Different actin affinities of human cardiac essential myosin light chain isoforms

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Abstract The N terminus of myosin light chain 1 (MLC-1) of skeletal muscle bind to the C terminus of actin. We investigated whether the N termini of human cardiac MLC-1 isoforms likewise bind to actin. Furthermore, we investigated whether the N-terminal sequence 5–15 (P5–14) of MLC-1 of human atrium (ALC-1) and ventricle (VLC-1) bind with different affinities to actin. Affinity beads were produced by covalently coupling a synthetic peptide corresponding to the N-terminal sequence 4–14 of human VLC-1 to aminohexylagarose in order to bind G-actin. We found, that G-actin specifically binds to the affinity beads. Furthermore, preincubation of G-actin with P5-14 of both ALC-1 and VLC-1 decreased the amount of G-actin recovered from the affinity beads in a concentration-dependent manner. The half-maximal effective concentrations, however were significantly ($p < 0.01$) different being 0.32 ± 0.02 μM and 0.71 ± 0.02 μM for the VLC-1 and ALC-1 peptide, respectively. The appropriate scrambled peptides were without effect up to 3 μM. These results demonstrate the specific interaction between the N-terminal domains of human cardiac MLC-1 isoforms and actin and reveal different actin affinities of MLC-1 isoforms. Weak binding of ALC-1 to actin could explain the higher cycling kinetics of cross-bridges with ALC-1 compared to those with VLC-1.

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Key words: Myosin light chain; Actin; Cardiac muscle

1. Introduction

The cardiac myosin molecule consists of two heavy chains (MHC; 200 kDa), each associated with two types of light chains (MLC) [1]. Two genes coding for MHC are expressed in the mammalian heart, namely the alpha-MHC and the beta-MHC located in tandem on chromosome 14 [2–4]. The MLC types are classified into essential MLC (MLC-1) and regulatory (phosphorylatable) MLC (MLC-2) [5,6]. Recent X-ray crystallographic analysis demonstrated that both essential and regulatory MLC are associated with the neck region of the myosin-S1 fragment [7]. Two MLC-1 isoforms are produced by two different genes, namely a ventricular-specific (VLC-1) which is the same isoform as the MLC-1 present in adult slow skeletal muscle, and an atrial-specific (ALC-1) isoform [8,9]. Human heart fibers with high ALC-1/VLC-1 ratio exhibited an increased shortening velocity (V_{max}) as well as an accelerated rate of tension development [10], thus improving the contractile state of the heart.

The molecular mechanism explaining the effects of essential MLC on the cross-bridges in the heart may reside in the mode of interaction between MLC-1 and actin. It has been demonstrated that the amino-terminal domain of MLC-1 isoforms interact with the carboxyl-terminal domain of actin [11–13]. This interaction could be of functional importance since inhibition of this interaction using synthetic peptides increased force production and shortening velocity of human heart fibers [14] and myofibrillar ATPase [15]. We proposed that MLC-1-actin interaction imposes a 'molecular load' on the myosin cross-bridge. Relieving this load accelerates cross-bridge cycling kinetics and may enhance tension output per cross-bridge thus increasing contractility. Interestingly, the primary structure of the most amino terminus of the ALC-1 and VLC-1 are different [8]. We hypothesized that binding of ALC-1 to actin is weaker than binding of VLC-1 to actin thus decreasing the 'molecular load' imposed on the cross-bridge. This would, in analogy to weakening MLC-1-actin interaction by peptides, explain the accelerated cross-bridge cycling kinetics and tension production by ALC-1 expression in the human ventricle [10].

2. Material and methods

2.1. Actin purification

Actin was prepared from rabbit psoas [16] and was a generous gift from Dr. P. Allison, Heidelberg, Germany. Lyophilized actin (100 mg) was suspended in 3 ml actin buffer consisting of 5 mM HEPES, pH 7.5, 1 mM ATP, 0.2 mM $CaCl_2$, 0.5 mM NaN_3 and was homogenized by a glass-teflon homogenizer. Following centrifugation at $200\,000 \times g$ for 1 h the supernatant was recovered as G-actin. The concentration was estimated by measurement of optical density at 290 nm: 0.63 = 1 mg/ml).

2.2. Synthetic peptide

The N-terminal amino acid sequences 5–14 of VLC-1 (KPEPKKDDAK) and ALC-1 (KPEPKKEAAK) (human heart sequence) [8] as well as the corresponding scrambled peptides (PKDKEAKPKD for VLC-1 and PKEKEAKPKA for ALC-1) were commercially synthesized and purified by BioTeZ (Berlin, Germany). The purity was greater than 98% as assessed by HPLC and mass spectroscopy.

2.3. MLC affinity beads

A synthetic peptide with the N-terminal sequence 4–14 (KKPEPKKDDAK) of the VLC-1 was covalently coupled to an agarose gel. To achieve covalent coupling, the peptide was synthetized with a N-terminal monochloro-acetyl-glycyl extension. This peptide was then immobilized to activated *w*-aminohexylagarose via activation by 2-iminothiolane hydrochloride as described [17]. Coupling reaction was performed in phosphate-buffered saline (PBS, pH 7.4) for 3 h at room temperature and at a peptide concentration of 6 mg/ml packed gel. The peptide resin was then incubated for 1 h with 5 ml of 40 mM iodoacetamide to block the remaining active groups. The MLC affinity bead was extensively washed with PBS and stored in the presence of 1% bovine serum albumine and 0.02% sodium azide.

2.4. Actin binding to affinity beads

MLC affinity beads (15 μl of packed gel) were incubated with 50 μg G-actin in a final volume of 500 μl actin buffer for 1 h at +4°C on a rotating wheel. Competition experiments were performed by incuba-

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tion of different concentrations of either ventricular MLC peptide 5–14 or atrial MLC peptide 5–14 or the corresponding scrambled peptides with G-actin for 12 h at +4°C prior binding to affinity beads for 1 h at +4°C as outlined above. Following actin binding the beads were washed three times with actin buffer, extracted with 20 ml SDS-sample buffer (5% SDS, 50 mM Tris-HCl, pH 7.5, 250 mM sucrose, 75 mM urea, 60 mM β-mercaptoethanol) and heated at 95°C for 2 min. The resulting supernatants were further processed by SDS-PAGE, while the affinity beads were discarded.

2.5. SDS-PAGE and immunoblot analysis

Samples were electrophoretically separated on 8% polyacrylamide gels at 4°C for 3 h and transferred to nitrocellulose (Hybond-C, 45 μm, Amersham, Germany) in a buffer containing 40 mM Tris, 300 mM glycin, 0.01% SDS, 20% (v/v) methanol. Briefly, the blots were incubated with the monoclonal antibody against a-sarcomeric actin (Sigma, Munich, Germany) at a dilution of 1:500 for 90 min and the secondary peroxidase-conjugated antibody (anti-mouse IgG, Bio-Genes, Germany, diluted 1:10000) for 1 h at room temperature. The 43-kDa actin band was visualized by enhanced chemoluminescence reaction kit (Amersham, Germany) using an X-ray film.

2.6. Statistical analysis

Statistical analysis was performed using the commercially available statistic program 'Instat2' on an IBM compatible PC (means, standard deviations (SD), Student's *t*-test for unpaired values). All values are expressed as means ± SEM.

3. Results

3.1. Binding of G-actin to affinity beads

As demonstrated in Fig. 1, the synthetic peptide corresponding to the N-terminal VLC-1 sequence 4–14 covalently linked to aminohexylagarose binds G-actin in a time-dependent manner. At +4°C, maximal actin binding could be detected after 1 h. Under the same experimental conditions, no G-actin binding was observed to the aminohexylagarose matrix alone (data not shown).

3.2. Binding of N-terminal ALC-1 and VLC-1 peptides to G-actin

To demonstrate that peptides derived from the N-terminal domains 5–14 (P5–14) of ALC-1 and VLC-1 bind to G-actin with different affinities, we incubated G-actin with different peptide concentrations for 12 h at +4°C prior incubation with the affinity beads. Different peptide affinities should produce different fractions of unbound G-actin concentrations which could subsequently be recovered from the affinity beads and detected by the actin antibody in the Western blot.

Pre-incubation of G-actin with 1 μM of P5-14 of VLC-1 saturated actin interaction sites preventing thereby subsequent binding to the affinity beads (Fig. 2. The corresponding VLC-1 scrambled peptide (3 μM) did not bind to actin. Please note from the Western blots shown in Fig. 2, that the G-actin signal obtained after incubation with 3 μM scrambled peptide was about the same as that obtained upon G-actin binding alone (cf. gels Nos. 5 and 7 in Fig. 2A). These results suggest that binding of the N-terminal domain 5–14 of VLC-1 to actin is site-specific. Half-maximal effective dosage of the VLC-1 peptide was 0.32 ± 0.02 μM (6 different experiments; Fig. 2B).

Likewise, the N-terminal ALC-1 sequence 5–14 removed G-actin from the equilibrium reaction in a concentration-dependent manner, while a corresponding scrambled peptide up to 3 μM was not effective (Fig. 2): Western blots (Fig. 2A) show, that the G-actin signal obtained after incubation with 3 μM scrambled peptide was about the same as that obtained upon G-actin binding alone (cf. gels Nos. 5 and 6 in Fig. 2A). However, binding of the ALC-1 peptide to G-actin was significantly weaker than binding of the VLC-1 peptide. Even at 3 μM ALC-1 peptide, a considerable amount of G-actin could be eluted from the affinity beads. Half-maximal effective concentration of the ALC-1 peptide was found to be 0.71 ± 0.02 (Fig. 2B) and was significantly ($p < 0.01$) higher than different from the VLC-1 peptide.

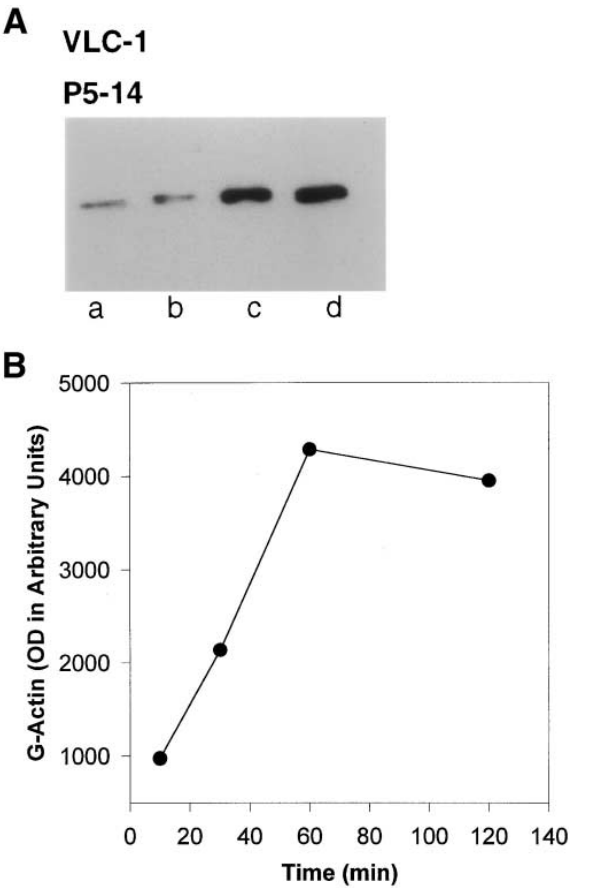


Fig. 1. (A) Time-dependent binding of G-actin to affinity beads as revealed upon elution and Western blot (ECL signal). A peptide corresponding to the human VLC-1 sequence 4–14 was covalently coupled to aminohexylagarose to bind G-actin. a, b, c and d correspond to the signals obtained after 10 min, 30 min, 60 min and 120 min, respectively. (B) ECL signals obtained from (A) (optical densities OD in arbitrary units) were plotted versus incubation time of affinity beads with G-actin.

4. Discussion

We developed an in vitro actin binding assay using aminohexylagarose covalently coupled with a synthetic peptide corresponding to the N-terminal actin binding domain 4–14 of VLC-1 [8]. G-actin was pre-incubated with free ALC-1 or VLC-1 peptides (or the corresponding scrambled peptides) to obtain equilibrium between bound and free actin. The equilibrium complex is than incubated with the affinity beads which bind the free G-actin. The prediction was that in the equilibrium reaction with low affinity peptides a higher fraction of free G-actin exist which can be recovered by subsequent incubation with the affinity beads.

We could demonstrate that G-actin binds to affinity beads.

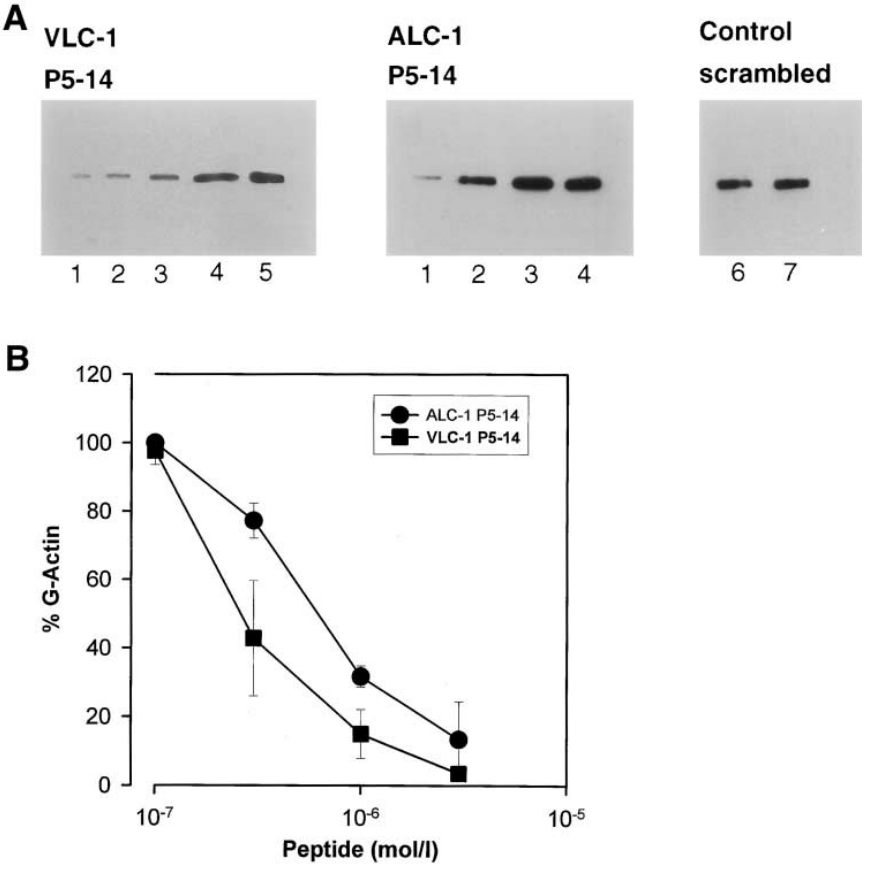


Fig. 2. (B) Recovery of G-actin from affinity beads (ECL signals). G-actin was incubated with different concentrations (in mol/l) of synthetic peptides corresponding to the N-terminal domains of 5–14 (P5–14) of the atrial and ventricular myosin light chain 1 (ALC-1 and VLC-1, respectively) of the human heart. 1, 2, 3 and 4, correspond to 3 μM, 1 μM, 0.3 μM and 0.1μM peptide, respectively; 5 corresponds to actin alone; 6 and 7 correspond to 3 μM ALC-1 and VLC-1 scrambled peptide, respectively. (B) Plot of G-actin recovered from the affinity beads (given in % of maximal ECL signal obtained without competing peptide) versus different concentrations of ALC-1 (closed circles) and VLC-1 (closed squares) peptides corresponding to the N-terminal domain 5–14 (P5–14) of the human heart.

This shows for the first time that the N-terminal sequence 4–14 of human cardiac VLC-1 indeed interacts with actin as recently demonstrated for skeletal muscle MLC-1 [11–13]. Pre-incubation of G-actin with the N-terminal peptide 5–14 of VLC-1 prevented actin from binding to the affinity beads at a peptide concentration of around 1 μM. The corresponding scrambled peptide at the same concentration was without effect demonstrating that the binding of the N-terminal MLC-1 peptides is highly specific. The main finding in this paper is that the actin affinity of the N-terminal peptides 5–14 derived from ALC-1 and VLC-1 was different: in our assay system, half-maximal effective dosage of P5-14 of VLC-1 was 0.32 μM while half-maximal effective dosage of P5-14 of ALC-1 was 0.71 μM. From our data it appears that amino acids 7 and 8 of MLC-1 regulate the affinity of the N terminus of MLC-1 to actin. This supports our initial hypothesis: the increased cross-bridge cycling kinetics in the presence of ALC-1 is due to a weak actin-binding of its N terminus. Thus, ALC-1 would impose a weaker 'molecular load' to the cross-bridge than VLC-1 increasing its kinetic and force generation. Furthermore, reconstitution of skinned skeletal muscle fibers with those MLC-1, in which charged amino acids were substituted by uncharged alanine at the most N terminus increased shortening velocity [18]. Please note, that the first 20 N-terminal amino acids of ALC-1 contains 7 charged amino acids while VLC-1 contains 9 charged amino acids within the first 20 N-terminal amino acids in the human heart [8]. Decreasing the amount of charged amino acid residues – by mutation or differential MLC-1 isoform expression – increases cross-bridge cycling kinetics.

In summary, the presented data support our hypothesis that the weak interaction between the N-terminal domains of ALC-1 with actin promotes cross-bridge cycling kinetics and force per cross-bridge thus improving the contractile state of the human heart.

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