Calsequestrin mutant D307H exhibits depressed binding to its protein targets and a depressed response to calcium

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Abstract

Objective: A point mutation in human cardiac calsequestrin (CSQ-D307H) is responsible for a form of polymorphic ventricular tachycardia (PVT). When overexpressed in heart cells, the mutated CSQ leads to diminished Ca\(^{2+}\) transients, consistent with defective regulation of intralumenal sarcoplasmic reticulum (SR) Ca\(^{2+}\).

Methods: To analyze the D307H mutant and determine whether the D307H mutation results in loss of normal protein–protein interactions, we prepared recombinant human wild-type (WT) and D307H forms of CSQ in mammalian cells.

Results: Although we found the two proteins to undergo similar glycosylation and phosphorylation, we discovered that Ca\(^{2+}\)-dependent binding of the D307H mutant to both triadin-1 and junctin was reduced by greater than 50% compared to WT. Reduced binding of the D307H mutant CSQ to target proteins was similar throughout a complete range of Ca\(^{2+}\) concentrations. To investigate the mechanism of reduced Ca\(^{2+}\)-dependent binding, Ca\(^{2+}\)-dependent changes in intrinsic fluorescence emission for the two protein forms were compared. Intrinsic fluorescence of the D307H mutant was highly reduced, reflecting significant alteration in the tertiary protein structure. Moreover, the changes in fluorescence caused by increasing the Ca\(^{2+}\) concentration were very significantly blunted, indicating that the Ca\(^{2+}\)-dependent conformational change was virtually lost.

Conclusions: We conclude that the point mutation D307H leads to a profoundly altered conformation that no longer responds normally to Ca\(^{2+}\) and fails to bind normally to triadin and junctin.

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1. Introduction

CSQ is concentrated inside the junctional SR lumen where it plays an important role in excitation–contraction coupling [1–5]. CSQ effects on Ca release appear to occur through interactions with lumenal SR sites on junctin and triadin, which are small membrane spanning proteins bound to the ryanodine receptor/SR Ca\(^{2+}\) release channel [6–12]. In heart, triadin-1 is the predominant isoform [12]; it appears on SDS-gels as a 40 kDa doublet comprised of glycosylated and unglycosylated molecules. Junctin, a smaller (26 kDa) SR CSQ-binding protein, bears only minor identity to triadin-1 [7]. When SR proteins are analyzed on blots using radiolabeled-CSQ overlays, both junctin and triadin are specifically labeled in a Ca\(^{2+}\)-dependent fashion [7,13]. Kobayashi et al. [14] showed that a KEKE motif within triadin-1 residues 210–224 accounted for CSQ binding. Moreover, even-numbered lysine residues on one side of a beta sheet were identified as sites of interaction with a presumed negatively charged CSQ tail [14], responsible for strong electrostatic binding of CSQ to triadin and junctin even after SDS-gel electrophoresis and transfer to nitrocellulose.

Lahat et al. [15] have shown that a point mutation in human cardiac calsequestrin (D307H) is the gene locus for autosomal recessive catecholamine-induced polymorphic ventricular tachycardia (PVT). These authors hypothesized

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that the histidine in the mutant form might disrupt a band of negative amino acids and could thus upset the protein’s charge balance, disrupting Ca²⁺ binding. In studies of isolated heart cells, Viatchenko-Karpinski et al. [16] reported that the D307H mutant produced a blunted response to cardiac cell depolarization. To determine whether these actions might reflect a loss of normal protein interactions of CSQ, we compared binding to junctin and triadin of WT and CSQ mutant D307H. In this paper, we report that the D307H mutation in CSQ produces an altered conformation that is less responsive to Ca²⁺ in the range of concentrations likely to exist inside the junctional SR lumen. Moreover, at all Ca²⁺ concentrations tested, CSQ-D307H exhibited greatly reduced binding to its target proteins.

2. Methods

2.1. Adenoviral DNA constructs

Human cardiac CSQ DNA was obtained from the EST clone 4289141 (American Type Culture Collection), and subcloned into pShuttle (Life Sciences) for recombination with replication-deficient adenoviral DNA pAd.CMV (Life Sciences) as previously described[17]. The D307H mutant was generated using Quick Change (Life Sciences). Substitution of the CAC codon with GAC resulted in a BamHI restriction site that was used along with complete sequencing of the DNA inserts to verify sequence.

2.2. Overexpression and purification of CSQ

Viral-mediated overexpression (MOI=25) of CSQ protein in COS cells was similar to that previously described for HEK cells [17]. Purified CSQ was concentrated and desalted in purified water using a Centicon-30 (Amicon) and stored frozen.

2.3. Radiolabeling of CSQ by [³²P]phosphorylation

Purified CSQ proteins were phosphorylated to very high specific activity by incubating 5 µg CSQ with 10 U purified CK2 (Promega) in a reaction containing 10 mM MOPS, pH 7.4, 10 mM MgCl₂, 150 mM NaCl, and 10 µM ATP (400,000 cpm/pmol) for 40 min at 30°C. The final purified proteins were phosphorylated to a stoichiometry of approximately 0.1 mol Pi/mol CSQ or 880,000 cpm/µg CSQ.

2.4. Binding to CSQ-binding proteins

Junctional SR membrane vesicles were purified from mongrel dogs by Ca oxalate loading and sucrose density centrifugation as previously described [18]. The investiga-

Fig. 1. Radiolabeled WT and D307H forms of CSQ. Human WT and D307H mutant CSQs were purified as described in Methods, and quantified by absorbance at 280 nm. (A) Protein samples (10 µg) were phosphorylated using purified CK2 and high-specific activity [γ-³²P]ATP. Aliquots (0.5 µg) of the phosphorylated proteins were analyzed by SDS-PAGE (upper panel) and the dried gel placed against BioMax film for 2 min. (lower panel). Scintillation counting of the two excised radioactive bands gave 144,000 and 147,000 cpm. (B) Purified CSQ proteins were analyzed by electrospray mass spectrometry. Mass spectra produced spectra characteristic of ER localization (predominant glycoform Man9GlcNAc2) and verified the D307H mutation.
tion conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Nitrocellulose-bound membrane protein was prepared by SDS-PAGE and electrophoretic transfer in amounts indicated in figure legends. CSQ binding was carried out essentially as described by Jones et al. [7] with minor modifications. Instead of labeling with $^{125}$I, a $^{32}$P-phosphorylated protein was used, prepared as described above. We previously showed that CSQ binding to triadin-1 and junctin is independent of the level of CSQ phosphorylation on CK2-sensitive sites [19]. Binding was carried out in buffer A (20 mM MOPS, pH 7.2, 150 mM KCl) containing 5 $\mu$L of $^{32}$P-labeled WT or D307H mutant CSQ for 90 min, then rinsed for up to several hours without significant removal of counts. Where indicated, buffer A was supplemented with varying levels of Ca$^{2+}$ throughout the procedure. Bound CSQ was visualized by autoradiography of dried nitrocellulose and quantified by digital scanning of Biomax film (Kodak) and application of NIH Image. Exposures were typically less than 30 min depending on the age of the probe. Multiple exposures were obtained to assure subsaturation of films.

2.5. Antibodies

Rabbit antiserum was raised to canine cardiac triadin-1 peptide (KGKHSEEVAGGSKR) and canine junctin (SKHTHSAKGNNQRKRN) coupled to keyhole limpet hemocyanin via an N-terminal Cys and affinity purified as previously described [20]. Immunoblotting was carried out as previously described [21].

2.6. Fluorescence emission

Equal amounts (20 $\mu$g) of purified WT or D307H mutant CSQ (based upon A$_{280}$) were added to 1 ml buffer B (5 mM MOPS, pH 8.0, 50 mM KCl) and fluorescence emission was determined as a function of wavelength (300–400 nm) using an Amino-Bowman Series 2 Luminescence Spectrometer. Excitation was at 295, photomultiplier voltage was 600 V, and triplicate scans were averaged for each Ca$^{2+}$ concentration analyzed. Values obtained at each wavelength were converted to data files, plotted in Excel (Microsoft), and retraced using Photoshop (Adobe) for maximum clarity.

3. Results

3.1. Preparation of purified recombinant human CSQ

Recombinant human CSQ WT and D307H mutant proteins were overexpressed in mammalian cells and purified using a two-step purification scheme involving Ca$^{2+}$-dependent chromatography on phenyl-agarose. Yields

![Fig. 2. Binding of $^{32}$PCSQ to SR proteins. (A) Electrophoretically separated canine junctional SR protein (50 $\mu$g) was transferred to nitrocellulose (lanes 1–2, sample blots stained with amido black). Protein mass standards (left) are expressed in kDa. Nitrocellulose strips were incubated with $^{32}$PCSQ WT (lane 3) or D307H mutant (lane 4) and rinsed, as described in Methods. One strip was cut in half and the two halves treated with radiolabeled WT (left) or D307H (right); only binding to junctin is shown in inset. One strip was cut into two pieces and treated with antibodies raised to canine junctin (JCT) or triadin-1 (TRD) (lane 5). (B) $^{32}$PCSQ WT or D307H mutant binding was carried out in four independent experiments and autoradiograms quantified by densitometry. Values of CSQ binding were determined for the upper (glycosylated, glyc) and lower (unglyc) triadin protein bands, and for junctin.

Values obtained for the D307H mutant form were expressed as a percentage (ordinate) of values obtained for WT in each experiment, which were normalized to 100%. Results are expressed as means $\pm$SE. Mann–Whitney test was used to compare group values; differences in binding to each of the three protein targets were statistically significant ($p<0.05$).
of the D307H mutant were slightly reduced (approximately 25%) relative to WT, a loss that occurred primarily at the Ca$^{2+}$-dependent elution step. Purified proteins were quantified by absorbance at 280 nm and levels verified by SDS-PAGE and Coomassie blue staining (Fig. 1A).

To assess the basic structures of the two CSQ forms, 20 µg of the purified proteins were analyzed by mass spectrometry as previously described [17]. Mass spectra verified the expected mass difference of 22 Da due to the D307H mutation (Fig. 1B), but showed no substantial differences in steady-state levels of phosphorylation on CK2-sensitive sites or glycosylation, indicating similar biosynthesis and trafficking in COS cells [17,22].

### 3.2. Binding to SR proteins

$^{32}$P labeling produced WT and D307H mutant CSQ of very high specific activity, and occurred equally well for both protein forms (Fig. 1B). Counting of $^{32}$P-labeled CSQs yielded 144,000 cpm and 147,000 cpm for 5 µl of the diluted WT and D307H proteins, respectively, diluted in binding buffer (average of three determinations).

Canine cardiac junctional SR vesicles were used as a source of junctin and triadin-1 protein, and binding of purified $[^{32}$P]CSQ to nitrocellulose-bound SR proteins was carried as described in Methods. Autoradiographic data showed two major sites of binding (Fig. 2A, lanes 3 and 4) corresponding to predicted masses for triadin-1 and junctin (40–45 kDa and 26 kDa), and protein binding sites comigrated with triadin-1 and junctin immunoreactivities (lane 5). Low amounts of binding were also observed at a protein mass around 30 kDa. Triadin-1 appeared as a well-separated doublet corresponding to glycosylated and unglycosylated forms. Comparison of junctin and triadin binding for WT and D307H mutant forms of CSQ (lanes 3 and 4) showed that D307H binding was lower by approximately 50% (Fig. 2B, average of four determinations). Based upon radioactive counts bound to junctin, levels of CSQ binding corresponded to less than 5% of the junctin in the SR sample, assuming that junctin represents 4.4% of junctional SR protein [7].

To determine whether the reduced binding corresponded to a change in relative Ca$^{2+}$ affinities, we measured CSQ binding with varying Ca$^{2+}$ concentrations. The reduced binding of the D307H mutant to junctin was similar at 0.0.2, and 1 mM Ca$^{2+}$ (Fig. 3). Reduced binding to triadin-1 appeared similarly independent of Ca$^{2+}$ concentration, although, compared to junctin binding, CSQ binding was difficult to measure at Ca$^{2+}$ concentrations above submillimolar levels.

### 3.3. Intrinsic fluorescence emission

Fluorescence emission spectra for the D307H mutant and WT CSQ revealed significantly different fluorescence spectra, consistent with a shift of intrinsically fluorescent residues to less polar environments (Fig. 4, inset). To more easily compare the responses of the two proteins to rising levels of Ca$^{2+}$, spectra were normalized by setting to 1.0 the values of emission at 330 nm in the absence of Ca$^{2+}$ (Fig. 4). The response to Ca$^{2+}$ for the D307H mutant was substantially altered relative to the WT protein. Plotting the changes in fluorescence as a function of [Ca$^{2+}$] revealed a clearly blunted response of CSQ to submillimolar Ca$^{2+}$ levels, exhibiting little change even at 1 mM Ca$^{2+}$. In contrast, WT CSQ had undergone most of what was its maximal change in conformation by 1 mM Ca$^{2+}$ (Fig. 5). The D307H mutant CSQ did not reproduce the intrinsic fluorescence pattern of WT CSQ at any concentration of Ca$^{2+}$. Even at 20 mM Ca$^{2+}$ there remained a roughly two-

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**Fig. 3. Ca$^{2+}$-dependence of CSQ binding.** Binding of CSQ WT or D307H mutant to junctin was determined as shown in Fig. 2. Binding was carried out on four strips and autoradiograms quantified by densitometry. Two of the four strips are shown (inset) for each condition. Results are expressed as means±SE. Values at each concentration of Ca$^{2+}$ were tested for significance using a t-test to compare group means, and differences were statistically significant ($p<0.005$).
fold change in fluorescence emission, consistent with a complete and irreversible loss of the WT conformation, as opposed to only a change in Ca$^{2+}$ affinity.

4. Discussion

In this study, we have shown that a point mutation in CSQ (D307H) leads to changes in protein conformation that are likely to explain the loss of function found in cultured cardiomyocytes, as well as in the phenotype of the disease. Loss of function by the D307H mutant in vitro appears to result from a highly altered conformation of the protein that can no longer undergo changes associated with the Ca$^{2+}$ bound state. This altered protein conformation not only altered its responsiveness to Ca$^{2+}$, it also resulted in loss of binding to junctin and triadin-1 throughout the same complete range of Ca$^{2+}$ concentrations. The D307H mutation is virtually insensitive to Ca$^{2+}$ in the range of concentrations most likely to occur within the SR lumen during cycles of systole and diastole, and thus incapable of normal function.

Changes in intrinsic fluorescence emission caused by the D307H mutation reveal complex changes in conformation that may be difficult to define in absolute terms; however, it appears likely that altered conformation and corresponding loss of its normal response to Ca$^{2+}$ are responsible for the loss in binding to junctin and triadin. Although the precise residues in CSQ that are responsible for this binding have not been defined, it is believed that acidic residues in the tail [23] region of the molecule bind to the series of positive lysine residues that make up the CSQ binding site within the KEKE motif in triadin-1 and junctin.

The major loss of binding to junctin and triadin observed for CSQ-D307H appeared inconsistent with the minor loss of binding to phenyl-agarose. Detailed structural features of the phenyl-CSQ interaction, however, have not been carefully investigated. It is generally assumed that the interaction involves the so-called trifluoperazine (TFP)-binding site S$^{113}$EEIVNFV, a hydrophobic domain also labeled in an Ca$^{2+}$-dependent manner in cardiac CSQ by the hydrophobic affinity label 3-(trifluomethyl)-3-m$^{[125]I}$iodophenyl diazirine [13,24].

![Graph showing Ca$^{2+}$ induced changes in fluorescence of WT and D307H mutant CSQ at 330 and 350 nm.](image)

Fig. 5. Ca$^{2+}$ induced changes in fluorescence of WT and D307H mutant CSQ at 330 and 350 nm. Changes in fluorescence observed at 330 and 350 nm for each of the Ca concentrations shown in Fig. 4 are shown plotted for each of the two protein forms: WT, closed circles; D307H mutant, open circles. Fluorescence changes observed in 20 mM Ca$^{2+}$ is shown on the left edge of each plot.
dissociation between conformational changes seen in fluorescence emission spectra and Ca\(^{2+}\) binding were previously examined by Hildago et al. [25] and these authors hypothesized that additional Ca\(^{2+}\) binding sites in CSQ may exist which do not participate in generating the intrinsic fluorescence changes. These same binding sites might correspond to those induced upon CSQ polymerization, as described by Kang et al. [26,27]. These authors hypothesize that CSQ polymer formation is an important feature of CSQ in vitro, and that stacking of CSQ monomers explains the superstoichiometric Ca\(^{2+}\) binding beyond what occurs for CSQ monomers. The fluorescence changes observed here occurred at low CSQ concentrations (20 \(\mu\)g/ml), suggesting that significant alterations in the response to Ca\(^{2+}\) binding by CSQ monomers might be expected to influence subsequent formation of CSQ polymers. While differences in equilibrium Ca\(^{2+}\) binding may exist in the D307H mutant, it seems likely that this point mutation produces alterations in protein folding, and that this altered conformation affects interactions with target proteins junctin and triadin-1, not reduced Ca\(^{2+}\) per se. In support of a more nonspecific effect on Ca\(^{2+}\) binding, we found that precipitation of the D307H mutant in the presence of insoluble Ca\(^{2+}\) salts was also substantially diminished compared to the WT protein (data not shown).

The CSQ point mutation D307H leads to a recessive form of catecholaminergic polymorphic ventricular tachycardia [15]. The first studies aimed at the molecular mechanism were carried out by Viatchenko-Karpinski et al. [16] using viral overexpression in cultured primary rat heart cells. These authors found that the D307H mutant overexpression led to loss of normal function in cultured heart cells. The observed phenotype resembled that of cells overexpressing the antisense protein, similar in effect to a down-regulation of CSQ function. In heart cells, a dominant-negative effect resulted in a suppressed ability of SR to store Ca\(^{2+}\). This was manifested by drastically reduced size and duration of Ca\(^{2+}\) transients. These authors have suggested that disrupted interactions of CSQ-D307H with the RyR channel complex might explain the altered regulation of Ca\(^{2+}\) release by luminal Ca\(^{2+}\). This hypothesis is consistent with our data, indicating that altered CSQ conformation disrupts its ability to bind to its targets junctin and triadin, which can explain its reduced ability to regulate Ca\(^{2+}\) release through the RyR following excitation. Moreover, the responses of the molecule to changes in Ca\(^{2+}\) appear to be profoundly blunted.

In conclusion, we have shown that the CSQ point mutation D307H produces a highly altered protein when biosynthesized in mammalian cells. The mutant CSQ exhibits biochemical and biophysical properties consistent with data obtained in intact heart cells, as well as in affected individuals. These data reveal a great sensitivity of CSQ conformation to its primary sequence, and an unexpected effect of conformation on its physiological function.

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