Inefficiency Glycosylation Leads to High Steady-state Levels of Actively Degrading Cardiac Triadin-1*

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Triadin-1 provides a mechanism by which the Ca2+-release channel/ryanodine receptor may link with calsequestrin to regulate Ca2+ release. Calsequestrin and triadin-1 both contain N-linked glycans, but only about half of triadin-1 in the heart remains unglycosylated. To investigate mechanisms for this incomplete glycosylation, we overexpressed triadin-1 as a series of glycoform variants in non-muscle cell lines and neonatal heart cells using plasmid and adenoviral vectors. We showed that the characteristic incomplete glycosylation stemmed from properties of the glycosylation sequence that are conserved among triadin splice variants, including the close proximity of Asn75 to the sarcoplasmic reticulum inner membrane. Although triadin-1 appeared by SDS-PAGE analysis as a 35/40-kDa doublet in all cells, variations occurred in the relative levels of the two glycoforms depending on the cell type and whether overexpression involved a plasmid or adenoviral vector. Treatment of triadin-1 with the proteasome inhibitor MG-132 led to striking changes in the relative levels of triadin-1 that indicated active breakdown of unglycosylated, but not glycosylated, triadin-1. Besides substantial increases in the relative levels of unglycosylated triadin-1, proteasome inhibition led to an accumulation of two new modified forms of triadin-1 that were seen with triadin-1 only when it is unglycosylated on Asn75. Effects of tunicamycin and endoglycosidase H confirmed that these novel isoforms represent two alternative N-linked glycosylation sites, indicating that an alternative topology occurs infrequently leading to yet other glycoforms with short half-lives.

During excitation-contraction coupling in heart cells, Ca2+ release from sarcoplasmic reticulum (SR) occurs through the ryanodine receptor that is localized and concentrated in junctional SR (1–4). Junctin and triadin are single-spanning membrane proteins that are concentrated at SR junctions and play a role in coupling of calsequestrin to the ryanodine receptor, perhaps by binding to each (5–10).

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2 The abbreviations used are: SR, sarcoplasmic reticulum; TRD, cardiac triadin-1; Ad.TRD, adenovirus encoding TRD; 3-(N-morpholino)propanesulfonic acid; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; ER, endoplasmic reticulum; MV, membrane vesicle.
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EXPERIMENTAL PROCEDURES

Materials—MG-132 (carbobenzoxy-l-leucyl-l-leucyl-l-leucinal) was purchased from Sigma and prepared as a 5 mM stock solution in Me2SO. Restriction enzymes were purchased from New England Biolabs and Invitrogen. PCR products were generated using Pfu Turbo polymerase (Stratagene), and PCR primers were from Invitrogen. COS and HEK cells were purchased from American Type Culture Collection, and cell culture reagents were obtained from Invitrogen. Plasmid DNA was isolated using the Midi Prep kit from Qiagen. 125I-Labeled protein A was obtained from PerkinElmer Life Sciences. Collagenase type II was from Worthington. Endoglycosidase H was from Roche Applied Science. All other reagents were purchased from Sigma.

SDS-PAGE and Immunoblotting—SDS-PAGE was carried out according to Laemmli (18) on 10% acrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore), and stained with Amido Black (Sigma). Purified rabbit anti-TRD antibodies raised to canine cardiac TRD C-terminal peptide (257KGKHSEVAGGSKR) were described previously (15), and 125I-protein A (2 µl/ml) was used to detect immune complexes (19) by autoradiography using Kodak BioMax MS film. In some cases, densitometry was carried out using NIH Image software.

TRD Constructs—The adenoviral construct for canine cardiac TRD (Ad.TRD) was described previously (15). Wild-type TRD cDNA was subcloned from pShuttle-CMV (Stratagene) into pcDNA3.1 (Invitrogen). Single amino acid substitutions F76A and S77T were carried out by QuikChange site-directed mutagenesis (Stratagene) of the wild-type sequence. For TRD(G−), Ser77 was mutated to Ala with the use of a single nucleotide substitution. For the TRD-shift(G+) construct, nine residues (YPYDVPIYA) were inserted into the canine TRD sequence after Asp72 to increase the distance between the glycosylation site and the ER/SR membrane. TRD-shift(G+) was generated by PCR of the original TRD-pcDNA3.1 template with the following primers: forward 5′-CTTCTATGCTAGCTGGCTAAAACACTTTTTGACC-3′; reverse 5′-CACATCATCATAAGGATACTACGTCAAACATACACAC-3′. The resulting product was a linear DNA that contained the inserted sequence as part of the two ends. The elongated DNA was religated before bacterial transformation. To generate TRD-shift(G−) from TRD-shift(G+), the then shifted Ser86 was mutated to Ala, as described above. Chief glycoform variants are summarized together in Table 1. Cytosolic lysine point mutants of TRD(G−), K20R, K26R, K30R, and K33R, were constructed using QuikChange. All constructs were verified by restriction enzyme mapping and DNA sequence analysis (Wayne State University Molecular Core Facility).

Cell Culture—HEK and COS cells were cultured in Dulbecco’s modified Eagle’s medium with 25 mM HEPES and supplemented with 10% fetal bovine serum and 100 units/ml penicillin G, 0.1 mg/ml streptomycin, and 0.25 µg/ml amphotericin B. Cells were cultured at 37 °C with 5% CO2.

Heart Cell/Tissue Preparations—Neonatal (2 day) rat heart cells were prepared according to Maki et al. (20). To reduce the non-myocyte population, cells were preplated on a 100-mm culture dish for 45 min at 37 °C. Canine cardiac homogenates and membrane vesicles were isolated from canine left ventricle, as described previously (21). Protein concentrations were determined according to Lowry et al. (22).

Plasmid-mediated Overexpression—DNA transfection of adherent cells was carried out using Lipofectamine 2000 reagent (Invitrogen), according to the manufacturer’s specifications. Twenty four hours before transfection, cells were seeded into 6-well plates with a cell density suitable to obtain ~70% confluence at the time of transfection. Equal cell densities were used in lieu of protein determinations. Equal protein loading for immunoblot analyses was verified by staining of polyvinylidene difluoride transfers with Amido Black. For transfection of a single plasmid, 3 µg of DNA/well was used with 2.5 µl of Lipofectamine 2000 in Opti-MEM (Invitrogen). Cells were harvested 72 h after transfection for immunoblot analyses, in a buffer containing 20 mM MOPS, pH 7.5, 4% CHAPS, 250 mM NaCl, and added to SDS for SDS-PAGE or centrifuged at 13,000 rpm for 10 min (Biofuge), as indicated in figure legends. Only minor levels of TRD remained in the detergent-insoluble pellet under these conditions.

Adenovirus-mediated Overexpression—Treatment with recombinant adenoviruses was carried out in cultured nonmuscle cells (HEK and COS) and primary rat neonatal heart cells, as described previously (23). Adenovirus addition to cultured cell lines was carried out 3 h post-plating; primary neonatal rat heart cells were treated 24 h post-plating. Multiplicity of infection values were as follows: HEK, 1; COS, 50; neonatal rat heart cells, 20. Virus treatments were allowed to proceed for 48 h before cells were harvested for biochemical analysis.

RESULTS

Partial Glycosylation of TRD in Heart and Nonmuscle Cells—In cardiac SR, TRD exists as two equally abundant protein glycoforms of 35 and 40 kDa (13). To determine whether the same TRD glycoform distribution occurs in heart homogenates, we compared cardiac microsomal (crude SR vesicle) preparations with homogenates from the same heart. Immunoblot analyses showed that although TRD was highly enriched in cardiac microsomes compared with homogenates, relative levels of unglycosylated (35 kDa) and glycosylated (40 kDa) TRD were similar, with roughly equal amounts of each glycoform (Fig. 1, A and C). Accumulation of unglycosylated TRD might be expected to occur only in rough ER where core Asn-linked oligosaccharide (Glc3Man9GlcNAc2) transfer occurs; yet it appears possible that unglycosylated TRD present in junctional SR could account for the steady-state levels of unglycosylated TRD found in whole heart tissue.

To determine whether partial glycosylation of TRD occurred in other mammalian cells, we treated HEK and COS cells with an adenovirus encoding canine TRD (Ad.TRD). Partial glycosylation was a feature of TRD even in these nonmuscle cells, although relative levels of the two glycoforms differed between the cell types. In HEK cells, relative levels of glycosylated and unglycosylated TRD were similar to those in canine heart tissue, whereas in COS cells the majority of biosynthesized TRD remained unglycosylated. Besides the slower mobility form that results from TRD glycosylation, we often observed another slower mobility form of TRD that appeared above the unglyco-
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Overexpression of TRD-shift(G+) in HEK and COS cells transformed the standard pattern of partial glycosylation into one in which only the fully glycosylated protein was observed (Fig. 2A, lane 2, for each cell type). Glycosylation site deletion mutants TRD(G−) (S77A, Fig. 2A, both lanes 3) or TRD-shift(G−) (S86A) (both lanes 4) produced only the unglycosylated form of TRD.

Having proven that changes in TRD sequence could reverse its characteristic incomplete glycosylation, we sought to determine whether other features of TRD sequence might also affect the extent of its glycosylation. We therefore generated two additional point mutations in the glycosylation sequence of canine wild-type TRD, based upon data shown from previous *in vitro* studies (25–27). We compared wild-type, shift (G+), F76A, and S77T overexpression in COS cells. The data indicated that, indeed, each of the three mutations affected the extent of glycosylation (Fig. 2, B and C). Nonetheless, only the shift (G+) mutation resulted in total reversal of incomplete glycosylation.

**TRD Glycosylation Effects on Turnover**—During the course of our experiments, we observed differences in the relative levels of glycosylated and unglycosylated TRD depending upon whether we used plasmid or adenoviral overexpression. We therefore compared directly overexpression of TRD using an adenoviral vector or expression plasmid in COS cells and HEK cells. Compared with the accumulation of primarily unglycosylated TRD seen using adenoviruses, expression plasmids produced a much greater amount of glycosylated relative to unglycosylated TRD (Fig. 3A, compare lanes 1 and 3). Plasmid overexpression periods were routinely compared 3 days with only 2 days for adenoviruses, but very little change in relative glycoform levels was seen for either overexpression vector during the time course of several days.

Differences in steady-state accumulation for the two biosynthetic methods prompted us to test whether there might be differences in the rates of protein breakdown for the two TRD glycoforms. Addition of the proteasome inhibitor MG-132 (20

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TABLE 1

| NAME    | cDNA     | GLYCOFORM            |
|---------|----------|----------------------|
| TRD     | wild-type| unglycosylated       |
| TRD(G−) | S77A     | glycosylated on N75   |
| TRD-shift(G+) | N(75→84) | unglycosylated       |
| TRD-shift(G−) | N(75→84), S86A | glycosylated on N84   |

N-terminus M1′TEITAEGKASTTTTVIDSNKGSVPKSGKVLRKTVEEDIVTTFSSPR
Transmembrane A6′AWLVIALITWSSAVAVMIR
C-terminus (trunc) D6′LVVDAYNSFLSKISDPLKLHVDAVEF

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sylated form (Fig. 1B, arrowhead). A slower mobility form can also be seen above the glycosylated TRD (Fig. 1B, middle lane).

Reversal of Partial Glycosylation of TRD—A central question regarding the accumulation of unglycosylated compared with glycosylated TRD at steady state is why some molecules escape the actions of the oligosaccharide transferase, which is thought to act during translocation through the ER protein pore Sec61 (24). We first tested the hypothesis that the proximity of the site of N-linked glycosylation to the inner membrane surface might decrease the efficiency of the core-oligosaccharide transfer. Nine amino acid residues were inserted after Asp72, increasing the distance between the inner membrane surface and the site of N-linked glycosylation from 7 to 16 residues. This insertion resulted in a mutant termed TRD-shift(G+) to designate movement of N-linked glycosylation from residues 75 to 84 (Table 1).
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overexpression of wild-type TRD in COS cells, for example, unglycosylated TRD (relative to glycosylated TRD) increased by 80 ± 20% (S.D., three separate experiments) (Fig. 3, cf. lanes 1 and 2). Larger increases were observed with adenovirus overexpression in COS cells (Fig. 3, lanes 3 and 4). Levels of unglycosylated relative to glycosylated TRD also increased in HEK cells (Fig. 3, lanes 5 and 6) and in neonatal heart cells (Fig. 3B). These changes in relative levels suggest that TRD undergoes active breakdown and that the greatest effect is on the unglycosylated form.

Identification of Novel TRD Mobility Forms—Equally pronounced in MG-132-treated cells was the appearance of two novel mobility forms of TRD, tentatively labeled α and β forms. These forms are very clearly observed in Fig. 3, lane 4, in which COS cells predominantly overexpress only the unglycosylated form in the absence of MG-132. Based upon their mobilities by SDS-PAGE, their mass differences would be roughly 3 and 6 kDa greater than the 35-kDa mass of unglycosylated TRD. Various combinations of unglycosylated, glycosylated, α, and β isoforms accumulate to varying degrees depending upon the method of overexpression, the amount of proteasome activity, and the cell type (Fig. 3).

It was apparent from the study of TRD glycoforms that α/β isoforms occurred with overexpression of unglycosylated but not for glycosylated TRD. When the glycoform variants previously characterized (Table 1 and Fig. 2) were treated with MG-132, a consistent picture developed. The fully glycosylated variant TRD-shift(G+) exhibited neither of the α/β isoforms following MG-132 treatment (Fig. 4, lanes 1 and 2), whereas the glycosylation site mutant TRD-shift(G−) showed both α/β isoforms (lanes 5 and 6), albeit at reduced levels compared with molecules in which the Asn75 had not been shifted to improve glycosylation (lanes 3 and 4). In fact, the glycosylation site mutant for wild-type, TRD(G−), exhibited the strongest response to MG-132, both in terms of its increase in unglycosylated TRD as well as its most marked levels of α/β isoforms.

The mobilities of the α form and the glycosylated form were very similar, making it difficult to resolve the α form under many conditions. Interestingly, although our TRD-shift(G+)
and its 9-amino acid extension, produces only fully glycosylated TRD in all cells tested, a very small amount of another TRD glycoform could be detected in the presence of MG-132 (Fig. 4, lane 2, asterisk). This TRD glycoform exhibited a mobility most consistent with that of the α isoform, although its identity as such could not be verified by SDS-PAGE alone. An identical pattern to that observed here in COS cells was observed when these glycoform variants were analyzed in HEK cells (data not shown).

Effect of Tunicamycin on TRD Modification—To further investigate the relationship between TRD glycosylation and the effects of proteasome inhibition, we treated cells with tunicamycin following 24 h of TRD overexpression. As expected, tunicamycin led to the loss of glycosylated TRD (Fig. 5, lanes 1 and 3). In addition, MG-132 increased the levels of this nonglycosylated TRD (Fig. 5, lanes 3 and 4). In contrast to the case of wild-type TRD and the glycosylation site mutant TRD(G−), however, α/β forms of TRD were not observed following MG-132 treatment of tunicamycin-treated cells (Fig. 5, compare lanes 2 and 4). Similar results were obtained for the glycosylation site mutant TRD(G−), where tunicamycin-treated COS cells (Fig. 5, lanes 7 and 8) exhibited the large increase in unglycosylated TRD following proteasome inhibition, but without the α/β forms seen in the absence of tunicamycin (lanes 5 and 6).

The absence of α/β forms of TRD with tunicamycin treatment was unexpected. A mechanism for loss of α/β forms because of tunicamycin treatment was not consistent with the fact that the only luminal ER site for N-linked glycosylation was removed by the S77A engineered into the TRD(G−) mutant. There remained a possibility that tunicamycin was somehow acting on the two glycosylation sites Asn9 and Asn21 (see Table 1), and that MG-132 was unmasking an otherwise transiently formed, unstable topology in which these two N-terminal glycosylation sites were modified.

To test this hypothesis, we treated TRD-containing samples containing the α/β forms, with the enzyme endoglycosidase H, which can remove high mannose-type N-linked glycans. These results showed that in vitro, the glycosylated form of native TRD and the α/β forms of TRD were sensitive to the effects of endoglycosidase H, verifying that the α/β forms of TRD corresponded to N-linked glycosylation sites Asn9 and Asn21 (Fig. 6).

DISCUSSION

An Inefficiency of Glycosylation—We previously characterized the glycan structures for cardiac calsequestrin from heart, a luminal protein also highly enriched in junctional SR, and we showed that calsequestrin molecules include a range of glycoforms differing only in mannose content (23). It was therefore surprising to us that TRD, which is in close proximity to calsequestrin in junctional SR, exists in heart tissue as both glycosylated and unglycosylated forms, the result of incomplete glycosylation on Asn75 within the ER lumen (13). Incomplete glycosylation, which is rarely reported in the literature, would not be predicted for TRD because its modification site is fully contained within the SR lumen and reportedly has a single topology (13). All triadin isoforms share this conserved
sequence for N-linked glycosylation at Asn^{75}, although alternative splicing in heart leads to TRD (cardiac triadin-1), a highly truncated form of the skeletal muscle form of the protein (13).

In this study, we have shown that incomplete glycosylation of TRD can be converted to complete glycosylation by a mutation (Asn^{75} to Asn^{84}) that shifts the glycosylation site from its native 7, to a new distance 16 amino acids away from the ER/SR membrane. The completeness of the loss of the unglycosylated form was evident in the lack of unglycosylated TRD even in the presence of proteasome inhibition (Fig. 4, lane 2). A minor amount of a higher mobility form present in this condition (Fig. 4, asterisk) exhibited a mass slightly greater than that of unglycosylated TRD and may correspond to the α isoform (discussed below). These data are analogous to findings of Popov et al. (28) who reported that efficient glycosylation of the anion exchanger band 3 required the site to be 12–14 residues from the transmembrane segment. Although two other glycosylation-site sequence modifications (F76A and S77T) increased the efficiency of glycosylation, only the shift(G+) mutant produced a fully glycosylated protein, even when the native site was left intact. Nevertheless, the glycosylation site mutants, taken together, support the hypothesis that TRD glycosylation in intact heart results from sequence constraints. Studies in cultured heart cells (15) and transgenic mice (30) in which TRD glycosylation has been found to vary from its native state suggest that regulation may occur at the level of biosynthesis.

It will remain to be shown whether fully glycosylated TRD (TRD-shift(G+)) is more functional than the native protein in supporting control of ryanodine receptor function. Indeed, it remains to be shown that this form is functional at all, because there may exist some critical role in heart for the selection of only a small subset of total TRD molecules to survive the biosynthetic process.

**TRD Glycoform Turnover and Steady State**—The structure of triadin proteins and its conserved site of N-linked glycosylation (Asn^{75}) ensures an inefficient glycosylation, which ensures a more rapid turnover. Because proteasome inhibition greatly increased levels of unglycosylated TRD, it is apparent that very significant levels of unglycosylated TRD must be synthesized without attached glycans to maintain the roughly equal levels of glycosylated and unglycosylated TRD that exist at steady state. Stimulation of unglycosylated TRD with proteasome inhibition was consistently observed for unglycosylated TRD, regardless of whether or not occurred because of the inherent inefficiency of wild-type TRD glycosylation (Fig. 3), as a result of glycosylation site mutation (Fig. 4), or as a result of treatment with tunicamycin (Fig. 5). Meanwhile, there was little indication for proteasome degradation of the (Asn^{75}) glycosylated form. Pulse-chase experiments will be needed to properly define the relative half-lives; however, our anti-TRD antibody was not capable of immunoprecipitating TRD.

Our data suggested that inefficient glycosylation of TRD results from a constraint caused by the closeness of the Asn^{75} site to the rough ER translocon and oligosaccharride transfer complex, but it does not explain why, under a variety of conditions, the unglycosylated form was the predominant form observed. For example, in all cells tested except HEK cells, Ad.TRD produced primarily the unglycosylated form of the protein (Fig. 3). This included neonatal rat heart cells (Fig. 3B) and, as shown previously, cultured adult rat heart cells (15). This is despite the fact that native rat heart TRD exists as a doublet of roughly equal amounts of both forms (15). The reason for this result is not intuitively obvious because a stochastic oligosaccharide transfer process should produce a similar ratio of the two glycoforms regardless of the biosynthetic rate. Among different species, the ratio of glycosylated and unglycosylated TRD does appear to vary (13), which might explain the divergence we report for HEK and COS cells. However, the effects of proteasome inhibition in heart cells may be needed to accurately determine the degree of actively degrading TRD underlying the steady-state levels determined by immunoblot analyses.

Thus, what cell biological features determine whether a given TRD protein molecule will undergo glycosylation remains to be determined. Asparagine-linked glycosylation occurs during nascent chain translocation, before protein folding (24); thus, inefficient glycosylation should not be a function of protein conformation or folding. It is also not simply a result of overexpression, because the pattern of glycosylation of overexpressed TRD recapitulates the pattern that exists in intact heart. It is unlikely that inefficient glycosylation is because of saturation of the cellular mechanism for N-linked glycosylation for several reasons. First, under identical conditions in both COS and HEK cells, calsequestrin can be overexpressed to very high levels with no detectable loss of glycosylation (17). Second, the lack of TRD glycosylation did not correlate with the level of overexpression; for a given cell type and gene transfer procedure, there was no significant change in the pattern of glycosylation observed by increasing the level of overexpression. Third, the fact that we were able to effectively prevent formation of unglycosylated TRD by increasing the distance of the site from the membrane surface using the TRD-shift(G+) mutant strongly suggests that loss of glycosylation is an inherent property of the cell biology and not of overexpression.

**Inverse Topology of TRD and Its Degradation by the Proteasome**—Based upon an extensive scientific literature on ER-associated degradation, or endoplasmic reticulum-associated degradation, we originally hypothesized that the α/β isoforms of TRD corresponded to mono-ubiquitinated and di-ubiquitinated forms, respectively. However, the masses of the α and β isoforms based upon their relative mobilities by SDS-PAGE do not match the 8600-Da change in mass corresponding to all known forms of mammalian ubiquitin. Moreover, immunological approaches to ubiquitin detection on TRD failed to provide evidence of ubiquitin, and point mutations of each of the four predicted cytosolic Lys residues in TRD (Lys^{20}, Lys^{26}, Lys^{30}, and Lys^{33}) had no discernible effect on α/β forms following MG-132 treatment (data not shown). Thus, ubiquitination of TRD may not be required for TRD degradation by the proteasome, although it is not ruled out by our studies.

Transient formation of apparently full-length TRD molecules having an alternative topology in which the N terminus becomes inserted into the lumen of the ER during translation (type I protein) indicates a level of infidelity or simply a variability in ER membrane insertion for the TRD polypeptide. One
possible cause of such a process might be a second signal sequence, similar to the case described for the prion protein (29). The fact that this topological form also undergoes a relatively rapid degradation by the proteasome might be an expected feature for an effective quality control mechanism. Levels of these topological forms observed in conjunction with overexpression of Asn75 (S77A) mutants exhibited minor differences depending upon the experimental system, but they generally represented less than 10% of the TRD molecules and were generally observed only after proteasome inhibition. Future detailed analysis of the relative synthetic rates of the various TRD glycoforms will be carried out more accurately by using radioactive pulse experiments.

Regulation of TRD Biosynthesis in Heart—Based upon our data from overexpression experiments, we predict that steady-state levels of unglycosylated TRD in heart represent only a fraction of the amount of unglycosylated protein being synthesized. Additionally, in heart there is an important question regarding how similar levels of glycosylated and unglycosylated TRD can exist in both heart homogenates and purified junctional SR vesicles (13) (also see Fig. 1), given that translation and proteasomal degradation are expected to both occur in rough ER. Maintenance of the site of glycosylation (Asn75) at a place in the protein that ensures an inherent inefficiency and an active breakdown of the majority of the molecules suggests that this feature may have a function in cardiac SR biology. These questions will require the reagents developed in these studies, along with adenoviruses and antibodies applicable in rat heart cells. Recently published studies on TRD overexpression in cultured rat heart cells (15) and using transgenic mice (30) have shown that TRD overexpressed in these experimental systems is glycosylated very differently than in native heart. How this affects the proper interpretation of these data, and how TRD glycosylation affects Ca\(^{2+}\)-release complex formation are important questions for future studies.

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