Role of essential light chain EF hand domains in calcium binding and regulation of scallop myosin

(muscle contraction/helix-loop-helix/ATPase/mutagenesis)

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ABSTRACT The specific Ca\(^{2+}\) binding site that triggers contraction of molluscan muscle requires the presence of an essential light chain (ELC) from a Ca\(^{2+}\) binding myosin. Of the four EF hand-like domains in molluscan ELCs, only domain III has an amino acid sequence predicted to be capable of binding Ca\(^{2+}\). In this report, we have used mutant ELCs to locate the Ca\(^{2+}\) binding site in scallop myosin and to probe the role of the ELC in regulation. Point mutations in domain III of scallop ELC have no effect on Ca\(^{2+}\) binding. Interestingly, scallop and rat cardiac ELC chimeras support Ca\(^{2+}\) binding only if domain I is scallop. These results are nevertheless in agreement with structural studies on a prototypic fragment of scopelous myosin, the regulatory domain. Furthermore, Ca\(^{2+}\) sensitivity of the scallop myosin ATPase requires scallop ELC domain I: ELCs containing cardiac domain I convert scallop myosin to an unregulated molecule whose activity is no longer repressed in the absence of Ca\(^{2+}\). Despite its unusual EF hand domain sequence, our data indicate that the unique and required contribution of molluscan ELCs to Ca\(^{2+}\) binding and regulation of molluscan myosins resides exclusively in domain I.

Muscle myosins are highly conserved in overall morphology and subunit composition, consisting of two heavy chains, two essential light chains (ELCs), and two regulatory light chains (RLCs). The C-terminal portions of the heavy chains are wrapped around each other in an α-helical coiled-coil “tail,” and the N termini form two pear-shaped globular “heads.” Each head contains the actin binding sites, ATPase activity, and binding sites for one of each type of light chain. Despite these similarities, myosins from different muscle types are functionally distinct. A distinguishing feature of molluscan myosins is that each head possesses one specific, high-affinity Ca\(^{2+}\) binding site that nonmolluscan (e.g., vertebrate skeletal, cardiac, and smooth muscle) myosins lack. Ca\(^{2+}\) binding to these sites triggers contraction in molluscan muscle (reviewed in ref. 1).

Although all three myosin subunits are required for Ca\(^{2+}\) binding and for regulation, hybrid studies with foreign light chains on scallop regulatory domain (RD), a prototypic fragment of scallop myosin, have indicated that the Ca\(^{2+}\) binding site may be located in the ELC. Scallop RD is composed of the two light chains together with a short fragment of the heavy chain (HCF) and lacks the actin and ATP binding sites of myosin but does retain the specific Ca\(^{2+}\) binding site. Ca\(^{2+}\) binding is retained by reconstituted RDs only if the source of ELC is from a Ca\(^{2+}\) binding myosin (2, 3).

Myosin light chains are members of the EF hand superfamily of proteins, and each consists of four EF hand-like domains (4–6). The EF hand is a protein sequence motif that potentially may bind divalent cation. The inability to do so is apparently due to deletions, insertions, or substitutions of critical amino acids. The canonical EF hand consists of two α-helices flanking a 12-amino acid Ca\(^{2+}\) binding “loop” (see Fig. 1A) (reviewed in ref. 7). Molluscan ELCs are predicted to be capable of binding divalent cation only in domain III, and therefore the contraction-triggering Ca\(^{2+}\) binding site has been expected to be located in this domain. In the predicted Ca\(^{2+}\) binding loop of domain III (see Fig. 1A), position −X is occupied by a conserved serine in molluscan ELCs, whereas in nonmolluscan ELCs this residue is methionine or leucine. Given the requirement of an oxygen-containing side group at this position in known Ca\(^{2+}\) binding EF hand domains, it has been proposed that this amino acid difference accounts for the inability of nonmolluscan myosins to bind Ca\(^{2+}\) (8). Despite these predictions based on sequence homology, recent crystallographic studies on scallop RD have indicated the presence of a divalent cation in domain I, but not domain III, of the ELC (9). The sequence of domain I is extremely divergent from the canonical EF hand loop (see Fig. 1A) and has been considered incapable of binding divalent cations (8, 10). The observation of a divalent cation in this domain in the crystal structure necessitates further biochemical studies on the ELC.

In this paper, we have probed the role(s) of scallop ELC EF hand domains in scallop myosin function by using mutant ELCs. Our results indicate that domain III is not the location of the Ca\(^{2+}\) binding site. In contrast, we find that Ca\(^{2+}\) binding by scallop RD and Ca\(^{2+}\) sensitivity of the scallop myosin ATPase require that domain I be scallop sequence. Preliminary reports of this work have appeared (11–14).

MATERIALS AND METHODS

Light Chain Constructs. Site-directed mutagenesis was done by PCR under conditions recommended by the manufacturers (Perkin–Elmer and Stratagene). Internal point mutations were introduced by the megaprimer method (13). Plasmid constructs were sequenced across the ELC coding region. All other methods for manipulating DNA were performed as described (16).

The scallop ELC cDNA (17) was subcloned into the NdeI site of pMW172 (18) to encode a nonfusion protein. Scallop ELCs with the amino acid substitutions D94A, S102M, and −ech (a fusion of EDE to the C terminus) were subcloned into the NdeI and EcoRI sites of pMW172. The rat cardiac ventricular ELC cDNA in pTZ19R (19) was subcloned between the NeoI and HindIII sites of pMW172. This construct encodes the cardiac ELC with a short fusion of 3 amino acids (MGS) at the N terminus.

To construct the scallop ELC and cardiac ELC chimeras, Apr II sites were introduced between domain I and domain II of each of the two ELCs, and Age I sites were introduced...
between domains II and III. An Nde I site overlapping the translation initiation codon was introduced at the 5' end of the cardiac ELC. The appropriate DNA fragments were ligated into pMW172 to generate chimeric ELCs expressed as non-fusion proteins. The amino acid sequences across the domain I–II junctions are scallop INPR (residues 42–46), cardiac QNPTQ (residues 89–93) ScI–CII,ScIII,CIV INPRQ, and CII–ScIII,ScIV,ScV QNPR. The sequences across the domain II–III junctions are scallop EQGT (residues 79–83), cardiac DTGY (residues 130–134), ScI,ScIV–CII,ScIV ETGT, and CII,ScIII–ScIV,ScV DTGT.

Light Chain Expression and Purification. ELCs were bacterially expressed and purified as described for the scallop RLC (20), except that all ELCs were further purified to >90% purity by reverse-phase chromatography (ProRPC, Pharmacia). Yields varied from 10 to 40 mg of pure protein per liter of cell culture.

Electrophoresis. Protein samples were electrophoresed on urea gels (12.5% polyacrylamide, pH 8.6) (21) and on SDS/15% polyacrylamide gels (22).

Reconstitution of Regulatory Domains and Ca2+ Binding. ELCs were reconstituted with scallop HCF and scallop or clam RLC as described (2, 23). Light chain/HCF stoichiometries were obtained from densitometry of SDS and urea gels. Ca2+ binding was measured under equilibrium conditions at 4°C either by gel filtration (2) or by equilibrium dialysis (23). Ca2+ binding measurements were performed at 10 mM and 1 μM free Ca2+ in the presence of 2 or 10 mM MgCl2. Ca2+ binding measurements by reconstituted RDs were normalized to native RD controls obtained in all experiments.

ELC Exchange. Desensitized myosin was prepared by removing RLCs from scallop myosin (24). Either 5-fold or 50-fold molar excess of reduced ELCs was added to desensitized myosin (typically 2–3 mg of myosin) in exchange buffer [600 mM NaCl/0.1 mM EDTA/2 mM dithiothreitol (DTT)/2 mM ATP/25% (vol/vol) glycerol/10 mM Hepes, pH 7.6] and incubated with gentle stirring for 4 min at 41–42°C. Samples were then placed on ice, diluted with 12 vol of 3 mM MgCl2/0.1 mM DTT/5 mM sodium P2, pH 6.5, and collected in a Beckman GPR tabletop centrifuge (5000 x g; 15 min). The myosin-containing pellets were resuspended gently and washed with 40 mM NaCl/3 mM sodium azide/1 mM MgCl2/5 mM sodium P2, pH 7.0. The myosin was recombined with RLC as described (20).

Other. The actin-activated ATPase rates were measured by proton liberation in the absence and presence of Ca2+ (20). Bay scallops (Argopecten irradians) were obtained from the Nantucket Marine Department (Nantucket, MA) and the Marine Biological Laboratory (Woods Hole, MA). Scallop myosin (25), native scallop RD (2), rabbit actin (26), and clam (Mercenaria mercenaria) RLC (27) were prepared as described. Protein amounts were determined by the method of Bradford (Bio-Rad) (28) with bovine serum albumin as a standard. ELC sequences were aligned by the PILEUP program of the University of Wisconsin Genetics Computer Group, version 7.0 (29).

RESULTS

Effects of Point Mutations in Domain III on Calcium Binding. Since the sequences of molluscan ELCs indicate the presence of a competent EF hand in domain III (Fig. 1A), we tested whether this domain constitutes the specific Ca2+ binding site by substituting alanine for aspartate at the +X position (D94A). Likewise, methionine was substituted for the molluscan-specific serine at the −X position in the scallop ELC (S102M).

Surprisingly, Ca2+ binding was neither reduced nor affected in the reconstituted RDs containing either D94A or S102M mutant ELCs (Fig. 2B), indicating that oxygen-containing side

![Fig. 1](https://example.com/fig1.png)

**Fig. 1.** ELC constructs. (A) Amino acid sequences of the predicted EF hand loops of the four domains of scallop ELC. Targets of amino acid substitutions described in the text are underlined. Consensus sequence for the canonical EF hand Ca2+ binding loop is shown below, with six liganding residues precisely spaced at positions x, y, z, −y, −x, −z. Amino acids shown are the required residues at the positions indicated. −O, oxygen-containing side group; +, hydrophobic residue, * amino acids with side-chain character not important for Ca2+ binding. (B) Schematic drawing of ELC chimeras (solid bars, scallop ELC; open bars, rat cardiac ELC). Roman numerals indicate EF hand domains. Amino acid positions of native scallop and rat cardiac ELCs that correspond to the domain junctions in the chimeras are indicated.
domain I of the scallop ELC, despite its unusual sequence (Fig. 1A), functions as the contraction-triggering Ca$^{2+}$ binding site of scallop myosin.

**Regulation by Mutant ELCs.** We have probed the role of the ELC in regulation by assaying mutant ELCs for their effects on Ca$^{2+}$ sensitivity of scallop myosin ATPase activity. We attempted to remove the ELC from scallop myosin by treatments that reversibly remove the ELC from skeletal muscle myosin (ammonium chloride) (30) or the ELC from smooth muscle myosin (trifluoperazine) (31). However, both of these methods denatured scallop myosin (data not shown). Therefore, we used molar excesses of mutant ELCs to exchange them onto scallop myosin. ELC exchange required removal of the RLC as observed (24). The different wild-type and mutant ELCs exchanged to various levels (Table 1). Ca$^{2+}$ sensitivity of ATPase activity was measured on a mixed population of myosin, containing both endogenous and exogenous ELCs. The net charges of the cardiac and chimeric ELCs all differed sufficiently from the endogenous scallop ELC such that exchange levels could be monitored by urea gel electrophoresis (Fig. 3A). We also constructed a scallop ELC with three additional negatively charged amino acids in order to follow the efficiency of exchange (−chg; Fig. 3A).

Exposure of scallop myosin to 41–42°C for 4 min was optimal for efficient ELC exchange without greatly interfering with RLC rebinding. Control experiments in which scallop myosin was reconstituted with less than stoichiometric amounts of the ELC established that full Ca$^{2+}$ sensitivity requires an RLC/ELC stoichiometry in excess of 0.80 and that Ca$^{2+}$ sensitivity is linearly proportional to RLC content (data not shown). Under these conditions, the −chg ELC exchanged to high levels (=54%), RLC was fully rebound, and Ca$^{2+}$ binding and Ca$^{2+}$ sensitivity were not affected (Fig. 3).

Levels of exchange sufficient to assay Ca$^{2+}$ sensitivity of the scallop myosin actin-activated MgATPase were obtained with all mutant ELCs tested. Only rat cardiac ELC failed to exchange to a measurable degree (<10%). Exchange of the ScI–CII,III,IV chimera was somewhat improved by increasing the amount of mutant ELC from 5-fold to 50-fold molar excess (Table 1). Exchange of the remaining mutant ELCs was done with 5-fold molar excess ELC. The limited exchange we obtained with the chimeric ELCs indicates that portions of the scallop ELC sequence throughout the entire protein contribute to the affinity of the scallop ELC for scallop heavy chain.

**Table 1.** Ca$^{2+}$ dependence of ATPase of hybrid myosins

<table>
<thead>
<tr>
<th>Myosin</th>
<th>n + Ca$^{2+}$</th>
<th>Ca$^{2+}$ Ratio</th>
<th>% ELC exchange</th>
</tr>
</thead>
<tbody>
<tr>
<td>Myosin</td>
<td>7 310 ± 60</td>
<td>31 ± 25</td>
<td>0.10</td>
</tr>
<tr>
<td>Myosin (− RLC)</td>
<td>7 177 ± 38</td>
<td>207 ± 60</td>
<td>1.17</td>
</tr>
<tr>
<td>Myosin (+ RLC)</td>
<td>6 249 ± 66</td>
<td>24 ± 25</td>
<td>0.10</td>
</tr>
<tr>
<td>−chg ELC</td>
<td>5 304 ± 62</td>
<td>25 ± 9</td>
<td>0.11 ± 4</td>
</tr>
<tr>
<td>ScI–CII,III,IV</td>
<td>4 277 ± 92</td>
<td>15 ± 5</td>
<td>0.05</td>
</tr>
<tr>
<td>ScI–CII,III,IV</td>
<td>4 286 ± 136</td>
<td>16 ± 7</td>
<td>0.06</td>
</tr>
<tr>
<td>ScI–CII,III,IV</td>
<td>3 253 ± 32</td>
<td>71 ± 9</td>
<td>0.28</td>
</tr>
<tr>
<td>ScI–CII,III,IV</td>
<td>2 225</td>
<td>140</td>
<td>0.62 ± 29</td>
</tr>
</tbody>
</table>

Myosin was desensitized by RLC removal and exchanged with mutant ELCs, and RLC was readded as described. Top three lines show data for control myosins exposed to exchange conditions without added ELC. Myosins containing mutant ELCs rebound RLC similarly to control myosin subjected to exchange conditions. RLC/ELC ratios exceeded 90% of controls with the exception of the ScI–CII,III,IV mutant, where RLC reuptake was only 70% and 72% of control values. Actin-activated ATPase is in mol of H+ per mg per min. Low ATPase ratios indicate great Ca$^{2+}$ sensitivity; increase in ratio signifies loss of Ca$^{2+}$ sensitivity. ELCs were exchanged with a 5-fold excess of mutant ELCs. Included is a single experiment where a 50-fold excess of ScI–CII,III,IV resulted in 27% exchange without diminishing Ca$^{2+}$ sensitivity. n, Number of experiments.
Sequence comparison to EF hand Ca\(^{2+}\) binding proteins suggested that domain III of the scallop ELC should form the high-affinity Ca\(^{2+}\) binding site. However, targeted substitution of amino acids thought to be important for Ca\(^{2+}\) binding by EF hand domains had no effect on Ca\(^{2+}\) binding. These results are supported by the crystal structure of scallop RD, where there is no indication of a divalent cation in domain III (9). In fact, the structure of this domain appears to be distorted relative to the canonical EF hand structure (9).

Although the sequence of domain I of the scallop ELC is unusual for a Ca\(^{2+}\) binding EF hand domain (see Fig. 1A), our results with chimeric ELCs indicate that the specific sequence contribution of the scallop ELC to Ca\(^{2+}\) binding by scallop myosin resides exclusively in this domain.

Ca\(^{2+}\) sensitivity of ATPase activity was retained by hybrid myosins containing chimeric ELCs with a scallop domain I (ScI-ScII,ScIII,ScIV or ScII-ScI,ScIII,ScIV) showed full Ca\(^{2+}\) sensitivity of the actin-activated MgATPase (open bars). Untreated scallop myosin (column 1), myosin after native scallop ELC exchange (column 2), after –chg exchange (column 3), after ScI-ScII,ScIII,ScIV exchange (column 4), after ScII-ScI-ScIII,ScIV exchange (column 5), after C32-ScII,ScIII,ScIV exchange (column 6), and after C32-ScII,ScIII,ScIV exchange (column 7). % Ca\(^{2+}\) sensitivity = (1 – EGTA rate/Ca\(^{2+}\) rate) × 100 (32).

After exchange, myosins containing chimeric ELCs with a scallop domain I (ScI-ScII,ScIII,ScIV or ScII-ScI,ScIII,ScIV) showed full Ca\(^{2+}\) sensitivity of the actin-activated MgATPase. In contrast, myosins containing chimeric ELCs with a cardiac domain I (C1-ScII,ScIII,ScIV or C2-ScII,ScIII,ScIV) showed reduced Ca\(^{2+}\) sensitivity, and the loss of sensitivity correlated with levels of exchange (Table 1 and Fig. 3). RLC was fully rebind by myosin containing the C32-ScII,ScIII,ScIV chimera. Although RLC rebind to myosin containing the C32-ScII,ScIII,ScIV chimera was somewhat reduced, the loss of Ca\(^{2+}\) sensitivity by myosin containing this chimera was significantly greater than that which could be accounted for by the lowered RLC content (Table 1 and Fig. 3B). The loss of Ca\(^{2+}\) sensitivity with the C32-ScII,ScIII,ScIV and C32-ScII,ScIII,ScIV chimeras was mainly due to an increase in the ATPase activity in the absence of Ca\(^{2+}\) (Table 1). The presence of Ca\(^{2+}\) did not greatly alter the ATPase activity of hybrid myosins containing these two chimeras, although small changes cannot be excluded because of the limited ELC exchange (Table 1).

**DISCUSSION**

Contraction of molluscan muscle is triggered by Ca\(^{2+}\) binding to myosin. The Ca\(^{2+}\)-dependent movement of scallop myosin *in vitro* (33) as well as Ca\(^{2+}\)-dependent tension development by permeabilized scallop muscle fiber bundles (34) has established that Ca\(^{2+}\) sensitivity of the myosin MgATPase is a good measure of regulation of contraction. In this report, we have explored the role of ELC EF hand domains in Ca\(^{2+}\) binding and regulation of scallop myosin.

![Fig. 3. ELC exchange onto scallop myosin. (A)](image)

**Fig. 3. ELC exchange onto scallop myosin. (A)** Urea/poly-acrylamide gel electrophoresis. Positions of scallop RLC and endogenous scallop ELC are indicated. Untreated scallop myosin (lane 1), myosin with scallop RLC removed (lane 2), myosin with scallop RLC readded (lane 3), myosin after –chg exchange (lane 4; –chg migrates faster than scallop ELC), after ScI-ScII,ScIII,ScIV exchange (lane 5; chimera migrates slower than scallop ELC), after ScII,ScI-ScIII,ScIV exchange (lane 6; chimera migrates faster than scallop ELC), and after C32-ScII,ScIII,ScIV exchange (lane 7; chimera migrates slower than both scallop ELC and RLC). (B) Percentage exchange of mutant ELCs (solid bars) and percentage Ca\(^{2+}\) sensitivity of actin-activated MgATPase (open bars). Untreated scallop myosin (column 1), myosin after native scallop ELC exchange (column 2), after –chg exchange (column 3), after ScI-ScII,ScIII,ScIV exchange (column 4), after ScII,ScI-ScIII,ScIV exchange (column 5), after C32-ScII,ScIII,ScIV exchange (column 6), and after C32-ScII,ScIII,ScIV exchange (column 7). % Ca\(^{2+}\) sensitivity = (1 – EGTA rate/Ca\(^{2+}\) rate) × 100 (32).

Sequence comparison to EF hand Ca\(^{2+}\) binding proteins suggested that domain III of the scallop ELC should form the high-affinity Ca\(^{2+}\) binding site. However, targeted substitution of amino acids thought to be important for Ca\(^{2+}\) binding by EF hand domains had no effect on Ca\(^{2+}\) binding. These results are supported by the crystal structure of scallop RD, where there is no indication of a divalent cation in domain III (9). In fact, the structure of this domain appears to be distorted relative to the canonical EF hand structure (9).

Although the sequence of domain I of the scallop ELC is unusual for a Ca\(^{2+}\) binding EF hand domain (see Fig. 1A), our results with chimeric ELCs indicate that the specific sequence contribution of the scallop ELC to Ca\(^{2+}\) binding by scallop myosin resides exclusively in this domain.

Ca\(^{2+}\) sensitivity of ATPase activity was retained by hybrid myosins containing chimeric ELCs with a scallop domain I (ScI-ScII,ScIII,ScIV or ScII-ScI,ScIII,ScIV), while Ca\(^{2+}\) sensitivity was lost by myosins containing chimeric ELCs with a cardiac domain I (C1-ScII,ScIII,ScIV or C2-ScII,ScIII,ScIV). These results indicate that, in addition to its crucial role in forming the specific Ca\(^{2+}\) binding site, domain I of the scallop ELC constitutes the unique and required contribution of the scallop ELC to regulation of scallop myosin.

In none of the mutant ELCs have we tested uncouple Ca\(^{2+}\) binding and regulation. Furthermore, the loss of Ca\(^{2+}\) sensitivity was the result of elevated activity at low Ca\(^{2+}\) concentrations. These observations contrast with previous studies on RLC function in scallop myosin, where the presence of an RLC that fails to restore the specific Ca\(^{2+}\) binding site inhibits ATPase activity in the absence and presence of Ca\(^{2+}\) (35). Our data suggest that a functional (i.e., molluscan) domain I is required for both suppression of the myosin ATPase in the absence of Ca\(^{2+}\) (the characteristic feature of the resting state in molluscan muscle) as well as activation of the ATPase by binding Ca\(^{2+}\).

The amino acid sequence of domain I of molluscan ELCs is divergent from the canonical EF hand, and crystallographic studies on scallop RD (9) indicate that the structure of domain I is also unusual. In the current structural model (9), the Ca\(^{2+}\) coordinating loop consists of 9 contiguous amino acids in domain I of the scallop ELC; residues 19–27 (DFWDGR-DGA) (see Fig. 1A). All three aspartate residues bind Ca\(^{2+}\) via their side chains, but an inordinately large number of bonds are via main-chain carbonyls (from D19, G23, and A27). We note that domain I of the intestinal Ca\(^{2+}\) binding protein is also anomalous, although in a different fashion, with four carbonyl oxygens contributing to the Ca\(^{2+}\) coordination (36). Many nonmolluscan ELCs (including the rat cardiac ELC) have aspartate (or glutamate) at the positions corresponding to D19 and D25; therefore, the preservation of these residues does not fully account for the unique ability of molluscan ELCs to bind Ca\(^{2+}\). We note, however, that five residues in the Ca\(^{2+}\) binding loop (FWDGDR) are fully conserved among, and unique to, the six molluscan ELC sequences known. Although the function of the tryptophan in this cluster is not known, it may account for the increase in intrinsic tryptophan fluorescence associated with the light chain binding domain (RD) upon Ca\(^{2+}\) binding to scallop myosin (37). We also note two residues (K32 and C39 in the scallop ELC) in the C-terminal helix of domain I (helix B) that are specific to molluscan ELCs. At least a subset of these conserved amino acids is likely to be the molecular basis for the functional differences between molluscan vs. nonmolluscan ELCs.

We have shown that the contraction-triggering Ca\(^{2+}\) binding site in scallop myosin is located in domain I, not domain III, of the scallop ELC. These findings complement crystallographic data on scallop RD (9). We also establish that the unique and required contribution of the scallop ELC to regulation of scallop myosin resides exclusively in domain I. The location of the Ca\(^{2+}\) binding site in an unusual EF hand
domain, and the lack of Ca\(^{2+}\) binding by domain III (a "normal" EF hand), calls into question previous assumptions about divalent cation capacity of EF hand domains based on their sequence. A more precise understanding of the molecular basis for the functional differences between molluscan and nonmolluscan ELCs may come from future mutagenesis and structural studies.

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