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Reconstitution of ventricular myosin with atrial light chains 1 improves its functional properties✪★

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Atrial light chain 1 (ALC-1) is expressed in embryonic and hypertrophied human ventricles but not in normal adult human ventricles. We investigated the effects of recombinant human atrial light chains (hALC-1) on the structure and enzymatic activity of synthetic filaments of ventricular myosin. The endogenous ventricular myosin light chain 1 (VLC-1) was partially replaced by recombinant hALC-1 yielding hALC-1 levels of 12%, 24% and 42%. This reconstitution of ventricular myosin with hALC-1 did not change the length of synthetic myosin filaments but led to more rounded myosin heads in comparison with those of control filaments. Actin-activated ATPase activity of myosin, a parameter of functional activity of molecular motor, amounted to 79.5 nmol P_i/mg per min in control myosin filaments. Reconstitution with hALC-1 caused a profound increase of the actin-activated myosin ATPase activity in a dose dependent manner, for example, synthetic myosin filaments formed with 12%, 24% and 42% hALC-1 reconstituted myosin revealed the actin-activated ATPase activity increased by 18%, 26% and 36%, respectively, as compared to control. These results strongly suggest that *in vivo* expression of ALC-1 enhances ventricular myosin function, thereby contributing to cardiac compensation.

Keywords: cardiac myosin, myosin light chains, reconstituted myosin, myosin filaments, actin-activated ATPase activity, dilated cardiomyopathy

The molecular motor myosin II consists of two α-helical heavy chains which fold at the C-terminus into a coiled-coil rod, and at the N-terminus into two pear-shaped heads (Rayment *et al*., 1993) with each containing both actin-binding and ATP-binding sites. An α-helical 'neck region' connects the two catalytic domains which are each associated with two types of myosin light chains (LC), an essential LC, or LC-1 and a regulatory LC, or LC-2.

In cardiac muscle, LC-1 and LC-2 have distinct isoforms specific for the atria (ALC-1, ALC-2) and ventricles (VLC-1, VLC-2). However, in human embryos large amounts of ALC-1, therefore termed embryonic LC-1, is also expressed in both ventricles (Auckland *et al*., 1986). In adults, this form of LC-1 is not widely expressed. Interestingly, in patients with hypertrophic and dilative cardiomyopathy (DCM) and valvular heart disease (Auckland *et al*., 1986; Sütsch *et al*., 1992; Morano *et al*., 1997; Ritter *et al*., 1999; Khalina *et al*., 2002), ALC-1 re-appears in ventricles. Previously, we demonstrated a 23% increase in the amount of ALC-1 expression during DCM as compared to control, indicating a possible physiological importance of this phenomenon (Khalina *et al*., 2002). After surgical intervention and subsequent normalisation of the hemodynamic state, there is a decreased expression of ALC-1 in ventricles (Sütsch *et al*., 1992) suggesting that this phenomenon has a compensatory effect. In addition, at the skinned fibre level it has been demonstrated that the maximal shortening velocity, rate of tension redevelopment, isometric force generation

✪Paper dedicated to the memory of Professor Witold Drabikowski and Professor Gabriela Sarzała-Drabikowska.

★Preliminary results of this investigation were presented as the abstract of Khalina Y et al. (2003) at the 32nd EMC (*J Muscle Res Cell Motil* **24**: 344–345).

Abbreviations: ALC-1, atrial light chain 1; ALC-2, atrial light chain 1; DCM, dilated cardiomyopathy; DTT, dithiothreitol; hALC-1, human atrial light chain 1; $hALC\text{-}1_{HIS}$, tagged human atrial light chain 1; LC, light chain; Ni-NTA, nickel-nitrilotriacetic; VLC-1, ventricular light chain 1; VLC-2, ventricular light chain 1.

per cross-section, and Ca^{2+} sensitivity of isometric force generation increased upon partial replacement of VLC-1 by ALC-1 in the human and mouse ventricles (Morano *et al.*, 1996; 1997; Fewell *et al.*, 1998; Ritter *et al.*, 1999). However, these results were obtained using systems in which myosin function and actin–myosin interaction is strongly affected by accessory proteins of the thick and thin filaments; and thus the direct effect of this reconstitution on myosin function remains unclear. In previous studies we presented data demonstrating that total ventricular myosin light chain (LC-1 and LC-2) replacement with the atrial isoforms results in an improvement of myosin properties (Khalina *et al.*, 2003). The data reported here identifies ALC-1 as an activator of the enzymatic activity of ventricular myosin and thereby provides a direct demonstration of the physiological importance of such a process at the compensatory stage of cardiac failure.

MATERIAL AND METHODS

Recombinant human ALC-1. Human ALC-1 was cloned using the previously established hemp4 clone kindly supplied by Anna Starzinski-Powitz (Zaimmermann et al., 1973). An 812 bp BstYI–HindIII fragment carrying the complete protein coding region of hALC-1 (594 nucleotides), plus additional 18 nucleotides from the 5′ UTR and 200 nucleotides from the 3′ UTR of hALC-1 was inserted into the BamH1–HindIII site of the expression vector pRSETA (Invitrogen GmbH, Karlsruhe, Germany) to yield hALC-1 as an N-terminal His-tagged fusion protein (hALC-1HIS). A single colony of BL21(DE3)pLysS cells (Invitrogen GmbH, Karlsruhe, Germany) transformed with the hALC-1HIS was grown in Luria-Bertani medium containing 100 μg/ml ampicillin and 50 μg/ml chloramphenicol. Protein expression was induced with 0.1 mM isopropyl-(ɒ)-thiogalactopyranoside (Diagnostic Chemicals Limited) for 3–4 h at 37°C. Following protein expression cells were harvested by centrifugation and re-suspended in 20 ml phosphate-buffered saline (PBS), pH 7.2, containing 1% Triton X-100 and the protease inhibitors 0.1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin A, 0.1 mM benzamidine, 1 mM iodoacetamide, 1% Triton X-100 and sonicated twice for 45 s (power level 70%, cycle 70) in a SonoPlus HD70 Sonifier (Bachofer Laboratoriumsgeräte). Sonicated material was then centrifuged (10000 r.p.m. for 10 min at 4°C in a Beckman JA-20 rotor) and the supernatant incubated with 1 ml of Ni-NTA-agarose beads (Quiagen, Germany) for 20 min at 4°C on a rotating wheel. Beads were extensively washed with binding buffer supplemented with 20 mM imidazole, then hALC-1HIS fusion protein was eluted with 200 mM imidazole. Purified hALC-1HIS had an apparent molecular mass of 35 kDa (Fig. 1, lane 1). The N-terminal His tag was then cleaved by incubation of 10 μg hALC-1HIS with 1 unit of enterokinase for 7–14 h at 4°C. Removal of enterokinase followed the manufacturer's protocol (EK-away resign, Invitrogen, Germany). Subsequently, the imidazole-containing buffer was replaced with 0.5 M KCl using a Sephadex G25 column (PD-10, Amersham Pharmacia Biotech AG, Sweden) and uncleaved hALC-1HIS as well as the His-tag sequence were captured on Ni-NTA-agarose. Final supernatant contained recombinant hALC-1 protein preparation which was >90% pure and showed an apparent molecular mass of 28 kDa (Fig. 1, lane 2), i.e. the molecular mass of normal hALC-1 without the about 8 kDa N-terminal His tag. The proteins were analyzed by SDS/PAGE using 15% polyacrylamide gels.

Reconstitution of ventricular myosin with ALC-1. For reconstitution experiments we used porcine ventricular myosin and human recombinant ALC-1. Human and porcine cardiac tissues have a high level of biochemical and immunological compatibility. Therefore, we believe the data collected in these experiments could be clinically relevant. Myosin was purified from porcine cardiac muscle obtained from the local slaughterhouse, as previously described (Offer *et al.*, 1973). Myosin was then subjected to dissociation of the heavy and light chains by addition of solid NH_4Cl to 4.7 M and was incubated for 10 min on ice (Wagner & Weeds 1977). Excess recombinant hALC-1 was added to this complex and NH_4Cl was rapidly removed by gel filtration using a Sephadex G25 Column (PD-10, Amersham Pharmacia Biotech AG, Sweden). Removal of NH_4Cl allowed the re-association of myosin subunits resulting in the formation of myosin with VLC-1 partially replaced by hALC-1. Reconstituted myosin was precipitated by ammonium sulfate saturation up to 43% and washed several times with the buffer (0.1 M KCl, 10 mM imidazole, 5 mM $MgCl_2$, 1 mM DTT, pH 7.0). In order to obtain different levels of VLC-1 substitution with ALC-1 (12%, 24% and 42% from total LC-1) different amounts of hALC-1 (2, 3 and 4-fold molar excess) were used. All preparative steps were carried out at 4°C. In order to exclude the effect of this treatment on myosin, ventricular myosin

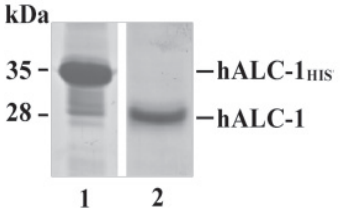

Figure 1. SDS/PAGE analysis of expressed and purified recombinant hALC-1 (15% gel).

Lane 1, recombinant hALC-1_{HIS}; lane 2, cleaved recombinant hALC-1.

treated as described above but without the addition of hALC-1 was used as a control in all experiments.

Formation of synthetic myosin filaments. Synthetic myosin filaments were formed by overnight dialysis against buffer (0.1 M KCl, 10 mM imidazole, 5 mM $MgCl_2$, 1 mM DTT, pH 7.0), as previously described (Podlubnaya *et al.*, 1999). The required level of Ca^{2+} ions (pCa 7.5) in solution was achieved by addition of EGTA up to 1 mM. These myosin filaments were used in structural studies and ATPase assays.

Actin was purified from rabbit back skeletal muscles, as previously described (Pardee & Spudich, 1982).

Actin-activated ATPase activity of myosin filaments. Actin-activated ATPase activity of synthetic myosin filaments was determined by the rate of P_i release using a malachite green containing colour reagent (Lanzetta *et al.*, 1979). To achieve saturation of myosin heads by actin, a myosin/actin molar ratio of 1:12 was used (0.1 mg/ml myosin, 0.1 mg/ml actin, in the buffer 0.1 M KCl, 10 mM imidazole-HCl, 5 mM $MgCl_2$, 1 mM EGTA, 1 mM DTT, 0.005% NaN_3, pH 7.0, pCa 7.5). The reaction was initiated by addition of ATP (Sigma-Aldrich Chemie GmbH, Germany) (to final concentration of 2 mM) to myosin and actin filaments and carried out at 25ºC.

Protein concentrations were determined by the Lowry (1951) method.

Electron microscopy. Synthetic myosin filaments were formed as described above, then applied on collodion or formvar coated grids and negatively stained with 2% uranyl acetate. Electron microscopy was performed on an EM-910 (LEO, Germany) electron microscope operated at 80 kV. Micrographs were taken at magnifications of 5000× and 25000×. DigiVision software (Münster, Germany) was used to determine the filament lengths.

Statistical analysis. Results were analyzed using the Student´s *t*-test and are expressed as means ± S.D.

RESULTS

Reconstitution of cardiac myosin molecules was achieved using chemical dissociation of myosin into its heavy and light chains and subsequent re-association in the presence of foreign light chains. The reconstitution experiments were performed using purified porcine ventricular myosin containing the 25 kDa ventricular light chain 1 and the 18 kDa ventricular light chain 2 (Fig. 2, lane 1) in combination with recombinant hALC-1 migrating at 28 kDa (Fig. 1, lane 2; Fig. 2, lane 3). This reconstitution process led to a partial replacement of endogenous porcine VLC-1 by hALC-1 as determined by SDS gel electrophoresis and densitometry analysis of VLC-1 and hALC-1 protein bands. In different sets of experiments, reconstitution efficiencies of 12%, 24% and 42% were obtained (values are given in percentage of total myosin light chain 1 isoforms, i.e. hALC-1 + VLC-1 amounted to 100%). Figure 2 depicts electrophoresis patterns of myosin light chain composition with different reconstitution efficiencies (lanes 4–6). Ventricular myosin chemically dissociated and re-associated with endogenous VLC-1 and VLC-2 (i.e., treated in the same way as reconstituted samples, but without exchange of the light chains) was used as a control (Fig. 2, lane 2) to exclude any possible artifacts due to the treatment with NH_4Cl. During reconstitution, the normal stoichiometry of the myosin subunits was retained as evidenced by the ratio between the myosin light chain 1 isoforms (hALC-1 + VLC-1) and the ventricular light chain 2 (VLC-2). Ratios of 0.95 ± 0.1 (n = 10) and 0.98 ± 0.09 (n = 10) were observed for reconstituted and control myosin, respectively, indicating that all heavy chain LC-binding sites were occupied.

The structure of the synthetic myosin filaments formed by reconstituted myosin and control myosin was analyzed by electron microscopy. The analysis revealed a bipolar shape and similar diameter of both types of myosin filaments. Filament lengths ranged from 348 nm to 2438 nm (n = 147, n = number of counted filaments) for hALC-1-reconstituted myosin and from 348 nm to 2217 nm (n = 134) for control myosin. The difference in the length between control and hALC-1-reconstituted myosin filaments is not surprising considering these are synthetic filaments whose length varies widely. However, there was a distinct change in the shape of some of the myosin heads upon reconstitution with hALC-1. As shown in Fig. 3, on the surface of synthetic filaments, the heads of purified ventricular myosin (Fig. 3A), purified atrial myosin (Fig. 3B) and control myosin (Fig. 3C) in the absence of Ca^{2+}, form narrow cross-stripes with typical periodicity of 14.5 nm (indicated by arrows) due to their elongated shape, which is distinctly shown in Fig. 3D (double

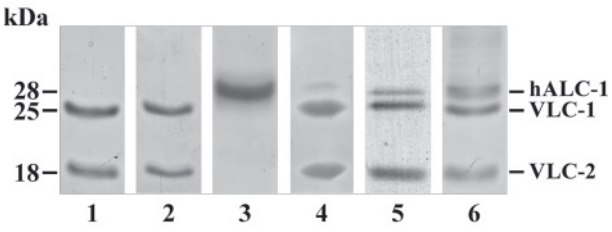

Figure 2. SDS/PAGE analysis, LC region (15% gel).

Lane 1, purified (untreated) porcine ventricular myosin; lane 2, control (NH_4Cl treated porcine ventricular myosin); lane 3, recombinant hALC-1; lane 4, hALC-1-reconstituted myosin (12% of hALC-1); lane 5, hALC-1-reconstituted myosin (24% of hALC-1); lane 6, hALC-1-reconstituted myosin (42% of hALC-1).

arrows point at the pairs of heads of single myosin molecules). In contrast, some heads of reconstituted myosin filaments exhibit spherical shape (Fig. 3F, arrows) and as a consequence they form wider cross-stripes with a periodicity of 14.5 nm (Fig. 3E, arrows) and about 29 nm (Fig. 3F, arrows).

To examine the functional activity of reconstituted myosin, the actin-activated ATPase activity of the synthetic myosin filaments was measured. This activity was determined for purified ventricular myosin, control myosin, and myosin reconstituted with different amounts of hALC-1 in the presence of saturating actin concentration (myosin/actin molar ratio of 1:12), which allows all myosin heads to be activated by actin.

A dose-dependent increase in ATPase activity of the reconstituted myosin filaments was found (Fig. 4). In the presence of 12%, 24% and 42% of hALC-1, ATPase rose by 18 ± 4.2% (n = 10; $P < 0.01$), 26 ± 3.5% (n = 10; $P < 0.001$) and 36 ± 4.1% (n = 10; $P < 0.001$), respectively, when compared to that of control myosin filaments. The mean ATPase activities of synthetic filaments formed by purified untreated ventricular myosin and control myosin (i.e. formed by ventricular myosin, dissociated and re-associated with endogenous light chains) were 90.3 ± 2.9 nmol Pi/mg/min (n = 20) and 72.5 ± 5.9 nmol P_i/mg/min (n = 10, $P < 0.01$) correspondingly. Thus, filaments of control myosin retained 80% of the activity of untreated myosin. Therefore, the increase in ATPase activity observed for reconstituted myosin is a direct effect of hALC-1 and is not due to the treatment with NH_4Cl.

DISCUSSION

Data obtained using the cardiac muscle fibres and transgenic animal models suggest that expression of ALC-1 in ventricles could accompany compensatory processes in the heart (Morano *et al.*, 1996; 1997; Fewell *et al.*, 1998). However, these studies do not identify the direct effects of such a reconstitution on the functional properties of myosin due to possible influence of accessory proteins of thick and thin filaments. Therefore the present study was undertaken to examine the direct effect of ALC-1 on the functional properties of purified ventricular myosin. Recombinant hALC-1 was expressed and purified and then used for ventricular myosin reconstitution. We investigated the structure and actin-activated ATPase activity of such hALC-1-reconstituted ventricular myosin as compared to control myosin. Our experiments indicate that myosin retains normal subunit stoichiometry, and the myosin filaments conserve their structural properties after chemical treatment and reconstitution, as demonstrated by the retention of the control LC-1/LC-2 ratio and dimensions of synthetic filaments. It is known that the loss of LC-2 affects the structural properties of myosin and results in the formation of filaments with destructive features, for example, synthetic filaments formed by

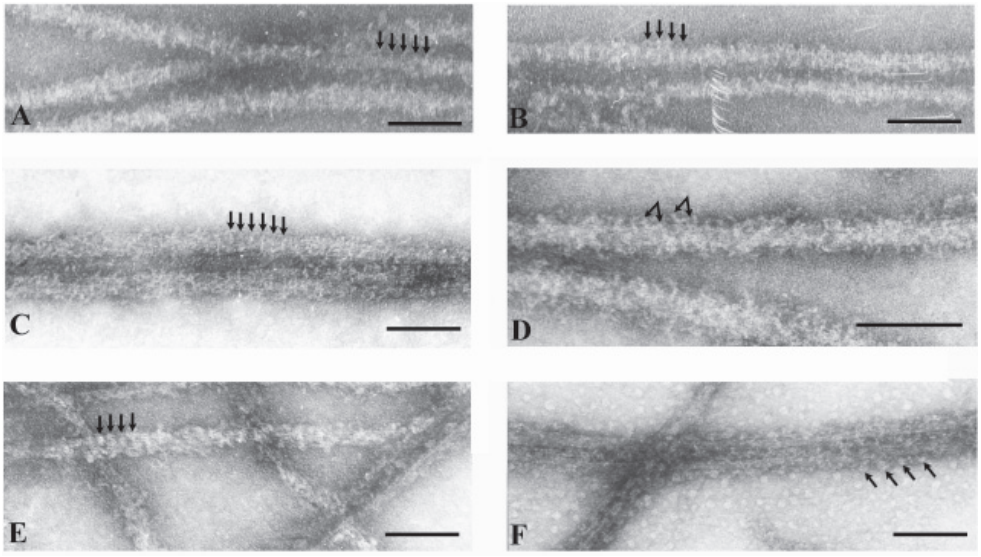

Figure 3. Electron micrographs of synthetic myosin filaments (pCa 7.5) negatively stained with 1% aqueous uranyl acetate.

(A) Filaments formed by purified ventricular myosin. Narrow cross stripes with period of about 14.5 nm are indicated by arrows. (B) Filaments formed by purified atrial myosin. Arrows indicate narrow cross stripes with period of about 14.5 nm. (C–D) Filaments formed by control myosin. Arrows indicate narrow cross stripes with period of about 14.5 nm. At larger magnification pairs of elongated heads are distinctly seen in some regions of the filament (D, double arrows). (E–F) Filaments formed by hALC-1-reconstituted myosin (42% of hALC-1). (E) Wider cross stripes with period of 14.5 nm formed by rounded myosin heads are indicated by arrows. (F) Rounded heads arranged with period of about 29 nm are distinctly seen at the surface of hALC-1-reconstituted myosin filament (arrows). Scale bar, 100 nm.

LC-2-deficient myosin are very short (near 500 nm) and thin (Chowrashi *et al.*, 1989). In our experiments, reconstituted myosin formed filaments with lengths up to 2400 nm. Moreover, LC-2-deficient myosin is reported to have strongly decreased actin-activated ATPase activity (Margossian *et al.*, 1992; 1999). Contrary to this, with reconstituted myosin we observed the formation of filaments with normal dimensions that was accompanied by an increase in actin-activated ATPase activity.

It is known that under normal conditions myosin heads have an elongated pear-shaped form (4–7 nm × 19 nm) (Knight & Trinick, 1984), as demonstrated in Fig. 3D. As described by Podlubnaya *et al.* (1999), in the absence of Ca^{2+} (i.e., under the conditions used in this work) myosin heads of synthetic vertebrate myosin filaments are predominantly located close to the filament backbone forming shelves termed 'crowns' unlike their helical arrangement in native vertebrate filaments (Levine, 1997). In agreement with this description, the most of negatively stained relaxed native and synthetic filaments of invertebrate myosin display similar crowns (Clarke *et al.*, 1986; Frado & Craig 1989; Levine, 1997). On electron micrographs these crowns are seen as periodic cross-striations (periodicity of about 14.5 nm). In our hands, synthetic filaments also show cross-striations with 14.5 nm periodicity. The width of such cross stripes reflects the size and shape of myosin heads. The cross stripes are narrow in the case of purified and control myosin filaments (Fig. 3A–C) since the heads of such a myosin have an elongated form. However, they are wider in the case of ALC-1-substituted myosin filaments (Fig. 3E), whose heads became rounded (Fig. 3F). On occasion myosin heads can be seen at a distance from the backbone as individual double elongated heads (Fig. 3D). In the case of ALC-1-reconstituted myosin, a portion of heads have distinct spherical form (Fig. 3F) as compared to that for ventricular and atrial myosin filaments.

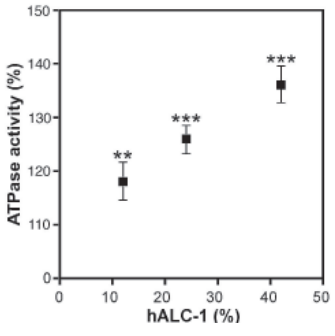

Figure 4. Increase of actin-activated ATPase activity of myosin filaments by reconstitution of ventricular myosin with hALC-1.

The activity of control myosin filaments corresponds to 100%. hALC-1 content is given as percentage of total LC-1. Results are means ±S.E.M., $^{**}P < 0.01$, $^{***}P < 0.001$ (compared to control).

We propose that the observed conformational change of myosin heads could originate from the charge redistribution due to incorporation of ALC-1 into ventricular myosin heads since the N-terminus of ALC-1 has additional positive charge as compared to VLC-1 (Fodor *et al.*, 1989). A similar phenomenon was observed under phosphorylation of cardiac LC-2 (Podlubnaya, unpublished) — a process which also causes charge redistribution in myosin heads. Moreover, the heads of phosphorylated myosin exhibited a tendency to associate with each other. This tendency could explain also the larger size of some heads in ALC-1-reconstituted myosin filaments. Thus the structural data obtained by us are also an evidence of incorporation of hALC-1 into ventricular myosin.

It is reasonable to suggest that charge redistribution, which takes place upon light chain replacement, modifies the structural properties of reconstituted myosin. Moreover, this modification might affect the active site of the myosin head leading to an increase of actin-activated myosin ATPase activity. Interestingly, a change of the myosin head shape under LC-2 phosphorylation, mentioned above, was also accompanied by activation of actomyosin ATPase activity. The fact that the hALC-1-induced change in the head shape does not cause a loss of function is demonstrated by the increase in ATPase activity and the preservation of the LC-1/LC-2 molar ratio of reconstituted myosin. Indeed, the reconstitution of ventricular myosin with hALC-1 significantly increased actin-activated ATPase activity of synthetic myosin filaments. Moreover, a strong positive correlation has been observed between the extent of hALC-1 incorporation and the increase in ATPase activity.

It can be supposed that the observed increase of ATPase activity is influenced by different filament lengths of reconstituted and control myosins. According to the experience of our colleagues (personal communication of Stepkowski and Moos), longer myosin synthetic filaments exhibit lower actomyosin ATPase activity than shorter filaments (by 3–4 times). The filaments of ALC-1-reconstituted myosin were longer than the control ones. Therefore, a decrease in actomyosin ATPase activity should be expected. On the contrary, an hALC-1-dependent increase in the actomyosin ATPase activity was in fact registered.

As stated above, a shift in isoform composition of LC-1 takes place in human ventricles during some cardiac diseases. The amount of ALC-1 in ventricles averages between 0–10% (Morano, 1999) and 0–23% (Khalina *et al.*, 2002) in DCM and 0–32% in hypertrophic cardiomyopathy (Morano, 1999). The content of hALC-1 in reconstituted myosin in one set of experiments (12%) corresponded to the reported values of ALC-1 expression in DCM ventricles.

The effect observed by us suggests that even a low level of reconstitution markedly improves the functional status of myosin and thereby could improve the contractility of the myocardium.

Our suggestion about the adaptive nature of ALC-1 re-expression in the failed heart is in agreement with the appearance of an embryonic cardiac isoform of another sarcomeric protein — titin — in DCM ventricles (Makarenko *et al.*, 2004). This exchange of titin isoforms is supposed to be involved in the adaptive response of the heart.

Therefore, our results strongly suggest that the replacement of VLC-1 by ALC-1 is a molecular mechanism which improves cardiac myosin function and thereby adapts the contractile apparatus of ventricular cardiomyocytes to an increased work load.

Acknowledgements

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