Skeletal Muscle Expression and Abnormal Function of β-Myosin in Hypertrophic Cardiomyopathy

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Abstract

Hypertrophic cardiomyopathy is an important inherited disease. The phenotype has been linked, in some kindreds, to the beta-myosin heavy chain (β-MHC) gene. Missense and silent mutations in the β-MHC gene were used as markers to demonstrate the expression of mutant and normal cardiac β-MHC gene message in skeletal muscle of hypertrophic cardiomyopathy patients. Mutant β-myosin, also shown to be present in skeletal muscle by Western blot analysis, translocated actin filaments slower than normal controls in an in vitro motility assay. Thus, single amino acid changes in β-myosin result in abnormal actomyosin interactions, confirming the primary role of missense mutations in β-MHC gene in the etiology of hypertrophic cardiomyopathy. (*J. Clin. Invest.* 1993. 91:2861–2865.) Key words: cardiac hypertrophy • beta-myosin heavy chain • molecular genetics • mutation • muscle

Introduction

Hypertrophic cardiomyopathy (HCM)[1] is an autosomal dominant inherited disease, and represents an important cause of sudden cardiac death particularly in otherwise healthy young individuals such as athletes. The disease has been defined as an increase in left ventricular wall thickness in the absence of another cause of cardiac hypertrophy. Associated left ventricular systolic and diastolic dysfunction, myocardial ischemia, and life-threatening arrhythmias frequently cause disabling symptoms. Recently, the phenotype has been linked, in some kindreds, to the beta-myosin heavy chain (β-MHC) gene located on the long arm of chromosome 14 (1–3). Several mutations resulting in substitutions of single evolutionary conserved amino acids in this gene have been described (4–8). These have all been detected in the head and head-rod junction regions of the β-MHC protein. The β-MHC gene, however, has been excluded as the disease locus in other kindreds (3, 9, 10). There is, thus, allelic and nonallelic genetic heterogeneity in HCM.

Evidence exists that β-myosin is also present in the slow fibers of skeletal muscle of some animals (11–13). In this study, we show that normal and mutant cardiac β-MHC gene message is also expressed in skeletal muscle of HCM patients with identified missense mutations in the β-MHC gene. The presence of the mutant cardiac β-MHC message in skeletal muscle indicates that the skeletal slow myosin heavy chain isoform is transcribed from the cardiac β-MHC gene. We further show that mutant β-myosin is present in skeletal muscle and has abnormal function in an in vitro assay in which actin filaments are translocated by myosin bound to a coverslip surface. These results demonstrate that some β-MHC gene mutations in patients with HCM are associated with an abnormal actomyosin interaction which may be the basis of their disease.

Methods

Amplification of exon 23 of β-MHC. DNA was extracted from peripheral blood lymphocytes (14). PCR product for sequencing was performed using intron 22 and intron 23 primers to avoid coamplification of the highly homologous α-MHC gene. We have previously described primers and PCR parameters (7). Sequencing of total PCR product, without subcloning, was performed with a Sequenase kit (U. S. Biochemical Corp., Cleveland, OH) on single-stranded template generated by alkaline denaturation of biotin labeled PCR products bound to streptavidin coated magnetic beads (Dynal, Inc., Great Neck, NY) (3). RNA was extracted (15) from fresh biopsies of skeletal muscle after homogenization in buffer with a Polytron (Brinkman Instruments, Westbury, NY). Reverse transcriptase PCR was performed using the GeneAmp RNA PCR kit (Perkin Elmer Cetus, Norwalk, CT). Random hexamers were used to prime the reverse transcription reaction. Cycle lengths of 30 s and temperatures of 94, 61, and 72°C were used for 30 cycles of denaturation, annealing, and extension, respectively. The 5'primer was 5'-GAGAAGAATGACCTGCAGCTCC-3'. The 3' primer, 5'-AGCTTGGCAATGATCTCATCC-3', had a 40-bp guanine-cytosine clamp affixed to the 5' end. The products digested by PvuII (BRL, Gaithersburg, MD) was electrophoresed by 10% PAGE and stained with ethidium bromide.

Amplification of exon 8 of β-MHC. The primers were chosen from intron 7 and intron 8. They were: 5'-CTGCCCTCCAAGGTCCTGTACC-3' and 5'-CCATTCCTCCACCAGTCCAAGTC-3', respectively. The cDNA library was constructed from skeletal muscle RNA using the Uni-ZAP vector (Stratagene Inc., La Jolla, CA). It was sequenced, in part, using the Sequenase kit (Stratagene Inc.) according to the double-stranded sequencing protocol of the manufacturer.

Purification of proteins. All procedures were performed at 4°C. Myosin was purified from human skeletal or cardiac muscle biopsies. Briefly, 100–150 mg of frozen tissue was pulverized and washed three times in 5 vol of buffer A containing 20 mM Mops (pH 7.0), 40 mM

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1. *Abbreviations used in this paper:* β-MHC, beta-myosin heavy chain; HCM, hypertrophic cardiomyopathy.

KCl, 5 mM EGTA, 2 mM DTT, 0.01% NaN_3, 0.1 mM PMSF, 10 mg/liter pepstatin, 10 mg/liter tosyl-L-phenylalamine chloromethyl ketone, 10 mg/liter leupeptin, 5 mg/liter chymostatin, and 10 mg/liter tosyl-L-lysine chloromethyl ketone. To purify myosin, the washed myofibrils were resuspended in buffer A and extracted for 15 min by the addition of NaCl, ATP, and $MgCl_2$ to final concentrations of 0.5 M, 10 mM, and 10 mM, respectively. The sample was sedimented for 15 min at 470,000 *g* using an ultracentrifuge (TL100; Beckman Instruments, Inc., Palo Alto, CA). Rabbit skeletal muscle actin was prepared as previously described (16).

Preparation of antibodies. To specifically recognize the β-MHC isoform from skeletal muscle tissue, a polyclonal antiserum was raised against a synthetic peptide corresponding to the unique carboxyl-terminus of the β-MHC sequence (amino acids 1921–1935, reference 17) and the specific IgG fraction was purified by affinity chromatography in which the antigen was coupled to Affi-Gel 15 (Bio-Rad Laboratories, Richmond, CA) according to manufacturer's instructions. The IgG did not cross-react with the fast skeletal MHC isoform, nor with the cardiac α-MHC in a Western blot analysis.

Another antibody was made to identify the expression of the mutated $403^{Arg \rightarrow Gln}$ β-MHC in the skeletal muscle tissue of HCM patients. This antibody was raised against a synthetic peptide (amino acids 392–402, reference 14) of the β-MHC sequence (which does not include the mutated residue) and purified as described above.

Identification of mutated β-MHC from skeletal muscle tissue. Human soleus muscle myosin was electrophoresed on 5% SDS-PAGE containing a 30:0.4 ratio of acrylamide:bis acrylamide, which allows the separation of the fast from the β/slow MHC isoform. The β-MHC band was excised from the gel, electroeluted using a micro-electroelutor (Amicon Corp., Danvers, MA), concentrated to a final volume of 50–100 μl and extensively digested with 2 mg/ml endoproteinase Arg-C (Calbiochem-Behring Corp., San Diego, CA). The digest was concentrated to 50-μl final vol, electrophoresed on a 20% SDS-PAGE, and blotted onto a PVDF membrane (Millipore Corp., Bedford, MA) using a Milliblot-SDE apparatus (Millipore). The blot was probed with the rabbit polyclonal IgG raised against a synthetic peptide extending from residues 392–402 in the β-MHC sequence.

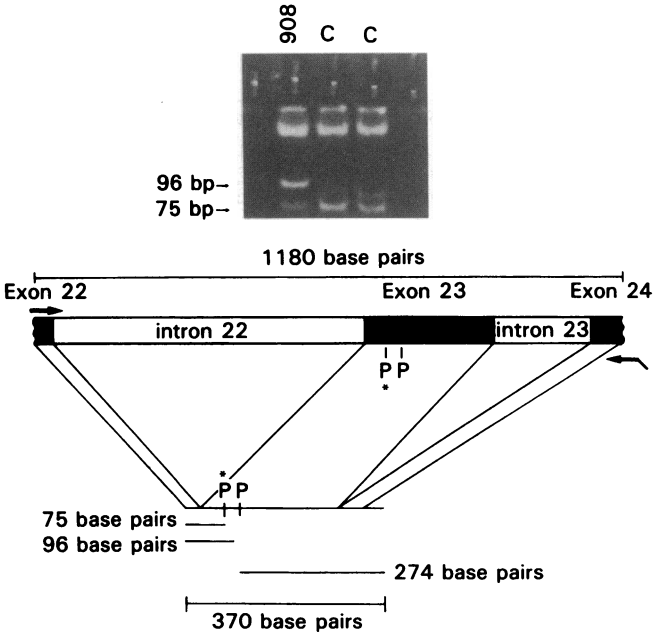

Figure 1. A PvuII digest of reverse transcriptase PCR fragments, amplified from skeletal muscle RNA, encompassing the 908 amino acid residue mutation in the β-MHC gene of the same affected individual and two controls. Below is a genomic map of the region showing the location of the two PCR primers, marked with horizontal arrows, which generated a 370-bp fragment from RNA, easily distinguished from a 1,180-bp fragment obtained from DNA. One of the PvuII sites in exon 23 is destroyed by the mutation and upon digestion yields an aberrant 96-bp fragment present only in the affected individual together with the predicted normal 75-bp fragment. P, PvuII site; *, PvuII site destroyed by the 908 Leu → Val mutation.

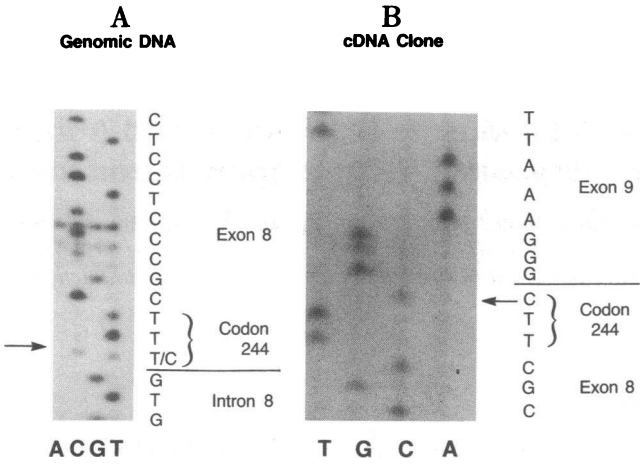

Figure 2. (*A*) A portion of a sequence from genomic DNA showing a silent mutation in codon $244^{o\ Phe}$ of exon 8 in the β-MHC gene of a patient with the 908 Leu → Val mutation. The arrow marks both a T and a C which are present in this patient, who is a heterozygote for the $244^{o\ Phe}$ mutation. (*B*) A portion of sequence of a clone obtained from a cDNA library constructed from skeletal muscle of the same individual. Only the C is present, demonstrating the segregation of one of the two alleles shown in genomic sequence of the β-MHC gene in *A*.

In vitro motility assay. The in vitro motility assay was performed in two different ways. In some instances either pure cardiac β-myosin or soleus skeletal muscle myosin was bound to the nitrocellulose-coated surface in monomeric form as described in Umemoto and Sellers (18). To selectively study the motility of β-myosin from soleus muscle without interference from the contaminating fast myosin, the anti-β-MHC COOH-terminal-specific antibody was used to affinity select the β-myosin. In this case the β-myosin-specific IgG was first introduced into the motility assay flow cell at a concentration of 0.1–0.5 mg/ml in buffer B containing 0.5 M NaCl, 10 mM Mops (pH 7.0), 0.1 mM EGTA, 1 mM DTT, and allowed to incubate for 20 min. The unbound antibody was washed out and the surface was coated with 0.5 mg/ml BSA in buffer B. After 1 min, soleus muscle myosin was added in buffer B. The same buffer was used, after 5 min, to remove the unbound material, which contains the fast myosin isoform. The in vitro motility assay was otherwise performed and the results quantified essentially according to Homsher et al. (19). The composition of the assay buffer was 20 mM KCl, 10 mM Mops (pH 7.2), 5 mM $MgCl_2$, 1 mM ATP, 0.1 mM EGTA, 10 mM DTT, 0.7% methylcellulose, 2.5 mg/ml glucose, 0.1 mg/ml glucose oxidase, and 0.02 mg/ml catalase.

Specificity of binding of β-myosin to the coverslip was determined by SDS-PAGE analysis of the unbound protein after washout and of the protein bound to the nitrocellulose surface following extraction of the surface with 2% SDS sample buffer.

Results

Expression of the β-MHC gene message in skeletal muscle. Skeletal muscle RNA was obtained from members of two kindreds in which we previously linked missense mutations in the genomic sequence of the cardiac β-MHC gene to HCM (3, 7). A C → G transversion was detected in a PCR fragment,

A

Arg-C Proteolytic Fragment of Wild Type Sequence - 3.5 kDa:

-EEQAEPDGTEEADKSAYLMGLNSADLLGGLCHP**R**-
↑ ↑
Arg-C Arg-C

B

Arg-C Proteolytic Fragment of 403$^{Arg \rightarrow Gln}$ Mutant Sequence - 6.6 kDa:

-EEQAEPDGTEEADKSAYLMGLNSADLLGGLCHP**Q**VKVGNEYVTKGQNVQQVIYATGALAKAVYER-
↑ ↑
Arg-C Arg-C

Figure 3. Schematic showing the wild-type (*A*) and mutant (*B*) sequences following enodoproteinase Arg-C cleavage. Cleavage sites are shown by arrows. The Arg → Gln substitution at amino acid residue 403 destroys a cleavage site, resulting in longer (6.6 kD vs 3.5 kD) sequence.

amplified from genomic DNA of an affected member of kindred 2755 (7). The mutation results in a Leu → Val substitution in codon 908 and destroys a PvuII site. Hence, a reverse transcriptase PCR fragment, amplified from skeletal muscle RNA of an affected member, yielded an aberrant band when digested with PvuII (Fig. 1). Comparison of the normal and aberrant bands shows that the ratio of mutant to normal message in pectoralis muscle is > 50%. Multiple cDNA clones from a library constructed from the same skeletal muscle RNA were sequenced in part, and matched the previously published cardiac β-MHC gene sequence obtained from cardiac cDNA (17) and a genomic clone (20), (data not shown). Sequence of a PCR fragment encompassing codon 244^{Phe} amplified from genomic DNA of the same individual shows the two alleles (C and T) of a polymorphism reflecting a silent mutation (Fig. 2 *A*). A cDNA clone from this individual contained the 908$^{Leu \rightarrow Val}$ mutation which cosegregated with the C in codon 244^{Phe} (Fig. 2 *B*), further confirming that the β-MHC message in human skeletal muscle is transcribed from the cardiac β-MHC gene on chromosome 14q.

Expression of normal and mutant β-myosin in the slow, type I, skeletal muscle fibers in a patient with the 403 Arg → Gln mutation. Loss of arginine in this mutation allowed discrimination of mutant from normal β-MHC by digestion with endoproteinase Arg-C, which specifically cleaves at the carboxyl-terminal site of arginine residues. An antibody was raised against a peptide corresponding to amino acid residues 392–402. In the control unmutated myosin the Arg-C peptide recognized by this antibody should have a molecular mass of ~ 3.5 kD, whereas in the 403$^{Arg \rightarrow Gln}$ mutation, the loss of Arg would result in a new, larger peptide with a molecular mass of 6.6 kD (Fig. 3).

Beta-myosin was extracted from soleus muscle biopsies of HCM patients with the 403$^{Arg \rightarrow Gln}$ mutation and normal controls. The β-myosin heavy chain was separated from the fast-myosin heavy chain isoform by 5% SDS-PAGE. The band corresponding to β-MHC was excised from the gel and extensively digested with endoproteinase Arg-C. The sample was then applied on a 20% SDS-PAGE, which allows the separation of very low molecular weight peptides, and was blotted to a PVDF membrane and probed with the antibody. A single peptide at the expected size of 3.5 kD was detected in control myosin, while an extra peptide of 6.6 kD, together with the wild-type peptide, was observed in the digested β-myosin from HCM patients due to the replacement of Gln for Arg at residue 403 (Fig. 4).

In vitro motility assay studies. Skeletal muscle biopsies from patients with identified mutations of the β-MHC gene were used to study the pathophysiology of the mutant myosin in an in vitro motility assay (21). This assay measures the rate of sliding of rhodamine phalloidin-labeled actin filaments translocated by myosin molecules bound to a nitrocellulose-coated surface. To study the function of β-myosin from individuals with the 908$^{Leu \rightarrow Val}$ and 403$^{Arg \rightarrow Gln}$ mutations, this isoform had to be separated from the fast-myosin isoform, also present in skeletal muscle. Beta-myosin was first enriched through the choice of the soleus muscle, as it contains 75–90% slow fibers. An antibody raised to the unique carboxyl-terminal end of β-

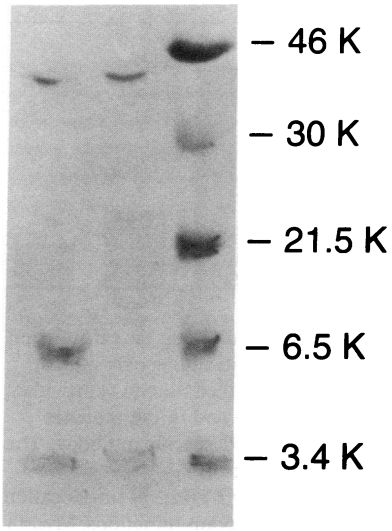

Figure 4. The 403 Arg → Gln mutant β-MHC is present in human soleus muscle. The Arg → Gln substitution at residue 403 allows for detection of mutant protein when the β-MHC is digested with arginine specific endoproteinase Arg-C. An antibody to a synthetic peptide flanking the mutation recognizes in Western blot the predicted 3.5-kD peptide in β-MHC from a normal individual (lane *B*). In the affected heterozygous individual an aberrant 6.6-kD peptide resulting from the loss of Arg at residue 403 as well as the normal 3.5-kD peptide is recognized (lane *A*). Prestained low molecular mass standards (Amersham Corp., Arlington Heights, IL) are shown in lane *C*.

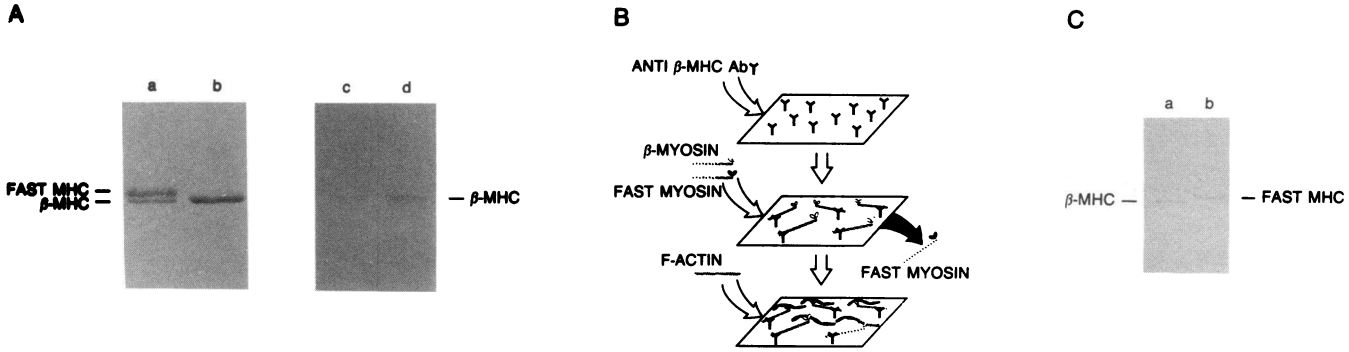

Figure 5. Use of anti-β-MHC carboxyl-terminal specific IgG to immunoabsorb β-myosin for in vitro motility assays. (*A*) 5% SDS-PAGE (*a, b*) and Western blot (*c, d*) probed with an antibody prepared against a synthetic peptide (RAKSRDIGTKGLNEE) derived from the unique carboxyl-terminal region of β-MHC. Lanes *a* and *c* contain myosin from soleus muscle; lanes *b* and *d* contain myosin from human ventricle. (*B*) Schematic diagram showing the strategy for using the carboxyl-terminal β-MHC specific antibody for immunoabsorbing the β-myosin isoform from myosin purified from soleus muscle which is a mixture of fast skeletal and β-myosin isoforms. (*C*) 5% SDS-PAGE showing the myosin that is bound to the coverslip via the carboxyl-terminal β-MHC specific antibody (lane *a*) and the flow through containing the fast skeletal muscle myosin isoform.

MHC sequence was used to immunoabsorb the β-myosin to the nitrocellulose-coated surface. This antibody specifically detects, in a Western blot analysis of myosin from skeletal and cardiac muscle, only the β-MHC (Fig. 5, *A* and *B*). SDS-PAGE analysis of both the flow through and immunoabsorbed fractions confirmed that only β-myosin was bound to the antibody-coated surface (Fig. 5 *C*). Immunoabsorbed β-myosin from normal individuals translocated actin filaments at a rate of 0.49±0.04 μm/s. The actin sliding velocity in individuals with the $908^{Leu \rightarrow Val}$ or the $403^{Arg \rightarrow Gln}$ mutations were only 0.17±0.01 and 0.09±0.02 μm/s, respectively (Fig. 6). Since both mutant and normal beta myosins were present, the effect of the mutation may be even larger than observed. The antibody did not affect the rate of actin filament translocation by pure β-myosin obtained from cardiac ventricle (Fig. 6).

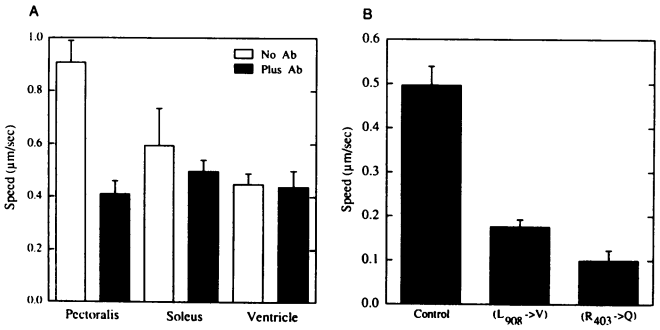

Figure 6. Mutations at residue 403 and 908 decrease the rate of actin filament sliding. (*A*) Velocity of actin filaments sliding over human pectoralis and soleus muscle myosin and purified human ventricular myosin, assayed in the absence (*white bars*) and in the presence (*black bars*) of the carboxyl-terminal β-MHC specific antibody. The data are expressed as the mean±SD of the rate of 20–100 actin filaments in a single representative experiment. (*B*) Rate of translocation of actin filaments over myosins with distinct point mutations. Myosin preparations from four individuals with the $403^{Arg \rightarrow Gln}$ mutation and five individuals with the $908^{Leu \rightarrow Val}$ mutations were analyzed. Myosin from five normal subjects were used as controls.

Discussion

We have determined that a mutant β-cardiac myosin is expressed in skeletal muscle of HCM patients with identified β-MHC mutations and has abnormal function. This strengthens the hypothesis that β-MHC mutations are indeed responsible for the disease in some kindreds. The consistency of the findings is underscored by the fact that two unrelated kindreds (Western European versus Asian origin) with the same $403^{Arg \rightarrow Gln}$ mutation had the same degree of abnormal myosin function. The cardiac findings in the two kindreds with the $403^{Arg \rightarrow Gln}$ and $908^{Leu \rightarrow Val}$ mutations have been similar. However, the natural histories are markedly different: the $403^{Arg \rightarrow Gln}$ mutation is characterized by 100% penetrance in adults, early onset, and a high frequency of sudden death (7). In contrast, in the $908^{Leu \rightarrow Val}$ mutation, HCM is not apparent in childhood, the penetrance is 63% in adults, and there is a low frequency of sudden death (7). Despite these differences, myosin from patients with either mutation translocates actin filaments with similar velocities in the in vitro motility assay. This may be due, in part, to the nature of the motility assay in its measurement of an unloaded velocity (19). The quantities of pure β-myosin isolated precluded other biochemical analyses such as ATPase activity or actin interaction. Measurement of these parameters and others such as loaded tension or ATP consumption may further discriminate between the functions of these two mutant β-myosins compared to normal myosin. Our experiments suggest that, in individuals with myosin-associated HCM, the disease may be explained by abnormal actomyosin interactions resulting in decreased myosin function and that the hypertrophy may be compensatory.

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