Cardiac myosin heavy chains lacking the light chain binding domain cause hypertrophic cardiomyopathy in mice

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Welikson, Robert E., Scott H. Buck, Jitandrakumar R. Patel, Richard L. Moss, Karen L. Vikstrom, Stephen M. Factor, Setsuya Miyata, Howard D. Weinberger, and Leslie A. Leinwand. Cardiac myosin heavy chains lacking the light chain binding domain cause hypertrophic cardiomyopathy in mice. Am. J. Physiol. 276 (Heart Circ. Physiol. 43): H2148–H2158, 1999.—Myosin is a chemomechanical motor that converts chemical energy into the mechanical work of muscle contraction. More than 40 missense mutations in the cardiac myosin heavy chain (MHC) gene and several mutations in the two myosin light chains cause a dominantly inherited heart disease called familial hypertrophic cardiomyopathy. Very little is known about the biochemical defects in these alleles and how the mutations lead to disease. Because removal of the light chain binding domain in the lever arm of MHC should alter myosin’s force transmission but not its catalytic function, we tested the hypothesis that such a mutant MHC would act as a dominant mutation in cardiac muscle. Hearts from transgenic mice expressing this mutant myosin are asymmetrically hypertrophied, with increases in mass primarily restricted to the cardiac anterior wall. Histological examination demonstrates marked cellular hypertrophy, myocyte disorganization, small vessel coronary disease, and severe valvular pathology that included thickening and plaque formation. Skinned myocytes and multicellular preparations from transgenic hearts exhibited decreased Ca2+ sensitivity of tension and decreased relaxation rates after flash photolysis of diazo 2. These experiments demonstrate that alterations in myosin force transmission are sufficient to trigger the development of hypertrophic cardiomyopathy.

Transgenic mice; valve disease; myosin heavy chain

MYOSIN IS A CHEMOMECHANICAL MOLECULE that catalyzes the hydrolysis of ATP. In the presence of actin, myosin converts chemical energy into mechanical force. Conventional myosin, or myosin II, is a hexameric molecule consisting of two heavy chains (MHCs) and two pairs of nonidentical light chains (MLCs). The MHC consists of a helically coiled tail and a globular head or motor region (34). The tail has been shown to be important for filament formation (25, 48), whereas the head contains the catalytic active site and actin and light chain binding domains. The MLCs wrap around a long helix of the MHC head, presumably stabilizing its structure. The regulatory MLC resides at the distal end of the helix, and the essential MLC abuts the regulatory MLC on the proximal end. It has been suggested that the light chain binding domain behaves as a lever arm, amplifying small movements in the myosin head (34, 43). Removal of MLCs from skeletal MHC leads to decreased velocity in an in vitro motility assay without significant decreases in ATPase activity (23). Dictyostelium MHC from which the light chain binding domains were deleted moves more slowly than intact myosin in an in vitro motility assay (43, 44), whereas an insertion mutant with an additional essential MLC binding domain moves more rapidly (43). To determine the relationship between the lever arm and the catalytic properties of the motor, the light chain binding domain of the Dictyostelium myosin head was replaced by structurally similar repeats of the α-actinin molecule. This chimera behaved similarly to wild-type myosin in ATP hydrolysis and movement, suggesting that the length of the lever arm is directly responsible for changes in motility, rather than having an indirect effect on catalysis (2).

In heart and skeletal muscle, myosin is the most abundant protein and plays a major role in contraction (9, 29). Mutations of MHC and MLC have been linked to familial hypertrophic cardiomyopathy (FHC) (13, 32). FHC is an autosomal dominant disease characterized by left ventricular hypertrophy, myofibril and myocyte disarray, and sudden death. Thus far, all of the genes that have been linked to FHC encode structural proteins of the sarcomere (6, 13, 32, 41). More than 40 different mutations in MHC have been described (see Ref. 46). Whereas the majority of the described mutations are found in regions of the myosin head required for enzymatic activity, five mutant alleles map to the lever arm of the MHC (33). Although the biochemical defects in most FHC alleles are not known, analysis of FHC MHC alleles demonstrated diminished actin-activated ATPase activity and in vitro motility in two FHC mutations, but a third mutant allele examined had normal activities (36). The contractile properties of soleus muscle fibers from patients with three different MHC mutations were determined and found to have three different phenotypes (22). Fibers from a patient with the Arg403Gln mutation (which is known to be defective in actin-activated ATPase) had reduced power...
output and velocity of shortening, whereas those with the Gly$^{256}$Glu mutation were indistinguishable from the wild type. A third mutation (Gly$^{741}$Arg) displayed diminished power output, decreased velocity of shortening, and decreased isometric force generation (22). To date, no mutations implicated directly in light chain binding have been described in FHC patients. However, two FHC mutations have been described that occur in the light chain binding domain (S782N and A797T) (28, 33).

The effects of most FHC alleles on the chemomechanical properties of myosin are not known. We wanted to test the hypothesis that a cardiac MHC with a known functional defect would behave as a dominant negative mutation, causing hypertrophic cardiomyopathy. The mutation we chose was one that would alter force transmission but not catalytic activity. Such analysis will also demonstrate whether such a mouse resembles the other previously described mouse models for FHC.

Mice expressing different MHC mutations demonstrate many features of the human disease, but they each exhibit distinct phenotypes (12, 45). For example, mice with theArg$^{626}$Gln mutation exhibit severe atrial hypertrophy but no ventricular hypertrophy (12), whereas mice with the actin binding domain deletion exhibit significant ventricular hypertrophy and no atrial hypertrophy (45). These previous results suggest that mutations in the MHC will result in cardiomyopathic phenotypes in transgenic mice but that these may be distinct from the human disease and from each other. We produced transgenic mice expressing MHC with a deletion in the light chain binding domain (ΔLCBD).

METHODS

Construction of ΔLCBD MHC Transgenic Construct

Nucleotides 2,381 to 2,563 were deleted from published rat α-cardiac MHC (27) by PCR. A 615-bp fragment was generated using the sense primer 5'-TCGGATCCCCACTATGCTG-3' containing BstX I and BamH I sites and the antisense primer 5'-CTTCTCTTGCTGCGCTCTTCTCATCTGGATCTGC-3', which spans the deletion. The resulting PCR product was used as a sense megaprimer with the antisense primer 5'-CTGAATCTGTAGCATTGCGCTGCAGCG-3' containing BstX I and EcoR I sites. The 824-nucleotide (nt) product was digested with BamH I and EcoR I, ligated into pTZ19 (USB), and sequenced to verify the deletion. A 797-bp BstX I/Bcl I fragment was then ligated into a BstX I/Bcl I digested pMTmyc (a COOH-terminal myc-tagged MHC) fragment to generate pMTΔLCBDmyc. The mutant myosin was then cloned into a transgenic construct driven by 3.3 kb of rat α-cardiac MHC promoter (15) followed by the mouse β-globin terminator (40). Included in the upstream sequence were the first two exons and the first intron of the rat α-cardiac MHC gene. The resulting construct was designated ΔLCBDmyc.

Production of Transgenic Mice

ΔLCBDmyc was digested with Xho I and Sac I to liberate the transgene from prokaryotic vector sequences. The DNA was separated on a 0.7% agarose gel, and the linear transgene DNA fragment was purified and then injected into the pronuclei of fertilized mouse eggs derived from an F1 cross between FVB/N and C57/B16 strains (17). Founder animals were identified by Southern blot analysis and then backcrossed to C57/B16 mice.

Protein and Myofibril Purification

Whole heart lysate was prepared by homogenizing tissue in 10 volumes of buffer A [50 mM KCl, 10 mM HEPES, 2 mM MgCl$_2$, 0.5 mM EDTA, 2 mM EGTA, 2 mM dithiothreitol (DTT), and 0.1 mM phenylmethylsulfonyl fluoride]. Crude myofibrils were prepared by pelleting the lysate at 10,000 g for 15 min at 4°C. Myofibrils from this pellet were purified using the Triton X-100 method (37).

SDS-PAGE and Western Blotting

Heart lysates and crude or purified myofibrils were prepared in Laemmli sample loading buffer (21). Gel samples were briefly sonicated and then boiled for 3 min before separation at 10 µg on a 10% SDS-PAGE gel followed by electrophoretic transfer to nitrocellulose membrane (42). The membranes were blocked in 5% nonfat dry milk in phosphate-buffered saline (PBS) for 2 h at room temperature and incubated overnight at 4°C with a monoclonal antibody against the c-myc epitope (9E10) (5) followed by a horseradish peroxidase-conjugated secondary antibody (Jackson ImmunoResearch, West Grove, PA). Bands corresponding to myc-tagged mutant myosin were visualized by chemiluminescence as previously described (47). To quantitate the amount of transgene protein, myc immunoreactivity of the tagged MHC was compared with a standard curve of myc-tagged purified desmoglein (provided by K. Green, Northwestern University Medical School, Chicago, IL). The amount of total myosin protein was determined by densitometric scanning of Coomassie blue-stained gels and was used to calculate the relative percentages of mutant and wild-type myosin.

Body, Heart, and Tibia Measurements

Mice were killed by cervical dislocation and weighed. Hearts were excised, washed in PBS, blotted dry, and weighed. The atria and left and right ventricles were dissected free of each other and weighed separately. The left hindlimbs of the mice were severed, and the skin and muscle were removed. The remaining muscle tissue was removed by overnight incubation (37°C) in 2% KOH. Tibias were rinsed with distilled water, air dried, and then measured.

Histology

Hearts were fixed in 4% paraformaldehyde in PBS overnight at 4°C, embedded in paraffin, and then sectioned on a microtome by using standard techniques. Sections were...
stained with either hematoxylin and eosin or Masson trichrome.

Echocardiography

Six-month-old female mice were sedated with an intraperitoneal injection of avertin (30–40 mg/100 g body wt), and their chests were shaved. Electrocardiogram leads were fastened to the right and left forelimb, and the ground was fastened to the tail. The mice were placed chest down on a Cincinnati Standoff acoustic gel pad. Images were obtained with a Vingmed CM F800 (Horten, Norway) echocardiography machine using a pediatric 7.5-MHz wide-band annular array transducer. With the use of two-dimensional guided M-mode echocardiography, left ventricular dimensions and wall thickness were measured in systole and diastole, and fractional shortening was calculated. Fractional shortening was calculated as [(LVD − LVS)/LVD] × 100, where LVD and LVS are diastolic and systolic measurements of left ventricular wall thicknesses and dimensions.

Statistical Analysis

Continuous data are expressed as means ± SE. The significance of differences between means was evaluated with unpaired t-tests. Values of P < 0.05 were considered statistically significant.

Skinned Myocardial Preparations

Hearts of adult (12 mo old) mice of either sex were rapidly excised and placed in ice-cold relaxing solution containing (in mM) 100 NaCl, 10 imidazole, 5 MgCl₂, 2 EGTA, and 4 ATP; pH 7.0, in which the atria were removed and the ventricular tissue was minced into five to six pieces. The ventricular pieces were then homogenized in ice-cold relaxing solution for 8 s with a Polytron homogenizer (Kinematica) to yield single myocytes and myocyte-sized fragments (100 × 20 µm). The cellular homogenate was centrifuged at 120 g for 1 min, and the resultant pellet was washed twice with relaxing solution and then resuspended in relaxing solution containing 0.3% Triton X-100 to permeabilize sarcolemmal and intracellular membranes. After 6 min, the skinned myocytes were washed twice with fresh relaxing solution and stored at 4°C until used within 8 h to determine the Ca²⁺ sensitivity of tension. Multicellular skinned myocardial preparations (600–900 µm long × 100–260 µm wide) for determining the kinetics of contraction and relaxation were prepared as for skinned myocytes, except that 1) hearts were excised in room temperature Ringer solution containing (in mM) 118 NaCl, 4.8 KCl, 2 NaH₂PO₄, 1.2 MgCl₂, 25 HEPES, 5 pyruvic acid, and 11 glucose, pH 7.4, and ventricular tissue was then rapidly frozen in liquid nitrogen; 2) the frozen pieces of ventricles were thawed and homogenized for ~4 s with the Polytron homogenizer; and 3) the washed homogenate was suspended for 30 min in relaxing solution containing 150 µM saponin and 1% Triton X-100 to permeabilize sarcolemmal and intracellular membranes. This minor modification of published methods (11) resulted in preparations that were homogenous and mechanically robust on the basis of uniform diameter, absence of branching, and uniformity of sarcomere pattern while relaxed and activated. The rundown of maximum force-generating capability was <15%, and systematic effects of rundown on results were minimized by randomization of intensities of ultraviolet (UV) flash in DM-nitrophen experiments. Minimal compliance of attachments was evidenced by maintenance of uniform striation pattern of the preparation while relaxed and activated.

Experimental Protocols

Determination of Ca²⁺ sensitivity of tension. Ca²⁺ sensitivity of tension of skinned single myocytes was determined as described previously (36). On the stage of an inverted microscope (Olympus), single skinned myocytes were attached with silicone adhesive (Dow Corning) to stainless steel pins (10-µm outer diameter) that were attached to the active element of the force transducer (model 403, Cambridge Technology) and a motor (model 6350, Cambridge Technology). After the silicone attachment (~40 min) cured, the myocytes were transferred to a PCa 9.0 solution and sarcomere length was adjusted to ~2.3 µm using on-line video microscopy.

Developed isometric force was measured in activating solution at 15°C containing a range of free Ca²⁺ concentration ([Ca²⁺]free) (38). Isometric forces measured at submaximal PCa (i.e., −log [Ca²⁺]) were expressed as a fraction of maximal force measured at PCa 4.5 (i.e., Prel = P/Po, where Prel is relative force, P is steady-state force, and Po is maximal force) and were plotted versus PCa. The data were analyzed by least-squares regression using the Hill equation: log (Prel/1 − Prel) = −log k + log (1/[Ca²⁺]°), where k is the Hill coefficient and k is the intercept (in pCa units) of the fitted line with the x-axis. Lines were fit to the tension-pCa curves by using constants derived from this analysis from the following equation: P = (1/[Ca²⁺]°)/(kH/([Ca²⁺]° + kH)), where kH denotes the [Ca²⁺] at which relative tension is half-maximal.

Determination of contraction and relaxation rates. Multicellular skinned ventricular preparations were transferred to relaxing solution in an experimental chamber as described (31). The ends of the preparation were attached to the force transducer and the arms of the motor. The experimental setup was mounted on the stage of an inverted microscope (Olympus) fitted with a ×40 objective and a closed-circuit television camera (model WV-BL 600, Panasonic). Light from a halogen lamp passing through a cutoff filter (transmission >620 nm) was used to illuminate the multicellular ventricular preparation. In this way it was possible to record and store video images of the preparation before, during, and after photolysis of the caged Ca²⁺ chelator (31). These video images were used to assess mean sarcomere length (SL) during the course of the experiment. Force changes were recorded on a chart recorder (Allen Datagraph; slow time base) and an oscilloscope (Nicolet 310; fast time base).

Once the temperature was stabilized at 15°C, preparations were stretched to a mean SL of ~2.35 µm. To determine maximum force (Pmax), the preparation was transferred from relaxing solution to preactivating solution for 2 min and was then transferred to maximal activating solution. Once force reached steady state, the preparation was rapidly slackened and returned to relaxing solution. To determine the rate of tension development, preparations were transferred to a loading solution containing 1 mM DM-nitrophen and 0.2 mM CaCl₂ for 5 min of equilibration. Preparations were then transferred to an 80-µl quartz-walled photolysis chamber filled with silicone oil (Dow Corning 200, viscosity = 10 cs), where they were exposed to a flash of UV light. Low, intermediate and high levels of postflash active force were achieved in random order by photolyzing DM-nitrophen with three different intensities of UV light flashes. After the postflash force (P) was recorded, preparations were transferred back to relaxing solution. Postflash forces were expressed relative to maximum force, i.e., P/Pmax. At the end of an experiment, maximum force at PCa 4.5 was again measured and used for correction of rundown of the preparations. Apparent rate constants of force development (kₗ) were characterized by linear transformation of the half-time estimate
[\( k_f = -\ln 0.5\left( t_{1/2}\right)^{-1} \)] and are expressed per second as previously described (8, 35).

A similar protocol was used to determine relaxation rates, except that 1) the preparations were incubated for 2 min in the loading solution containing 2 mM diazo 2 and either 0.85 mM CaCl\(_2\) (transgenic myocardial preparations) or 0.80 mM CaCl\(_2\) (wild-type myocardial preparations); and 2) the relaxation from steady state was initiated by photolyzing diazo 2 with a single flash of high-intensity UV light. Rate constants of relaxation \( k_r \) were characterized by linear transformation of the half-time of force decay \( [k_f = -\ln 0.5\left( t_{1/2}\right)^{-1}] \) expressed per second.

Solutions

Chemicals were purchased from Sigma Chemical (St. Louis, MO) except for CaCl\(_2\) (Orion Research, Beverly, MA), propionic acid (Fluka, Milwaukee, WI), DM-nitrophen and creatine kinase (Calbiochem, La Jolla, CA), and diazo 2 (Molecular Probes, Eugene, OR). Solution compositions were calculated using the computer program of Fabiato (10) and the stability constants (corrected to pH 7.0 and 15°C) listed by Godt and Lindley (14). The apparent stability constants \( K_{Ca} \) and \( K_{Mg} \) used for nonphotolyzed DM-nitrophen were \( 2.0 \times 10^9 \text{ M}^{-1} \) and \( 4.0 \times 10^8 \text{ M}^{-1} \), respectively (18), and those for nonphotolyzed diazo 2 were \( 4.55 \times 10^9 \text{ M}^{-1} \) and \( 1.82 \times 10^9 \text{ M}^{-1} \), respectively (1). The pCa 9.0 solution contained 20 mM imidazole, 7 mM EGTA, 14.5 mM creatine phosphate, 0.02 mM CaCl\(_2\), 5.42 mM MgCl\(_2\), and 4.74 mM ATP. The pCa 4.5 solution contained 20 mM imidazole, 7 mM EGTA, 14.5 mM creatine phosphate, 7.0 mM CaCl\(_2\), 5.26 mM MgCl\(_2\), and 4.81 mM ATP. The ionic strength of both solutions was adjusted to 180 mM with KCl. A range of solutions containing different [Ca\(^{2+}\)]_free (i.e., pCa 6.4–5.5) for determining Ca\(^{2+}\) sensitivity of tension was prepared by mixing solutions of pCa 9.0 and pCa 4.5. Relaxing and maximal activating solutions used in flash-photolysis experiments were similar to these solutions, except that both contained 100 mM N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid instead of imidazole, both contained 5 mM DTT, and ionic strength was adjusted to 180 mM with potassium propionate. The solution used to determine the rate of force development contained 1 mM DM-nitrophen, 0.20 mM CaCl\(_2\), 5.94 mM MgCl\(_2\), 4.75 mM ATP, 15 mM creatine phosphate, and 100 U/ml creatine kinase. The solution used to determine the rate of relaxation contained 2 mM diazo 2, 0.80 or 0.85 mM CaCl\(_2\) (experiments with wild-type or transgenic mice, respectively), 5.37 mM MgCl\(_2\), 4.79 mM ATP, 15 mM creatine phosphate, and 100 U/ml creatine kinase.

RESULTS

Generation of \( \triangle \)LCBD MHC Transgenic Mice

Transgenic mice were created that express a rat cardiac \( \alpha \)-MHC lacking the light chain binding domain (residues 782–846 in rat cardiac \( \alpha \)-MHC) (Fig. 1) (26, 32). Expression was directed by 2.9 kb of 5'-flanking DNA from the rat cardiac \( \alpha \)-MHC gene (15) that, as we have previously shown (45), directs cardiac-specific expression. To distinguish the transgene from the wild-type or transgenic hearts. Western blots of heart protein samples (10 µg each) were reacted with the myc antibody 9E10. Protein (10 µg) was loaded in each lane as follows: lane 1, nontransgenic whole heart lysate; lane 2, homogenously transgenic whole heart lysate; lane 3, insoluble fraction resulting from homogenization in a low-salt buffer followed by a low-speed spin; and lane 4, soluble fraction from same preparation in lane 3. All of myc-tagged \( \triangle \)LCDB MHC pelleted with the insoluble myofibril fraction in lane 3.

Fig. 2. Transgene protein expression. Whole heart homogenates from nontransgenic animals (lane 1: 10 µg total protein), heterozygously transgenic animals (lane 2: 5 µg total protein), and homozygously transgenic animals (lane 3: 5 µg total protein) were separated on SDS-PAGE and either stained with Coomassie blue (A) or transferred to nitrocellulose for Western blot analysis with the anti-myc antibody (B). C: Western blot analysis of total heart lysates and crude myofibrils from transgenic hearts. Western blots of heart protein samples (10 µg each) were reacted with the myc antibody 9E10. Protein (10 µg) was loaded in each lane as follows: lane 1, nontransgenic whole heart lysate; lane 2, homogenously transgenic whole heart lysate; lane 3, insoluble fraction resulting from homogenization in a low-salt buffer followed by a low-speed spin; and lane 4, soluble fraction from same preparation in lane 3. All of myc-tagged \( \triangle \)LCDB MHC pelleted with the insoluble myofibril fraction in lane 3.
endogenous MHC, the last 33 nt of the rat α-MHC coding region were replaced by a sequence specifying the epitope recognized by the anti-human c-myc antibody 9E10 (5). Transgenic mice in which nearly 40% of the cardiac myosin was an myc-tagged wild-type α-MHC transgene appear completely normal and exhibit no cardiac pathology (see Fig. 6). After founders of ΔLCBD mice were identified by Southern analysis and their progeny were tested for protein expression, one line was obtained and backcrossed to C57/Bl6 for several generations. A heterozygous line was maintained, and a homozygous line was derived. Expression of ΔLCBD MHC was detected by probing a Western blot of total heart lysate with the human c-myc-specific antibody 9E10 (5). Figure 2A shows a Coomassie-stained gel of the heart lysate. A band corresponding to the size of MHC (200 kDa) was detected by the myc antibody only in the transgenic heart lysate samples and not in the wild-type sample (Fig. 2B). ΔLCBD MHC protein constituted 4 and 7% of the total MHC in heterozygotes and homozygotes, respectively, as determined by comparison with an myc-tagged standard and a myosin standard (see METHODS; data not shown). To test whether this mutant myosin could incorporate into sarcomeres, the ability of ΔLCBD MHC to copurify with the myofibril fraction was assessed. Hearts were homogenized in 50 mM KCl and centrifuged at 10,000 g to pellet myofibrils. ΔLCBD MHC copurified with the myofibril fraction, indicating that the transgene protein incorporated into myofibrils (Fig. 2C).

Purification of myofibrils also allows a precise examination of the relative amounts of various cardiac sarcomeric proteins by SDS-PAGE. To determine whether expression of this mutant transgene protein induced any alteration in isoform content or stoichiometry, myofibrils were purified from wild-type and homozygous transgenic mouse hearts and examined by SDS-PAGE (Fig. 3). When Coomassie-stained gels were examined by laser densitometry, the ratio of actin to MHC remained unchanged in the transgenic mouse hearts. However, because the transgene did not have the light chain binding domain of MHC, there was a significant (P < 0.05) reduction in the ratios of MLC 1 and MLC 2 to MHC in homozygous transgenic mouse hearts (data not shown).

**ΔLCBD MHC Mice Have Cardiac Hypertrophy**

Pathological states often lead to cardiac hypertrophy as a secondary response (24). FHC patients characteristically have cardiac hypertrophy of varying degrees, although the anatomic location of the hypertrophy varies. ΔLCBD mice were examined for increases in heart mass (Fig. 4). Because FHC is a progressive disease, measurements were taken from young (2 mo) and old (10 mo) mice (Fig. 4). The heart mass of 2-mo-old transgenic mice was equivalent to that of wild-type mice. However, at 10 mo of age, the heart mass of transgenic mice was 40% greater than that of wild-type animals (Fig. 4). This demonstrates that ΔLCBD MHC mice develop and maintain cardiac hypertrophy. This hypertrophy was restricted to the left ventricle and was not seen in the right ventricle or atria (data not shown).
ΔLCBD MHC Results in Anterior Wall Hypertrophy

The majority of patients with FHC exhibit hypertrophy of the septum and of the anterior wall, with the posterior wall being less affected (24). Two-dimensional guided M-mode echocardiograms of 6-mo-old female ΔLCBD MHC mice demonstrated asymmetric anterior wall hypertrophy, mimicking that seen in the majority of FHC patients (Fig. 5, A and B). The degree of hypertrophy was greater in the homozygous animals, suggesting that the hypertrophy was proportional to the amount of mutant protein. Systolic anterior wall thickness of heterozygous and homozygous ΔLCBD mice was greater than that of wild-type 6-mo-old females. In contrast, there was no hypertrophy of the posterior wall in systole or diastole in heterozygous or homozygous mice (Fig. 5, C and D). Systolic function, as assessed by left ventricular shortening fraction, did not differ between ΔLCBD MHC and wild-type mice (Fig. 5E).

Hearts of ΔLCBD MHC Mouse Hearts Develop Cellular and Valvular Pathology

The histopathological features of hypertrophic cardiomyopathy include cellular and nuclear hypertrophy, small vessel coronary disease, myocellular disarray, and interstitial fibrosis (24). Hearts from ΔLCBD transgenic mice were examined to determine whether a deletion in the mechanical domain of MHC would also lead to FHC-type cellular pathology. Figure 6A shows a section from the heart of a wild-type animal. Figure 6B shows a section of a heart from a transgenic line that expressed a wild-type, myc-tagged MHC. No histopathological changes were evident in this line of animals. The hearts from heterozygous and homozygous mice of the
ΔLCBD line exhibited cellular hypertrophy, myocyte disorganization, and small vessel pathology (Fig. 6, C and D). The histopathological changes were more severe in homozygotes than in heterozygotes (data not shown). One striking phenotype in both heterozygous and homozygous mice was a severe valve pathology. Valves were significantly thickened with fibrous plaques, and this was frequently accompanied by thrombus formation (Fig. 6E). Figure 6E demonstrates a mitral valve from a transgenic animal, and Fig. 6F shows a mitral valve from a wild-type animal. Of six transgenic animals examined, five had mitral valve pathology and one had tricuspid valve pathology. Of the abnormal mitral valves, four involve the anterior and posterior leaflets, but the anterior leaflet was more involved than the posterior leaflet in three of five animals. The pathology in the tricuspid valve showed involvement of the septal leaflet with a fibrous plaque on the right ventricular septal wall.

Myocytes From ΔLCBD Mice Have Decreased Ca^{2+} Sensitivity of Tension

Calcium sensitivities of tension in single ventricular myocyte preparations from transgenic and wild-type
mice are compared in Fig. 7. The pCa for half-maximal force (pCa50) for transgenic preparations (n = 6) was 5.63 ± 0.02, and that for wild-type preparations (n = 4) was 5.68 ± 0.02. Compared with wild-type preparations, transgenic preparations had decreased Ca2+ sensitivity of tension as evidenced by reduced developed tension at given sub maximal [Ca2+]free. Developed tension (P/Po) at pCa 5.7 of transgenic myocytes (0.37 ± 0.3) was significantly less than that of wild-type myocytes (0.47 ± 0.3; P < 0.05). This shift in the tension-pCa relationship was also evident in the flash-photolysis data (Fig. 8): developed tensions achieved in response to release of identical amounts of [Ca2+]free from DM-nitrophen were significantly less in transgenic than in wild-type preparations, as described below. The Hill coefficient was 3.11 ± 0.22 for transgenic preparations and 3.42 ± 0.22 for wild-type preparations, indicating no difference in apparent cooperativity of force development.

Myocardial Preparations From ΔLCBD Mice Have Reduced Rates of Relaxation

Rates of force development of multicellular preparations from transgenic (n = 5) and wild-type (n = 5) mice after flash photolysis of DM-nitrophen are compared in Fig. 8. Transgenic and wild-type preparations exhibited similar maximum rates of force development (k1) and similar activation-dependencies of k1. However, transgenic preparations generated less force than wild-type preparations after UV flashes that produced identical [Ca2+]free. This difference was observed at both low- (0.29 ± 0.03 vs. 0.40 ± 0.02 P < 0.05) and intermediate-intensity (Po 0.63 ± 0.02 vs. 0.74 ± 0.903 P < 0.05) UV flashes. This difference indicated that Ca2+ sensitivity of tension was reduced in the transgenic myocardium. There was also no difference in maximum tension/cross-sectional area between transgenic and wild-type ventricular myocytes.

Relaxation rates after flash photolysis of diazo 2 in multicellular preparations from transgenic (n = 4) and wild-type (n = 4) mice are compared in Fig. 9. Steady-state force before photolysis of wild-type and transgenic preparations was similar (0.457 ± 0.017 and 0.437 ± 0.027 P < 0.05, respectively). In both wild-type and transgenic preparations there was a >90% decrease in steady-state force due to rapid chelation of Ca2+ after photolysis. The relaxation rates (k1) in transgenic myocardial preparations (n = 4) were significantly slower (P < 0.05) than in wild-type preparations (n = 4) (i.e., 13.0 ± 0.5 and 14.6 ± 0.4 s⁻¹, respectively). Thus there was a significant decrease in relaxation rate in transgenic compared with wild-type preparations.

DISCUSSION

Missense mutations in the cardiac MHC have a profound effect on cardiac structure and function (for a review, see Ref. 46). More than 40 mutations in MHC that cause FHC have been identified. However, few have been studied for their impact on the functions of cardiac myosin (36, 39). Although several FHC mutations have been mapped on the three-dimensional structure of the myosin head, it is not possible to predict the functional defect of most mutations, especially those found in regions of the molecule not associated with catalytic activity. Two mutant alleles have been shown to exhibit decreased actin-activated ATPase activity in vitro (36, 39), suggesting that at least a subset of FHC alleles have functional defects in myosin’s catalytic activity. One of these mutations (Arg50Gln), has been demonstrated to function by a dominant negative mechanism (39). The multimeric...
nature of the cardiac sarcomere is likely to explain the prevalence of dominant mutations in FHC. However, depending on the severity of the mutation, the threshold and time for the onset of hypertrophic cardiomyopathy may be quite variable.

Analysis of the contractile properties of soleus fibers obtained from FHC patients with a mutation in the lever arm (Gly741Arg) of the MHC are most relevant to the mutation described here (22). Fibers from these patients are impaired in maximum velocity of shortening, isometric force generation, and power output. We predicted that deletion of the MLC binding domain of MHC would be a dominant negative mutation that would result in an FHC-like phenotype when expressed in the hearts of mice. Because the light chain binding domain is in the force-generating portion of the molecule, and distant from the filament forming portion in the rod, we expected the assembly of ΔLCBD MHC to be normal and that it would incorporate into sarcomeric structures and, therefore, would behave as a dominant mutation. The ability of this protein to assemble was assessed by a COS cell transfection assay (47), and it was shown to be indistinguishable from wild-type myosin in its assembly properties (S. Miyata, R. Thompson, R. and L. Leinwand, unpublished observations). In the context of the transgenic heart, this mutant myosin is a very strong dominant mutation, because mice develop anterior wall hypertrophy, myocyte hypertrophy, disarray, and severe valvular pathological changes when >10% of their cardiac MHC is the mutant ΔLCBD MHC. In addition, the phenotype is dose responsive, because the pathology and hypertrophy are much more severe in homozygously transgenic mice than in heterozygously transgenic mice. The phenotype seen in these mice is not due to large differences in myofibrillar protein content. Examination of the myofibrillar protein profile (Fig. 3) revealed no gross changes in the content of MHC, actin, or tropomyosin. Compensatory decreases in the MLC content of the myofibril were documented in the homozgyously transgenic mice but not in heterozygotes. The low level of transgene expression in the heterozygotes (4% of the total myosin vs. 7% in the homozygotes) most likely precluded the demonstration of a decrease in MLC content in these animals without the analysis of an extremely large number of animals.

Compared with wild-type single myocyte and multicellular preparations, transgenic myocardial preparations exhibited a decreased Ca2+ sensitivity of force. In a two-state kinetics model of cross-bridge interaction by Brenner (7), steady-state force (P) is described by

\[ P = N \times F \times \left[ \frac{f_{\text{app}}}{\left( f_{\text{app}} + g_{\text{app}} \right)} \right], \]

where \( N \) is the number of cycling cross bridges, \( F \) is the average force per cross bridge, \( f_{\text{app}} \) is the rate constant for the transition from the non-force-generating state to the force-generating state, and \( g_{\text{app}} \) is the rate for the reverse. Thus a decrease in Ca2+ sensitivity of tension as observed in the present study may be due to a reduction in the number of cycling cross bridges, the force per cross bridge, or the proportion of cross bridges in the force-generating state as a result of a decrease in \( f_{\text{app}} \), an increase in \( g_{\text{app}} \) or both.

Further analysis of the kinetics data was done in the context of this model to address these possibilities because, in the model, \( k_f = f_{\text{app}} + g_{\text{app}} \). Wild-type and transgenic multicellular preparations yielded identical relationships between \( k_f \) and steady Ca2+-activated force, i.e., \( k_f \) increased with increased activation (indicated by greater isometric force). The relationship between \( k_f \) and Ca2+-activated force is similar to that reported previously for rat myocardium using photolabile Ca2+ chelators (3) or rapid release and restretch maneuvers in steadily activated preparations (4). Thus, because the rate of force development was unchanged, it appears that the cross-bridge attachment rate (i.e.,
Thus, transgene expression decreased both Ca\(^{2+}\) relaxation rate, which is opposite to the effect observed. Expected to increase both Ca\(^{2+}\) sensitivity of tension and relaxation rate, which is opposite to the effect observed. Thus transgene expression decreased both Ca\(^{2+}\) sensitivity of tension and relaxation rate, which cannot be explained using a simple kinetics scheme. Regardless of the underlying molecular mechanism(s), such mechanical effects would tend to reduce myocardial power production (decreased Ca\(^{2+}\) sensitivity) and reduce diastolic filling (slowed relaxation) in the transgenic myocardium.

The phenotypic changes observed in the \(\Delta LCBD\) mice are seen in some proportion of FHC patients (24). One of the most striking phenotypes seen in these animals is the valve pathology. It has been reported that \(\approx 66\%\) of FHC patients exhibit abnormal valve morphologies (19, 20). However, the presence of mitral valve pathology varies even within a single family, and no genotypic information is available in the published report (19). The greater penetrance of the valve phenotype in the \(\Delta LCBD\) mice is likely to be accounted for by their inbred genetic background. The valve pathology includes inflammation, thickening, sclerosis, and plaque and thrombus formation, which are preferentially associated with the anterior leaflet of the mitral valve. In fact, thrombi have even been observed in the right ventricle. These pathological phenomena must be attributed to secondary or tertiary effects because cardiac valves do not contain myocardium and presumably do not express the transgene. The valvular damage may result from trauma of the leaflet against the adjacent endocardium and may be comparable to the damage of the mitral valve apparatus secondary to systolic anterior motion in some hypertrophic cardiomyopathy patients. This valve phenotype has not been described in the other two FHC mouse models, despite the prevalence of this phenotype in patients; therefore, these mice will be extremely useful in deciphering the pathogenesis of these various clinical phenotypes.

The basis for the difference between the phenotypes of the previously reported MHC mutant mice and those reported here is unclear but is likely tied to distinct biochemical properties of the different mutations. The biochemical defects in the Arg\(^{430}\)Gln MHC are diminished actin-activated ATPase activity and reduced in vitro motility. Skeletal muscle fibers from patients with this mutation showed decreased maximum velocity of shortening and decreased force-to-stiffness ratio (22). The mutation produced in this report is in a different region of the myosin molecule and would be expected to have a very different functional impact on force generation, presumably affecting stability and mobility of the head/neck region of myosin. In summary, a myosin heavy chain that cannot bind light chains is a dominant negative mutation in mice and provokes many aspects of the pathogenesis of the human disease.

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