Functional Consequences of a Mutation in an Expressed Human α-Cardiac Actin at a Site Implicated in Familial Hypertrophic Cardiomyopathy*

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Point mutations in human α-cardiac actin cause familial hypertrophic cardiomyopathy. Functional characterization of these actin mutants has been limited by the lack of a high level expression system for human cardiac actin. Here, wild-type (WT) human α-cardiac actin and a mutant E99K actin have been expressed and purified from the baculovirus/insect cell expression system. Glu-99 in subdomain 1 of actin is thought to interact with a positively charged cluster located in the lower 50-kDa domain of the myosin motor domain. Actin-activated ATPase measurements using the expressed actins and β-cardiac myosin showed that the mutation increased the $K_m$ for actin 4-fold ($4.7 \pm 0.7 \mu M$ for WT versus $19.1 \pm 3.0 \mu M$ for the mutant), whereas the $V_{max}$ values were similar. The mutation slightly decreased the affinity of actin for S1 in the absence of nucleotide, which can partly be accounted for by a slower rate of association. The in vitro motility for the E99K mutant was consistently lower than WT over a range of ionic strengths, which is likely related to the lower average force supported by the mutant actin. The thermal stability of the E99K was comparable to that of WT-actin, implying no folding defects. The lower density of negative charge in subdomain 1 of actin therefore weakens the actomyosin interaction sufficiently to decrease the force and motion generating capacity of E99K actin, thus providing the primary insult that ultimately leads to the disease phenotype.

The majority of heritable cardiac conditions are either hypertrophic cardiomyopathy (HCM)7 or dilated cardiomyopathy (DCM). HCM, a frequent cause of sudden cardiac death in young athletes, is characterized by an increase in the ventricular mass (hypertrophy) of the heart muscle, whereas DCM is typified by thinning of the ventricular wall. HCM is transmitted as an autosomal dominant trait, and molecular genetic studies show that there are over 200 missense mutations in eight different sarcomeric contractile protein genes (reviewed in Ref. 1). To date there are nine recognized actin mutations implicated in cardiomyopathies. Seven are associated with HCM ([E99K, P164A, Y166C, A230V, A295S, M305L, and A331P] (2–5), and two are found in DCM (V925I and H9251) (6). Patients carrying these actin mutations display a phenotype ranging from severe cardiac disease to being asymptomatic. The conserved nature of the amino acid sequence of actin suggests that mutated residues will likely affect basic actin functions, including actomyosin interactions, actin-actin interactions involved in polymerization, interactions with the Z line, or the binding of regulatory proteins. The current study focuses on the HCM actin mutation E99K, which is located in a surface loop in subdomain 1 of actin (Fig. 1A). Individuals with this HCM mutation manifest with a later onset of the disease, and they display a variable degree of hypertrophy (4).

During the cross-bridge cycle, myosin makes contact with actin at several interfaces in a multistep process. The initial weak interaction is mainly ionic in nature and transitions into stronger binding using hydrophobic contacts. Each myosin head interacts with two adjacent actin monomers along the F-actin helix. The primary binding site is with subdomain 1 of the upper actin monomer, whereas the secondary binding site occurs with subdomain 1 of the actin monomer below. Glu-99 is believed to be part of this secondary binding site, which is also ionic in nature (Fig. 1, B and C). It is one of three acidic residues (Glu-93, Glu-99, and Glu-100), which are thought to interact with a positively charged loop in the lower 50-kDa domain of the myosin motor domain (7–9). A charge reversion such as that occurring in the E99K mutant is likely to have an impact on actomyosin interactions.

To ultimately understand the functional effects that actin mutations have in cardiac disease, it is critical to have an expression system capable of producing mutant actins in quantities abundant and pure enough to allow investigation of its primary defect at the molecular level. Attempts to express actin in Escherichia coli resulted in insoluble aggregates due to the lack of the required cytosolic chaperonin containing TCP-1 in prokaryotes (10, reviewed in Ref. 11). Mutated cardiac actin can be synthesized in cell-free coupled transcription/translation systems (12), but only picogram quantities of protein are expressed, making it difficult to obtain the necessary biochemical and biophysical information. Yeast is a viable expression system (reviewed in Ref. 13), but yeast actin shares only 87% identity with human α-cardiac actin, making it less than ideal to study human disease. Likewise, Dictostelium discoideum and Drosophila can be used to express actin, but the functional consequences of mutations implicated in human disease need to be investigated in the correct isoform.

Our laboratory has succeeded in expressing actin in high yields in the baculovirus/Sf9 cell system (14). Here we cloned and expressed human α-cardiac actin (WT) and the charge reversal mutant E99K implicated in HCM, in the baculovirus expression system. Our results suggest that the primary defects of E99K actin are slower motility, reduced average force, and a weakened interaction with cardiac myosin in the presence of ATP. These deficits at the molecular level appear to be sufficient to trigger the disease phenotype.
Effects of E99K Mutation in Human Cardiac Actin

EXPERIMENTAL PROCEDURES

Plasmid Construction—The Drosophila 5C actin gene (generous gift from Loy Volkman) was used as a template to produce the coding sequence of wild-type human α-cardiac actin (WT) by site-directed mutagenesis. The resulting gene was cloned into the baculovirus transfer vector pAcUW51 (BD Biosciences) behind the p10 promoter. An HCM actin mutant (E99K) was made using WT as the template. Both constructs were fully sequenced to verify mutagenesis and the absence of PCR-induced errors. The numbering system corresponds to that of human α-cardiac muscle actin.

Expression and Purification of Recombinant Actin—Recombinant baculovirus encoding for WT and E99K were prepared using established protocols (15). Infected Sf9 cells in suspension were harvested at 72 h and lyed in 1 M Tris-HCl, pH 7.5, 0.6 M KCl, 0.5 mM MgCl2, 4% Triton X-100, 1 mg/ml Tween 20, 0.5 mM NaN3, 1 mM DTT, 0.5 mM 4-(2-Aminoethyl)-benzenesulfonfluoride hydrochloride, 5 mM benzamidine, and 5 μg/ml leupeptin. The cell lysate was clarified and dialed overnight into 15 mM MOPS, pH 7, 0.2 mM CaCl2, 0.2 mM DTT, 0.25 mM NaN3, and 1 μg/ml leupeptin. The lysate (150 ml from a 3 billion cell preparation of 3 billion cells) was incubated with 60 ml of SP-Sepharose (Amersham Biosciences) for 1 h. The flow through was collected and dialyzed into G buffer, and concentrated with an Amicon Ultra centrifugal filter device (Millipore Corp.). The actin was polymerized overnight on ice by addition of 0.1 M KCl and 2 mM MgCl2 and spun at 328,000 × g for 2 h. The pellet was resuspended in actin storage buffer (5 mM NaCl, 10 mM Imidazole, pH 7, 1 mM MgCl2, 1 mM DTT). Concentration was determined from absorbance at 290 nm using an extinction coefficient of 0.63 ml mg⁻¹ cm⁻¹. Typical yield was ~3 mg of protein/billion cells. Actin that was not used immediately (within 5 days) was dialyzed back into G buffer for 3 days and stored in liquid nitrogen after adding 2 mg of sucrose/mg of G-actin.

Purification of Other Proteins—Rabbit β-cardiac myosin was prepared from frozen rabbit hearts with atria removed (Pel Freez Biologicals) following the method described for skeletal muscle myosin (16) with minor modifications (17). The purified myosin was stored at −20 °C in 50% glycerol for up to 6 months. This myosin was used to prepare cardiac S1 (cS1) by chymotryptic cleavage as described (18). Tissue-purified chicken skeletal actin was used in many assays as a control due to its availability and similarity to cardiac actin. Skeletal actin was prepared from chicken pectoralis acetone powder as described (19).

Gels and Immunoblots—Proteins were separated on a 4–12% Bis-Tris NuPAGE gel (Invitrogen) run in MES buffer (per NuPAGE technical guide). Immunoblots were incubated with either monoclonal anti-α-sarcomeric actin (Sigma clone 5C5, 1:500 dilution) or monoclonal anti-β-cytoplasmic actin (Sigma clone AC-15, 1:1000 dilution). The secondary antibody was anti-mouse horseradish peroxidase, and detection was achieved by using a 3,3′-diaminobenzidine peroxidase substrate tablet set (Sigma).

Actin-activated ATPase—The actin-activated ATPase activity of β-cardiac rabbit myosin (137 μg/ml) was determined at 30 °C using various concentrations of the two recombinant actins (WT and E99K) in 10 mM imidazole, pH 7.0, 50 mM NaCl, 1 mM MgCl2, 1 mM NaN3, and 1 mM DTT. The assay was initiated by the addition of 2 mM MgATP and stopped with SDS at six time points every 10 min for 1 h. The inorganic phosphate was determined colorimetrically. Details were described previously (20). For β-cardiac rabbit S1 (30 μg/ml), the buffer conditions were 10 mM imidazole, pH 7.0, 5 mM NaCl, 1 mM MgCl2, 1 mM NaN3, and 1 mM DTT at 30 °C. The lower salt concentration was needed to keep the Kᵢ values as low as possible.

Rigor Binding Affinity—Pyrene-labeled chicken skeletal actin was prepared as described (20) and stored in 10 mM HEPEs, pH 7.0, 0.1 M NaCl, 5 mM MgCl2, 1 mM EGTA, 1 mM NaN3, and 1 mM DTT. Protein concentration was determined by Bradford reagent (Pierce) with bovine serum albumin as a standard. The competing and pyrene-labeled actins were stabilized by the addition of equimolar phallolidin for 24 h before use. Nucleotide stocks were prepared with equimolar amounts of magnesium. Pyrene-actoS1 (15 nM pyrene-labeled actin, various concentrations of the unlabeled competing actin, and 250 nM cS1) in 10 mM HEPEs, pH 7.0, 0.2 M KCl, 5 mM MgCl2, 1 mM EGTA, 1 mM NaN3, and 1 mM DTT was mixed with 6 μM MgATP at 20 °C using a Kin-Tek stopped flow spectrophotometer. 100 nM cS1 was used in similar experiments containing 0.1 M KCl. Pyrene actin fluorescence was excited at 360 ± 10 nm, and emission was detected with a 400-nm cutoff filter. All transients are the average of at least five to eight independent mixings.

Data were fitted to a competitive binding curve essentially as described before (21). The y-axis value was not fixed when fitting the curve but allowed to float. The Kᵢ of cS1 for the pyrene-labeled actin was determined by fitting to a curve but allowed to float. The Kᵢ of cS1 for the pyrene-labeled actin was...
set at 12 nM in 100 mM KCl, and 200 nM in 200 mM KCl, based on independent direct binding assays. Pyrene actin concentration was 15 nM, and cS1 concentration was 100 nM at 100 mM KCl and 250 nM at 200 mM KCl.

Rates of Binding to Actin—Binding of actin to cS1 was measured in the presence or absence of ADP. Various actin concentrations in 10 mM HEPES, pH 7.0, 25–100 mM KCl, 5 mM MgCl$_2$, 1 mM EGTA, 1 mM NaN$_3$, and 1 mM DTT, ± 1 mM ADP was mixed with 0.5 µM cS1. For 90° light scattering, the exciting beam was 294 ± 10 nm and the emission was detected with a 294 nm interference filter. At least five to eight transients were averaged before being fitted to an exponential using Kin-Tek software.

In Vitro Motility Assay—Actin filament velocity was measured at 30 °C in motility buffer (25 mM imidazole, pH 7.5, 0–100 mM KCl, 1 mM EGTA, 4 mM MgCl$_2$, 10 mM DTT, 1 mM MgATP, 2.9 mg/ml glucose, 0.125 mg/ml glucose oxidase (Sigma), and 0.023 mg/ml catalase (Sigma)) (20). If used, methylcellulose was 0.5% or 0.7%. Cardiac myosin was applied to nitrocellulose-coated flow cells at 66 µg/ml. In early assays 200 µg/ml cardiac myosin was combined with equimolar actin and 1 mM MgATP and centrifuged for 20 min at 350,000 × g to remove myosin that was unable to dissociate from actin in the presence of ATP. Myosin was adhered to the nitrocellulose-coated flow cell, and the surface was blocked with 0.5 mg/ml bovine serum albumin. An actin/ATP wash was then performed by adding 1 µM vortexed actin (to create small filaments) for 30 s, followed by a 1 mM MgATP wash, to remove any remaining rigor heads. In later motility assays, the cardiac myosin was purified by hydrophobic interaction chromatography immediately preceding the assay, and the actin/ATP wash was eliminated. The velocities of at least 55 filaments and up to as many as 250 filaments were averaged to obtain the mean ± S.D. Data were analyzed using the program described previously (22).

Average Force Assay—Isometric force was measured in vitro motility by adding α-actinin in varying amounts to create an internal load on the actin until velocity was stopped. The assay was performed as described (17) at 30 °C, using motility buffer containing 50 mM KCl and 0.5% methylcellulose. In all cases, cardiac myosin was purified by hydrophobic interaction chromatography immediately before the assay was performed. Myosin was infused in the nitrocellulose flow cells and allowed to incubate for 1 min, followed by three washes with motility buffer containing no ATP or methylcellulose. Varying concentrations of α-actinin were then added for 1 min, followed by blocking with three washes of rigor motility buffer containing 0.5 mg/ml bovine serum albumin. Fluorescently labeled actin was added twice for 30 s each. Rigor motility buffer was added twice, followed by two washes of motility buffer with ATP and methylcellulose.

Thermal Denaturation—DNase-I inhibition was performed as described (23). Briefly, actin was heated and held at increasing temperatures (1 °C/min) in a Mastercycler gradient 5331 (Eppendorf). The actin samples were removed from the heat at specific temperature points (25–65 °C) and placed on ice until all samples were collected. The actin samples were mixed with salmon sperm DNA (Sigma) and held at 25 °C for at least 5 min. Final actin concentration in the assay was 1.2 µg/ml, and DNA was 40 µg/ml (100 mM Tris, pH 7.6, 4 mM MgCl$_2$, 1.8 mM CaCl$_2$, and 2 mM NaN$_3$). Immediately before following the absorbance change, 0.6 µg/ml DNase-I (from bovine pancreas, Grade I, Roche Applied Science) was added to the actin-DNA mixture in the cuvette. The optical density at 260 nm was followed continuously for 1 min at 25 °C using a Hitachi U-2001 spectrophotometer. The slopes were determined at each time point. Percent inhibition was determined using the formula [1 − (slope at a given temperature/average slope at 65 °C)] × 100. The melting temperature ($T_m$) was determined as the 50% point between 25 °C (100% inhibition of DNase-I and 0° slope) and 65 °C (0% inhibition and the maximum slope).

RESULTS

Expression of Human α-Cardiac Actin—Wild-type (WT) and a mutant (E99K) human α-cardiac actin were expressed in S9 cells. Actin was clearly the predominant protein expressed by the infected S9 cells. Following purification by ion exchange chromatography and a functional polymerization step, the expressed actin showed no major contaminants (Fig. 2). Yields of purified protein were similar for the WT and mutant actins (~3 mg/billon S9 cells). Western blotting with antibodies specific for mammalian skeletal or cardiac muscle actins (Sigma clone 5C5) recognized both recombinant cardiac actins but not endogenous S9 cell actin. Western blotting with a monoclonal antibody specific to β-cytoplasmic actin (Sigma clone AC-15) showed a small car- ryeover of endogenous S9 cell actin (data not shown). Negatively stained images of the expressed actins showed normal filament morphology and no evidence of bundling (data not shown), as had been reported for the Dictyostelium E99H/E100H actin mutant (24).

In some of the experiments described below, actin purified from chicken skeletal tissue was included as an additional species for comparison. Human and avian skeletal actin are identical, whereas cardiac and skeletal actin differ at only four positions (D2E, E3D, L299M, and S358T).

The E99K Mutation Increases the $K_m$ for Cardiac Myosin—The steady-state actin-activated ATPase activity of β-cardiac myosin as a function of increasing actin concentrations was determined for WT and E99K actins at 50 mM NaCl (Fig. 3A). The $K_m$ for actin was 4-fold higher for E99K actin than for WT-actin, while the $V_{max}$ values were similar (Table 1). Data obtained with tissue purified actin was indistinguishable from expressed WT-actin (data not shown). Thus, one consequence of the charge reversal mutation is a large increase in the $K_m$ of cardiac actin.
myosin for actin, without an appreciable change in the maximum acto-myosin ATPase activity.

Actin-activated ATPases were also performed with cardiac S1 at 5 mM NaCl (Fig. 3B). Similar to the experiments described above with cardiac myosin, the $V_{\text{max}}$ values for WT-actin and E99K were similar (Table 1). Note that the $V_{\text{max}}$ values obtained with cS1 are ~4-fold higher than obtained with whole myosin, a characteristic typical of chymotryptic S1 from striated muscle myosins. In contrast to the data obtained with myosin, both actins showed a similar $K_m$ value (Table 1).

As a control, the same actin preparation used for the S1 assays still showed a large difference in $K_m$ when full-length myosin was used. Pelletting assays with cS1 and the two actins in the presence of ATP also revealed no differences in affinity (data not shown). Possible explanations for the differences between the S1 and myosin results are addressed under “Discussion.”

**TABLE 1**

**Actin activated ATPase activity**

Conditions: 30 °C in 10 mM imidazole, pH 7.0, 0.1 M NaCl, 1 mM MgCl$_2$, 1 mM NaN$_3$, and 1 mM DTT.

| Actin   | Cardiac myosin | Cardiac S1 |
|---------|----------------|------------|
|         | $V_{\text{max}}$ | $K_m$     | $V_{\text{max}}$ | $K_m$     |
| WT      | 0.81 ± 0.04    | 4.7 ± 0.7  | 4.4 ± 0.2        | 23.3 ± 3.3 |
| E99K    | 0.98 ± 0.09    | 21.6 ± 3.7 | 4.6 ± 0.4        | 29.6 ± 5.9 |

As a control, the same actin preparation used for the S1 assays still showed a large difference in $K_m$ when full-length myosin was used. Pelletting assays with cS1 and the two actins in the presence of ATP also revealed no differences in affinity (data not shown). Possible explanations for the differences between the S1 and myosin results are addressed under “Discussion.”

**Affinity of S1 for Actin in the Absence of Nucleotide Is Affected by the Point Mutation**—A competitive binding assay was used to assess the affinity of cardiac S1 for the mutant E99K, WT, and tissue-purified actins in the absence of nucleotide (21). cS1 and pyrene-labeled tissue-purified actin were incubated with increasing concentrations of unlabeled competing actins (WT, E99K, or tissue-purified). The actomyosin mixture was dissociated in the stopped flow by mixing with MgATP (Fig. 4A). The amplitude of the pyrene signal decreased as the concentration of the unlabeled competing actin increased, consistent with greater competition of unlabeled actin for cS1. The amplitude of the signal was plotted as a function of the concentration of the unlabeled actin (Fig. 4B). When the data were fitted to a competitive binding equation to derive the $K_d$ values (21), E99K actin showed only a slightly decreased affinity for cS1 compared with WT-actin (Table 2).
The rate of association of actin and cS1 in the presence or absence of MgADP was examined using transient kinetics. The rate of association of cS1 to E99K actin, both in the presence of ADP and in rigor, was ~60% of that observed with WT-actin (Table 3). Because the change in the equilibrium binding constant in rigor was smaller than the observed change in the association rate, the dissociation rate constant must also be affected by the mutation.

Cardiac Myosin Moves E99K Actin at Slower Velocities Than WT-actin—The effect of the mutation on the motor properties of actomyosin was assessed by the in vitro motility assay. All the actin filaments in the field (WT, E99K, and tissue-purified) were moved by β-cardiac myosin. The mutant E99K showed a reduced velocity of between 13 and 21% relative to WT and tissue-purified actin in the presence of methylcellulose, over a range of KCl concentrations (Fig. 5A). The difference between E99K and either the WT or tissue-purified actins was statistically different (p < 0.01) at all KCl concentrations but was most pronounced at 100 mM. There was no statistical difference between WT and tissue-purified actin. The lower velocity of the E99K actin was not due to sub-saturating methylcellulose concentrations, because the velocity at which the mutant actin moved remained the same from 50 to 200 μM methylcellulose (50 mM KCl).

Methylcellulose prevents diffusion of actin away from the myosin substrate, so in vitro motility was also performed without this additive (Fig. 5B). Interactions between actin and myosin weaken with increasing salt concentration, and at some point the actin filaments diffuse away from the myosin-coated surface. At <10 mM KCl, the velocity of WT and E99K actin was similar. As the KCl concentration increased, the velocity of the E99K actin was ~10% slower than that of WT-actin. Consistent data could only be obtained at 35 mM KCl for E99K actin, and at 45 mM KCl for both WT and tissue-purified actins before total detachment occurred. (Data for tissue-purified actin are not shown.) For each data point shown, the velocities of at least 5 filaments and up to as many as 250 filaments were averaged to obtain the mean ± S.D.

![FIGURE 5. In vitro motility assay. Velocity of E99K (●), WT (○), and tissue-purified (△) actins sliding over β-cardiac rabbit myosin at varying KCl concentrations. A, in the presence of methylcellulose, lower velocities (between 8 and 21%) were seen at all KCl concentrations with the E99K mutant actin. There was a statistical difference (p < 0.01) between E99K and both WT and tissue-purified actins at all KCl concentrations. There was no statistical difference between tissue-purified and WT-actins. Conditions: 25 mM imidazole, pH 7.5, 0–100 mM KCl, 1 mM EGTA, 4 mM MgCl2, 0.1 mM DTT, and 1 mM ADP at 20 °C. B, in vitro motility in the absence of methylcellulose, other conditions the same as in A. At less than 10 mM KCl the velocity of WT and E99K actin were similar, but with increasing KCl concentrations the velocity of E99K was ~10% slower than WT. Directed movement ceased at a lower KCl concentration for E99K than for WT. Consistent data could only be obtained at 35 mM KCl for E99K actin, and at 45 mM KCl for both WT and tissue-purified actins before total detachment occurred. (Data for tissue-purified actin are not shown.) For each data point shown, the velocities of at least 5 filaments and up to as many as 250 filaments were averaged to obtain the mean ± S.D.](Image 218x26 to 246x38)

#### TABLE 2

Dissociation constants of cardiac S1 for actin in the absence of nucleotide

| Conditions     | E99K | WT             | Tissue-purified |
|----------------|------|----------------|----------------|
| ADP, 25 mM KCl| 2.58 (n = 2) | 3.75 (n = 2) | 3.83 (n = 3) |
| Rigor, 100 mM KCl| 1.40 (n = 2) | 1.92 (n = 2) | 3.0 (n = 3) |

The rate of association of actin and cS1 in the presence or absence of MgADP was examined using transient kinetics. The rate of association of cS1 to E99K actin, both in the presence of ADP and in rigor, was ~60% of that observed with WT-actin (Table 3). Because the change in the equilibrium binding constant in rigor was smaller than the observed change in the association rate, the dissociation rate constant must also be affected by the mutation.

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Methylcellulose prevents diffusion of actin away from the myosin substrate, so in vitro motility was also performed without this additive (Fig. 5B). Interactions between actin and myosin weaken with increasing salt concentration, and at some point the actin filaments diffuse away from the myosin-coated surface. At <10 mM KCl, the velocity of WT and E99K actin was similar. As the KCl concentration increased, the velocity of the E99K actin was ~10% slower than that of WT-actin. Consistent data could only be obtained at 35 mM KCl for E99K actin, and at 45 mM KCl for both WT and tissue-purified actins before total detachment occurred. (Data for tissue-purified actin are not shown.) For each data point shown, the velocities of at least 5 filaments and up to as many as 250 filaments were averaged to obtain the mean ± S.D.
Effects of E99K Mutation in Human Cardiac Actin

The concentration of actin was purified by hydrophobic interaction chromatography and used at 150 μg/ml, whereas both WT and tissue-purified actin needed a concentration of 1.75 μg/ml actin to stop movement. β-Cardiac rabbit myosin was purified by hydrophobic interaction chromatography and used at 150 μg/ml. Conditions: 25 mM imidazole, pH 7.5, 50 mM KCl, 1 mM EGTA, 4 mM MgCl₂, 10 mM DTT, 1 mM MgATP, and oxygen scavengers at 30 °C.

FIGURE 6. Average isometric force generated by myosin against the various actins. The actin-binding protein α-actinin was added to the in vitro motility assay to create a load on the actin. The concentration of α-actinin needed to stop actin filament motion is a measure of the force-generating capacity of myosin. E99K actin was immobilized at α-actinin concentration of 1.25 μg/ml, whereas both WT and tissue-purified actin needed a concentration of 1.75 μg/ml α-actinin to stop movement. β-Cardiac rabbit myosin was purified by hydrophobic interaction chromatography and used at 150 μg/ml. Conditions: 25 mM imidazole, pH 7.5, 50 mM KCl, 1 mM EGTA, 4 mM MgCl₂, 10 mM DTT, 1 mM MgATP, and oxygen scavengers at 30 °C.

FIGURE 7. Thermal stability of G-actin. A DNase-I binding assay (see “Experimental Procedures”) was used to assess the melting temperature (T_m) of the various actin species, after 24 h on ice. Both the expressed actsns (●, E99K; ◦, WT) and the tissue-purified actin (□) showed a similar T_m of ~60 °C. T_m was defined as the temperature that produced a 50% decrease in the ability of actin to inhibit the endonuclease activity of DNase-I. Conditions: 1.2 μM actin, 40 μg/ml DNA, 100 mM Tris, pH 7.6, 4 mM MgCl₂, 1.8 mM CaCl₂, 2 mM Na₃VO₄, and 0.6 μg/ml DNase-I at 25 °C.

cate that the mutation does not affect the intrinsic stability of the mutant actin monomers. Daily repetition of the assay further demonstrated that the stability of the expressed actins was unchanged for 5 days when stored on ice (Table 4). After this time, the T_m of the expressed actins slowly started to decline to 55 °C over the course of 3 weeks. The tissue-purified actin maintained its stability when stored on ice at 4 °C. The tissue-purified actin remained unchanged for 5 days on ice at 4 °C. The tissue-purified actin showed a very slow decline in stability after 3 weeks storage, whereas the expressed actins showed a very slow decline in stability after 5 days.

Table 4

| Days on ice | E99K         | WT           | Tissue-purified |
|------------|--------------|--------------|----------------|
| 1          | 59.8 ± 0.11  | 60.5 ± 0.15  | 60.0 ± 0.01    |
| 2          | 59.1 ± 0.12  | 59.9 ± 0.06  | 59.9 ± 0.01    |
| 3          | 60.2 ± 0.16  | 59.3 ± 0.08  | 59.9 ± 0.01    |
| 5          | 59.1 ± 0.50  | 58.7 ± 0.13  | 59.9 ± 0.01    |
| 7          | 58.2 ± 0.23  | 59.4 ± 0.18  | 60.7 ± 0.10    |
| 9          | 57.5 ± 0.10  | 57.3 ± 0.19  | 60.6 ± 0.18    |
| 14         | 56.6 ± 0.21  | 56.8 ± 0.30  | 60.0 ± 0.16    |
| 21         | 55.5 ± 0.43  | 55.2 ± 0.33  | 59.0 ± 0.03    |

DISCUSSION

Mutations in α-cardiac, α-skeletal, and γ-cytoplasmic actsns all lead to human disease (cardiomyopathies, nemaline myopathies, and deafness, respectively). The high sequence conservatism among actin isoforms implies that a change in any amino acid will likely prove to be detrimental to actin function. Studying the effect of these mutations in vitro has been slowed by the lack of a high yield expression system for human actsns. Our laboratory has previously expressed a nonpolymerizable cytoplasmic actin mutant from Drosophila in the baculovirus/Sf9 cell expression system (14). Here we developed a method for purification of overexpressed, untagged human α-cardiac actin that provides functional recombinant actin in good yield and with only trace contaminants. Our ability to express actin in Sf9 cells implies that this cell line must have the necessary eukaryotic cytosolic chaperonin containing TCP-1, or a close homologue, to properly fold actin (reviewed in Ref. 11).

Expressed versus Tissue-purified Actin—In some assays the expressed WT-actin was compared with tissue-purified chicken skeletal actin. Human and avian skeletal actin are identical, but there are four amino acid differences between cardiac and skeletal actin isoforms (D2E, E3D, L299M, and S358T). Thus there is no a priori reason why the two isoforms should give identical results. Nonetheless, the two actsns displayed very similar properties with regard to steady-state ATPases, the rate of movement by cardiac myosin in a motility assay, affinity for myosin, and the power stroke is unaffected.

Post-translational modifications can potentially differ between expressed and tissue-purified actsns. Results of mass spectrometry analysis (data provided by the Keck facility at Yale) indicated that Sf9 cells were able to perform at least some of the post translational modifications seen in higher cell systems. Specifically, His-73 of the expressed cardiac and Drosophila cytoplasmic actsns were both methylated to approximately half-maximal levels. We speculate that the large degree of overexpression overwhelmed the ability of the Sf9 cell to completely modify all the expressed protein. We were unable to firmly establish if N-terminal acetylation had been achieved, even in the control tissue-purified sample.

Defects Displayed by E99K Actin—Three Glu residues (Glu-93, Glu-99, and Glu-100) in or adjacent to loop 94–101 of subdomain 1 of actin have been proposed to interact with a cluster of Lys residues in the lower 50 kDa of myosin, forming a secondary actin-binding site (Fig. 1B) (9). The increased K_m caused by the +2 net charge change of the E99K mutation is consistent with the idea that this residue plays a role in this interface. Despite the increased K_m of the mutant actin, there was no significant difference in V_max implying that the weak-to-strong transition associated with phosphate release and the power stroke is unaffected.

The K_m obtained from steady-state ATPases in which double-headed myosin species are used is not a measure of intrinsic affinity of actin for myosin in the presence of ATP, so the assay was repeated using cS1. Surprisingly, the WT and E99K actin now showed the same K_m values (Fig. 3 and Table 1). How can the difference between myosin and S1 be...
Effects of E99K Mutation in Human Cardiac Actin

reconciled? It should be noted that the mutated residue is only one of many residues that participate in the actomyosin interaction. Perhaps the lower salt concentration used by necessity in the S1 assay (5 mM NaCl), compared with that used in the myosin assay (50 mM NaCl), allowed all the other electrostatic interactions to prevail and essentially “swamp out” the mutant phenotype. This would be consistent with the data in Fig. 5B, which showed that at <10 mM KCl, the velocity of WT and E99K actin was similar. In this view, we would have missed an important phenotype of this mutant if we had not used the more physiologically relevant myosin filaments at the higher salt concentration. An alternative view is that the actin mutation in some way renders the two-headed myosin molecule to act “one-headed,” which would shift the $K_m$ to a higher value only in the case of myosin. Consistent with this idea, the $K_m$ for single-headed filamentous myosin is ~10-fold higher than double-headed filamentous myosin (27).

In vitro motility is slowed by ~20% near physiological ionic strength. The slower motility despite the same $V_{max}$ for the actin-activated ATPase activity could be due to a lower average force, which would allow drag forces to more readily slow movement. In agreement with this idea, the average force supported by the mutant actin is ~30% lower than WT-actin. Average force could be reduced either because there is a lower unitary force per cross-bridge when it interacts with E99K actin, or because fewer force-producing cross-bridges are formed. Single-molecule studies could in principle distinguish between these possibilities.

Equilibrium binding in the absence of nucleotide showed that the affinity of actin for S1 is only modestly decreased by the point mutation, consistent with the fact that the strong binding interface is composed of many structural elements. Transient kinetic studies showed that the mutant actin binds cardiac S1 with ~2-fold lower rate than WT-actin under these conditions.

Intrinsic Stability of the Mutant Actin—Expression yields of the WT and E99K actin preparations were consistently similar when tallied after one round of polymerization, indicating that the WT and mutant actin monomers were equally processed and folded in the S97 cell. In contrast, E99K human α-cardiac actin expressed using a cell-free reticulocyte transcription/translation system was less efficiently folded than WT-actin, and thus mutant monomer incorporation into filaments was reduced (12). This observation led to the idea that effects on folding may play a role in the disease process. Our preparation would exclude improperly folded molecules, because the purification procedure involves a functional polymerization step. Vang et al. (12) also found that a significant portion of the E99K mutant was unable to bind DNase-I at 37 °C, whereas our expressed actins bound DNase fully until heated to 60 ± 4 °C. Because misfolding was reported to be more pronounced at 37 °C (12), the results might be reconciled by the 10 °C temperature difference used during expression between the two systems (37 °C for the cell-free system versus 27 °C for the baculovirus/S9 cell expression).

Comparison with Other Mutations in This Region of Actin—Three other actin mutants in the secondary binding loop have been expressed and characterized to varying extents: E99H/E100H in Dictyostelium actin (24), E99A/E100A in yeast actin (28–30), and E93K in Drosophila Act88F (31). The properties of these mutants shared a number of similarities with E99K human cardiac actin. E99H/E100H Dictyostelium actin showed a decreased rate of in vitro motility without methylcellulose, but this was the only assay performed, because the filaments tended to bundle (24). The yeast mutant E99A/E100A showed an increased $K_m$ in actin-activated ATPase assays, but no difference in strong binding affinity, similar to our E99K mutant. Motility was abolished in the absence of methylcellulose but was the same rate as WT in its presence. An almost 30% reduction in average force was also observed by a motility assay (29). Motility results for the E93K Drosophila actin were similar to ours, including velocity reductions in the presence or absence of methylcellulose and dissociation of actin filaments away from the surface at a lower salt concentration than WT-actin (31).

The decrease in velocity seen with these mutants varied considerably as one would expect when performing the in vitro motility assay with different buffer conditions, temperature, and myosin isoforms. It has been noted that decreased temperatures decreased the number of filaments sliding and exacerbated the velocity differences between the mutant and the WT-actins (24, 32). Our data were collected at 30 °C, and we chose to use cardiac myosin to rule out any difference that might result when mixing proteins from different tissue types.

When compared with their respective WT-actins, two major differences were seen between E99K cardiac actin and E99A/E100A yeast actin, even though both mutants have a net charge change of +2. The yeast mutant actin showed no motility in the absence of methylcellulose, while ours moved over cardiac myosin with only a slight decrease in velocity. The yeast mutant actin had a decreased $V_{max}$ in an actin-activated ATPase assay (28), as opposed to our E99K mutant, which has the same $V_{max}$ as WT. These differences are likely a result of the fact that yeast actin has only two acidic residues at its N terminus, compared with four in cardiac actin. In agreement with this interpretation, the motility, $V_{max}$, $K_m$, as well as average force of E99A/E100A yeast actin were restored to WT values when two additional negative charges were added to the N terminus (30). Thus in yeast actin, four negative charges in subdomain 1 are more important for WT-actin function than their exact location. An implication of this result is that a charge ablation mutation in cardiac actin (e.g. E99A) would not result in a disease phenotype, whereas a charge reversal mutation such as E99K reduces charge density in subdomain 1 despite the presence of four acidic residues (DDEE) at the N terminus.

A finding that differed considerably for the Drosophila E93K mutant compared with all others was its strikingly large 10-fold reduction in affinity in the strong binding state in the absence of nucleotide (31). Preliminary data with a human cardiac E93K mutant showed a more modest decrease in rigor affinity (~3-fold), so the location of the mutation is unlikely to be the sole reason for the different results.

Impaired Actomyosin Interactions Account for the Disease Phenotype in Humans—We have demonstrated that the E99K actin mutation reduced the affinity of myosin for actin and decreased force generation. How this mutation ultimately leads to cardiac disease and what determines the severity of the disease are still unanswered questions. A dominant mutation could result in several different scenarios depending upon the availability of sufficient amounts of functional protein. Haploinsufficiency could occur if the gene product of one actin allele is rendered totally unusable, because it had been improperly processed after translation, resulting in too few thin filaments available to support the system. A more modest scenario was presented by Vang et al. (12) who report that the E99K mutant is not folded as rapidly and properly as the WT at physiological temperatures; therefore, the mutant allele might supply less actin than that obtained from the WT allele. The degree of mutant incorporation might explain the varying penetrance seen in the cardiomyopathies. Consistent with this idea, motility studies in which the relative amounts of WT and E99A/E100A mutant yeast actin were varied showed that a 50/50 mix restored nearly normal binding and motility (28). Families carrying the E99K mutation display variable hypertrophy, suggesting that the mutation can be equalized or exacerbated by interactions with another protein. Our next step is to
increase the complexity of the system by reconstituting a regulated thin filament containing the mutant actin to more closely mimic the in vivo situation.

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