Identification of Sequences Necessary for the Association of Cardiac Myosin Subunits

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Abstract. To begin to understand the nature of myosin subunit assembly, we determined the region of a vertebrate sarcomeric myosin heavy chain required for binding of light chain 1. We coexpressed in *Escherichia coli* segments of the rat alpha cardiac myosin heavy chain which spanned the carboxyl terminus of subfragment 1 and the amino terminus of subfragment 2 with a full-length rat cardiac myosin light chain 1. A 16 amino acid region of the myosin heavy chain (residues 792–808) was shown to be required for myosin light chain 1 binding in an immunoprecipitation assay.

MYOSIN is an asymmetric hexamer comprised of two heavy chains (210 kD) and two pairs of light chains (18–25 kD). In smooth muscle, invertebrate muscle and vertebrate nonmuscle myosin, myosin light chain 2 (MLC2)[1] regulates the actin-activated ATPase of myosin in response to the calcium concentration of the cell (for reviews see Kamm and Stull, 1985; Citi and Kendrick-Jones, 1988). The role of MLC1 in myosin function is less clear (see Wagner and Giniger, 1981; Sivaramakrishnan and Burke, 1982). However, isoforms of MLC1 have been shown to be associated with different shortening velocities of skeletal muscle (Sweeney et al., 1988).

The interactions of the subunits of myosin are not precisely defined, but both biochemical and electron microscopic studies suggest that MLCs bind near the junction of the subfragments 1 and 2 (S1 and S2). Scallop myosin reacted with anti-light chain antibodies demonstrates MLC2 binding near the head–rod junction (Flicker et al., 1983). Similar positions were noted for vertebrate MLC2, with MLC1 binding distally to MLC2, in the S1 head (Tokunaga et al., 1987; Katoh and Lowey, 1989). Proteolytic peptides of myosin heavy chain (MHC) from the carboxyl terminus of the S1 head bind MLC (Szentkiralyi, 1984; Mitchell et al., 1986). Burke et al. (1983) also implicated the carboxyl terminus of the S1 head by abolishing MLC1 binding with proteolytic digestion of a 3-kD segment from the S1–S2 junction. Sellers and Harvey (1984) used gel overlay to assign the light chain binding sites to 26 kD at the carboxyl terminus of smooth muscle myosin S1. However, Okamoto et al. (1986) have shown that MLC1 binds near the site of ATP binding on smooth muscle myosin. In the scallop it has been shown that exchange of MLC1 with exogenously added MLC1 can occur only in the absence of MLC2 (Ashiba and Szent-Gyorgyi, 1985). This finding, coupled with the observation that the sulfhydryl groups of scallop MLC1 are only available for labeling in the absence of MLC2, suggests that MLC1 lies between MLC2 and MHC (Walliman and Szent-Gyorgyi, 1981; Hardwicke et al., 1982).

The use of recombinant DNA methods to produce myosin segments that have precisely defined sequences would be of great use in mapping the interactions of myosin subunits. A portion of the *Caenorhabditis elegans unc54* MHC gene corresponding to S1 was expressed in *Escherichia coli* and shown to interact with MLCs in a gel overlay system (Mitchell et al., 1989). This approach identified the extreme carboxyl terminus of S1, (residues 848–886 on the *C. elegans unc54* MHC) as the region of the MHC which is responsible for the MHC:MLC interaction. This approach, while useful, studies the association of MLC with a denatured MHC and, therefore, may not reflect all the possible interactions between MHC and MLC. We have shown previously that coexpression of soluble MHC and MLC1 results in their coassembly with the appropriate stoichiometry (McNally et al., 1988). In the present study, the interaction of a nondenatured MHC and MLC1 was studied using *E. coli* coexpression of the rat alpha cardiac MHC along with the full-length rat ventricular MLC1. Deletions spanning S2 and the 20-kD region of S1 were used to identify a region responsible for MLC1 binding on the MHC. This approach, which examines the binding of two proteins in solution which are associated in vivo, identified 16 amino acids toward the carboxyl end of S1 as necessary for MLC1 binding.

Materials and Methods

Construction of Expression Plasmid

A cDNA encoding the first 3111 bp of the rat alpha cardiac MHC (McNally et al., 1989*a*) was digested with BglI, treated with T4 DNA polymerase, digested with HindIII, and ligated into the expression vector PINIAI that had been digested with EcoRI, blunted, and cut with HindIII (Masui et al.,

1. *Abbreviations used in this paper*: MHC, myosin heavy chain; MLC, myosin light chain; S1 and S2, subfragment 1 and 2, respectively.

1983). This expression construct was then digested with XbaI, treated with Klenow fragment, digested with HindIII, and ligated into pKK233-2 (Pharmacia Fine Chemicals, Piscataway, NJ) that had been digested with EcoRI, treated with Klenow, and then digested with HindIII. This placed the expression of the MHC sequences under the control of the *tac* promoter (deBoer et al., 1983). This resulted in alteration of the first amino acids of the alpha cardiac MHC from MTDAQM to MLGNLA. The termination codons are provided by the expression vector.

A full-length cDNA for the rat ventricular MLC1 cDNA (McNally et al., 1989*b*) was engineered by site-directed mutagenesis with the oligonucleotide 5'GATTACAGCTCCATGGCCCCC3' to contain an NcoI site at its amino-terminal methionine codon. The mutagenized plasmid was digested with NcoI and HindIII and ligated into a similarly digested pKK233-2 (Pharmacia Fine Chemicals); a native MLC1 sequence was generated. The portion of this expression construct containing the promoter, coding regions, and terminator sequences was placed on the same plasmid with the sequences expressing the alpha cardiac MHC by digesting both with SphI, purifying the appropriate fragments and ligating them.

Construction of Deletion Mutants

Deletions of the 3' end of the 3.1-kb plasmid of the alpha cardiac MHC were constructed using an exonuclease III/S1 nuclease system (Erase-a-base; Promega Biotec, Madison, WI), (Henikoff, 1984), and sequenced (Kraft et al., 1988). Those deletions that retained a HindIII site and terminated in the desired region, spanning the 20-kD terminal portion of S1 and the amino terminus of S2, were selected and subcloned into the expression vector to produce the deletions of the MHC along with the full-length MLC1 protein.

Cell Growth and Induction

E. coli carrying the expression construct plasmids were grown in LB and 50 μg/ml ampicillin at 37°C until reaching an OD_{595} of 0.7 at which point IPTG (isopropylthiogalactoside) was added to a final concentration of 0.2 mM. The *E. coli* were grown an additional 30 min and then harvested and washed as previously described (McNally et al., 1988). The *E. coli* were resuspended in a lysis buffer containing the following: 50 mM Tris-HCl, pH 7.5, 100 mM KCl, 5 mM EDTA, pH 7.5, 5% (wt/vol) sucrose, 0.3 mM PMSF, 0.5 μg/ml leupeptin, 0.7 μg/ml pepstatin, 0.2 mM TPCK, 0.2 mM TLCK, and 1 mM DTT. 500 ml of *E. coli* culture (~1 g wet weight) was resuspended in 15 ml of lysis buffer. The cell suspension was passed three times through a chilled French pressure cell (40 ml size) at 1,000 lb/in^2 The lysate was then centrifuged at 30,000 *g* for 45 min to remove the insoluble debris. The quantity of MHC expressed by the various bacterial strains was determined by an ELISA assay with F59 antibody (see below). MLC1 quantities in bacterial strains were determined by immunoblot analysis with MF5 (see below) compared to known quantities of rabbit S1.

Immunoprecipitations

Immunoprecipitations were made in two ways. The first used Pansorbin formalin-fixed *Staphylococcus aureus* cells (Calbiochem-Behring Corp., La Jolla, CA). The Pansorbin cells were prepared by mixing 30 μl of cells with 1 ml 0.1% BSA in TBS (150 mM NaCl, 50 mM Tris, pH 7.7) twice, pelleting for 1 min in a microfuge to collect the cells. The blocked cells were then incubated with 25 μl of 1 mg/ml rabbit anti-mouse IgG (Cappel-Organon, West Chester, PA) for 1 h on ice. These cells were washed once with 200 μl of TBS and then resuspended in 100 μg of purified F59 antibody (Miller et al., 1989; generous gift of F. Stockdale, Stanford University) for a minimum of 2 h on ice. The cells were washed twice with 250 μl of TBS and then incubated with lysate supernatants of *E. coli* expressing deletions of MHC and full-length MLC1 overnight on ice. The immunoprecipitations were pelleted and washed three times with TBS plus 1 mM DTT. The final pellet was resuspended in SDS-PAGE sample buffer and electrophoresed on a 15% Laemmli (1970) gel. A duplicate gel was transferred to nitrocellulose by standard techniques (Towbin et al., 1979), and immunoblotted with MF5, an anti-MLC2 monoclonal antibody that also cross-reacts with cardiac MLC1 (generous gift of D. Fischman, Cornell University Medical School, New York), (Shimizu et al., 1985).

For measurements of the stoichiometry of MHC:MLC1, partial fractionation of the bacterial extracts was carried out before incubation with an anti-myosin immunoaffinity resin. After French press lysis of 500 ml culture of *E. coli* cells into 10 ml of lysis buffer (6% sucrose, 225 mM NaCl, 5 mM Tris pH 7.7, 0.5 mM DTT, protease inhibitors as above) a high speed centrifugation step was carried out (100,000 *g* for 37 min at 4°C). The supernatant was incubated with 20 ml of DE52 resin (equilibrated with lysis buffer) to remove nucleic acid. The material not bound to DE52 was subjected to high speed centrifugation (100,000 *g* for 96 min at 4°C). 6 ml (corresponding to ~35% of the original culture) of this supernatant was incubated overnight at 4°C in batch with 0.5 ml of Sepharose covalently linked to the anti-MHC IgG, F59, at a concentration of 1.9 mg/ml. The resin was washed with 15 ml of a buffer containing 0.4 M $(NH_4)_2SO_4$, 0.5 mM DTT, 0.25 mM EDTA, 50 mM Tris pH 7.7 at 4°C. 300 μl of 1× Laemmli buffer was added to the resin. The mixture was heated at 100°C for 5 min before loading (25 μl) on a polyacrylamide gel. Each sample was run on duplicate gels, along with chymotryptic S1 from rabbit skeletal myosin as a control. One gel was stained with Coomassie brilliant blue and the other was immunoblotted with MF5 and F59 (see above). The lanes of the Coomassie brilliant blue–stained gel were scanned with a quantitative scanning laser densitometer (Pharmacia LKB UltroScan, Piscataway, NJ). The areas under the peaks were evaluated using the Gelscan XL software program. The MHC and MLC1 peaks were obtained for chymotryptic S1 and their ratio compared to those obtained from various bacterial constructs. The ratio for chymotryptic S1 is 1.1 (range 0.9–1.2). The ratios for D4, D3, D2, D6, and D6 and MLC1 are indistinguishable and show a range of 0.9–1.1

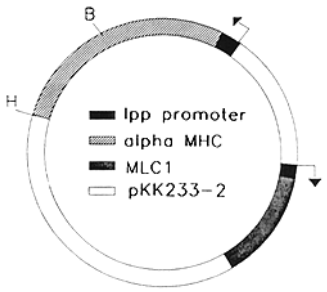


Figure 1. Plasmid for coexpression of rat alpha cardiac MHC and rat ventricular MLC1. (McNally et al., 1989*a*,*b*). Two separate copies of the IPTG-inducible *tac* promoter are present on the plasmid and drive expression of the two subunits of myosin. Deletions of the rat alpha cardiac MHC were generated by exonuclease III digestion followed by subcloning at the BglII and HindIII sites shown in the MHC. *B*, BglII; *H*, HindII.

Results

Coexpression of MLC1 with Deletions of MHC

An *E. coli* expression vector was constructed to express deletions of the rat alpha cardiac MHC along with the full-length rat ventricular MLC1. Both of the subunits of myosin were expressed from a single plasmid with two copies of the isopropylthiogalactosidase (IPTG)-inducible *tac* promoter (deBoer et al., 1983). A diagram of this plasmid is shown in Fig. 1. A series of deletions was generated using exonuclease III digestion of a 3.1-kb cDNA for the rat alpha cardiac MHC. Progressive deletions that retained the proper cloning

Table I. Deletion Expression Plasmids of the Rat Alpha Cardiac MHC to Map the MLC1 Binding Site on the MHC

Clone	bp	AA	Predicted molecular weight	Binds MLC1
D-1	2,145	715	81,185	−
D0	2,205	735	83,532	−
D1	2,376	792	89,805	−
D6	2,424	808	93,326	+
D2	2,496	832	95,058	+
D3	2,622	874	100,042	+
D4	3,096	1,032	118,310	+

Listed above is the name of the deletion of the rat alpha cardiac MHC cDNA, its length in nucleotides (*bp*) and amino acids (*AA*) and the predicted molecular weight of the expressed segment. Shown at the far right are the results of MLC1 binding determinations.

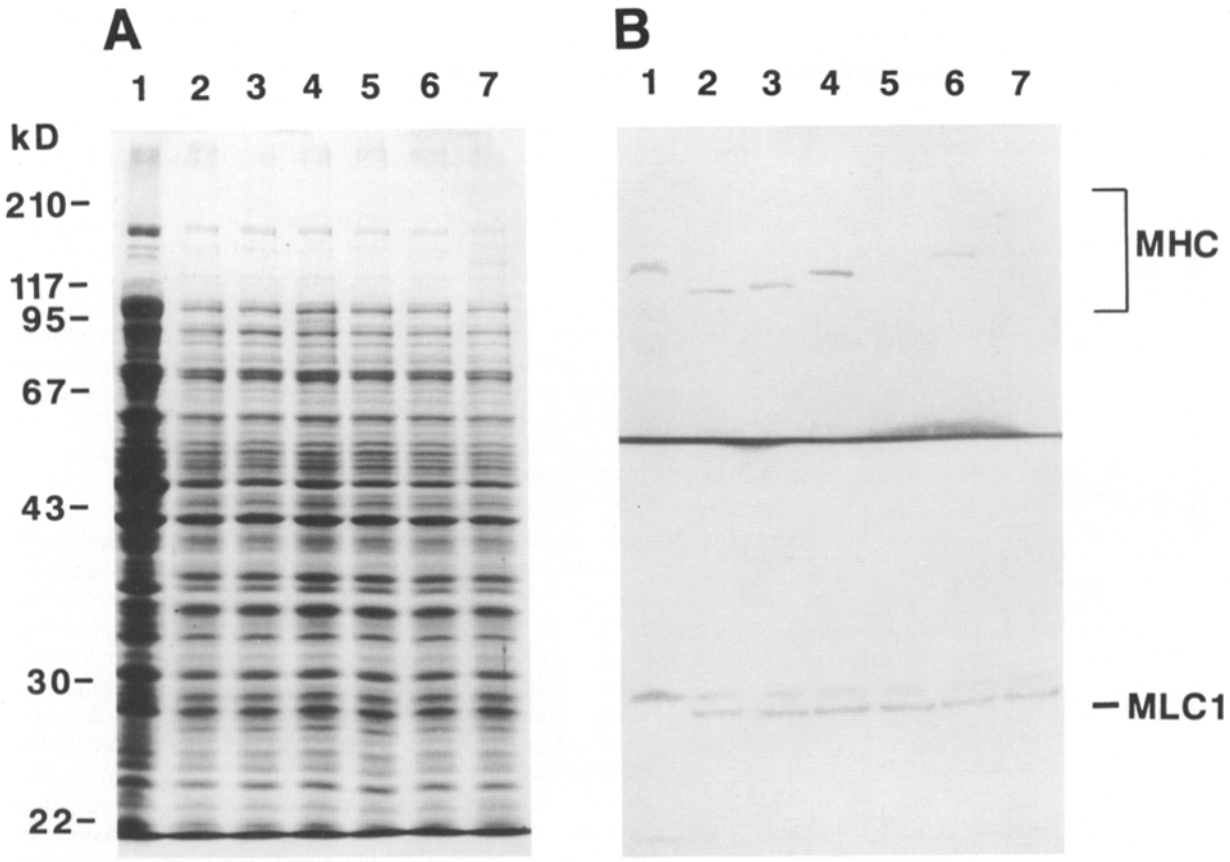


Figure 2. Expression of the deletions of the rat alpha cardiac MHC and rat ventricular MLC1 in *E. coli*. Shown are whole cell lysates of bacteria expressing MHC deletions and full-length MLC1. (*A*) 10% Coomassie blue-stained SDS-PAGE. (*B*) Immunoblot of 10% SDS-PAGE where the top half was reacted with F59, an anti-MHC monoclonal antibody (Miller et al., 1989). The lower half of the immunoblot was reacted with MF5, an anti-MLC monoclonal antibody (Shimizu et al., 1985). MF5 also recognizes a bacterial protein that migrates just above the MLC1 band. Lane *1*, a bacterial strain expressing the D2 MHC construct, but not expressing MLC1; Lanes *2–7* are extracts of *E. coli* expressing the deletions of the MHC with the full-length MLC1 (−1D, D0, D1, D2, D3, and D4, respectively).

sites were expressed in *E. coli*. Seven deletions were constructed to span the region (2,145–3,096 bp), which corresponds to the carboxyl terminus of the 20-kD tryptic region of the S1 head to the middle of the S2 region (amino acid residues 715–1,032). Table I summarizes the lengths of the MHC deletions and the predicted molecular weights.

A substantial portion of both MHC and MLC1 were found in the soluble fraction of an *E. coli* lysate after a nondetergent lysis (data not shown). The MLC1 protein was produced at ~5 mg/liter of *E. coli* (2 g wet weight) which was in molar excess of the MHC deletions (also ~5 mg/liter of *E. coli*). The various MHC deletions were produced at levels which varied only slightly from each other. Expression of the MHC and MLC1 from these constructs is shown in Fig. 2. The deletions of the alpha cardiac MHC all appear to migrate at the molecular weights predicted from their amino acid sequence.

E. coli expressing each of the MHC deletions and the full-length MLC1 were grown, induced and lysed under nondetergent conditions. The cell debris was pelleted, and the supernatant was used to study the association of MHC and MLC1. Immunoprecipitations with an anti-MHC monoclonal antibody, F59 (Miller et al., 1989) were used to study the interaction of MHC and MLC1. The shortest three constructs, −1D, D0, and D1 were found not to be associated with any MLC1 as determined by Coomassie blue-stained SDS-PAGE as well as immunoblot analysis with an anti-MLC monoclonal antibody, MF5 (Shimizu et al., 1985). The four longer constructs, D6, D2, D3, and D4, were found to associate with MLC1. The results for the D6 construct compared with D0 can be seen in Fig. 3. D6 (amino acids 1–808) is the shortest segment of the MHC which can bind MLC1. The ratio of MHC to MLC1 was quantitated by densitometric scans of Coomassie blue-stained gels, using chymotropic S1 as a standard (see Materials and Methods). From this analysis, we conclude that the region between D1 and D6 is necessary for the association of MHC and MLC.

Comparison of the MLC1 Binding Site among MHCs

A comparison of the sequence of residues 792–808 on the rat alpha cardiac MHC, with MHCs from lower eukaryotes, vertebrate nonmuscle, smooth muscle, and striated muscles revealed only one invariant residue (Fig. 4). On the other hand, 8 of the 16 residues in the putative light chain binding site are conserved in all of these vertebrate striated muscle MHC's.

Discussion

The deletion analysis presented here suggests that amino

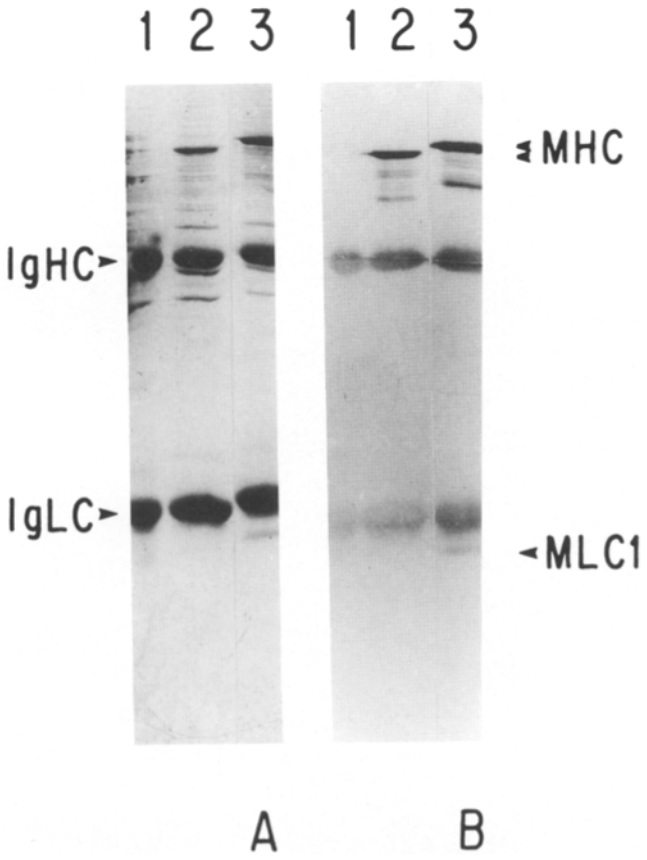
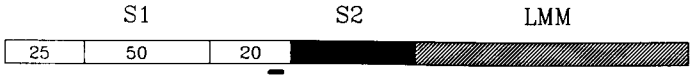


Figure 3. Analysis of MHC deletions for association with MLC1. Shown are duplicate 15% polyacrylamide gels stained with Coomassie brilliant blue (*A*) or blotted and reacted with monoclonal antibodies directed against MHC (*top* panel) and MLC1 (*bottom* panel) (*B*). Lane *1*, immunobeads with no bacterial extract. Lanes *2* and *3* are immunoprecipitations of bacterial extracts (D0 and D6, respectively, both coexpressing MLC1) with F59 antibody coupled to Sepharose. Vertical arrows mark the positions of the immunoglobulins from the monoclonal antibody (*IgHC* and *IgLC*, immunoglobulin heavy chain and light chain, respectively) and the myosin subunits retained by the MHC antibody.

acids 792–808 of cardiac MHC are necessary for its interaction with MLC1. One implication of our results is that other regions of S1 and the amino terminus of S2 are not necessary for the association with MLC1. It should be kept in mind that it has not been established that these 16 residues are sufficient for MLC1 binding. It is possible that the inability of the shorter MHC constructs (−1D, D0, and D1) to bind MLC1 is due to conformational changes resulting from removal of amino acids 792–808. However, studies of Szentkiralyi (1984) showed that a 14-kD peptide is capable of binding MLC1, suggesting that very distal sequences are not required for the stoichiometric binding. The identification of this region of the MHC as the MLC1 binding site is in good agreement with previous studies on invertebrate myosin which used different approaches (Mitchell et al., 1986, 1989), as well as earlier proteolytic cleavage studies (see Burke et al., 1983; Szentkiralyi, 1984).

The MLC1 binding site identified by this study lies 30 amino acids amino-terminal the the proline residue thought to be the junction between S1 and S2 as defined by McLachlan and Karn (1982). The D6 deletion, which terminates at residue 808, migrates electrophoretically similarly to cardiac muscle S1 derived from proteolysis by chymotrypsin (data not shown) and corresponds exactly to the terminal residue of chymotryptic S1 from chicken skeletal muscle as determined by Maita et al. (1987). This confirms the prediction that the MLC1 binding site is near the S1–S2 junction. Immunoelectron microscopy studies that use anti-MLC antibodies have identified the MLC1 binding region to be somewhat distal to the MLC2 binding site at the head–rod junction when viewing the rotary shadowed myosin image (Katoh and Lowey, 1989). This would argue that the 16 amino acid region identified by the present experiments would lie upstream of the head–rod junction on the rotary shadowed image. *E. coli* expression experiments with amino-terminally truncated portions of the *Acanthamoeba* myosin II sequence have predicted that the S1-S2 junction may actually lie within the head of myosin when viewed as a rotary-shadowed image in the electron microscope (Rimm et al., 1989). Their results would place the MLC1 binding site within the head as op-



R K T A M R K Y Y Y E V K K G G	*Acantha my I*
R A F L A R R M Y D K M R E Q T	*Acantha my II*
R G W I A R K V Y K Q A R E H T	*Dictyo my II*
R W H L G L K D R K R R M E Q R	*C. elegans*
R G Y L A R K A F A K R Q Q Q L	*chick smooth*
R G Y L A R K A F A K R Q Q Q L	*human nonmuscle*
R G F L M R V E F K K M M E R R	*chick embryonic*
R G F L M R V E F Q K M V Q R R	*human embryonic*
R G F L M R V E Y Q K M L Q R R	*human perinatal*
R G F L M R V E F Q K M M Q R R	*rat embryonic*
R G V L S R M E F K K L L E R R	*rat beta*
R G Q L M R I E F K K M V E R R	*rat alpha*
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Figure 4. Evolutionary comparison of the MLC1 binding site. A linear schematic of the MHC is shown above with a line indicating the location of the 16 amino acid MLC1 binding site. This region, residues 792–808, is shown below from several MHC sequences. Shown are the *Acanthamoeba* MHCI (Hammer et al., 1986); *Acanthamoeba* MHCII (Hammer et al., 1987); *Dictyostelium mhca*, (Warrick et al., 1986); *C. elegans unc54* (McLachlan and Karn, 1982); chicken smooth muscle MHC (Yanigisawa et al., 1987); human nonmuscle MHC (Saez et al., 1990); chicken embryonic skeletal MHC (Molina et al., 1987); human embryonic (Eller et al., 1989); human perinatal skeletal MHC (Karsch-Mizrachi et al., 1990); rat embryonic MHC (Strehler et al., 1986); rat beta cardiac MHC (Kraft et al., 1989), and rat alpha cardiac MHC (McNally et al., 1989*a*). Dots are shown below the hydrophobic residues.

posed to at the head–rod junction. It may be that MLCs, while interacting strongly with one portion of the MHC, span a large region of the MHC.

Of the 16 amino acids identified by the deletion analysis to be necessary for MLC1 binding, 6 are hydrophobic and 8 are charged. Since half of the residues are charged, it is possible that the interaction between MHC and MLC1 is ionic, as has been suggested by the work of Zaager and Burke (1988*a*,*b*). The sequence of this region is highly conserved among vertebrate striated muscle MHC's where 8 of the 16 amino acids are identical. However, only one residue is absolutely conserved among all sequenced MHC's. This comparison included MHC's from evolutionarily distant organisms, such as the lower eukaryotes, *Acanthamoeba* and *Dictyostelium*. The *Acanthamoeba* MHC I is thought to be associated with only one MLC (for review see Korn and Hammer, 1988). The different light chain binding properties of the MHC I class of molecules may be reflected in the divergence of their sequences in the MLC1 binding region.

All three myosin subunits are encoded by multiple genes in vertebrates. Multiple isoforms of MHC and MLC may be present in a single cell (for a review see Bandman, 1985). The work presented here suggests that the assembly of these proteins may be dictated only by their primary sequence. To understand further the assembly of myosin, experiments are in progress to determine the binding site on the myosin molecule for MLC2 by constructing bacterial strains coexpressing all three myosin subunits.

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