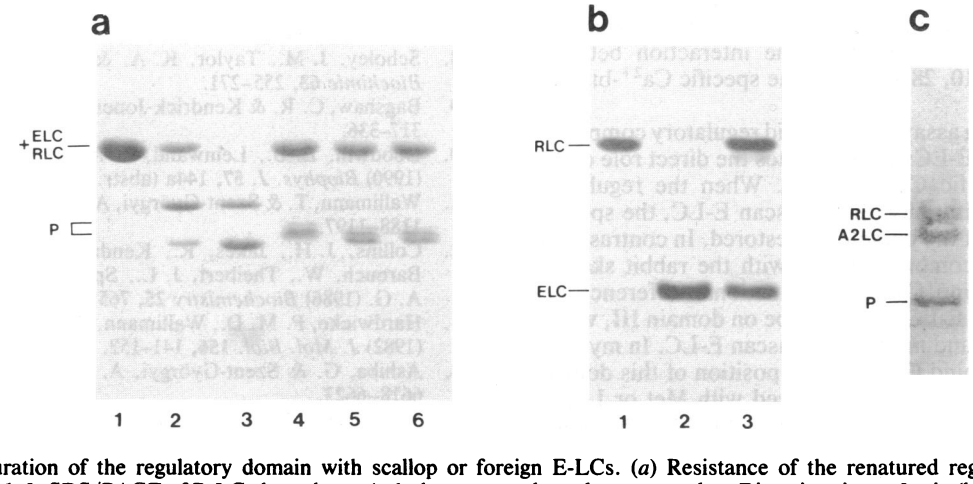

FIG. 3. Renaturation of the regulatory domain with scallop or foreign E-LCs. (*a*) Resistance of the renatured regulatory complex to proteolysis. Lanes 1–3, SDS/PAGE of R-LC alone; lanes 4–6, the renatured regulatory complex. Digestion times: 0 min (lanes 1 and 4), 10 min (lanes 2 and 5), 30 min (lanes 3 and 6). (*b*) Urea/polyacrylamide gel of the renatured regulatory complex. Lane 1, R-LC; lane 2, E-LC; lane 3, the renatured regulatory complex. Heavy chain fragment was not detected in the urea gel. (*c*) SDS/PAGE of the renatured hybrid regulatory complex with rabbit skeletal A2-LC and scallop R-LC. The ratio of heavy chain fragment:A2-LC:R-LC was determined by densitometry as 1:0.89:0.95. P, heavy chain fragment.

chain is similar in E-LCs of regulated and unregulated myosins.

DISCUSSION

Our results indicate that the regulatory domain of scallop myosin may consist of a 10-kDa region of the heavy chain together with the R-LC and E-LC in 1:1 stoichiometry. This domain is in the neck region of the myosin head (15). It has an elongated structure that may extend as far as 100 Å from the head/rod junction toward the tip of S1 as judged from electron microscopic studies of myosins treated with polyclonal antibodies against LCs (26). It contains the specific Ca^{2+}-binding site of scallop myosin that is responsible for Ca^{2+} activation in the intact molecule. The mutual protection of the heavy chain and the LCs from proteolysis allows the isolation of this domain from S1. Since the Ca^{2+}-binding affinity of the regulatory domain was completely preserved in this fragment (the regulatory complex), long-range interactions within the heavy chain are not necessary to produce the Ca^{2+}-binding site and the structural integrity of the Ca^{2+}-binding site does not appear to be influenced by the other functional sites of the heavy chain such as those involved in actin binding and ATPase activity. The regulatory complex remains soluble even at high protein concentrations (20 mg/ml) without significant aggregation and is well suited for functional and structural studies of the specific Ca^{2+}-binding site of scallop myosin.

The regulatory complex dissociates into its components in the presence of denaturing agents; the heavy chain fragment can thus be isolated from the LCs. Upon renaturation, the heavy chain fragment recombines stoichiometrically with the LCs. The heavy chain fragment alone aggregates during renaturation and the binding of LCs is required to keep it soluble. This solubilizing action can be taken as a measure of the rebinding of LCs. The renatured regulatory complex fully regains the specific Ca^{2+}-binding site and the solubility properties of the untreated complex. The preparation is therefore uniquely suited for studying the role of E-LC in the specific Ca^{2+} binding since it has not been possible previously to remove E-LC from scallop myosin without denaturing the heavy chain.

Both LCs have four homologous regions that are related to the Ca^{2+}-binding domain (E-F hand motif) found in parvalbumin and troponin C (2, 3, 27). In R-LCs, deletions and nonconservative substitutions at critical positions have altered divalent cation-binding sites of three domains, so that only the N-terminal domain binds divalent cations. Although the divalent cation-binding site in the N-terminal domain of R-LC is essential for regulating the interaction between myosin and actin (10, 28), it is not the specific Ca^{2+}-binding site of myosin (9).

The Ca^{2+}-binding assay of the hybrid regulatory complexes containing foreign E-LCs demonstrates the direct role of the E-LC in the specific Ca^{2+} binding. When the regulatory complex is reconstituted with molluscan E-LC, the specific Ca^{2+}-binding site of the complex is restored. In contrast, the hybrid regulatory complex formed with the rabbit skeletal A2-LC does not bind Ca^{2+}. The functional difference between two types of E-LCs seems to be on domain III, which has an intact E-F hand motif in molluscan E-LC. In myosins that are unable to bind Ca^{2+}, the -X position of this domain has a critical mutation: Ser is replaced with Met or Leu, a mutation that destroys the E-F hand motif (12). Therefore, the role of the E-LCs in the specific Ca^{2+} binding is most likely to provide the specific Ca^{2+}-binding site. This is further supported by the finding that the amino acid sequence of the heavy chain fragment deduced from cDNA clones of scallop adductor myosin does not appear to contain an E-F hand motif (29).

The Ca^{2+}-binding site in scallop myosin is unusual since three peptides are needed to form it. It is possible that Ca^{2+} is bound directly to all three peptides. Another possibility is that Ca^{2+} is bound only to the E-F hand present in the molluscan E-LC and that the other two peptides stabilize this configuration. The stabilization interaction is very specific since R-LCs from unregulated myosins will not substitute.

It is not yet clear whether the differences between regulated and unregulated myosins are fully accounted for by the demonstrated differences in their R-LCs and E-LCs. The scallop heavy chain fragment may also provide a unique—and as yet unknown—contribution to the specific Ca^{2+}-binding site and Ca^{2+} regulation. It is possible, however, that its major structural role is to bind to and position the LCs near each other to create the recognition site required for these functions.

The LCs have previously been localized near the head/rod junction in a variety of muscle myosins (26, 30, 31). The binding sites of R-LCs for myosin heavy chain are preserved among different species, and the R-LC of scallop myosin can be replaced with the R-LCs from all of the myosins so far tested (7, 8, 19, 32). It is also likely that E-LCs have common binding sites since the rabbit skeletal A2-LC and *Mercenaria* E-LC can combine stoichiometrically with the scallop heavy chain fragment and R-LC. The regulatory domain may be a common structural feature of many myosins. Except for the Ca^{2+} regulation of molluscan myosin, no other functions of this domain have yet been demonstrated. Structural and functional studies of this region will be of particular interest since this region is the only physical connection of the actin-binding sites to the head/rod junction.

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