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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 (θ) 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-

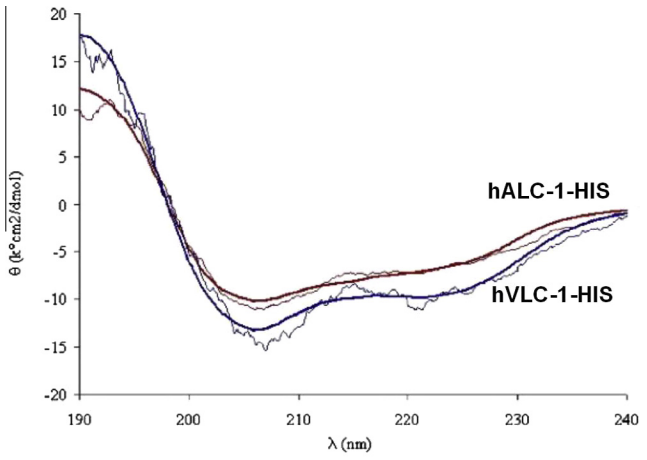

Fig. 1. Circular dichroism spectra (calculated fitted curve) of hALC-1-HIS or hVLC-1-HIS (each 4 μM). θ is 1/1000 of the mean residual molar ellipticity. Noisy curve represents original signals, smooth curve represents the corresponding calculated fit.

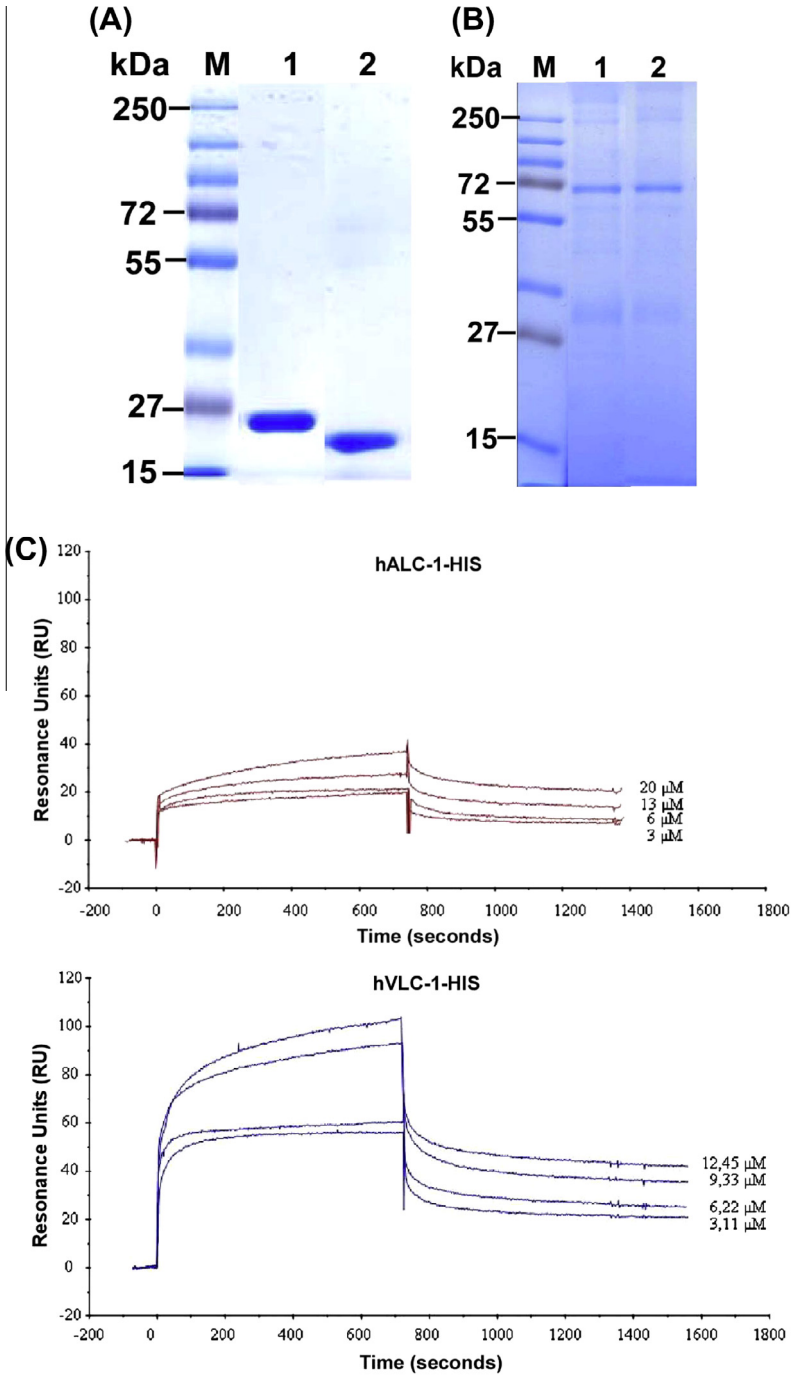

Fig. 2. Protein analysis and protein–protein interactions by surface plasmon resonance spectroscopy (A) SDS–PAGE of hALC-1-HIS (lane 1) and hVLC-1-HIS (lane 2), (B) SDS–PAGE of GST-α-actin (lane 1) and GST-α-actin^{ala3} (lane 2) and (C) Representative original registration of surface plasmon resonance signals of the interactions between hALC-1-HIS or hVLC-1-HIS (3–20 μM each) with cardiac GST-α-actin. The signals shown correspond to the specific interaction (difference signal) with wild-type α-actin (GST-α-actin), since the interaction signal obtained with the mutated GST-α-actin^{ala3} was monitored simultaneously as control and became automatically subtracted.

HPO_4), pH 7.4 were used as analyte and injected into the flow cells at a perfusion rate of 10 μl/min. The analyte concentrations ranged from 0.125 to 15 μM. Between sample injections the surface was regenerated with 5 μl of buffer containing 133 mM NaCl, 8 mM NaOH, 0.05% CHAPS, 0.05% Tween-80, 0.05% Tween-20, and 0.05% Triton X-100. For data analysis, rate constants were calculated by global fitting using the BIAevaluation 3.2 RC 1 program (Biacore AB). Curves were fitted to a single-site interaction model. Equilibrium K_D values were determined from the rate constants k_{on} and k_{off} according to $K_D = k_{off}/k_{on}$. The analysis software corrects for systematic drift in baseline that occurred during measurements.

Table 1
Surface plasmon resonance analysis of hALC-1-HIS and hVLC-1-HIS (13 μM each) with GST-α-actin. The signals obtained upon interaction with the mutated GST-α-actin^{ala3} was used as control and became automatically subtracted.

	k_{on} (M^{-1} s^{-1})	k_{off} (s^{-1})	K_D (nM)	n
hALC1-HIS	1018 ± 233**	3.9 10^{-4} ± 0.6 10^{-4}	575 ± 178**	8
hVLC1-HIS	2908 ± 874	6.7 10^{-4} ± 3.8 10^{-4}	186 ± 55	8

n = number of experiments.
** $P < 0.01$.

3. Statistics

Values are means ± SEM. Statistical difference between mean values was calculated using Student's *t*-test for two-tailed unpaired values. Data were considered significant at *p*-values of <0.05.

4. Results and discussion

In this study we investigated for the first time the binding constants of human cardiac A1 myosin light chain isoforms (human atrial and ventricular essential myosin light chains, hALC-1 and hVLC-1, respectively) with α-actin. For these investigations, we generated recombinant proteins of the binding partners. CD-spectroscopy was applied to study the secondary structures of the A1 isoforms (Fig. 1). They revealed CD-spectra having negative bands at 222 and 208 nm and a positive band at 193 nm (Fig. 1) which is typical for α-helical proteins [34]. hALC-1-HIS and hVLC-1-HIS revealed similar α-helicity/random-coil ratio, which were estimated to about 22%/46% and 36%/43%, respectively (Fig. 1). The high proportions of α-helical secondary structure of both A1 isoforms are in accordance with the known EF-hand structure of myosin light chains [1,2]. Recombinant A1 isoforms showed the expected molecular masses, with hALC-1-HIS and hVLC-1-HIS around 28 kDa and 25 kDa, respectively [8,9,27] (Fig. 2A). Both recombinant GST-α-actin molecules revealed the same molecular mass of around 70 kDa (Fig. 2B) corresponding to the combined molecular masses of α-actin (ca. 42 kDa) with the GST-Tag (26 kDa). We did not analyse recombinant α-actin molecules by CD-spectroscopy since their large GST portions could obscure the data on actin structures.

We found, that similar to the interaction of small N-terminal A1 peptides with α- actin [11,14,17,18], binding of cardiac A1 isoforms to actin is reversible. Specific complex formation of hALC-1-HIS or hVLC-1-HIS with GST-α-actin was measured by surface plasmon resonance spectroscopy (SPR; Fig. 2C). We used cardiac α-actin with Ala-mutated A1 binding sites (GST-α-actin^{ala3}) as control. The SPR signals herein represent the difference signals, i.e. SPR signals automatically corrected for unspecific binding of A1 to the control GST-α-actin^{ala3} giving the specific interaction signals of GST-α-actin with the A1 isoform. We observed K_D values of the A1/actin complexes in the upper nanomolar range (Table 1). One main finding in this paper is that the affinity of the hALC-1/α-actin complex (K_D = 575 nM) was significantly ($p < 0.01$) lower compared with the hVLC-1/α-actin complex (K_D = 186 nM), i.e. a 3-fold higher K_D (Fig. 2C; Table 1). The lower affinity of hALC-1 to actin was mainly due to a significantly ($p < 0.01$) depressed k_{on} which was around 3fold smaller ($p < 0.01$) compared with the hVLC-1/α-actin interaction (Table 1). The specific binding of complete A1 to actin is much stronger than actin-interaction of small synthetic N-terminal A1 peptides which revealed K_D values in the micromolar range (K_D 25–54 μM) [34]. However, in both sets of experiments – actin interaction with N-terminal A1 peptides or complete A1 isoforms – actin affinity of hALC-1 was around 3fold lower than that of hVLC-1. The major actin binding activity of A1 is mediated by the first 11 N-terminal residues [14] which bind to a C-terminal cluster of negatively charged amino acids, i.e. 359-EYDE-364 on actin [7,12,36]. The complementary characteristics of the identified residues suggests ionic binding between four sticky lysine residues K3, K4, K8, and K9 of cardiac A1 isoforms with negatively charged E360, D362, and E363 of cardiac α-actin, respectively [7]. Distinct actin binding affinities of cardiac A1 isoforms is surprising, since primary sequences of hALC-1 and hVLC-1 within the critical actin-binding lysine residues K3–K9 are identical (hALC-1: MAPKKPEPKK***EA***AK***P;*** hVLC-1: MAPKKPEPKK***DD***AK***A***). Thus, the distinct primary sequences just down-stream to the actin-binding lysine residues (c.f. amino acids in ***bold italic***) may critically modulate the actin affinities of A1 isoforms.

There is a large body of evidence showing that binding of A1 to actin slows down shortening velocity of muscle preparations [11,14–18] probably by decreasing the ADP release rate from the catalytic core of the myosin motor domain [22]. Since A1 interaction with actin is an equilibrium reaction with a K_D in the upper nanomolar range, myosin cross-bridges (XBs) may exist in two states, i.e. one with faster mobility (no A1/actin interaction), and a state with slower mobility (A1 bound to actin). In this concept, weakening A1/actin interaction, e.g. by A1 isoforms could well modify XB function and, therefore muscle properties. This hypothesis predicts that in muscle with essential myosin light chains without (A2 light chain) or weakened (hALC-1) actin affinity, the fraction of XBs in a fast mobility state should increase. In fact, shortening velocity of muscle preparations with increasing amounts of ALC-1 [27,31,32] or the A2 isoform [15,16] rose significantly We conclude, that expression of ALC-1, e.g. in the hypertrophied human ventricle, increases cardiac contraction velocity by reducing the binding of the N-terminus to actin. Re-expression of the ALC-1, therefore, seems to be an auto-regulatory mechanism of the human heart to adapt to an increased work demand.

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