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Structure-Function Relationships of the Two Surface Loops of Myosin Heavy Chain Isoforms from Thermally Acclimated Carp

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The structure-function relationships of fast skeletal myosin isoforms remain poorly understood. To shed some light, we constructed chimeric myosins comprised of *Dictyostelium* myosin heavy chain backbone with carp loop sequences and analyzed their functional properties. A loop 2–10 chimeric myosin having the loop 2 sequence of the fast skeletal isoform predominantly expressed in carp acclimated to 10°C showed V_{max} in actin-activated Mg^{2+}-ATPase activity 1.4-fold higher than a loop 2–30 chimera constructed from the loop 2 sequence of the dominant isoform in carp acclimated to 30°C. These two chimera exhibited no significant differences in sliding velocity of actin filaments in *in vitro* motility assay. Contrastingly, both loop 1-associated chimeras, loop 1–10 and loop 1–30, did not differ in both ATPase activity and in sliding velocity of actin filaments.

Myosin is an actin-based molecular motor which transduces chemical energy obtained by ATP hydrolysis into mechanical work. It consists of two heavy chains of about 200 kDa and four light chains of about 20 kDa. The four light chains are classified into two distinct functional types: each of two essential (ELC) and regulatory light chains (RLC). N-terminal halves of two heavy chains form a globular domain, subfragment 1 (S1), with ELC and RLC, whereas C-terminal halves form extended rod-like structures of α-helical coiled-coil (1). S1 contains ATP- and actin-binding sites, and is fully equipped with factors essential for motor function of myosin (2, 3). Although it has recently been suggested that two surface loops of S1 modulate motor function of myosin, precise role of each loop is still ambiguous. Objective of this study was to reveal the structure-function relationships of these loops using carp fast skeletal myosin heavy chain isoforms.

Previously, our group has shown that carp expresses fast skeletal myosin isoforms having different motor activities in association with temperature acclimation (4–9). Hwang *et al.* (4) first showed that myosin preparation from carp acclimated to 10°C had V_{max} for actin-activated Mg^{2+}-ATPase activity 1.6-fold higher than that from fish acclimated to 30°C. Later, Chaen *et al.* (8) reported that myosin from the 10°C-acclimated carp moved actin filaments in *in vitro* motility assay faster than that from the 30°C-acclimated fish. However, since myosins purified from thermally acclimated carp were mixtures of different isoforms, it was difficult to evaluate isoform-specific functions. Recently, we have isolated cDNA clones encoding fast skeletal myosin heavy chain isoforms from carp, where major isoforms were the 10°C and 30°C types predominantly expressed at 10°C and 30°C, respectively (9–11).

These two isoforms generally resembled each other in the primary structure, showing more than 90% sequence identity. However, the sequences of two surface structures, loops 1 and 2, were diverged between the isoforms (Fig. 1A). Recent studies on myosin from other species have shown that these loops are implicated in modulating motor functions of the myosin molecule (12, 13). Loop 1, which connects the 25 kDa and 50 kDa tryptic fragments of S1 heavy chain, is located near the nucleotide-binding pocket and likely to tune the rate constant for ADP release. On the other hand, loop 2 connects the 50 kDa and 20kDa S1 tryptic fragments and is one of the actin-binding sites, possibly regulating V_{max} of actin-activated Mg^{2+}-ATPase activity. Thus, it seems reasonable to speculate that structural differences in the two surface loops of carp myosin heavy chain isoforms affect their motor activities.

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In this study we expressed chimeric myosins comprised of *Dictyostelium discoideum* myosin backbones with loop sequences of carp S1 heavy chain isoforms. These chimeras were subjected to Mg^{2+}-ATPase activity measurements and *in vitro* motility assays to reveal structure-function relationships of carp myosin isoforms.

MATERIALS AND METHODS

Construction of expression vector for chimeric myosin heavy chain. The expression vectors for chimeric *Dictyostelium* myosin heavy chains having loop sequences of carp, *Cyprinus carpio*, were constructed according to the method described by Uyeda *et al.* (14) using an extrachromosomal expression vector pTIKL-myosin (15). Substituted regions are boxed in Fig. 1A.

Transformation of expression vectors into Dictyostelium cells. Transformation of expression vectors into *Dictyostelium* myosin null cells (16) was carried out as described by Egelhoff *et al.* (17). While transformed cells were selected by adding 10 μg/ml geneticin (G418), the growth rate was determined with increased cell numbers in suspension culture. The culture medium containing cells were shaken at 150 rpm at 22°C and developments of the transformed cells were accomplished on agar plates covered with a lawn of *Escherichia coli* cells.

Purification of proteins. Phosphorylated chimeric myosins were purified from transformed *Dictyostelium* cells as described by Shimada *et al.* (18). Rabbit fast skeletal muscle α-actin was purified according to Spudich and Watt (19).

Measurements of actin-activated Mg^{2+}-ATPase activity. Actin-activated Mg^{2+}-ATPase activities of expressed chimeric myosins were determined by the malachite green method (20) by measuring released Pi. Reaction mixtures contained 18 mM MOPS (pH 7.4), 12.5 mM KCl, 4 mM $MgCl_2$, 1 mM ATP, 0.1 mM DTT, 0.03 mg/ml myosin, and various concentrations (0–1 mg/ml) of F-actin. The reaction was initiated by the addition of ATP and the mixture was incubated at 20°C, from which 100 μl aliquots were taken at 5-min intervals. The reaction was stopped by the addition of 100 μl ice-cold 0.6 M perchloric acid.

In vitro motility assay. The motile activities of chimeric myosins were measured by *in vitro* motility assay, observing F-actin filaments sliding on a cover glass coated with myosin as reported previously (21–24).

RESULTS

Expression and Purification of Chimeric Myosins

Figure 1A shows the deduced amino acid sequences of two surface loops of carp myosin heavy chain isoforms (11). The 10°C and 30°C types represent fast skeletal isoforms predominantly expressed in carp acclimated to 10°C and 30°C, respectively. Loop 1–10 and loop 1–30 denote *Dictyostelium* chimeric myosins with loop 1 of the 10°C- and 30°C-type carp myosin heavy chains, respectively, whereas loop 2–10 and loop 2–30 indicate their counterparts comprising loop 2, respectively (Fig. 1B). Transformed cells with chimeric myosin heavy chain genes showed slightly slower growth rate in suspension culture than the control cells of *Dictyostelium* wild-type. However, all the transformants could rescue myosin null phenotype in cytokinesis and grew up to $\sim 1 \times 10^7$ cells/ml. In addition, all the transformed cells formed normal fruiting bodies under starving conditions (data not shown). These results indicate that the present chimeric myosins of *Dictyostelium* had normal functions.

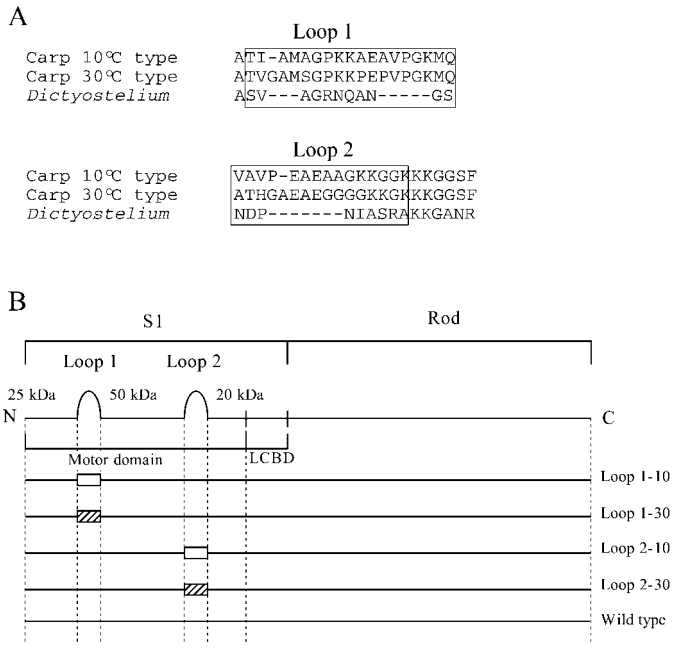

FIG. 1. (A) Amino acid sequences of loop 1 and loop 2 of carp fast skeletal myosin heavy chain isoforms together with those of *Dictyostelium discoideum*. The 10°C and 30°C types are dominant isoforms expressed in carp acclimated to 10°C and 30°C, respectively (11). The highly diverged regions are boxed. LCBD, light chain binding domain. (B) Schematic representation of *Dictyostelium* chimeric myosin heavy chains. *Dictyostelium*, carp 10°C-type, and carp 30°C-type sequences are represented by lines, open boxes, and hatched boxes, respectively. Loop 1–10 and loop 1–30 denote *Dictyostelium* chimeric myosins with loop 1 from carp 10°C- and 30°C-type myosin heavy chains, respectively; loop 2–10 and loop 2–30 indicate those with loop 2 from corresponding isoforms of carp.

For purification of chimeric myosins, actomyosin was first extracted from 500 ml of cell culture by a conventional method. Actin and myosin were dissociated by adding 5 mM Mg^{2+}-ATP to actomyosin solution and crude myosin was obtained by ultracentrifugation. DEAE-5PW ion-exchange column chromatography was performed after treating with catalytic subunit of recombinant myosin light chain kinase (25). Chimeric myosins thus obtained were of highly purified state with almost no contaminating proteins (data not shown).

Actin-activated Mg^{2+}-ATPase Activities of Chimeric Myosins

Actin-activated Mg^{2+}-ATPase activity was measured for chimeric myosins as well as for the wild-type coun-

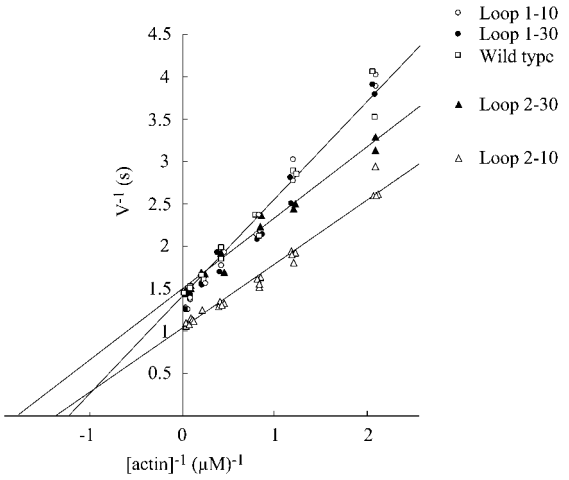

FIG. 2. Double-reciprocal plots of actin-activated Mg^{2+}-ATPase activity of *Dictyostelium* chimeric myosins containing carp loop 1 and loop 2 together with those for *Dictyostelium* wild-type. Refer to the legend of Fig. 1 for definition of chimeric myosins.

terpart at 20°C in the absence and presence of various concentrations of F-actin up to 1 mg/ml. V_{max} in the activity and K_m for actin were calculated by double-reciprocal plots (Fig. 2, Table 1). The two loop 1-associated chimeric myosins, loop 1–10 and loop 1–30, showed V_{max} and K_m values similar to those of *Dictyostelium* wild-type myosin. These results were consistent with the data reported by Murphy and Spudich (26) for *Dictyostelium* chimeric myosins having loop 1 of rabbit skeletal or *Acanthamoeba* myosin, which showed that chimeric substitutions of loop 1 did not affect V_{max} of actin-activated Mg^{2+}-ATPase activity. However, in the present study on carp, the V_{max} in actin-activated Mg^{2+}-ATPase activity of the loop 2–10 myosin was 1.4-fold higher than the loop 2–30 myosin, but without marked variation in K_m for actin. Uyeda *et al.* (14) constructed chimeras of *Dictyostelium* myosin by substituting loop 2 region with those from other animal species and demonstrated that the chimeric myosin having rabbit skeletal loop 2 showed actin-activated Mg^{2+}-ATPase activity 5.1-fold higher than that of *Dictyostelium* wild-type in the presence of 1 mg/ml F-actin. Although chimeric myosins containing carp loop 2 sequences in this study did not show such high activity, the differences in the activity between loop 2–10 and loop 2–30 chimera were significant (Fig. 2, Table 1).

TABLE 1

The Maximal Initial Velocity (V_{max}) of Actin-activated Mg^{2+}-ATPase Activity and Apparent Affinity to F-actin (K_m) at 20°C of the *Dictyostelium* Chimeric Myosins Containing Carp Loop 1 and Loop 2 together with Those for *Dictyostelium* Wild-type

Myosin*	V_{max} (s^{-1})**	K_m (μM)**
Loop 1–10	0.80 ± 0.04	1.1 ± 0.2
Loop 1–30	0.80 ± 0.02	0.8 ± 0.2
Loop 2–10	0.95 ± 0.02[a]	0.7 ± 0.2
Loop 2–30	0.68 ± 0.02[a]	0.6 ± 0.3
Wild type	0.71 ± 0.01	0.9 ± 0.1

* Loop 1–10 and loop 1–30 denote *Dictyostelium* chimeric myosins with loop 1 from the 10°C- and 30°C-type myosin heavy chains, respectively, whereas loop 2–10 and loop 2–30 indicated those with loop 2 from corresponding isoforms of carp.

** Means ± SE from 4 determinations for two preparations.

[a] Difference is significant at $P < 0.001$ level.

TABLE 2

Sliding Velocities of Fluorescent F-actin over the *Dictyostelium* Chimeric Myosins Containing Carp Loop 1 and Loop 2 at 25°C Together with Those of *Dictyostelium* Wild-type

Myosin*	Sliding velocity (μm/s)**
Loop 1–10	1.30 ± 0.24
Loop 1–30	1.29 ± 0.23
Loop 2–10	1.27 ± 0.17
Loop 2–30	1.29 ± 0.29
Wild type	1.29 ± 0.22

* Loop 1–10 and loop 1–30 denote *Dictyostelium* chimeric myosins with loop 1 from the 10°C- and 30°C-type myosin heavy chains, respectively, whereas loop 2–10 and loop 2–30 indicated those with loop 2 from corresponding isoforms of carp.

** Means ± SE for sliding velocity of 40 actin filaments.

In Vitro Motility Assay

Table 2 shows the results obtained from *in vitro* motility assay for chimeric myosins. The *Dictyostelium* wild-type moved actin filaments at 1.29 ± 0.22 μm/s, which was slightly slower than those reported previously (18, 27). However, all chimeric myosins showed similar sliding velocities, unlike the changes in actin-activated Mg^{2+}-ATPase activity described before.

DISCUSSION

We have recently found that carp expresses fast skeletal myosin isoforms having different motor activities following temperature acclimation (4–9). Although these isoforms closely resembled each other in the primary structure, the sequences in loop 1 and loop 2 regions were markedly different among the isoforms (11). So far, it was somewhat difficult to prepare recombinant skeletal myosin molecules, which facilitate an evaluation of sequence diversity in the functional domain. Recently, Uyeda *et al.* (14) have successfully constructed *Dictyostelium* myosins having loop 2 sequences of foreign origins and showed that resulting chimeric substitution affected actin-activated Mg^{2+}-

ATPase activity. Murphy and Spudich (26) also claimed that loop 1 structure affected ADP release rate in the cross-bridge cycle using the same expression system.

Such developments stimulated us to investigate the structure-function relationships of carp myosin heavy chain isoforms focusing on the two surface loops by using *Dictyostelium* myosin expression system. The loop 2–10 myosin showed V_{max} 1.4-fold higher than the loop 2–30 counterpart. These results with chimeric myosins were consistent with our previous observation that fast skeletal myosin prepared from the 10°C-acclimated carp showed V_{max} 1.6-fold higher than that of the 30°C-acclimated carp (4). It should be noted that the 10°C- and 30°C-type myosin heavy chains are dominant isoforms in the 10°C- and 30°C-acclimated carp, respectively (9–11). Recent studies on myosin loop 2 have revealed that it has a critical role in modulating actin-activated Mg^{2+}-ATPase activity. This loop is located at one of the actin-binding sites, positively charged amino acids of which are considered to interact with the negatively charged N-terminal part of the actin molecule (28, 29). However, amino acid sequences of loop 2 are highly diverged among various myosins including carp isoforms. Spudich (12) claimed that loop 2 possibly tunes the rate of phosphate release and thus affect V_{max} for actin-activated Mg^{2+}-ATPase activity. Although three positively charged residues of lysine were contained in both loop 2 sequence from the two carp isoforms, the overall sequences were markedly different between the isoforms (Fig. 1A).

On the other hand, no significant differences in V_{max} were observed between the present two loop 1 chimeric myosins, loop 1–10 and loop 1–30. Moreover, the loop 1–10 and loop 1–30 constructs, as well as the two loop 2-associated chimeras, moved actin filaments in *in vitro* motility assay at similar sliding velocities. These results reported in this study were clearly different from previous findings that loop 1 structure affected the sliding velocity of actin filaments (26, 30, 31). As described before, myosin preparation from carp acclimated to 10°C moved actin filaments faster than that from the 30°C-acclimated carp (8). Therefore, differences in motile activity of carp myosin isoforms are probably caused by amino acid substitutions in other regions. However, it should be noted that *Dictyostelium* and skeletal myosins may have different properties.

In summary, the present study strongly suggests that the differences in V_{max} values for actin-activated Mg^{2+}-ATPase activity observed in myosin preparations from thermally acclimated carp were mostly due to the different amino acid sequences of their loop 2 regions predominantly expressed at respective acclimation temperatures. In addition, our data showed that two surface loops of carp myosin heavy chain isoforms apparently did not affect the change in sliding velocity of actin filaments. However, we could not identify regions responsible for such change in sliding velocity.

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