Analysis of Myosin Heavy Chain Functionality in the Heart*

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Comparison of mammalian cardiac α- and β-myosin heavy chain isoforms reveals 93% identity. To date, genetic methodologies have effected only minor switches in the mammalian cardiac myosin isoforms. Using cardiac-specific transgenesis, we have now obtained major myosin isoform shifts and/or replacements. Clusters of non-identical amino acids are found in functionally important regions, i.e. the surface loops 1 and 2, suggesting that these structures may regulate isoform-specific characteristics. Loop 1 alters filament sliding velocity, whereas Loop 2 modulates actin-activated ATPase rate in Dicyostelium myosin, but this remains untested in mammalian cardiac myosins. α → β isoform switches were engineered into mouse hearts via transgenesis. To assess the structural basis of isoform diversity, chimeric myosins in which the sequences of either Loop 1 + Loop 2 or Loop 2 of α-myosin were exchanged for those of β-myosin were expressed in vitro. 2-fold differences in filament sliding velocity and ATPase activity were found between the two isoforms. Filament sliding velocity of the Loop 1 + Loop 2 chimera and the ATPase activities of both loop chimeras were not significantly different compared with α-myosin. In mouse cardiac isoforms, myosin functionality does not depend on Loop 1 or Loop 2 sequences and must lie partially in other non-homologous residues.

Myosin, the molecular motor of the heart, generates force and motion by coupling its ATPase activity to its cyclic interaction with actin. Myosin is a hexamer protein and is composed of two heavy chains (MHC) and two essential and two regulatory myosin light chains. Structurally, MHC is composed of a number of discrete domains: a helical rod necessary for thick filament formation, and a globular head that contains the actin-binding site, catalytic, and motor domains (1). In the mammalian heart, two functionally distinct MHC isoforms, termed V1 and V3, are present. V1 is a homodimer of two α-MHC molecules, whereas V3 is a β-homodimer. Expression of V1 and V3 is controlled both developmentally and hormonally. In the mouse, β-MHC expression in the ventricles predominates prenatally. However, via thyroid hormone regulation, β-MHC expression is silenced at birth, and α-MHC is transcribed (2). The functional differences between V1 and V3 myosin in terms of shortening velocity, force generation, and ATPase activity are profound. For example, rabbit V1 myosin has a 2–3-fold faster actin filament sliding velocity than V3, but generates only half the average isometric force (3, 4). Likewise, both the Ca2+-stimulated and actin-activated ATPase activities of rabbit V1 myosin are ~2–3 times greater than for V3 myosin (3, 5). Similar differences in actin velocity and myofibrillar ATPase activity have been observed between mouse V1 and V3 myosin, but there is no difference in their average force generation (6). Although the proteins are functionally distinct, the primary amino acid sequences of mouse α- and β-MHC are 93% identical. Thus cardiac isoform diversity must lie in the non-identical residues (127 of 1388 amino acids in mice). The differences in the enzyme kinetics and mechanics of the myosin interactions that are observed between the two cardiac isoforms are believed to reside in two, hypervariable “loops,” so called because their structures cannot be defined via x-ray crystallography due to their relative disorder. Loop 1 (L1), which is located between residues 213 and 223, is at the mouth of the nucleotide pocket while Loop 2 (L2), at positions 624–646, cradles the long pocket while Loop 2, at positions 624–646, cradles the long pocket. These loops are therefore thought to interact with the actin-binding site and perhaps the nucleotide. Loop 2 is also implicated in the regulation of actin-activated ATPase rate.

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† The abbreviations used are: MHC, myosin heavy chain; hGH, human growth hormone; L1, Loop 1; L2, Loop 2; NTG, non-transgenic; m.L/s, muscle lengths per second; TG, transgenic; PTU, propylthiouracil; UTR, untranslated region.
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pending on the sequence of Loop 2. However, a number of studies indicate that the loops may not influence myosin kinetics and mechanics as proposed and thus have varying roles depending on the structure of the myosin backbone. Rat and pig β-MHC, which have identical Loop 1 sequences apart from a single conservative substitution, have 3–4-fold differences in ATPase activity and ADP dissociation (12). Sweeney et al. (13) showed that the properties of Loop 1 chimeras with a smooth muscle backbone are a function of loop size/flexibility rather than related to the properties of the myosin from which Loop 1 was derived. Furthermore, chimeric myosins that consisted of a Dictyostelium MHC backbone with carp loop sequences did not exhibit changes in sliding velocity if Loop 1 was substituted, although Loop 2 substitution did lead to the expected modulation of actin-activated ATPase activity (14). Taken together, these studies indicate that the role of the surface loops for MHC functionality depends on the interplay of the surface loops with other regions important for myosin mechanics and kinetics.

In contrast to the abundance of detailed studies on in vitro function of various MHC isoforms, our current knowledge of how differences found on the single molecule level are reflected in in vivo cardiac function is limited. Cardiac isoform shifts can be achieved by endocrine intervention but hypothyroidism not only results in a nearly complete V_i → V_s shift in rodent hearts, it also induces a number of structural changes including mitochondrial swelling, as well as rupture and loss of continuity of the myofilaments (15). Thus one cannot dissect MHC isoform shift induced functional changes from contractile impairment due to structural damage. Transgenesis avoids these issues. To date, only a single transgenic study has dealt directly with cardiac myosin isoform substitution, showing that contractile function of TG mouse hearts with low-level expression of MHC was reduced by 15% (16). This disproportionate impairment of contractile function might be due to the presence of a heterologous species (rat) cDNA being placed into the mouse context, resulting in a dominant negative effect of the TG protein.

The present study is the first to investigate directly the functional significance of cardiac isoform diversity by using TG mice in which ventricular V_i is largely replaced by V_s. This approach has the advantage of effecting isoform replacement in the heart without the pleiotropic stimuli that are normally used to induce MHC isoform transitions, such as pressure-overload induced hypertrophy or changes in hormonal status (17, 18). A significant V_i → V_s shift resulted in the expected changes in the heart at the single motor and biochemical levels as well as in fiber mechanics and kinetics. However, in contrast to a hypothyroidism-effected replacement, cellular structure appeared normal and whole organ function was preserved with relatively minor effects on systolic and diastolic hemodynamics in the intact animal. Furthermore, we generated transgenic (TG) mice in which we substituted the sequences of Loop 1 and/or Loop 2 of mouse α-MHC with the respective sequences of β-MHC and assessed the mechanical and enzymatic characteristics of the chimeric MHCs. These experiments were designed to test whether a sequence substitution in the Loop 1 and/or Loop 2 region is sufficient to confer β-like activity to the α-MHC molecule. Complete replacement of the endogenous α-MHC protein with the chimeric myosin resulted in surprisingly minor differences in enzyme kinetics indicating that, for these cardiac isoforms, other variable regions or residues must play a predominant role in determining overall ATPase activity and velocity of shortening.

EXPERIMENTAL PROCEDURES

Generation of TG Animals—TG mice expressing full-length mouse β-MHC were generated. β-MHC cDNA was produced using a combina-
regulated and replaced proportionally by the TG protein. Therefore, we could achieve partial or even complete replacement of the endogenous MHC with TG proteins. When the highest expressing line (Line 102) was bred to homozygosity, 75% of the total MHC was β-MHC (Fig. 1D).

We reasoned that if a phenotype were likely to be present, it would be most easily detected in line 102, and this line became the focus of our analyses. Immunohistochemical staining using a V3-specific antibody derived from the hypervariable Loop 2 region of β-MHC (Fig. 5B) showed only traces of this isoform in NTG cardiomyocytes (Fig. 2A). Confocal analysis confirmed that the PTU-treated animals showed significant accumulation of β-MHC (Fig. 2B), but the characteristic striated pattern was somewhat blunted, consistent with the major effects that hypothyroidism has on cardiomyocyte morphology. In contrast with the PTU-treated mice, striated morphology was well conserved in the β-MHC TG cardiomyocytes, with the pattern of staining confirming the correct incorporation of TG protein into the sarcomere (Fig. 2C). Cardiac histology was examined using young adult animals (8–12 weeks) (Fig. 2, D and E) and aged animals (1 and 2 years). No significant differences in the gross morphology of either heterozygous or homozygous TG hearts were observed, and no differences in heart rate or chamber
weight could be detected (Table I). Quantification of the molecular markers of hypertrophy, which we have found to be a very sensitive marker of any response at the cellular level, was carried out at the transcript level as described previously (34), and no differences could be detected.2 Similarly, we could not detect any obvious differences in the cardiomyocytes from homozygous TG mouse hearts using either light or electron microscopy (Fig. 2, D–G). No early deaths or overt ill health was noted in any of the TG animals during the first year and a half of life as compared with the NTG experimental cohorts. We conclude that the α → β transition is benign in terms of the animals overall cardiac morphology of the animals and that no early mortality or morbidity presents under normal animal husbandry conditions.

**Consequences of Isoform Replacement in Intact Fibers**—In light of the unremarkable phenotype at the whole animal level, we wished to confirm that changes in isoform content had affected the mechanical and kinetic properties of the skinned myofibers. Ventricular papillary muscles were isolated from line 102, PTU-treated and NTG mice. Line 102 heterozygotes have ~40% β-MHC while the homozygotes show ~73% replacement. The skinned fiber is a complex system in which the contractile machinery operates against the internal cytoskeletal structures in both the cardiomyocytes and connective tissue. Therefore, V\text{max} in a fiber is never truly unloaded, as is assumed to be the case in the *in vitro* actin motility assay (see below).

We first wished to compare the effects of ~40% replacement versus the fibers derived from PTU-treated animals, in which ~95% of the cardiac myosin consisted of β-MHC. Fibers were isolated from 9-week-old animals in order to minimize the effects of any secondary pathology that might develop later in life, and the unloaded shortening and maximum shortening velocities, as well as the relative power that the fibers developed, were measured (Fig. 3). As expected on the basis of the degree of α-MHC replaced by β-MHC, the values derived from line 102 heterozygotes were intermediate between the NTG (100% α-MHC) and PTU (90% β-MHC) data. Significant, graded decreases in the unloaded shortening velocity were noted (Fig. 3A) from NTG (3.80 ± 0.14 m. l./s, n = 7) to line 102 (2.72 ± 0.26 m. l./s, n = 4) to the PTU-derived fibers (1.51 ± 0.24 m. l./s, n = 3). The same gradual decreases were also observed in the force-velocity data used to derive the maximum shortening velocities (Fig. 3B). The power-force relationships, and maximum power produced followed the same trend (Fig. 3C), and the data show that the shift in MHC isoform content leads directly to changes in crossbridge cycling rates.

We confirmed both the trend and stability of these changes by developing a cohort of heterozygotes and homozygotes over the course of a year and subsequently carrying out fiber measurements comparing these two populations to NTG fibers (Fig. 4). Similar graded decreases in the unloaded shortening velocity (Fig. 4A), maximum shortening velocity (Fig. 4B), and maximum power produced (Fig. 4C) were noted when the NTG, heterozygotes, and homozygotes were compared. No changes in the calcium-force relationship could be observed in any of the TG fibers.2

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Fig. 4. Contractile properties of isolated ventricular fibers II. A, slack test comparison between NTG, TG line 102 heterozygotes, and TG line 102 homozygotes. The change in length (Δlength) was plotted versus the time lag between the onset of a release and the onset of tension recovery (Δtime). Straight lines were then fitted by the least-squares method. The maximum shortening velocities were also determined using the slack test. Units are in muscle lengths per s (m.l./s). B, force-velocity relationships and maximum shortening velocities determined by isotonic quick releases under constant load at pCa = 5. C, relative power was extrapolated from the force-velocity relationships. Multiple fiber preparations were derived from 2 to 4 animals per group. Power output is defined as the relative power (P/π) multiplied by the velocity (m.l./s). Values are expressed as means ± S.E. *, p < 0.05; **, p < 0.01; ***, p < 0.001 versus NTG. Absolute values differ slightly from the data set in Fig. 3 because of minor alterations in the fiber apparatus. However, relative differences are conserved.

above data clearly showed that the cardiomyocyte is tolerant of significant MHC isoform shifts that are transgenically imposed. We next explored the structural basis of the different cardiac myosins' unique functionalities by replacing the endogenous MHC with α/β chimeras, the working hypothesis being that the functional differences between the isoforms presumably are caused by the different loop sequences. The structure of Loop 1 is thought to modulate the rate of Mg²⁺-ATP binding and Mg²⁺-ADP release while the structure of Loop 2 affects the rate of myosin attachment to actin (9–11). Two constructs, in which the sequences of either Loop 1 and Loop 2 (L₁+L₂) or only Loop 2 (L₂) of mouse α-MHC were substituted by the corresponding β-MHC sequences were made and used to generate TG mice (Fig. 5A). In order to detect the TG protein, an antibody to the α-MHC sequence was generated (Fig. 5B). Western blots of myofibrillar proteins derived from NTG, PTU-treated, or TG mice carrying the chimeric myosins (L₁+L₂ or L₂) using the Loop 2 β-MHC antibody. In the L₁+L₂ TG hearts, replacement was 100%, in the L₂ TG hearts, 84%.

In Vitro Motility Assays.—To determine the effects on motor function, in vitro actin motility assays were performed using MHC that had been isolated from heterozygous line 102 β-TG mice (40% replacement), from high-replacement (100%) L₁+L₂ TG hearts, and from NTG as well as from PTU-treated hearts (Fig. 6). V₁ → V₃ replacement had a clear effect on molecular motor velocity, with the isoform switches in the PTU and β-MHC TG preparations significantly decreasing the sliding velocity of myosin. For the 40% replacement of V₁ with V₃ in line 102 heterozygotes, the observed values were intermediate between the NTG and PTU-derived samples, as expected considering the results of our previous studies in which we compared filament sliding velocities of mouse V₁/V₃ mixtures in varying proportions, and observed a linear relationship between relative isoform content and filament sliding velocity (6). In the present study, actin filament sliding velocity of the L₁+L₂ chimeric MHC was not significantly different from that of α-MHC, indicating that the loops did not confer "β-like" activity on the molecule.

ATPase Assays.—The different myosins are characterized by their unique enzymatic activities. We determined both the myofibrillar Ca²⁺-stimulated Mg²⁺-ATPase and actin-activated ATPase activities of myosins purified from NTG, L₁+L₂, L₂, and β-TG hearts. As expected, a 2-fold difference in myofibrillar Ca²⁺-stimulated Mg²⁺-ATPase activity was observed between NTG and PTU hearts (Fig. 7A). Myosin isolated from the β-MHC-expressing TG line 102 homozygote hearts exhibited depressed activities that were consistent with a 75% replacement of V₁ with V₃ (Fig. 7B). The chimeric myosins, while displaying somewhat diminished activities at the two highest calcium concentrations tested, had myofibrillar ATPase values
Fig. 6. In vitro motility assays. The bars indicate the absolute velocities of actin filaments (micrometers per second) translocated by the respective myosins. Values are expressed as means ± S.E. *, p < 0.001 versus NTG.

that were closer to V₁ than V₃ (Fig. 7C). For the L₁+L₂-TG hearts in which a 100% replacement had been effected, enzymatic activity of this myosin was more like V₁ than V₃, indicating that the loops did not confer full "β-like" activity on the molecule. There were no significant differences in the pCa₅₀ values between any of the groups. The slightly different ATPase values between L₁+L₂ and L₂ myofibrils is most likely due to the higher degree of protein replacement in the L₁+L₂-TG mouse hearts.

Fig. 7. In vitro Ca²⁺-stimulated ATPase activities. A–C, Ca²⁺-stimulated Mg²⁺-ATPase activities were determined from NTG and PTU-treated (A), β-TG (B), and L₁+L₂ and L₂-TG (C) hearts. Panels B and C show the NTG and PTU data as dotted lines to facilitate comparison. A 2-fold difference in myofibrillar Ca²⁺-stimulated Mg²⁺-ATPase activity was observed between the NTG and PTU hearts. ATPase activity of L₁+L₂-TG myofibrils was reduced, but not to the level of the PTU group. ATPase activity of L₂-TG myofibrils was also different from the PTU group, but not from the NTG group. In contrast, β-TG myofibrils showed significantly reduced ATPase activity close to that seen in the PTU group. *, p < 0.05 versus NTG; †, p < 0.05 versus PTU, n = 3–5, myofibrils from 3- to 6-month-old hearts.

To explore this phenomenon further, data were obtained for the more physiologically relevant actin-activated Mg²⁺-ATPase activity (Fig. 8). While MHC isolated from line 102 homozygotes exhibited the expected decrease in enzymatic activity (Fig. 8A) neither L₁+L₂ nor L₂ of/β chimeric MHCs showed any significant differences from the V₁ enzyme at any actin concentration (Fig. 8, B and C).

Cardiac Hemodynamics in Isolated Working Hearts and in Vivo—Although the V₁ → V₃ TG animals appeared overly healthy in the unstressed state and showed no signs of morbidity or increased mortality, we reasoned that whole organ function must be affected because of the slower motor velocity. To that end, we determined cardiac hemodynamics for the line 102 homozygotes using the isolated working heart preparation and in the intact animal (Table II). The unpaced isolated working hearts, with ∼75% V₃ displayed a significantly reduced heart rate with concomitant reductions in both systolic and diastolic parameters (Table II). We repeated the measurements under paced conditions (397 bpm) so that heart rate would not directly affect systolic or diastolic function and again noted significant decreases in the values of both dP/dtₘᵟᵢₙ and dP/dtₘₐₓ.

In vivo data from intact mice were also consistent with a decrease in systolic and diastolic function in β-TG mice. Mean arterial pressure, LV systolic pressure, and LV dP/dtₘₐₓ tended to be lower in TG animals compared with wild type (Table II). Furthermore, dP/dtₘᵟᵢₙ (dP/dt at 40 mm Hg developed pressure) was also significantly lower in TG mice, suggesting that the decreased rate of contraction could not be accounted for by the observed differences in afterload and is more likely due to actual differences in myocardial contractility. Likewise, both dP/dtₘᵟᵢₙ and the time constant of relaxation (tau, τ) also dem...
onstrate significant impairment of relaxation in the TG animals, consistent with data from the isolated hearts. Interestingly, in contrast to the isolated unpaced working heart, there was no difference in heart rate between the NTG and β-TG mice, presumably because of compensation via neurohumoral mechanisms in the intact animal. Supporting this hypothesis, we found that administration of the β-adrenergic blocker propranolol revealed a difference in heart rate between TG and wild type mice (434 ± 29 versus 474 ± 11). Finally it is important to note that out of the 6 mice in the β-TG group, 2 were hemodynamically unstable under anesthesia and died before completion of the protocol, further underscoring their cardiovascular deficit.

**DISCUSSION**

The aim of this study was to investigate the functional consequences of myosin isoform diversity and how the different motor abilities influence cardiac contractile function. In mammalian adult hearts, alterations in MHC isoform expression occur in response to various pleiotropic stimuli such as hypertrophy, failure, hypo- or hyperthyroidism. While these partial/complete isoform switches correlate with changes in cardiac performance, there are a myriad of other structural and functional changes that accompany these processes (17, 18), such that it is impossible to ascribe the changes in heart function to modifications in MHC isoform content alone. To test the hypothesis that changes in isoform content in the absence of such global processes could alter heart function, we used transgenesis to generate mice that overexpressed specifically in the heart the β-MHC-encoded isoform.

Cardiac-specific transgenesis also was effective in replacing the endogenous myosin with α/β chimeric myosins, which could then be used to investigate whether myosin functionality could be critically altered by exchanging loop sequences between the cardiac MHC isoforms. Based on chimeric studies in Dictyostelium myosin II, Spudich et al. (9–11) postulated that the structures of the myosin surface loops 1 and 2 could serve as modulators of the enzymatic and mechanical properties of the MHC. However, a number of other investigators reported evidence that the structure of the myosin backbone could profoundly influence surface loop function (12, 13). Rat and pig β-MHC, which have identical Loop 1 sequences apart from a single conservative substitution, have 3–4-fold differences in ATPase activity and ADP dissociation (12). The present study addresses this controversy in full-length mammalian cardiac MHC. Our data demonstrate that the sequences of the surface loops of mouse cardiac MHC, in isolation, are of minor importance in determining isoform-specific characteristics. In light of the previous chimeric studies, the sequences of the α- and β-loops in mouse cardiac MHC may be dependent upon other variable amino acids for their ability to influence both the kinetics and mechanics of cardiac muscle contraction.

Changes in charge and length of Loop 2 in Dictyostelium myosin can modulate MHC function (35). Neither mouse α- and β-Loop 1 nor mouse α- and β-Loop 2 differ in net charge (Fig. 5B), but charge distribution and spacing varies considerably between the two isoforms. These differences in the three-dimensional arrangement of charges may influence myosin-nucleotide and actin-myosin interactions (8). This concept is supported by data demonstrating that the structural differences between the rat α- and β-loops are sufficient to alter Dictyostelium MHC function (11). Loop 2 of Dictyostelium myosin II was exchanged for Loop 2 of rat α- or β-MHC, resulting in actin-activated ATPase activities of the two chimeras that closely reflected ATPase activities of rat α- and β-MHC. Mouse and rat α-Loop 2 are identical, and mouse and rat β-Loop 2 differ only in one amino acid (mouse A631V rat), a conservative substitution. Our data indicate that Loop 2 exchanges between mouse α- and β-MHC do not affect the physiologically relevant actin-activated Mg2+-ATPase activity: neither the L1+L3 nor L2 chimeras were different from that of α-MHC. Similarly, ATPase activity of myofibrils from L2-TG hearts was not altered. Only at non-physiologically high calcium concentrations was the ATPase activity of L1+L3 myofibrils reduced, indicating that Loop 2 plays only a very minor role in regulating this aspect of isoform functionality. Moreover, the present chimeric study also demonstrates that switches in Loop 1 sequence do not alter ADP release rate and thereby actin filament sliding velocity in mouse cardiac MHC, in contrast to the previous Dictyostelium data (10). The data, together with other chimeric-based studies indicate that the structure of the MHC backbone influences whether or not loop exchanges between isoforms can affect MHC function.

These seemingly contradictory data can be reconciled by hypothesizing that the neighboring structures of the myosin backbone direct the flexible loops into certain conformations and thereby promote or ameliorate their influence on actin-myosin interactions or nucleotide binding and release. Alignment of the amino acid sequences of the backbones of Dictyostelium myosin II and mouse cardiac α-MHC reveals only 33% identity (64% homology). Consequently, it is not surprising that the Dictyostelium myosin II backbone could provide different atomic interactions with a chimeric loop than would the mouse cardiac MHC backbone. Furthermore, it is well known that within the myosin head even widely separated regions can critically influence one another, and this may underlie a partial explanation for the importance of the backbone

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**TABLE II**

|                | Isolated working heart (unpaced) | Isolated working heart (paced) | In vivo |
|----------------|----------------------------------|--------------------------------|---------|
|                | NTG n = 5                        | β-TG n = 6                      | NTG n = 5 | β-TG n = 6 |
| Heart rate (bpm) | 378 ± 300                        | 317 ± 8                        | 397 ± 100 | 397 ± 100   |
| MAP (mm Hg)     | 116 ± 200                        | 96 ± 3                        | 103 ± 200 | 95 ± 2        |
| LVP syst (mm Hg) | 6,580 ± 1,730                    | 4,566 ± 130                    | 5,611 ± 1,912 | 4,914 ± 114 |
| dP/dt max (mm Hg/s) | -5,138 ± 1,000                | -2,834 ± 105                   | -4,107 ± 11,100 | -3,244 ± 60 |
| dP/dt min (mm Hg/s) | -3,244 ± 60                    | -5,240 ± 400                   | -7,784 ± 3,020 | -5,091 ± 367 |
| Tau             | ND                               | ND                             | 9.7 ± 0.3 | 12.9 ± 0.8   |

a p < 0.05 vs. NTG.

b p = 0.09.

c p < 0.07.

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All results are given as mean ± S.E. 4-month-old mice were used for working heart experiments; 3-month-old mice were used for in vivo studies. Line 102 homozygotes were used (β-TG). The unpaced and paced models were carried out on slightly different apparatus and absolute values between these two groups cannot be directly compared; however, NTG and TG cohorts within the unpaced or paced groups can be compared directly. ND, not determined; MAP, mean arterial pressure; LVP sys*, left ventricular systolic peak pressure; dP/dt, change in pressure in relation to time; dP/dt max, dP/dt at 40 mm Hg developed pressure; Tau, time constant of relaxation.
sequence for loop function. Therefore, alignment of more closely related MHC isoforms in order to find regions that could influence isoform-specific characteristics appears to be the most feasible approach for determining the critical amino acids that underlie the differing functionalities of the unique isoforms. By comparing MHC isoform sequences across various mammalian species in the context of all available functional data, we identified only 8 non-conservative amino acid substitutions in α-MHC (residues 2, 210, 442, 452, 801, 1092, 1637, and 1681) and only 4 residues in the β-MHC (residues 424, 573, 1201, and 1368) that may be responsible for species-specific MHC-isoform functionality rather than surface Loops 1 and 2 (6). Functional analysis of these residues may tell us which regions of the backbone are of importance for MHC function and would provide the basis for further structure-function studies comparing α-MHC and β-MHC.

Functional differences between the cardiac MHC isoform were manifested at the single motor, biochemical, and fiber levels, in a manner that reflected the altered $V_t/V_N$ ratios. Thus, it is clear that changes in myosin enzymatic activity are reflected by concomitant changes in motor velocity, which, in turn, lead to changes in fiber contractility. These changes are all consistent with the correlations that have been previously noted (36–38). Comparing the data with those studies, the unloaded shortening velocity decreased 30% in TG mice exhibiting 40% replacement with β-MHC, by 56% in TG mice with 73% replacement, and by 60% in the PTU-treated animals (Figs. 3 and 4), a value in the same range as measured by Fitzsimons et al. (39), who found an 80% decrease in rat single cardiac myocytes that express essentially pure β-MHC. In our hands, substituting the majority of α-MHC with β-MHC either via transgenesis or by inducing hypothyroidism resulted in a similar impairment in fiber mechanics. This indicates that, in this setting, the MHC isoform switch has a predominant effect compared with other pleiotropic effects of PTU.

Our data also demonstrate how the in vitro mechanical and kinetic differences of the MHC isoforms are reflected at the whole organ and intact animal levels. Although the unstressed heart is overly healthy, the 2-fold differences in actin filament sliding velocity and ATPase activity between α- and β-MHC observed on the molecular level resulted in altered systolic and diastolic function in isolated hearts and in vivo. In unpaced isolated hearts, a 17% decrease in left ventricular pressure and a 31% decrease in dP/dt max together with a 45% increase in dP/dt min was seen, and when differences in heart rate were removed by atrial pacing, these differences persisted. Left ventricular pressure measurements from intact animals revealed similar contractile deficits in β-TG mice.

Recently, contractile function of TG mouse hearts with low-level expression of Myc-tagged rat β-MHC was measured in Langendorff preparations (16). In those hearts, dP/dt max was reduced by 15% although replacement of the endogenous MHC with the tagged β-MHC was only 12%. In light of our data, the relationship between contractility and relative isoform content may be non-linear and supporting this hypothesis, in hypothyroid hearts predominantly expressing β-MHC, a small amount of α-MHC expression can significantly augment myocyte power output (41).

The spontaneous heart rate of the isolated hearts, as well as heart rate in propranolol-treated mice, was reduced in β-TG mice as compared with NTG animals. A trend toward lower heart rates in β-TG isolated hearts was also reported by Tardiff et al. (16). These data support the concept that there might be an intrinsic feedback mechanism adapting heart rate to the kinetic properties of the MHC (42). In vivo, it is the autonomic nervous system that regulates sinus node firing, even in anesthetized animals, and the system is regulated through both the sympathetic and parasympathetic neurons (43). Based on the normal histology, unremarkable sarcomeric structure of the cardiomyocytes and the lack of hypertrophy in the β-TG mice, we conclude that as long as the heart is able to adequately respond to autonomic regulation, and contractile function is sufficiently compensated to maintain cardiac output, no overt or subclinical phenotype will present.

While it has been generally accepted that isoform shifts can result in different functional endpoints, until very recently the potential of a $V_1 \rightarrow V_2$ transition to affect human heart disease remained problematic, as it was thought that the human ventricle contained only the $V_2$ isoform in either the normal or diseased state. However, evidence now exists that an isoform shift does occur in the failing human ventricle, with α-MHC mRNA accounting for as much as 34% of the total MHC transcript in the normal heart (44). Down-regulation of α-MHC in the failing human ventricle at both the RNA and protein levels occurs (45, 46). Our data show that a $V_1 \rightarrow V_2$ shift in mouse hearts reduces contractile function. Taken together with the potential energy-conserving effect of a $V_1 \rightarrow V_2$ shift and the fact that even small amounts of $V_1$ might impact favorably on cardiac function, it is now critical to understand what role an isoform shift might play in disease onset and progression. Crossing the $V_2$ TG mice into different mouse models of hypertrophy and failure should provide insight into the potential role(s) the different cardiac MHCs may play in the pathogenesis of cardiac disease.

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REFERENCES

1. Raymont, I., Holden, H. M., Whitaker, M., Yohn, C. B., Lorenz, M., Holmes, K. C., and Milligan, R. A. (1996) Science 268, 58–65
2. Morin, K. (1993) Circulation 87, 1451–1460
3. Harris, D. E., Work, S. S., Wright, R. K., Alpert, N. R., and Warshaw, D. M. (1994) J. Muscle Res. Cell Motil. 16, 11–19
4. Palmiero, K. A., Tyska, M. J., Dupuis, D. E., Alpert, N. R., and Warshaw, D. M. (1999) J. Physiol. 519, 669–678
5. Litten, B. Z., 3rd, Martin, B. J., Low, R. B., and Alpert, N. R. (1982) Circ. Res. 50, 856–864
6. Alpert, N. R., Brousseau, C., Federico, A., Krenz, M., Robbins, J., and Warshaw, D. M. (2002) Am. J. Physiol. Heart Circ. Physiol. 283, H1446–H1454
7. Murphy, C. T., and Spudich, J. A. (2000) J. Muscle Res. Cell Motil. 21, 139–151
8. Weiss, A., Schiaffino, S., and Leinwand, L. A. (1999) J. Mol. Biol. 290, 61–75
9. Spudich, J. A. (1994) Nature 372, 515–518
10. Murphy, C. T., and Spudich, J. A. (1990) Biochemistry 29, 6738–6744
11. Uyeda, T. Q., Ruppel, K. M., and Spudich, J. A. (1994) Nature 368, 567–569
12. Pereira, J. S., Pavlov, D., Nili, M., Greaser, M., Homsher, E., and Moss, R. L. (2001) J. Biol. Chem. 276, 4409–4415
13. Sweeney, H. L., Rosenfeld, S. S., Beal, F., Faust, L., Smith, J., Xing, J., Stein, L. A., and Sellers, J. R. (1998) J. Biol. Chem. 273, 6262–6270
14. Hirayama, Y., Sutoh, K., and Watabe, S. (2000) Biochem. Biophys. Res. Commun. 269, 237–241
15. Lopes, A. C., Furlanetto, R., Sasso, W. S., and Didio, L. J. (1993) Submicroscopic Cytol. Pathol. 25, 203–266
16. Tardiff, J. C., Hewett, T. E., Factor, S. M., Vykstrom, K. L., Robbins, J., and Leinwand, L. A. (2000) Am. J. Physiol. Heart Circ. Physiol. 278, H412–H419
17. Chien, K. R., Knowlton, K. U., Zhu, H., and Chien, S. (1991) FASEB J. 5, 3037–3046
18. Inamurra, S., Matsuya, S., Hiratsuka, E., Kimura, M., Nakanishi, T., Nishikawa, T., Furutani, Y., and Takao, A. (1991) Am. J. Physiol. 260, H73–H79
19. Sanchez, A., Jones, W. K., Gulkic, J., Doetschman, T., and Robbins, J. (1991) J. Biol. Chem. 266, 22419–22426
20. Subramanian, A., Gulkic, J., Neumann, J., Knotts, S., and Robbins, J. (1993) J. Biol. Chem. 268, 4331–4336
21. Tyska, M. J., Hayes, E., Giewat, M., Seidman, C. E., Seidman, J. G., and Warshaw, D. M. (2000) J. Biol. Chem. 275, 737–744
22. Pardee, J. D., and Spudich, J. A. (1982) Methods Cell Biol. 24, 271–289
23. McKinlie, J. J., Gan, L. Z., and Solaro, R. J. (1990) Circ. Res. 66, 1204–1216
24. Fowers, F. M., and Solaro, R. J. (1990) Am. J. Physiol. 258, C1348–C1351
25. Bloug, E. R., Rennie, E. R., Zhang, F., and Reiser, P. J. (1996) Anal. Biochem. 233, 31–35
26. Reiser, P. J., and Klins, W. O. (1998) Am. J. Physiol. 274, H1048–H1053
27. Sanhue, A., Gulkic, J., Jewell, J., and Robbins, J. (2001) J. Biol. Chem. 276, 32682–32686
28. Nguyen, T. T., Hayes, E., Mulieri, L. A., Leavitt, B. J., ter Keurs, H. E., Alpert, R. M., and Spudich, J. A. (1994) Science 264, 139–142
29. Lorenz, J. N., and Robbins, J. (1997) Am. J. Physiol. 272, H1137–H1146
30. Gulick, J., Hewett, T. E., Klevitsky, R., Buck, S. H., Moss, R. L., and Robbins, J. (1997) Circ. Res. 80, 655–664
31. Fewell, J. G., Hewett, T. E., Sanbe, A., Klevitsky, R., Hayes, E., Warshaw, D., Maughan, D., and Robbins, J. (1998) J. Clin. Invest. 101, 2630–2639
32. James, J., Osinska, H., Hewett, T. E., Kimball, T., Klevitsky, R., Witt, S., Hall, D. G., Gulick, J., and Robbins, J. (1999) Transgenic Res. 8, 9–22
33. Palermo, J., Gulick, J., Colbert, M., Fewell, J., and Robbins, J. (1996) Circ. Res. 78, 504–509
34. Jones, W. K., Grupp, I. L., Doetschman, T., Grupp, G., Osinska, H., Hewett, T. E., Boivin, G., Gulick, J., Ng, W. A., and Robbins, J. (1996) J. Clin. Invest. 98, 1966–1917
35. Furch, M., Geeves, M. A., and Manstein, D. J. (1998) Biochemistry 37, 6317–6326
36. Sugiura, S., Kobayakawa, N., Fujita, H., Momomura, S., Chaen, S., and Sugi, H. (1998) Adv. Exp. Med. Biol. 453, 125–130
37. Reiser, P. J., Moss, R. L., Giulian, G. G., and Greaser, M. L. (1985) J. Biol. Chem. 260, 9077–9080
38. Bottinelli, R., Schiaffino, S., and Reggiani, C. (1991) J. Physiol. 437, 655–672
39. funnel, D. P., Patel, J. R., and Moss, R. L. (1998) J. Physiol. 513, 171–183
40. Metzger, J. M., Wahr, P. A., Michele, D. E., Albayya, F., and Westfall, M. V. (1999) Circ. Res. 84, 1310–1317
41. Herron, T. J., and McDonald, K. S. (2002) Circ. Res. 90, 1150–1152
42. Rouslin, W., and Broge, C. W. (1996) Am. J. Physiol. 270, C1271–C1276
43. Rosen, M. R. (2001) Lancet 357, 468–471
44. Nakao, K., Minobe, W., Roden, R., Bristow, M. R., and Leinwand, L. A. (1997) J. Clin. Invest. 100, 2362–2370
45. Miyatake, S., Minobe, W., Bristow, M. R., and Leinwand, L. A. (2000) Circ. Res. 86, 385–390
46. Reiser, P. J., Portman, M. A., Ning, X. H., and Schomisch Moravec, C. (2001) Am. J. Physiol. Heart Circ. Physiol. 280, H1814–H1820
