Published in final edited form as:

Biochem J. 2013 August 1; 453(3): . doi:10.1042/BJ20130028.

Molecular Determinants of Co- and Post-translational N-glycosylation of Type I Transmembrane Peptides

Heidi L. H. Malaby and William R. Kobertz*

Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2324

Programs in Neuroscience and Chemical Biology, University of Massachusetts Medical School, 364 Plantation Street, Worcester, MA 01605-2324

Abstract

Type I transmembrane peptides acquire N-linked glycans during and after protein synthesis to facilitate anterograde trafficking through the secretory pathway. Mutations in N-glycosylation consensus sites (NXT and NXS, where X = P) that alter the kinetics of the initial N-glycan attachment have been associated with cardiac arrhythmias; however, the molecular determinants that define co- and post-translational consensus sites in proteins are not known. Here, we identified co- and post-translational consensus sites in the KCNE family of K⁺ channel regulatory subunits to uncover three determinants that favor co-translational N-glycosylation kinetics of type I transmembrane peptides that lack a cleavable signal sequence: threonine containing-consensus sites (NXT), multiple N-terminal consensus sites, and long C-termini. The identification of these three molecular determinants now makes it possible to predict co- and post-translational consensus sites in type I transmembrane peptides.

Keywords

N-glycosylation; oligosaccharyltransferase; kinetics; consensus sites; KCNE

Introduction

The folding, assembly and trafficking of eukaryotic membrane proteins to the cell surface is highly dependent on asparagine-linked (N-linked) glycosylation. During membrane protein biogenesis in the ER, the oligosaccharyltransferase (OST) attaches a 14-sugar oligosaccharide to the nascent polypeptide at N-linked consensus sites: N-X-T/S-Y, where X and Y can be any residue other than proline [1, 2]. Until recently, the initial attachment of N-linked glycans to polypeptides was thought to be exclusively a co-translational process; however, kinetic investigations have shown that post-translational N-glycosylation occurs at select consensus sites in both water soluble [3, 4] and membrane-embedded proteins [5, 6]. Although either threonine or serine is required at the (n + 2) position [7], previous studies have shown that serine-containing consensus (NXS) sites are less efficiently N-glycosylated than the analogous threonine-containing consensus (NXT) sites [8-10]. This difference in N-

Address correspondence to: Dr. William R. Kobertz, Department of Biochemistry and Molecular Pharmacology, University of Massachusetts Medical School, 364 Plantation Street, Worcester MA 01605-2324, 508.856.8861 (phone), 508.856.8867 (fax), william.kobertz@umassmed.edu.

Author contributions: HM and WRK designed the experiments and wrote the manuscript. HM performed all of the experiments and data analysis. Both authors have approved the final version of the manuscript.

The authors declare that they have no competing financial interests.

glycosylation efficiency has been attributed to the physiochemical differences of the serine and threonine hydroxyl groups; however, the recent crystal structure of a bacterial OST indicates that the hydroxyl residue is not directly involved in the catalytic transfer of the N-linked glycan to the asparagine residue [11]. Thus, the molecular and cellular bases for the serine and threonine differential remain unclear.

One family of membrane-embedded proteins that contain multiple serine and threonine N-linked glycosylation sites is the KCNE family of type I transmembrane peptides (E1 – E5) (Figure 1A). These N-terminally glycosylated membrane proteins co-assemble with and modulate several different voltage-gated K⁺ channels [12]. Mutations that destroy N-linked glycosylation sites in E1 and E2 give rise to Long QT syndrome (LQTS) [13, 14], a disorder of the cardiac rhythm that is sometimes accompanied with neural deafness [15]. We have recently shown that the E1 LQTS mutant, T7I, is particularly deleterious because it prevents N-glycosylation at both consensus sites, yielding unglycosylated regulatory subunits that do not traffic to the cell surface [5]. This long range N-glycosylation defect occurs because direct ablation of one E1 consensus site shifts the N-glycosylation kinetics from co-translational to post-translational glycan attachment. Interestingly, the kinetics of N-glycan attachment could be converted to co-translational by simply switching the hydroxyl residue from serine to threonine in the remaining intact consensus site, rescuing both N-glycosylation and the anterograde trafficking defect of the T7I mutation.

Because N-glycosylation kinetics have been implicated in the diseases of the cardiac rhythm, we set out to identify co- and post-translational consensus sites in the KCNE family in order to define the molecular determinants that affect the kinetics of N-glycosylation in type I transmembrane peptides. Using rapid, radioactive pulse-chase experiments, we determined the N-glycosylation kinetics of wild type E2 – E5 and a panel of N-glycosylation mutants. By comparing the N-glycosylation kinetics of the consensus sites in the KCNE family, we uncovered three determinants of N-glycan attachment to type I transmembrane peptides: (1) substituting serine for threonine in consensus sites significantly reduces co-translational N-glycosylation; (2) multiple consensus sites in the N-terminus of a type I transmembrane peptide increase N-glycan attachment efficiency; (3) longer cytoplasmic C-termini favor co-translational glycosylation. The identification of these three determinants of N-glycan attachment provides a schema to predict the consensus sites in type I transmembrane peptides that are susceptible to the long range glycosylation defects observed with the T7I LQTS mutation.

Experimental

Cloning and plasmids

Human KCNE1 – KCNE5 were cloned into pcDNATM3.1⁽⁻⁾ vector such that five additional methionine residues and an HA tag (YPYDVPDYA) were added to the C-terminus of each KCNE peptide [5]. Mutations were introduced using either the Quikchange Site Directed Mutagenesis Kit or by traditional PCR cassette mutagenesis (Syzygy Biotech Taq 2x MeanGreen Master Mix) between the 5' *KpnI* and 3' *BglII* sites. Single N-glycosylation consensus site constructs were generated by mutating the asparagine residue in the other consensus sites to glutamine (e.g. “E2 N6” contains a N29Q mutation). All constructs were confirmed by DNA sequencing the entire gene.

Cell culture and transfection

Chinese hamster ovary-K1 (CHO) cells were sustained in Gibco-F12K Nutrient Mixture, Kaighn's Modification (with L-glutamine) media, supplemented with 10% FBS (Sigma) and 1% penicillin/streptomycin. Cells were passaged using 0.5% Trypsin-EDTA and plated onto

20 mm dishes at 80% confluency. After 24 h, the cells were transiently transfected with a mixture of 3 μ g KCNE DNA and 80 μ L Lipofectamine in 2 mL OptiMem. After 6 h at 37°C, the transfection cocktail was removed, 10 mL of F12K media was added and the cells were incubated for 15-20 h.

Pulse-chase assays and cell lysis

Transfected cells were washed with PBS (2 $\times$ 4 mL) and incubated for 35 min at 37°C in Gibco DMEM High Glucose Media (4.5 g/L D-glucose, lacking L-methionine and L-cysteine), supplemented with 10% FBS, 1% Pen Strep, and 2 mM L-glutamine. The media was removed and the cells were incubated at 37°C for 2 min in (4 mL) of DMEM High Glucose Media containing 100 μ Ci/mL EasyTag EXPRESS [³⁵S] Protein Labeling Mix (Perkin Elmer). The radioactive media was removed and the cells washed with PBS (2 $\times$ 4 mL) and chased with F12K media for 3, 6, 9, or 12 min at 37°C. The cells were then washed with PBS (2 $\times$ 4 mL), and lysed with 750 μ L of low salt lysis buffer (in mM): 50 TRIS-HCl, pH 7.4, 150 NaCl, 20 NaF, 10 Na₃VO₄, 1% NP-40, 1% CHAPS, which was supplemented with protease inhibitors: 1 mM phenylmethylsulfonyl chloride (PMSF) and 1 μ g/mL each of leupeptin, pepstatin, and aprotinin (LPA). Cells were lysed for 30 min with vigorous shaking at 4°C, and the cell debris scraped and pelleted at 14,000 rpm for 10 min at room temperature.

Radioimmunoprecipitation and electrophoresis

Protein G agarose beads (Pierce) were prepared by washing (3 $\times$ 750 μ L) in low salt lysis buffer. After pelleting the cell debris, the supernatant was precleared with 50 μ L beads for 2 hours at 4°C on a roller drum. The beads were then spun down, and the supernatant transferred to new tubes containing 25 μ L beads pre-incubated with 1 μ L monoclonal anti-HA antibody (Sigma). After an overnight incubation at 4°C, the beads were pelleted, the supernatant removed and the beads were subjected to 5 washes: low salt lysis buffer (3 $\times$ 750 μ L); high salt buffer (1 $\times$ 750 μ L): 50mM TRIS-HCl, pH 7.4, 500 mM NaCl, 1% NP-40, 1% CHAPS, 20 mM NaF, 10 mM Na₃VO₄, and a final wash with low salt lysis buffer (1 $\times$ 750 μ L). For enzymatic deglycosylation assays, 1 μ L Endo H_f (New England Biolabs) was added to beads in 50 μ L low salt lysis buffer and incubated at 37°C for 1 hour. Peptides were eluted from the beads with (50 μ L) of 100 mM DTT and 2x SDS gel loading buffer at 55°C for 15 min. Samples were analyzed by SDS-PAGE (10% or 15%) and the gels were dehydrated for 1 h in a 30% Ethanol, 2% glycerol solution. Gels were dried for 2 h at 80°C, applied to film, and analyzed for autoradiography by Typhoon FLA-9000 phosphoimager after 7 – 14 d.

Determination of co- and post-translational N-glycosylation

All signals were quantified using Image Gauge software (Fujifilm). For all constructs (except wtE3), the percent maximally glycosylated was calculated by dividing the signal of the maximally glycosylated species by the total signal at each time point. For the wtE3 constructs with three consensus sites, all glycoforms were used; thus, the percent glycosylated is the sum of the signal from all three glycoforms divided by the total signal at each time point. The total glycosylation in Table 1 is the maximally glycosylated species at 12 min except for the triply glycosylated constructs, where all three glycoforms were used. To determine the percent co-translational N-glycosylation for each construct, we divided the percent glycosylated at 0 min (co-translational) by the 12 min time point (total glycosylation). This simplified calculation assumes that the small KCNE peptides are fully synthesized before the 3 min chase point and that the kinetics of co- and post-translational N-glycosylation are substantially different, both of which have been previously substantiated in the literature [3, 5, 16]. The percent post-translational glycosylation was calculated by

taking the difference between co- and total glycosylation and dividing it by total glycosylation for each construct.

Results

N-glycosylation of the N-terminus of type I transmembrane peptides occurs within seconds to minutes upon insertion into the ER lumen [5]. To observe these rapid kinetics in radioactive pulse-chase experiments, extremely short (2 min) pulses of [³⁵S] methionine and cysteine are required. However, this experimental time constraint significantly reduces the amount of metabolically-labeled protein that can be isolated by immunoprecipitation. Therefore, we sought experimental conditions that would allow us to observe and compare the kinetics of N-glycosylation of wild type and a panel of mutant KCNE peptides. Because both OST isoforms are conserved in all metazoan except *Caenorhabditis* [6], any readily transfectable cell line is sufficient as long as exogenous expression of the KCNE peptide does not deplete cellular substrates or saturate the ER machineries in the cell. Therefore, we chose to heterologously express the different KCNE constructs in CHO cells, which we have previously shown are not overwhelmed by exogenous expression of type I transmembrane peptides [5]. In addition, the subsequent Golgi modifications in CHO cells mirrors both the N- and O-glycosylation of E1 observed in cardiomyocytes [17] where all five KCNE peptides are expressed [18, 19]. To increase and normalize the radioactive signal in our experiments, we used methionine-rich constructs that contained five methionine residues and an HA-tag (for immunoprecipitation) at the C-terminus of each KCNE peptide. Together, these experimental conditions yielded enough metabolically labeled KCNE protein to observe co- and post-translational N-glycosylation.

Using this experimental paradigm, we first examined wild type E4 (wtE4), which contains a single NXT consensus site (N8, T10) in its N-terminus (Figure 2). Cells were metabolically labeled with [³⁵S], chased with cold media, and the wtE4 proteins were isolated at various times (0 – 12 min). For every time point, two closely spaced bands were observed (Figure 2A). Endo H treatment (which removes immature N-glycans found in the ER) of the 12 min sample resulted in a single, darker band that identified the upper and lower bands as glycosylated and unglycosylated protein, respectively (Figure 2A, wtE4: EH lane). Time points were limited to 12 min because longer chase times resulted in further glycan processing, which changed the mobility and decreased the signal of the glycosylated species (data not shown). Plotting the data (Figure 2B) showed that the amount of glycosylated material slowly increased over the entire time course. As expected, we observed similar results in other mammalian cell lines (Supplementary Figure 1).

To calculate the amount of co- and post-translational N-glycosylation, we exploited the kinetic difference between these two unique mechanisms of N-glycan attachment (Figure 1B). Although both modes of N-glycan attachment are rapid, post-translational N-glycosylation is substantially slower than co-translational [3, 5]. This kinetic disparity allows for a simplified convention where co-translational is defined as N-glycan attachment occurring during the radioactive pulse (zero time point) and all subsequent N-glycosylation during the chase periods as post-translational (see Experimental). Using this convention, only 16% of the N-glycosylated E4 protein is post-translationally glycosylated (Figure 2B); thus, the NXT site in wtE4 is a strong co-translational site. Because wtE4 contains an NXT site, we compared its N-glycosylation kinetics to the T10S mutant (NXS site). In stark contrast to wtE4 (Figure 2A), the majority of the T10S mutant protein was unglycosylated after the radioactive pulse (0 min). After 12 min, however, the amount of N-glycosylated material nearly doubled, resulting in 50% post-translational glycosylation of the T10S mutant (Table 1). These results with E4 show that the hydroxylated residue in the consensus site strongly influences the kinetics of N-glycosylation.

We next determined the N-glycosylation kinetics of E2 because it has two NXT consensus sites (N6, T8 and N29, T31). Similar to the singly-glycosylated E4 construct, the NXT sites in wtE2 were co-translationally N-glycosylated (Figures 3A and 3C). Endo H treatment identified the unglycosylated and glycosylated bands, which was confirmed as the doubly glycosylated species when we examined the NXS mutants (T8S and T31S). Both NXS mutations reduced the amount of co-translational N-glycosylation, resulting in the appearance of a singly glycosylated species. Although the consensus site mutations were identical (threonine to serine), the reduction of co-translational N-glycosylation compared to wtE2 was different for the two mutants. Therefore, we examined the E2 consensus sites individually using singly glycosylated constructs (Figures 3B and 3D). The N-glycosylation kinetics at N6 was nearly identical to wtE2 with a ~ 30% loss in co-translational and concomitant increase in post-translational glycosylation when the native threonine was mutated to serine (Table 1). In contrast, the threonine to serine mutation at N29 was more detrimental in the singly glycosylated construct—essentially inhibiting both co- and post-translational N-glycosylation even though a bona fide consensus site was present. Thus, the co-translational efficiency of NXS sites also depends on their location in the N-terminus. Moreover, these results with the singly glycosylated constructs (Figure 3B) revealed a second determinant of N-glycan attachment to type I transmembrane peptides: multiple consensus sites increase co-translational glycosylation efficiency.

In contrast to E2, E5 contains two NXS consensus sites (N2, S4 and N25, S27). Surprisingly, these consensus sites in wtE5 were co-translationally N-glycosylated (80%), yielding only two bands that were confirmed as unglycosylated and doubly glycosylated using Endo H (EH lane) and the singly glycosylated E5 mutants (Figure 4A). Moreover, conversion of either consensus site into an NXT site had no effect on the glycosylation kinetics. Because the multiple glycosylation sites in wtE2 partially masked the effect of threonine to serine mutation, we compared the glycosylation kinetics at the two E5 consensus sites separately (Figures 4B and 4C). Using these singly glycosylated E5 constructs, we observed a small, but significant increase in co-translational N-glycosylation for the N25 consensus site when it was converted to NXT. Post-translational N-glycosylation varied between 20 – 35% for the E5 single consensus site mutants (Table 1), with the N25, S27 site (Figure 4C) showing the most N-glycan attachment after protein translation. These results showed that the N-glycosylation kinetics are affected by the serine to threonine mutation; however, the measured effects were modest because the wild type NXS sites in E5 are efficiently co-translationally N-glycosylated.

Lastly, we examined E3, which is the smallest of the KCNE peptides (103 aa), yet it contains the most N-glycosylation sites (Figure 5, NXT: N5, N22 and N41). Despite having three NXT sites, wtE3 is a poor substrate for N-glycosylation (Figure 5A), with over half of the metabolically-labeled protein remaining unglycosylated after 12 min. Of the N-glycoforms (confirmed by Endo H, Supplemental Figure 2), only the singly and doubly glycosylated species were observed at early time points. Subsequent post-translational N-glycosylation significantly increased the amount of the doubly glycosylated form; however, very little fully glycosylated material was observed. To determine whether one or more of the consensus sites were refractory to N-glycosylation, we compared the glycosylation kinetics of the single site mutants. Similar to wtE3, all three single site constructs were poor substrates for co-translational N-glycosylation (Figures 5A and 5B: open symbols) and thus the majority of the glycans were added post-translationally.

The paucity of co-translational N-glycosylation for all of the NXT consensus sites in E3 suggested that another factor was influencing the N-glycosylation kinetics. For type I transmembrane peptides, the length of the C-terminus should affect co-translational N-glycosylation because longer C-termini will increase both the probability of insertion into

the Sec61 complex (translocon) by the signal recognition particle (SRP) complex and the time the peptide spends in the translocational tunnel [20]. Our results with E3 are consistent with this hypothesis because it has the shortest C-terminus (23 aa). Therefore, we made chimeras with E4 (which has the longest C-terminus) to determine whether co-translational N-glycosylation of the NXT sites in E3 could be improved. In both the wild type and single site constructs, extending the length of the C-terminus (XL constructs) significantly increased the amount of co-translational and total N-glycosylation for all consensus sites except N41, which had comparable amounts of total glycosylation (Figures 5A and 5B: closed symbols). The unglycosylated and differently glycosylated bands were identified by comparing gel mobilities to a singly-glycosylated construct (Supplemental Figure 2). For the wtE3 construct, the improvement in N-glycosylation also reduced the amount of unglycosylated protein and increased the fully glycosylated form such that it and the doubly glycosylated species were the predominant glycoforms after 12 min. To confirm that the length of the C-terminus influences the kinetics of N-glycosylation, we also made and examined the reverse E4/E3 chimera (XS), which has a shorter C-terminus than wtE4 (Figure 5C). As expected, shortening the E4 C-terminus reduced the amount of co-translational N-glycosylation, resulting in an increase in post-translational N-glycosylation (Figure 5D). In total, these results demonstrated that co-translational N-glycosylation efficiency depends on the length of the C-terminus in type I transmembrane peptides.

Discussion

Using rapid radioactive pulse-chase experiments to measure the kinetics of N-glycosylation, we determined that the consensus sites in E4, E2, and E5 peptides are efficiently co-translationally N-glycosylated. In contrast, all three consensus sites in E3 are predominately post-translationally N-glycosylated. From these results, we identified three determinants of N-glycosylation kinetics for type I transmembrane peptides:

The hydroxyl residue in the consensus site (NXT vs NXS). Our results show that substituting serine for threonine in co-translational sites significantly reduces co-translational and overall efficiency of N-glycosylation (Figures 2 and 3). A threonine preference has been previously observed *in vitro* with hexapeptides, where a threonine to serine substitution results in a 40-fold decrease in N-glycan attachment [10]. Although we observed a threonine preference with type I transmembrane peptides, a similar preference has not been observed with water soluble proteins [3]. This discrepancy can be attributed to the insertion mechanism of type I transmembrane peptides into the ER translocon (Sec 61) [20]. For type I transmembrane peptides that lack a signal sequence, the N-terminal N-linked consensus sites are inserted into the ER lumen en masse after the transmembrane domain exits the ribosomal tunnel (Figure 1B). Thus, the lifetime of the type I transmembrane peptide in the translocon, as well as the time the OST has to modify the NXT and NXS consensus sites, is dictated by the length of the C-terminus (Figure 1A). Because the KCNE family of peptides has relatively short C-termini (~ 20 – 120 aa), the OST's preference for NXT sites becomes evident, resulting in skipped NXS sites that are subsequently modified by the post-translational machinery, which was recently identified as the OST isoform STT3B [3] (Figure 1B). Recently, a hydroxyl residue preference was also discovered for type II transmembrane proteins [6], indicating that an NXT preference may be general for all single pass transmembrane proteins. Although a threonine in the consensus site increases co-translational N-glycosylation, not all NXS sites are poor co-translational sites. Both NXS sites in wtE5 (Figure 4) and the E1 N-terminal consensus site (Supplemental Figure 2) are co-translationally glycosylated.

Multiple Consensus Sites. In addition to a threonine preference, N-glycan attachment is more efficient with type I transmembrane peptides that have multiple consensus sites

(Figure 3). The direct ablation or partial reduction of co-translational N-glycosylation at one site (T8S and T31S E2 mutants) leads to the appearance of unglycosylated material that may or may not (N29, T31S) be post-translationally glycosylated. For wtE5, this coupling effect between glycosylation sites overrides the threonine preference (Figure 4A), yielding the same amount of glycosylated protein regardless of the hydroxyl groups in the consensus sites.

Length of C-terminus. By manipulating the number of amino acids in the E3 and E4 C-termini, we also showed that co-translational N-glycosylation efficiency is dependent on the length of the C-terminus. Elongation of a short C-terminus can increase co-translational efficiency by two mechanisms. The first mechanism, as mentioned above, simply involves increasing the resident time of the type I transmembrane peptide within the ER translocon (Sec 61) by increasing the time it takes for the translocon-docked ribosome (Figure 1B) to complete protein translation. The second mechanism involves the initial insertion of the type I transmembrane domain into the ER membrane. For KCNE peptides with relatively longer C-termini, the bolus of N-glycosylation at the zero time points indicates that the transmembrane domain of these peptides is recognized by the signal recognition particle (SRP) and delivered and inserted into the ER translocon before protein translation is complete [20]. However, for wild type E3 peptides with extremely short C-termini (23 aa), the reduced amount of co-translational and total N-glycosylation hints that these peptides may not enter the ER membrane via the translocon, but by a post-translational ER insertion machinery similar to what has been described for type II transmembrane peptides [21]. Thus, elongation of the E3 C-terminus may increase co-translational N-glycosylation by improving insertion into the ER translocon. To perform our pulse-chase studies (Experimental), the C-terminus of all constructs were actually 18 aa longer; therefore, for true wild type E3—which is N-glycosylated [22]—the likelihood for co-translational N-glycosylation is greatly diminished.

Because the insertion mechanism of single pass TM peptides into the translocon dictates how N-glycosylation sites enter the ER lumen, these three determinants of N-glycosylation kinetics should be general for all type I transmembrane peptides that lack a cleavable ER signal sequence. A re-examination of the KCNE family using these three determinants also reveals how the individual family members are designed to achieve maximal N-glycosylation. The majority of the consensus sites in KCNE peptides contain a threonine residue, which are readily co-translationally modified. Because the attachment of one N-glycan enhances subsequent modifications, all human KCNE peptides except E4 contain at least two consensus sites. For E4, its elongated C-terminus increases co-translational N-glycosylation, which alleviates the need for consensus site coupling. Conversely, E3 has three consensus sites to compensate for its extremely short C-terminus. Thus, the KCNE peptides are genetically-encoded to ensure that their N-termini are N-glycosylated.

Although KCNE peptides are optimized to acquire N-glycans early and often during biogenesis to promote assembly and anterograde trafficking with K⁺ channel subunits [23], mutations can derail the process, leading to un- and hypo-glycosylated proteins. We have previously shown that the inherited LQTS mutant, T7I, exposes a flaw in E1, where direct ablation of one consensus site indirectly reduces N-glycosylation at the remaining NXS site, resulting in unglycosylated E1 protein [5]. The determinants of N-glycosylation in this study directly explain this observation with E1—the ablation of one consensus site eliminates consensus site coupling, leaving a lone NXS site that is neither co-translationally or post-translationally N-glycosylated. This compounded hypoglycosylation was recapitulated with the N29, T31S E2 mutant in this study (Figure 3B). However, the similar LQTS mutation in E2 (T8A) is not predicted to cause catastrophic loss of N-glycosylation in healthy cells because the remaining N29 consensus site in wild type E2 contains a threonine. Satisfyingly,

the results with singly glycosylated E2 mutant (N29, T31) are in agreement this prediction (Figure 3B).

The strong co-translational preference for NXT sites indicates that the loss of a single methyl group in the consensus site may not be benign in type I transmembrane peptides. Under certain circumstances (i.e. drug-induced, stress, etc.), the shift to post-translational N-glycosylation and mild hypoglycosylation caused by a threonine to serine mutation (Figures 2 and 3) may be sufficient to result in a disease state. Consistent with this mild hypoglycosylation/haploinsufficiency hypothesis are the Brugada syndrome (E3: T4A) [24] and antibiotic-induced long QT syndrome mutations (E2: T8A, Q9E) [14, 25] that eliminate or flank consensus sites in other KCNE peptides. For the drug-induced mutations that increase hERG K⁺ channel sensitivity to macrolide antibiotics, the additional presence of the drug could perturb the already delicate balance of co- and post-translational N-glycosylation of these mutant E2 proteins. Similarly, the T4A mutation that immediately precedes the first consensus site in E3 is in a position to alter the kinetics of N-glycosylation. Future kinetic studies that examine the central and flanking consensus site residues in type I transmembrane peptides will further refine the determinants of N-glycan attachment to better predict the disease-causing mutations that effect N-glycosylation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Dr. Reid Gilmore for his advice and guidance on all aspects of N-linked glycosylation. This work was supported by a grant to WRK from the National Institutes of Health (GM-070650).

References

1. Silberstein S, Gilmore R. Biochemistry, molecular biology, and genetics of the oligosaccharyltransferase. *Faseb J*. 1996; 10:849–858. [PubMed: 8666161]
2. Mohorko E, Glockshuber R, Aepli M. Oligosaccharyltransferase: the central enzyme of N-linked protein glycosylation. *Journal of inherited metabolic disease*. 2011; 34:869–878. [PubMed: 21614585]
3. Ruiz-Canada C, Kelleher DJ, Gilmore R. Cotranslational and posttranslational N-glycosylation of polypeptides by distinct mammalian OST isoforms. *Cell*. 2009; 136:272–283. [PubMed: 19167329]
4. Bolt G, Kristensen C, Steenstrup TD. Posttranslational N-glycosylation takes place during the normal processing of human coagulation factor VII. *Glycobiology*. 2005; 15:541–547. [PubMed: 15616124]
5. Bas T, Gao Y, Lvov A, Chandrasekhar KD, Gilmore R, Kobertz WR. Post-Translational N-Glycosylation of Type I Transmembrane KCNE1 Peptides: Implications for Membrane Protein Biogenesis and Disease. *J Biol Chem*. 2011; 286:28150–28159. [PubMed: 21676880]
6. Shrimall S, Trueman SF, Gilmore R. Extreme C-terminal sites are posttranslocationally glycosylated by the STT3B isoform of the OST. *J Cell Biol*. 2013; 201:81–95. [PubMed: 23530066]
7. Breuer W, Klein RA, Hardt B, Bartoschek A, Bause E. Oligosaccharyltransferase is highly specific for the hydroxy amino acid in Asn-Xaa-Thr/Ser. *FEBS Lett*. 2001; 501:106–110. [PubMed: 11470266]
8. Kasturi L, Chen H, Shakin-Eshleman SH. Regulation of N-linked core glycosylation: use of a site-directed mutagenesis approach to identify Asn-Xaa-Ser/Thr sequons that are poor oligosaccharide acceptors. *Biochem J*. 1997; 323(Pt 2):415–419. [PubMed: 9163332]
9. Shakin-Eshleman SH, Spitalnik SL, Kasturi L. The amino acid at the X position of an Asn-X-Ser sequon is an important determinant of N-linked core-glycosylation efficiency. *J Biol Chem*. 1996; 271:6363–6366. [PubMed: 8626433]

10. Bause E, Legler G. The role of the hydroxy amino acid in the triplet sequence Asn-Xaa-Thr(Ser) for the N-glycosylation step during glycoprotein biosynthesis. *Biochem J.* 1981; 195:639–644. [PubMed: 7316978]
11. Lizak C, Gerber S, Numao S, Aebl M, Locher KP. X-ray structure of a bacterial oligosaccharyltransferase. *Nature.* 2011; 474:350–355. [PubMed: 21677752]
12. McCrossan ZA, Abbott GW. The MinK-related peptides. *Neuropharmacology.* 2004; 47:787–821. [PubMed: 15527815]
13. Schulze-Bahr E, Wang Q, Wedekind H, Haverkamp W, Chen Q, Sun Y, Rubie C, Hordt M, Towbin JA, Borggrefe M, Assmann G, Qu X, Somberg JC, Breithardt G, Oberti C, Funke H. KCNE1 mutations cause Jervell and Lange-Nielsen syndrome. *Nat Genet.* 1997; 17:267–268. [PubMed: 9354783]
14. Park KH, Kwok SM, Sharon C, Baerga R, Sesti F. N-Glycosylation-dependent block is a novel mechanism for drug-induced cardiac arrhythmia. *Faseb J.* 2003; 17:2308–2309. [PubMed: 14563681]
15. Tyson J, Tranebjaerg L, McEntagart M, Larsen LA, Christiansen M, Whiteford ML, Bathen J, Aslaksen B, Sorland SJ, Lund O, Pembrey ME, Malcolm S, Bitner-Glindzicz M. Mutational spectrum in the cardioauditory syndrome of Jervell and Lange-Nielsen. *Hum Genet.* 2000; 107:499–503. [PubMed: 11140949]
16. Hershey JW. Translational control in mammalian cells. *Annu Rev Biochem.* 1991; 60:717–755. [PubMed: 1883206]
17. Chandrasekhar KD, Lvov A, Terrenoire C, Gao Y, Kass RS, Kobertz WR. O-Linked Glycosylation of the Cardiac I_{Ks} Complex. *J Physiol.* 2011; 589:3721–3730. [PubMed: 21669976]
18. Bendahhou S, Marionneau C, Haurogne K, Larroque MM, Derand R, Szuts V, Escande D, Demolombe S, Barhanin J. In vitro molecular interactions and distribution of KCNE family with KCNQ1 in the human heart. *Cardiovasc Res.* 2005; 67:529–538. [PubMed: 16039274]
19. Lundquist AL, Manderfield LJ, Vanoye CG, Rogers CS, Donahue BS, Chang PA, Drinkwater DC, Murray KT, George AL Jr. Expression of multiple KCNE genes in human heart may enable variable modulation of I_{Ks} . *J Mol Cell Cardiol.* 2005; 38:277–287. [PubMed: 15698834]
20. Shao S, Hegde RS. Membrane protein insertion at the endoplasmic reticulum. *Annual review of cell and developmental biology.* 2011; 27:25–56.
21. Stefanovic S, Hegde RS. Identification of a targeting factor for posttranslational membrane protein insertion into the ER. *Cell.* 2007; 128:1147–1159. [PubMed: 17382883]
22. Gage SD, Kobertz WR. KCNE3 Truncation Mutants Reveal a Bipartite Modulation of KCNQ1 K^+ Channels. *J Gen Physiol.* 2004; 124:759–771. [PubMed: 15572349]
23. Chandrasekhar KD, Bas T, Kobertz WR. KCNE1 Subunits Require Co-assembly with K^+ Channels for Efficient Trafficking and Cell Surface Expression. *J Biol Chem.* 2006; 281:40015–40023. [PubMed: 17065152]
24. Nakajima T, Wu J, Kaneko Y, Ashihara T, Ohno S, Irie T, Ding WG, Matsuura H, Kurabayashi M, Horie M. KCNE3 T4A as the Genetic Basis of Brugada-Pattern Electrocardiogram. *Circulation journal : official journal of the Japanese Circulation Society.* 2012; 76:2763–2772. [PubMed: 22987075]
25. Abbott GW, Sesti F, Splawski I, Buck ME, Lehmann MH, Timothy KW, Keating MT, Goldstein SA. MiRP1 forms IKr potassium channels with HERG and is associated with cardiac arrhythmia. *Cell.* 1999; 97:175–187. [PubMed: 10219239]

Figure 1.

Depiction of the KCNE family and the co- and post-translational N-glycosylation pathways. (A) All five KCNE members are depicted to scale; residue numbers for the beginning and ending of the transmembrane region (TM) and the end of the C-terminus are labeled. The approximate location and type of glycosylation site (NXT/S) are shown in the N-terminus. (B) Cartoon of co- and post-translational N-glycosylation of type I transmembrane peptides. OST: oligosaccharyltransferase; Sec 61: ER translocon; TM: transmembrane domain

Figure 2.

N-glycosylation kinetics of E4 reveals a threonine preference for co-translational glycosylation. **(A)** Cartoon of E4 N-terminus depicting the consensus site and representative fluorographs of wild type (wtE4: filled circles) and mutant (T10S: open circles) E4. Cells were pulsed for 2 min and chased for the indicated times. Immunoprecipitated E4 proteins were separated by electrophoresis and detected by autoradiography. EH denotes the endoglycosidase H-treated sample to identify the unglycosylated species. Unglycosylated (0-Gly) or singly glycosylated (1-Gly) are labeled. **(B)** Plots of the maximally glycosylated (1-Gly) species at each time point. The visible error bars that protrude beyond the data points are $\pm$ s.e. from 3 – 4 experiments. Co- and post-translational glycosylation percentages are shown for wtE4.

Figure 3.

Multiple consensus sites enhance N-glycosylation of E2. (A) Cartoon of E2 N-terminus depicting the consensus sites and representative fluorographs from pulse-chase experiments of wild type (wtE2: filled circles) and serine mutants (T8S: right-filled circles; T31S: left-filled circles). EH designates endoglycosidase H treated lane. (B) Representative pulse-chase results for the singly glycosylated constructs. *Top panels:* N-glycosylation kinetics at the first N-glycosylation consensus site (N6) containing a threonine (T8: closed squares) or serine (T8S: open squares). *Bottom panels:* N-glycosylation kinetics at the second N-glycosylation consensus site (N29) containing a threonine (T31: closed triangles) or serine (T31S: open triangles). Unglycosylated (0-Gly), singly (1-Gly) and doubly glycosylated (2-Gly) species are labeled in the fluorographs where detected. (C, D) Plots of the of maximally glycosylated species from A: 2-Gly; B: 1-Gly. Error bars are $\pm$ s.e. from 3 experiments.

Figure 4.

Consensus site coupling in E5 obscures a mild co-translational preference for threonine. **(A)** Cartoon of E5 N-terminus depicting the consensus sites and representative fluorographs from pulse-chase experiments of wild type (wtE5: open circles) and threonine mutants (S4T: left-filled circles; S27T: right-filled circles). The maximally glycosylated species (2-Gly) is plotted on the right. Co- and post-translational N-glycosylation at the **(B)** N2 and **(C)** N25 consensus sites. *Left panels:* Representative fluorographs of the NXS (S4: open squares; S27: open triangles) and NXT (S4T: closed squares; S27T: closed triangles) consensus sites. *Right:* Plots of the of maximally glycosylated species (1-Gly). Unglycosylated (0-Gly), singly (1-Gly) and doubly glycosylated (2-Gly) species are labeled in the fluorographs where detected. Error bars are $\pm$ s.e. from 3 – 5 experiments.

Figure 5. C-terminal tail length affects E3 N-glycosylation kinetics. **(A)** Representative fluorographs from pulse-chase experiments. *Top panels:* wild type (wtE3: open circles) and the single glycosylation site mutants (N5: open squares; N22: open triangles; N41: open diamonds); *Bottom panels:* C-terminal elongation (XL) of the above constructs via an E3/E4 chimera (filled symbols). **(B)** Plots of (*left*) the glycosylated material (1-Gly, 2-Gly, and 3-Gly forms) for the wtE3 constructs and (*right*) maximally glycosylated species (1-Gly) of the single site mutants. **(C)** Representative fluorographs from pulse-chase results for a C-terminal shortened E4 construct (XS). wtE4 data from Figure 2 are shown for comparison. **(D)** Percent co-translational N-glycosylation (zero time point) of the wild type and altered tail length forms. wt: solid bars; XL and XS: hatched bars; * $p < 0.05$, unpaired two-sided Student's *t* test; error bars in panels **B** and **D** are $\pm$ s.e. from 3 – 4 experiments. Cartoon of the E3 N-terminus depicting the three consensus sites.

Table 1KCNE N-glycosylation¹

	% Total	% Co-	% Post-
E4			
wt	68.6 ± 3.9	83.6 ± 7.0	16.4 ± 7.7
T10S	44.2 ± 1.3*	49.9 ± 4.5*	50.1 ± 5.3*
E4 (XS)	78.0 ± 1.0	50.1 ± 1.3*	49.1 ± 1.8*
E2			
wt	92.9 ± 2.0	82.1 ± 5.9	17.9 ± 6.0
T8S	69.5 ± 2.7*	64.1 ± 3.6*	35.9 ± 4.9*
T31S	38.4 ± 1.1*	65.3 ± 3.7*	34.7 ± 4.3*
N6, T8	92.5 ± 2.5	80.5 ± 3.7	19.5 ± 4.1
N6, T8S	59.0 ± 1.2*	52.5 ± 5.4*	47.5 ± 5.7*
N29, T31	82.8 ± 1.6	71.0 ± 1.8	29.0 ± 2.3
N29, T31S	10.5 ± 1.8*	ND	ND
E5			
wt	71.4 ± 1.0	79.0 ± 2.8	21.0 ± 3.0
S4T	70.9 ± 2.5	81.8 ± 3.2	18.2 ± 3.9
S27T	74.2 ± 2.8	79.0 ± 3.6	21.0 ± 4.3
N2, S4	83.4 ± 2.0	72.5 ± 2.3	27.5 ± 2.9
N2, S4T	86.5 ± 0.7	77.4 ± 3.7	22.6 ± 3.7
N25, S27	86.4 ± 0.8	67.0 ± 1.8	33.0 ± 2.0
N25, S27T	86.7 ± 1.1	80.7 ± 2.8*	19.3 ± 2.9*
E3			
wt [#]	40.6 ± 2.1	63.4 ± 7.6	36.6 ± 8.8
wt (XL) [#]	58.2 ± 2.0*	87.4 ± 4.8*	12.6 ± 5.1
N5	30.3 ± 3.8	30.1 ± 8.1	69.9 ± 16.9
N5 (XL)	38.2 ± 1.0*	61.8 ± 3.5*	38.2 ± 4.2*
N22	33.7 ± 2.2	43.4 ± 3.3	56.6 ± 7.5
N22 (XL)	56.0 ± 2.7*	75.8 ± 6.2*	24.2 ± 7.1*
N41	37.4 ± 5.1	14.2 ± 6.0	85.8 ± 18.8
N41 (XL)	30.6 ± 0.2	71.4 ± 4.6*	28.6 ± 4.6*

¹Total glycosylation determined from the maximally glycosylated species at the concluding chase time point

except for the wtE3 and wtE3 (XL) constructs where all three glycoforms were used. The percentages of co- and post-translational N-glycosylation were determined as described in the Experimental section. Values are mean $\pm$ s.e. from 3 – 5 pulse-chase experiments. ND, not determined;

* $p < 0.05$ (one-way ANOVA with a Tukey's multiple comparison test). Comparisons were performed for each KCNE member and only between constructs with the same consensus sites.