In Vivo Analysis of an Essential Myosin Light Chain Mutation Linked to Familial Hypertrophic Cardiomyopathy

Atsushi Sanbe, David Nelson, James Gulick, Elizabeth Setser, Hanna Osinska, Xuejun Wang, Timothy E. Hewett, Raisa Klevitsky, Eric Hayes, David M. Warshaw, Jeffrey Robbins

Abstract—Mutations in cardiac motor protein genes are associated with familial hypertrophic cardiomyopathy. Mutations in both the regulatory (Glu22Lys) and essential light chains (Met149Val) result in an unusual pattern of hypertrophy, leading to obstruction of the midventricular cavity. When a human genomic fragment containing the Met149Val essential myosin light chain was used to generate transgenic mice, the phenotype was recapitulated. To unambiguously establish a causal relationship for the regulatory and essential light chain mutations in hypertrophic cardiomyopathy, we generated mice that expressed either the wild-type or mutated forms, using cDNA clones encompassing only the coding regions of the gene loci. Expression of the proteins did not lead to a hypertrophic response, even in senescent animals. Changes did occur at the myofilament and cellular levels, with the myofibrils showing increased Ca\(^{2+}\) sensitivity and significant deficits in relaxation in a transgene dose-dependent manner. Clearly, mice do not always recapitulate important aspects of human hypertrophy. However, because of the discordance of these data with data obtained in transgenic mice containing the human genomic fragment, we believe that the concept that these point mutations by themselves can cause hypertrophic cardiomyopathy should be revisited. (Circ Res. 2000;87:296-302.)

Key Words: myosin ■ heart ■ muscle ■ mouse ■ hypertrophy

Familial hypertrophic cardiomyopathy (FHC) is a dominant disease of the cardiac sarcomere.\(^1\) Multiple mutations in the different thick- and thin-filament proteins have been linked to the disease, including the \(\beta\)-myosin heavy chain (\(\beta\)-MHC), the essential and regulatory myosin light chains (ELC and RLC), myosin binding protein C, \(\alpha\)-tropomyosin, cardiac troponin T, cardiac troponin I, actin, and titin.\(^2\) The disease is clinically heterogeneous, with some mutant alleles resulting in a poor clinical prognosis and others being relatively benign.\(^3\) That so many of the FHC mutations are linked to genes encoding sarcomeric proteins suggests that the pathogenesis of the disease likely involves a common mechanism of contractile dysfunction.

Mutations in the human ELC (Met149Val) and RLC (Glu22Lys, Pro94Arg), which result in midventricular cavity obstruction due to papillary muscle hypertrophy, have been described,\(^4\) and the Met149Val mutation has been modeled in mice by inserting entire human mutant or wild-type genetic loci into transgenic (TG) mice.\(^5\) The authors hypothesized that the mutant light chain hypertrophic cardiomyopathy resulted from abnormal movement of the neck region and alterations of the myosin in a stretch-activation response.\(^5\) However, 2 novel missense mutations, Phe18Leu in exon 2 and Arg58Gln in exon 4 of RLC, were later found in the French population. These mutations presented a classic phenotype of hypertrophic cardiomyopathy without mid left ventricular obstruction and papillary muscle hypertrophy.\(^6\)

Considering the disparity in the 2 groups of data obtained from the human populations concerning the occurrence of hypertrophy resulting in a midventricular obstruction, we decided to pursue the question of actual causality of the point mutations. That is, are the point mutations located in the myosin light chains sufficient for the hypertrophy? Rather than using the genomic sequences of a heterologous species, the murine cDNA, free of all intronic DNA, was used to produce the TG mouse lines to create mouse models in which partial replacements of cardiac ELC and RLC with proteins carrying the Met149Val and Glu22Lys mutations, respectively, could be studied. Both epitope-tagged and untagged constructs were used to establish the degree of replacement, allowing us to determine dose-dependent effects of the mutant transgenes. Unlike the murine model carrying the human ELC\(^{\text{Met149Val}}\) gene locus, mice with the mutated murine ELC cDNA or RLC\(^{\text{Glu22Lys}}\) failed to exhibit either overt hypertrophy or midventricular cavity obstruction.

Materials and Methods
An expanded Materials and Methods section is available online at http://www.circresaha.org.
Constructs have also been described\(^1\); a Glu→Lys substitution at position 22 in the cognate protein was made (RLC2\(^{v\mu}\)), recapitulating the human gene mutation.\(^4\) MyHC indicates myosin heavy chain.

A fourth construct containing the RLC2\(^{v\mu}\) endogenous isoform (RLC2\(^{v\alpha}\)) and TG expression levels of 3.8-fold and 4.2-fold was made: clone 97 contained a single amino acid substitution; clone 38 contained the single amino acid change, as well as a VSV tag at the carboxyl terminus; and clone 21 consisted of normal ELC1\(^{v}\) with the VSV tag at the carboxyl terminus. Line 66, in which TG-encoded, wild-type ELC1\(^{v}\) is expressed at levels sufficient for replacement of the endogenous protein, has been described.\(^7\) The wild-type regulatory myosin light chain construct has also been described\(^6;\) a Glu→Lys substitution at position 22 in the cognate protein was made (RLC2\(^{v\mu}\)), recapitulating the human gene mutation.\(^4\) MyHC indicates myosin heavy chain.

Results

Generation of Myosin Light Chain TG Mice

An experimental strategy was devised that called for 4 constructs to be used to generate ELC1\(^{v}\) TG mice and 2 constructs for the RLC2\(^{v}\) analyses (Figure 1). Expression of a cDNA encoding the full-length ELC1\(^{v}\) transgene in lines carrying copy numbers of <90 leads to complete replacement of the endogenous isoform with TG protein but no overt changes in heart function or structure.\(^7\) Line 66, which displays these characteristics,\(^7\) was used to generate a control cohort of mice to rule out any effects that might be due to TG overexpression of a contractile protein. We modified the wild-type cDNA construct by adding a VSV tag at the carboxyl terminus, which appears to be innocuous,\(^8\) but, as a precaution, the TG lines generated with this construct were not used for functional analyses because of the possible confounding effects of the epitope. These lines were used both for confirming that TG protein was incorporated normally and to generate a curve in which transcript levels could be standardized and used to estimate the degree of replacement at the protein level. This strategy does, however, preclude us from directly assessing the degree of protein substitution.\(^9\)

Quantification of atrial replacement showed between 60% and 80% substitution of the ELC1\(^{a}\) with ELC1\(^{v}\) Met158Val. As the human genomic fragment used to generate TG mice contained the ELC1\(^{v}\) Met158Val mutation, our initial analyses focused on the lines generated with the analogous mouse cDNA (Figure 2A). Overall protein stoichiometry of the myofibril was not affected by TG expression of a contractile protein, and thus, TG expression led to a concomitant decrease in the endogenous protein species such that normal light chain stoichiometry was maintained (Figure 2B). Different lines showed varying levels of expression that corresponded to the number of TG copies; copy number varied from 2 to 15 (data not shown). TG transcript integrity was confirmed by sequencing (data not shown).

The degree of replacement could be estimated directly in the atria (Figure 2B). We focused on lines 95 and 6, which displayed TG expression levels of 3.8-fold and 4.2-fold relative to the endogenous transcript, respectively, and which in the past resulted in 50% to 75% replacement.\(^10\) Quantification of atrial replacement showed between 60% and 80% substitution of the ELC1\(^{a}\) with ELC1\(^{v}\) Met158Val. Line 66 represents the wild-type ELC1\(^{v}\) construct; this line shows >90% replacement in the atrial compartment. The corresponding tagged lines, lines 83 and 55, respectively, show similar levels of replacement. As can be observed in the atrial sample, the tag results in a slightly slower mobility under the electrophoresis conditions used (Figure 2B).
Four lines expressing varying amount of wild-type protein and an additional 4 lines expressing ELC1v Met158Val tagged protein were used to generate a standard curve, as a linear relationship between the degree of atrial versus ventricular replacement was apparent. Replacement was more complete in the ventricles than in the atria, possibly as a result of the stronger affinity of ELC1v for its endogenous contractile apparatus. Ventricular replacement was estimated at 60% in line 95 and 80% in line 6. All histological analyses using the tagged proteins indicated that they were incorporated normally into the sarcomere and that the overall organization of the contractile apparatus was unperturbed by TG replacement. When tested at 8 weeks for activation of the fetal transcriptional program, which serves as a sensitive marker for cardiac dysfunction and/or hypertrophy, none could be detected (data not shown).

**ELC1v Mutant Mice Exhibit Myocyte Disarray and Interstitial Fibrosis**

The histopathological findings for FHC reflect the genetic heterogeneity of the disease and can be quite diverse. Common findings are myocellular disarray, foci of disorganized cells, and interstitial fibrosis. Trichrome staining of the ELC1v mutant lines at 10 to 12 months showed varying degrees of histopathology (Figure 3). Sections from the apex of line 95 showed mild fibrosis, whereas line 6 shows a more severe pathology. Line 6 also shows relatively more myocyte disarray, and interstitial spacing is obviously affected compared with nontransgenic (NTG) or line 66 mice, demonstrating that the degree of pathology was dependent on transgene dose.

2B). Four lines expressing varying amount of wild-type protein and an additional 4 lines expressing ELC1vMet158Val tagged protein were used to generate a standard curve, as a linear relationship between the degree of atrial versus ventricular replacement was apparent. Replacement was more complete in the ventricles than in the atria, possibly as a result of the stronger affinity of ELC1v for its endogenous contractile apparatus. Ventricular replacement was estimated at ~60% in line 95 and ~80% in line 6. All histological analyses using the tagged proteins indicated that they were incorporated normally into the sarcomere and that the overall organization of the contractile apparatus was unperturbed by TG replacement. When tested at 8 weeks for activation of the fetal transcriptional program, which serves as a sensitive marker for cardiac dysfunction and/or hypertrophy, none could be detected (data not shown).

Transmission electron microscopy bore out the abnormal histopathology. In the NTG animals, the regular arrangement of sarcomeres was obvious, with both A and I bands easily distinguishable and Z bands aligned transversely. The intercalated discs show a characteristic steplike structure (Figure 4A). In contrast, sections taken from the LV (Figure 4C) or septum (Figure 4D) of multiple 7-month-old hearts derived from an ELC1vMet158Val animal (line 95) show numerous ultrastructural abnormalities and cell vacuolization (F, asterisk). The interstitium (E) contains numerous collagen fibers (arrows).
ELC1vMet158Val Mice Exhibit Decreased Ventricular Mass

The histopathology of mice carrying the mutated transgene was consistent with the clinical cellular pathology observed in a majority of FHC cases studied. However, as noted above, in young adult animals we were unable to detect activation of the fetal transcriptional programs characteristic of a hypertrophic response. In light of those data, the heart weight/body weight ratios of the TG cohorts were examined (Table). Both lines 95 and 6, which expressed the mutant protein, displayed reduced rather than increased values at 7 and 25 weeks when compared with either the NTG or ELC1v wild-type–expressing cohorts. The decreases in heart mass were restricted to the LV (9%, line 95; 15%, line 6). A similar phenomenon was observed previously, when a mutated troponin T protein was expressed in mice. Decreased ventricular mass was due to smaller myocyte size, consistent with the hypothesis that the primary deficit in FHC is myocellular in nature. Cell size was determined using isolated cardiomyocytes from line 6 and compared with those derived from age- and sex-matched wild-type animals. ELC1vMet158Val cardiomyocytes showed a decrease of 12% in cell volume as compared with the NTG wild-type controls, a result consistent with the tissue weight data. Even in senescent animals (1.5 years) derived from either line 95 or 6 ELC1vMet158Val mice, no hypertrophy at any level was observed, nor were differences in chamber volume, as determined by echocardiography, observed throughout the animals’ lifetimes (data not shown).

ELC1vMet158Val Mutation Leads to Functional Deficits

The structural and ultrastructural deficits described above indicated that either a primary or secondary pathology was occurring. To determine whether the mutant protein substitution had functional consequences before any detectable pathology presented, skinned fibers were derived from the papillary muscles of young adults (6 to 7 weeks) and subjected to both mechanical and enzymatic analysis (Figure 5). At this age, TG protein levels are at steady state, but no overt pathology is detected (data not shown). Thus, any changes detected in the fiber kinetics should be due to the primary isoform change and should not merely reflect secondary compensatory processes such as fibrosis. The ELC1vMet158Val fibers exhibited leftward shifts in both ATPase activity (Figure 5A) and the pCa-force curve (Figure 5B). Significant decreases in the power-force relationships were also noted (Figure 5C) and, although maximum shortening velocity and maximum force were unchanged (Figure 6), maximum power was decreased as a result of changes in the shape of the F:V curve (Figures 5C and 5D). The amplitudes of the shifts in myofibrillar Mg$^{2+}$ATPase activity, the pCa-
force relationship, and the decrease in maximum power from line 6 hearts were all significantly greater than those observed for line 95. Thus, the functional abnormalities of the fibers from line 6 are consistent with the more severe pathology (Figures 3 and 4) that eventually develops.

The initial studies using human-derived material showed that the ELC1v Met158Val mutation led to an increase in the velocity of actin translocation, as measured by the in vitro motility assay in human. Furthermore, alterations in crossbridge cycling of ventricular skinned fibers derived from TG mice, which expressed the human mutation, were also detected. To determine whether the point mutation led directly to changes in crossbridge cycling rates, unloaded and maximum shortening velocities were measured. No significant differences could be detected in either the unloaded shortening velocity (Figure 6A) or the maximum shortening velocity (Figure 6B) of either ELC1v Met158Val line, as compared with the NTG cohort. Finally, the in vitro motility assay was used to determine the speed at which ELC1v Met158Val myosin derived from line 6 was able to translocate actin; consistent with the fiber data, no significant differences were observed (Figure 6C).

Are the functional changes in the fibers manifested at the whole-organ level? On the basis of the leftward shifts obtained for the pCa\(^2+\)-Mg\(^2+\)-ATPase and pCa\(^2+\)-force relationships, we hypothesized that the hearts might show near normal or even hypercontractility, but relaxation would be affected. FHC patients commonly exhibit increased left ventricular ejection fractions, but this is usually attributed to either the hypertrophy itself or to isoform shifts in the contractile proteins. Ex vivo cardiac functional analyses using the working heart preparation were carried out on mature adults (3 to 4 months old) from NTG, ELC1v wild-type (line 66), and ELC1v Met158Val (line 95 and line 6) cohorts (Table 1 online, available at http://www.circresaha.org). Both relaxation and contractile function were slightly increased in line 66 animals, compared with NTG controls. Hearts from line 95 showed a 25% increase in contractility as measured by \(+\)dP/dt, but a 22% deficit in relaxation \((-\)dP/dt). Although \(+\)dP/dt was unaffected in the experimental cohort derived from line 6 under conditions of moderate load, \(-\)dP/dt values were decreased by \(\approx\)50%. Thus, the ELC1v Met158Val mutant hearts demonstrate a significant impairment in relaxation and some hypercontractility (in line 95) as compared with NTG or line 66 (ELC1v wild-type) mice. This divergence between diastolic and systolic function in the different lines probably reflects secondary epiphenomena occurring during development of the pathogenesis that was observed via light and electron microscopy.

**RLC Glu22Lys Mutation**

The lack of an overt hypertrophic response in the ELC1v Met158Val mice was puzzling, and we wished to confirm these results with an independent mutation. Therefore, another myosin light chain mutation, the Glu\(\rightarrow\)Lys substitution found at position 22 in the RLC, was also modeled in TG mice. The construct (Figure 1) was similarly linked to the \(\alpha\)-MHC promoter and used to generate 4 TG lines that showed varying degrees of protein replacement in a copy number–dependent fashion (data not shown). Initially, we focused on lines 19, 79, and 32, which showed 27, 55, and >95% replacement of the corresponding endogenous isoform with the TG species, respectively (Figure 7). However, when preliminary analyses showed no detectable phenotype, only line 32, in which essentially all of the RLC2v consists of the RLC\(\text{Glu22Lys}\) species as based on the degree of replacement observed in the atria, was used. No hypertrophy could be detected in mature adult animals either when chamber weights were determined (Table 2 online, available at http://www.circresaha.org) or at the cellular level (Figure 7B). The histology was also unremarkable, and at neither the gross cellular or molecular levels could any response be detected in either sex (data not shown). Thus, even when replacement with the TG species is much greater than 50%, a hypertrophic response is not elicited.

**Discussion**

The function(s) of the cardiac myosin light chains is beginning to be elucidated. Both bind to a critical region of the heavy chain, and expression of a cardiac MHC lacking the light chain binding site resulted in a hypertrophic cardiomyopathy. With these data in mind, it is logical to suppose that point mutations in either cardiac light chain could have
for the in vitro motility data discrepancies may lie in the structural differences between the human and the mouse proteins. The MHC isoforms with which the light chains necessarily interact are also different; in the human the β-MHC is present, whereas in the adult mouse, only α-MHC is expressed. The alanine- and proline-rich N-terminal domain is significantly shorter in human ELC1v as compared with the mouse (a 9-amino acid deletion, Figure 2A), and this different sequence, when coupled with the Met149Val substitution, might result in a phenotype unique to the human sequence. This region appears to be important in ELC and MHC interactions and/or interactions between ELC and actin.15 Although the normal human gene, when placed into the mouse, did not yield a hypertrophic phenotype, the major differences in structure preclude an unambiguous assessment of the phenotype, as differences were observed in the stretch-activated response of isolated fiber preparations. Mutations in other regions of the human locus or even aberrant splicing resulting in a protein containing additional mutations were not ruled out.4,5 Other differences present in the 12-kb human genomic fragment could also act synergistically with the ELC1vMet149Val mutation. Those changes obviously would not be present in the murine cDNAs used in our studies. The effects of ELC1vMet149Val might be within tolerable limits in this particular mouse strain (FVB/N) as opposed to the mice carrying the human transgene (C57B/J6). Strain-specific variation of phenotype does occur, and modifying factors can play a major role in the hypertrophic response.16 The present study does show, however, that in this genetic background, these mutations are not sufficient for induction of hypertrophy.

The mutations are not completely benign. ELC1vMet158Val fibers revealed a transgene dose–dependent decrease in power output as measured by the force-velocity relationship. Isolated heart analyses were consistent with the fiber data, as relaxation was impaired significantly in ELC1vMet158Val mice, whereas contractility was increased in line 95. The fiber studies were performed at 7 weeks, minimizing the possibility that the functional defects observed reflected a developing secondary pathology. It has been established that changes in the light chain can have dramatic effects on the Ca2+ sensitivity of a myofiber,11 and the most straightforward interpretation is that both the decrease in power output and the increase in Ca2+ sensitivity are directly due to the functional consequences of the altered ELC1v structure.

Acknowledgments
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References


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Supplementary Material

MATERIALS AND METHODS

Transgene Construction
The full-length murine ELC1v cDNA was used as a template for polymerase chain reactions in order to make the additional constructs used in this study (Figure 1). Three ELC constructs were made. Clone 97 contained a valine substitution for a methionine residue at position 158 (corresponding to Met149 in the human sequence). Clone 38 contained the single amino acid change as well as the vesicular stomatitis virus (VSV) epitope at the carboxy terminus while clone 21 consisted of the wild-type sequence with the VSV-tag (Figure 1A). The mouse RLC2v cDNA was isolated using RT-PCR and the mutation made using standard PCR mutagenesis. The fragments were linked to the mouse alpha myosin heavy chain (α-MyHC) promoter, freed from the plasmid backbone by digestion with Not I, purified from agarose, and used to generate TG mice.

Measurement of Cardiomyocyte Size
Left ventricular myocytes were enzymatically isolated at 3 months using standard perfusion methodology. The dissociated myocytes were immediately filtered through a 250-µm mesh into centrifuge tubes containing 2.5% glutaraldehyde and used for cell morphometry. Isolated myocyte preparations with >90% rod-shaped cells were obtained. Myocyte size was measured with a Z2 COULTER® Sizer (Beckman Coulter Co.) using sample sizes of approximately 6000. The median value of the sample was utilized for statistical analysis.
References

**Figure 1.** Cardiomyocyte size. Myocytes derived from 3 month ELC1\textsuperscript{Met158Val} (line 6) and NTG hearts were isolated and cell volumes determined with the Coulter Sizer as described in **MATERIALS AND METHODS**. Approximately 6000 myocytes from each sample were measured in each experiment (n=6), mixed gender. A, Shown are the distributions from the Coulter Sizer. B, Histogram. The median value of the sample was used for statistical analysis. ***P<0.001.
Fig. 1 (Supplementary Material)
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<th>Heart rate (beats per min)</th>
<th>dp/dt (mmHg/sec)</th>
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<tr>
<td>390 ± 1</td>
<td>2323 ± 98**</td>
<td>6724 ± 278</td>
</tr>
<tr>
<td>380 ± 1</td>
<td>3518 ± 66**</td>
<td>8209 ± 209**</td>
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<tr>
<td>368 ± 3</td>
<td>5512 ± 122**</td>
<td>7776 ± 227**</td>
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<tr>
<td>379 ± 2</td>
<td>4711 ± 87</td>
<td>6814 ± 115</td>
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Table 1. Isolated Working Hearts (Supplementary Material)
To determine EC₉₀ values and the Hill coefficients, pCa-force and M_ATPase data were fitted to logistic equations with Origin software in order.

<table>
<thead>
<tr>
<th>pCa</th>
<th>M_ATPase</th>
<th>pCa</th>
<th>M_ATPase</th>
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Ntc (n=5)

Table 2. Shaken Tissue Measurements (Supplementary Material)

EC₉₀ Hill Coefficient 

M_ATPase pCa-Force Relationship
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<table>
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<tr>
<td>0.39 ± 0.02</td>
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<td>0.41 ± 0.04</td>
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<td>0.28 ± 0.03</td>
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Data are mean ± SEM. *p < 0.05 vs. NTG. **p < 0.05 vs. SEM.

Table 3: Normalized RTC2 (μm) and LV Mass (mg).

(Supplementary Material)