Rapid Communication *J. Biochem.* 131, 641–645 (2002)

Essential Light Chain Modulates Phosphorylation-Dependent Regulation of Smooth Muscle Myosin[1]

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Received March 15, 2002; accepted March 29, 2002

To examine the functional role of the essential light chain (ELC) in the phosphorylation-dependent regulation of smooth muscle myosin, we replace the native light chain in smooth muscle myosin with bacterially expressed chimeric ELCs in which one or two of the four helix-loop-helix domains of chicken gizzard ELC were substituted by the corresponding domains of scallop (*Aquipecten irradians*) ELC. All of these myosins, regardless of the ELC mutations or regulatory light chain (RLC) phosphorylation, showed normal subunit constitutions and NH_4^+/EDTA-ATPase activities, both of which were similar to those of native myosin. None of the ELC mutations changed the actin-activated ATPase activity of myosin in the absence of RLC phosphorylation. However, in the presence of RLC phosphorylation, the substitution of domain 1 or 2 in the ELC significantly decreased the actin-activated ATPase activity, whereas the substitution of both of these domains did not change the activity. In contrast to myosin, the domain 2 substitution in the ELC did not affect the actin-activated ATPase activity of single-headed myosin subfragment 1. These results suggest an interhead interaction between domains 1 and 2 of ELCs which is required to attain the full actin-activated ATPase activity of smooth muscle myosin in the presence of RLC phosphorylation.

Key words: actin-activated ATPase, essential light chain, phosphorylation-dependent regulation, smooth muscle, smooth muscle myosin.

The smooth muscle myosin molecule comprises two heavy chains, two essential light chains (ELC), and two regulatory light chains (RLC). These polypeptide chains form a double-headed structure with two globular heads connected to an α-helical coiled coil tail. Each head consists of the N-terminal half of a heavy chain and both types of light chains. The heavy chain comprises a motor domain containing an ATPase site and an actin binding site, an ELC binding domain, and an RLC binding domain from the N-terminus. Both light chains are members of the calmodulin superfamily that contains four helix-loop-helix domains (referred to as domains 1–4 from the N-terminus), and bind to the heavy chain with an N-terminal lobe composed of domains 1 and 2, and a C-terminal lobe composed of domains 3 and 4 (*1*). The tail is further divided into two domains, the subfragment-2 (S2) domain composed of the N-terminal one-third of the tail and the light meromyosin domain composed of the rest of the tail, which is responsible for the filament assembly.

The motor function of smooth muscle myosin is regulated through RLC phosphorylation. In the absence of RLC phosphorylation, myosin exhibits low actin-activated ATPase activity and is unable to move actin filaments. The phosphorylation enhances the ATPase activity more than 20-fold and enables the movement of actin filaments (for a review, see Ref. *2*). The phosphorylation-dependent regulation of smooth muscle myosin is thought to require various intramolecular interactions between its domains. A double-headed fragment of myosin, heavy meromyosin (HMM), which lacks a light meromyosin domain, fully retains the phosphorylation-dependent regulation, whereas a single head fragment, subfragment-1 (S1), has completely lost the regulation and is always in the on state (*3*). Single-headed constructs of which the tails are not long enough to stabilize the dimeric forms also show poor regulation (*4–6*). Similarly, single-headed myosin in which one of the heads is removed by proteolysis is impaired in the phosphorylation-dependent regulation (*7–9*). In addition, a truncated myosin lacking a specific length of the S2 domain from the head–tail junction shows partial regulation with an incomplete off state in the absence of RLC phosphorylation (*6*). These results suggest that head–head and/or head–S2 interactions are required for the completely off state of smooth muscle myosin. Recently, single motor domain HMMs with two RLCs were reported to show the phosphorylation-dependent regulation (*10, 11*). The actin-activated ATPase activities of these constructs are sufficiently low in the absence of RLC phosphorylation and are enhanced in its presence, indicating that the interactions between two RLCs are important in the regulation of smooth muscle myosin. However, the ATPase activities of these constructs in the presence of RLC phosphorylation are significantly

[1]This work was supported in part by a grant from the Akiyama Foundation, Japan.

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Abbreviations: ATPγS, adenosine 5′-*O*-(3-thiotriphosphate); ELC, essential light chain; HMM, heavy meromyosin; RLC, regulatory light chain; S1, myosin subfragment 1; S2, myosin subfragment 2; WT, wild-type.

lower than that of double-headed HMM, suggesting that an interaction between the two motor domains is required for the high actin-activated ATPase activity. A head–head interaction necessary for the activation is also indicated by the observation that both heads are partially activated in HMM with one phosphorylated head (*12*).

In contrast to the RLC that is directly involved in the phosphorylation-dependent regulation of smooth muscle myosin, the ELC appears to be non-essential for the regulation. Myosin lacking the ELC actually shows the phosphorylation-dependent regulation as to the actin-activated ATPase activity and the ability to move actin-filaments, but its activities in the presence of RLC phosphorylation are significantly lower than those of myosin having ELC (*4, 13*). In addition, the substitution of scallop (*Patinopecten yessoensis*) ELC for the native ELC in smooth muscle myosin impairs the phosphorylation-dependent regulation of actin-activated ATPase activity (*14*), indicating a possible implication of ELC in the regulation. In this study, we produced a series of chimeric ELCs in which one or two helix-loop-helix domains of smooth muscle ELC were substituted by the corresponding domains of scallop (*Aquipecten irradians*) ELC, and probed the role of ELC domains in the phosphorylation-dependent regulation of smooth muscle myosin using these chimeric ELCs. Our results suggest that the ELC mediates an interaction between the two motor domains that is required to obtain the fully activated state of smooth muscle myosin in the presence of RLC phosphorylation.

Myosin was prepared from porcine aorta smooth muscle (*7*), and calmodulin and myosin light chain kinase from chicken gizzard smooth muscle, and actin from rabbit skeletal muscle as described previously (*15*). RLC was prepared from chicken gizzard as described by Yoshida and Yagi (*16*). Myosin and isolated RLC were thiophosphorylated with smooth muscle myosin light chain kinase, calmodulin, and MgATP-γS in the presence of Ca^{2+} as described (*17*). Myosin and RLC were completely thiophosphorylated under the conditions, as judged on urea gel electrophoresis (*18*).

The chicken gizzard ELC cDNA cloned into the *Eco*RI/*Pst*I sites of pT7-7 (thus, a fusion of ARIL before the native N-terminus (*19*)) was provided by Dr. K.M. Trybus. The cDNA of scallop (*Aquipecten irradians*) adductor ELC (a fusion of EDE to the C-terminus) cloned into the *Nde*I/*Eco*RI sites of pMW172 (*20*) was provided by Dr. A.G. Szent-Györgyi. The cDNAs of these chicken and scallop ELCs were subcloned into the *Xba*I/*Pst*I and *Xba*I/*Eco*RI sites of pBluescript II SK(+) (Stratagene), respectively, for mutagenesis. Four chimeric ELCs, in which one or two of the helix-loop-helix domains of the gizzard ELC were substituted by the corresponding domains of the scallop ELC, were constructed (Fig. 1). The cDNAs for these chimeric ELCs were constructed by the polymerase chain reaction using the overlap extension method (*21, 22*). The cDNAs of C(Sc12), C(Sc1), and C(Sc2) were subcloned into the *Xba*I/*Pst*I sites of pBluescript II SK(+), and that of C(Sc34) into its *Xba*I/*Eco*RI sites, and then they were sequenced (Hitachi SQ-5500 DNA autosequencer). The cDNAs of C(WT), C(Sc2), and C(Sc34) were cloned into the *Xba*I/*Hin*dIII sites of an expression vector, pET-30b(+) (Novagen), and those of C(Sc1), C(Sc12), and Sc(WT) into its *Xba*I/*Eco*RI sites.

A colony of *Escherichia coli* BL21(DE3) containing an ELC construct in pET-30b(+) was grown overnight at 37°C in 20 ml of LB medium containing 30 μg/ml kanamycin. The cell culture was inoculated into 1 liter of LB medium containing 30 μg/ml kanamycin at 37°C to an OD_{600} of 1, and then induced with 1 mM isopropyl β-D-thiogalactoside and grown overnight. The cells were harvested and washed, and then lysed by freezing and thawing after lysozyme treatment, the insoluble inclusion bodies being obtained as described by Trybus and Chatman (*19*). The inclusion bodies were washed with 20 ml of 1 mM EDTA and 25 mM Tris-HCl (pH 8.0), and then solubilized in 10 ml of 6 M guanidine hydrochloride, 5 mM EDTA, 20 mM DTT, and 0.1 M Tris-HCl (pH 8.0). The solution was dialyzed against 1 mM EDTA, 1 mM DTT, and 20 mM Tris-HCl (pH 8.0), clarified at 140,000 ×*g* for 1 h (Hitachi SCP55H), and then subjected to anion-exchange HPLC on a Poros HQ/M column (4.6 × 100 mm; PerSeptive Biosys-

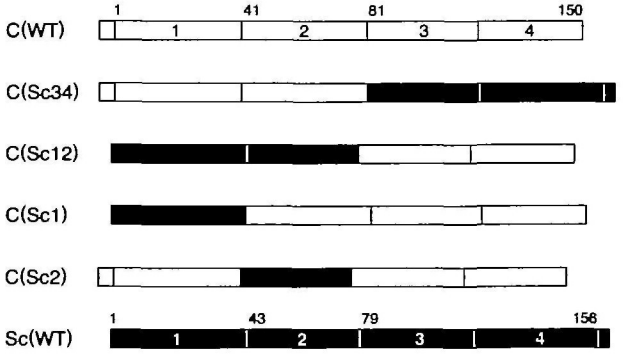

Fig. 1. **Schematic drawing of ELC constructs.** The open and solid bars represent sequences derived from chicken gizzard smooth muscle and scallop striated muscle ELC, respectively. The numbers in the bars indicate helix-loop-helix domains. The numbers above the bars indicate the positions of amino acid residues of the native chicken and scallop ELCs that correspond to the domain junctions in the chimeric ELCs. The light and dark gray bars at the N-terminus of chicken ELC and the C-terminus of scallop ELC, respectively, represent small numbers of non-native residues.

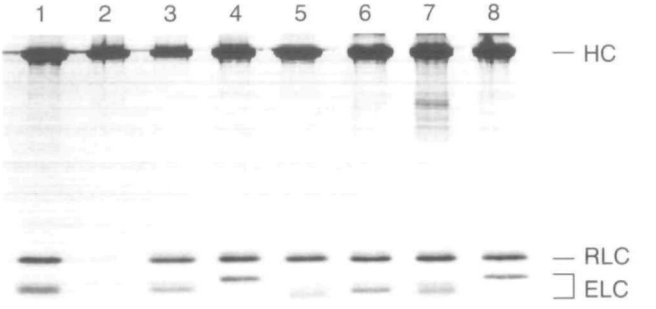

Fig. 2. **SDS gel electrophoresis of reconstituted myosin.** The light chain–deficient myosin was mixed with the recombinant ELC and unphosphorylated RLC (2 mol each light chain/mol head). The unbound light chains were removed with a Sephacryl S-300HR spun column and the obtained myosin samples were analyzed by SDS gel electrophoresis (*33*). Native myosin (lane 1), LC-deficient myosin (lane 2), and myosin reconstituted with C(WT) (lane 3), C(Sc34) (lane 4), C(Sc12) (lane 5), C(Sc1) (lane 6), C(Sc2) (lane 7), or Sc(WT) (lane 8). HC represents myosin heavy chain. Essentially the same results were obtained with thiophosphorylated RLC instead of the unphosphorylated form.

tems). ELC was eluted with a concentration gradient of 0–1 M NaCl (0.1 M/min) in 1 mM EDTA, 1 mM DTT, and 20 mM Tris-HCl (pH 8.0) at 4.0 ml/min, and then dialyzed against 1 mM DTT and 40 mM imidazole (pH 7.0). The molecular weight of 18,000 was used for the recombinant ELCs. The other methods used were as described previously (*13*).

To introduce the recombinant ELC into aorta smooth muscle myosin, the native light chains were removed by the use of trifluoperazine with 4.6 M NH_4Cl (*13*). The light chain-deficient myosin (Fig. 2, lane 2) was reconstituted with the recombinant ELC and the unphosphorylated or thiophosphorylated RLC (Fig. 2). Each reconstituted myosin contained the heavy chain, ELC, and RLC, with a stoichiometry of 1.0:0.9–1.1:0.8–1.0, as determined by gel densitometry. Myosins reconstituted with both the ELC and RLC showed similar NH_4^+/EDTA-ATPase activities to each other and the values were comparable to that of native myosin, regardless of the ELC mutation or RLC phosphorylation, whereas myosins lacking ELC showed significantly lower activities (Table I). These results indicated stoichiometric reconstitution for these myosins containing the recombinant ELCs.

The actin-activated ATPase activities of the reconstituted myosins, in the absence of RLC phosphorylation, were as low as that of native myosin, regardless of the ELC mutation (Fig. 3A). The low ATPase activity of unphosphorylated myosin containing Sc(WT) was inconsistent with the previous results for smooth myosin containing ELC of sea scallop (*Patinopecten yessoensis*) (*14*). The discrepancy might be due to five amino acid substitutions between the ELCs of bay scallop (*Aquipecten irradians*) and sea scallop (*23, 24*).

In the presence of RLC phosphorylation, myosin containing C(WT) showed high actin-activated ATPase activity and the degree of phosphorylation-dependent regulation was more than 20-fold, *i.e.* comparable to that observed for native myosin (Fig. 3A). Similar results were obtained for myosins containing C(Sc12), C(Sc34), and Sc(WT). In contrast, myosins containing C(Sc1) and C(Sc2) in the presence of RLC phosphorylation showed significantly lower activities (Fig. 3A), and thus their degrees of regulation decreased to ~13-fold and ~7-fold, respectively. However, myosins containing C(Sc12) and Sc(WT), both of which contain domains 1 and 2 of scallop ELC, unexpectedly showed normal high activities in the presence of RLC phosphorylation, as described above. To rule out the possibility that the low activity is due to low affinities of C(Sc1) and C(Sc2) for the heavy chain, the relative affinities were estimated through ELC exchange experiments. The ELC exchange of myosin was carried out in duplicate at 6°C with 4 μM myosin and a 3-fold molar excess of the recombinant ELC over the endogenous native ELC as described previously (*25*), and the relative binding affinity of the recombinant ELC for the heavy chain was calculated from the starting concentrations and the relative degree of exchange determined by gel densitometry. The relative affinities of these chimeric ELCs and Sc(WT) for the heavy chain were ~10-fold lower than that of C(WT) but similar to each other. In addition, the NH_4^+/EDTA-ATPase activity of myosin lacking ELC was significantly low but the activities of myosins containing C(Sc1) and C(Sc2) were similar to those of myosins containing the other recombinant ELCs (Table I). These results indicate that the low actin-activated ATPase activities of the myosins containing C(Sc1) and C(Sc2) were not due to the low affinities of these ELCs for the heavy chain. The substitution of domain 1 or 2 in gizzard ELC by that of scallop ELC therefore impaired the activation of smooth muscle myosin upon RLC phosphorylation but the substitution of both of these domains did not. An interaction that is required to obtain the fully activated state of myosin would occur between domains 1 and 2 of gizzard ELC, and the interaction would occur between these domains of scallop ELC as well. Scallop myosin itself is also regulated and may have the same activation mechanism based on the interdomain interaction of ELC as smooth muscle myosin.

TABLE I. **NH_4^+/EDTA-ATPase activity of reconstituted myosin.** NH_4^+/EDTA-ATPase activity was measured at 25°C with 0.02–0.04 μM myosin head in 0.6 M NH_4Cl, 20 mM EDTA, 1 mM ATP, 1 mM DTT, 0.2 mg/ml bovine serum albumin, and 40 mM Tris-HCl (pH 8.0) (*15*). Values are the means ± SD for 3–5 independent preparations. LC-def., light chain-deficient; Reconst., reconstituted.

Myosin	ATPase activity (s^{-1}·$head^{-1}$)	
	Unphosphorylated	Thiophosphorylated
Native	7.10 ± 0.70	7.63 ± 1.06
LC-def. myosin	3.28 ± 0.78	
Reconst. myosin with		
RLC	3.78 ± 0.98	3.97 ± 0.78
C(WT) + RLC	6.62 ± 0.53	6.78 ± 0.65
C(Sc34) + RLC	6.96 ± 0.37	6.70 ± 0.54
C(Sc12) + RLC	6.34 ± 0.52	6.53 ± 0.59
C(Sc1) + RLC	6.33 ± 0.32	6.41 ± 0.43
C(Sc2) + RLC	6.29 ± 0.48	6.46 ± 0.36
Sc(WT) + RLC	6.90 ± 0.55	6.89 ± 0.55

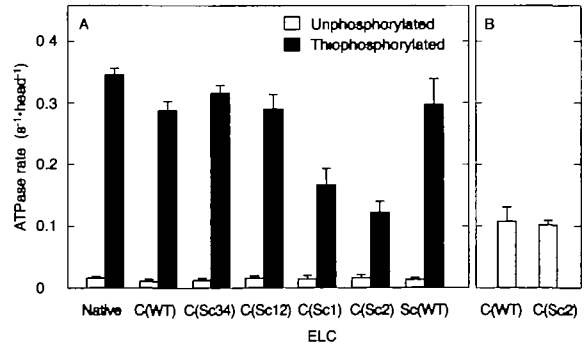

Fig. 3. **Actin-activated ATPase activity of reconstituted myosin and S1.** Actin-activated ATPase activity was obtained by subtracting the ATPase activity in the absence of actin from that in its presence. In the absence of actin, the ATPase activities of unphosphorylated myosin and S1 were 0.003–0.012 s^{-1} $head^{-1}$, and those of phosphorylated myosin were 0.014–0.024 s^{-1}·$head^{-1}$. A, actin-activated ATPase activity of myosin reconstituted with the recombinant ELC and unphosphorylated (open bars) or thiophosphorylated RLC (shaded bars). B, actin-activated ATPase activity of S1 prepared from myosin reconstituted with the recombinant ELC and unphosphorylated RLC. Values are the means ± SD for three independent preparations. The ATPase activities were measured as described previously (*13*) under the conditions of 0.2 μM myosin head, ±40 μM actin, 50 mM NaCl, 6 mM $MgCl_2$, 1 mM ATP, 1 mM EGTA, 1 mM DTT, and 20 mM imidazole (pH 7.0) at 25°C, except that 100 μM actin, 25 mM NaCl, and 5 mM $MgCl_2$ were used for S1 ATPase. At these actin concentrations the ATPase activities would be close to the V_{max} values since the K_{actin} value for aorta myosin is reported to be ~5 μM under similar conditions (*34*), and the K_{actin} value for a single-headed fragment of smooth muscle myosin is reported to be ~2-fold higher than that of the double-headed form (*3, 5, 10, 11, 28*).

The substitution of domain 1 or 2 of ELC would thus impair the interaction between these domains and prevent myosin from attaining the fully activated state.

Does such an interaction between these domains occur in each ELC or between the ELCs in the two different heads? To answer this question, a single head fragment of myosin, S1, was prepared from the reconstituted myosin containing C(WT) or C(Sc2) by proteolysis with *Staphylococcus aureus* V8 protease (*3*). Note that no proteolysis of light chains was detected in these S1 preparations on SDS gel electrophoresis. The actin-activated ATPase activity of S1 containing C(Sc2) was essentially the same as that of S1 containing C(WT) (Fig. 3B). The results indicated that in myosin the interaction occurs between domain 1 of ELC in one head and domain 2 of ELC in the other head. The ELC–ELC interaction is likely to mediate an interaction between the two motor domains that leads smooth muscle myosin to the fully activated state in the presence of RLC phosphorylation, in agreement with previous reports that showed low levels of actin-activated ATPase activity for single motor domain HMMs (*10, 11, 27, 28*). Indeed, the actin-activated ATPase activity of S1 containing C(WT) was significantly lower than that of myosin containing the same ELC (Fig. 3). Previous studies also showed 2–6 times lower activity for S1, monomeric short-tailed HMMs or single motor domain HMMs than for double-headed HMM (*3, 4, 6, 26*). Thus, S1 is always in the on state regardless of RLC phosphorylation but may not be in the fully activated state because of the lack of the head–head interaction. Based on the head–head interaction needed for the fully activated state, the two heads in phosphorylated myosin would function asymmetrically at the phosphate release step in the actomyosin ATPase cycle. One head, which may not bind to actin, would interact with the other head bound to actin and accelerate the phosphate release from the latter head, and thus these two heads would have different kinetic properties. The functional asymmetry of the two heads may explain the two populations with different turnover rates observed for the phosphorylated smooth muscle HMM ATPase in the presence of actin (*12*).

Is such an interaction between heads possible in the smooth muscle myosin molecule? The crystal structure of myosin head (*29, 30*) indicates that in the N-terminal half of ELC, domain 1 interacts with both the heavy chain and domain 2 of ELC, and thereby the major part of domain 1 is sandwiched between them. From the overlapping region, residues 2–13 of domain 1 and residues 54–62 of domain 2 project in opposite directions. In the myosin molecule these projected parts of ELC and the clefts made by these projected parts and the C-terminal half of the ELC, the RLC and the heavy chain could provide sites for the ELC–ELC interaction. Such an interaction would mediate an interaction between the two motor domains, which facilitates the phosphate release, leading myosin to the fully activated state. In fact, a disulfide bridge formed between the two ELCs with their Cys1s, which would prevent the above ELC–ELC interaction, reduces the actin-activated ATPase activity of smooth muscle myosin in the presence of RLC phosphorylation (*15*). For *Dictyostelium* myosin, the substitution of Ala for charged residues corresponding to residues 57, 58, and 60 of gizzard ELC has been reported to result in a significant reduction in the actin-activated ATPase activity (*31*). These projected regions of ELC contain many amino acid substitutions between gizzard and scallop ELCs: 10 out of residues 2–13 of gizzard ELC and 6 out of residues 54–62 of gizzard ELC are substituted in scallop ELC and more than 50% of the substitutions are non-conservative (*23, 32*). The high frequency of amino acid substitution observed in these regions is consistent with the impairment of the ELC–ELC interaction on the substitution of one of these domains, and some of these substituted residues are likely to be responsible for the ELC–ELC interaction, but more studies are needed to determine exactly the residues that participate in the interaction.

We wish to thank Drs. Ken-ichi Nakashima and Akiko Nakatomi for their technical assistance. We also thank Dr. Hitoshi Fujisawa for the helpful discussions.

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