Human essential myosin light chain isoforms revealed distinct myosin binding, sarcomeric sorting, and inotropic activity

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Aims
In this paper, we tested the hypothesis that different binding affinities of the C-terminus of human cardiac alkali (essential) myosin light chain (A1) isoforms to the IQ1 motif of the myosin lever arm provide a molecular basis for distinct sarcomeric sorting and inotropic activity.

Methods and results
We employed circular dichroism and surface plasmon resonance spectroscopy to investigate structural properties, secondary structures, and protein–protein interactions of a recombinant head-rod fragments of rat cardiac β-myosin heavy chain aa664–915 with alanine-mutated IQ2 domain (rβ-MYH664–915Qala4) and A1 isoforms [human atrial (hALC1) and human ventricular (hVLC-1) light chains]. Double epitope-tagging competition was used to monitor the intracellular localization of exogenously introduced hALC-1 and hVLC-1 constructs in neonatal rat cardiomyocytes. Contractile functions of A1 isoforms were investigated by monitoring shortening and intracellular-free Ca²⁺ (Fura-2) of adult rat cardiomyocytes infected with adenoviral (Ad) vectors using hALC-1 or β-galactosidase as expression cassettes. hALC-1 bound more strongly (greater than three-fold lower K_D) than did hVLC-1. Sorting specificity of A1 isoforms to sarcomeres of cardiomyocytes rose in the order hVLC-1 to hALC-1. Replacement of endogenous VLC-1 by hALC-1 in adult rat cardiomyocytes increased contractility while the systolic Ca²⁺ signal remained unchanged.

Conclusion
Intense myosin binding of hALC-1 provides a mechanism for preferential sarcomeric sorting and Ca²⁺-independent positive inotropic activity.

Keywords
Essential myosin light chains  •  Heart  •  Cardiomyocyte contraction

1. Introduction
Type II myosin, which consists of two heavy chains (MYH) and four light chains (MLCs), is the motor protein of muscle which interacts cyclically with the thin (actin) filament, thus producing force and shortening while consuming ATP. MYH (i.e. MYH7, rat cardiac β-MYH, accession NP_058936 in this study) can be separated into: (i) an N-terminal ‘head’ or subfragment 1 (S-1) (aa1–837) consisting of a pear-shaped 8 nm motor domain with ATPase and actin-binding sites, connected to an α-helical 8.5 nm lever arm; (ii) a 150 nm α-helical rod (aa838–1935), consisting of a 60 nm subfragment 2 (S-2), and a C-terminal the 90 nm light meromyosin (LMM) which forms a coiled-coil with a second MYH.¹ ² Two types of myosin light chains (MLCs), essential (ELCs) and regulatory (RLCs), are associated with the lever arm of the MYH. The lever arm contains two IQ motifs in tandem.³ ⁴ IQ1 binds ELCs, IQ2 binds RLCs.¹ ² The full-length ELC is designated as alkali-1 MLC (A1), while alternatively spliced ELCs with deleted N-terminal 46aa are called alkali-2 MLC (A2).² ³–⁸ The primary structure of A1 isoforms is built of an actin-binding-lysine-rich N-terminus,⁹ a proline-rich antenna-like spacer, and a C-terminal lever arm-binding region consisting of four helix-loop-helix EF-hand domains which form a dumbbell-like shape. Binding of ELC to the lever arm is non-covalently, mainly through van der Waals contacts.³ ⁴ ELC binding to IQ1 of the lever arm is...
critical for myosin function: analysis of the mechanics of single myosin molecules without ELC using ultracompact glass needles showed only about one-third of the force generated by native myosin. 10

In the normal adult mammalian 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. Most patients with cardiac hypertrophy and congenital heart diseases re-express hALC-1 in their ventricles, partially replacing the VLC-1 isoform. This VLC-1-to-ALC-1 isoform shift induced a pronounced positive inotrop effect. 11 Likewise, transgenic overexpression of murine ALC-1 in the mouse ventricle or hALC-1 in the rat ventricle replaced the VLC-1 isoform in the sarcomere and increased cardiac contractility and myosin motor properties. 12–14

Replacement of endogenous VLC-1 by newly expressed ALC-1 could be elicited by two mechanisms, namely (i) a suppression of VLC-1 expression by ALC-1 and (ii) by different binding affinities of the A1 isoforms with the IQ1 motif of the myosin lever arm. The first possibility assumes, that the A1-1 molecule executes a direct regulatory effect on the expression of the VLC-1 gene, which seems to be unlikely with the present state of knowledge. In this paper, we tested the hypothesis that different A1 isoforms could have distinct binding properties with the myosin lever arm. We, therefore, produced three recombinant fusion proteins, namely: a rat β-myosin head-rod fragment aa664–915 (β-MYH664–915IQala4), human ALC-1 (h(ALC-1)), and human VLC-1 (h(VLC-1)). Protein–protein interaction between A1 isoforms and MYH was analysed by surface plasmon resonance measurements.

To show that distinct affinities of A1 isoforms with MYH correlate with different sarcomeric sorting properties, we applied a double epitope-tagging competition method. In this experimental system, any combination of two isoforms tagged with two different epitopes is co-expressed from their cDNAs within the same cell. Visualization of exogenous proteins is achieved with antibodies specific to each tag. 15

In addition, we investigated if the intense binding of hALC-1 to MYH and its preferential sorting capacity could influence the contractile properties of intact cardiomyocytes, which ought to be independent from systolic Ca2+ activation levels. We tested this hypothesis by simultaneously monitoring both the contractile and the Ca2+ handling properties of adult rat cardiomyocytes infected with an adenoviral (Ad) vector with hALC-1 as expression cassette.

In this paper, we show that hALC-1 binds significantly stronger (with a 3.3-fold lower Kd) to the myosin lever arm than does hVLC-1. This may provide the molecular basis for its preferential sarcomeric sorting specificity observed in neonatal cardiomyocytes if compared with hVLC-1. Furthermore, we found that Ad-driven over-expression of hALC-1 in primary adult rat cardiomyocytes significantly increased contraction amplitude while the systolic-free Ca2+ levels and Ca2+ cycling kinetics remained unchanged. This provides evidence that myosin molecules with intense-binding hALC-1 generate more force than myosin with VLC-1.

2. Methods

2.1 Recombinant proteins

Cloning and generation of recombinant fusion proteins of human ELC isoforms and a myosin head-rod fragment of aa 665–915 with N-terminal hexahistidine (HIS)-tag/40 amino acid (aa) spacer and N-terminal glutathione S-transferase (GST)-tag/I4aa spacer, respectively, were performed as described recently. 16 To eliminate unspecific binding to the IQ2 domain and to provide a stretched rather than a collapsed myosin head-rod structure, we deteriorated the IQ2 domain of rβ-MYH664–915 by alanine-scanning site-directed mutagenesis (rβ-MYH664–915IQala4). 16 All constructs were checked by restriction site mapping and DNA sequencing.

2.2 Construction of epitope-tagged hALC-1 and hVLC-1 expression plasmids

hALC-1 and hVLC-1 were cloned with a C-terminal HIS and myc tag, respectively, to differentiate both recombinant forms from each other in immunofluorescence after co-transfection of neonatal cardiomyocytes. To verify that the presence of the tags does not influence intracellular sorting properties of A1 isoforms, hALC-1 and hVLC-1 with myc and HIS tag, respectively, were cloned and co-transfected. To prepare eukaryotic plasmids expressing hALC-1 and hVLC-1, the corresponding cDNA clones (Ima-Genes, Berlin, Germany) were used as template and amplified by PCR. To perform the PCR, two pairs of primers were synthesized: i.e. for hALC-1, sense primer: 5′-CCaagcgtGGCCACCATGCATCATCATCATCATCATATGGCTCTCAAAGAAGCT-3′, anti-sense primer: 5′-ACaaggctTCCACCTGACATGTGCTTGACAAA-3′, for hVLC1, sense primer: 5′-CCaagcgtGGCCACCATGCATGGAGAACAAGGAATTCT.GCCACCATG-3′ and the restriction site HindIII (italic letters) was incorporated into primers to facilitate subcloning into the eucaryotic expression vector pcDNA3.1(+) (Invitrogen GmbH, Karlsruhe, Germany). In each sense primer, following HindIII site, a Kozak consensus sequence (GCCACCATG)17 for generating a ribosome-binding site and an ATG start sites were added to initiate the expression in eukaryotic cells. PCR products were cut with HindIII and ligated with HindIII-cut pcDNA3.1(+) (+). These plasmids were then transfected into Top10 cells and purified using the Invitrogen Plasmid Maxi Kit (Invitex, Berlin, Germany). All constructs were confirmed by DNA sequencing T7 promoter and T7 terminator sequencing primers.

2.3 Analysis of protein–protein interactions by surface plasmon resonance

Binding studies of the recombinant fusion proteins were carried out in a BIAcore 2000 Instrument (Uppsala Sweden) at 25°C using the sensor chip CMS (BIAcore AB) as recently described. 16 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 Kd values were determined from the rate constants koff and kon according to Kd=koff/kon. The analysis software corrects for systematic drift in baseline that occurred during measurements.

2.4 Circular dichroism spectroscopy

Circular dichroism (CD) spectra of ELC fusion proteins were recorded in 1 mm quartz cuvettes (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 into reference spectra obtained from proteins of known structures.
2.5 Construction and propagation of adenovirus vectors

For all experiments, replication-deficient adenovirus vectors of the first generation were used which are based on a deletion of the E1 region of an adenovirus type 5 (Ad vector). The Ad vectors, AdCMV.hALC-1 and AdCMV.β-gal, were constructed by cloning a cDNA of the human essential atrial light chain 1 and Escherichia coli β-galactosidase (each without tag) into an adenovirus backbone of the AdEasyTM system as described recently. All vectors were propagated on HEK 293 cells, purified, and stored at −80°C. The titers of stocks measured by plaque assays were 7.7 × 10^8 pfu/mL, with a particle/pfu ratio of 30/. Recombinant adenoviruses were tested for the absence of wild-type virus by polymerase chain reaction of the early transcriptional unit E1 and E1a.

2.6 Adult rat cardiomyocytes

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). Approval was granted by a local ethics review board (LAGeSo, Berlin, Germany). Male Wistar rats aged 3 months were anesthetized with isoflurane, followed by intraperitoneal injection of 8 μg xylazine and 35 μg ketamine. Hearts were rapidly removed, transferred into isotonic NaCl solution containing heparin (1000 U), and connected to a canula in a Langendorff perfusion system. Hearts were perfused at 37°C for 3 min with Ca2+-free Krebs Henseleit buffer (KHb) and 10 mM butanedione monoxime (BDM) gassed with carbogen. After that, perfusion was switched to recirculation with KHb containing 0.04% collagenase (Worthington Biochemical Corp, Lakewood, NJ, USA), 0.2% bovine serum albumin (BSA), 10 mM BDM, and 8.3 mM glucose for 30 min. The ventricles were minced and incubated in the digestion medium for another 10 min at 37°C. After filtration through a nylon mesh (200μm pore size) and centrifugation, cells were resuspended in Ca2+-free medium. Ca2+ concentration was increased stepwise to 500 μM to obtain Ca2+-tolerant cardiomyocytes. After final washes, cardiomyocytes were resuspended in M199 medium completed with 0.2% BSA, 5% fetal calf serum (FCS), 5 mM creatine, 5 mM taurine, 2 mM carnitine, 10 μM cytosine-α-arabinofuranoside, and antibiotics. Before preparation of adult rat cardiomyocytes, cells were resuspended in Ca2+-free medium. M199 medium completed with 0.2% BSA, 5% FCS, 5 mM creatine, 5 mM taurine, 2 mM carnitine, 10 μM cytosine-α-arabinofuranoside, and antibiotics, and cultivated for 48 h. Cardiomyocytes were homogenized with a motor-driven glass-teflon homogenizer in SDS-sample buffer (5% SDS, 50 mM Tris–HCl pH 7.5, 75 mM urea, 10 mM dithiothreitol), denatured for 3 min at 95°C, and cleared by centrifugation. The supernatant containing the total SDS-extracted proteins was removed and the protein concentration was determined by a modified Lowry method. SDS-solubilized proteins were separated by SDS–PAGE on a 12% resolving gel and transferred to nitrocellulose filters according to standard procedures. The transfers were incubated overnight with different primary antibodies. Recombinant proteins were analysed by specific antibodies which recognize the HIS-tag (Quigen, Germany) or the GST-tag (Invitrogen, Germany). HAC-1 expression in AdV-infected adult rat cardiomyocytes was monitored using a peptide-directed, affinity-purified anti-hALC-1 antibody raised against the amino acid sequence 29–43 (PAPAEKPEAFDKPS). The secondary antibody was a peroxidase-conjugated anti-mouse (for HIS and GST tags; Biogenes, Germany) or anti-rabbit antibody (Biogenes, Berlin, Germany) (for HAC-1) for 1 h at room temperature. M2H were detected for the absence of wild-type virus by polymerase chain reaction of the early transcriptional unit E1 and E1a.

2.7 Measurement of cardiomyocyte shortening and Ca2+ transients

Attached adult rat cardiomyocytes infected with Ad vectors after 48 h cultivation were washed with Hank’s balanced salts solution (mM concentrations: MgSO4 0.81, KCl 5.36, KH2PO4 0.44, NaCl 137, Na2HPO4 0.82, glucose 5.55, CaCl2 1.26, Sigma H1387) buffered with 10 mM HEPES at pH 7.4 (HBSS). Attached cardiomyocytes were loaded with Fura-2-AM dissolved in HBSS in the dark at room temperature for 15 min. Dye solution was removed, and cells were left on HBSS for another 13 min. Only cardiomyocytes of optically intact rod-shaped morphology with clear cross striation were analysed. Cardiomyocytes were electrically stimulated until a stable steady-state contraction and Fura-2 signal could be monitored. Cell shortening and Fura-2 signals were simultaneously measured at 30°C on an Ionoptix Contractility and Fluorescence System (Ionoptix). Cardiomyocytes were electrically stimulated with bipolar pulses of 5 ms duration at 1 Hz. Cell shortening, expressed as percentage of resting cell length, was measured using the video-edge technique at a sampling rate of 240/s. Ca2+ transients were monitored as ratio of fluorescence emission at 510 nm obtained by alternate excitation at 340 and 380 nm (340/380 ratio). Data files from 15 consecutive beats recorded at intervals were averaged for the analysis.

2.8 Neonatal cardiomyocytes

Neonatal cardiomyocytes were prepared from 1 to 3 days old Sprague–Dawley rats and cultivated as described previously. A total of 10 × 10⁶ neonatal rat cardiomyocytes were seeded into each well of a 12-well plate. After 18 h incubation, the medium was replaced with 1 mL SM20-I (Biochrome, Germany). One microgram of plasmid DNA (in 100 μL SM20-I) was mixed with 4 μL Lipofectamine (in 100 μL SM20-I) and incubated for 20 min at room temperature. The resultant complex mixture was added to the cells seeded in a 12-well plate. The mixture was replaced with fresh medium 6 h after transfection and after 18 h cells were washed twice with PBS. Cells were fixed with 3.7% parformaldehyde for 10 min, washed with PBS, and permeabilized with 0.5% Triton X-100 (in PBS) for 10 min, followed by another PBS wash step. Subsequently, 0.2% fish skin gelatin was added for 30 min to block non-specific binding sites, followed by incubation at 4°C over night with monoclonal antibodies anti-HIS (Qiagen) and anti-c myc (Invtrogen), respectively. After washing three times with PBS with 0.1% Nonidet NP40, the secondary antibodies, Alexa488 and Alexa568 (Invitrogen) in 0.2% fish-skin gelatin, were added for 1 h. Cells were washed three times with PBS with 0.1% NP40 again and finally fixed with Moviold. Cells were observed using confocal laser microscopy (Axiovert 40 CFL).

2.9 Western blot analysis

SDS-solubilized proteins were separated by SDS–PAGE on a 12% resolving gel and transferred to nitrocellulose filters according to standard procedures. The transfers were incubated overnight with different primary antibodies. Recombinant proteins were analysed by specific antibodies which recognize the HIS-tag (Quigen, Germany) or the GST-tag (Invitrogen, Germany), HAC-1 expression in AdV-infected adult rat cardiomyocytes was monitored using a peptide-directed, affinity-purified anti-hALC-1 antibody raised against the amino acid sequence 29–43 (PAPAEKPEAFDKPS). The secondary antibody was a peroxidase-conjugated anti-mouse (for HIS and GST tags; Biogenes, Germany) or anti-rabbit antibody (Biogenes, Berlin, Germany) (for HAC-1) for 1 h at room temperature. M2H were detected using the monoclonal antibody 2C9, which detects both α- and β-M2H (gift from D. Morret, Montpellier). Immunoreactive proteins were visualized with the enhanced chemiluminescence’s reaction Kit (ECL, Amersham) and X-ray films (X-Omat, Kodak, Rochester, NY, USA). Signals were scanned densitometrically using BIO–RAD GS-710 (calibrated imaging densitometry, San Diego, CA, USA).
2.10 Statistics
Statistical difference between mean values was calculated using Student’s
t-test for unpaired or paired values and was considered significant at
P-values of <0.05.

3. Results
3.1 hALC-1 binds more strongly to the
myosin lever arm than hVLC-1
We could recently show, that alanine mutation of the IQ2 domain of rβ-
MYH664–915 (I814, Q815, R819, and W827, i.e. rβ-MYH664–915IQala4)
stretched the head-rod-domain and increased more than 30-fold the
binding affinity of A1 to the IQ1 domain if compared with the functional
and structural features of rβ-MYH664–915.16 rβ-MYH664–915IQala4
remained monomeric in dilute solution.16 That is why we used
rβ-MYH664–915IQala4 in the presented work to study functional differ-
ences between human cardiac A1 isoforms.
Affinity of the hALC-1/rβ-MYH664–915IQala4 complex was significantly
(P < 0.001) higher compared with the hVLC-1/ rβ-MYH664–915IQala4
complex interaction, i.e. 3.3-fold lower K_D (Figure 2, Table 2). The
lower affinity of hVLC-1 to the myosin lever arm was mainly due to a sig-
nificantly lower k_on which was around three-fold slower (P < 0.001)
compared with hALC-1/rβ-MYH664–915IQala4 (Figure 1, Table 1).

3.2 Circular dichroism spectra
of recombinant A1 isoforms
CD-spectra were measured to study the secondary structures of
recombinant A1 isoforms (Figure 1). Human cardiac A1 isoforms

![Figure 1](https://academic.oup.com/cardiovascres/article-abstract/90/3/513/276293/attachment/1)

Table 1 Interaction between rβ-MYH664–915IQala4 and the two A1 isoforms human atrial myosin light chain (hALC-1) and
human ventricular myosin light chain (hVLC-1)

<table>
<thead>
<tr>
<th>Ligand</th>
<th>k_on (M⁻¹s⁻¹)</th>
<th>k_off (s⁻¹)</th>
<th>K_D (nM)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>hALC-1</td>
<td>2.2 × 10⁴ ± 0.5 × 10⁴*</td>
<td>1.5 × 10⁻⁴ ± 0.3 × 10⁻⁴</td>
<td>11.2 ± 2.8*</td>
<td>12</td>
</tr>
<tr>
<td>hVLC-1</td>
<td>7.4 × 10³ ± 0.2 × 10³</td>
<td>1.9 × 10⁻⁴ ± 0.2 × 10⁻⁴</td>
<td>37.0 ± 7.3</td>
<td>12</td>
</tr>
</tbody>
</table>

k_on, association rate; k_off, dissociation rate; K_D, dissociation constant. n is the number of experiments.

*P < 0.001 hALC-1 vs. hVLC-1.
revealed CD-spectra having negative bands at 222 and 208 nm and a positive band at 193 nm which is typical for mainly α-helical proteins. hALC-1 and hVLC-1 revealed similar degrees of α-helicity, which were estimated to amount to 57 and 55%, respectively. The high proportions of α-helical secondary structure are in accordance with the known EF-hand structure of MLCs 1–4 (Figure 1).

3.3 Intracomartmental sorting of exogenous A1 isoforms in rat neonatal cardiomyocytes

Cultured neonatal rat cardiomyocytes were co-transfected with equal amounts of hALC-1 and hVLC-1 cDNA with HIS-tag and myc-tag, respectively. Incorporation into sarcomeres was detected by using immunofluorescence microscopy with antibodies detecting the HIS or the myc tag. The intracompartmental sorting pattern of hALC-1 and hVLC-1 are clearly different. Figure 2 shows the localization of hALC-1-HIS in many sarcomeres, while hVLC-1 revealed diffuse expression with weak sarcomeric integration. Likewise, we observed preferential incorporation of hALC-1 into sarcomeric structures while hVLC-1 revealed a diffuse expression with weak sarcomeric integration. (C) Immunofluorescence analysis of sarcomeres of a neonatal cardiomyocyte transfected with hALC-1-HIS cDNA and double-stained with a HIS-antibody (green, hALC-1), and α-actin (red) antibody for Z-Line detection. Please note the absence of hALC-1 staining in the H-zone, i.e. the middle of the sarcomeres.

Figure 2 Confocal images depicting the integration of the myosin light chain isoforms into neonatal rat cardiomyocytes 18 h after transfection with lipofectamine. (A) Sarcomeres were stained for the HIS-tag to detect hALC-1 (green) and for the myc-tag to demonstrate hVLC-1 (red). Please note the preferential incorporation of hALC-1 into sarcomeric structures (small arrows) while hVLC-1 revealed a diffuse cellular expression with weak sarcomeric integration. (B) Sarcomeres were stained for the myc-tag to detect hALC-1 (green) and for the HIS-tag to demonstrate hVLC-1 (red). Likewise, we observed preferential incorporation of hALC-1 into sarcomeric structures while hVLC-1 revealed a diffuse cellular expression with weak sarcomeric integration. (C) Immunofluorescence analysis of sarcomeres of a neonatal cardiomyocyte transfected with hALC-1-HIS cDNA and double-stained with a HIS-antibody (green, hALC-1), and α-actin (red) antibody for Z-Line detection. Please note the absence of hALC-1 staining in the H-zone, i.e. the middle of the sarcomeres.

Essential light chain isoform function
Table 2 Analysis of (A) contraction and (B) intracellular-free Ca$^{2+}$ concentrations monitored as Fura-2 signal of adult rat cardiomyocytes infected with AdCMV.hALC-1 (hALC-1), AdCMV.β-gal (β-gal) (50 MOI each), or control infected (mock).

<table>
<thead>
<tr>
<th></th>
<th>Mock (n = 31)</th>
<th>hALC-1 (n = 40)</th>
<th>β-gal (n = 40)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(A) Contraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>F5 (% cell length)</td>
<td>4.1 ± 0.3</td>
<td>5.2 ± 0.5*</td>
<td>3.9 ± 0.4</td>
</tr>
<tr>
<td>dS/dt$_{max}$ (μm/s)</td>
<td>64.8 ± 5.4</td>
<td>74 ± 5.5*</td>
<td>60.2 ± 6.1</td>
</tr>
<tr>
<td>+dS/dt$_{max}$ (μm/s)</td>
<td>45.4 ± 3.8</td>
<td>54.8 ± 4.1*</td>
<td>43.5 ± 5.2</td>
</tr>
<tr>
<td>(B) Fura-2 signal (R$_{340/380}$nm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>R$_{max}$</td>
<td>0.47 ± 0.04</td>
<td>0.58 ± 0.04</td>
<td>0.61 ± 0.05</td>
</tr>
<tr>
<td>+dR/dt$_{max}$ (RIS)</td>
<td>11.4 ± 0.9</td>
<td>14.3 ± 1</td>
<td>15.2 ± 1.2</td>
</tr>
<tr>
<td>+dR/dt$_{max}$ (RIS)</td>
<td>2.9 ± 0.16</td>
<td>3.5 ± 0.2</td>
<td>3.4 ± 0.2</td>
</tr>
</tbody>
</table>

(A) F5, fractional shortening amplitude expressed in % diastolic cell length; –dS/dt$_{max}$, maximal rate of shortening; +dS/dt$_{max}$, maximal rate of relaxation; R$_{340/380}$: fluorescence ratio measured at 340 and 380 nm, respectively; (B) R$_{max}$, maximal amplitude of the fluorescence ratio during systole; +dR/dt$_{max}$, maximal rate of fluorescence development during systole; –dR/dt$_{max}$, maximal rate of fluorescence cessation during diastole; n is the number of cardiomyocytes investigated. *P < 0.05 compared with β-gal infected cardiomyocytes.

4. Discussion

In this study, we investigated structural and functional features of human cardiac A1 isoforms. The myosin head-rod fragment with alanine mutated IQ2 domain, i.e. rβ-MYH664–915IQalan4 used in this work for protein–protein interaction studies remained monomeric in dilute solution, revealed a stretched conformation with mainly α-helical secondary structures, and showed strong interactions with A1 light chains, as demonstrated recently.16

Protein complex formation of both hVLC-1 and rat VLC-1 (rVLC-1; cf.15) with the MYH664–915IQalan4 turned out to have almost identical Kd values. In contrast, we observed a highly significantly (greater than three-fold) lower Kd of the hALC-1/MYH664–915IQalan complex than of the VLC-1/MYH664–915IQalan complexes. The distinct lever arm binding properties of hALC-1 and hVLC-1 isoforms are supported by different primary structures (84% identity; cf. NCBI Blast). The different binding properties could be due to different amounts of A1 interaction sites along the IQ1 motif. In this model, hALC-1 would have more binding sites than the VLC-1 isoforms. In fact, chicken ELC revealed 24, while scallop ELC only 14 interaction sites with IQ1.1,4

The higher affinity of hALC-1 compared with the hVLC-1 predicts distinct integration properties into the sarcomeres of cardiomyocytes. We tested this hypothesis by co-expression of hALC-1 and hVLC-1 in neonatal cardiomyocytes. We could indeed show herein that the hALC-1 preferentially integrated into the sarcomeres of neonatal cardiomyocytes. This is in line with recent works reporting on distinct sarcomeric sorting specificities of different MLC isoforms, which increased in the order MLC3 nm, MLC1sa, MLC1sb, MLC1f, and MLC3f.21 In particular, the second EF-hand domain seems to be responsible for the isoform-specific sorting of ELC.22

We could exclude any influence of the different tags on sarcomeric sorting properties by exchanging the tags for the A1 isoforms. ELC isoforms regulate both force and contraction velocity of muscle. During human cardiac hypertrophy VLC-1 is partially replaced by ALC-1, a process which seems to be facilitated by the intense lever arm affinity of ALC-1. Partial VLC-1/ALC-1 replacement showed significant functional effects. Thus, maximal shortening velocity (V$_{max}$) and force per cross-sectional area of human ventricle increased.11 In accordance, transgenic overexpression of ALC-1 in the hearts of murine14 and rats12 increases force generation and contraction velocity.

The molecular mechanism of ALC-1 inotropy is not fully understood. V$_{max}$ is determined by $\beta_{fop}$22,24 which equals the ADP release rate from the nucleotide binding site of the motor domain.25,26 Accordingly, an increased $\beta_{fop}$ upon ALC-1 expression would predict a reduced rather than enhanced force generation. Hence, additional mechanisms must exist which independently of cross-bridge cycling kinetics may regulate cross-bridge force generation by ELC isoforms.
Force generation per myosin molecule depends on the elastic properties of the lever arm, i.e. its resistance to bending forces. Increasing rigidity of the lever arm, therefore, would power up force generation,\(^\text{27}\) assuming the distance the lever arm moves (10 nm) remains unchanged. It seems reasonable to assume, that association of the lever arm with the MLC increases lever arm rigidity and at the same time force generation per cross-bridge. Note, that a 10 nm poly-alanine α-helix (mimicking the lever arm) revealed a lateral stiffness of 1.2 pN/nm\(^\text{28}\) while stiffness of a cross-bridge with associated MLC comes close to 3.1 pN/nm,\(^\text{27,28}\) suggesting that association of MLC with the lever arm could substantially increase rigidity of an α-helix. In this concept, the strong binding of hALC-1 to myosin could increase the spring constant of the lever arm and therefore force generation of the individual myosin cross-bridge above the value obtained upon hVLC-1 or rVLC-1 binding. Thus, two separated α-helices with associated MLC represent an interesting alternative to a coiled-coil lever arm, since they are stiff enough for efficient force transmission while omitting steric hindrance of the two head domains of native myosin molecule.

An increased force generation per single cross-bridge upon enhanced lever arm rigidity in the presence of hALC-1 suggests improved muscle

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**Figure 3** (A) Phase contrast (left) and immunofluorescence (right) of AdCMV.hALC-1 infected adult rat cardiomyocytes (ARC; MOI 50) after 2 days of culture by confocal microscopy. Cells were characterized by incubation with an antibody raised against hALC-1 (green). (B) Double-labeling immunofluorescence of sarcomeres of AdCMV.hALC-1 infected ARC (MOI 50) after 2 days of culture by confocal microscopy. Cells were characterized by incubation with two specific antibodies raised against cardiac myosin heavy chain (α-MYH; green) and hALC-1 (α-hALC-1; red), or merged. Bar indicates 2 μm. (C) Western blot using anti-hALC-1. (1) 1 μg recombinant HIS-tagged hALC-1 as expression standard; (2) mock infected adult rat cardiomyocytes (ARC; 10 μg); (3) AdCMV.β-gal infected ARC (10 μg); (4) AdCMV.hALC-1 infected ARC (10 μg) after 2 days of culture.
contraction without additional activating free Ca\textsuperscript{2+}. We tested this hypothesis by the analysis of the contractile properties of adult rat cardiomyocytes infected with an Ad vector with hALC-1 as expression cassette. We found that overexpression of hALC-1 in primary adult rat cardiomyocytes powered by the Ad vector increased significantly contraction amplitude while the systolic-free Ca\textsuperscript{2+} levels and Ca\textsuperscript{2+} cycling kinetics remained unchanged. This provides direct evidence, that in the intact cardiomyocyte, myosin molecules with hALC-1 generate more force than myosin with rVLC-1.

Increased force generation per single cross-bridge upon enhanced lever arm rigidity via ELC interaction has an energetical aspect, since both high-force- and low-force-generating myosin molecules would consume comparable amounts of ATP. Thus, it is plausible that through its high lever arm affinity ALC-1 could cause increased tension generation without additional activating free Ca\textsuperscript{2+}.

In summary, we showed that hALC-1 forms a significantly stronger complex with the myosin lever arm than does hVLC-1 (or rVLC-1\textsuperscript{16}). This may explain the observed preferential sarcomeric sorting of hALC-1 in mammalian cardiomyocytes. ALC-1 inotropy is elicited without concomitant increase in activating Ca\textsuperscript{2+} which may be caused by increasing tension output per cross-bridge with hALC-1 bound to IQ1.

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