Published in final edited form as:

J Mol Cell Cardiol. 2010 February ; 48(2): 286. doi:10.1016/j.yjmcc.2009.09.014.

Human actin mutations associated with hypertrophic and dilated cardiomyopathies demonstrate distinct thin filament regulatory properties *in vitro*

Edward P. Debold^{*,#}, Walid Saber[†], Yaser Cheema[†], Carol S. Bookwalter^{*}, Kathleen M. Trybus^{*}, David M. Warshaw^{*}, and Peter VanBuren^{*,†,±}

^{*} Department of Medicine, University of Vermont, College of Medicine, 149 Beaumont Drive, Burlington, VT 05405

[†] Department of Molecular Physiology & Biophysics, University of Vermont, College of Medicine, 149 Beaumont Drive, Burlington, VT 05405

Abstract

Two cardiomyopathic mutations were expressed in human cardiac actin, using a *Baculovirus*/insect cell system; E99K is associated with hypertrophic cardiomyopathy whereas R312H is associated with dilated cardiomyopathy. The hypothesis that the divergent phenotypes of these two cardiomyopathies is associated with fundamental differences in the molecular mechanics and thin filament regulation of the underlying actin mutation was tested using the *in vitro* motility and laser trap assays. In the presence of troponin (Tn) and tropomyosin (Tm), β -cardiac myosin moved both E99K and R312H thin filaments at significantly ($p < 0.05$) slower velocities than wild type (WT) at maximal Ca^{++} . At submaximal Ca^{++} , R312H thin filaments demonstrated significantly increased Ca^{++} sensitivity (pCa_{50}) when compared to WT. Velocity as a function of ATP concentration revealed similar ATP binding rates but slowed ADP release rates for the 2 actin mutants compared to WT. Single molecule laser trap experiments performed using both unregulated (i.e. actin) and regulated thin filaments in the absence of Ca^{++} revealed that neither actin mutation significantly affected the myosin's unitary step size (d) or duration of strong actin binding (t_{on}) at 20 μM ATP. However, the frequency of individual strong-binding events in the presence of Tn and Tm, was significantly lower for E99K than WT at comparable myosin surface concentrations. The cooperativity of a second myosin head binding to the thin filament was also impaired by E99K. In conclusion, E99K inhibits the activation of the thin filament by myosin strong-binding whereas R312H demonstrates enhanced calcium activation.

Keywords

laser trap; motility assay; heart failure; single molecule biophysics

[±]to whom correspondences should be address. tel. 802-656-2450, fax. 802-656-0747.

[#]present address: Muscle Biophysics Laboratory, Department of Kinesiology, 158 Totman Building, 30 Eastman Lane, University of Massachusetts, Amherst MA 01003

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

Introduction

Point mutations in the contractile proteins of the myocardium are linked to both hypertrophic (HCM) and dilated (DCM) cardiomyopathies, pathologies characterized by distinctly different patterns of ventricular dysfunction and remodeling, where remodeling is the change in cardiac chamber morphology associated with a pathophysiologic state. Specifically, HCM (a leading cause of sudden death in people under 35 years old [1]) is characterized by a thickening of the ventricular walls with maintained or even enhanced contractility [2]. DCM, in contrast, is characterized by an increase in left ventricular chamber dimensions, a thinning of ventricular walls, and a depression of myocardial contractility, ultimately progressing to clinically overt heart failure [3]. Although the pathway from sarcomeric point mutation to pathology is unclear, this differential remodeling response presents a unique opportunity to characterize the primary insult to the contractile protein machinery associated with these mutations.

In contrast to the better characterized effects of human cardiomyopathy associated mutations in myosin and the muscle regulatory proteins, troponin (Tn) and tropomyosin (Tm), much less is known about the functional significance of the 9 mutations identified in actin, 7 of which cause HCM and 2 of which cause DCM [4–7]. Given the highly conserved nature of actin and its integral role in muscle contraction, a mutation in a key functional domain of actin could specifically affect the mechanics, kinetics and/or regulation of the actomyosin interaction. A recent study with expressed human actin revealed that the HCM-causing mutation, E99K, significantly affected the dynamics of the actomyosin interaction [8]. Specifically, both a decrease in the binding affinity of the myosin S1-subfragment to E99K actin, and decreased actin filament velocity in an *in vitro* motility assay were observed. This amino acid residue is located in subdomain 1 of actin and in the absence of Ca^{++} is proximate to Tm [9]. As such this mutation could affect the regulatory properties of Tn and Tm, particularly at low calcium levels.

The DCM associated mutation (R312H) may also affect thin filament regulation. This mutation is located in subdomain 3, a region of actin that is postulated to make important electrostatic contacts with Tm. The neighboring residue (D311) forms one of the strongest interactions between Tm and actin [10]. Thus a mutation that results in a charge change, such as is seen with this mutation, could perturb interactions with Tm and dynamically affect the azimuthal movement of Tm as a function of thin filament activation.

Recent advances in optical trapping methods have provided a unique opportunity to study and quantify the regulatory process of muscle activation at the molecular level. Our laboratory [11] has shown that this assay can be used to quantify the degree to which Tn and Tm inhibit the actomyosin interaction and to determine the relative effects of cooperative activation by myosin strong-binding. Given the different subdomain locations of these two actin mutations and their potential to differentially affect thin filament activation, we have chosen to study the regulatory effects of these two actin mutations. As the thin filament is activated by both calcium and myosin strong-binding, the *in vitro* motility and the laser trap assay are used to investigate these facets of thin filament activation. Thus, by directly comparing the molecular regulatory properties of the HCM mutation (E99K) with the DCM mutation (R312H), we may gain insight in the molecular triggers that initiate the divergent patterns of pathological ventricular remodeling associated with these two distinct clinical phenotypes.

Methods

Expression and purification of recombinant actin

Site-directed mutagenesis was used to change the coding sequence of WT human ACTC1 to either E99K or R312H. The constructs were fully sequenced to verify mutagenesis and the

absence of PCR-induced errors. Expressed, purified protein was obtained using the baculovirus/Sf9 cell system according to previously reported protocols [8]. Expressed actin was stored in liquid nitrogen with 2 mg of sucrose per mg of G-actin. Before use, thawed G-actin was clarified via centrifugation at 95K for 20 minutes at 4°C. Further purification of G actin was performed via chromatography with a Superdex 200 gel filtration column (10/300 GL, GE Healthcare). Actin polymerization was performed with the addition of 0.1 M KCl and 2 mM MgCl₂. The polymerized actin was then labeled with tetramethylrhodamine isothiocyanate (TRITC) phalloidin at a 1:1 molar ratio. To characterize the ATP-dependence of actin filament velocity, purified tissue isolated bovine cardiac actin (Cytoskeleton Inc., Denver, CO) was used, which is identical in primary sequence to human cardiac actin. After chromatography and polymerization, actin was used within 4 days.

Regulated thin filaments

Troponin (Tn) and Tropomyosin (Tm) were isolated from bovine cardiac muscle and prepared as previously described [12]. These proteins were combined with the actin filaments to form calcium regulated thin filaments as previously detailed [13;14].

Myosin

Rabbit cardiac muscle myosin was isolated using the protocol described in Margossian and Lowey [15] with modification [16]. Cardiac myosin was stored in 50% glycerol at -20°C. Before each experiment a 10mg aliquot of myosin was further purified using hydrophobic interaction chromatography [16]. This final purification step helped ensure that only active myosin heads were used in the *in vitro* assays.

Buffers

For the laser trap and *in vitro* motility experiments the myosin was diluted in a high salt buffer (0.3 M KCl, 25 mM imidazole, 1 mM EGTA, 4 mM MgCl, and 10 mM DTT, pH 7.4). The fluorescently labeled thin filaments were maintained in a low salt (actin) buffer (25 mM KCl, 25 mM imidazole, 2 mM EGTA, 5 mM MgCl, 10 mM DTT, pH 7.4). The final motility solutions contain the low salt buffer, an oxygen scavenging system (0.1 mg·ml⁻¹ glucose oxidase, 0.018 mg·ml⁻¹ catalase, and 2.3 mg·ml⁻¹ glucose), 2 mM ATP, 0.375 % methylcellulose and an additional 100 nM Tn and 100 nM Tm (to maintain thin filament regulation). The calcium concentration in the motility assay buffers was varied from pCa 10 to pCa 4 as previously described [17].

In vitro motility

Actin filament velocity was determined for WT and each mutant actin moving over a coverslip surface coated with rabbit β -cardiac myosin (loading buffer concentration 100 μ g·ml⁻¹) as previously described [17]. In a typical experiment the velocities of more than 250 thin filaments were averaged. The pCa: velocity curve was performed 4–6 times for each experimental condition. Mean values were plotted and fit to the Hill equation by the least squares method (Sigma Plot, SPSS Inc.), to provide the parameters: $V_{\max}$ (maximal velocity), pCa₅₀, and the Hill coefficient. Regulated thin filament velocity as a function of ATP concentration (range 1–2000 μ M) was determined at pCa 4.

Laser trap assay

A three-bead laser trap assay was performed as previously described in detail [18;19] with minor modifications [11]. Contractile proteins and solutions were introduced into the experimental chamber (a 20 μ l flow cell) in the following sequence: a) cardiac myosin (1–40 μ g·ml⁻¹) was added to the chamber and allowed to incubate for 2 min; b) 20 μ l of 0.5 mg ml⁻¹ BSA in actin buffer was then added and incubated for 5 min; c) this was followed by

three, 20 μ l washes with actin buffer, and; d) the final assay buffer contained actin buffer with the following added: 20 μ M MgATP, either unregulated TRITC-labeled actin or regulated TRITC-labeled thin filaments, *N*-ethylmaleimide-modified skeletal myosin-coated beads, and 100 nM excess Tn and Tm (to maintain complete regulation of the reconstituted thin filaments). Experiments were performed at room temperature ($\sim 20^{\circ}\text{C}$).

Manipulating the microscope stage enabled the capture of the NEM myosin-coated beads (1 μ m diameter) in two separate laser traps. The ends of a single fluorescently-labeled actin or regulated thin filament were then attached to these beads and the filament pretensioned to ~ 4 pN. A 3 μ m diameter bead, serving as a pedestal, on the myosin coated surface was then brought into contact with the actin filament. The actin filament displacement was digitally recorded (4 kHz) using the bright-field image of one of the actin-attached beads that was projected onto a quadrant photodiode. Typically 1–2 min of data were recorded for each interaction with the myosin coated surface. These data were analyzed by mean-variance [18;20] to determine the step size of myosin's unitary displacement, d , and the duration of strong actin binding, t_{on} and the total number of events within a data record. At high binding event frequencies (0.5–2 events per second), the frequency or attachment rate can be determined by dividing the total record time by the number of events [11]. However, at very low binding event frequencies ($\sim 0.01 - 0.1 \text{ s}^{-1}$), we manually scanned through each displacement record generating an MV histogram every 2–5 sec to guide detection of binding events (see Figure 1). If the displacement variance dropped $\geq 50\%$ compared to baseline for at least 10 ms it was considered to be a binding event. These events were then totaled and divided by the duration of the data record to determine binding event frequency.

Statistical analysis

Values reported are the mean $\pm$ standard error unless otherwise noted. All measures were compared using a one-way ANOVA followed by post-hoc analysis to determine differences.

Results

The HCM-(E99K) and DCM-causing (R312H) mutations were successfully expressed in *Baculovirus* Sf/9 cells using a human cardiac actin sequence. Both actins readily polymerized and were successfully labeled with TRITC-phalloidin and fully regulated thin filaments were reconstituted with bovine cardiac Tn and Tm.

In vitro motility

The velocities of unregulated and regulated thin filaments propelled by β -cardiac myosin were determined for WT and the two mutant actin (Table 1). For unregulated actin, both the E99K and R312H mutants exhibited significantly reduced velocities compared to WT ($P < 0.05$; Table 1). With the addition of regulatory proteins, thin filament velocity for both actin mutations increased in a cooperative fashion as a function of free calcium with complete cessation of motility in the absence of calcium. The lack of movement at pCa 10 indicates that troponin and tropomyosin are able to bind to both E99K and R312H actin and regulate motility in a calcium-dependent manner. At maximal calcium activation, thin filament velocities for all actins were higher than unregulated filaments (Table 1), consistent with previous studies where the presence of regulatory proteins enhances thin filament velocities [21]. As with unregulated filaments, the velocity for the E99K thin filaments was significantly decreased at maximal Ca^{++} activation compared to WT ($P = 0.03$; Figure 2A, Table 1). However, the E99K mutation did not affect the calcium sensitivity of thin filament activation, as indicated by the pCa₅₀ (Table 1, Figure 2A inset). Incorporating the R312H actin mutation into a regulated thin filament resulted in a 25% reduction ($P = 0.008$) in velocity at maximal Ca^{++} activation relative to WT (Figure 2B; Table 1). In addition, the R312H thin filaments exhibited a significant

increase in calcium sensitivity (pCa_{50}) when compared to WT thin filaments ($P=0.016$; Figure 2B, Table 1). There was no significant difference between Hill coefficients for all actins in the regulated motility assay (Table 1).

Actin filament velocity is limited by myosin detachment [22] and with detachment dependent on the rate of ADP release (k_{-ADP}) and the subsequent rate of ATP binding (k_{+ATP}) to the myosin active site [23], it was possible to assess the contributions of these two kinetic parameters to the slowed mutant velocities by characterizing the ATP-dependence of velocity at maximal calcium activation ($pCa\ 4$) [24]. As demonstrated in Figure 3A, velocity increases hyperbolically with ATP concentration. Fitting these data to Michaelis-Menten kinetics (Sigma Plot, SPSS Inc.) yielded maximal velocities of $1.2 \pm 0.06\ \mu\text{m/s}$ for WT, $1.0 \pm 0.07\ \mu\text{m/s}$ for E99K and $1.0 \pm 0.06\ \mu\text{m/s}$ for R312H ($P<0.05$ for both mutants when compared to WT). The K_m for ATP was similar for all actins: WT, $29 \pm 6\ \mu\text{M}$; E99K, $27 \pm 9\ \mu\text{M}$; and R312H, $27 \pm 7\ \mu\text{M}$ ($P=NS$). See Discussion below and Table 3 for further analysis and estimates for k_{-ADP} and k_{+ATP} that were derived from of these data.

Single molecule experiments

Estimates of myosin step size, d , were determined by mean- variance analysis (see Methods). When unregulated WT actin was characterized using the three-bead laser trap assay, d for β -cardiac myosin was $7 \pm 2\ \text{nm}$ (Table 2, Figure 4A), in agreement with previous estimates using a rabbit cardiac myosin [24]. Both E99K and R312H exhibited similar unitary step sizes when compared to WT actin in either the presence or absence of regulatory proteins at $pCa\ 10$ (Table 2; Figure 4). In addition, the duration of strong actin binding (t_{on}) was not different for either actin mutation at $20\ \mu\text{M}$ ATP using either unregulated or regulated actin when compared to WT (Table 2).

In the absence of Tn and Tm, the myosin binding event frequency to actin was similar for WT, R312H, and E99K with 1 event occurring approximately every 2 seconds using a sparsely coated myosin surface ($1\ \mu\text{g}\cdot\text{ml}^{-1}$; Figures 4A, 5). However, the probability of myosin strong-binding events was reduced more than 10-fold using regulated thin filaments in the absence of calcium ($pCa\ 10$) when compared to actin alone (Figures 4C, 5), consistent with previous observations [11]. The fact that myosin can still bind to regulated thin filaments at $pCa\ 10$ where actin filament velocity is completely inhibited (see Figure 2) suggests that the equilibrium position of the Tn-Tm complex on actin under these conditions allows for a finite but low probability that the myosin can still bind productively to actin [11;25]. To overcome this inhibitory effect, the surface concentration of myosin was increased until the frequency of binding was restored to that observed using unregulated actin (Figures 4D, 5). This increase in myosin concentration is a direct measure of the degree of inhibition provided by the regulatory proteins [11]. A similar increase in myosin concentration using unregulated actin resulted in continuous actin filament motility (Figure 4B), thus further emphasizing the inhibitory effect of the regulatory proteins.

Regulated thin filaments containing WT actin at $pCa\ 10$ required that the myosin concentration be increased 12.5-fold to achieve the myosin binding frequency rate observed using unregulated actin (Figures 4D, 5). A similar increase in myosin concentration was required for R312H regulated thin filaments suggesting that thin filament inhibition in the absence of calcium is not affected by this mutation (Figures 4D, 5). In contrast, the E99K mutation greatly increased the degree of thin filament mediated inhibition of myosin strong-binding, as a 30-fold increase in myosin surface density was required to achieve the binding frequency of unregulated actin (Figures 4D, 4E, 5).

Thin Filament Cooperative Activation

Further increases in the surface concentration of myosin beyond that required to restore the unregulated binding frequency, elicits events that are staircase-like in appearance (Figure 6). These short bursts of motility result from multiple myosins sequentially binding to the thin filament in rapid succession. Such data provide information about the cooperative activation of the thin filament resulting from myosin strong-binding [11]. Using thin filaments reconstituted with WT actin, short bursts of motility were produced when the myosin surface density was increased from $12.5 \mu\text{g}\cdot\text{ml}^{-1}$ to $15 \mu\text{g}\cdot\text{ml}^{-1}$ with similar results obtained using regulated R312H thin filaments. However thin filaments reconstituted with E99K actin did not demonstrate these short bursts of motility until the myosin surface concentration was increased to $40 \mu\text{g}\cdot\text{ml}^{-1}$. This observation suggests that the presence of E99K actin in the thin filament inhibits myosin strong-binding activation of the thin filament to a greater extent than WT.

Discussion

This is the first study to examine the effects of human cardiomyopathic actin mutations on thin filament regulation. Both the HCM (E99K) and DCM (R312H) human actin mutations resulted in significant reductions in maximal Ca^{++} -regulated thin filament velocity. However the effects of these two actin mutations on thin filament regulation were quite different when characterized using the laser trap and motility assay. The E99K mutation exhibited a marked depression in thin filament activation by myosin strong-binding in the absence of calcium (pCa 10). In contrast when compared to WT actin, the R312H mutation increased Ca^{++} -sensitivity for velocity in the motility assay without effecting myosin strong-binding activation at pCa 10. These results highlight the fundamental differences in thin filament activation by myosin-strong binding and calcium. Such disparate alterations in thin filament regulation are likely key biomechanical triggers, initiating the molecular events that lead to the distinct patterns of pathological HCM and DCM ventricular remodeling.

Actin filament velocities

At the molecular level, actin filament velocities in the motility assay (V) are defined by myosin's step size, d , and myosin's attached lifetime, t_{on} , to actin following the step, i.e. $V \approx d/t_{\text{on}}$ [22]. Since both the E99K and R312H mutations had no effect on myosin's inherent motion generating capacity (i.e. step size) (see Table 2) using either unregulated or regulated thin filaments, the reduction in velocity for both mutants is likely the result of an alteration in the kinetics of the actomyosin interaction that determines t_{on} . We have shown previously [24;26] that t_{on} is limited by the rate of ADP release ($k_{-\text{ADP}}$) and the subsequent rate of ATP binding ($k_{+\text{ATP}}$) to myosin's active site so that:

$$t_{\text{on}} = (k_{-\text{ADP}} + ((k_{+\text{ATP}})[\text{MgATP}]))/((k_{+\text{ATP}})[\text{MgATP}](k_{-\text{ADP}})). \quad (\text{Eq. 1})$$

Using the 7 nm myosin step size determined in the laser trap, we are able to convert the velocity data in Figure 3A to a plot of t_{on} (i.e. $t_{\text{on}} = d/V$) as a function of ATP concentration (Figure 3B). Fitting this relation to Eq. 1 provides estimates of $k_{+\text{ATP}}$ and $k_{-\text{ADP}}$ (Table 3). The predicted $k_{+\text{ATP}}$ were similar for all 3 actins and in good agreement with biochemically derived values for bovine cardiac muscle [27]. Since $k_{+\text{ATP}}$ is the primary determinant of t_{on} at limiting ATP concentrations in the laser trap, the similarity in $k_{+\text{ATP}}$ would also explain why t_{on} in the laser trap (at 20 μM ATP) were similar for all 3 actins. In contrast, at saturating ATP, t_{on} is largely dependent on $k_{-\text{ADP}}$ such that $t_{\text{on}} \approx 1/k_{-\text{ADP}}$. Using the velocities at 2 mM ATP to estimate t_{on} and thus $k_{+\text{ATP}}$, the calculated $k_{-\text{ATP}}$ for the E99K and R312H were significantly lower than WT (Table 3). Although a similar trend was predicted by the fits to Eq. 1, the estimates for $k_{-\text{ATP}}$ are not as well defined (Table 3) and most likely reflects the fact that

k_{-ATP} is not constant over the range of velocities due its strain dependence [28]. Without any demonstrable effect on k_{+ATP} , these data suggest that both the E99K and the R312H mutation result in a decrease in ADP release kinetics (Table 3), slowing myosin detachment from actin and thus slower thin filament velocities. In addition, with velocities reduced for both unregulated and regulated thin filaments, it appears that both mutations directly modulate the actomyosin dissociation kinetics.

E99K thin filament regulation

The laser trap assay presents a unique opportunity to examine how mutant actins may impact the capacity of the thin filament in the absence of Ca^{++} to inhibit myosin strong-binding under low ATP concentrations (see Results; [11]). With unregulated E99K actin filaments, the frequency of myosin binding events was no different than for WT actin (see Figures 4A,5). In contrast, myosin strong-binding event frequencies with regulated thin filaments at pCa 10 were reduced over 3-fold for E99K at intermediate myosin concentrations (e.g. $10\text{--}20\ \mu\text{g}\cdot\text{ml}^{-1}$) when compared to WT thin filaments (Figures 4D,5). While a decrease in the strength of myosin strong-binding to E99K actin [8] may contribute in part to the increased inhibition of myosin binding in the absence of Ca^{++} , our data suggest that the E99K mutation directly modulates the regulatory behavior of the thin filament likely through the interaction of actin with both Tn and/or Tm.

We interpret these data using a three-state model of thin filament activation [29], where Tm transitions between the blocked, closed and open states. In this model in the absence of Ca^{++} , Tm predominantly occupies the blocked state. Ca^{++} binding to Tn then permits the transition of Tm from the blocked to closed state. With myosin strong-binding, Tm azimuthally shifts on actin to the open or fully active state [30]. Therefore, both Ca^{++} and myosin strong-binding are critical activators of the thin filament [31;32]. In the regulated thin filament, Tm is positioned over subdomain 1 and 2 in the absence of both calcium and myosin strong-binding [9]. The positive charge change associated with the E99K mutation may enhance the electrostatic interactions of Tm and actin, favoring the blocked state on Tm in the absence of Ca^{++} , similar to the proposed effect for a nearby actin mutation [33]. Accordingly, a higher myosin concentration on the motility surface would be required to activate the E99K thin filament by myosin strong-binding.

The cooperative activation of the thin filament, indicated by short bursts of motility in the laser trap at low ATP concentrations, is the mechanical equivalent of rigor activation observed in solution studies [34]. We have previously modeled this cooperative behavior as one myosin head initially binding to the thin filament, altering the Tm position on actin to increase the binding probability of a second myosin head [11;35]. The minimum myosin concentration required for the E99K ($40\ \mu\text{g}\cdot\text{ml}^{-1}$) to exhibit such short bursts of motility is approximately 2–3 times higher than both the WT and R312H ($12.5\text{--}15\ \mu\text{g}\cdot\text{ml}^{-1}$). Assuming that myosin is evenly dispersed across the motility surface and equally available to interact with the thin filament, and that once attached to actin in the laser trap, myosin's t_{on} is similar for the 3 actins (Table 2), these data indicate that the myosin heads must be closer together on the motility surface in order to cooperatively activate the E99K thin filament compared to WT filaments. In other words, the cooperative effect of myosin strong-binding to an E99K thin filament is not transmitted as well along the length of the thin filament as it is for WT and R312H thin filaments. The length of thin filament over which this increased binding probability extends has been termed a cooperative unit [31]. The present data suggests that the E99K cooperative unit size is reduced in comparison to WT thin filaments. As muscle activation is a highly cooperative process involving both calcium and myosin binding, a reduction in the cooperative unit length could slow the rate of thin filament activation along the filament, directly affecting the dynamic properties of muscle particularly at the initiation of myocardial contraction.

If the frequency of E99K myosin strong-binding events and its cooperative activation of regulated thin filaments in the absence of calcium are reduced, then why is the sensitivity of the pCa:velocity relation unaffected (Figure 2A)? It must be emphasized that the myosin binding events observed in the laser trap relate only to myosin's ability to activate the thin filament in the absence of calcium. At higher calcium, thin filament activation involves the transition of Tn-Tm from the blocked/closed to the open states, which is critically dependent on both calcium and myosin strong-binding [29;32]. The ability of myosin strong-binding to modulate the calcium sensitivity (pCa₅₀) of cardiac thin filaments in skinned myocardium was demonstrated by a leftward shift in the pCa:force relations with addition of a myosin strong-binding analogue (i.e. NEM-modified S1) [36]. While force is a function of the number of attached cross-bridges, maximal actin filament velocity and pCa₅₀ in these motility experiments are independent of the myosin surface density until myosin concentrations on the surface are reduced more than 60% of saturating levels [17]. At higher calcium levels, the calcium-dependent shift in the Tn-Tm equilibrium is likely sufficient to allow the high density of myosin molecules on the motility surface to fully activate the thin filament thus yielding the normal sensitivity to calcium for the E99K.

R312H thin filament regulation

In contrast to the E99K thin filaments, the frequency of myosin strong-binding events using regulated R312H thin filaments at pCa 10 was similar to WT regulated thin filaments (Figure 5). As strong-binding to a regulated thin filament requires myosin isomerization from the weak to the strongly bound state, these data would indicate that this kinetic transition is not affected by the R312H mutation. However, it is probable that the R312H mutation affects the actin conformation and/or its interaction with Tn-Tm leading to the observed increase in sensitivity of velocity to calcium (see Figure 2B, Table 1). To this point, the residue R312 in actin is thought to make strong contacts with Tm [10], thus charge changes in this region of actin may alter the equilibrium position of Tm and its resultant effects on thin filament regulation. Similar to our findings, regulated thin filaments in yeast, having the double E311A/R312A actin mutant, displayed a greater Ca⁺⁺ sensitivity in the motility assay but interestingly did not demonstrate a decrease in maximal velocity, indicating fundamental differences between these mutations in yeast actin and the human R312H mutation [37].

Role of functional alteration in cardiomyopathy

The complex mechanisms that lead from mutation to cardiomyopathy remain enigmatic, likely involving several intracellular signaling pathways [38]. Previous studies by us and others have demonstrated divergent functional effects of sarcomeric protein mutations that are causal in the development of a hypertrophic or dilated cardiomyopathic phenotype [39–41]. The present investigation highlights changes in the molecular function of two cardiomyopathy-causing mutations in human actin that likely trigger the pathological remodeling of the ventricle. While E99K demonstrated a reduced myosin strong-binding activation of the thin filament, R312H exhibited enhanced calcium activation of the thin filament. As the actively beating heart is in a constant state of activation and deactivation such divergent effects on the activation of the myofilament may be directly attributable to the different patterns of remodeling in these two disease associated mutations.

Acknowledgments

We would like to thank Samantha Beck and Kelly Begin for their superb technical assistance and Guy Kennedy of the Instrumentation and Model Facility at the University of Vermont for his mechano-optical expertise. This work was funded by a grant from the NIH HL59048 to DMW, PVB, and KMT.

Reference List

1. Maron BJ, Epstein SE, Roberts WC. Hypertrophic cardiomyopathy: a common cause of sudden death in the young competitive athlete. *Eur Heart J* 1983;4 (Suppl F):135–44. [PubMed: 6686529]
2. Ho CY, Sweitzer NK, McDonough B, Maron BJ, Casey SA, Seidman JG, et al. Assessment of diastolic function with Doppler tissue imaging to predict genotype in preclinical hypertrophic cardiomyopathy. *Circulation* 2002;105(25):2992–2997. [PubMed: 12081993]
3. Seidman JG, Seidman C. The genetic basis for cardiomyopathy: from mutation identification to mechanistic paradigms. *Cell* 2001;104(4):557–567. [PubMed: 11239412]
4. Mogensen J, Klausen IC, Pedersen AK, Egeblad H, Bross P, Kruse TA, et al. Alpha-cardiac actin is a novel disease gene in familial hypertrophic cardiomyopathy. *J Clin Invest* 1999;103(10):R39–R43. [PubMed: 10330430]
5. Mogensen J, Perrot A, Andersen PS, Havndrup O, Klausen IC, Christiansen M, et al. Clinical and genetic characteristics of alpha cardiac actin gene mutations in hypertrophic cardiomyopathy. *J Med Genet* 2004;41(1):e10. [PubMed: 14729850]
6. Olson TM, Doan TP, Kishimoto NY, Whitby FG, Ackerman MJ, Fananapazir L. Inherited and de novo mutations in the cardiac actin gene cause hypertrophic cardiomyopathy. *J Mol Cell Cardiol* 2000;32(9):1687–694. [PubMed: 10966831]
7. Van Driest SL, Ellsworth EG, Ommen SR, Tajik AJ, Gersh BJ, Ackerman MJ. Prevalence and spectrum of thin filament mutations in an outpatient referral population with hypertrophic cardiomyopathy. *Circulation* 2003;108(4):445–451. [PubMed: 12860912]
8. Bookwalter CS, Trybus KM. Functional consequences of a mutation in an expressed human alpha-cardiac actin at a site implicated in familial hypertrophic cardiomyopathy. *J Biol Chem* 2006;281(24):16777–6784. [PubMed: 16611632]
9. Brown JH, Zhou Z, Reshetnikova L, Robinson H, Yammani RD, Tobacman LS, et al. Structure of the mid-region of tropomyosin: bending and binding sites for actin. *Proc Natl Acad Sci U S A* 2005;102(52):18878–8883. [PubMed: 16365313]
10. Lorenz M, Poole KJ, Popp D, Rosenbaum G, Holmes KC. An atomic model of the unregulated thin filament obtained by X-ray fiber diffraction on oriented actin-tropomyosin gels. *J Mol Biol* 1995;246(1):108–19. [PubMed: 7853391]
11. Kad NM, Kim S, Warshaw DM, VanBuren P, Baker JE. Single-myosin crossbridge interactions with actin filaments regulated by troponin-tropomyosin. *Proc Natl Acad Sci U S A* 2005;102(47):16990–6995. [PubMed: 16287977]
12. Potter JD. Preparation of troponin and its subunits. *Methods Enzymol* 1982;85(Pt B):241–263. [PubMed: 7121270]
13. Homsher E, Kim B, Bobkova A, Tobacman LS. Calcium regulation of thin filament movement in an in vitro motility assay. *Biophys J* 1996;70(4):1881–892. [PubMed: 8785348]
14. VanBuren P, Alix SL, Gorga JA, Begin KJ, LeWinter MM, Alpert NR. Cardiac troponin T isoforms demonstrate similar effects on mechanical performance in a regulated contractile system. *Am J Physiol Heart Circ Physiol* 2002;282(5):H1665–H1671. [PubMed: 11959629]
15. Margossian SS, Lowey S. Preparation of myosin and its subfragments from rabbit skeletal muscle. *Methods Enzymol* 1982;85(Pt B):55–71. [PubMed: 6214692]
16. Malmqvist UP, Aronshtam A, Lowey S. Cardiac myosin isoforms from different species have unique enzymatic and mechanical properties. *Biochemistry* 2004;43(47):15058–5065. [PubMed: 15554713]
17. Gorga JA, Fishbaugher DE, VanBuren P. Activation of the calcium-regulated thin filament by myosin strong binding. *Biophys J* 2003;85(4):2484–2491. [PubMed: 14507711]
18. Guilford WH, Dupuis DE, Kennedy G, Wu J, Patlak JB, Warshaw DM. Smooth muscle and skeletal muscle myosins produce similar unitary forces and displacements in the laser trap. *Biophys J* 1997;72(3):1006–021. [PubMed: 9138552]
19. Kad NM, Rovner AS, Fagnant PM, Joel PB, Kennedy GG, Patlak JB, et al. A mutant heterodimeric myosin with one inactive head generates maximal displacement. *J Cell Biol* 2003;162(3):481–488. [PubMed: 12900396]
20. Patlak JB. Measuring kinetics of complex single ion channel data using mean- variance histograms. *Biophys J* 1993;65(1):29–42. [PubMed: 7690261]

21. Homsher E, Lee DM, Morris C, Pavlov D, Tobacman LS. Regulation of force and unloaded sliding speed in single thin filaments: effects of regulatory proteins and calcium. *J Physiol* 2000;524(Pt 1): 233–243. [PubMed: 10747195]
22. Huxley HE. Sliding filaments and molecular motile systems. *J Biol Chem* 1990;265(15):8347–8350. [PubMed: 2187860]
23. Siemankowski RF, Wiseman MO, White HD. ADP dissociation from actomyosin subfragment 1 is sufficiently slow to limit the unloaded shortening velocity in vertebrate muscle. *Proc Natl Acad Sci U S A* 1985;82(3):658–662. [PubMed: 3871943]
24. Palmiter KA, Tyska MJ, Dupuis DE, Alpert NR, Warshaw DM. Kinetic differences at the single molecule level account for the functional diversity of rabbit cardiac myosin isoforms. *J Physiol* 1999;519(Pt 3):669–678. [PubMed: 10457082]
25. Schaertl S, Lehrer SS, Geeves MA. Separation and characterization of the two functional regions of troponin involved in muscle thin filament regulation. *Biochemistry* 1995;34(49):15890–5894. [PubMed: 8519745]
26. Tyska MJ, Warshaw DM. The myosin power stroke. *Cell Motil Cytoskeleton* 2002;51(1):1–5. [PubMed: 11810692]
27. Marston SB, Taylor EW. Comparison of the myosin and actomyosin ATPase mechanisms of the four types of vertebrate muscles. *J Mol Biol* 1980;139(4):573–600. [PubMed: 6447797]
28. Jackson, dR, Jr; Baker, JE. The energetics of allosteric regulation of ADP release from myosin heads. *Phys Chem Chem Phys* 2009;11(24):4808–4814. [PubMed: 19506755]
29. McKillop DF, Geeves MA. Regulation of the interaction between actin and myosin subfragment 1: evidence for three states of the thin filament. *Biophys J* 1993;65(2):693–701. [PubMed: 8218897]
30. Galinska-Rakoczy A, Engel P, Xu C, Jung H, Craig R, Tobacman LS, et al. Structural basis for the regulation of muscle contraction by troponin and tropomyosin. *J Mol Biol* 2008;379(5):929–935. [PubMed: 18514658]
31. Geeves MA, Lehrer SS. Dynamics of the muscle thin filament regulatory switch: the size of the cooperative unit. *Biophys J* 1994;67(1):273–282. [PubMed: 7918995]
32. Vibert P, Craig R, Lehman W. Steric-model for activation of muscle thin filaments. *J Mol Biol* 1997;266(1):8–4. [PubMed: 9054965]
33. Cammarato A, Craig R, Sparrow JC, Lehman W. E93K charge reversal on actin perturbs steric regulation of thin filaments. *J Mol Biol* 2005;347(5):889–894. [PubMed: 15784249]
34. Bremel RD, Weber A. Cooperation within actin filament in vertebrate skeletal muscle. *Nat New Biol* 1972;238(82):97–01. [PubMed: 4261616]
35. Lehman W, Rosol M, Tobacman LS, Craig R. Troponin organization on relaxed and activated thin filaments revealed by electron microscopy and three-dimensional reconstruction. *J Mol Biol* 2001;307(3):739–744. [PubMed: 11273697]
36. Fitzsimons DP, Patel JR, Moss RL. Cross-bridge interaction kinetics in rat myocardium are accelerated by strong binding of myosin to the thin filament. *J Physiol* 2001;530(Pt 2):263–272. [PubMed: 11208974]
37. Gerson JH, Bobkova E, Homsher E, Reisler E. Role of residues 311/312 in actin-tropomyosin interaction. In vitro motility study using yeast actin mutant e311a/r312a. *J Biol Chem* 1999;274(25): 17545–7550. [PubMed: 10364188]
38. Heineke J, Molkentin JD. Regulation of cardiac hypertrophy by intracellular signalling pathways. *Nat Rev Mol Cell Biol* 2006;7(8):589–600. [PubMed: 16936699]
39. Morimoto H, Hirose M, Takahashi M, Kawaguchi M, Ise H, Kolattukudy PE, et al. MCP⁻¹ induces cardioprotection against ischaemia/reperfusion injury: role of reactive oxygen species. *Cardiovasc Res* 2008;78(3):554–562. [PubMed: 18267955]
40. Lombardi R, Bell A, Senthil V, Sidhu J, Noseda M, Roberts R, et al. Differential interactions of thin filament proteins in two cardiac troponin T mouse models of hypertrophic and dilated cardiomyopathies. *Cardiovasc Res* 2008;79(1):109–17. [PubMed: 18349139]
41. Debold EP, Schmitt JP, Moore JR, Patlak JB, Beck SE, Seidman JG, et al. Hypertrophic and dilated cardiomyopathy mutations differentially affect the molecular force generation of mouse {alpha}-cardiac myosin in the laser trap assay. *Am J Physiol Heart Circ Physiol*. 2007

Figure 1. Myosin binding event detection by mean-variance analysis

At low myosin binding event frequencies, the mean-variance (MV) analysis technique was used to identify individual binding events in the laser trap assay (see [18] for details of the analysis). In brief, a moving window (20 ms in duration) was passed over the raw displacement trace (lower traces) to calculate a displacement variance and mean at every time point for consecutive 2–5 s periods. This analysis generated MV histograms (upper panels). For displacement traces without any myosin binding events (baseline), the MV histogram was described by a single high variance population centered at 0 nm displacement (left upper panel). When a binding event occurred, the MV histogram for the corresponding displacement trace (event) was described by a high variance population and a separate low variance population with a mean displacement equal to the myosin step size (right upper panel).

Figure 2. *In vitro* motility data

Velocity:pCa relationships for regulated thin filaments using WT, E99K, and R312H mutated human cardiac actin. Each point represents the mean $\pm$ SEM, WT, $n = 6$; E99K, $n = 5$; R312H, $n = 4$; where n is the number of experiments. The velocity:pCa data were fit to the Hill equation. (A) E99K demonstrated slower maximal velocities ($P < 0.05$). As demonstrated in the inset where velocity is normalized to the maximum of the Hill fit, no change in half maximal activation (pCa_{50}) was observed, see Table 1. (B) R312H showed a decrease in maximal velocity ($P < 0.05$) and a leftward shift in the velocity:pCa relation ($P < 0.05$) as emphasized in the inset.

Figure 3. The ATP dependence of velocity and t_{on}

(A) Velocity as a function of ATP concentration for WT, E99K, and R312H. The regression lines depict the fit of the data to the Michaelis-Menten equation for WT (solid), E99K (dotted) and R312H (dashed). (B) t_{on} (determined from the relation $V=d/t_{on}$) as a function of ATP, assuming $d=7$ nm (see results). Regression line depict the fit of the data to Equation 1 using the k_{ADP} values derived from t_{on} values at high ATP concentrations for WT (solid), E99K (dotted) and R312H (dashed), (see Table 3 for parameters of the fit).

Figure 4. Myosin binding events to both unregulated and regulated thin filaments in the laser trap assay

(A) Raw displacement traces highlighting myosin binding events (arrowheads) using unregulated WT, E99K, and R312H actin at $1 \mu\text{g}.\text{ml}^{-1}$ myosin concentration and $20 \mu\text{M}$ ATP. (B) Unregulated filaments regardless of the expressed actin exhibited continuous motility when the myosin concentration was increased to $12.5 \mu\text{g}.\text{ml}^{-1}$. (C) Thin filaments reconstituted with Tn and Tm reduced the frequency of binding events in the absence of calcium by an order of magnitude at the same myosin and ATP concentrations as in panel A. (D) The frequency of binding events was restored to that observed for unregulated thin filaments for the WT and R312H regulated thin filaments by increasing the myosin concentration on the surface 12.5-fold ($12.5 \mu\text{g}.\text{ml}^{-1}$ myosin). This was not the case for the E99K. (E) Only when the myosin concentration was increased to $35 \mu\text{g}.\text{ml}^{-1}$ was the binding event frequency restored for E99K regulated thin filaments to unregulated levels as in panel A.

Figure 5. Binding event frequency vs. myosin concentration

Single binding event frequency data (mean $\pm$ SEM) are plotted as a function of the myosin concentration on the cover-glass surface. The event frequency was determined from 2–20 displacement records of 1–2 min in length for WT (circle), E99K (triangle), and R312H (square) filaments. The event frequency for unregulated actin was not different among the three actin variants used (open symbols) at 1 $\mu\text{g}\cdot\text{ml}^{-1}$ myosin concentration. With unregulated actin, using myosin concentrations greater than 1 $\mu\text{g}\cdot\text{ml}^{-1}$ resulted in continuous motility (indicating multiple myosin binding events) as exemplified in Figure 4B. The frequency of binding for all variants of regulated thin filaments (RTF) (closed symbols) was significantly less than that for unregulated actin and only upon increasing the myosin surface density was the binding frequency restored to unregulated values. R312H mutant RTF elicited binding event frequencies that were similar to WT actin at the same myosin concentrations. Myosin concentrations beyond the highest concentrations plotted for each actin was no longer associated with single binding events but rather bursts of motility were observed, indicating multiple, sequential binding events (see Results, Figure 6). All experiments were performed at 20 μM ATP. * indicates that the frequency of binding events is significantly less than the observed frequency for WT RTF at 12 $\mu\text{g}\cdot\text{ml}^{-1}$ ($P < 0.05$).

Figure 6. Cooperative activation of the thin filament in the laser trap

Using WT regulated thin filaments (RTF), short bursts of motility were elicited at a myosin surface concentration of $15 \mu\text{g}.\text{ml}^{-1}$. This indicates that two or more heads of myosin were bound to the thin filament and provides a measure of the activation length along the thin filament (i.e. cooperative unit) [11]. Using mutant E99K RTF almost three-fold ($40 \mu\text{g}.\text{ml}^{-1}$) more myosin was required to observe short bursts of motility. This suggests that the length of cooperative activation is reduced for E99K actin. (see Discussion).

Table 1

In vitro motility parameters of regulated WT, E99K, and R312H thin filaments.

Actin	Unregulated Actin Velocity ($\mu\text{m}\cdot\text{s}^{-1}$)	Maximum RTF Velocity ($\mu\text{m}\cdot\text{s}^{-1}$)	pCa ₅₀	Hill Coefficient
WT (n = 5)	1.0 ± 0.1	1.3 ± 0.1	6.34 ± 0.04	2.9 ± 0.8
E99K (n = 6)	0.7 ± 0.04 [*]	1.1 ± 0.1 [*]	6.39 ± 0.09	1.7 ± 0.6
R312H (n = 4)	0.5 ± 0.03 [*]	1.0 ± 0.1 [*]	6.64 ± 0.08 [*]	1.7 ± 0.6

Maximum velocity for regulated thin filaments (RTF), pCa₅₀, and Hill coefficient determined from the fit of the Hill equation, see Figure 2.

^{*} indicates significantly different than WT at P≤0.05.

Table 2

Laser trap assay analysis of unitary myosin binding events for WT, E99K, and R312H actin filaments.

	Actin	<i>d</i> (nm)	<i>t</i> _{on} (ms)
Unregulated	WT	7 ± 2	29 ± 14
	E99K	9 ± 2	23 ± 10
	R312H	8 ± 3	46 ± 12
Regulated (pCa 10)	WT	7 ± 3	34 ± 18
	E99K	8 ± 4	38 ± 24
	R312H	9 ± 3	29 ± 14

Mean variance analysis of the raw displacement trace [18] was used to determine the unitary step size of myosin (*d*) and the duration of strong actin binding (*t*_{on}). Unregulated actin and regulated thin filaments were used in this assay. The values represent the mean ± SD of between 6 and 20, 1–2 minute segments of a laser trap displacement record at 20μM. No significant differences were observed between WT and either of the mutant actin filaments.

Table 3

ADP release and ATP binding rates

Actin	k_{+ATP} ($\mu\text{M}^{-1}\text{s}^{-1}$) (fit)	k_{-ADP} (s^{-1}) (fit)	k_{-ADP} (s^{-1}) (derived)
WT	2.9 ± 0.1	236 ± 106	180 ± 15
E99K	3.5 ± 0.3	102 ± 26	$133 \pm 13^*$
R312H	2.8 ± 0.2	115 ± 52	$140 \pm 11^*$

ADP release rate (k_{-ADP}) and ATP binding rate (k_{+ATP}) were determined from the fit of the t_{on} versus [ATP] in Figure 3B to Equation 1 (see Discussion). A derived value for k_{-ADP} was determined from t_{on} values at 2 mM ATP (see Discussion).

* indicates significantly different than WT at $P \leq 0.05$.