Selective Requirement of Myosin Light Chain 2v in Embryonic Heart Function*

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Ju Chen‡, Steven W. Kubalak§, Susumu Minamisawa‡, Robert L. Price§, K. David Becker‡,
Reed Hickey‡, John Ross, Jr.‡‡, and Kenneth R. Chien‡***‡‡

From the ‡Department of Medicine and **Center for Molecular Genetics, University of California at San Diego, School of Medicine, La Jolla, California 92093-0613, and ¶Department of Cell Biology & Anatomy, Medical University of South Carolina, Charleston, South Carolina 29425-2204, and ‡Department of Developmental Biology and Anatomy, School of Medicine, University of South Carolina, Columbia, South Carolina 29208

Muscle myosin, the highly conserved molecular motor, contains one pair of heavy chains and two pairs of light chains, the essential myosin light chains (MLC-1 or -3)1 and the regulatory myosin light chain-2 (MLC-2) (1). The three-dimensional structure of myosin indicates that the light chains are arranged in tandem, with MLC-1/-3 in the amino-terminal half of the neck and MLC-2 in the neck/tail junction (2–4). MLC-2 plays an essential role in regulating vertebrate smooth muscle contraction. The phosphorylation of a single serine residue (Ser-19) of smooth muscle MLC-2 is the switch for turning on the actin-activated myosin ATPase and hence, contraction. However, the acto-myosin interaction in vertebrate striated muscle is mainly regulated through the troponin-tropomyosin complex, and MLC-2 is thought to have only a modulatory effect (5). It has been shown in an in vitro motility assay that removal of MLC-2 from myosin markedly reduces actin filament sliding velocity without significantly reducing myosin ATPase activity (6, 7). Although mutation of the single MLC-2 gene results in a flightless phenotype in Drosophila (8), the precise in vivo physiological function of myosin light chains in vertebrate striated muscle is unclear. Furthermore, there is no direct evidence that the highly conserved structure of individual MLC-2 isoforms reflects a unique functional requirement in distinct muscle cell types. Recently, alterations in MLC-2 expression have been correlated with the onset of cardiac morphogenetic defects during embryogenesis (9–11), point mutations in MLC-2v have been shown to be associated with a genetic form of human cardiomyopathy (12), and induction of the atrial MLC-2 isoform has been shown to occur in cardiac hypertrophy and failure (13). Our efforts have been directed toward understanding the biological and physiological roles of the MLC-2v, which is the ventricular isoform of MLC-2, by disrupting the gene through homologous recombination in mice.

MATERIALS AND METHODS

Gene Targeting—A 12-kilobase genomic mlc-2v fragment was isolated from a mouse 129svj genomic DNA library (Stratagene, La Jolla). PCR-based mutagenesis was used to convert the 1.4-kilobase pair fragment between three base pairs 5’ of translational start codon ATG and intron 2 BamHI site into an Xhol site (see Fig. 1a). The deleted 1.4-kilobase fragment contained part of exon 1, intron 1, exon 2, and part of intron 2. Cre recombinase cDNA (15), internal ribosomal entrance sequence (16), green fluorescent protein cDNA (17), and pGKneo-tk cassettes were inserted into the Xhol site. The linearized construct was electroporated into J1 ES cells. G418-resistant ES clones were screened for homologous recombination by Southern blotting with probe A, B, C. Chimeric males were tested for germ-line transmission of the agouti coat phenotype of 129-derived ES cells by crossing with Black Swiss female breeders. For PCR analysis, oligonucleotides for Cre cDNA (Cre1, 5’-GGCTGCCAGAACCCATGAGGACA-3’; Cre2, 5’-CTAGAGCATGC-ATTTGCAAGTTC-3’) and the mlc-2v gene (MLC2v-P1, 5’-GGCA-ACTGGCCTCAGACACCAT-3’; MLC2v-P4, 5’-TGTGGAGCCTCTGG-3’) were used. Total protein and myofibrillar protein extracts were prepared (18) from single day 12.0 embryonic heart ventricles, of which 25% were subjected to Western blotting analysis with MLC-2 and MLC-2a polyclonal antibodies and tropomyosin monoclonal antibody.

Histological and Immunohistochemical Analysis—Histological and immunohistochemical studies were performed by a modification of previously described methods (19, 20, 21).

Transmission Electron Microscopy—Embryos were processed for transmission electron microscopy as described by Price et al. (22) and Reynolds (23).

In Vivo Videomicroscopy of Embryos—Mice pregnant at E11.5 and...
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FIG. 1. Targeting the MLC-2v gene. a, targeting strategy. A restriction map of mlc-2v genomic region of interest is shown on top, the targeting construct is shown in the center, and the mutated locus after homologous recombination is shown at the bottom. ATG is the translational start site. The arrow indicates the primers (MLC-2v-P1, MLC-2v-P4, Cre1, and Cre2) used for PCR analysis of genomic DNA. B, BamHI; E, EcoRI; H, HindIII; S, SstI; X, XbaI; *X*, methylated XbaI site in bacterial host strain. Kb, kilobases. IRES, internal ribosomal entrance sequence, GFP, green fluorescent protein. b, detection of MLC-2v wild type (+) and mutant (−) alleles by Southern blot analysis. DNAs from 11.5 days post-coitum embryos derived from an intercross between heterozygote mice were digested with XbaI and analyzed by Southern blot with probe A, c, detection of wild type (+) and mutant (−) alleles by PCR analysis. DNAs used for Southern blot analysis were subjected for PCR with oligonucleotides for the mlc-2v gene (MLC2v-P1, 5′-GGCAACTGGCCTCAGACACCAT-3′; MLC2v-P4, 5′-TGTGGAGCCTCTGGATCAGGAC-3′) and the Cre cDNA (Cre1, 5′-GTTTCGAAGAACCCTGATGAGCA-3′; Cre2, 5′-CTAGAGCCTGTATTTGCACATGC-3′), d, detection of MLC-2v protein by Western blot analysis. Myofilament protein prepared from day 12 embryonic heart ventricles were analyzed with anti-MLC-2v polyclonal antibody and anti-tropomyosin C monoclonal antibody. Genotype designations are +/+ wild type; +/− heterozygous; −/− homozygous.

RESULTS AND DISCUSSIONS

Lack of MLC-2v Results in Embryonic Lethality at E12.5—The mlc-2v gene targeting construct is depicted in Fig. 1. The linearized targeting construct was electroporated into J1 ES cells, and the cells were selected with G418. Of a total of 47 colonies screened, 3 were identified as homologous recombinants by Southern blot hybridization analysis of genomic ES cell DNA. Two of the three colonies were injected into C57BL/B6 blastocysts, both resulting in the generation of chimeras, one of which displayed germ-line transmission. Heterozygous (MLC-2v+/−) offspring appeared normal in all respects and were crossed to generate MLC-2v homozygote (MLC-2v−/−) mice. Southern blot and PCR analyses were used to genotype offspring (Fig. 1, b and c). The absence of MLC-2v proteins in MLC-2v−/− embryos was confirmed by Western blot analysis, utilizing a polyclonal anti-MLC-2v antibody (Fig. 1d). No viable MLC-2v−/− offspring were obtained in litters from MLC-2v+/− intercrosses (Table I), indicating that the MLC-2v−/− null mutation was embryonic lethal. To investigate the timing of the lethality, mice from E9.5 to 13.5 were genotyped. All of the MLC-2v−/− embryos died at approximately E12.5, and all MLC-2v−/− died by E13.5 (Table I), thereby indicating that the MLC-2v−/− embryos die at approximately
E12.5. **MLC-2v−/− Embryos Die from Heart Failure**—All MLC-2v−/− embryos that died just before dissection displayed massive cardiac enlargement, wall thinning, chamber dilation, and pleural effusions. Histologic examination revealed hepatic congestion and an engorged vena cava consistent with congestive heart failure (data not shown). No significant abnormalities in other organs were found. Analysis of global cardiac function revealed that the left ventricular ejection fraction of MLC-2v−/− embryos averaged 33%, which was significantly reduced when compared with wild type and heterozygous littermates at E11.5 (46%, 49%) and at E12.5 (50%, 53%) (Fig. 2a).

Left ventricular end diastolic volumes were similar among the three groups at E11.5, but by E12.5, they were significantly elevated in MLC-2v−/− embryos (Fig. 2b). Collectively, these results suggest that global cardiac function is already severely impaired by E11.5 in MLC-2v−/− embryos and that cardiac dysfunction results in progressive embryonic heart failure around E12.5. The resulting phenotype is similar to mid-gestational embryonic heart failure seen in a wide variety of other gene-targeted murine embryos (14).

**MLC-2v−/− Embryonic Hearts Display Abnormal Myofibrillar Organization**—Ultrastructural analysis of comparable areas from the left ventricular free walls of wild type and mutant mice revealed abnormalities in sarcomeric assembly in the MLC-2v−/− embryos (Fig. 3). Myocytes from E12.5 MLC-2v−/− embryonic hearts displayed prominent interruptions and myofibrillar disorganization of the normal parallel alignment of thick and thin filaments (Fig. 3, a and b). Additionally, total fiber width was narrower within mutant ventricular myocytes, and overall distances between Z-bands was greater than in wild type specimens. There were also several areas in mutant hearts where the organization and alignment of Z-bands between sarcomeres was not conserved (Fig. 3c). These observations could also be observed in ventricular samples from younger MLC-2v−/− embryos (E10.5 and E11.5) during earlier stages of myofibrillogenesis (data not shown).

Taken together, these data indicate that there is a selective requirement for MLC-2v in the normal development of ventricular cardiac myocyte structure and function. The observed disruption of myofibrillar organization in the absence of MLC-2v leads to reduced myocyte contractility and cardiac function that results in death at approximately E12.5 and suggests a specific requirement of MLC-2v in the interaction between thick and thin filaments during cardiac sarcomere assembly.

In this regard, **in vitro** motility assays have previously shown that removal of MLC-2 reduces actin filament sliding velocity by about 63% without significantly reducing the myosin ATPase activity (6, 7). It has also been proposed that MLC-2 can act to stiffen the myosin neck (3, 4). If each head of the thick filament is responsible for the arrangement of proper hexagonal packing of the thick filament and thin filament during myogenesis, then the disorganized and improperly functioning myosin head due to the lack of MLC-2v would result in improper packing and organization of thick and thin filaments during cardiac sarcomere assembly.

**MLC-2a Cannot Compensate for the Deficiency of MLC-2v**—Two different myosin light chain 2 genes, *mlc-2v* and *mlc-2a*, are abundantly expressed in the ventricular chamber at early stages of murine cardiogenesis, raising the issue as to whether there is a unique role for MLC-2v versus MLC-2a during the maintenance of heart function and morphogenesis. *mlc-2v* is initially expressed at about E8 and continues to be restricted to the ventricular chamber throughout embryonic development and into adulthood (27, 28). The expression of *mlc-2a* is uniform in the E8 linear heart tube and then becomes down-regulated at the RNA level in the ventricular chamber by E12.5 (29). To investigate whether MLC-2a can compensate for
MLC-2v in mutant embryos, we examined MLC-2a mRNA and protein levels in MLC-2v−/− embryos at E12. Although there were no significant differences in the MLC-2a mRNA levels between wild type and MLC-2v−/− embryos (Fig. 4a), Western blot analysis (Fig. 4b) demonstrated that MLC-2a protein content in the ventricular chamber was dramatically increased in both total cellular protein and myofibril protein extracts of these embryos. A quantitative study of myofilament proteins isolated from E12 embryonic ventricles indicated that levels of MLC-2a protein in MLC-2v−/− ventricles were comparable to the MLC-2v protein levels in wild type litter mates (Fig. 4c). This result is consistent with the findings in a transgenic mouse model in which overexpression of cardiac MLC-2v does not result in an increase in ventricular MLC-2v protein levels despite a significantly higher level of MLC-2v mRNA in these transgenic mice (30). In both cases, the MLC-2 protein level appears to be regulated at the post-transcriptional level. Thus, the current study indicates that during early stages of cardiac chamber development, MLC-2a protein levels increase in the ventricles of mutant animals, implying that MLC-2a may par-

**FIG. 4.** MLC-2v and MLC-2a mRNA and protein analysis in wild type and MLC-2v−/− embryos. a, RNase protection assay on isolated ventricles of E12 embryos using riboprobes for MLC-2v, MLC-2a, and control probe EF-1a. b, Western blot analysis of total proteins (lanes 1–4) and purified myofilament proteins (lanes 5–8) from E12.0 embryonic ventricles using a polyclonal antibody for MLC-2a and a monoclonal antibody for tropomyosin. c, myofilamental proteins from E12 embryonic ventricle were analyzed by SDS-glycerol polyacrylamide gel electrophoresis and stained with Coomassie Blue. d–i, immunohistochemical analysis of MLC-2v (panels d and f) and MLC-2a (panels e, g, h, and i) in wild type (panels d, e, and h) and MLC-2v−/− embryos (panels f, g, and i) by confocal microscopy. Panels h and i were taken from the left ventricular free wall rough trabecular area, similar to that shown in Fig. 3.
tially replace MLC-2v in the developing ventricular myocyte. In this regard, immunohistochemical analysis by confocal microscopy of MLC-2v expression in MLC-2v −/− embryos demonstrated that MLC-2a protein was indeed elevated in the ventricles of mutant embryos (Fig. 4g) and incorporated into nascent myofibrils (Fig. 4i). However, MLC-2a cannot completely compensate for the deficiency of MLC-2v, as shown by the lack of sarcomeric structure (Fig. 3) and depressed cardiac contractility (Fig. 2). We conclude that there is an important qualitative difference between MLC-2v and MLC-2a proteins, reflecting a unique requirement for MLC-2v during functional maturation of the ventricular chamber, thereby underscoring the potential importance of MLC-2 in the in vivo regulation of cardiac contractility. Furthermore, these studies provide the first direct evidence that alterations in MLC-2v can directly lead to overt heart failure, which will become of interest to explore in the post-natal setting of the adult heart.

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