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# Distinct interactions between actin and essential myosin light chain isoforms

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ABSTRACT

Binding of the utmost N-terminus of essential myosin light chains (ELC) to actin slows down myosin motor function. In this study, we investigated the binding constants of two different human cardiac ELC isoforms with actin. We employed circular dichroism (CD) and surface plasmon resonance (SPR) spectroscopy to determine structural properties and protein–protein interaction of recombinant human atrial and ventricular ELC (hALC-1 and hVLC-1, respectively) with α-actin as well as α-actin with alanin-mutated ELC binding site (α-actin$^{ala3}$) as control. CD spectroscopy showed similar secondary structure of both hALC-1 and hVLC-1 with high degree of α-helicity. SPR spectroscopy revealed that the affinity of hALC-1 to α-actin ($K_D$ = 575 nM) was significantly ($p < 0.01$) lower compared with the affinity of hVLC-1 to α-actin ($K_D$ = 186 nM). The reduced affinity of hALC-1 to α-actin was mainly due to a significantly ($p < 0.01$) lower association rate ($k_{on}$: 1018 $M^{-1}$ $s^{-1}$) compared with $k_{on}$ of the hVLC-1/α-actin complex interaction (2908 $M^{-1}$ $s^{-1}$). Hence, differential expression of ELC isoforms could modulate muscle contractile activity via distinct α-actin interactions.



## 1. Introduction

Type II myosins, the motor proteins which drive muscle contraction, are composed of two heavy chains (MYH) and four non-covalently linked light chains (MLC) [1]. The lever arm of the MYH contains two IQ motifs in tandem. IQ1 binds the essential myosin light chain (ELC), whereas IQ2 binds the regulatory myosin light chain (RLC) [1,2]. The full-length ELC is designated as the A1 light chain isoform [3]. The ELC gene transcript of fast skeletal muscle is alternatively spliced [4–6]. This leads to a N-terminally 42aa truncated ELC isoform designated as the A2 [3]. ELC in cardiac, slow skeletal, and most of the fast-twitch muscle is of the A1 type [3]. The primary structure of A1 isoforms are built of an N-terminus (aa1–46) and a large C-terminus (aa47–≈200) consisting of four helix-loop-helix EF-hand domains which binds to the myosin lever arm [1,2,7]. Molecular modeling of the N-terminal A1 segment showed a rod-like antenna structure with a length of 91 Å [7].

The utmost N-terminus of A1 (aa1–15) contains a "sticky" element of several charged amino acids, in particular lysines (K3, K4, K8, K9) and down-stream a repetitive Ala-Pro-rich segment (aa ≈ 15–28) [8,9]. The sticky N-terminus, but not the Ala-Pro segment of A1 [10,11] binds to a cluster of acidic residues at the C-terminus of actin (aa 360–364) [12,13]. Weakening the A1/actin interaction by a variety of experimental interventions and models increased myosin motor activity, i.e. actin-activated myosin ATPase activity, *in vitro* motility of actin filaments, or maximal shortening velocity of skinned muscle fibers [11,14–21]. Ala-replacement of all four N-terminal lysines was more effective than replacement of the first two N-terminal lysines in increasing shortening velocity [16]. In line, recent transgenic overexpression of an N-terminally truncated ventricular A1 ($A1^{\Delta 1-43}$) in the heart accelerated the ADP-dependent cross-bridge detachment step [22] which critically determines maximal shortening velocity [23]. The same study [22] showed increased rigor stiffness, providing evidence that the N-terminus of A1 tethers myosin with the actin filament. Furthermore, the sarcomere-length dependency of cardiac force generation was blunted in $A1^{\Delta 1-43}$ [24]. Hence, myosin motor activity and contractility regulation of the whole heart may be tuned by the interaction between the N-terminus of A1 and actin. In the normal adult human heart two A1 isoforms are expressed in a tissue-specific manner, namely an atrial-specific (MYL4, hALC-1, accession NP_001002841) and a ventricular-specific (MYL3, hVLC-1, accession NP_000249) A1 isoform [25]. Human embryos express large amounts of ALC-1 both in the whole heart and in skeletal muscle [26]. hALC-1 protein levels decrease in

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the ventricle to undetectable levels during early postnatal development but persisted in the atrium throughout the whole life [26]. The hypertrophied right ventricle of children with Tetralogy of Fallot express large amounts of hALC-1 in the ventricle, up to adulthood [27,28]. Similarly, the hypertrophied left ventricle of patients with ischemic, dilative, and hypertrophic cardiomyopathy express hALC-1 [29,30]. Surgical intervention and subsequent normalization of the hemodynamic state decrease hALC-1 [30]. The VLC-1-to-ALC-1 shift in the hypertrophied human heart induced a pronounced positive inotropic effect. i.e. increased force generation as well as shortening velocity [27]. Likewise, transgenic overexpression of ALC-1 in the rodent ventricle replaced VLC-1 in the sarcomeres and increased maximal shortening velocity and force generation [31,32]. The molecular mechanism of hALC-1 inotropy may be based on its strong myosin lever arm binding [33] as well as its weaker actin- binding properties [34] compared with the hVLC-1. In fact, dissociation constants ($K_D$) of synthetic peptides derived from the utmost N-terminus (aa 1–15) of hALC-1 was significantly lower compared with the corresponding N-terminal peptide from hVLC-1 [34]. However, there are no information yet on the interaction properties of full-length A1 with actin. To obtain more detailed information on the properties of the A1/actin complexes, we investigated protein–protein interaction of recombinant hALC-1 and hVLC-1 with recombinant α-actin as well as α-actin with eliminated A1-binding site (α-actin$^{ala3}$) as control. In contrast to the values obtained with N-terminal A1 peptides ($K_D$ in the micromolar range), we observed $K_D$-values in the upper nanomolar range, with actin binding of hALC-1 being significantly weaker than actin binding of hVLC-1.

## 2. Material and methods

### 2.1. Cloning and generation of recombinant proteins

We cloned and generated recombinant fusion proteins of human cardiac ELC isoforms (hALC-1 and hVLC-1) as well as and alanin-mutated cardiac α-actin. All constructs were checked by restriction site mapping, and DNA sequencing using T7 promoter and T7 terminator sequencing primers. hALC-1 and hVLC-1 were cloned with a C-terminal HIS tag. To prepare eukaryotic plasmids expressing hALC-1 and hVLC-1, the corresponding cDNA clones (ImaGenes, Berlin, Germany) were used as template and amplified by PCR using following primers for hALC-1: (sense primer) 5′-ATGGCTCCCAAGC CTGAGCCTAAG-3′, and (anti-sense primer) 5′-TAGCATGATGTGCTT GACAAAGGCTT-3′. For hVLC1: (sense primer) 5′-ATGGCCCCCAAAA AGCCAGAGCCCAAG-3′, and (anti-sense primer) 5′-GCTGGACATGAT GTGCTTCACAAATGCTT-3′. PCR-products were ligated into pEXP5-Topo (Invitrogen, Karlsruhe, Germany) containing a 6xHIS tag (hALC-1-HIS, hVLC1-HIS).

α-actin with a N-terminal glutathione S-transferase (GST) tag was expressed using the pReceiver-BO4 (GeneCopoeia Inc. Maryland, USA) (GST-α-actin). To monitor specific interaction of recombinant A1 isoforms with α-actin, we mutated α-actin 359-EYDE-364 to 359-AYAA-364 (GST-α-actin$^{ala3}$) using the QuickChange site-directed mutagenesis kit (StratageneEurope, Amsterdam, Netherlands) according to the manufacturers protocol. Ala was used as the substituting amino acid because its small side chain would be expected to minimally perturb the structure of the protein. The pEXP5-Topo containing cDNAs of hALC-1-HIS or hVLC1-HIS, and the pReceiver-BO4 expression vectors containing the cDNAs of GST-α-actin and GST-α-actin$^{ala3}$ constructs were used to transform BL21 (DE3) pLysE cells (Invitrogen GmbH, Karlsruhe, Germany). Protein expression was induced with 0.1 mM isopropyl(-D)-thiogalactopyranoside (IPTG; Diagnostic Chemicals Ltd.) for 3 h at 37 °C. Cells were then sonicated, centrifuged, and the supernatant prepared for purification of the different recombinant proteins:

Recombinant hALC-1-HIS or hVLC1-HIS were incubated for 50 min. at 4 °C with 0.5 ml of Ni–NTA-agarose beads (Qiagen, Hilden, Germany). Fusion proteins were eluted with 100 mM imidazole, 300 mM NaCl, 50 mM $NaH_2PO_4$ pH 8.0. Recombinant GST-α-actin and GST-α-actin$^{ala3}$ proteins were incubated with glutathione-Sepharose beads for 60 min at room temperature. Proteins were eluted with G-actin elution buffer (2 mM Tris, 0.2 mM ATP, 0.5 mM β-mercaptoethanol, 0.2 mM $CaCl_2$, 20 mM L-glutathione, 1.4 mM CHAPS (pH 8.5).

### 2.2. Circular dichroism spectroscopy

Circular dichroism (CD) spectra of hALC-1-HIS or hVLC-1-HIS fusion proteins were recorded in a 1 mm quartz cuvette (Hellma, Müllheim, Germany) on a J-720 spectrometer (Jasco, Tokyo, Japan) at 25 °C using a scanning speed of 50 nm/min, a bandwidth of 1 nm, and a response time of 2 s. Proteins were dissolved at concentrations of 4 or 6 μM in 10 mM Tris, 120 mM NaF, pH 7.4. Presented spectra give the mean residual molar ellipticity ($\theta$) of one out of four independent experiments. Secondary structure compositions were estimated by deconvoluting CD spectra in the range of 205–240 nm [35] into reference spectra obtained from proteins of known structures.

### 2.3. Analysis of protein–protein interaction by surface plasmon resonance spectroscopy (SPR)

Binding studies of the recombinant fusion proteins were carried out in a BIAcore 2000 Instrument (Uppsala, Sweden) at 25 °C using the sensor chip CM5 (BiAcore AB). Sensor chips were chemically activated by the injection of 90 μl of a 1:1 mixture of N-hydroxysuccinimide (NHS, 100 mM) and *N*-ethyl-*N*′-(3dimethylaminopropyl)-carbodi-imide (EDC, 400 mM) at a flow rate of 10 μl/min. The recombinant proteins GST-α-actin (test) and GST-α-actin$^{ala3}$ were diluted in a 10 mM acetate buffer, pH 4.5, and immobilized on separate lanes on the chip at a binding level of 2 ng/mm$^2$, which was based on the assumption that a SPR response of 1000 relative units (RU) translates to 1 ng/mm$^2$ immobilized protein. The remaining matrix sites were blocked by the injection of 70 μl of 1 M ethanolamine, pH 8.5. Purified recombinant hALC-1-HIS or hVLC-1-HIS diluted in PBS (100 mM NaCl, 1 mM EGTA, 5 mM $Na_2$-

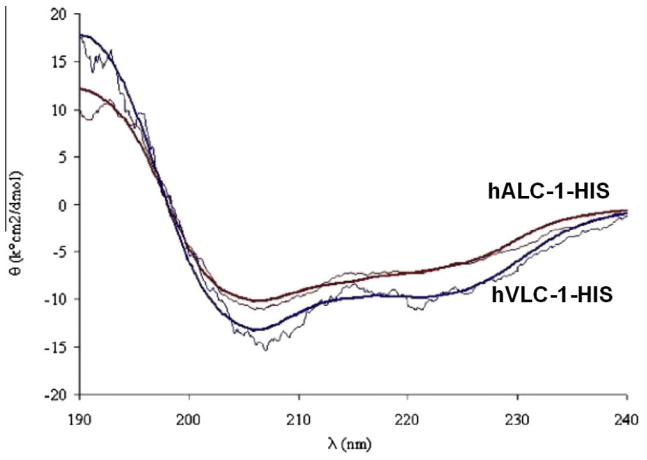


**Fig. 1.** Circular dichroism spectra (calculated fitted curve) of hALC-1-HIS or hVLC-1-HIS (each 4 μM). $\theta$ is 1/1000 of the mean residual molar ellipticity. Noisy curve represents original signals, smooth curve represents the corresponding calculated fit.