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Regulatory light chain mutations associated with cardiomyopathy affect myosin mechanics and kinetics

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ABSTRACT

The myosin regulatory light chain (RLC) wraps around the alpha-helical neck region of myosin. This neck region has been proposed to act as a lever arm, amplifying small conformational changes in the myosin head to generate motion. The RLC serves an important structural role, supporting the myosin neck region and a modulatory role, tuning the kinetics of the actin myosin interaction. Given the importance of the RLC, it is not surprising that mutations of the RLC can lead to familial hypertrophic cardiomyopathy (FHC), the leading cause of sudden cardiac death in people under 30. Population studies identified two FHC mutations located near the cationic binding site of the RLC, R58Q and N47K. Although these mutations are close in sequence, they differ in clinical presentation and prognosis, with R58Q showing a more severe phenotype. We examined the molecular based changes in myosin that are responsible for the disease phenotype by purifying myosin from transgenic mouse hearts expressing mutant myosins and examining actin filament sliding using the *in vitro* motility assay. We found that both R58Q and N47K show reductions in force compared to the wild type that could result in compensatory hypertrophy. Furthermore, we observed a higher ATPase rate and an increased activation at submaximal calcium levels for the R58Q myosin that could lead to decreased efficiency and incomplete cardiac relaxation, potentially explaining the more severe phenotype for the R58Q mutation.

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1. Introduction

Familial hypertrophic cardiomyopathy (FHC), the leading cause of sudden cardiac death in people under 30 [1–4], is characterized by myofibrillar disarray and thickening of the left ventricle, papillary muscles, or septum. Individuals bearing FHC mutations sometimes experience shortness of breath or chest pain although often sudden cardiac death occurs with no clinical symptoms. FHC is caused by mutations in cardiac sarcomeric proteins including the myosin heavy chain (MHC), the regulatory (RLC) and essential light chains of myosin, troponins I, T, and C, titin, tropomyosin, actin, and myosin binding protein C (Reviewed in [3,5]). The clinical presentation and prognosis of the disease depend on the specific mutation.

Several mutations in the myosin regulatory light chain (RLC) have been implicated in FHC (For reviews, see [1,6]). The RLC wraps around the alpha helical neck of the myosin head by binding to a 35 amino acid IQ motif on the MHC. The neck region of the MHC has been proposed to act as a lever arm, amplifying small conformational changes that originate at the catalytic site in the myosin head into large movements, allowing myosin to generate motion and force [7]. Furthermore, this neck region has been proposed to serve as the compliant element in the myosin crossbridge with the RLC playing a structural role, modulating the stiffness of the lever arm [8]. The RLC also contains a highly conserved phosphorylatable serine [9–12] that plays an important role in the activation and modulation of myosin (Reviewed in [13,14]).

In addition to a phosphorylation site, the RLC also contains an N-terminal divalent cation binding site [15,16]. The absence of bound cation in the RLC binding site has been shown to alter the structural properties of the RLC and consequently, the contractile properties of the cation-free myosin [16–18]. Furthermore, binding of nucleotides to the myosin head has been shown to alter the conformation of the RLC [19]. Given the important role of the RLC in muscle contraction, one can hypothesize that FHC mutations in the RLC affect myosin mechanics or kinetics. This report addresses this hypothesis by examining FHC mutant myosins using the *in vitro* motility assay.

Two of the FHC associated mutations in the RLC [20–22] that have been identified in population studies are located either near (R58Q) or at (N47K) the Ca^{2+} binding site (Fig. 1). Although these mutations are close to each other in sequence, they differ in both clinical presentation and prognosis, with R58Q showing a more severe

Abbreviations: FHC, Familial hypertrophic cardiomyopathy; RLC, Regulatory light chain; MHC, Myosin heavy chain; Tg, Transgenic; NTg, Non-transgenic.

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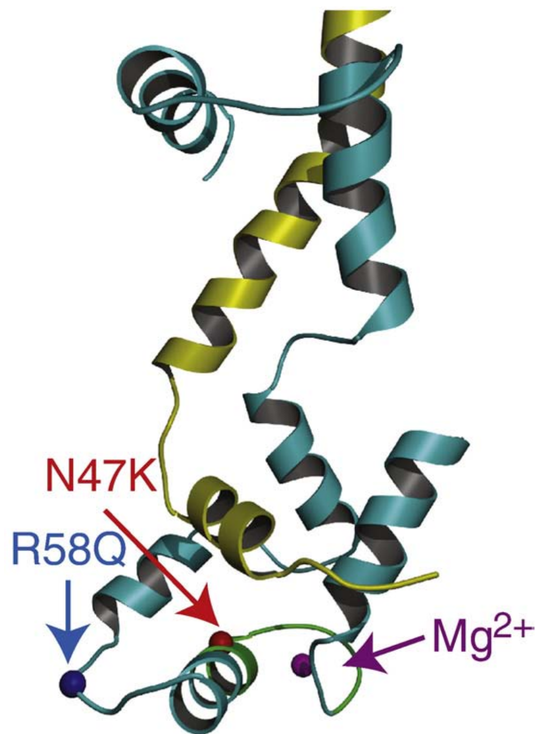

Fig. 1. FHC mutations mapped onto the chicken skeletal RLC structure (Accession # 2MYS [7]). The C-terminal region of the myosin heavy chain is shown in yellow and the RLC is shown in cyan. The locations of R58, N47, Mg^{2+} and the cation binding site are highlighted in blue, red, magenta and green respectively.

phenotype. N47K has been associated with rapidly progressing late onset mid-ventricular hypertrophy and papillary muscle hypertrophy whereas R58Q has been associated with thickening of the left ventricular wall and multiple cases of sudden cardiac death [20–22]. Studies of isolated RLC and myosin reconstituted with mutant RLCs have shown that both R58Q and N47K mutations deactivate RLC calcium binding however, phosphorylation of R58Q restores this ability [18,23]. While effects of the mutations have previously been studied in muscle fibers [18,24], fibers are complicated structures, making it is difficult to dissect the mutation dependent changes in the acto-myosin interaction at the molecular level. In this study, we examine the phenotypic properties of the R58Q and N47K mutations at the molecular level by utilizing myosin purified from transgenic mice and employing the *in vitro* motility assay [25]. We measured the velocity, duty cycle, ATPase activity, isometric force, and calcium sensitivity of both R58Q and N47K mutant myosins. We found several differences in the properties of mutant myosins compared to the wild type (WT) myosin, indicative of the mutation dependent effects in the actin-myosin interaction. Both R58Q and N47K showed a reduction in isometric force that could result in compensatory hypertrophy of the heart. Also, based on our data, we hypothesize that the more severe phenotype of R58Q results from: (1) a decrease in efficiency as evidenced by a higher ATPase rate with a decrease in force and (2) increased rate of activation at submaximal calcium levels that could lead to diastolic dysfunction due to incomplete relaxation of the heart.

2. Materials and methods

2.1. Protein preparation

Alpha cardiac myosin was isolated from the hearts of non-transgenic mice (NTg), transgenic wild type mice expressing the human cardiac RLC (Tg-WT), and transgenic mutant mice expressing either the N47K or R58Q RLC mutations, denoted respectively as Tg-N47K and Tg-R58Q. As described in Wang et al. [24], all transgenic mice (WT L2, N47K L6 and R58Q L8) express ~100% transgene. Myosin was purified from 5–6 hearts from each group of mice, as described in detail in Szczesna-Cordary et al. [26], The hearts were collected from female and male mice of 4–7 months of age. At least 3 different myosin preparations obtained at different time intervals were used for experiments.

Pig cardiac tropomyosin was purified from pig hearts according to Eisenberg and Kielley [27]. Recombinant human cardiac troponin subunits (TnT, TnI, and TnC) were prepared according to standard methods [28] and the formation of troponin complex was carried out as described in Szczesna et al. [29] and Gomes et al. [30].

Actin was prepared from chicken pectoralis muscle acetone powder using the method of Straub [31] with the modification of Drabikowski and Gergely [32]. The actin was suspended in actin buffer (25 mM KCl, 1 mM EGTA, 10 mM DTT, 25 mM imidazole, 4 mM $MgCl_2$). TRITC phalloidin labeled actin was prepared by incubating a 1:1 molar ratio of TRITC phalloidin and actin in actin buffer overnight at 4 °C.

2.2. Unregulated motility assay

The quality of the myosin preparation was assessed by measuring the actin filament sliding velocity in the motility assay. Unless otherwise noted, all experiments were performed at 24 °C. The average actin filament sliding velocity for NTg murine cardiac myosin at 24 °C was 1.2±0.5 μm/s, however, when the flow cell was incubated with 100 μg/ml of NTg myosin and heated to 35 °C, the velocity was 4.2±1.3 μm/s, consistent with previously reported values [33]. The *in vitro* motility assays were performed as previously described with some subtle modifications [26]. Approximately 200 μg of myosin in 50% glycerol was suspended in 1 ml of 10 mM DTT in water and allowed to precipitate for 1 h on ice. This step removed the glycerol and, if present, any damaged myosin molecules unable to form thick filaments. The myosin was then centrifuged at 16,000 ×*g* for 30 min at 4 °C. The supernatant was then discarded and the pellet was resuspended in 200 μl of myosin buffer (300 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM $MgCl_2$, 10 mM DTT). Any damaged myosin heads that were unable to bind and release from actin were removed by mixing the myosin, 1 mM ATP, and 1.1 μM actin and centrifuging in an Airfuge for 30 min at 100,000 ×*g*. The myosin concentration after centrifugation was determined using a Bradford assay (Bio-rad Labs. Hercules, CA) and diluted to the desired concentration in myosin buffer.

Flow cells were constructed by forming a channel between nitrocellulose coated coverslips and a standard glass slide with double stick tape (100 μm width 3M Corp., St. Paul, MN). Myosin was adsorbed to the coverslip surface by incubating 30 μl of myosin (100 μg/ml) in myosin buffer for 1 min. Any remaining surface lacking myosin was blocked by adding 30 μl of 0.5 mg/ml bovine serum albumin (BSA) in myosin buffer followed by a 60 μl wash with actin buffer (25 mM KCl, 25 mM imidazole, 1 mM EGTA, 4 mM $MgCl_2$, 10 mM DTT). As an additional measure to minimize the effects of damaged myosin heads, 30 μl of 1 μM unlabeled actin (myosin : unlabeled actin molar ratios varied from 40:1 to 400:1) in actin buffer was vortexed and added to the flow cell. After incubating for 2 min, the flow cell was washed with 60 μl of actin buffer containing 1 mM ATP and then 120 μl of actin buffer without ATP. 30 μl of 5 nM TRITC labeled actin was then added to the flow cell and allowed to incubate for 1 min. Motility was initiated by the addition of motility buffer (actin buffer with the addition of 0.5% methyl cellulose, 1 mM ATP, 2 mM dextrose, 160 units glucose oxidase, and 2 μM catalase) to the flow cell.

To determine relative changes in duty cycle defined as the fraction of the myosin biochemical cycle spent attached to actin, actin filament sliding velocities were measured at several myosin surface densities

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