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Functional analysis of myosin missense mutations in familial hypertrophic cardiomyopathy

(thick filaments/sarcomere/myofibril)

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ABSTRACT **To analyze potential functional consequences of myosin heavy chain (MHC) mutations identified in patients with familial hypertrophic cardiomyopathy (FHC), we have assessed the stability of the mutant MHCs and their ability to form thick filaments. Constructs encoding wild-type rat α MHC and seven corresponding FHC missense mutants were transfected into COS cells. Immunoblot analysis suggested that FHC mutations do not grossly alter protein stability. Wild-type α MHC transfected into COS cells forms structures previously shown to be arrays of thick filaments, which also resemble myosin structures observed early in differentiation of muscle cells. Surprisingly, up to 29% of COS cells transfected with the FHC mutants failed to form filamentous structures. To assess whether this phenotype was specific for the FHC mutants and not generalizable to any myosin mutation, COS cells were transfected with a construct encoding an MHC with a 168-amino acid deletion of the hinge/rod region. This deletion construct formed filamentous structures with the same frequency as wild-type MHC. Biochemical analysis of one FHC mutant (Arg-249 → Gln) demonstrates that the structures formed by the mutant are solubilized at a lower ionic strength than those formed by wild-type MHC. We conclude that although the FHC mutant MHC is not labile, its assembly properties may be impaired.**

Familial hypertrophic cardiomyopathy (FHC) is an autosomal dominant disorder characterized by asymmetric ventricular hypertrophy, myocyte disarray, and a wide spectrum of clinical symptoms (1, 2). Syncope, cardiac arrhythmias, and sudden death can occur in both symptomatic and asymptomatic individuals (3–5). Molecular genetic studies have recently demonstrated seven distinct missense mutations in the β cardiac myosin heavy chain (MHC) gene on chromosome 14 of affected individuals in 12 different families (6–8).

Although genetic studies have yielded compelling evidence that myosin mutations are responsible for FHC, the precise molecular basis and pathogenesis of this disease remain unknown. The autosomal dominant inheritance pattern could result either from a dominant negative interference of the mutant protein with the wild-type myosin or from a reduced expression of myosin. Precedents for both of these mechanisms have been described in myosin mutations found in the eukaryotes *Drosophila melanogaster* (9–12) and *Caenorhabditis elegans* (13–17).

Myosin is a hexamer which is both an enzyme and a structural protein (18). Its enzymatic activity is characterized by ATP hydrolysis following actin binding, while its structural role is characterized by the self-assembly of several hundred molecules into a bipolar thick filament. Subsequent incorporation of thick filaments into the sarcomere requires the participation of other contractile proteins in a complex process which is not fully understood. The missense mutations in the β MHC gene identified thus far occur in the globular enzymatic head domain and the head/rod junction of the molecule. Although the FHC mutations occur in amino acids that are extremely well conserved in evolution, they are not located in regions of known functional significance (19).

We have examined several functional characteristics of the myosin molecule through transient transfections of nonmuscle cells in culture. With a view toward developing an animal model for FHC, we have introduced seven of the FHC mutations into the rat α cardiac MHC gene, the major ventricular MHC molecule in the adult rat heart. The rat α and β MHC genes are extremely homologous to each other and are both identical to the human β MHC gene in those residues affected by the known FHC mutations (20, 21). Specifically, we examined the following properties of mutant MHCs: (*i*) the stability of the mutant polypeptides, (*ii*) their ability to form filaments *de novo*, and (*iii*) solubilization of the mutant MHC from a cell lysate. The results reported here demonstrate that although all seven FHC mutant MHC molecules appear stable when expressed in a nonmuscle cell, their ability to form filaments or the stability of those filaments may be impaired.

MATERIALS AND METHODS

Construction of Expression Vectors. A complete rat wild-type α MHC cDNA and seven corresponding FHC mutants were cloned in the pMT21 expression vector, which utilizes the adenovirus major late promoter (Genetics Institute, Cambridge, MA) and a Rous sarcoma virus (RSV) expression vector. The details of the construction of pMT21-α wild type were previously described (22). The RSV wild-type construct consists of a full-length α MHC cDNA with the simian virus 40 small t intron and polyadenylylation sequence. The polymerase chain reaction was used to amplify sequences encoding missense mutations of the β MHC gene as indicated (Fig. 1). These mutations were then introduced into the corresponding sequence of the two α MHC expression vectors. The identity of each clone was confirmed by DNA sequencing.

Cell Lines. COS-1 African green monkey kidney cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 2 mM L-glutamine, penicillin G at 50 units/ml, streptomycin at 50 μg/ml, and 10 mM Hepes buffer at pH 7.0. A 100% confluent culture was split 1:10 12–20 hr prior to transfection. Transfections were performed according to the DEAE-dextran procedure described by Levy-Mintz and Kielian (23), using DNA at 4 μg/ml and DEAE-dextran at 250

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Abbreviations: MHC, myosin heavy chain; FHC, familial hypertrophic cardiomyopathy; RSV, Rous sarcoma virus.

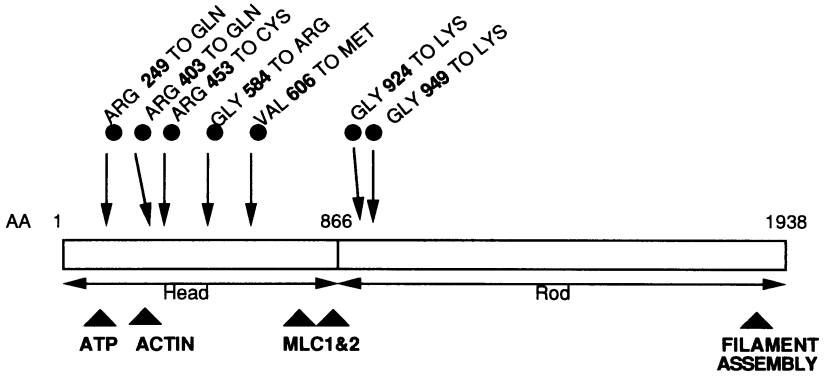

FIG. 1. Location and identity of the seven missense mutations of the β MHC gene in FHC. These mutations are located in the head and head/rod junction of the molecule. These seven mutations were introduced into the corresponding sequences of both full-length α MHC expression constructs. MLC, myosin light chain.

μg/ml. Cells were harvested 48 hr after transfection for immunocytochemistry and immunoblot analysis.

Indirect Immunofluorescence. COS cells on coverslips were fixed for 5 min at room temperature with 3.7% formaldehyde in pH 7.4 phosphate-buffered saline (PBS) containing 6 mM sodium phosphate, 6 mM potassium phosphate, 170 mM NaCl, and 3 mM KCl. Fixation was completed by a 2-min incubation in 100% ice-cold acetone. Nonspecific binding sites were blocked with 10% normal goat serum in PBS in a high-humidity chamber for 30 min at 37°C. Supernatant from cultures of mouse cells producing F59 (24), a monoclonal antibody which recognizes an epitope in the MHC head, was used as primary antibody in incubation with fixed cells in a high-humidity chamber for 30 min at 37°C. The secondary antibody, fluorescein-conjugated goat anti-mouse IgG (The Jackson Laboratory), was diluted 1:40 with 10% normal goat serum in PBS prior to incubation for 30 min at 37°C. The cells were examined by indirect immunofluorescence and under polarized light.

Immunoblots. Transfected cells were assessed for protein expression by Western blotting 48 hr after transfection. The cells from a 100-mm tissue culture plate were washed in 5 ml of PBS, pelleted, and lysed with 100 μl of Laemmli sample buffer (25). One-fifth of the lysate was electrophoresed on an SDS/8% polyacrylamide gel, transferred to a 0.2-μm pore nitrocellulose filter (26), and blocked with 5% nonfat dried milk in PBS. Primary antibody was F59 and secondary antibody was horseradish peroxidase-conjugated goat anti-mouse IgG (Sigma). Immunoreactive species were visualized by chemiluminescence.

Myosin Solubilization Curve. COS-1 cells transfected with the wild-type pMT21-α construct and FHC mutant pMT21 (Arg-249 → Gln) were lysed 48 hr after transfection in modified Seller's buffer (27). This solution contained KCl concentrations ranging from 0 to 600 mM, 0.5% Triton X-100, 50 mM Mops at pH 7.5, 5 mM EGTA, 2 mM dithiothreitol, 2 mM $MgCl_2$, 5 mM ATP, aprotinin at 20 μg/ml, pepstatin at 5 μg/ml, chymostatin at 10 μg/ml, leupeptin at 10 μg/ml, and 0.4 mM phenylmethanesulfonyl fluoride. Crude lysates harvested from 100-mm plates in 500 μl of buffer at each KCl concentration were treated with DNase (SIGMA; 1 mg/ml) for 2 hr at 4°C to disrupt actin filaments. Lysates were centrifuged at 140 × g for 5 min at 4°C. Equal proportions of pellets and supernatants at each KCl concentration were electrophoresed on SDS/8% polyacrylamide gels and transferred to nitrocellulose as described above. Densitometric laser scanning was performed to quantify the proportion of myosin remaining in the soluble fraction compared with the amount in the pellet.

Electron Microscopy. Transfected cells were fixed for 30 min at room temperature in 2.5% glutaraldehyde in PBS, permeabilized with 0.1% Triton X-100 in 0.2% cold water fish gelatin, and allowed to react with antibodies (F59 and horseradish peroxidase-conjugated goat anti-mouse IgG) as described above. Transfected MHC species were visualized by staining cells with a solution of diaminobenzidine and horseradish peroxidase (Vector Laboratories). Cells were post-fixed with 1% osmium tetroxide in 1 M sodium cacodylate, pH 7.4. COS cell monolayers were embedded, stained, and sectioned as previously described (22, 28). Sections were cut parallel to the plane of the substratum and examined in a JOEL 1200EX electron microscope at an accelerating voltage of 80 kV.

RESULTS

To identify possible molecular defects of the FHC mutant myosins, we transiently transfected into nonmuscle cells DNA constructs encoding wild-type and mutant MHC (Fig. 1). One testable hypothesis concerning the molecular defect is that the FHC mutant MHCs are inherently unstable, resulting in hearts with half the normal amount of MHC. The stability of FHC mutant protein was examined after trans-

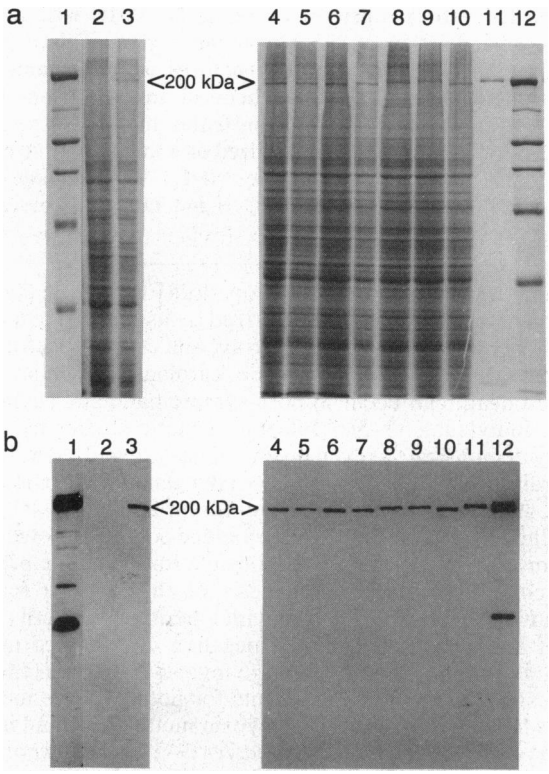

FIG. 2. Coomassie blue-stained SDS/8% polyacrylamide gels (*a*) and the corresponding immunoblots (*b*) of whole-cell lysates of COS cells transiently transfected with pMT21-α and the seven corresponding FHC mutants under the direction of the adenovirus late promoter. Lane 1, high molecular mass markers for lanes 2 and 3; lane 2, mock-transfected cells; lane 3, pMT21-α wild type; lane 4, Arg-249 → Gln; lane 5, Arg-403 → Gln; lane 6, Arg-453 → Cys; lane 7, Gly-584 → Arg; lane 8, Val-606 → Met; lane 9, Gly-924 → Lys; lane 10, Gly-949 → Lys; lane 11, 0.83 μg of purified rat cardiac myosin; lane 12, high molecular mass markers for lanes 4–11.

fection into COS cells. These cells do not express any other muscle-specific MHCs, permitting detection of the transfected cardiac myosin by a sarcomeric MHC-specific antibody. The pMT21 expression constructs encoding the wild-type rat α cardiac MHC and seven FHC mutants (Fig. 1) were transiently transfected into COS cells, and the transfectants were assessed for protein expression 48 hr afterward. Fig. 2 shows Coomassie blue-stained SDS/8% polyacrylamide gels (*a*) and the corresponding immunoblots (*b*) of whole-cell lysates derived from the pMT21 constructs encoding α wild-type MHC and the seven FHC mutants. A single immunoreactive band of 200 kDa is visible for each mutant and not present in mock-transfected cells. A full-length protein is produced by each expression construct; no obvious degradation is seen for any of the mutants. A total of 4–6 μg of MHC protein was obtainable from a 100-mm tissue culture plate transfected with the pMT21 vector; approximately 0.1–0.2 μg of MHC was produced from the same number of cells transfected with the RSV vector.

The defect in FHC may be related to a known assayable function of the myosin molecule such as its ability to form thick filaments. We therefore examined the organization of transfected MHC in COS cells by light and electron microscopy. Previous work has shown that wild-type rat α cardiac MHC in transfected COS cells forms arrays of thick filaments by the following criteria. They are birefringent; their formation depends on an intact myosin rod; and electron microscopic analysis demonstrates groups of thick filament structures (22). These structures show no apparent association with the endogenous COS cell cytoskeleton (C. G. Saez and L.A.L., unpublished observations) and therefore allow analysis of thick filament formation in the absence of other sarcomeric components.

COS cells were transfected with each DNA construct (wild type and each of the seven FHC mutants) under the direction of two different strength promoters. We assessed the distribution of the wild-type and mutant myosins by immunofluorescence in at least 200 transfected COS cells for each DNA construct 48 hr after transfection. Fig. 3 shows a COS cell transfected with wild-type myosin (*a*) compared with cells transfected with FHC mutant Arg-249 → Gln (*b–d*) in the RSV vector. The majority of cells transfected with FHC constructs demonstrate the filamentous phenotype seen with wild type. The bundles of thick filaments are variable in width and in length, ranging from 1 to 20 μm long. However, a significant proportion of cells transfected with the FHC mutants failed to form filamentous structures. Fig. 3*c* shows two different cells transfected with mutant Arg-249 → Gln. One cell shows the filamentous phenotype, while the other shows mutant myosin with a diffuse pattern of distribution. While only 2% of cells transfected with wild-type MHC failed to form filamentous structures, up to 29% of cells transfected with the FHC mutants failed to form these structures (Fig. 4). To address the possibility that the phenotypic difference was related to random variations in the amount of expressed protein, these experiments were repeated with a different viral promoter [RSV long terminal repeat (LTR)] which directs the synthesis of less than 1/40th as much protein. The same percentages of nonfilamentous structures were obtained for both the RSV LTR and late adenovirus promoters, and regardless of the efficiency of the transfection. Similar results were also obtained when transfections were examined at later times (data not shown).

As further validation that analysis of these structures is relevant to processes which occur during normal muscle development, we asked whether similar structures form during the early stages of muscle cell differentiation. We stained C_2C_{12} myoblast cell cultures for endogenous sarcomeric MHC after the cells had been placed in differentiation medium. At early time points (40 hr), most of the sarcomeric MHC-positive cells resembled the cell shown in Fig. 3*e*. At

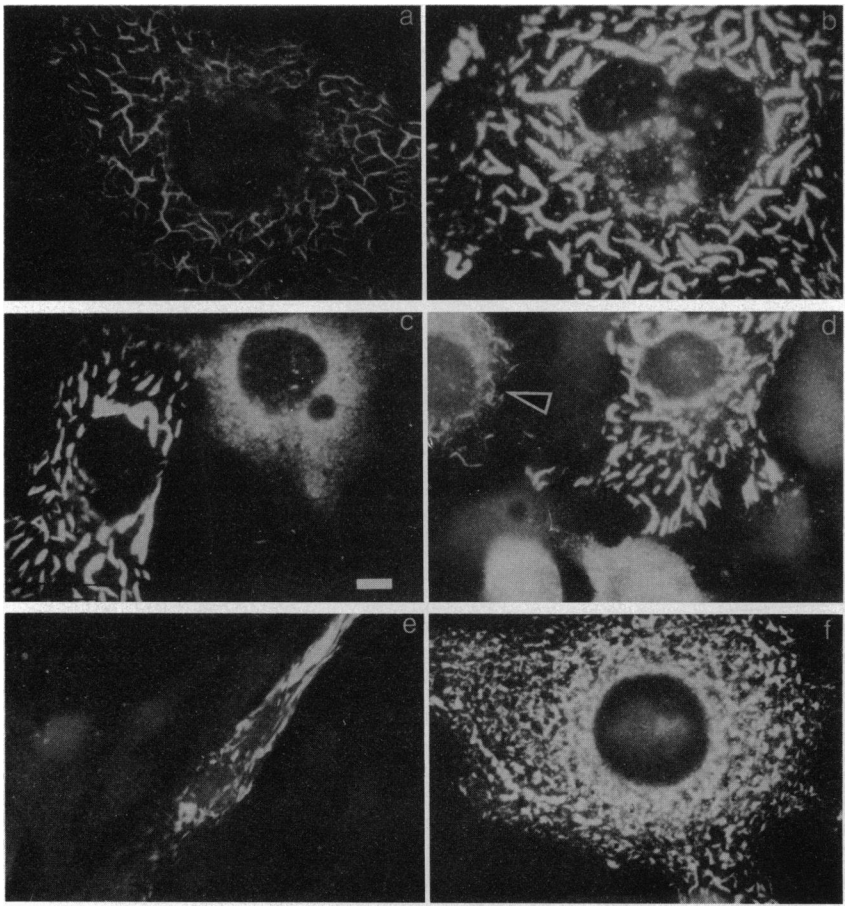

FIG. 3. Indirect immunofluorescence of COS cells transiently transfected with wild-type MHC (*a*), FHC mutant Arg-249 → Gln (*b–d*), and α MHC with a 168-amino acid deletion of the hinge/rod region (*f*); indirect immunofluorescence of C_2C_{12} cells 40 hr after induction of differentiation (*e*). All cells have been allowed to react with a monoclonal antibody specific for sarcomeric MHC (F59). Transfected COS cells in *c* and *d* show a diffuse nonfilamentous distribution of the mutant myosin. The cell to the left of the arrowhead in *d* demonstrates both filamentous and nonfilamentous structures. The C_2C_{12} cell in *e* shows structures similar to those observed in the transfected COS cells. (Bar = 10 μm.)

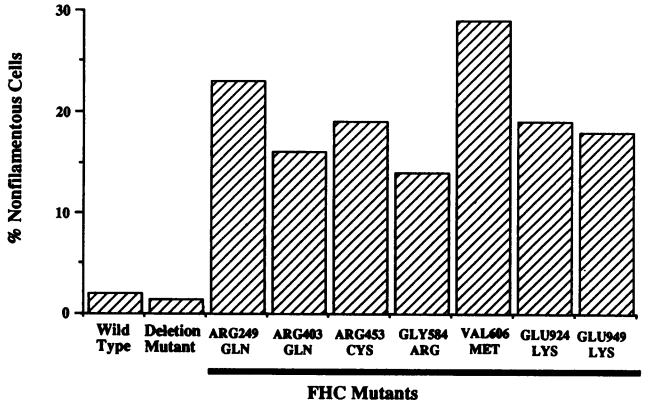

FIG. 4. Bar graph shows the percentage of transfected COS cells demonstrating the diffuse nonfilamentous phenotype. At least 200 transfected cells were examined for each expression construct. All seven FHC mutant myosins show a defect in the formation of filamentous structures in transfected COS cells.

this level of resolution, there is obvious similarity between the MHC organization in C_2C_{12} myotubes and in COS cells transfected with α MHC.

To assess the specificity of this "diffuse" phenotype, we examined a non-FHC mutant MHC construct which has undergone a 168-amino acid deletion of the hinge/rod region of the myosin molecule from residue 972 to residue 1139. Cells transfected with this deletion construct show a phenotype which is distinct from that seen in either the wild-type or FHC mutant MHCs (Fig. 3*f*). These structures appear shorter (1–7 μm in length) than those observed for the wild-type MHC (1–20 μm in length) but do not exhibit a diffuse staining pattern (Fig. 4). These results suggest that the phenotype observed with the FHC mutants is specific and not generalizable to any mutation of the MHC molecule.

To examine the ultrastructure of these filamentous structures, transfected COS cell monolayers were examined by negative stain electron microscopy. Bundles of thick filaments were present in the majority of transfected cells containing wild-type or FHC mutant MHCs (Fig. 5). In some cells, striated structures with a 40- to 50-nm repeat were observed. Fig. 5*a* demonstrates structures typically seen in cells transfected with wild-type MHC, while Fig. 5*b* demonstrates structures more prevalent in cells transfected with FHC mutant Arg-249 → Gln.

Two pairs of nonidentical myosin light chains bind to the head region of the MHC and have been implicated in modulating the ATPase activity of the molecule (18). In transfected COS cells, wild-type α MHC is expressed in a 100-fold molar excess relative to the endogenous nonmuscle heavy and light chains (22). Furthermore, partial purification and immunoprecipitation do not demonstrate any detectable nonmuscle light chain (22). Therefore, the majority of the MHCs do not have myosin light chains associated with them. To characterize any phenotypic effect of the cognate essential myosin light chain on the formation of the α cardiac filamentous structures, cotransfections were performed with an expression construct encoding rat ventricular myosin light chain 1 and the α cardiac MHC constructs. When cotransfected cells were examined by immunofluorescence, the apparent length and organization of the filamentous structures for both the wild-type α MHC and the FHC mutants were unaffected (data not shown).

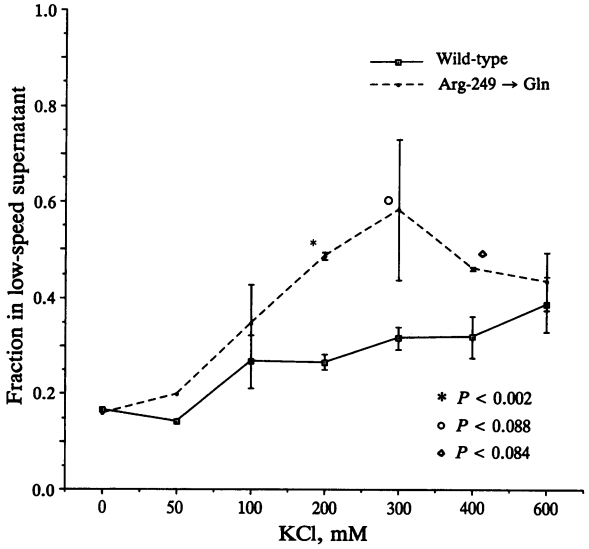

FIG. 6. Solubility curves for wild-type MHC and FHC mutant Arg-249 → Gln. Standard error bar indicated for a data point represents at least three separate determinations.

Purified myosin has characteristic patterns of solubility in solutions of different ionic strengths, reflecting the formation of thick filaments at low ionic strength and their disassembly at higher ionic strength (29, 30). If the diffuse distribution of FHC mutant MHC reflects a loose interaction between myosin molecules, we might predict solubilization at a lower ionic strength relative to the wild-type MHC. To test this hypothesis, we exposed mutant and wild-type myosin from whole cell lysates to solutions of increasing ionic strength and

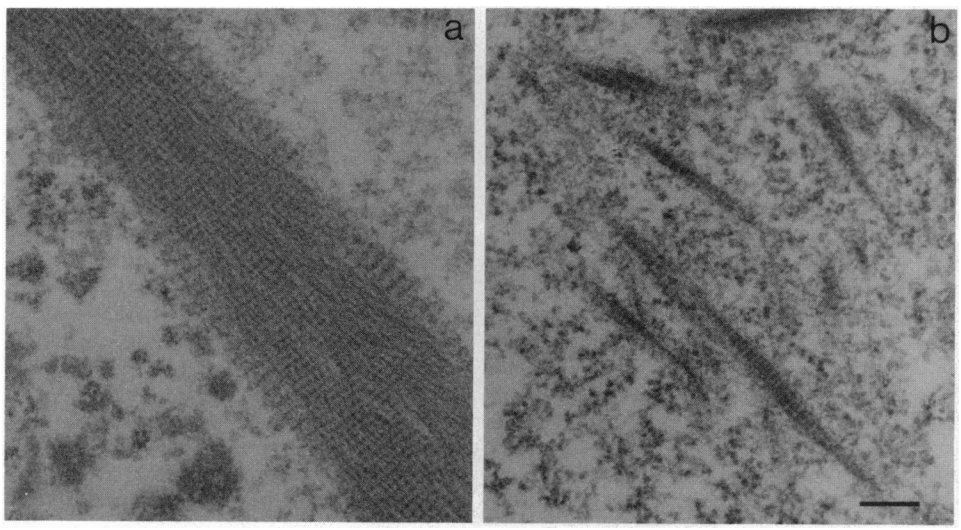

FIG. 5. Transmission electron micrographs of COS cells transfected with wild-type MHC (*a*) and FHC mutant Arg-249 → Gln (*b*), demonstrating striated structures with 40- to 50-nm repeat. (Bar = 200 nm.)

tested for solubility of the MHC. COS cells transfected with either wild-type myosin or FHC mutant Arg-249 → Gln were lysed in a buffer solution containing 0.5% Triton X-100, DNase, ATP, and KCl concentrations ranging from 0 to 600 mM. The lysate was subjected to a low-speed centrifugation and a solubility curve was generated by quantifying the proportion of myosin contained in the supernatant and pellet. Fig. 6 shows that a higher proportion of the FHC mutant Arg-249 → Gln was solubilized at a lower ionic strength relative to wild type.

DISCUSSION

The molecular genetic dissection of FHC has yielded compelling evidence that mutations in the MHC play a role in the disease. Structural and functional analyses of the MHC protein are imperative to understanding the pathogenesis of FHC. An important advantage of introducing mutant and wild-type contractile protein gene constructs into well-defined cultured cells is the opportunity to specify the components under analysis on a background of limited or absent sarcomeric constituents.

One potential effect of the mutations identified in FHC might be to alter protein half-life. However, seven FHC mutant proteins are not grossly unstable in these cells. This result is in concert with the finding of normal MHC composition and MHC/actin ratios in cardiac biopsy specimens taken from two individuals with FHC mutation Arg-403 → Gln (31). However, differences in the specific turnover rates for MHC proteins between nonmuscle cells and cardiocytes may exist; regional differences within the heart in protein processing and degradation rates cannot be excluded.

In the current study, we examined at least 200 transfected cells for each DNA construct (wild type and seven FHC mutants) and found that a reproducible significant proportion of cells transfected with the FHC mutants failed to form filamentous structures. Although sequences in the MHC rod have been shown to be responsible for thick filament assembly, mutations in the MHC head could have a long-range effect on assembly, as has been suggested in the nematode (17). This phenotypic pattern was true for all the FHC mutants tested but was not seen for an expression construct encoding MHC with a 168-amino acid deletion. The fact that this large mutation in the MHC did not produce the same phenotype as that seen in the FHC mutants suggests that the FHC mutant phenotype is not generalizable to any alteration in the myosin molecule. Further, the filamentous structures seen in transfected COS cells appear similar at the light microscope level to endogenous sarcomeric MHC observed in differentiating C_2C_{12} mouse myotubes. Electron micrographs shown in Fig. 5 demonstrate that the striated structures formed by the transfected wild-type and mutant MHCs are orderly arrays despite the substoichiometric amounts of myosin light chains. Our interpretation of the smaller structures (seen in Fig. 5*b*) which are more prevalent in the mutants is that they represent the diffuse staining pattern seen at the light microscope level.

To assess any possible effect of the FHC mutants on the biochemical properties of myosin, transfected cell lysates were exposed to increasing ionic strength and tested for solubility of the MHC. An alteration in the properties of the filament bundles might be predicted to affect solubility relative to wild-type myosin. Within the context of a crude lysate, the FHC mutant Arg-249 → Gln was solubilized at a lower ionic strength than wild-type MHC. Neither wild-type nor FHC mutant MHCs are completely solubilized at 600 mM KCl; this result might be expected, since solubility is being assessed in a complex mixture of proteins and not with purified MHC. This behavior may indicate that the structures formed by the FHC mutant MHCs are less tightly packed. Among a dimorphic population of myosin molecules, some might be preferentially incorporated into higher structures. Alternatively, an MHC with altered assembly properties might be incorporated normally but have an altered interaction with other contractile proteins potentially involved in ATP hydrolysis, alignment of thick/thin filaments, and/or determination of sarcomere length. The effect of the mutation does not appear to be dominant negative with respect to filament formation, as a considerable proportion of the transfected cells are still capable of forming these filamentous structures with the same phenotype as wild-type myosin.

Thick filament formation and assembly into sarcomeres are necessary but not sufficient for myosin function *in vivo*. Actin/light chain binding and ATP hydrolysis are also crucial in the role of myosin as an enzyme. Future experiments to assess the effect of the mutants on enzymatic activity and myocyte contractility are necessary for a more complete understanding of the molecular defect in FHC.

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