Glycosylation of Skeletal Calsequestrin

IMPLICATIONS FOR ITS FUNCTION

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Emiliano J. Sanchez, Kevin M. Lewis, Gerhard R. Munske, Mark S. Nissen, and ChulHee Kang

From the School of Molecular Biosciences, Washington State University, Pullman, Washington 99164-4660 and the Department of Chemistry, Washington State University, Pullman, Washington 99164-4630

Background: Calsequestrin serves as a calcium storage/buffer protein in sarcoplasmic reticulum and undergoes a post-translational modification.

Results: The specific site, degree, structure, and effects of glycosylation were determined.

Conclusion: The glycosylation prevented premature polymerization of calsequestrin ensuring mobility to the SR.

Significance: The glycosylation establishes a functional high capacity calcium binding polymer and allows calsequestrin to be retained in SR.

Calsequestrin (CASQ) serves as a major Ca\(^{2+}\) storage/buffer protein in the sarcoplasmic reticulum (SR). When purified from skeletal muscle, CASQ1 is obtained in its glycosylated form. Here, we have confirmed the specific site and degree of glycosylation of native rabbit CASQ1 and have investigated its effect on critical properties of CASQ by comparison with the non-glycosylated recombinant form. Based on our comparative approach utilizing crystal structures, Ca\(^{2+}\) binding capacities, analytical ultracentrifugation, and light-scattering profiles of the native and recombinant rabbit CASQ1, we propose a novel and dynamic role for glycosylation in CASQ. CASQ undergoes a unique degree of mannose trimming as it is trafficked from the proximal endoplasmic reticulum to the SR. The major glycoform of CASQ (GlcNAc\(_2\)Man\(_n\)) found in the proximal endoplasmic reticulum can severely hinder formation of the back-to-back interface, potentially preventing premature Ca\(^{2+}\)-dependent polymerization of CASQ and ensuring its continuous mobility to the SR. Only trimmed glycans can stabilize both front-to-front and the back-to-back interfaces of CASQ through extensive hydrogen bonding and electrostatic interactions. Therefore, the mature glycoform of CASQ (GlcNAc\(_2\)Man\(_{1-4}\)) within the SR can be retained upon establishing a functional high capacity Ca\(^{2+}\) binding polymer. In addition, based on the high resolution structures, we propose a molecular mechanism for the catecholaminergic polymorphic ventricular tachycardia (CPVT2) mutation, K206N.

The sarcoplasmic reticulum (SR)\(^2\) in muscle cells serves as a Ca\(^{2+}\) storage/release system that controls the state of the actin-myosin fibrils by regulating cytosolic Ca\(^{2+}\) concentrations (1). Release of Ca\(^{2+}\) from the SR stimulates muscle contraction, and reuptake of cytosolic Ca\(^{2+}\) into the SR returns muscles to a relaxed state. In this pump-storage-release of Ca\(^{2+}\) by skeletal and cardiac SR, calsequestrin (CASQ) plays a major role through buffering free Ca\(^{2+}\) levels within the lumen of the SR together with other luminal acidic proteins (2). Both cardiac CASQ (CASQ2) and skeletal CASQ (CASQ1) do not act as a passive Ca\(^{2+}\) buffer but rather play an active role in regulating Ca\(^{2+}\) levels and facilitating its release from the SR lumen (1–3). Through localizing CASQ and its bound Ca\(^{2+}\) proximal to theryanodine receptor, diffusion times for Ca\(^{2+}\) release can be drastically reduced (4).

Each CASQ molecule binds large numbers of Ca\(^{2+}\) ions with low affinity (\(K_d \sim 1\) mM) over the physiological range of Ca\(^{2+}\) concentrations and releases it with a high off-rate (5–11). High capacity Ca\(^{2+}\) binding by CASQ is largely known to be nonspecific (12, 13). This high capacity and low affinity Ca\(^{2+}\) binding by CASQ has been directly linked to its unique Ca\(^{2+}\)-dependent precipitation (4, 10, 14, 15). According to our widely accepted model of dynamic polymerization (4, 10, 16, 17), Ca\(^{2+}\) fills the electronegative pockets formed within the front-to-front and back-to-back intermolecular interfaces of CASQ, which cross-bridges CASQ molecules through intermolecular cooperative binding of Ca\(^{2+}\) and eventually leads to polymer formation. Evidence of dynamic polymerization associated with physiologic Ca\(^{2+}\) signaling has been confirmed in muscle in vivo (18).

Both CASQ1 and CASQ2 are often purified as glycosylated and/or phosphorylated isoforms. Phosphorylation of CASQ2 results in a drastic increase of its helical content and Ca\(^{2+}\) binding by providing a highly ordered polyanionic network (19). However, the consequence of glycosylation of CASQ is not clearly understood. Although glycosylation is considered the most abundant protein modification found in nature, occurring

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\(^b\) This article contains supplemental Fig. 1.

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\(^c\) To whom correspondence should be addressed. Fax: 509-335-9688; E-mail: chkang@wsu.edu.

\(^d\) The abbreviations used are: SR, sarcoplasmic reticulum; ER, endoplasmic reticulum; CPVT, catecholaminergic polymorphic ventricular tachycardia; rCPVT, rabbit CPVT; CASQ, calsequestrin; rCASQ, rabbit CASQ.
Glycosylation of Skeletal CASQ, Implications for Its Function

across all kingdoms of life (20), there is no general model or consensus as to how glycosylation affects protein function. Inhibition and/or suppression of glycosylation often results in protein aggregation or misfolding, leading to non-functional final states (21). Likewise, in a canine tachycardia-induced model of hypertrophy that leads to heart failure, CASQ2 glycosylation and phosphorylation are significantly altered (22). The cellular processes underlying these changes have remained uncertain due to the lack of understanding of CASQ trafficking (23).

Although CASQ does not contain any known targeting sequences, such as the C-terminal KDEL tetrapeptide, CASQ uniquely evades secretion and is retained in junctional SR (24–26). It has been proposed that the unique degree of glycan trimming could regulate its intracellular trafficking and retention in the junctional SR (26). Understanding the molecular mechanism for the unique trafficking mechanism and high capacity Ca$^{2+}$ binding of CASQ is critical for proper understanding of Ca$^{2+}$ handling in myocytes and its genetic abnormalities such as malignant hyperthermia or catecholaminergic polymorphic ventricular tachycardia (CPVT) (3, 27–29). Of particular note is the inhibition and/or suppression of glycosylation often results in protein aggregation or misfolding, leading to non-functional binding capacity in causing its lethal phenotype (23).

To estimate the fractional occupancy (Y) = [bound Ca$^{2+}$]/[total rCASQ1]) for recombinant and native rCASQ1, equilibrium dialysis and atomic absorption spectrophotometry were as described previously in Sanchez et al. (19).

MALDI Mass Spectrometry of rCASQ1—Native rCASQ1 protein at 1 mg ml$^{-1}$ was chemically cleaved by the addition of 1 M CNBr in the presence of 4 mM CsI and 70% TFA at 25 °C for 2 h. This chemical cleavage allowed for the isolation of both the C-terminal peptide and the peptide containing the N-linked glycoside. After treatment with CNBr, the sample was dried and then dissolved in 1:1 water:sinopinic acid. The sample was then dried on a MALDI plate and analyzed in negative mode with an Applied Biosystem 4800 mass analyzer. For whole protein mass spectrometry, native rCASQ1 and peptide N-glycosidase F-treated samples at a concentration of 0.5 mg ml$^{-1}$ in 50 mM NaPi, pH 7.0 were dissolved in 1:1 sinopinic acid with 0.1% TFA and analyzed in positive mode with an Applied Biosystem 4800 mass analyzer. The peptide N-glycosidase F treatment for native rCASQ1 was done at a concentration of 0.5 mg ml$^{-1}$ in 50 mM NaPi, pH 7.0, and was treated with 500 units of peptide N-glycosidase F (glycerol-free, New England Biolabs) in a reaction volume of 20 μl overnight at 37 °C. Sample was then analyzed with SDS-PAGE electrophoresis to determine completeness of cleavage.

Electrospray Ionization Mass Spectrometry of rCASQ1 Peptide Fragments—Recombinant and native rCASQ1 preparations were digested with either CNBr or trypsin to produce unique desired peptides. These samples were chromatographically separated and analyzed by a Bruker Esquire HCT mass spectrometer as described before (35). Peptides were then subjected to a Mascot search (Matrix Science). Mascot search parameters included tryptic modification to include both of the dioxindolyalanines as well as asparagine modifications for glycosylation. Other parameters included were chemical alterations to methionine and included oxidative products generated by CNBr treatment. These modifications were both the sulfoxide and sulfone forms of methionine as well as homoserine and homoserine lactone. Serine/threonine phosphorylation was also searched as a variable.

Circular Dichroism (CD)—CD spectra of recombinant and native rCASQ1 were measured using an AVIV 202SF spectropolarimeter (AVIV Biomedical, Inc.) at 25 °C. Spectra of each protein at a concentration of 0.25 mg ml$^{-1}$ in 20 mM MOPS (pH 7.2) and 300 mM KCl was recorded from 200 to 260 nm.

Thermostability of Recombinant and Native rCASQ1—The thermostability of both recombinant and native rCASQ1 was monitored as a function of CD signal at 222 nm using an AVIV 202SF spectropolarimeter (AVIV Biomedical, Inc.) and temperature. Briefly, samples containing 5 μM concentrations of either native or recombinant rCASQ1 in 20 mM KP, pH 7.2, and 300 mM KCl were heated in a stepwise fashion by 2.5 °C per step with a 4-min equilibration time and a 3-s scan time. Temperature was regulated with a Peltier device (AVIV Biomedical), and samples were heated from 25 to 85 °C. To determine if the sam-
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RESULTS

CD of rCASQ1—As previously reported (4, 10), a buffer containing 300 mM KCl was chosen for the CD spectra because at this concentration of K⁺ ion, CASQ1 is driven to a fully folded monomeric structure. Under these conditions both recombinant and native rCASQ1 exhibited identical far-UV CD spectra (Fig. 1A), indicating that there is no overall change in secondary structure between glycosylated and unglycosylated rCASQ1 monomers. The estimated Tm values for the native and the recombinant rCASQ were very similar, 49.25 and 48.75 °C, respectively (Fig. 1B).

Analytical Ultracentrifugation—Both recombinant and native rCASQ1 (1 mg ml⁻¹ in 20 mM MOPS, 300 mM KCl, pH 7.2) containing various concentrations of mxt Ca²⁺ (0.5, 1, 2, 5, 10) were used to determine sedimentation velocity coefficients using a Beckman Coulter XL-I analytical ultracentrifuge with an An50-Ti rotor spun at 50,000 rpm at 25 °C for 14–16 h. Each analysis incorporated 100 scans, and the values for the density and viscosity of the buffer relative to water were 1.038 and 1.0227, respectively.

Molecular Mass Determination by Multiangle Light Scattering—The chromatography and light-scattering experiments were performed as previously described (19).

Overall Structures of rCASQ1—We were also able to crystallize both recombinant and native rCASQ1 under the same conditions, which allowed us a detailed comparison between the native and recombinant form of rCASQ1 (Table 1). The α-carbons of the native and recombinant rCASQ1 were superimposable with a root mean square deviation of 0.16 Å without including two N-terminal and four C-terminal residues, which displayed different conformations between two rCASQ1 structures (Fig. 2A). In addition, the electron density for the triose originating from the carboxamide side chain of Asn-316, GlcNAc,Mn₁, was identified in the crystal structure of native rCASQ1 (Fig. 2B). Two structures showed a minor difference in the vicinity of the glycosylation site. The temperature factors of the β-loop near the Asn-316 in native rCASQ1 were driven to a predominant dimeric state similar to native rCASQ1 (Fig. 3). With an increase to 1 mM Ca²⁺, native rCASQ1 was driven to a predominantly dimeric state, whereas recombinant rCASQ1 appeared in an almost equimolar monomer:dimer ratio (Fig. 3). As the Ca²⁺ concentration increased to 2 mM, recombinant rCASQ1 was driven to a dimeric state similar to native rCASQ1 (Fig. 3). These results indicated a significant difference between native and recombinant rCASQ1 at 222 nm are shown. Recombinant measurements are shown in blue, and native measurements are shown in red.

![CD spectra of rCASQ1](image-url)

**FIGURE 1.** CD spectra of rCASQ1. A, shown are wavelength scans of both native and recombinant rCASQ1 from 260 to 200 nm. The blue line represents recombinant rCASQ1, whereas the red line represents native rCASQ1. B, thermostability of rCASQ1 is shown. Molar ellipticity measurements of native and recombinant rCASQ at 222 nm are shown. Recombinant measurements are shown in blue, and native measurements are shown in red.

**TABLE 1**

Crystallographic data for the native and recombinant rCASQ1

| Data collection statistics | Native rCASQ1 | Recombinant rCASQ1 |
|----------------------------|--------------|-------------------|
| Spacegroup                 | C222₁        | C222              |
| Resolution range (Å)       | 30.70–1.76   | 38.91–1.74        |
| No. of reflections (>)     | 40,970       | 41,273            |
| Completeness (%)           | 97.9% (90%)  | 97.9% (90%)       |

| Refinement statistics      | Native rCASQ1 | Recombinant rCASQ1 |
|----------------------------|---------------|-------------------|
| Rwork (%)                  | 0.18          | 0.19              |
| Rfree (%)                  | 0.21          | 0.23              |
|Bond lengths (Å)            | 0.012         | 0.015             |
|Bond angles (°)             | 0.81          | 0.91              |
|Wilson B-factor             | 22.0          | 25.3              |
|Average B-value (Å)         | 28.51         | 31.1              |
|No. of atoms                | 2972          | 2922              |
|Ramachandran plot           | 558           | 430               |
|Favored                     | 98.30%        | 98.6%             |
|Allowed                     | 100.00%       | 100.00%           |

* Rwork = ∑ ||Fobs||−||Fcalc||/∑ ||Fobs||; r.m.s.d. for main chain atoms are the root mean-squared deviations of the bond lengths and bond angles from their respective ideal values as implemented in PHENIX.

* Rfree is calculated with removal of =5% of the data as the test set at the beginning of refinement.
and recombinant rCASQ1 in their oligomeric response to low levels of Ca\(^{2+}\).

**Analytical Ultracentrifugation**—Both CASQ1 proteins precipitated at Ca\(^{2+}\) concentrations higher than \(\sim 3\) mM, which disabled any HPLC-based experimental approach. Therefore, to characterize the propensity of oligomerization at higher Ca\(^{2+}\) concentrations, sedimentation velocity experiments were performed using analytical ultracentrifugation. As shown in Fig. 4, the sedimentation coefficient of recombinant rCASQ1 (4.3) was smaller than that of native rCASQ1 (6.0) in the presence of 1 mM Ca\(^{2+}\). This was indicative of a lower oligomeric state, supporting the results of dynamic light-scattering experiments (Fig. 3). However, at 5 mM Ca\(^{2+}\), the sedimentation velocities of recombinant and native rCASQ1 were almost identical, with native slightly higher at 6.6 than that of recombinant at 6.5 (Fig. 4).

**Ca\(^{2+}\) Binding Capacity Assay**—To study the potential effect of glycosylation on the Ca\(^{2+}\) binding capacity of rCASQ1, Ca\(^{2+}\) binding properties of native and recombinant proteins were analyzed by atomic absorption spectroscopy using the same buffer condition as that of multiangle light scattering. As shown in Fig. 5, native and recombinant rCASQ1 showed a multiphasic curve that was indicative of the stepwise formation of dimeric rCASQ1 to tetrameric and then higher-ordered polymeric structures as Ca\(^{2+}\) increases. Consistent with multiangle light scattering results, native rCASQ1 underwent a transition at lower Ca\(^{2+}\) concentrations (0.86 mM Ca\(^{2+}\)) relative to recombinant rCASQ1 (1.25 mM Ca\(^{2+}\)). However, at the Ca\(^{2+}\) concentrations above \(\sim 5\) mM, both recombinant and native CASQ1 bound similar amounts of Ca\(^{2+}\) ions, which indicated that glycosylation did not change Ca\(^{2+}\) binding capacity at those concentrations.

**Mass Spectrometry of rCASQ1**—Previously, electrospray ionization mass spectroscopy was used to determine the degree of CASQ1 glycosylation. However, this determination was ambiguous due to the mass of a phosphate ion being almost identical
to half the mass of a mannose residue. Our novel approach was to chemically cleave a peptide fragment that contains only Asn-316 and none of the potential phosphorylation sites (Thr-229, Thr-189, and Thr-353). By treating the protein sample with 1 M CNBr with 4 mM CsCl, we were able to selectively cleave a peptide spanning residues 300–324, which contained the N-linked glycan attached to Asn-316. However, due to chemical modification of Trp-324 (36), there was a mass increase of 13.99 Da through its conversion into dioxindolylalanine lactone (33). With this modified mass, the expected molecular mass of a glycan-free peptide would be 2731.31 Da, and with the expected glycan triose core of GlcNAc2Man1, the overall mass would correspond to 3299.54 Da. With the increase of additional mannose residues (22), the mass should increase incrementally by 160.0 Da (Calculated masses for 2, 3, and 4 mannose: 3461.59, 3623.64, and 3785.69, respectively). Therefore, as seen in Fig. 6, the molecular mass peak corresponding to 3299.49 Da was assigned to the GlcNAc2Man1, 3461.5 Da peak to GlcNAc2Man2, 3623.57 Da peak to GlcNAc2Man3, and finally the 3785.59 peak to GlcNAc2Man4.

We were also able to generate a unique C-terminal peptide under the same cleavage conditions that contains Thr-353, the proposed major phosphorylation site in CASQ1 (37). Although other phosphorylation sites exist within the C-terminus of other CASQs, these sites are not found within the C-terminal of rCASQ1 and were, therefore, excluded as possible outcomes. The expected molecular mass for the unphosphorylated peptide would be seen at 2868.0 Da in negative mode and at 2948.0 Da for its phosphorylated form. As seen in Fig. 6, there was no corresponding peak for the phosphorylated form of this peptide. Instead, two peaks were seen at 2850.0 and 2832.0 Da, which correspond to the loss of one and two waters from the peptide. Those peaks were not due to a loss of phosphate group, as the relative peak intensity increased with increased laser power, and the apparent loss of water has been seen with other acid-rich peptides analyzed in negative mode. We also independently confirmed that CNBr/CsI treatment did not acid-hydrolyze any phosphate from the phosphorylated sites by analyzing casein-derived phosphopeptides using electrospray ionization mass spectroscopy and the same MASCOT search parameters as described under “Experimental Procedures” with the addition of tryptic cut sites (supplemental Fig. 1).

We also used MALDI mass spectrometry to investigate the heterogeneity of rCASQ1 glycosylation by comparing the native rCASQ1 proteins with/without peptide N-glycosidase treatment. The two major peaks of native rCASQ1 were 42,925.5 and 43,090.2, with a broad degree of heterogeneity. After treatment with peptide N-glycosidase F, the determined molecular mass was reduced to 42,243.10 Da, with a much narrower peak indicating a more homogenous sample (Fig. 6, inset).

In addition, tryptic digestion of the same protein preparation followed by a MASCOT search yielded peptides that are unique to rCASQ2, which indicated coexistence of rCAQ1 and rCASQ2 in the skeletal SR. Two unique peptides were found with the expected scores of 0.0057 for a peptide experimentally
determined at 1524.96 Da and a calculated mass of 1524.73 Da and an expected value of 1.2E-6 for a peptide of experimental mass 3189.85 Da and a calculated mass of 3189.52. With an adjusted p value of 0.05, both of these peptides are significant and unique for rCASQ2 (supplemental Fig. 1).

**DISCUSSION**

Posttranslational modifications are known to regulate the function of proteins, often by modulating their physical characteristics (38). Among those modifications, phosphorylation and glycosylation are the most common. The attachment of glycans during or after protein synthesis introduces profound consequences on both structure and function of a protein. Inhibition of glycosylation often results in aggregation or misfolding of the corresponding proteins, leading to non-functional and/or disease states (20). Phosphorylation is another essential regulatory mechanism in nearly every aspect of eukaryotic cellular functions, affecting more than 30% of all proteins (39, 40). After phosphorylation, the disordered regions around the modification site of the target protein often become highly ordered (41).

During its transit through the secretory pathway to the SR, CASQ undergoes a yet uncharacterized glycosylation and phosphorylation (37, 42, 43, 45–50). Previously, both glycosylation and phosphorylation have been linked to a junctional SR trafficking mechanism (19, 37, 50–52). Therefore, altered or impaired post-translational modifications could cause serious pathological symptoms (22). Previously, we have shown that phosphorylation at the C terminus of human CASQ2 produces a disorder-to-order transition by providing a more stable network of anions, which increases its Ca$^{2+}$ binding capacity (19, 37).

Consequently, phosphorylation could not only affect trafficking due to its effect on solubility but could also regulate a level of Ca$^{2+}$ binding capacity of CASQ and, hence, a SR Ca$^{2+}$ release amount in response to physiological needs (19, 37).

**Glycosylation**—Both CASQ1 and CASQ2 of various species contain an N-glycosylation consensus sequence (Asn-X-Ser/Thr) at their C terminus. Our mass spectometry data showed that the predominant glycan of rabbit CASQ1 is a triose, GlcNAc$_2$Man$_n$, with the presence of lesser, but approximately equal, amounts of GlcNAc$_2$Man$_2$, GlcNAc$_2$Man$_3$, and GlcNAc$_2$Man$_4$ (Fig. 6), which differs from that of canine CASQ (42, 53). Changes in the mannose content of N-linked glycans are often observed during trafficking through distinct ER and Golgi compartments (54, 55). However, trimming of N-linked glycans to Man$^1$, Man$^3$, and Man$^4$, which is the case in rCASQ1, is still poorly understood (23).

In native rCASQ1, GlcNAc$_2$Man$_{1–4}$ was attached to Asn$^{316}$, which is located at the beginning of a short β-turn comprised of residues 316–319. The temperature factors for those residues were substantially reduced upon glycosylation. However, consistent with CD spectra (Fig. 1), those glycans did not cause any significant secondary structural change. On the other hand, consistent with the results of both sedimentation (Fig. 4) and light-scattering studies (Fig. 3), the presence of the glycans significantly stabilizes intermolecular interactions. The com-

**FIGURE 6. Mass spectra of rCASQ1.** Mass spectra were obtained using the linear high mass negative method supplied by Applied Biosystem/SEIEX. Spectra were obtained from CNBr digestion of native rCASQ1. Mass peaks were fitted according to the glycan composition of the peptide fragment. For clarity, GlcNAc composition is represented by circles, and Man composition is represented by squares. The dotted line at (m/z) of 2894.32 represents the expected m/z for a phosphorylated C-terminal peptide. Inset, shown is the whole protein mass spectra obtained for recombinant (solid line) or native (dashed line) rCASQ1 using the same method as described above. m/z corresponding to the highest intensity peaks are indicated.
parison of crystal structures between native and recombinant rCASQ1 showed that the interaction with neighboring N-terminal residue of a partner subunit was substantially strengthened by those glycans through both direct and water/ion-mediated indirect interactions (Fig. 7). For example, the carboxyl side chain of Glu-2 of a dimeric partner CASQ1 molecule was within a hydrogen-bonding distance with the N-acetyl group of the second GlcNac (Fig. 7). In addition, one Na\textsuperscript{+} ion was tightly coordinated by the side-chain oxygens of Glu-1, Asn-316, and Thr-318, the backbone oxygen of Gly-248, and the N-acetyl group of the first GlcNac.

Although the major glycans according to our mass spectrometry data, GlcNac\textsubscript{2}Man\textsubscript{1–4}, would not cause any steric hindrance in both front-to-front and back-to-back interfaces (Fig. 8, A, B, and D), the expected bulky glycan moiety of CASQ1 in the proximal ER, GlcNac\textsubscript{2}Man\textsubscript{8,9}, can certainly interfere with the back-to-back interaction due to severe steric clash (Fig. 8E). However, the same bulky glycans (GlcNac\textsubscript{2}Man\textsubscript{8,9}) do not interfere with the formation of the front-to-front interface (Fig. 8C); instead they can accelerate dimer formation through enforced front-to-front interaction between two subunits, as mentioned above. Therefore, it is likely that high mannosylation of CASQ (Man\textsubscript{8,9}) is a device to prevent premature oligomerization or aggregation while at the same time stabilizing the dimer and keeping CASQ in solution through mimicking the role played by many molecular chaperones (44). As with other proteins, CASQ is progressively modified as it moves from the entry side (cis) to the exit side (trans) of ER. As CASQ is processed, interference in back-to-back interactions on part of the glycan chain is reduced through successive trimming. Glycan moieties in medium levels of glycosylation, such as Man\textsubscript{5} or Man\textsubscript{6}, are no longer inhibitory. Eventually the matured form of glycans (GlcNac\textsubscript{2}Man\textsubscript{1–4}) allows CASQ molecules to polymerize in the SR (Fig. 8A). Therefore, through the time- and space-controlled mannose trimming, Ca\textsuperscript{2+}-dependent polymerization causes CASQ to deposit itself at its target site. Considering the similarity of the Ca\textsuperscript{2+} binding capacity and sedimentation coefficients between native and recombinant CASQ1 in high Ca\textsuperscript{2+} concentration, it is likely that the major glycan form in

![FIGURE 7. Observed molecular interactions of glycans in front-to-front dimer interfaces.](https://example.com/figure7.png)

![FIGURE 8. Observed and predicted dimer interaction of native rCASQ1.](https://example.com/figure8.png)
mature CASQ1, GlcNAc$_2$Man$_1$, might play a passive role once it reaches the SR.

Although canine cardiac CASQ localizes to the proximal ER cisternae of cells, canine skeletal CASQ1 escapes that proximal site and is found in the distal regions of the ER, which is an ER-GolgI intermediate compartment. A distinct difference has been noticed between CASQ1 and CASQ2 glycoforms, and mannose trimming from the attached glycoside has been proposed as the step responsible for shuttling CASQ2 into the cardiac SR (17). In general, CASQ1 is more negatively charged than CASQ2, although CASQ2 has a more negatively charged C terminus. Previously we have shown CASQ2 forms a polymer at lower Ca$^{2+}$ levels relative to CASQ1 (10). It is highly plausible that the negative charge of CASQ1 delays the productive collision between monomers. In addition, the higher degree of glycosylation observed in cardiac CASQ2 (Man$_n$ or Man$_{n+1}$) can further stabilize the dimer and polymer, resulting in earlier polymerization at lower Ca$^{2+}$ concentrations when compared with cardiac CASQ1 (Man$_1$). Therefore, it is tempting to speculate that differences in both mannose trimming and net charge are the determinant for the observed differential targeting of CASQ1 and CASQ2.

Implication on K206N-CPVT2 Mutation—The recently discovered K206N-CPVT2 mutation has been shown to incorporate additional glycans, which results in lower Ca$^{2+}$ binding capacity and altered polymerization in CASQ2 (30). Neumann et al. suggested that the alteration in function is due to the amino acid substitution and not the altered glycosylation. Indeed, as evidenced in the crystal structure, the corresponding Lys–187 in rCASQ1 was critical in stabilizing two salt-bridges (Fig. 9). Those symmetrical salt-bridge pairs stabilized the front-to-front dimer together with N-terminal arm-exchange interactions in the opposite side of the dimer. As shown in Fig. 9 inset, the formation of a salt bridge between Arg-134 and Asp-261 was dependent on the interaction between Glu-133 and Lys-187 (Lys-206). Thus, K206N mutation and consequent glycosylation in this solvent-exposed area will disrupt the network of salt bridges and weaken the front-to-front interaction without introducing much steric hindrance (Fig. 9).

Conclusion—The observed mature forms of oligosaccharide (GlcNAc$_2$Man$_{1-4}$) did not have any significant effect on the structure and stability of monomeric rCASQ1. Instead, through further stabilizing the N-terminal arm-exchange of a partnering subunit, the glycans significantly enhanced or stabilized formation of a front-to-front dimer. Coupled with the body of evidence in this article, we propose that dynamic mannose-trimming in CASQ is a primary mechanism for intracellular trafficking/targeting. The polymerization of CASQ is a consequence of Ca$^{2+}$-dependent back-to-back interaction among CASQ dimers (4, 10, 16, 17). The formation of this interface is prevented by the bulky glycan moiety in the proximal ER (GlcNAc$_2$Man$_{8,9}$) but is later enabled by progressive mannose-trimming.

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REFERENCES

1. Royer, L., and Rios, E. (2009) Deconstructing calsequestrin. Complex buffering in the calcium store of skeletal muscle. J. Physiol. 587, 3101–3111
2. MacLennan, D. H., Abu-Abed, M., and Kang, C. (2002) Structure-function relationships in Ca$^{2+}$ cycling proteins. J. Mol. Cell. Cardiol. 34, 897–918
3. MacLennan, D. H., and Chen, S. R. (2009) Store overload-induced Ca$^{2+}$ release as a triggering mechanism for CPVT and MH episodes caused by mutations in RYR and CASQ genes. J. Physiol. 587, 3113–3115
4. Park, H., Wu, S., Dunker, A. K., and Kang, C. (2003) Polymerization of calsequestrin. Implications for Ca$^{2+}$ regulation. J. Biol. Chem. 278, 16176–16182
5. Meissner, G., Conner, G. E., and Fleischer, S. (1973) Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca$^{2+}$-pump and Ca$^{2+}$-binding proteins. Biochim. Biophys. Acta 298, 246–269
6. Ikemoto, N., Nagy, B., Bhatnagar, G. M., and Gergely, J. (1974) Studies on a metal-binding protein of the sarcoplasmic reticulum. J. Biol. Chem. 249, 2357–2365
7. MacLennan, D. H., and Wong, P. T. (1971) Isolation of a calcium-seques-tering protein from sarcoplasmic reticulum. Proc. Natl. Acad. Sci. U.S.A. 68, 1231–1235
8. Slupsky, J. R., Ohnishi, M., Carpenter, M. R., and Reithmeier, R. A. (1987) Characterization of cardiac calsequestrin. Biochemistry 26, 6539–6544
9. Mitchell, R. D., Simmerman, H. K., and Jones, L. R. (1988) Ca$^{2+}$-binding effects on protein conformation and protein interactions of canine cardiac calsequestrin. J. Biol. Chem. 263, 1376–1381
10. Park, H., Park, I. Y., Kim, E., Youn, B., Fields, K., Dunker, A. K., and Kang, C. (2004) Comparing skeletal and cardiac calsequestrin structures and their calcium binding. A proposed mechanism for coupled calcium binding and protein polymerization. J. Biol. Chem. 279, 18026–18033
11. Kim, E., Youn, B., Kemper, L., Campbell, C., Milting, H., Varsanyi, M., and Kang, C. (2007) Characterization of human cardiac calsequestrin and its deleterious mutants. J. Mol. Biol. 373, 1047–1057
12. MacLennan, D. H., and Reithmeier, R. A. (1998) Ion tamers. Nat. Struct. Biol. 5, 409–411
13. Hidalgo, C., Donoso, P., and Rodriguez, P. (1996) Protons induce calsequestrin conformational changes. Biophys. J. 71, 2130–2137

FIGURE 9. Structural impact of K206N mutation. Shown is a hypothetical model of glycan positioning in K206N mutation. Normal glycosylation moieties at Asp-316 are shown in green and red for each monomer, and expected glycosylation moieties introduced by mutation at K206N are shown in blue. The inset represents the salt bridge that stabilizes the front-to-front interface.
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14. Gatti, G., Trifari, S., Meseali, N., Parker, J., Michalak, M., and Moldeslo, J. (2001) Head-to-tail oligomerization of calsequestrin. A novel mechanism for heterogeneous distribution of endoplasmic reticulum luminal proteins. J. Cell Biol. 154, 525–534

15. Cho, J., Ko, K. M., Singaravelu, G., Lee, W., Kang, G. B., Rho, S. H., Park, B. J., Yu, J. R., Kagawa, H., Eom, S. H., Kim do, H., and Ahn, J. (2007) Functional importance of polymerization and localization of calsequestrin in C. elegans. J. Cell Sci. 120, 1551–1558

16. Wang, S., Trumble, W. R., Liao, H., Wesson, C. R., Dunker, A. K., and Kang, C. H. (1998) Crystal structure of calsequestrin from rabbit skeletal muscle sarcoplasmic reticulum. Nat. Struct. Biol. 5, 476–483

17. Millstein, M. L., Houle, T. D., and Cala, S. E. (2009) Calsequestrin isoforms localize to different ER subcompartments. Evidence for polymer and hetero-oligomer-dependent localization. Exp. Cell Res. 315, 523–534

18. Laukonis, B. S., Zhou, J., Royer, L., Shannon, T. R., Brum, G., and Rios, E. (2006) Depletion “skraps” and dynamic buffering inside the cellular calcium store. Proc. Natl. Acad. Sci. U.S.A. 103, 2982–2987

19. Sanchez, E. J., Munske, G. R., Criswell, A., Milting, H., Dunker, A. K., and Kang, C. (2011) Phosphorylation of human calsequestrin. Implications for calcium regulation. Mol. Cell. Biochem. 353, 195–204

20. Larkin, A., and Imperiali, B. (2011) The expanding horizons of asparagine-linked glycosylation. Biochemistry 50, 4411–4426

21. Mitra, N., Sinha, S., Ramya, T. N., and Surolia, A. (2006) N-Linked oligosaccharides as outfitters for glycoprotein folding, form, and function. Trends Biochem. Sci. 31, 156–163

22. Kiarash, A., Kelly, C. E., Phinney, B. S., Valdivia, H. H., Abrams, J., and Cala, S. E. (2004) Defective glycosylation of calsequestrin in heart failure. Cardiaco. Res. 63, 264–272

23. McFarland, T. P., Millstein, M. L., and Cala, S. E. (2010) Rough endoplasmic reticulum to junctional sarcoplasmic reticulum trafficking of calsequestrin in adult cardiomyocytes. J. Mol. Cell. Cardiol. 49, 556–564

24. Campbell, K. P., MacLennan, D. H., Jorgensen, A. O., and Mintzer, M. C. (1983) Purification and characterization of calsequestrin from canine cardiac sarcoplasmic reticulum and identification of the 53,000-dalton glycoprotein. J. Biol. Chem. 258, 1197–1204

25. Frangini-Armstrong, C., Kenney, L. J., and Varriano-Marston, E. (1987) The structure of calsequestrin in triads of vertebrate skeletal muscle. A deep-etch study. J. Cell Biol. 105, 49–56

26. Houle, T. D., Ram, M. L., McMurray, W. J., and Cala, S. E. (2006) Different endoplasmic reticulum trafficking and processing pathways for calsequestrin (CSQ) and epitope-tagged CSQ. Exp. Cell Res. 312, 4150–4161

27. Priori, S. G., and Chen, S. R. (2011) Inherited dysfunction of sarcoplasmic reticulum Ca2+ handling and arrhythmogenesis. Circ. Res. 108, 871–883

28. Chopra, N., Kannankeril, P. J., Yang, T., Hlaing, T., Holinstat, I., Ettenson, K., Pfeifer, K., Atkins, B., Jones, L. R., Franzini-Armstrong, C., and Knollmann, B. C. (2007) Modest reductions of cardiac calsequestrin increase calcium store. J. Biol. Chem. 282, 43635–43641

29. Kirchhefer, U., Wehrmeister, D., Postma, A. V., Pohlentz, G., Mormann, B. C. (2007) Modest reductions of cardiac calsequestrin increase calcium store. J. Biol. Chem. 282, 43635–43641

30. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, Y. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX. A comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221

31. Nob, S. M., Brayton, K. A., Brown, W. C., Norimine, J., Munks, G. R., Davitt, C. M., and Palmer, G. H. (2008) Composition of the surface proteome of Anaplasma marginale and its role in protective immunity induced by outer membrane immunization. Infect. Immun. 76, 2219–2226

32. Domingues, M. R., Domingues, P., Reis, A., Fonseca, C., Amado, F. M., and Ferrer-Correia, A. J. (2003) Identification of oxidation products and free radicals of tryptophan by mass spectrometry. J. Am. Soc. Mass Spectrom. 14, 406–416

33. Emsley, P., and Cowtan, K. (2004) Coot. Model-building tools for macromolecular graphics. Acta Crystallogr. D Biol. Crystallogr. 60, 2126–2132

34. Adams, P. D., Afonine, P. V., Bunkóczi, G., Chen, Y. B., Davis, I. W., Echols, N., Headd, J. J., Hung, L. W., Kapral, G. J., Grosse-Kunstleve, R. W., McCoy, A. J., Moriarty, N. W., Oeffner, R., Read, R. J., Richardson, D. C., Richardson, J. S., Terwilliger, T. C., and Zwart, P. H. (2010) PHENIX. A comprehensive Python-based system for macromolecular structure solution. Acta Crystallogr. D Biol. Crystallogr. 66, 213–221

35. Shoshan-Barmatz, V., and Ashley, R. (1998) The structure, function, and cellular regulation of ryanodine-sensitive Ca2+ release channels. Int. Rev. Cytol. 183, 185–270

36. Shental-Bechor, D., and Levy, Y. (2008) Effect of glycosylation on protein folding. A close look at thermodynamic stabilization. Proc. Natl. Acad. Sci. U.S.A. 105, 8256–8261

37. Johnson, L. (2009) The regulation of protein phosphorylation. Biochem. Soc. Trans. 37, 627–641

38. Iakoucheva, L. M., Radivojac, P., Brown, C. J., O’Connor, T. R., Sikes, I. G., Obradovic, Z., and Dunker, A. K. (2004) The importance of intrinsic disorder for protein phosphorylation. Nucleic Acids Res. 32, 1037–1049

39. Shoshan-Barmatz, V., Orr, I., Weil, S., Meyer, H., Varsanyi, M., and Heilmeyer, L. (1996) The identification of the phosphorylated 150/160-kDa proteins of sarcoplasmic reticulum, their kinase, and their association with the ryosode receptor. Biochim. Biophys. Acta 1283, 89–100

40. Varsanyi, M., and Heilmeyer, L. (1980) Autocatalytic phosphorylation of calsequestrin. FEBS Lett. 122, 227–230

41. Ram, M. L., Kiarash, A., Marsh, J. D., and Cala, S. E. (2004) Phosphorylation and dephosphorylation of calsequestrin on CK2-sensitive sites in heart. Mol. Cell. Biochem. 266, 209–217

42. Szegedi, C., Sárközi, S., Herzog, A., Jona, I., and Varsanyi, M. (1999) Calsequestrin. More than “only” a luminal Ca2+ buffer inside the sarcoplasmic reticulum. Biochem. J. 337, 19–22

43. Herzog, A., Szegedi, C., Jona, I., Herberg, F. W., and Varsanyi, M. (2000) Surface plasmon resonance studies prove the interaction of skeletal muscle sarcoplasmic reticular Ca2+ release channel/ryanodine receptor with calsequestrin. FEBS Lett. 472, 73–77

44. Houle, T. D., Ram, M. L., and Cala, S. E. (2004) Calsequestrin mutant D307H exhibits depressed binding to its protein targets and a depressed response to calcium. Cardiovasc. Res. 64, 227–233

45. Hellenius, A., and Aebl, M. (2001) Intracellular functions of N-linked glycan. Science 291, 2364–2369

46. Moremen, K. (2002) Golgi α-mannosidase II deficiency in vertebrate systems. Implications for asparagine-linked oligosaccharide processing in mammals. Biochim. Biophys. Acta 1573, 225–235