Post-translational N-Glycosylation of Type I Transmembrane KCNE1 Peptides

IMPLICATIONS FOR MEMBRANE PROTEIN BIOGENESIS AND DISEASE

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N-Glycosylation of membrane proteins is critical for their proper folding, co-assembly and subsequent matriculation through the secretory pathway. Here, we examine the kinetics of N-glycan addition to type I transmembrane KCNE1 K⁺ channel β-subunits, where point mutations that prevent N-glycosylation at one consensus site give rise to disorders of the cardiac rhythm and congenital deafness. We show that KCNE1 has two distinct N-glycosylation sites: a typical co-translational site and a consensus site ~20 residues away that unexpectedly acquires N-glycans after protein synthesis (post-translational). Mutations that ablate the co-translational site concomitantly reduce glycosylation at the post-translational site, resulting in unglycosylated KCNE1 subunits that cannot reach the cell surface with their cognate K⁺ channel. This long range inhibition is highly specific for post-translational N-glycosylation because mutagenic conversion of the KCNE1 post-translational site into a co-translational site restored both monoglycosylation and anterograde trafficking. These results directly explain how a single point mutation can prevent N-glycan attachment at multiple sites, providing a new biogenic mechanism for human disease.

Asparagine-linked (N-linked) glycosylation is a highly conserved protein modification that eukaryotic cells utilize for the proper folding, assembly, and trafficking of membrane and secreted proteins. The initial attachment of N-linked glycans to polypeptides occurs in the endoplasmic reticulum (ER), where the oligosaccharyltransferase (OST) efficiently transfers a high mannose oligosaccharide onto asparagine residues within the primary sequence N-X-T/S-Y (“sequon”, where X and Y can be any natural amino acid other than proline) (1, 2). Although commonly referred to as a post-translational protein modification, N-linked glycosylation in mammalian cells typically occurs during translation as the nascent polypeptide is threaded through the translocation channel (translocon) into the ER lumen (3). Recently, however, one of the mammalian isoforms (STT3B) of the active site subunit of the ER resident OST complex has been shown to mediate post-translational N-glycosylation of a secreted protein, human blood coagulation factor VII (4). To date, factor VII is the only reported full-length protein that is post-translationally N-glycosylated in mammalian cells with intact N-glycosylation machinery (5). Factor VII curiously contains two distinct sequons: one sequon is modified co-translationally whereas the second sequon is modified post-translationally, yet the biological significance of a protein harboring both a co- and post-translational sequon is unclear.

Besides the structurally distorting proline residue, three other factors can significantly reduce or eliminate N-glycosylation of sequons (1). Residues at the X-position: NXS sequons with negatively-charged (Asp and Glu) and hydrophobic (Trp and Leu) amino acids at the X-position are poorer substrates for the OST complex (6, 7) (2). Proximity to the C terminus: sequons within ~60 residues of the C terminus often elude the OST complex, as the chain-terminated protein is believed to more rapidly enter the ER lumen through the translocon (8) (3). Proximity to a transmembrane domain: sequons that are less than 12 residues away from a transmembrane segment are inaccessible to the OST active site (9, 10). Bioinformatic interrogation of the residues flanking sequons (up to 20 residues) has not identified any other sequence motifs that have long range effects on N-glycosylation efficiency (11, 12).

KCNE1 (E1) is the founding member of a family of type I transmembrane K⁺ channel β-subunits that have two or three sequons in their extracellular N termini. E1 subunits have two sequons at N5 and N26, both of which are obligatorily glycosylated in native tissues as well as in standard electrophysiological expression systems (13–15). E1 co-assembles with KCNQ1 (Q1) K⁺ channels to produce the slowly activating cardiac Iks current (16, 17) and to recycle potassium in apical membranes of strial marginal and vestibular dark cells in the inner ear (18). A mutation that disrupts the sequon at N5 (T7I), gives rise to an inherited autosomal recessive form of Long QT Syndrome (LQTS), a disorder of the cardiac rhythm that is accompanied with neural deafness, Jervell-Lange-Nielsen Syndrome (JLNS) (19). An SNP in the equivalent threonine in KCNE2 (T8A) provokes drug-induced Long QT Syndrome with the commonly prescribed antibiotic, sulfamethoxazole (20, 21). However, the biogenic mechanism that underlies the importance for glycosylation at this absolutely conserved sequon remains to be elucidated.
Given the disease linkage between N-glycosylation and KCNE biology, we determined the kinetics and efficiency of N-glycosylation of the two sequons in E1 and the effects of N-glycan occupancy on co-assembly with K+ channel subunits and cell surface expression. By comparing wild type to a panel of E1 N-glycosylation mutants in metabolic labeling experiments, we found that the sequon adjacent to the E1 N terminus (N5) acquires its N-linked glycan during translation whereas the second glycan is primarily added to the N26 sequon post-translationally. Post-translational N-glycosylation was significantly affected by long range mutations since ablation of the N5 sequon with a disease-associated mutation inhibited post-translational attachment to the N26 sequon, which resulted in a bolus of unglycosylated E1 subunits. This long range inhibition was readily reversed by converting the N26 sequon into a cotranslational site, yielding monoglycosylated E1 subunits that formed complexes with Q1 channels, which were functionally indistinguishable from wild type complexes. These results reveal a new biogenic mechanism for channelopathies and provide insight into the structural requirements for post-translational N-glycosylation of type I transmembrane proteins.

EXPERIMENTAL PROCEDURES

Plasmids and cDNAs—Human Q1 and E1 were subcloned into pcDNA3.1(−) (Invitrogen). E1 constructs (wild type and mutants) possessed a C-terminal HA (YPYDVPDYA) epitope tag (14). To improve detection of pulse-labeling, five methionine residues were inserted immediately before the HA epitope tag. The different N-glycosylation mutants were generated by site-directed mutagenesis using the QuikChange system (Stratagene). The different mutants) possessed a C-terminal HA (YPYDVPDYA) epitope

Cell Culture and Transfections—Chinese Hamster Ovary (CHO-K1) cells were cultured in F-12K nutrient mixture (Invitrogen). HeLa cells and HEK cells were cultured in Dulbecco’s modified Eagle’s medium (Sigma). All of the media were supplemented with 10% fetal bovine serum (Hyclone) and 10² units/ml penicillin/streptomycin (Invitrogen). Cells were plated at 60–75% confluency in 35 mm dishes for all experiments except pulse-chase (100 mm). After 24 h, cells were transiently transfected at room temperature with Lipofectamine (Invitrogen) 8 µl per ml of Opti-MEMI (Invitrogen) for CHO or with 10 µl of Lipofectamine 2000 (Invitrogen) per ml of Opti-MEMI for HEK and HeLa, and returned to fresh media after 6 h (2 h for electrophysiology). DNA ratios (in µg): E1/empty pcDNA 3.1 plasmid: 1.5/0.75; Q1/E1 (Western blots): 0.75/1.5; Q1/E1 (pulse-chase): 0.75/1.5; Q1/E1 + pEGFP-C3 (electrophysiology): 0.5/2 + 0.25. Cells were used 48 h post-transfection for Western blots; 24 h for pulse-chase and electrophysiological experiments.

Cell Lysis and Western Blot Analysis—Cells were washed in ice-cold PBS (3 × 2 ml) and lysed at 4 °C in RIPKA buffer (in mM): 10 Tris·HCl, pH 7.4, 140 NaCl, 10 KCl, 1 EDTA, and 1% Triton-X, 0.1% SDS, 1% sodium deoxycholate, and supplemented with protease-inhibitors: 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 µg/ml each of leupeptin, pepstatin, and aprotinin (LPA). Lysates were diluted with SDS-PAGE loading buffer containing 100 mM DTT, loaded on a 15% SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and blocked in Western blocking buffer (5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween-20 (TBS-T)) for 30 min at RT. Membranes were incubated overnight at 4 °C with rat anti-HA (Roche) (1:750) in Western blocking buffer and washed in TBS-T next day and incubated with goat anti-rat horseradish peroxidase (HRP)-conjugated antibody (Jackson ImmunoResearch Labs, Inc.) (1:2000) in Western blocking buffer for 45 min at room temperature. Membranes were washed with TBS-T and incubated with SuperSignal West Dura Extended Duration Substrate (Pierce) for 5 min. HRP-bound proteins were detected by chemiluminescence using Fujifilm LAS-3000 CCD camera and quantified using the Image Gauge V2.1 software (Fujifilm).

Enzymatic Deglycosylation Assays—Nonidet P-40, BME, and reaction buffer (G5 for Endo H; G7 for PNGase F) were added to the cell lysates (20 µl), which were diluted with water such that the final concentrations were 1% for Nonidet P-40 and BME. Endo H (2 µl) or PNGase F (1 µl) (New England BioLabs, Inc.) were added to the samples and incubated at 37 °C for 30 min. The samples were brought to a final concentration of 100 mM DTT and 1.3% SDS and were separated on an SDS-PAGE (15% gel) and analyzed by Western blot as described above.

Cell Surface Biotinylation—Transfected cells were rinsed with ice-cold PBS²⁻ buffer (PBS containing 1 mM MgCl₂, 0.1 mM CaCl₂), incubated with 1 mg/ml sulfo-NHS-SS-biotin (Pierce) in PBS²⁻ buffer for 2 × 15 min at 4 °C. The biotin reagent was quenched by washing with (3 × 2 ml) with quench solution (PBS²⁻ containing 100 mM glycine) and then incubated with quench solution for 2 × 15 min at 4 °C. Cells were lysed in RIPKA buffer and cell debris was removed by centrifugation. For each sample, 75 µg of total protein was quantitated by BCA analysis and incubated with 25 µl of Immobilpur® Immobilized streptavidin beads (Pierce) overnight at 4 °C. One-half of the input (37.5 µg of total protein) was saved as a control for each sample. Beads were washed 3× with 0.1% SDS buffer (500 µl) and biotinylated proteins were eluted first with 30 µl 2× SDS-PAGE and 200 µM DTT mix for 15 min at 55 °C, then with 30 µl of 200 µM DTT for 5 min at 55 °C and the two elutions were combined to achieve 60 µl final volume. Half of the input and bead-eluted proteins were separated by SDS-PAGE, analyzed by Western blot as described above, and quantified in the linear range. To compare the cell surface expression of WT and mutant E1 subunits, the band intensities in the beads lanes were divided by twice the band intensity in the corresponding calnexin ½ input lanes and WT was normalized to 1. Because some cell lysis and intracellular labeling of proteins occurs during biotinylation, the amount of calnexin (CNX) labeling was first subtracted from each biotinylated E1 sample.

Pulse-chase Experiments—Cells were washed in PBS (2 × 4 ml) and starved in Dulbecco’s modified Eagle’s medium (DMEM) high glucose (Invitrogen) supplemented with 10% fetal bovine serum (Hyclone) and 10² units/ml penicillin/streptomycin (Invitrogen - Invitrogen) and 2 mM glutamine (Invitrogen) for 1 h at 37 °C. Then, 100 µCi/ml [¹³C]methionine and [¹⁵N]cysteine (MP Biomedicals, Inc.) was added to the starved media and the cells were pulsed 10 min for decay and co-im-
munoprecipitation experiments and 2 min for post-translational glycosylation studies. After the radioactive pulse, cells were washed in PBS (2 × 4 ml) and chased in standard medium for various times. The cells were washed in PBS (2 × 4 ml) and lysed for 30 min at 4 °C in 750 μl of low salt lysis buffer consisting of (in mM): 50 Tris-HCl, pH 7.4, 150 NaCl, 20 NaF, 10 Na3VO4, and 1% Nonidet P-40, 1% CHAPS supplemented with protease-inhibitors (PMSF and LPA).

Radioimmunoprecipitation Assays—Lysates were pelleted at 16,100 × g for 10 min at room temperature and supernatant was precleared with a slurry of Immobilized Protein G Beads (Pierce) in lysis buffer rotating for 2 h at 4 °C. The beads were pelleted and the precleared supernatants were rotated overnight at 4 °C with either a 100 μl of Protein G Beads/(2 μl) rat anti-HA (Roche) antibody mix, a 100 μl of Protein G Beads/(4 μl) rat anti-Q1 (Sigma) antibody mix, or 25 μl of Protein G Beads/(1 μl) goat anti-human cathepsin C (R&D Systems) antibody mix. The beads were pelleted at 16,100 × g for 10 min at room temperature and washed three times in low salt lysis buffer and then with 1× in high salt buffer consisting of (in mM): 50 Tris-HCl, pH 7.4, 500 NaCl, 20 NaF, 10 Na3VO4, and 1% Nonidet P-40, 1% CHAPS, followed by a final wash with low salt lysis buffer. For enzymatic deglycosylation of procathepsin C, the Protein-G bound immunoprecipitates were resuspended in 400 μl of low salt lysis buffer with Endo H (20 μl) (New England BioLabs, Inc.) and incubated at 37 °C for 1 h followed by a final wash with low salt lysis buffer. The washed and pelleted beads were eluted in 50 μl of 2× SDS and 100 μl DTT mix at 55 °C for 15 min. Supernatants were separated by SDS-PAGE (15%) and visualized by autoradiography. Signals were captured on a FLA-3000 phosphorimager and quantified using the Image Gauge V2.1 software (Fujifilm).

Perforated Patch Whole-cell Recordings—I KCNQ1 and I Ks were recorded in the whole-cell perforated patch configuration. Briefly, on the day of the experiment the cells were seeded on the surface of cover glass and placed into a custom recording bath filled with modified Tyrode’s solution contained (in mM) 145 NaCl, 5.4 KCl, 10 HEPES, 5 CaCl2 (pH 7.5 with NaOH). Transfected (eGFP-expressing) cells were selected using an Axiovert 40 CFL inverted light microscope (Zeiss). For the perforated patch configuration, a glass electrode (pipette resistance: 2.5–40 MΩ) filled with internal electrode solution contained (in mM) 126 KCl, 0.5 CaCl2, 5 EGTA, 4 K2-ATP, 0.4 GTP, 25 HEPES (pH 7.5 with CsOH), and 60 μg/ml Amphoterinicin B (Sigma; prepared in DMSO) was attached to the cell. Once a GΩ seal was achieved and access resistance achieved (<15 MΩ), Tyrode’s solution was replaced with the extracellular bath solution that contained (in mM) 160 NaCl, 2.5 KCl, 2 CaCl2, 1 MgCl2, 8 glucose, and 10 HEPES (pH 7.5 with NaOH).

Initially, the electrical access to the inside of the cell was monitored using 3-s depolarizing test pulse from a holding potential of −80 mV to +20 mV every 15 s. Cells with pronounced rundown were discarded, and only those that expressed stable currents were used. The I KCNQ1 and I Ks currents were elicited using a family voltage protocol described in the legend to Fig. 4. All measurements were performed at room temperature (24 ± 2 °C).

RESULTS

KCNE1 Subunits Are Post-translationally N-Glycosylated—To follow the rapid kinetics of N-linked glycosylation, we used very brief (2 min) pulses of radioactive methionine and cysteine to metabolically-label a C-terminally, HA-tagged E1 construct in CHO cells. CHO cells have two significant advantages over other traditional cell lines: (1) The absence of endogenously expressed voltage-gated K+ currents, which permits the study of the biogenesis of E1 regulatory subunits in the absence of presence of cognate K+ channel subunits (2). The N-glycosylation and anterograde trafficking of E1 subunits observed in native cells (cardiomyocytes and inner ear cells) is preserved in this cell line (14, 15, 22). Therefore, CHO cells expressing E1 subunits were metabolically labeled with 35S, chased with cold media, and the E1 proteins were isolated by immunoprecipitation at various times (Fig. 1A). At the early time points (0 and 3 min), an equal distribution of the un-, mono-, and di-glycosylated forms of E1 was observed. However, after the synthesis of the pulse-labeled E1 protein was complete (~3 min chase) (23), the signal intensity of the diglycosylated form continued to increase over time (Fig. 1B, open circles) such that it became the predominant form of E1. This increase in the diglycosylated E1 over time was also accompanied with a decrease in the un- and mono-glycosylated forms, which was readily observed in the gel image (Fig. 1A). These results indicated that N-linked glycans were being attached to E1 long after protein translation was complete.

Surprised by this apparent post-translational attachment of N-glycans in the ER, we wondered whether the increase in the diglycosylated form of E1 was due to preferential degradation of hypoglycosylated E1. Repeating the metabolic labeling in the presence of proteasome inhibitors (MG-132 or lactacystin) had no significant effect on post-translational N-glycosylation of E1.
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FIGURE 2. Post-translational N-glycosylation of KCNE1 occurs primarily at the N26 sequon. A, schematic representation of E1. The positions of the N-linked glycosylation sites and the transmembrane domain (TM) are denoted. Representative fluorographs of the E1 N-glycosylation mutants expressed alone (B) or with Q1 subunits (C). Cells were pulsed for 2 min to observe post-translational N-glycosylation, and chased for the indicated times. The immunoprecipitated E1 proteins were separated by electrophoresis and detected by autoradiography. N26Q (squares), NSQ (inverted triangles), and T7I (triangles). D, graphs of densitometric analysis of the E1 N-glycosylation mutants expressed alone (open symbols) or with Q1 subunits (filled symbols). The percentage of the maximally glycosylated forms with respect to total protein is plotted for each time point. Data plotted (n = 4–5) are mean ± S.E. for each chase point. After 3 min, the maximally glycosylated forms of N5Q and T7I increased whereas N26Q remained relatively constant. Dotted line segregates the N5 and N26 sequon data.

(Fig. 1, diamonds: MG-132). The abundance of unglycosylated WT and mutant E1 subunits hinted that overexpression was compromising protein translocation or N-glycosylation. To determine whether exogenous expression of E1 was depleting dolichol-linked oligosaccharides or saturating the co-translational glycosylation machinery, we overexpressed E1 subunits and monitored the N-glycosylation of procathepsin C, an endogenous glycoprotein, which normally acquires four N-glycans co-translationally (4). If E1 overexpression was artificially inducing post-translational N-glycosylation, we would expect to observe hypoglycosylated intermediates at early time points and/or post-translational N-glycosylation of cathepsin C. However, neither overexpression of E1 nor transient transfection of an empty vector reduced co-translational N-glycosylation of cathepsin C, as only the fully glycosylated protein was observed at all time points (supplemental Fig. S1A). Thus, E1 overexpression does not perturb N-glycosylation, but it may be taxing the protein translocation machinery, leading to an increased number of unglycosylated subunits. In total, these results demonstrated that the increase in the diglycosylated form of E1 was due to native post-translational N-glycosylation.

Because E1 peptides are regulatory subunits that co-assemble with K⁺ channel subunits in the ER in various cells and tissues (14, 24), we next determined whether the presence of K⁺-conducting subunits affected post-translational N-glycosylation of E1 subunits. Cells co-expressing Q1 channel and E1 regulatory subunits were pulsed for 2 min and chased at different times to observe post-translational N-glycosylation of E1 (Fig. 1, circles). Both the rate of post-translational N-glycosylation and the percentage of fully glycosylated E1 were similar in the presence or absence of Q1 subunits, indicating that either post-translational N-glycosylation of E1 is faster than co-assembly with Q1 subunits or Q1-E1 co-assembly does not inhibit post-translational N-glycosylation. Regardless of which mechanism was operational, these results show that post-translational N-glycosylation of E1 occurs in cells where K⁺ channel subunits are present. In addition, post-translational N-glycosylation of E1 was observed in several standard mammalian cell lines (supplemental Fig. S1B), suggesting that this post-translational modification in the ER is general and not cell-type specific.

Identification of a Post-translational N-Glycosylation Site in KCNE1 Subunits—To identify the sequon(s) on E1 that was post-translationally N-glycosylated, we made a panel of mutations that would result in glycan addition to either the N5 or N26 sequon and followed N-glycan addition with metabolic labeling (Fig. 2A). For N-glycan attachment to N5 sequon (N26Q mutant), the fully glycosylated form was the most abundant at the earliest time point, consistent with a typical co-translational N-glycosylation reaction (Fig. 2B). Quantification revealed minimal post-translational N-glycosylation of the N5 sequon (Fig. 2D). Co-expression with K⁺ channel subunits (Q1) had only a modest affect on co- and post-N-glycosylation of the N5 sequon (Fig. 2, C and D). In contrast, the N26 sequon (N5Q and T7I mutants) was poorly glycosylated during the protein translation time window (0–3 min), resulting in predom-
that primarily depend on post-translational $N$-glycosylation (N5Q and T7I) are severely hypoglycosylated whereas E1 subunits that utilize co-translational $N$-glycosylation (N26Q) are efficiently glycosylated.

Because WT acquires both of its $N$-glycans efficiently during and after protein translation, we initially thought the steady state glycoprotein differences that we observed were due to preferential degradation of the monoglycosylated E1 subunits. To our surprise, all three $N$-glycosylation mutants and WT had very similar degradation rates in standard radioactive pulse-chase experiments (supplemental Fig. S2). Taken together, the steady state and pulse labeling experiments indicate that the efficiency of post-translational $N$-glycosylation at the N26 sequon was enhanced by the presence of an $N$-linked glycan over 20 residues away. This long range disruption of $N$-glycan attachment was specific for post-translational $N$-glycosylation because the efficiency of glycosylation of the N5 sequon was not reduced by elimination of the N26 sequon.

Although the kinetics of post-translational $N$-glycosylation and degradation of N5Q, T7I, and WT E1 subunits was similar, there was a statistically significant difference between the steady state levels of monoglycosylated N5Q and T7I. Additional point mutations were introduced into the N5 sequon to determine whether the hydrophobicity or structure of the altered residue impacts $N$-glycosylation efficiency (Fig. 3). Substitution of asparagine for isoleucine (N5I) reduced $N$-glycosylation at the N26Q sequon similarly to T7I. Mutation to a less hydrophobic residue than isoleucine (T7A or T7Q) afforded slightly more glycosylated E1 protein than N5I or T7I; however, these differences were not statistically significant (supplemental Table S1). Distorting the N5 sequon with proline (T6P) had an intermediate reduction upon $N$-glycosylation, falling significantly between the isoleucine mutants and N5Q. This trend (T7I~N5I < T6P < N5Q < N26Q~WT) shows that disruption of the N5 sequon inhibits post-translational glycosylation of the N26 sequon, and that hydrophobic substitutions that disrupt the N5 sequon have a secondary effect upon the steady state levels of monoglycosylated E1.

**Functional and Cellular Consequences of KCNE1 Hypoglycosylation**—We subsequently determined whether the compounded hypoglycosylation of the E1 mutants altered their ability to traffic to the cell surface with Q1 subunits. Given the contrasting differences in the current profiles between unpartnered Q1 channels and Q1/E1 complexes (Fig. 4A), we initially used electrophysiology to measure the function of WT and mutant Q1/E1 complexes. Unpartnered Q1 channels give rise to small currents that rapidly activate (Fig. 4A) as well as inactive upon depolarization. In contrast, Q1/E1 complexes have larger currents that slowly activate over many seconds and show no measurable signs of inactivation. Thus, co-assembly with E1 bestows the Q1 channel with the appropriate properties to maintain the rhythmicity of the heartbeat and provide salt and water transport in the inner ear. Co-expression of Q1 with the $N$-glycosylation mutants afforded currents that were an amalgam of unpartnered Q1 channels (Fig. 4B) and Q1/E1 complexes. For T7I and N5Q, which are hypoglycosylated, there was consistently a larger amount of unpartnered Q1 currents (Fig. 4B, arrowheads) compared with N26Q, which
efficiently acquires its N-glycans co-translationally. Likewise, the relative mean peak currents for the hypoglycosylated mutants (N5Q, T7I) were significantly reduced compared with WT and N26Q complexes (Fig. 4C), which is consistent with E1 subunits needing at least one N-glycan to efficiently assemble and traffic with Q1 channels to the plasma membrane.

To directly measure the plasma membrane expression of the mutant E1 peptides co-expressed with Q1 channel subunits, we used cell surface biotinylation. This biochemical approach also allowed for the identification of the E1 glycoforms present on the plasma membrane. Cells expressing WT and mutant Q1/E1 complexes were labeled with a membrane impermeant, amine-reactive biotin reagent at 4 °C to prevent membrane recycling and minimize labeling of intracellular proteins. The biotinylated proteins were isolated with streptavidin beads (Beads) and normalized to their respective endogenous CNX signal (1/2 Input) to compare the cell surface expression of WT to the E1 mutants (Fig. 5A). To verify that the cells remained intact during biotinylation, we also monitored for labeling of the ER-resident protein, CNX (Beads), and subtracted out this background intracellular labeling to calculate the normalized cell surface expression in Fig. 5B. E1 subunits with a single glycan attached to the N5 sequon (N26Q) had cell surface expression that was similar to WT and required co-expression with Q1 (supplemental Fig. S3, A and B). In contrast, the E1 mutants lacking the N5 sequon (N5Q, T7I) were scarcely present at the plasma membrane even when they were co-expressed with Q1.

We next used enzymatic deglycosylation to determine which glycoforms of the mutants were present at the plasma membrane. As we have previously shown with WT, co-expression with Q1 subunits results in a strong, but diffuse band centered between 37 and 50 kDa (Fig. 5A), which is due in part to N-glycan maturation in the Golgi (14) as well as an additional modification that occurs in the Golgi (32). Using deglycosylation enzymes (supplemental Fig. S3C), we identified the unglycosylated, immaturely and maturely N-glycosylated forms of WT and mutant E1 subunits, which are denoted in Fig. 5A. Although the unglycosylated and immature forms of E1 were

![FIGURE 4. Current properties of KCNQ1 channels co-expressed with KCNE1 N-glycosylation mutants. A, representative families of Iq1 and Is currents elicited by the pulse protocol shown. The interpulse interval was 30 s. B, representative families of currents recorded from cells expressing Q1 and the E1 N-glycosylation mutants (+N5Q, +T7I, or +N26Q). Arrows mark the rapid activation that is indicative of unpartnered KCNQ1 channels (IQ1). C, relative mean peak currents (I/I_max) were normalized to the maximal WT I_s (+E1) and plotted as a function of the pulse voltage (V). Data (n = 3–5) are mean ± S.E.](image)

![FIGURE 5. Compounded hypoglycosylation of KCNE1 reduces cell surface expression via an anterograde trafficking defect. Cell surface labeling of E1 subunits co-expressed with Q1. A, representative immunoblots of cell surface labeling. Lanes denoted as (1/2 Input) are half the sample lysate that was set aside to quantitate the total amount of biotinylated proteins. Beads, lanes represent the cell surface biotinylated proteins that were isolated with streptavidin and separated by SDS-PAGE. The CNX immunoblots were used both to determine the amount of background lysis and to compare the cell surface expression of the mutants to WT. The mature (m), immature (im), and unglycosylated (un) forms were identified by enzymatic deglycosylation (supplemental Fig. S3C). B, quantification of the E1 proteins on the cell surface, which was calculated as described under “Experimental Procedures.” Error bars are S.E. from n = 3–4 immunoblots. C, all glycoforms of E1 equally co-assemble with Q1. Left panel: immunoprecipitation of radiolabeled E1 using antibodies specific for E1, Q1, or non-immune control antibody (−). The ratio of the glycoforms precipitated was the same whether Q1 or E1 antibody was used. Right panel: radiolabeled N-glycosylation mutants were co-immunoprecipitated with α-Q1 antibodies. Co-immunoprecipitations required the presence of Q1 channels, (−)/WT.](image)
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present in whole cell extracts (Fig. 5A, ½ Inputs), only the maturely \( N \)-glycosylated protein was detected at the cell surface over background calnexin labeling. In total, these results demonstrate that mutations that directly prevent co- and indirectly reduce post-translational \( N \)-glycosylation result in a large population of unglycosylated E1 subunits that do not reach the cell surface even when co-expressed with Q1 subunits.

Because E1 cell surface expression is dependent on co-assembly with Q1 (14), the absence of unglycosylated E1 subunits at the cell surface could arise from either an inability to co-assemble with Q1 subunits or an anterograde trafficking defect of the unglycosylated Q1/E1 complex. Therefore, we used co-immunoprecipitation to identify which E1 glycoforms co-assemble with Q1 subunits (Fig. 5C). Cells co-expressing Q1 and E1 subunits were metabolically labeled with \( ^{35} \text{S} \) for 10 min, chased with cold media for 6 min, and the proteins were isolated by immunoprecipitation. Immunopioloation of Q1 co-precipitated all three forms of E1: un-, mono-, and di-glycosylated. The co-immunoprecipitation was specific, as it required both the antibody and the presence of the Q1 subunit. Moreover, the ratio of the E1 glycoforms precipitated was similar whether E1 or Q1 protein was immunopreisolated, demonstrating that hypoglycosylation does not prevent co-assembly with Q1. Finally, both the un- and mono-glycosylated forms of the E1 mutants lacking the N5 sequon (N5Q, T7I) also co-immunoprecipitated with Q1, which indicates that the reduced cell surface expression of these hypoglycosylated E1 mutants is due to a post-assembly defect in anterograde trafficking.

The Serine/Threonine Residue in the KCNE1 N26 Sequon Specifies Co- and Post-translational N-Glycosylation—We next asked whether the trafficking defects of the N5Q and T7I mutants might be explained by a specific requirement for a glycan on the N5 sequon, or instead indicate that KCNE1 must have at least one glycan, irrespective of location (e.g. N5 or N26). Several studies have shown that threonine-containing sequons (NXT) are more efficiently glycosylated than serine-containing sequons (NXS)(7,25). Because the N26 sequon in all mammalian E1 subunits is NXS, we wondered whether the hydroxylated residue in this sequon was dictating whether the \( N \)-glycan was added to E1 during or after protein synthesis. If our posite were correct, then converting the N26 sequon into a co-translational site would not only rescue the observed hypoglycosylation defect, but restore cell surface expression of the Q1/E1 complex. Fig. 6A shows that replacement of the serine with a threonine residue (N5Q + S28T) results in efficient co-translational \( N \)-glycosylation of the N26 sequon. Moreover, switching the timing of \( N \)-glycan attachment to the N26 sequon was sufficient to restore cell surface expression and wild type Q1/E1 function (Fig. 6, B and C). Remarkably, there was little evidence of unpartnered Q1 channels functioning in cells expressing N5Q + S28T. Thus, the timing and efficiency of glycan attachment to the N26 sequon is specified by the hydroxy-amino acid residue within the consensus sequence.

DISCUSSION

Motivated by the genetic evidence that the sequon adjacent to the KCNE N terminus plays an important role in cardiac biology (19–21), we individually examined the kinetics and extent of \( N \)-linked glycosylation of the two E1 sequons. The results from our investigation provide four new mechanistic insights into the biogenesis and \( N \)-glycosylation of these type I transmembrane peptides (1). The two \( N \)-linked consensus sites on E1 are handled differently in the ER: glycans are added to the N5 sequon during translation whereas the glycan attachment to the N26 sequon occurs primarily after protein translation has been completed (2). The hydroxyamino acid in the N26 sequon dictates whether \( N \)-linked glycans are co- or post-translationally attached to E1 subunits (3). Post-translational \( N \)-glycosylation of E1 subunits lacking a co-translational site is less efficient, compounding hypoglycosylation and resulting in accumulation of unglycosylated peptides, which are competent for assembly with Q1 (4). Because unglycosylated Q1/E1 complexes do not reach the cell surface, post-translational \( N \)-glycosylation’s dependence on \( N \)-glycan occupancy explains how improper E1 subunit biogenesis can cause Long QT and Jervell-Lange Nielsen Syndromes.

Post-Translational Glycosylation of Properly Folded KCNE Subunits—Post-translational \( N \)-glycosylation has been historically associated with cells that have compromised glycosylation machinery (26) or that express truncated glycoproteins (27). All E1 constructs used here are full-length. Moreover, post-translational \( N \)-glycosylation is required to afford the predominant glycoform of E1 (the diglycosylated form), which folds and assembles with Q1 subunits to afford \( K^+ \) channel complexes that generate the hallmark cardiac \( I_{\text{Ks}} \) current (Fig. 5). Similarly, post-translational \( N \)-glycosylation of E1 is neither a cell-specific artifact nor a result of depleting the key components of the \( N \)-glycosylation pathway. Post-translational \( N \)-glycosylation of E1 was observed in several standard mammalian cell lines (supplemental Fig. S1B), consistent with the conservation of the STT3B subunit, which has been recently shown to attach \( N \)-glycans to a secreted proteins after translation (4). We also tried to detect post-translational \( N \)-glycosylation in native cells (cardiomyocytes); however, the low endogenous expression of E1 combined with the short radioactive pulse (2 min) did not produce enough labeled protein from an entire rat heart (data not shown). Although exogenous expression of E1 was needed to generate a detectable protein signal, it did not saturate the cotranslational machinery or deplete the dolichol-linked oligosaccharides because co-translational \( N \)-glycosylation of endogenous cathepsin C was unaffected (supplemental Fig. S1A). Taken together, our results suggest that post-translational \( N \)-glycosylation of E1 is a native mechanism that affords fully glycosylated regulatory subunits essential for proper electrical excitability.
timing of the lateral entry of a transmembrane segment into the ER membrane based on photo-cross-linking studies. The first model requires the completion of translation before exiting the translocation pore (28) whereas the second model suggests that certain transmembrane segments can enter the lipid environment before translation is terminated (29, 30). Thus, the observation of N-glycosylation events 10–15 min after translation of a 150 residue type I transmembrane peptide (Fig. 1) is consistent with post-translational N-glycosylation. Moreover, post-translational N-glycosylation of E1 subunits is not inhibited by co-assembly with Q1 channels (Figs. 1 and 5C). Because the translocon cannot simultaneously house a six transmembrane segment Q1 subunit and an E1 subunit, these data support the conclusion that post-translational N-glycosylation of type I transmembrane peptides occurs after E1 has been integrated into the membrane.

Proteins with Co- and Post-translational Sites—To date, only one full-length protein has been identified that has distinct co- and post-translational N-linked glycosylation sites, yet the spacing between the two sites in this secreted, water soluble protein is over 170 residues (5). For type I transmembrane peptides with closely spaced N-linked consensus sites, the cellular mechanisms that define co- and post-translational N-glycosylation sequons are likely to be different. In particular, the small size of KCNE subunits (~100–170 aa) and their short C termini (~20–100 amino acids) severely limits the time (1–5 s) the OST complex has to attach an N-glycan while the protein is in the translocon. For the N26 sequon of E1, we have shown that the hydroxyamino acid (serine versus threonine) within the consensus sequence (Fig. 6) is a determining factor for co- versus post-translational N-glycosylation. Because the OST active site has a higher affinity (25) for NXT sequons and more effi-

![Figure 6](https://example.com/image6.png)

**FIGURE 6.** Co- and post-translational N-glycosylation of the N26 sequon depends on the hydroxyamino acid. A, left: representative fluorograph of the N5Q + S28T (open hexagons) N-glycosylation mutant expressed alone. The 2 min pulse-chase labeling was performed as denoted in Fig. 2. Right: percentage of the maximally glycosylated forms with respect to total protein at each time point is compared with a predominately co-translational mutant (N26Q line) and post-translational mutant (NSQ line). Data plotted (n = 4) are mean ± S.E. for each chase point. B, cell surface labeling of N5Q + S28T subunits co-expressed with Q1. Left: representative immunoblots of cell surface labeling. Lanes denoted as ’(1/2 input)’ are half the sample lysate that was set aside for quantization. Beads, lanes represent the isolated biotinylated proteins. The CNX immunoblots were used to determine the amount of background lysis. The mature (m), immature (im), and unglycosylated (un) forms were identified as described in supplemental Fig. S3C. Right: quantification of E1 proteins on the cell surface which was calculated as described under “Experimental Procedures.” Error bars are S.E. from n = 3 immunoblots. C, left: representative family of currents from cells co-expressing N5Q + S28T with Q1. Voltage pulse protocol is shown in Fig. 4A. Arrow marks the absence of rapid activation that is indicative of unpartnered Q1 channels (Fig. 4A). Right: comparison of the relative mean peak currents (I/I_{max}) of the Q1/N5Q mutants with a serine (triangles) or a threonine (hexagons) residue in the N26 sequon (NKS versus NXT).
Post-translational N-Glycosylation

**FIGURE 7. Model of KCNE1 biogenesis, N-glycosylation, and co-assembly with KCNQ1 channels.** N-linked glycans are added to the N5 sequon of E1 subunits during translation (co-translational) and laterally exit the protein translocation channel to integrate into the membrane. Post-translational N-glycosylation of WT subunits at N26 occurs either before (a) or after (b) co-assembly with Q1 channel subunits. Once fully glycosylated, the Q1/E1 complex exits the ER and traffics to the plasma membrane. For the Long QT mutation, T7I, the subunit exits the translocon unglycosylated, and is a poor substrate (compared with WT) for post-translational N-glycosylation. Unglycosylated T7I subunits readily co-assemble with Q1 subunits, resulting in complexes that have an anterograde trafficking defect, which significantly reduces cell surface expression.

Ciently N-glycosylates these sequons versus their serine-containing versions (7), our results with E1 suggest that these two competing rates: N-glycan attachment by the OST and translocation exit determine the degree of co- and post-translational N-glycosylation at sequons in type I transmembrane peptides.

**Efficiency of Post-translational N-Glycosylation of Type I Transmembrane Peptides**—Unlike co-translational N-glycosylation, post-translational N-glycosylation is affected by long distance mutations. In particular, post-translational N-glycosylation efficiency of type I transmembrane peptides was dependent on the presence of an N-glycan over 20 residues away, as elimination of the N5 sequon reduced post-translational glycosylation at the N26 sequon compared with WT (Figs. 1 and 2). Ablation of the N5 sequon with more hydrophobic residues significantly decreased the steady state levels of monoglycosylated E1 (Fig. 3), indicating that the hydrophilicity of the N-glycan may be important for post-translational N-glycosylation at N26. However, a further kinetic investigation is needed to fully understand the role hydrophobicity plays in post-translational N-glycosylation. A similar dependence on N-glycan occupancy was also observed at the N5 sequon, as post-translational N-glycosylation of the N26Q mutant (Fig. 2) was barely detectable. Steady-state data support the notion that post-translational N-glycosylation is efficient at both sequons because the predominant forms of WT are 0 and 2 glycans. Selective degradation of the monoglycosylated WT species is unlikely because E1 subunits with an N-glycan at either sequon (N26Q and N5Q + S28T) are perfectly stable proteins. However, directly testing this supposition is hindered by the inability to individually monitor the rate of N-glycan attachment to the two different sequons in WT. Nonetheless, these data suggest that glycan occupancy directly affects post-translational N-glycosylation efficiency of type I transmembrane peptides.

**Cellular Advantages of Multiply Glycosylated Type I Transmembrane Peptides**—The spacing of the N-linked glycosylation consensus sites in E1 is absolutely conserved among vertebrates. Furthermore, 4 out of 5 members of the human KCNE family have at least two N-linked sites and the spacing between the sequons is relatively consistent (16–23 aa). A potential benefit of having two or three sequons is to increase the probability that KCNE subunits are at least monoglycosylated in the ER since having one N-glycan is necessary and sufficient for proper Q1/E1 complex anterograde trafficking (Fig. 6). Thus, unglycosylated KCNE subunits having two or three sequons that elude the co-translational N-glycosylation machinery could still acquire at least one N-linked glycan post-translational. Another potential advantage of a diglycosylated KCNE subunit is that it would improve the interactions (via multivalency) between the lectin family of chaperones in the ER (31), which have been hypothesized to interact with E1 (14). The initial delay in the decay of WT subunits that we observed is consistent with the notion that the chaperones that recognize N-linked glycans, calnexin and calreticulin, may be more avidly interacting with newly synthesized E1 subunits harboring two glycans (supplemental Fig. S28).

**Biogenic Model for Long QT Syndrome**—From our results, we propose a model for E1 subunit biogenesis, co-assembly with Q1 channel subunits, anterograde trafficking, and its implications for Long QT and Jervell-Lange Nielsen Syndromes (Fig. 7). In this model, WT subunits co-translationally acquire an N5 glycan before exiting the translocon. Once free from the proteinaceous environment of the translocon, post-translational N-glycosylation of E1 subunits either occurs before or after co-assembly with Q1 channel subunits (pathways a and b, respectively). Both pathways appear to be operational since all glycoforms of WT E1 (Fig. 5C) assemble with Q1 and co-assembly with Q1 does not inhibit post-translational N-glycosylation (Fig. 1). Once fully glycosylated, Q1/E1 complexes exit the ER and traffic to the plasma membrane. In contrast to WT, T7I subunits exit the translocon unglycosylated and thus are poor substrates for post-translational N-glycosylation, resulting in a large population of unglycosylated T7I subunits that assemble with Q1 subunits (Fig. 7, T7I). Because the anterograde trafficking of unglycosylated Q1/E1 complexes is compromised, this compounded hypoglycosylation severely decreases the number of Q1/E1 complexes functioning at the cell surface. In the heart, this would lead to a reduction in the cardiac IkS current and a prolongation of the QT interval, increasing the probability of an
arrhythmic event. A similar reduction of Q1/E1 complexes in
the developing ear would prevent the proper potassium flux
into and thus formation of the endolymphatic space. Given that
the spacing and number of N-glycosylation consensus sites in
KCNE subunits is conserved, we expect mutations that disrupt
the balance of co- and post-translational N-glycosylation will
lead to channelopathies for the KCNE family of K⁺ regulatory
subunits and be the underlying cause of disease for other mul-
tiply N-glycosylated type I transmembrane peptides.

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