Examining the *in Vivo* Role of the Amino Terminus of the Essential Myosin Light Chain*

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The functional significance of the actin binding region at the amino terminus of the cardiac essential myosin light chain (ELC) remains obscure. Previous experiments carried out *in vitro* indicated that modulation of residues 5–14 could induce an inotropic effect, increasing maximal ATPase activity at submaximal Ca**2**⁺ concentrations (Rarick, H. M., Opagenorth, T. J., von Geldern, T. W., Wu-Wong, J. R., and Solaro, R. J. (1996) *J. Biol. Chem.* 271, 27039–27043). Using transgenesis, we effected a cardiac-specific replacement of ELC with a protein containing a 10-amino acid deletion at positions 5–14. Both the ventricular (ELC1vΔ5–14) and atrial (ELC1aΔ5–14) isoforms lacking this peptide were stably incorporated into the sarcomere at high efficiencies. Surprisingly when the kinetics of skinned fibers isolated from the ELC1vΔ5–14 or ELC1aΔ5–14 mice were examined, no alterations in either unloaded shortening or maximum shortening velocities were apparent. Myofibrillar Mg**2**⁺-ATPase activity was also unchanged in these preparations. No significant changes in the fiber kinetics in the cognate compartments were observed when either deletion-containing protein replaced endogenous ELC1v or ELC1a. The data indicate that the previously postulated importance of this region in mediating critical protein interactions between the cardiac ELCs and the carboxyl-terminal residues of actin *in vivo* should be reassessed.

Myosin is the major motor protein in the contractile apparatus of all muscle types and cyclically interacts with the thin filament to generate force (1). The different "conventional" myosins, or myosin IIIs, are thought to underlie the contractile properties of the different muscle types. Myosin II is a hexameric protein made up of two heavy chains (MyHCs)1 (Mr ~229,000) and four light chains (MLCs) (Mr ~18,000–27,000). The MyHCs consist of two separate domains: a globular head region and a rod region that corresponds to the globular head and neck of the molecule. Also associated with this heavy chain domain are the MLCs (2). Myosin-actin cross-bridge interactions play an important role in determining cardiac systolic and diastolic function. Cardiac MyHC is encoded by two genes, α- and β-MyHC, and each heavy chain is associated with two MLC types, the regulatory MLC and essential MLCs (ELCs). X-ray crystallographic analyses have demonstrated that both the ELC and regulatory MLC are associated with the neck region of the MyHC (2, 3). Striated muscle can contain two ELC isoforms, ELC1f and ELC3f. The sequences of the two are quite similar except that the ELC1f isoform contains an amino-terminal extension of 40 amino acids. The cross-bridge kinetics of ELC3f fibers are significantly faster than ELC1f fibers (4), and the functional significance of the amino-terminal extension has been the subject of multiple investigations (5–9). We have shown previously that transgenic (TG) substitution of the atrial ELC1 isoform (ELC1a) for the ventricular isoform results in faster myocardial fiber kinetics as well as increased contractile function at the whole organ level (10). Because the amino-terminal alanine- and proline-rich region of the ELC1a is shorter than that of the ELC1v, the amount of interaction between the ELC and actin may be reduced, effectively attenuating the tether action and leading to increased function (11, 12). Thus, different ELC isoforms can regulate aspects of contractility in cardiac muscle. *In vitro* and NMR data showed that the amino-terminal region of ELC, encompassed within the first 11 residues of the ELC1f, ELC1a, and ELC1v isoforms, can bind to the carboxyl-terminal residues of actin where it presumably affects myosin-actin cross-bridge cycling (4, 12–15). Using a skinned fiber system, this ELC-actin interaction can be ablated by incubation with a synthetic peptide corresponding to amino acid residues 5–14 with a concomitant increase in the rate of fiber shortening (7). Similar experiments carried out with isolated rat cardiac myofibrils showed that exogenous peptide led to a stimulation of Mg**2**⁺-ATPase activity at submaximal Ca**2**⁺ levels (8). The ELC-actin interaction depends on the regional charges; in particular, ELC lysines 3, 4, 8, and 9 appear to be important and interact with actin carboxyl-terminal residues Glu-360, Glu-361, Asp-363, and Glu-364 (4). However, mutational analysis of actin in *Dictyostelium* revealed that Glu-360, Glu-361, Asp-363, and Glu-364 were not necessary for cross-bridge cycling (16). Thus, *in vivo* the functional significance of the interaction between the ELC amino-terminal region and the actin carboxyl-terminal region is unclear.

Using cardiac-specific promoters to drive expression in TG animals is an effective means of replacing endogenous sarcomeric components with transgenically encoded protein. The α-MyHC promoter can drive cardiac-specific TG expression such that ventricular and atrial sarcomeric isoforms can be efficiently exchanged with the TG-encoded species (17, 18). Replacement of endogenous species with proteins containing

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The abbreviations used are: MyHC, myosin heavy chain; MLC, myosin light chain; ELC, essential myosin light chain; TG, transgenic; NTG, nontransgenic; ELC1a, atrial isoform of ELC1; ELC1v, ventricular isoform of ELC1; BES, N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid.
Single site mutations have also been reported (10), allowing the structure-function relationships of single residues to be explored in vivo. To clarify the potential importance of the amino-terminal light chain-actin interactions within the whole organ and whole animal contexts, we created TG mice with cardiac-specific expression of mutated ELC1v and ELC1a isoforms. Lines of TG mice in which the endogenous ELC was replaced with a protein containing a 10-amino acid amino-terminal deletion between residues 5–14 were made. The data show that interactions mediated by this region of the protein are not essential for cross-bridge cycling in the heart.

**EXPERIMENTAL PROCEDURES**

**Transgene Construction**—Previously isolated full-length murine ELC1a and ELC1v cDNAs were used as starting templates (17, 19). Primers with SalI sites engineered at the termini were made to the 3′ and 5′-untranslated regions of the cDNA. The 5′ primer also contained the mutated bases necessary to delete the amino-terminal amino acid residues KPEPKKETAK in ELC1a and KPEPKKDDAK in ELC1v (Fig. 1).
that EGTA was substituted with 5 mM Ca-EGTA. The free Ca$^{2+}$ concentration was obtained by mixing the relaxing and contraction solutions in the appropriate proportions. Strip tension (millinewton/mm$^2$) was calculated by dividing force by fiber cross-sectional area calculated from widths measured at the major axis. Activating solution had the same ionic composition as relaxing solution. The solutions were formulated by solving a set of simultaneous equations describing the multiple equilibrium of ions in the solutions.

**RESULTS AND DISCUSSION**

A total of four constructs were used in these studies (Fig. 1A). To rule out any phenotype due to overexpression of either the atrial- or ventricular-specific ELC isoforms, lines carrying the wild type cDNAs (18, 19) were used as controls along with NTG animals. Although previous *in vitro* studies focused exclusively on the amino terminus of the ventricular isoform, we extended these studies to include the atrial isoform as well. The aminoterminal extension is shorter in ELC1a but also contains the 5–14-residue region (KPEPKKD/D/E/D/T/DAK) that has been implicated in actin binding (Fig. 1B). Multiple lines were generated for each TG construct; for the purposes of this study we focused on lines that yielded high levels of replacement. For each of the constructs expressing the wild type proteins, lines were in which replacement in the ectopic cardiac compartment was >90%; both the ELC1a and ELC1v “wild type” lines have been reported previously (18, 19). The ELC1vΔ5–14 TG line 188 showed significant levels of replacement (Fig. 1C). Analyses of ventricular RNA levels showed that line 188 had a 15.6-fold increase as compared with the endogenous transcript (data not shown) resulting in ~85% replacement (Fig. 1C). The ELC1vΔ5–14 line used in the studies below (line 22) had 21.7-fold higher atrial transcript levels as compared with the endogenous ELC1a. This level of TG expression resulted in >95% replacement of ELC1a in the atria (Fig. 1C).

The overall appearance, behavior, morbidity, and mortality of the Δ5–14 lines were all unremarkable. Histological analyses revealed normal cell morphology, the striations characteristic of cardiac muscle, and no fibrosis (data not shown). Over the animals’ lifetimes, they were monitored carefully for cardiac disease using both invasive (e.g. sacrificing animals at 6, 12, and 15 months) as well as noninvasive means such as echocardiography (20). The only response noted was a slight increase in ventricular weights in the ELC1aΔ5–14 animals. Whereas all heart chamber weights in ELC1vΔ5–14 TG mice were unremarkable, the ELC1aΔ5–14 TG mice did show a statistically significant increase in the left ventricular weight (Table I). Previously we showed that an ELC1v→ELC1a protein replacement has no effect on chamber mass (17), so that the minor hypertrophic response may in fact be because of the deletion at the amino terminus. However, it should be noted that both ELC1v→ELC1vΔ5–14 and ELC1a→ELC1aΔ5–14 caused no hypertrophy, so it appears as if the deletion must also be coupled with an isoform switch to produce the effect.

On the basis of the *in vitro* data, which were obtained using fiber preparations in which varying amounts of inhibitory peptides were added prior to any measurements, we were able to see changes in either the enzymatic and/or mechanistic parameters in the EL1vΔ5–14 TG mice and possibly in the ELC1aΔ5–14 animals as well. To this end we first examined the Mg$^{2+}$-ATPase activities and Ca$^{2+}$–force relationships using skinned fiber preparations derived from the left ventricular papillary muscles of the relevant TG lines (Fig. 2 and Table II). In contrast to the data obtained using the peptide inhibitors, no differences in either the Mg$^{2+}$-ATPase activity (Table II) or in maximum force development as a function of the Ca$^{2+}$ concentration (Fig. 2A) were detected in the ELC1vΔ5–14 TG mice. Previously we have shown that expression of ELC1a in the ventricle did not lead to changes in these parameters (10).
Similar results were obtained when the (atrial) fibers containing essentially complete replacement of the endogenous protein with ELC1a/NTG were examined (Table II and Fig. 2B).

We next examined the unloaded shortening velocities, maximum shortening velocities, and maximum relative power in the TG experimental cohorts. Previous experiments have indicated that expression of ELC1v in the heart does not lead to changes in these ventricular parameters (11). Surprisingly, replacement of the endogenous protein with the ELC1v/NTG species resulted in values identical to the NTG controls (Fig. 3, A, B, and C). Similar results were obtained when the (atrial) fibers containing essentially complete replacement of the endogenous protein with ELC1a/NTG/NTG–14 were examined (Fig. 3, D, E, and F).

The amino-terminal residues that were deleted in both ELC1v and ELC1a contain an actin-binding site (7, 15), and deletion of these residues might affect ELC-actin interactions, markedly attenuating the actin affinities of the proteins. Incubation of chemically skinned human ventricular fibers with the 5–14 amino-terminal peptide, KPEPKKDDAK, presumably saturated the carboxyl-terminal binding site of actin, resulting in an increase in isotonic tension generation at both submaximal and maximal Ca$^{2+}$ activation states (7). Additional experiments carried out with similar methodology showed that the ELC1v amino-terminal fragment induced a supramaximal increase in fiber Mg$^{2+}$-ATPase activity at submaximal Ca$^{2+}$ levels with no effect at low and maximum Ca$^{2+}$ levels (8). Although our data do not bear directly on the potential of an exogenous peptide to modulate fiber inotropy, all the data strongly indicate that the protein interactions between the amino-terminal 5–14 ELC region and the carboxyl terminus of actin are not essential for myosin motor function in vivo. These results are consistent with data that were obtained when charge reversal mutations at the actin carboxyl terminus acidic residues (E360H, E361H, D363H, and E364H) were made; these did not significantly affect either sliding velocities or force generation in actin motility analyses (16). Based on the stoichiometric ratios of peptide to actin or the thin filament that were used (8), the amount of peptide required for the stimulatory effect is relatively high (4 peptides:1 thin filament). Yet cross-linking studies showed that the amino terminus of the ELC could only reach the carboxyl terminus of actin when MyHC binds to two actin protomers (23).

Our data argue against the physiological importance of the proposed ELC-actin binding in regulating cross-bridge kinetics or the development of force with respect to Ca$^{2+}$ concentrations. In vitro data suggested that the ELC1v amino-terminal peptide directly activates Mg$^{2+}$-ATPase activity through an interaction with actin. This in turn results in a cooperative activation of the entire thin filament (8). Under in vivo conditions, where the fiber stoichiometry and integrity are maintained, we were unable to discern any effect on enzymatic, kinetic, or mechanistic parameters. Confirming the lack of any effect, a similar mutation made in the atrial isoform also resulted in normal fiber parameters. Because replacement was almost complete in the ELC1v/NTG/NTG–14 line 188 mice and essentially complete (>95%) in the ELC1a/NTG–14 line, it appears that residues 5–14 are not vital for maintenance of fiber integrity or function in vivo. Conservation of function over the lifetime of the animal is confirmed by the complete lack of increased morbidity or mortality in the TG cohorts. We believe it probable that the faster cycling rates that are observed in ELC1a and ELC3f fibers relative to ELC1v-containing fibers may reflect differential stabilizing effects in the neck region of MyHC.

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FIG. 3. Contractile properties of permeabilized ventricular fibers. Mechanical analysis of skinned fiber strips from left ventricular papillary muscles of NTG, ELC1v wild type, and ELC1v/NTG–14 (line 188; A, B, and C) and from atrial trabecular muscles of NTG, ELC1a wild type, and ELC1a/NTG–14 mice (line 22; D, E, and F). A and D, the unloaded shortening velocities and maximum shortening velocities. B and E, maximum shortening (units in muscle lengths/s (m.l.s)) was determined by the slack test as described previously (10). The data for each panel were collected under identical conditions of room temperature, humidity, and arbitrary machine settings. However, between panels, these conditions varied leading to slight variations between NTG values. C and F, maximum relative power. No significant differences in each of the three experimental cohorts presented. 1v wild, ELC1v wild type; 1a/NTG–14, ELC1a/NTG–14; 1a/NTG–14, ELC1a/NTG–14.

|                     | A                  | B                  | C                  | D                  | E                  | F                  |
|---------------------|--------------------|--------------------|--------------------|--------------------|--------------------|--------------------|
|                     | Unloaded shortening | Maximum shortening  | Maximum relative   | NTG                | 1v wild            | 1a/NTG–14          |
|                     | velocity (mI/Mg)   | velocity (mI/Mg)   | power (pI/Mg)      | 1v/NTG–14          | 1a/NTG–14          |                    |
| NTG                 | 214 ± 25           | 232 ± 4            | 0.64 ± 0.01        | 1.0 ± 0.1          | 1.0 ± 0.1          |                    |
| ELC1v (TG)          | ND                 | ND                 | ND                 | ND                 | ND                 | ND                 |
| ELC1v/NTG–14        | ND                 | 234 ± 13           | ND                 | ND                 | ND                 | ND                 |
| ELC1a (TG)          | 227 ± 2            | ND                 | ND                 | ND                 | ND                 | ND                 |
| ELC1a/NTG–14        | 239 ± 13           | ND                 | ND                 | ND                 | ND                 | ND                 |

TABLE II
Ca$^{2+}$-stimulated Mg$^{2+}$-ATPase activities
All values are mean ± S.E. (n = 4). Ca$^{2+}$-stimulated Mg$^{2+}$-ATPase activity (nmol/min/mg of protein) was determined by subtracting the activity at pCa 5.0 from the activity at pCa 8.0. ND, not determined.
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