Probing Conformational Changes within LC2 Domains of Cardiac Myosin

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ABSTRACT: Nine monoclonal antibodies were used to test calcium and EDTA effects on the molecular conformation of ventricular VLC2 within myosin. Antibody epitopes were located in six domains of VLC2 using recombinant proteins. The apparent association constants of these antibodies were measured in solution in the presence of calcium or EDTA. An immunofluorescence study was performed to establish whether the observed effects would occur in more integrated systems, as compared to isolated proteins in solution. Our results showed (1) a slight effect of calcium on isolated VLC2, located in the aa 29–45 domain, (2) a clear-cut effect of calcium on VLC2 within myosin, only in the aa 45–59 domain, and (3) in the presence of EDTA, antibody affinities for VLC2 within myosin similar to the affinities for isolated VLC2. These results are discussed in terms of spatial arrangements and binding mechanisms between HC and VLC2. They suggest that there are two processes for stabilizing HC/VLC2 complex formation: one binding *via* calcium chelation and another involving hydrophobic interactions.

The myosin molecule, the major constituent of muscle thick filaments, is composed of two heavy chains (HC)[1] and two pairs of light chains (LC1 and LC2). In adult human heart, various isoforms are present: atrial and ventricular LC1 and LC2 and α and β HC. The relative proportions of these isoforms vary according to tissue localization, aging, hormonal status, or pathology.

Recently, Lowey et al. (1993) dissociated and reassociated different myosin chains selectively and demonstrated that light chains of skeletal myosin are essential for transduction of ATP hydrolysis energy into rapid movement. Addition of the two LCs to the HC allowed 95% recovery of native myosin motility. From 8% residual motility for the myosin HC alone, a recovery of 17% was obtained by the addition of LC2 and 35% by the addition of LC1. In contrast, the truncated HC (SF1) (to Gln^{758}) of *Dictyostelium* myosin, unable *per se* to bind any LC, has the same motility as normal SF1 (Itakura et al., 1993), suggesting that *Dictyostelium* myosin LCs are not essential for filament sliding. More recently, in *Dictyostelium* myosin, Uyeda and Spudich (1993) concluded that a complex between RLC and ELC (equivalent to LC2 and LC1, respectively, in muscle myosins) and the HC has an inhibitory effect on ATPase activity, suggesting a structural role for the movement of myosin along the actin molecule. There is not yet a clear consensus on the function of each LC in striated muscles.

Various experimental techniques have been used to study myosin LC2s from muscles and nonmuscles of various organisms. LC2s are subject to phosphorylation by a specific calmodulin-dependent MLC kinase [for a review, see Sweeney et al. (1993)]. LC2s are located within the myosin head at the head/rod junction (Tokunaga et al., 1987; Katoh & Lowey, 1989; Mitchell et al., 1986; Saraswat & Lowey, 1991; Saraswat et al., 1992). Sequence comparisons between various calcium-binding proteins show that four EF-loop putative calcium binding sites exist in LC2s (Collins, 1991). The first loop in LC2 is the most potent calcium binding site. Deletions and nonconservative amino acid substitutions at critical positions in the three other loops alter their divalent cation binding capacities. Calcium and phosphorylation induce conformational changes in LC2s, as demonstrated by fluorescence studies and circular dichroism of isolated skeletal muscle light chains (Alexis & Gratzer, 1978). Calcium and EDTA, also by inducing conformational changes in LC2, influence chymotryptic cleavage of skeletal myosin LC2 (Kardami & Gratzer, 1982; Roulet et al., 1993). Using an anti-LC2 monoclonal antibody, Shimizu et al. (1985) showed that Ca^{2+} or Mg^{2+} cause a conformational change around the first helical domain of the calcium binding site of LC2 from chicken skeletal myosin. The recently reported atomic structure resolution of the chicken skeletal muscle SF1 crystal (Rayment et al., 1993) and that of the scallop regulatory domain (Xie et al., 1994; Trayer, 1994) have provided now direct molecular information on interactions between LC2 (or RLC) and the HC. It has thus been deduced that the N-terminal domain (helixes A–D) of scallop myosin RLC interacts through hydrophobic contacts with the 825–842-residue HC segment, whereas its C-terminal domain (helixes E–G) interacts through electrostatic bonds with the 811–824-residue HC segment containing Ile-Gln (IQ) sequence motifs.

Cardiac ventricular myosin VLC2 is a 164-residue protein (Dalla Libera et al., 1989), and there is little information on its precise function in heart contraction. VLC2 phosphorylation has been shown to increase actomyosin ATPase activity (Noland & Kuo, 1993) and modify calcium-sensitive force development (Morano, 1992), possibly during the myosin cross-bridge cycles (Metzger & Moss, 1992). In vertebrate heart, LC2 seems to be involved in controlling the length of thick filaments during myosin assembly processing (Margossian et al., 1987). Some pathologic situations confirm the functional importance of cardiac LC2. In baboons,

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[1] Abbreviations: HC, myosin heavy chain; VLC1, myosin ventricular light chain 1; VLC2, myosin ventricular light chain 2; ELC, scallop myosin essential light chain; RLC, scallop myosin regulatory light chain; MLCK, myosin light chain kinase; Mab, monoclonal antibody; EDTA, ethylenediaminetetraacetic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid; SDS, sodium dodecyl sulfate; SF1, subfragment 1; HMM, heavy meromyosin; ELISA, enzyme-linked immunosorbent assay; PCR, polymerase chain reaction.

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experimental myocardial hypertrophy is correlated with overexpression of the VLC2 (and βHC) isoform which replaces atrial-type LC2 (Henkel et al., 1993). In dilated cardiomyopathy, cardiac LC2 content is reduced, whereas LC1 or HC contents remain steady. LC2-deficient myosin was also found to possess a lower V_m for actin MgATPase activities, and thick filaments prepared from such hearts tended to be shorter than control filaments (Margossian et al., 1992).

Investigations of molecular mechanisms through dynamic modifications (i.e., conformation changes under various conditions) should clarify LC2 function. In this study, we analyze VLC2 conformations using LC2 that is isolated or within myosin, in the presence of calcium or EDTA. This involved a large set of mapped monoclonal antibodies raised against VLC2. Association constant measurements and immunofluorescence detection in the presence of calcium or EDTA were performed to compare the functional and structural roles of VLC2 binding to cardiac myosin HC.

MATERIALS AND METHODS

Protein Preparations. Cardiac myosin was prepared from bovine ventricle according to the method of Kielly and Bradley (1956). In some experiments, calcium-free myosin was prepared from usual myosin solutions by extensive dialysis against 50 mM Tris-HCl (pH 8.0), 0.3 M NaCl, 5 mM EDTA, and 5 mM EGTA and then against water. Pelleted myosin was dissolved in 50 mM Tris-HCl (pH 8.0) and 0.3 M NaCl, with further extensive dialysis against water to eliminate trace amounts of EDTA. Finally, precipitated myosin was redissolved in 0.12 M NaCl and 20 mM Tris-HCl (pH 7.5) buffer containing either 5 mM EDTA or 2 mM $CaCl_2$. Myosin LCs were purified by treating cardiac myosin with 5 M guanidine as described by Holt and Lowey (1975). VLC2 was separated on DEAE-cellulose with a KCl gradient (Franck & Weeds, 1974). Protein concentrations were determined by Bradford's method. The VLC2 content of the myosin preparations was densitometrically determined from SDS–polyacrylamide gels.

Production of Monoclonal Antibodies. Monoclonal antibodies were obtained according to the method of Köhler and Milstein (1975). The procedures for mouse immunization with human ventricular LCs, fusion, screening, production, and purification of antibodies have been described previously (Dechesne et al., 1985; Cornillon et al., 1992).

Association Constant Measurements. Apparent association constants (K_d) of the antigen–antibody equilibrium in solution were measured as initially reported by Friguet et al. (1985) and extensively discussed by Cornillon et al. (1992). Various doses of antigen were mixed with constant quantities of Mab in 0.12 M NaCl and 20 mM Tris-HCl (pH 7.5) buffer. Equilibrium in solution was reached after 2 h at room temperature, but the samples were occasionally incubated overnight. Free Mab was determined by direct solid-phase ELISA with isolated VLC2-coated wells (20 ng per well) after 15 min incubation at room temperature.

According to Friguet et al. (1985), if the total antigen concentration exceeds the total antibody concentration by 10-fold, the apparent dissociation constant K_d can be estimated by linear regression using the simplified Scatchard and Klotz equation $A_0/(A_0 - A) = 1 + K_d/a_0$, where a_0 is the total concentration of antigen, A the absorbance measured by ELISA for Mab at each antigen concentration, and A_0 the absorbance in the absence of antigen. The final results were means (±SE) calculated from three to seven experiments carried out with the same antigen and Mab preparations in the same conditions. Affinities were expressed by apparent association constants K_a, i.e., the inverse of K_d.

Different control experiments were routinely performed (1) to calibrate the Mab dilution range within which there is linear dependence between the absorbance signal and the Mab content, (2) to determine the amount of VLC2 within myosin in the myosin preparations under each measurement condition, and (3) to determine the antibody concentration of the culture supernatant by comparing results from purified antibody and the culture supernatant.

Electrophoresis and Western Blots. SDS–polyacrylamide gel electrophoresis was performed according to the method of Laemmli (1970). Electrophoretic gel to nitrocellulose transfers were performed as described by Matus et al. (1980). Antigens were revealed with hybridome culture supernatants. Mab fixation was detected by a goat anti-mouse antibody coupled with alkaline phosphatase (from Jackson Immuno Research).

Protein Expression and Epitope Mapping. The full cDNA of human ventricular LC2 was a generous gift of Dr. L. Dalla Libera (Padova, Italy). Different fragments of this cDNA were obtained by PCR and inserted in expression vector pUEx (Amersham). The constructions were controlled by sequencing with a T7 sequencing kit (Pharmacia). Expression in an *Escherichia coli* JM 109 strain and fusion protein isolation were described previously (Cornillon et al., 1992).

Myofibril Preparation and Immunofluorescence Microscopy. Bovine cardiac myofibrils were prepared as described by Solaro et al. (1971). They were run in a cytospin centrifuge to make them adhere to glass slides. They were then treated as cryostat sections for immunofluorescence analyses. Indirect immunofluorescence was performed on cryostat sections (10 μm) of frozen ventricle muscle according to Dechesne et al. (1985). Briefly, the sections were preincubated (30 min, 37 °C) with 0.15 M NaCl and 20 mM Tris-HCl (pH 7.5) containing 2 mM $CaCl_2$ or 10 mM EDTA and 10 mM EGTA. Then the hybridome culture supernatant was twice diluted in the same buffer and added to the sections. An FITC-coupled anti-mouse IgG antibody (from Sigma) was used to detect bound Mab. Fluorescent sections or myofibrils were examined under an Olympus BHS microscope equipped for epifluorescence.

RESULTS

Epitope Mapping of Mabs. To localize epitopes of the Mabs used in the present study, seven different fragments of VLC2 cDNA were amplified, cloned, and expressed in *E. coli*: Ala^1–Asp^{164}, Ala^1–Asn^{99}, Ala^1–Val^{59}, Ala^1–Ser^{129}, Lys^{29}–Asp^{164}, Lys^{45}–Asp^{164}, and Val^{79}–Asp^{164}. These proteins, fused with β-galactosidase, were analyzed by Western blotting using nine monoclonal antibodies. The reactivities of the different overlapping recombinant proteins allowed us to map the Mab epitopes. The results are summarized in Figure 1. The epitopes of the nine Mabs were located on five VLC2 domains: Mab 3B11 in the aa 29–45 domain (domain B), three antibodies (Mabs 3E1, 7D2, and 7D8) in the aa 45–59 domain (domain C), Mabs 1D3 and 1F5 in the aa 59–79 domain (domain D), Mab 1F6 in the aa 79–99 domain (domain E), and Mab 13G9 in the aa 99–129 domain (domain F). All reacted with the full-length recombinant VLC2, but neither of them recognized the control β-galactosidase.

Mab 12A8 reacted with natural isolated VLC2 but not with any recombinant LC2 fragment. Its epitope was previously determined as being within the common sequence of the N-extremity of VLC1 and VLC2, i.e., Ala^1–Lys^4

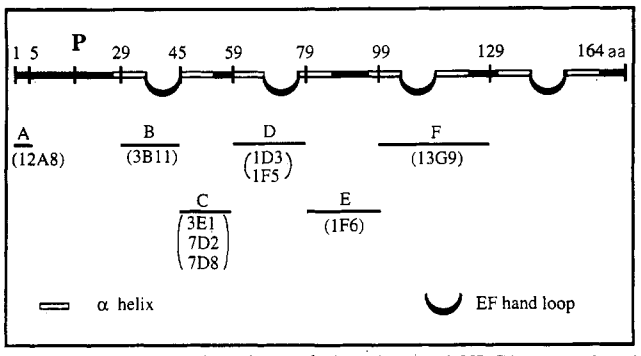

FIGURE 1: Epitope locations of the nine anti-VLC2 monoclonal antibodies. Top: the primary structure of human VLC2 is schematically represented with the phosphorylation site (P) and divalent cation binding sites (α helix and EF-loops). Bottom: the six epitope domains are outlined and denoted by letters (A–F). Designations of antibodies are in parentheses.

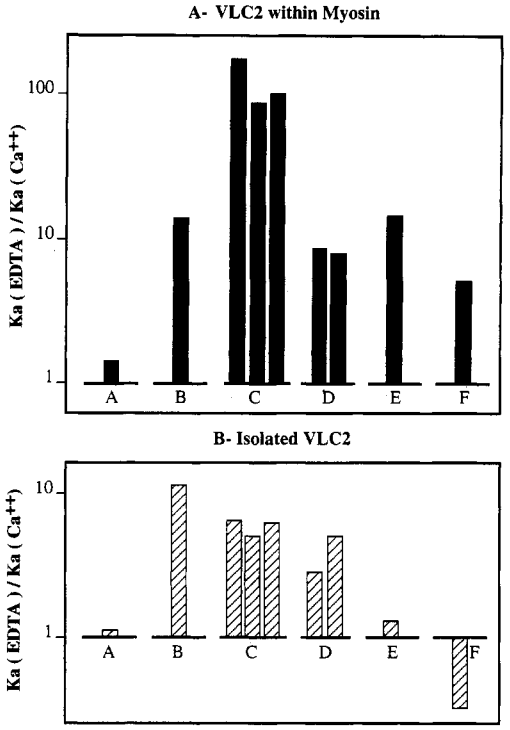

FIGURE 2: Calcium and EDTA effects on Mab affinities to VLC2 within myosin and to isolated VLC2. The relative ratios of apparent association constant values of anti-LC2 antibodies in the presence of 5 mM EDTA vs 2 mM calcium have been calculated from the values given in Table 1. Values obtained with VLC2 within myosin are represented by solid bars (A) and those with isolated VLC2 by hatched bars (B). Epitope domains of antibodies are denoted below with capital letters as in Figure 1.

(domain A) (Cornillon et al., 1992). This nonreactivity was previously explained by fusion with β-galactosidase or by an absence of Ala[1] trimethylation. Recombinant nonfused full-length VLC2 was not recognized by Mab 12A8 (data not shown). We thus conclude that this antibody specifically recognized the trimethylated N-extremity of VLC2, as previously described for another antibody by Boey et al. (1992).

EDTA/Calcium Effects. The affinities in solution of the nine Mabs studied with both native VLC2 within myosin and with isolated VLC2 molecules were determined at low ionic strength ($\mu = 0.12$) under two conditions: with 2 mM calcium or 5 mM EDTA. The Mab affinities for VLC2 within myosin with calcium or with EDTA are summarized in Table 1. The relative ratios between the Mab affinities with calcium and with EDTA were plotted to analyze the effects of calcium on the conformation of VLC2 within myosin (Figure 2A). The

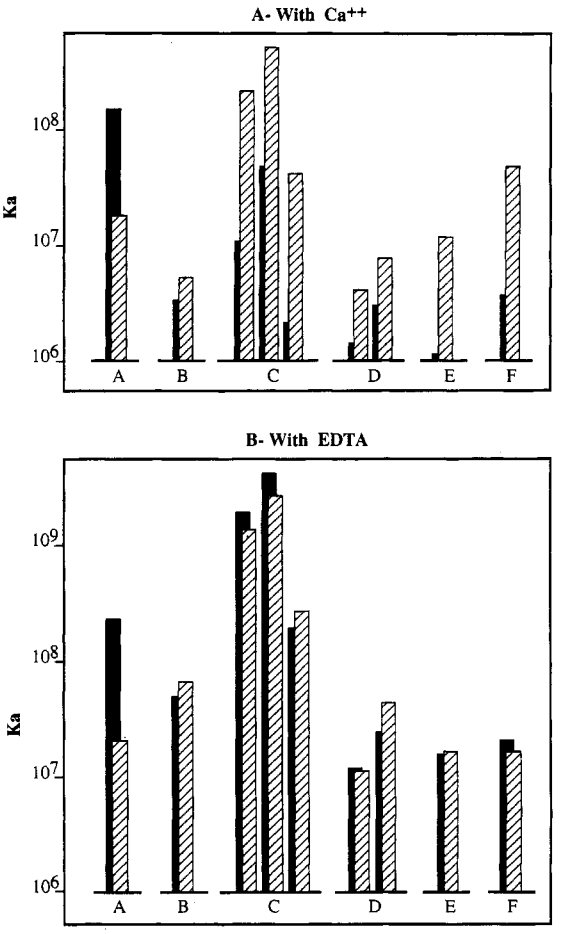

FIGURE 3: HC binding effect on Mab affinities to VLC2 in the presence of 2 mM calcium (A) or 5 mM EDTA (B). Apparent association constant values ($K_a \times 10^{-7}$ M^{-1}) of anti-LC2 antibodies to isolated VLC2 (hatched bars) or to VLC2 within myosin (solid bars). Each antibody is represented by a bar pair. Their epitope domains are denoted below with capital letters as in Figure 1.

anti-VLC2 Mab affinities, according to the presence of calcium or EDTA, differed in five out of six VLC2 domains investigated. The C domain was particularly affected: the affinities of the three Mabs analyzed were dramatically decreased in the presence of calcium (roughly 100-fold or more) as compared to the affinities in the presence of EDTA. The affinity decreases were moderate (about 10-fold) for domains B, D, and E or weak (5-fold) for domain F. No significant variations were observed for Mab 12A8 affinity in domain A.

When Mab affinities were measured on isolated VLC2 (Table 1 and Figure 2B), the affinities in the presence of calcium vs those in the presence of EDTA were moderately decreased for domains B and C (from 5- to 10-fold) and slightly decreased for domain D (from 3- to 5-fold). They were slightly increased for domain F (from 3-fold) and not affected for the N-terminal domain A and domain E. The affinity differences between calcium and EDTA conditions mainly depended on the association state of VLC2: affinity variations were clear-cut when VLC2 was within myosin.

We also analyzed the same affinity values in terms of the association state of VLC2 to assess the effect of HC binding on VLC2 conformation (Figure 3). Comparison of the affinity measurements in the presence of calcium (Figure 3A) for isolated VLC2 or VLC2 within the native myosin molecule revealed that all Mabs except Mab 12A8 behaved in the same way. They had a higher affinity with isolated VLC2 than with VLC2 within the myosin molecule: 3-fold higher for

Table 1: Apparent Association Constants ($K_a \times 10^{-7}$ M^{-1}) of Anti-LC2 Mab to Myosin and VLC2

epitope domain	Mab	myosin		VLC2		
		Ca^{2+} (2 mM)	EDTA (5 mM)	Ca^{2+} (2 mM)	EDTA (5 mM)	no addition
A	12A8	15 ± 1	22 ± 2	1.8 ± 0.6	2.0 ± 0.4	1.8 ± 0.3
B	3B11	0.34 ± 0.08	4.8 ± 1.7	0.6 ± 0.2	6.7 ± 1.4	5.0 ± 1.6
C	3E1	1.10 ± 0.07	189 ± 52	21 ± 3	135 ± 29	112 ± 11
	7D2	4.8 ± 0.2	417 ± 100	53 ± 9	278 ± 99	333 ± 106
	7D8	0.20 ± 0.04	20 ± 6	4.2 ± 0.6	26 ± 4	22 ± 5
D	1D3	0.14 ± 0.04	1.2 ± 0.3	0.4 ± 0.1	1.1 ± 0.4	1.1 ± 0.4
	1F5	0.3 ± 0.03	2.3 ± 0.5	0.8 ± 0.5	4 ± 0.9	1.9 ± 0.5
E	1F6	0.11 ± 0.04	1.60 ± 0.08	1.20 ± 0.08	1.6 ± 0.1	2 ± 1
F	13G9	0.37 ± 0.12	2.0 ± 0.6	4.8 ± 1.6	1.6 ± 0.7	2.0 ± 0.9

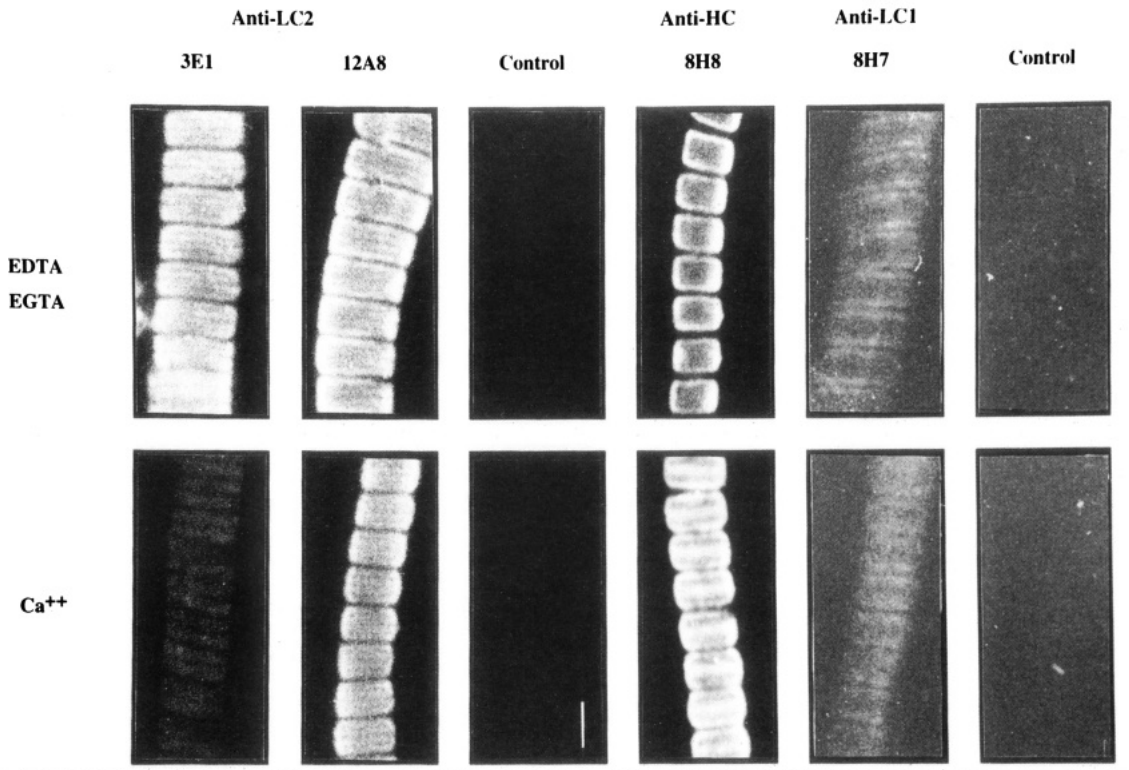

FIGURE 4: Immunohistofluorescence staining of cardiac myofibrils in the presence of 10 mM EDTA and 10 mM EGTA or in the presence of 2 mM $CaCl_2$. The Mabs used were 3E1, 12A8, 8H8 (anti-HC), and 8H7 (anti-VLC1). Bar = 2 μm.

Mabs located within the B and D domains and 10–20-fold higher for Mabs located within C, E, and F domains. Mab 12A8, whose epitope is located at the amino-terminal extremity, also had distinct reactivity with isolated VLC2 or VLC2 within myosin: it reacted with substantially higher affinity on myosin than on isolated VLC2. This is similar to previously reported results for VLC1: Mabs directed to the N-extremity of VLC1 better recognized VLC1 within myosin than isolated VLC1 (Cornillon et al., 1992).

EDTA did not have an effect *per se*, since no affinity differences for the isolated light chain were reported when 5 mM EDTA or none was added (see the two last columns in Table 1). Comparison of Mab affinities for VLC2 within myosin or isolated VLC2 in the presence of EDTA (Figure 3B) also showed no affinity differences between Mabs whose epitopes were located between residues 30 and 130. As indicated in Materials and Methods, calcium-free cardiac myosin solutions were prepared to test whether EDTA treatment of native myosin solutions irreversibly induced significant VLC2 releases before or during the affinity measurements. We observed that 90–100% of the initial VLC2 content was repeatedly recovered in the pelleted myosin solution after low salt precipitation. Mab 3E1 affinities for EDTA-treated, extensively dialyzed, and precipitated myosin solutions were similar to those obtained with standard native myosin preparations measured in the presence of 5 mM EDTA. The reversibility of the calcium effect on VLC2 within myosin was controlled by adding 2 mM calcium to the calcium-free myosin solution. Accordingly, Mab 3E1 affinity then decreased about 150-fold compared to that in the absence or presence of 5 mM EDTA. We noted that the N-terminal extremity of VLC2 was insensitive to calcium or EDTA: Mab 12A8 affinity only depended on the binding state of VLC2 (Table 1 and Figure 3).

Immunofluorescence Studies. Series of experiments were designed to establish whether the calcium/EDTA effect persisted in more integrated systems than isolated proteins in solution. Cardiomyofibrils and heart sections were stained with antibodies and observed by fluorescence microscopy. Mabs 12A8, 3B11, 3E1, 1D3, 1F6, and 13G9 directed against VLC2 were used. Only representative results with Mabs 3E1 and 12A8 are presented in Figure 4.

Myofibril staining in the presence of EDTA was brighter than with calcium for all of these Mabs, but not for 12A8. The most clear-cut images were obtained with Mab 3E1, probably because it had the highest affinities for VLC2 within myosin and EDTA-treated myosin. The EDTA effect was fully reversible with calcium. No difference was observed with

Mab 12A8, thus confirming the affinity results. An anti-HC rod (Mab 8H8) and an anti-VLC1 (Mab 8H7) were used as controls. No influence of EDTA or calcium was noted for these control Mabs (Figure 4).

Binding of anti-VLC2 was detected in the A band. No staining was observed at the center of the A band, at the Z band, or in the I band. Moreover, staining was brightest on each side of the bare zone, in the putative nonoverlap zone, and at the tapered end of the filament. In the presence of calcium, these striations disappeared and residual fluorescence was more homogeneous, probably because the antibody affinities had decreased.

DISCUSSION

The present study was aimed at examining how VLC2 interacts with the cardiac myosin heavy chain within the native molecule and whether calcium influences binding. More than 50 different Mabs were initially screened on isolated bovine ventricular LCs, using isolated human ventricular LCs as antigens. Nine Mabs reacting with isolated VLC2 were selected and their epitopes located. These Mabs were used to test the conformation of about two-thirds of the VLC2 molecule (residues 1–130). As previously demonstrated with isolated VLC1 (Cornillon et al., 1992), affinity differences for a Mab reacting with the same antigen in different states could be generally explained by conformation changes in the Mab epitope (shape change or accessibility change for some residues).

The present results show that the calcium ion slightly modified the conformation of isolated VLC2. The conformational changes seemed to be located on the VLC2 segment between residues 29 and 45. The calcium-induced conformational changes in VLC2 were dramatically enhanced when VLC2 was bound to the HC within the native myosin molecule. Three Mabs, reacting within the putative calcium site in VLC2, demonstrated that the calcium-induced conformational changes essentially occurred between residues 45 and 59 in the C domain of VLC2 (Figure 1). The Mab affinities with calcium were found to be 2 orders of magnitude lower than with EDTA for the three Mabs with epitopes located in the C domain (Figure 2A). The other VLC2 domains seemed to be much less or not at all affected by the conformation change, indicating that the calcium effect was relatively confined to a limited VLC2 segment. The precise location of the strongest calcium effect was not surprising since the putative calcium binding site in VLC2 stretches from residue 26 to residue 56 (an EF-loop from residue 36 to residue 46 flanked by two α helices, aa 26–36 and aa 46–56) (Collins, 1991). Figure 5 shows the concerned LC2 part on a structural model derived from Rayment's representation (Rayment et al., 1993). The present study involving nine Mabs located along VLC2 extends to cardiac myosin the calcium-induced conformational changes in fast skeletal myosin with a Mab to LC2 (Shimizu et al., 1985). The previous analysis was restricted to a narrow LC2 domain since the epitope of the Mab used was located around residues 35–36 (Reinach & Fischman, 1985) and the Mab reacted with fast skeletal LC2 but not with slow skeletal or cardiac VLC2 (Shimizu et al., 1985).

The effects of calcium and EDTA on VLC2 within the myosin molecule suggest that part of the binding mechanism of VLC2/HC could be dependent on the calcium ion. The moderate effect of calcium on antibody affinities in isolated VLC2, as compared to the substantial effects in myosin, suggests that isolated VLC2 alone could not provide all of the residues required for calcium binding. The calcium effects

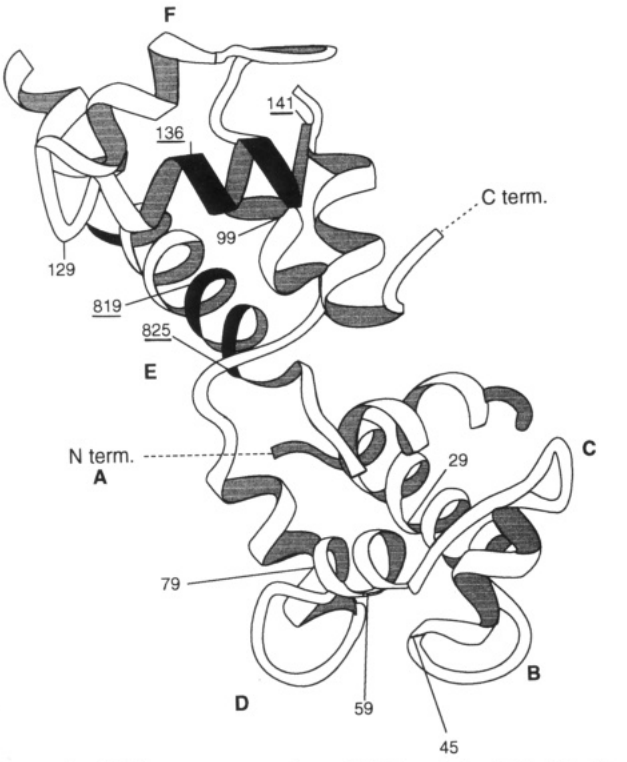

FIGURE 5: Ribbon representation of LC2 and the 800–843 S1 fragment derived from Rayment et al. (1993). Letters (A–F as in Figure 1) represent defined VLC2 domains, numbers represent VLC2 residue positions, and underlined numbers represent residue positions of the hydrophobic clusters (VLC2 and HC). Black areas represent the reported hydrophobic clusters. The location of the VLC2 N-extremity is arbitrarily shown since the segment was not resolved in the crystal.

on the binding of VLC2 to cardiac myosin β HC that we observed should be compared with the now available molecular information on muscle and nonmuscle myosins. The X-ray study of Rayment et al. (1993) on the chicken fast skeletal muscle SF1 crystal provides information on residues and segments possibly involved in VLC2/HC binding. The very recently reported three-dimensional structure of the regulatory domain of scallop myosin, which contains part of HC, together with ELC and RLC (Xie et al., 1994), points out the residues that are probably involved in interchain stabilization by the calcium ion. The X-ray study of Rayment et al. (1993) reveals that the small α-helix ending at HC residue 843 is facing the first EF-loop of VLC2. However, the distance between the two regions seems too large for divalent cation binding (Figure 5). The unsuccessful chemical cross-linking between LC2 and SF1, as compared to the successful cross-linking between LC2 and HMM (Mocz et al., 1982), confirms that correct native binding with LC2 cannot occur when the HC ends at residue 843. However, in the native myosin molecule, the IQ motif sequence within the myosin HC and the VLC2 EF loop could be close enough to build a common calcium binding site. The Xie structure on the regulatory domain of scallop myosin established that (1) one cation binding site is within the loop of the first EF hand of RLC, (2) another ELC calcium binding site requires cooperative interactions of the three chains, Gln^{812} of HC, Met^{116}, Gly^{117}, and Asp^{118} of RLC, and Phe^{20}, Gly^{21}, and Arg^{24} of ELC, and (3) the IQ motifs of the HC (aa 811–824) form the suitable bonds between residues at the end of the F helix and those of the linker between the F and G helices of RLC (i.e., Met^{116}, Gly^{117}, and Asp^{118}). Examination of the corresponding sequences in human ventricular myosin subunits (Table 2) revealed many differ-

Table 2: Sequence Comparisons of Scallop and Cardiac Human Myosins[a]

IQ motifs of HC (residues 811-824 of Scallop HC)	
IQ**R**NI**R**K**W**L**V**L**R**N**W**	Scallop heavy chain
IQWNIRG FMGVKNW	Human ß heavy chain
F, G α helix of RLC, (residues 108-126), and VLC2	
Y IKDLLEDM **G**D**N**FN TDEMR	Scallop RLC
YVREMLTTQ AE RFS KE EVD	Human VLC2
α helix F linker α helix G	
(aa 19-25) domains of ELC and VLC1	
D**F**W**D**G**R**D	Scallop ELC
DR T P KCE	Human VLC1

[a] Sequence comparisons between domains in the heavy and light chains from scallop and cardiac human β ventricular myosins involved in conformational stabilization of the calcium binding site are shown. Bold characters refer to the key residues according to Xie et al. (1994).

ences between muscle and nonmuscle myosins. IQ motifs occur in the β HC, but the LC residues involved in the interchain stabilization in scallop myosin are different in cardiac LCs. No calcium binding site has been described in VLC1, and the present work is only dealing with the putative calcium binding site in VLC2 analogous to that observed in the first EF hand of RLC. A sophisticated calcium/RLC/ELC/HC interaction network, as observed in scallop myosin, is improbable in ventricular myosin.

Mab affinity measurements in the presence of EDTA showed that removal of the calcium ion from cardiac myosin allowed VLC2 within myosin to bind most antibodies in the same way as isolated VLC2. This increased accessibility of the weakly bound VLC2 within cardiac myosin in the presence of EDTA was also demonstrated by immunofluorescence observations on myofibrils and tissue sections. This is in agreement with previous images obtained by Shimizu et al. (1985) on skeletal fast myofibrils. We determined that VLC2 was not (or only in a very low proportion) dissociated from the myosin HC after successive precipitations of EDTA-treated myosin solutions. In fact, cardiac VLC2 is probably more tightly attached to the cardiac myosin HC as compared to LC2 within fast skeletal myosin. More stringent conditions such as 37 °C and 5 mM ATP together with EDTA were shown to be required to dissociate or exchange significant amounts of skeletal LC2 but not VLC2 (Pastra-Landis & Lowey, 1986; Rajasekran et al., 1991). The present experiments, showing that VLC2 within cardiac myosin remained bound to the myosin HC in the presence of EDTA, suggest the existence of an additional binding region between VLC2 and the myosin HC along with the calcium-dependent site. The putative second binding site would be beyond residue 129 since Mab 13G9 had the same affinity for myosin and for isolated VLC2 in the presence of EDTA. Between residues 129 and 164, there is a stretch of seven adjacent hydrophobic amino acid residues (residues 136–142). This hydrophobic cluster is the longest of the whole VLC2 molecule. It could permit a hydrophobic interaction with the HC. The chicken skeletal SF1 crystal (Rayment et al., 1993) indicates that the LC2 domain, spanning residues 136–142, is near the HC part around residues 819–825 (Figure 5). There are five hydrophobic residues on the human β heavy chain, between residues 819 and 825. A hydrophobic interaction is thus possible between the 136–142 VLC2 domain and the 819–825 HC domain. It is generally accepted that contraction movement requires a shape or angle change in the myosin heads. HC molecular conformation control is thus a key characteristic of the contraction mechanism. Generally, conformation stabilization can be obtained in three main ways: (1) by hydrophobic interchain contacts, such as rod/rod interactions of two HC molecules, (2) by intrachain contacts, such as with different parts of the SF1 domain, and (3) by a latching mechanism with other peptides, as could be the case with light chains and the head/rod junction of the myosin HC molecule.

This latter mechanism is suggested by the SF1 ribbon representation proposed by Rayment et al. (1993). Specifically, LC2 embraces an SF1 α helix in two domains: HC 815–825 by LC2 95–164 and HC 828–843 by LC2 19–85. In each of these two domains, there can be an interaction between the two chains which could confer a blocking role of the 815–843 HC domain: the 815–825 domain by a hydrophobic interaction and the 825–843 domain by a calcium site. There have been extensive investigations on a calcium regulation role via LC2 for cardiac VLC2, but it has not been confirmed. The divalent cation could thus only have a structural function. In the same way, phosphorylation of Ser^{15} could modify all or part of the LC2 conformation and subsequently 815–843 HC flexibility. Mutagenesis experiments to modify the VLC2 hydrophobic cluster or the calcium site could provide some information on these hypothetical cardiac myosin HC latching mechanisms.

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