Functional Analysis of Myosin Mutations That Cause Familial Hypertrophic Cardiomyopathy

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ABSTRACT We have studied the actin-activated ATPase activities of three mutations in the motor domain of the myosin heavy chain that cause familial hypertrophic cardiomyopathy. We placed these mutations in rodent α-cardiac myosin to establish the relevance of using rodent systems for studying the biochemical mechanisms of the human disease. We also wished to determine whether the biochemical defects in these mutant alleles correlate with the severity of the clinical phenotype of patients with these alleles. We expressed histidine-tagged rat cardiac myosin motor domains along with rat ventricular light chain 1 in mammalian COS cells. Those myosins studied were wild-type α-cardiac and three mutations in the α-cardiac myosin heavy chain head ($Arg^{249}Gln$, $Arg^{403}Gln$, and $Val^{606}Met$). These mutations in human β-cardiac myosin heavy chain have predominantly moderate, severe, and mild clinical phenotypes, respectively. The crystal structure of the skeletal myosin head shows that the $Arg^{249}Gln$ mutation is near the ATP-binding site and the $Arg^{403}Gln$ and $Val^{606}Met$ mutations are in the actin-binding region. Expressed histidine-tagged α-motor domains retain physiological ATPase properties similar to those derived from cardiac tissue. All three myosin mutants show defects in the ATPase activity, with the degree of enzymatic impairment of the mutant myosins correlated with the clinical phenotype of patients with the disease caused by the corresponding mutation.

INTRODUCTION

Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant cardiac disease characterized by asymmetrical interventricular and left ventricular hypertrophy and myocellular disarray (Davies, 1984; Maron et al., 1987). The disease is clinically variable, ranging from benign to severe, and often results in sudden death in young athletes (Maron et al., 1978, 1986). FHC is also genetically heterogeneous, because it is caused by multiple mutations in at least seven different genes (Solomon et al., 1990; Dausse and Schwartz, 1993; Watkins et al., 1995c). All mutations found to cause FHC are structural proteins of the sarcomere: β-myosin heavy chain (MyHC) (Geisterfer-Lowrance et al., 1990; Watkins et al., 1993), α-tropomyosin (Watkins et al., 1995b), troponin T (Thierfelder et al., 1994), myosin binding protein-C (Watkins et al., 1995a; Bonne et al., 1995), ventricular light chains, light chain 1 (VLC1) and ventricular light chain 2 (VLC2) (Poetter et al., 1996), and troponin I (Kimura et al., 1997). The fact that all FHC-causing genes thus far identified are structural proteins suggests that FHC is a disease of the sarcomere (Thierfelder et al., 1994).

Over 40 different MyHC alleles have been described (reviewed in Vikstrom and Leinwand, 1996). These mutations account for ~30% of the reported FHC cases (Watkins et al., 1995c). Some of these mutations appear in clusters near functionally significant regions of the myosin motor domain (S1), such as the actin-binding domain, the ATP catalytic domain, and the LC-binding domain (Fig. 1) (Rayment et al., 1995). A number of mutations have also been described at the junction between S1 and the S2 (Rayment et al., 1995). It is becoming increasingly clear that specific amino acid residues play crucial roles in the ATPase function of the myosin molecule (reviewed in Rayment et al., 1996). Studies with the crystal structure of S1 and actin have led to the proposal that specific residues, loops, α-helices, and β-sheets may be directly involved in the mechanism of force transduction (reviewed by Vale, 1996; Milligan, 1996). Interestingly, none of the known FHC myosin mutations occur in proposed critical regions of force production, but the proximity of some of the mutations suggests that the motor function of the mutant myosin may be impaired.

The FHC mutations in this study ($Arg^{249}Gln$, $Arg^{403}Gln$, and $Val^{606}Met$, highlighted in Fig. 1) were chosen to attempt to identify the biochemical lesion and to determine whether the lesion correlates with the clinical prognosis. The $Arg^{249}Gln$ mutation has a moderate phenotype with respect to incidence and age of sudden death (Watkins et al., 1992). The $Arg^{403}Gln$ mutation is associated with a high frequency of sudden death in young adults and heart failure of adults in their fourth decade (Epstein et al., 1992; Watkins et al., 1992; Marian et al., 1995a). The $Val^{606}Met$ mutation is typically associated with a benign form of the disease, and most patients survive beyond the sixth decade (Watkins et al., 1992; Marian et al., 1995a). However, in another study it was reported that 50% of the patients with this allele in another family died between the ages of 15 and 27 years, suggesting a poor survival rate for $Val^{606}Met$ mutation in that family (Fananapazir and Epstein, 1994). These data make it clear that genetic modifiers undoubtedly exist and affect the clinical outcome of diseased patients.

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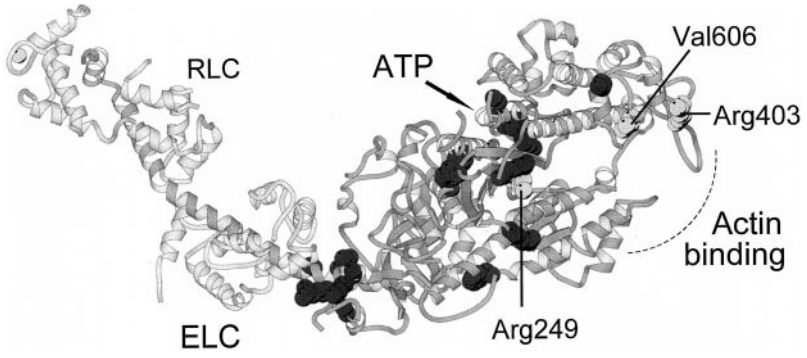

FIGURE 1 The crystal structure of chicken skeletal S1 showing the location of 21 FHC mutations (Rayment et al., 1995). The mutations are located near the ATP-, actin-, and light chain-binding region of the myosin head (discussed in Rayment et al., 1995). The residues in gray and Arg^{249}, Arg^{403}, and Val^{606} are those studied in this paper. The figure was prepared using the program MOLSCRIPT, version 2.0 (Kraulis, 1991).

However, possible general prognostic implications can be based on the identified alleles, and it is important to determine whether it is possible to correlate the clinical phenotype with the biochemical defects.

We studied the functional effects of three FHC mutations by creating mutations in rat cardiac α-S1 because of our interest in studying the FHC disease by using cultured rodent cardiac myocytes and transgenic rodent models (see Vikstrom et al., 1996). The dominant myosin isoenzyme of the left ventricle is α-MyHC in rodents and β-MyHC in humans. The amino acid identity between the motor domain of the rat cardiac α-MyHC and human β-MyHC is 93%. None of the FHC mutations that we are studying occur in sequences that are different, either between α- and β-MyHC or between rodent and human MyHC. A transgenic rodent model of FHC has shown that the presence of the Arg^{403}Gln mutation in the cardiac α-MyHC results in some phenotypic characteristics of the human Arg^{403}Gln-β-MyHC FHC disease (Geisterfer-Lowrance et al., 1996). This mutation, in the context of an additional mutation in the actin-binding domain, results in additional phenotypic similarities such as significant left ventricular hypertrophy (Vikstrom et al., 1996). Results obtained in transgenic mice expressing FHC mutations in the α cardiac MyHC suggest that the same mutation in either α or β MyHC may have similar functional defects.

In the present study, we have expressed and purified rat α-cardiac myosin S1, Arg^{249}Gln-, Arg^{403}Gln-, and Val^{606}Met-S1 mutants in cultured mammalian cells. We engineered a histidine tag at the C-terminal of S1 for affinity purification with a nickel-bound agarose. We report the V_{max} and K_m of the actin-activated ATPase activities of the expressed myosin S1s (a preliminary account was reported in abstract form in Roopnarine and Leinwand, 1996, 1997). Our results are consistent with other biochemical studies of these FHC alleles from an insect cell expression system of either rat α-cardiac HMM (Sweeney et al., 1994) or human β-cardiac HMM (Sata and Ikebe, 1996).

MATERIALS AND METHODS

Reagents

Oligonucleotides for the construction of S1 C-terminus with a histidine peptide were purchased from Gibco-BRL (Gaithersburg, MD). Ni-NTA agarose resin and DNA plasmid purification kits were purchased from Qiagen (Chatsworth, CA). Reagent-grade chemicals were purchased from Sigma (St. Louis, MO) and Fisher Scientific (Pittsburgh, PA).

Plasmid construction

Rat α-MyHC cDNA was previously cloned into the pMT21 expression vector to yield pMTα expression vector (Vikstrom et al., 1993). The FHC missense mutations were previously introduced into the α-MyHC sequence (Straceski et al., 1994). Cardiac α-S1 DNA plasmids were designed by inserting eight histidines in α-S1, α-Arg^{249}Gln-, α-Arg^{403}Gln-, and α-Val^{606}Met-S1, followed by a stop codon after the 808 a.a. in the full-length MyHC DNA sequence. The His tag is used to purify the S1 from COS cells by using the Ni-NTA agarose resin. DNA fragments encoding the C-terminal + His tag were synthesized by polymerase chain reaction methodology, cloned into PCR-Script vector (Stratagene, La Jolla, CA), sequenced with a Sequenase reagent kit (USB, Cleveland, OH), and subcloned into pMTα expression vector. Rat VLC1 cDNA was cloned into pRC-RSV (Invitrogen, Carlsbad, CA), an expression vector with a Rous sarcoma virus promoter (cloned by Dr. Robert E. Welikson, Cornell University).

Purification of proteins

The cardiac S1s were expressed in a mammalian cell line from African green monkey kidney (COS-1 cells), using DEAE-dextran transfection to introduce the DNA into the cells (Straceski et al., 1994). For each S1 construct, 30 100-mm culture dishes at 50% confluency of COS cells were transiently transfected and cultured at 37°C for ~60 h. Each dish was transfected with the S1 (12 μg) and VLC1 (6 μg) plasmids at a ratio of 2:1, which gave the highest number of cells expressing both S1 and VLC1 in the same cell.

The COS cells were washed with phosphate-buffered saline solution (0.17 M NaCl, 6 mM potassium phosphate, 3 mM KCl) to remove serum proteins, then incubated with lysis solution (50 mM sodium phosphate (pH 8.0), 50 mM NaCl, protease inhibitors (2 μg/ml of leupetin, aprotinin, chymostatin, and pepstatin), 0.1 mM phenylmethylsulfonyl fluoride, 7 mM β-mercaptoethanol (β-ME), 0.5% Triton X-100, 1 mM $MgCl_2$, and 1 mM NaN_3) on ice for 30 min. The cells were scraped off the dishes, sonicated for 10 s, incubated in 5 mM MgATP for 10 min, and then centrifuged at 100,000 rpm to remove cellular debris, nonmuscle myosin filaments, and nonmuscle actin. The S1 in the supernatant was purified on Ni-NTA resin by batch purification at 4°C. The supernatant was mixed with nickel-nitrilo-triacetic acid (Ni-NTA) agarose resin for 2–3 h and centrifuged at 5000 $\times$ g for 5 min to pellet S1-bound resin. The S1-bound resin was then washed twice (10 min each) with a high-ionic-strength solution (50 mM sodium phosphate (pH 7.0), 0.5 M NaCl, 1 mM $MgCl_2$, 0.5 mM β-ME), twice with a low-ionic-strength solution (5 mM sodium phosphate (pH 7.0), 50 mM NaCl, 1 mM $MgCl_2$, 0.5 mM β-ME) and eluted with 0.5 M imidazole (pH 7.0) in the previous buffered solution. The purified S1 was concentrated with a Amicon Centricon concentrator (mol. wt. 10,000) and

dialyzed in S1 solution (S1S: 5 mM imidazole (pH 7.0), 1 mM EGTA, 2 mM $MgCl_2$, and 0.1 mM dithiothreitol) at 4°C. The S1 sample was centrifuged at 14,000 × *g* in an Eppendorf centrifuge for 10 min at 4°C to remove residual nonmuscle myosin filaments. Rabbit skeletal acetone powder was prepared as previously described (Thomas et al., 1979) (some acetone powder was a gift from Dr. David D. Thomas and Dr. John J. Matta, University of Minnesota, Minneapolis, MN). F-actin was prepared as described by Thomas et al., (1979) and was dialyzed before the ATPase assays twice with S1S for 3 h to reduce ATP hydrolysis by actin during the assays.

Total cellular protein concentration was determined with the Biorad DC colorimetric assay. The S1 concentration was determined by either Western blots or slot blots, using an antibody specific to sarcomeric myosin as described below. The concentration of G-actin was determined spectrophotometrically from the expression $(A_{290} - A_{320})/E_{290}$ (where E_{290} is the extinction coefficient (1 mg/ml = 0.63) (Thomas et al., 1979). Molar concentrations were determined using molecular weights of 120,000 for cardiac chymotryptic S1 and 42,000 for actin (Thomas et al., 1979).

Detection of proteins

Cells grown on glass coverslips were prepared for immunofluorescence as previously described (Straceski et al., 1994). The following antibodies were used: sarcomeric MyHC specific monoclonal antibody, F59 (a gift from Frank Stockdale, Stanford University), or rabbit polyclonal MyHC antibody (made by Dr. Art S. Rovner, University of Vermont College of Medicine, Burlington, VT); nonmuscle myosin rabbit polyclonal antibody (a gift from Dr. Robert Adelstein, National Institutes of Health, Bethesda, MD); LC specific monoclonal antibody, F310. Immunofluorescence was detected with a secondary antibody (goat anti-mouse IgG) conjugated to either fluorescein or rhodamine. Colocalization of S1 and VLC1 was determined by immunoprecipitation of purified expressed S1 proteins and cardiac myosin (as a control) with a polyclonal myosin antibody and protein A (Sigma), as described by L'Ecuyer and Fulton (1993) for Staph A immunoprecipitation (except that the samples were not radioactive and magnetic separation was not used).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (7% acrylamide for myosin and S1 resolution, 12% acrylamide for LC1 resolution, and 7–12% acrylamide gradient for both S1 and LC1) and Western blots (Straceski et al., 1994) were used to determine the approximate molecular weights of the myosin fragments. The primary antibodies described above and secondary antibodies (goat anti-mouse horseradish peroxidase) were used to visualize the immunoblotted proteins with chemiluminescence reagents (Amersham, Arlington Heights, IL). Dog cardiac chymotryptic S1 (a gift from Dr. Sarkis Margossian, Albany Medical College, Albany, NY) was used to determine the concentration of purified S1 on either Western or slot blots. Protein quantitation was done with a Biorad gel documentation system (Gel Doc 1000) controlled by the Biorad Molecular Analyst program.

ATPase assays

The actin-activated ATPases were done in S1S (~15 μM ionic strength) at 25°C in a range of actin concentrations (10–100 μM). Reaction aliquots were removed at 1-, 3-, and 5-min intervals, quenched in 1 M HCl, centrifuged at 14,000 × *g*, and the supernatants were assayed for inorganic phosphate from the hydrolysis of ATP, using the malachite green colorimetric assay as described by Lanzetta et al. (1979). The ATPase activities were calculated in s^{-1}. The Mg-ATPase activity (in the absence of actin) was subtracted from each actin-activated ATPase. The V_{max} (maximum ATPase rate) and the K_m (the actin concentration where the ATPase activity is half V_{max}) were determined from the ordinate and abscissa intercepts of double-reciprocal plots of 1/ATPase activity (s^{-1}) versus 1/[actin] (μM). The reported values are the mean ± SD of either four (for α- and Arg^{403}Gln-S1) or three (Arg^{249}Gln- and Val^{606}Met-S1) different preparations. The hydrolysis of ATP by control samples of untransfected cells was minimal. In some cases contaminating nonmuscle myosin in the samples resulted in some hydrolysis of ATP, which was subtracted from the S1 samples before calculation of ATPase values.

RESULTS

The strategy for biochemically characterizing myosin motor domains was to cotransfect COS cells with expression constructs for S1 of the cardiac MyHC and myosin VLC1. Affinity chromatography was used to isolate the heavy chain and its associated LC1, and this complex was then assayed for ATPase activity over a range of actin concentrations. This approach depends on obtaining motor domains with an appropriate 1:1 stoichiometry of heavy chains: light chains. We chose to express amino acids 1–808 of rat α-cardiac MyHC because it was previously shown to bind to VLC1 with appropriate stoichiometry and to have a molecular weight similar to that of chymotryptically cleaved myosin S1 from heart tissue (McNally et al., 1991). Cotransfection of S1 and VLC1 cDNAs resulted in expression of both S1 and VLC1 in the same cells (Fig. 2). There was no obvious difference in the immunofluorescence staining pattern between wild-type S1 and FHC mutant S1 in the COS cells (not shown).

The expressed wild-type, FHC-mutant S1's, and VLC1 had molecular masses similar to that of chymotryptic S1 prepared from heart tissue (~120 kDa for S1 and ~30 kDa for VLC1) and migrated as single protein bands on a West-

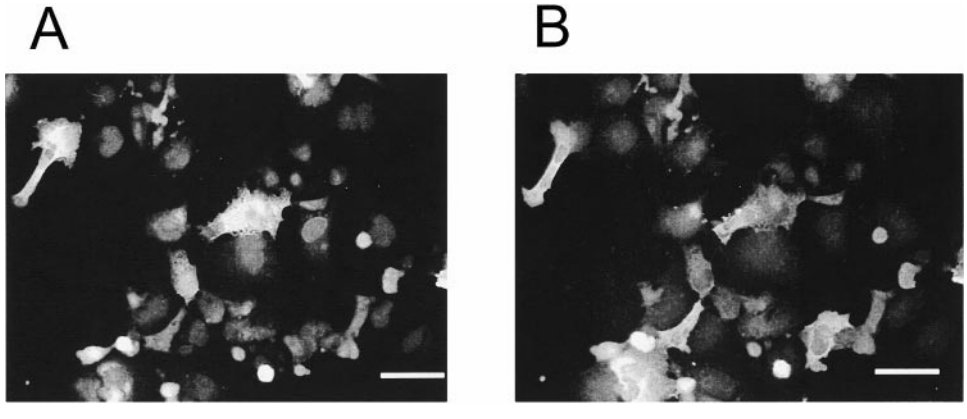

FIGURE 2 Indirect immunofluorescence detection of S1 and VLC1 cotransfected in COS cells. Antibodies against sarcomeric MyHC (*A*) and VLC1 (*B*) were used to visualize the expressed proteins. Bar = 100 μM.

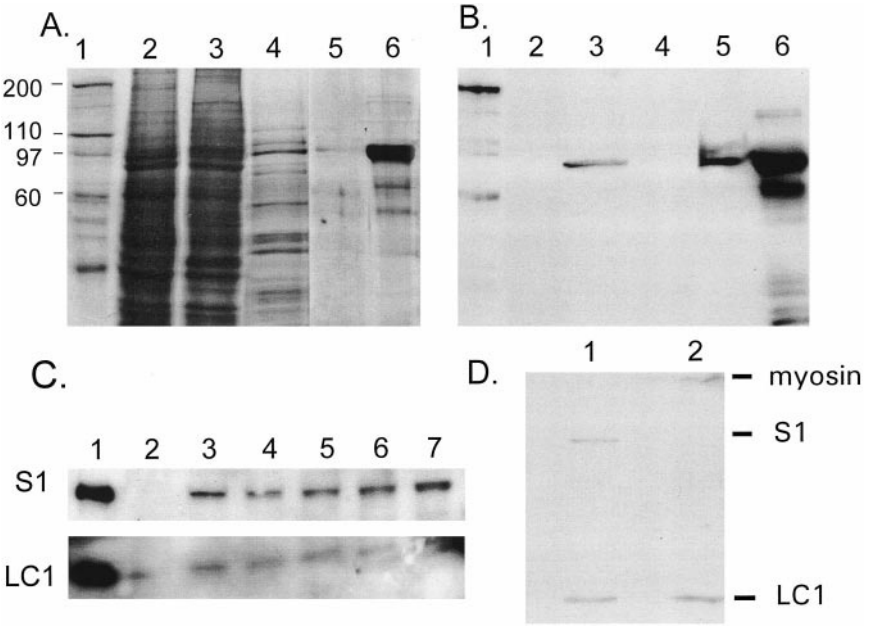

FIGURE 3 Purification and immunoprecipitation of expressed S1s. (*A*) Coomassie and (*B*) Western blot of 7% SDS-PAGE showing purification of S1. *Lane 1*: High-molecular-weight markers; *lane 2*: untransfected COS lysate (COS control); *lane 3*: S1 and LC1 transfected COS lysate; *lane 4*: supernatant after reacting S1 with Ni-NTA agarose resin; *lane 5*: S1 eluted from resin with 0.5 M imidazole; *lane 6*: cardiac S1 from dog heart. (*C*) Western blot of 7–12% gradient SDS-PAGE of resin-purified samples showing presence of S1 and LC1. *Lane 1*: Dog cardiac S1; *lane 2*: COS control; *lane 3*: α-S1; *lane 4*: β-S1; *lanes 5 and 6*: Arg^{249}Gln-, Arg^{403}Gln-, and Val^{606}Met-S1, respectively. (*D*) Western blot of 7–12% gradient SDS-PAGE of immunoprecipitated resin-purified expressed α-S1 (*lane 1*) and cardiac myosin (*lane 2*) probed with a sarcomeric myosin antibody and monoclonal light-chain antibody.

ern blot of a SDS-PAGE (Fig. 3, *B* and *C*). The similarity in the quantity of purified S1 between wild-type and mutant S1 samples suggests that the stability of the protein is not grossly affected by the FHC mutations, as previously observed for full-length wild-type and FHC mutant MyHC (Straceski et al., 1994). Quantitation of expressed proteins showed that a 100-mm culture dish had ~1–2 μg of expressed S1 per 2 mg of total cellular protein. The major reasons for the limited quantity of expressed proteins from COS cells were that 1) the transfection efficiency of plasmid DNA was relatively low (~40%), 2) the cells were grown on a monolayer (by necessity) instead of suspension media, and 3) the expression of high levels of sarcomeric myosin in nonmuscle cells appears to have some toxicity because attempts to establish stable cell lines have been unsuccessful. The supernatant from the mixture of COS cell lysate and Ni-NTA agarose resin did not show any S1, suggesting that all of the S1 bound to the resin (Fig. 3, *A* and *B*, *lane 4*). The resin-bound S1 was eluted with 0.5 M imidazole (Fig. 3, *A* and *B*, *lane 5*). Affinity purification of expressed S1 with the Ni-NTA agarose resin resulted in similar yields of wild-type and mutant S1 (not shown). Coomassie blue staining of the resin-purified samples showed that several other contaminating proteins were copurified (Fig. 3 *A*, *lane 5*). VLC1 is copurified with the resin-purified S1 (Fig. 3 *C*, *lower panel*). Immunoprecipitation with an anti-myosin antibody showed that VLC1 is associated with S1 (Fig. 3 *D*, *lane 1*). Quantitation of the proteins on the Western blot indicates that the stoichiometry of S1:VLC1 was 1:1, similar to that of full-length myosin (Fig. 3 *D*, *lane 2*). We previously showed that there is minimal association between the nonmuscle light chains and introduced muscle MyHC in this expression system (Vikstrom et al., 1993).

The main advantage of using S1 rather than the full-length myosin is that it is soluble under the low-ionic-strength conditions that are required for optimal S1-actin binding during the actin-activated ATPases. The V_{max} of the actin-activated ATPase of the expressed α-S1 was 11.61 ± 0.5 s^{-1} (Fig. 4, *left*), and the K_m was 13.28 ± 0.56 μM. This agrees well with ATPase values of tissue purified rat cardiac S1 that had a V_{max} of 14.3 s^{-1} and a K_m of 12.20 μM (Lauer et al., 1989). The V_{max} of Arg^{249}Gln-, Arg^{403}Gln-, and Val^{606}Met-S1 were 6.82 ± 0.19 s^{-1}, 3.29 ± 0.36 s^{-1}, 9.93 ± 0.42 s^{-1}, respectively (Fig. 4, *left*). Similarly, the K_m of the FHC mutations were also inhibited, as reflected in an increase compared to α-S1 of 24.5 ± 1.5 μM, 45.4 ± 0.5

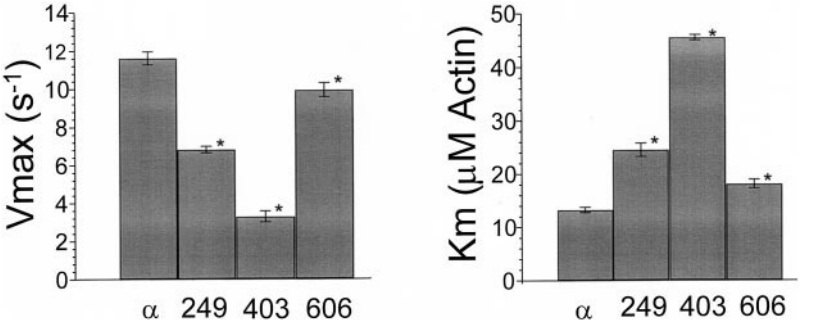

FIGURE 4 Actin-activated ATPases of expressed S1s. V_{max} (*left*) and K_m (*right*) of actin-activated ATPases of α-, Arg^{249}Gln-, Arg^{403}Gln-, and Val^{606}Met-S1 purified from COS cells. The values are the mean ± SD of three or four independent data sets. *For V_{max} and K_m values $p < 0.005$ (paired Student-*t*-test) compared with wild-type expressed α-S1.

μM, and 18.3 ± 1.0 μM, for Arg^{249}Gln-, Arg^{403}Gln, and Val^{606}Met, respectively (Fig. 4, *right*). When compared to wild-type α-S1, all mutant S1s showed statistically significant reductions in V_{max} and increases in K_m for actin. The limited quantity of S1s prevented detailed kinetic experiments to determine the specific rate constants that may be affected by the FHC mutation.

DISCUSSION

Interpretation of data and relationship to other work

The ATPase activities of the expressed wild-type α-S1 (Fig. 3) were similar to S1 purified from rat cardiac tissue (Lauer et al., 1989). The V_{max} of Arg^{249}Gln-S1 decreased by 1.7-fold, and the K_m increased by 1.85-fold compared with wild-type α-S1, suggesting that an actin-dependent kinetic rate constant of the ATPase cycle may be affected. Arg^{249} is located at the base of the ATP-binding pocket, ~29 Å from the phosphate-binding loop, and is exposed to the surface of the molecule as it faces the cleft that splits the 50-kDa domain of S1 (Fig. 5) (Rayment et al., 1995). It is possible that the Arg^{249}Gln mutation may perturb movements in the lower 50-kDa domain (*highlighted in black* in Fig. 5), which possibly involves a closure of the cleft during transition from the prehydrolysis state (myosin·ATP) to a metastable state (myosin·ADP·P_i) (Fisher et al., 1995). It was also suggested that the movement of the lower domain relative to the upper domain is directly coupled to a structural change in the LC domain region of the molecule that may be responsible for the power stroke during force generation (Fisher et al., 1995). Alternatively (or additionally), the proximity of Arg^{249} to residues that are directly involved in the mechanism of ATP binding and hydrolysis via charge-dependent interactions suggests that any charge changes in Arg^{249} may result in a negative influence on the overall myosin ATPase. Arg^{249} (the equivalent residue in chicken skeletal S1 is Arg^{251}) is 11.6 Å from $cAsp^{463}$, which forms a hydrogen bond with $cThr^{186}$ ($cThr^{186}$ is involved in the coordination of Mg^{2+} ion of the bound ATP) (Smith and Rayment, 1996). (The corresponding residues in the crystal structure of chicken skeletal S1 are preceded by "c", e.g., $cArg^{251}$.) Arg^{249} is close to $cLys^{248}$, which may be directly involved in a salt bridge with $cAsp^{463}$ during ATP binding (Fig. 5) (Smith and Rayment, 1996). Our observation that Arg^{249}Gln decreases the V_{max} is consistent with recently published data (Sata and Ikebe, 1996), in which there was a twofold decrease in V_{max} of human Arg^{249}Gln-β-HMM. However, we observed a 1.85-fold increase in K_m, whereas Sata and Ikebe (1996) reported that K_m was unchanged but the actin motility decreased by 1.6-fold.

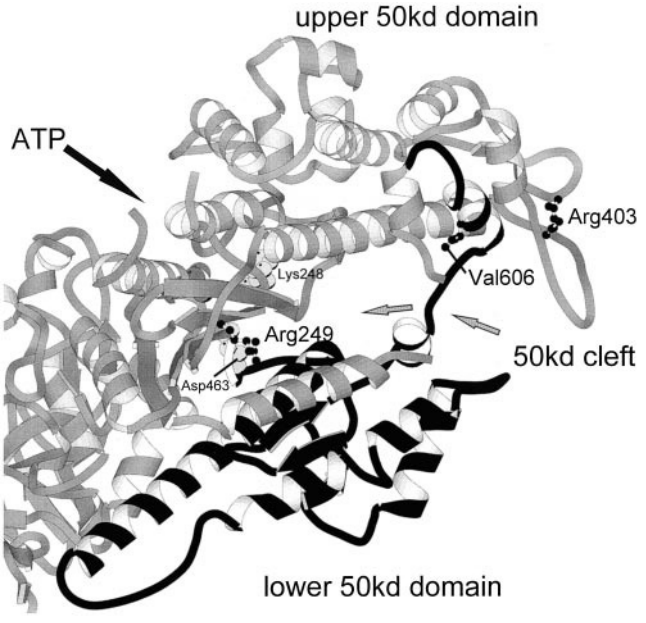

FIGURE 5 Location of FHC mutations in this study. An expanded region of the ATP- and actin-binding site (from Fig. 1) showing the location of the FHC mutations of this study. Arg^{249} faces into the cleft between the upper and lower 50-kDa domains and is proximal to residues $cLys^{248}$ and $cAsp^{463}$, which appear to form a salt bridge during ATP binding. Arg^{403} is at the base of a loop ($cArg^{405}$-$cLys^{415}$) that appears to interact with actin. Val^{606} appears to be buried in the upper 50-kDa domain. The figure was prepared using the program MOLSCRIPT, version 2.0.

A more severe effect on ATPase activity is observed with the Arg^{403}Gln mutation. The V_{max} of Arg^{403}Gln-S1 decreased by 3.5-fold and the K_m increased by 3.4-fold compared with α-S1, suggesting that the actin-myosin interaction is weakened substantially. Arg^{403} (equivalent to $cArg^{405}$) is at the base of a loop ($cArg^{405}$-$cLys^{415}$) that appears to play an important role during the stereospecific actin-myosin interaction (Fig. 5) (Milligan, 1996; Rayment et al., 1993). Molecular docking studies with the crystal structures of skeletal actin and S1 reveal that the myosin loop ($cArg^{405}$-$cLys^{415}$) is close to the actin residues Pro^{332}-Glu^{334} during the actin-myosin interaction (Milligan 1996; Rayment et al., 1993). It is proposed that the early weakly binding interaction between myosin and actin involves charged residues, whereas the later strong-binding interaction involves hydrophobic interactions (Rayment et al., 1993; Schroder et al., 1993). The Arg^{403}Gln mutation decreases the positive charge by one and may be likely to weaken the actomyosin interaction during force generation. The importance of Arg^{403} in the myosin function is also demonstrated in two other independent mutations of this residue that cause FHC, Arg^{403}Leu and Arg^{403}Trp (Dausse et al., 1993; Posen et al., 1995). Individuals with the Arg^{403}Leu mutation show either a high incidence of sudden death in young adults or survival into and past the sixth decade (Dausse et al., 1993). However, the Arg^{403}Trp mutation is quite mild and shows long-term survival of patients (Dausse et al., 1993; Posen et al., 1995), suggesting that a change of charge is not solely responsible for the clinical features of the disease, a notion that has long been discarded as a prognostic indication for the FHC disease (Vikstrom and Leinwand, 1996). Our results agree with previously published reports on the impaired function of expressed

$Arg^{403}Gln$-myosin. The V_{max} and K_m of $Arg^{403}Gln$ in the context of rat α-HMM (Sweeney et al., 1994), human β-HMM (Sata and Ikebe, 1996), and *Dictyostelium* myosin (Fujita et al., 1997) decreased by ~3.4-fold. Myosin from a soleus muscle biopsy from a patient with the $Arg^{403}Gln$ mutation showed a threefold decrease in actin motility and a 1.4-fold decrease in mechanical force of isolated fibers compared with normal fibers (Cuda et al., 1993a; Lankford et al., 1996).

The V_{max} and K_m of $Val^{606}Met$-S1 were affected to a lesser extent compared with the wild-type α-S1. The V_{max} decreased by 1.2-fold, and the K_m increased by 1.4-fold compared with wild-type S1, suggesting a slightly decreased actomyosin interaction. Val^{606} lies in the actin-binding domain and appears to be buried in the upper 50-kDa domain (Fig. 5). Our results differ slightly with a previous report on actin-activated ATPase of human cardiac β-HMM-$Val^{606}Met$, which did not demonstrate statistically significant impairment in actin-activated ATPase (Sata and Ikebe, 1996). There is also some heterogeneity in data from patients with this mutation. Force measurements of soleus muscles from a patient with this mutation were normal in one report (Thedinga et al., 1996). However, myosin isolated from soleus muscle from a diseased patient in another family showed that the mutant allele reduced the actin filament sliding velocity by 2.3-fold (Cuda et al., 1993b). These differences suggest that a small perturbation in the motor biochemical function may result in a variable prognosis in patients. It is possible that this difference is due to differences in the amount of mutant allele in the muscle fiber or cardiac myocyte. It is also likely that other factors such as genetic, environmental, and/or exercise influence the phenotype and onset of the FHC disease.

Our results agree with the moderate prognosis associated with the $Arg^{249}Gln$ diseased patients (Watkins et al., 1992). The severe impairment of myosin functional properties by the $Arg^{403}Gln$ mutation correlates with the poor prognosis displayed by diseased patients (Watkins et al., 1992; Epstein et al., 1992; Marian et al., 1992). The mild effect of the $Val^{606}Met$ mutation on the ATPase activity correlates well with the benign prognosis for affected patients in two different families (Watkins et al., 1992; Marian et al., 1995a).

Mechanism for FHC disease

The mechanism by which the FHC mutations in sarcomeric proteins cause hypertrophy and myocyte disarray is still unknown. It seems increasingly evident that mutations in sarcomeric proteins are likely to compromise sarcomere function (in terms of motor function, force production, and/or protein structure), resulting in hypertrophic cardiomyopathy. Cardiac hypertrophy can be the result of a number of stimuli, including exercise, pressure, and volume overload. It is possible that the mutant sarcomeric protein (myosin in this report) decreases myocardial contractility of the myocyte and imposes stress on the cardiac myocytes, which induces a hypertrophic response. The introduction of human β-$Arg^{403}Gln$-MyHC into feline cardiac myocytes resulted in disruption of sarcomeres, which may be due to a increased protein turnover rate or impaired mutant myosin binding to actin (Marian et al., 1995b). The latter agrees with our results of impaired actomyosin interaction of the $Arg^{403}Gln$-S1, suggesting that the biochemical defects of the motor domain may affect myosin filament assembly in the sarcomere. More recently, it was reported that human β-$Arg^{249}Gln$- and $Arg^{403}Gln$-MyHCs assembled normally into the thick filaments of rat cardiac myocytes, suggesting that the mutant proteins did not disrupt myofilament assembly (Becker et al., 1997). The differences between these two reports (Marian et al., 1995b; Becker et al., 1997) may be due to differences in the cardiac myocytes (feline versus rat) and/or the amount of mutant protein incorporated into a sarcomere because different expression vectors with different promoters were used in these studies. Interestingly, intact myofibrillar organization of heart tissue section from a deceased patient with the $Arg^{403}Gln$ mutation was previously observed (Vybiral et al., 1992).

CONCLUSIONS

We have shown that missense mutations ($Arg^{249}Gln$, $Arg^{403}Gln$, and $Val^{606}Val$) in the context of rat α-MyHC impaired the V_{max} and K_m of the actin-activated myosin ATPases. Our results suggest that the degree of disruption of the actomyosin interaction by the FHC mutations may be directly correlated with the severity of the disease. Future biochemical analysis of several other FHC mutations will be needed to establish a definite correlation between the enzymatic impairment between different mutants and their clinical phenotype of the heart disease. Our results with the FHC mutations in the background of rat α-MyHC will be very useful in interpreting studies performed on experimental laboratory small animal models.

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