Identification of Molecular Determinants that Shift Co- and Post-Translational N-Glycosylation Kinetics in Type I Transmembrane Peptides: A Dissertation

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IDENTIFICATION OF MOLECULAR DETERMINANTS THAT SHIFT CO- AND POST-TRANSLATIONAL N-GLYCOSYLATION KINETICS IN TYPE I TRANSMEMBRANE PEPTIDES

A Dissertation Presented

By

Heidi Laura Hafemann Malaby

Submitted to the faculty of the University of Massachusetts Graduate School of Biomedical Sciences, Worcester in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

April 7, 2014

BIOCHEMISTRY AND MOLECULAR PHARMACOLOGY
IDENTIFICATION OF MOLECULAR DETERMINANTS THAT SHIFT CO- AND POST-TRANSLATIONAL N-GLYCOSYLATION KINETICS IN TYPE I TRANSMEMBRANE PEPTIDES

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Biochemistry and Molecular Pharmacology
April 7, 2014
For all the teachers who fanned the flames
of inquisition and creativity
ACKNOWLEDGEMENTS

A large number of people have helped shaped me into the scientist and person that I am today that I wish to offer my sincerest gratitude. First, my PI Bill Kobertz, who has been a fantastic mentor and offered immeasurable guidance on science, writing, and life. I would also like to thank my committee members, Reid Gilmore, Tony Carruthers, Dan Bolon, and Chris Sassetti for their enlightening feedback and suggestions, as well as Karen Colley for serving as my external examiner.

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members Maria and André, who kept me company in the wee hours of the morning on those long experiment days. Jill Zitzewitz and Osman Bilsel deserve special thanks for their mentorship and friendship since I was a lowly roton. Your dedication to communicating science in your community is inspiring.

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Asparagine (N)-linked glycosylation occurs on 90% of membrane and secretory proteins and drives folding and trafficking along the secretory pathway. The N-glycan can be attached to an N-X-T/S-Y (X,Y ≠ P) consensus site by one of two oligosaccharyltransferase (OST) STT3 enzymatic isoforms either during protein translation (co-translational) or after protein translation has completed (post-translational). While co-translational N-glycosylation is both rapid and efficient, post-translational N-glycosylation occurs on a much slower time scale and, due to competition with protein degradation and forward trafficking, could be detrimental to the success of a peptide heavily reliant on post-translational N-glycosylation. In evidence, mutations in K⁺ channel subunits that shift N-glycosylation kinetics have been directly linked to cardiac arrhythmias. My thesis work focuses on identifying primary sequence factors that affect the rate of N-glycosylation.

To identify the molecular determinants that dictate whether a consensus site acquires its initial N-glycan during or after protein synthesis, I used short (~100-170 aa) type I transmembrane peptides from the KCNE family (E1-E5) of K⁺ channel regulatory subunits. The lifetime of these small membrane proteins in the ER translocon is short, which places a significant time constraint on the co-translational N-glycosylation machinery and increases the resolution between co- and post-translational events. Using rapid metabolic pulse-chase experiments
described in Chapter II, I identified several molecular determinants among native consensus sites in the KCNE family that favor co-translational N-glycosylation: threonine containing-consensus sites (NXT), multiple N-terminal consensus sites, and long C-termini. The kinetics could also be shifted towards post-translational N-glycosylation by converting to a serine containing-consensus site (NXS), reducing the number of consensus sites in the peptide, and shortening the C-termini.

In Chapter III, I utilized an E2 scaffold peptide to examine the N-glycosylation kinetics of the middle X residue in an NXS consensus site. I found that large hydrophobic and negatively charged residues hinder co-translational N-glycosylation, while polar, small hydrophobic, and positively charged residues had the highest N-glycosylation efficiencies. Poorly N-glycosylated NXS consensus sites with large hydrophobic and negatively charged X residues had a significantly improved co-translational N-glycosylation efficiency upon conversion to NXT sites.

Also in Chapter III, I adapted a siRNA knockdown strategy to definitively identify the OST STT3 isoforms that perform co- and post-translational N-glycosylation for type I transmembrane substrates. I found that the STT3A isoform predominantly performs co-translational N-glycosylation while the STT3B isoform predominantly performs post-translational N-glycosylation, in agreement with the roles of these enzymatic subunits on topologically different substrates.
Taken together, these findings further the ability to predict the success of a consensus site by primary sequence alone and will be helpful for the identification and characterization of N-glycosylation deficiency diseases.
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<table>
<thead>
<tr>
<th>Symbol</th>
<th>Description</th>
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<tbody>
<tr>
<td>≠</td>
<td>Cannot be</td>
</tr>
<tr>
<td>Å</td>
<td>Angström</td>
</tr>
<tr>
<td>α</td>
<td>Alpha</td>
</tr>
<tr>
<td>β</td>
<td>Beta</td>
</tr>
<tr>
<td>μ</td>
<td>Micro</td>
</tr>
<tr>
<td>³⁵S</td>
<td>Radioisotope of sulfur</td>
</tr>
<tr>
<td>CDG</td>
<td>Congenital disorder of glycosylation</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary</td>
</tr>
<tr>
<td>CTX</td>
<td>Charybdoxin</td>
</tr>
<tr>
<td>Dol</td>
<td>Dolichol</td>
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<tr>
<td>E1</td>
<td>KCNE1</td>
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<tr>
<td>E2</td>
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<tr>
<td>E4</td>
<td>KCNE4</td>
</tr>
<tr>
<td>E5</td>
<td>KCNE5</td>
</tr>
<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>ERAD</td>
<td>Endoplasmic reticulum associated degradation</td>
</tr>
<tr>
<td>HA</td>
<td>Hemagglutinin A</td>
</tr>
<tr>
<td>hERG</td>
<td>Human <em>Ether-à-go-go</em>-Related Gene</td>
</tr>
<tr>
<td>IP</td>
<td>Immunoprecipitation</td>
</tr>
<tr>
<td>LLO</td>
<td>Lipid-linked oligosaccharide</td>
</tr>
<tr>
<td>LQTS</td>
<td>Long QT Syndrome</td>
</tr>
<tr>
<td>K⁺</td>
<td>Potassium ion</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger RNA</td>
</tr>
<tr>
<td>N-glycosylation</td>
<td>Asparagine-linked glycosylation</td>
</tr>
<tr>
<td>OST</td>
<td>Oligosaccharyltransferase</td>
</tr>
<tr>
<td>PM</td>
<td>Plasma membrane</td>
</tr>
<tr>
<td>SRP</td>
<td>Signal recognition particle</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering RNA</td>
</tr>
<tr>
<td>SMX</td>
<td>Sulfamethoxazole</td>
</tr>
<tr>
<td>TA</td>
<td>Tail-anchored</td>
</tr>
<tr>
<td>TEVC</td>
<td>Two electrode voltage clamp</td>
</tr>
<tr>
<td>TM</td>
<td>Transmembrane</td>
</tr>
<tr>
<td>Q1</td>
<td>KCNQ1</td>
</tr>
<tr>
<td>WT</td>
<td>Wild type</td>
</tr>
</tbody>
</table>

Standard one letter or three letter code was used to abbreviate amino acids. Mutated residues were abbreviated by position number and one letter amino acid codes.
PREFACE

The experimental work in Chapter II has been published in a peer-reviewed journal. Reference to publication that represent the work contained within this chapter:


The experimental work in Chapter III has been written up as a manuscript for publication at the time of this thesis publication. I created all peptide constructs and performed all assays. Dr. Natalia Cherepanova is credited with designing the STT3 siRNAs used in CHO cells for the work in Chapter III. I designed the scrambled STT3 siRNAs also presented in Chapter III.

Dr. Karen Mruk developed the terpyridine linker strategy used in Appendix II. Dr. Mruk also provided the terpyridine precursors used in the linker synthesis.
Asparagine (N)-linked glycosylation is an essential physiological process driving glycoprotein trafficking to the plasma membrane and along the secretory pathway. This process begins either during or after protein translation, when a 14-sugar lipid-linked oligosaccharide (LLO) is attached at a protein consensus site of N-X-T/S-(Y), where X and Y can be any amino acid other than proline [1]. The timing and affinity of these attachment steps has been shown to be instrumental for efficient processing further along the trafficking and secretory pathways, and an inability to meet the temporal demands on forward trafficking can result in disease [2,3]. Discussed here is an overview of the proteins and systems involved in trafficking a newly synthesized peptide from translation to LLO attachment, protein folding, and endoplasmic reticulum (ER) exit along the secretory pathway. Ideal peptide candidates for studying N-glycosylation are the KCNE family, small K+ channel regulatory subunits that are heavily N-glycosylated. Their physiological relevance and known N-glycosylation deficiency diseases will be discussed in length.

**Co-translational translocation and membrane insertion**

Co-translational translocation begins by relocating an actively translating ribosome to the cytosolic face of the ER membrane. During protein translation, the signal recognition particle (SRP) reads and binds to an ER signal sequence
on a growing peptide (nascent) chain in the cytosol [4,5] (Figure I-1A). This signal sequence can be either a cleavable N-terminal sequence composed of ~10-30 predominantly hydrophobic amino acids, or the hydrophobic sequence of the first transmembrane domain [5]. A cryo-EM structure of the mammalian SRP showed that the SRP54 subunit is responsible for binding and protecting the nascent chain by lining the exposed surface with hydrophobic residues [6] (Figure I-1B). Further, a kink in the RNA chain of the SRP allows for a sharp angle in the complex. This places the SRP9 and SRP14 proteins near binding sites for elongation factors on the 60S ribosome, which supports the observation that translation is slowed while the SRP is bound to the ribosome and peptide nascent chain [4,6].

The SRP relocates the ribosome/nascent chain complex to the ER membrane and attaches to an SRP binding site on the SRP receptor, hydrolyzing GTP to GDP. This interaction shifts the SRP ribosome complex, which is thought to allow a portion of the ribosomal exit tunnel to bind a nearby Sec61 complex (translocon) and cause the SRP to dissociate from the ribosome/nascent chain (translocon) and cause the SRP to disassociate from the ribosome/nascent chain [7,8]. There is also evidence that TRAM (translocating chain-associated membrane protein) binds the peptide signal sequence after the SRP has hydrolyzed GTP and attaches to the ribosome/nascent chain [7].

What happens to the peptide next depends on the topology of the peptide being translated. A water-soluble peptide will move through the translocon tunnel into the ER lumen by ribosomal translational driving force. Once translation is complete, the peptide is released into the ER lumen. A preprosequence can be either a cleavable N-terminal sequence composed of ~10-30 predominantly hydrophobic amino acids, or the hydrophobic sequence of the first transmembrane domain [5]. A cryo-EM structure of the mammalian SRP showed that the SRP54 subunit is responsible for binding and protecting the nascent chain by lining the exposed surface with hydrophobic residues [6] (Figure I-1B). Further, a kink in the RNA chain of the SRP allows for a sharp angle in the complex. This places the SRP9 and SRP14 proteins near binding sites for elongation factors on the 60S ribosome, which supports the observation that translation is slowed while the SRP is bound to the ribosome and peptide nascent chain [4,6].
Figure I-1. Co-translational translocation and peptide targeting by the SRP

(A) Cartoon depiction of co-translational translocation. (I) SRP binds to and protects a hydrophobic signal sequence once it leaves the translation tunnel of the ribosome and contacts the ribosome to stall translation. (II) The SRP relocates the complex to the ER membrane where the SRP docks with the SRP receptor via GTP, which allows (III) the ribosome to dock with the translocon and the peptide to diffuse into the translocon tunnel. GTP hydrolysis releases SRP.

(B) Molecular model of the SRP as determined by cryo-EM. SRP54 = purple (M-domain dark purple, NG-domain light purple); SRP19 = orange; SRP9 = green; SRP14 = yellow; peptide nascent chain = blue; gray = RNA. Inset. Hydrophobic residues of SRP54 are colored yellow.

complete, the peptide will move completely out of the translocon through the lumen for folding and assembly [10]. Transmembrane proteins will initially proceed similarly; however, since the hydrophobic ER signal sequence recognized by the SRP is the transmembrane domain, the transmembrane of the nascent peptide is fed into the translocon tunnel upon ribosomal docking (Figure I-2). The nascent peptide then most likely samples orientations of the transmembrane sequence until a favored outcome with the lowest free energy is achieved [8]. To aid in the process, many transmembrane protein sequences contain a string of positively charged residues located on the destined cytosolic domain and adjacent to the transmembrane domain [11]. This may also serve as an ER retention signal depending on the residues proximity to the C-terminus [12]. For type I transmembrane peptides, where the N-terminus is located in the ER lumen, this results in the bolus of the N-terminus unfolding all at once into the ER lumen. For type II transmembrane peptides, where the C-terminus is located in the ER lumen, this results in the N-terminus diffusing back out into the cytosol, and the remaining nascent chain being fed through the tunnel into the ER lumen [8]. Once the hydrophobic residues making up the transmembrane region are favorably oriented in the translocon tunnel, this section of the nascent chain will diffuse laterally out of the translocon and into the ER membrane [8,13].
Figure I-2. Membrane orientation determination for type I and type II transmembrane peptides. Once the ribosome is docked with the Sec61 translocon complex, the peptide is inserted TM domain first. The TM then samples orientation through diffusion in the translocon tunnel. Type I TM peptides (right) often contain a string of positively charged residues at the beginning of the C-terminus, disfavoring diffusion into a hydrophobic tunnel. The N-terminus then preferentially diffuses into the ER lumen. Type II TM peptides (left) proceed similarly, but these often contain the positively charged residues at the end of the N-terminus, disfavoring entry into the translocon, while the C-terminus diffuses through the translocon tunnel.

Schematic generated by H. Malaby.
**The Oligosaccharyltransferase (OST) and Co-translational N-glycosylation**

Regardless of the final topology of a nascent chain through the translocon, once the peptide enters the ER lumen it is recognized by the oligosaccharyltransferase (OST), which scans the nascent chain for N-glycosylation consensus sites and attaches a preassembled oligosaccharide onto the consensus site asparagine [14,15]. N-glycosylation that occurs adjacently to Sec61 complex translocation and ribosomal translation of a peptide is termed co-translational N-glycosylation [16], and is conserved from arachea and eubacteria to fungi, insects, plants and vertebrates. All of these organisms contain at least an enzymatic homolog of the OST [17]. The mammalian OST is composed of seven subunits, each thought to perform a unique function for this highly specialized complex (Figure I-3). STT3 is the main catalytic subunit actually orchestrating the glycan attachment to the peptide and has two isoforms designated STT3A and STT3B that share 60% sequence identity but have distinct enzymatic properties [18,19]. Ribophorin I and Ribophorin II may contact the ribosome and the translocon [20] and OST48 may act as a linking bridge between Ribophorin I and II. DADI is thought to provide structural integrity to the complex as a whole [21]. N33/Tusc3 or IAP/MagT1 have oxidoreductase capability that might aid in oxidative folding of peptide substrates for efficient N-glycan attachment [22], and OST4 is thought to regulate the incorporation of N33/Tusc3 and IAP/MagT1 [21,23].
Figure I-3. Subunits of the Oligosaccharyltransferase (OST). Subunits composing the mammalian OST are depicted. Multiple isoforms indicated where appropriate.


Although the OST subunits have been identified, many of the details for assembly and function of the OST have yet to be determined [24]. It is known that the OST complex exists in heterogeneous populations in yeast and mammalian cells (because of the STT3A/STT3B and Tusc3/MagT1 subunit isoforms there might be as many as four possible mammalian OST isoforms), and these isoform ratios are uniquely distributed in different tissues [18]. What subunits are present in which isoforms and how the complex is arranged has yet to be elucidated.

Despite these uncertainties, the conservation of an enzymatic subunit from vertebrates to bacteria and the recently determined crystal structures of the OST STT3A/B homologs for bacterial *C. lari* PglB [25] (Figure I-4) and arachaeal *A. fulgidus* AgIB [26] allow for a proposed general mechanism of LLO attachment to a peptide. As the nascent chain leaves the translocon tunnel, it is thermodynamically bound by a region bridging the transmembrane and periplasmic domains of the catalytic protein, which causes an external loop (EL5) to become ordered and pin the peptide against the periplasmic domain to restrict motion [25]. When an N-linked consensus site of N-X-T/S-Y, where X and Y can be any amino acid besides proline, orients in the peptide binding site (Figure I-4, top inset), the hydroxyl residue (T/S) is coordinated through hydrogen bonding between a number of tryptophan and aspartic acid OST residues creating a binding pocket that is thought to help stabilize the nascent chain [27]. There is also NMR evidence that this binding pocket is highly flexible, which might
**Figure I-4. The bacterial OST homolog structure PglB.** Crystal structure of PglB, homolog to STT3, the enzymatic subunit of the OST shown in green. The ten amino acid long peptide crystalized in the peptide binding site is depicted in blue with highlights for the consensus site: N (red), A (yellow), T (purple). **Top inset:** View rotated 90° counterclockwise and zoomed. Note the curvature of the peptide around the consensus site. **Left bottom inset:** Sphere fill of top image. **Right bottom inset:** View rotated 180° clockwise. Note how Asn is the only amino acid of the peptide that is exposed to the LLO binding and catalytic site.

facilitate efficient scanning of the peptide [28]. When the peptide is properly aligned, only the functional group of the consensus site asparagine is accessible from the LLO binding and catalytic site (Figure I-4, bottom insets). The asparagine is coordinated between a divalent cation (usually Mg$^{2+}$ or Mn$^{2+}$), a glutamic acid, an arginine, and three aspartic acid OST residues. Though the precise mechanism is yet to be fully understood, this group is thought to undergo hydrogen swapping, resulting in an activated amide nitrogen in the consensus site asparagine, holding the asparagine functional group in a triplanar orientation. The nitrogen is then able to undergo a nucleophilic attack on the adjacently positioned C1 carbon of a LLO (presumably coordinated by the OST as well), creating a peptide bond and attaching the oligosaccharide to the asparagine [25].

Once an oligosaccharide is attached to the consensus site asparagine residue, the steric tension caused by the newly bonded glycan is thought to cause the OST to release the glycopeptide, specifically by displacing the EL5 which rapidly becomes unstructured causing disassembly of the peptide and LLO binding sites [25]. At this point the OST can repeat the process for additional consensus sites until translation is complete and the peptide moves out of range of the translocon and OST. For the two mammalian OST enzymatic isoforms, STT3A has been shown to be primarily associated with co-translational N-glycosylation, and is believed to interact directly with the translocon [29]. The STT3B isoform has been associated with post-translational N-glycosylation [30].
**Post-translational N-glycosylation**

*A “clean up” mechanism for skipped N-linked consensus sites*

The first evidence for post-translational N-glycosylation was identified with a number of peptide fragments *in vivo* [31] and larger proteins treated with N-glycosylation inhibitors [32,33]. However, since these studies were not of full-length proteins under homeostatic conditions, it was not understood if post-translational N-glycosylation was a normal cellular process. Post-translational N-glycosylation was also found to occur for a truncated peptide processing enzyme PAM; while the peptide truncation is a normal part of PAM processing, this happens downstream of N-glycosylation leaving the elusive question for a physiological role of post-translational N-glycosylation unanswered [34]. The first physiologically relevant post-translational N-glycosylation substrates identified were Wg, a Wnt secreted glycoprotein in *Drosophila* that needs a Porc chaperone to prevent folding and keep the N-glycosylation site available [35], and Factor VII, a human coagulation protein [36]. Factor VII was identified as a post-translational N-glycosylation substrate using a rapid pulse-chase assay to observe the kinetics of N-glycan attachment. This study found that the glycoforms of factor VII significantly increased after only 15 minutes of chase, indicating that N-glycans can be attached relatively quickly after protein translation has been completed [36]. Since then, a number of other proteins have also been found to contain post-translational N-glycosylation sites [2,37,38,39,40,41].
Recently, the OST enzymatic isoform STT3B was implicated in the mechanism of post-translational N-glycan attachment for water soluble, type II, and multi-spanning transmembrane proteins (Figure I-5) [30,41,42]. Using a series of selected OST isoform siRNA knockdowns, it was shown that only the STT3B isoform performs post-translational N-glycan attachment [30]. The STT3A isoform largely performs co-translational N-glycan attachment, but interestingly the STT3B isoform can also N-glycosylate in a co-translational manner if the STT3A isoform is knocked down, albeit at a reduced efficiency [30].

Overall, post-translational N-glycosylation by the STT3B isoform appears to contribute to quality control [43]. While STT3A attaches N-glycans at high efficiency, there are some sites that, for poorly understood reasons, are skipped by the co-translational N-glycosylation machinery. Presumably, these are the sites that the OST isoform STT3B attempts to find and glycosylate. While there is no evidence that the STT3B isoform actually attaches N-glycans at a reduced rate, in fact it seems to process oligosaccharides much faster [18], it is most likely hindered by the distance needed for diffusion to find proteins with skipped N-glycosylation consensus sites. While the STT3A isoform has very high local concentration of potential peptide substrate from association with the translocon, the STT3B isoform must work against potentially vast luminal space, protein folding, forward trafficking, and protein degradation. As such, the OST STT3B isoform appears less efficient in in vivo kinetic assays [40].
Figure I-5. Post-translational N-glycosylation. (A) Schematic for post-translational N-glycosylation of water-soluble proteins. While normal co-translational translocation occurs and the OST STT3A enzymatic isoform is present, some consensus sites are not N-glycosylated by this machinery. Instead, the protein exits the translocon into the ER lumen and these consensus sites are later N-glycosylated by the OST STT3B isoform. (B) A similar mechanism is proposed for TM proteins. Here, the protein exits the translocon by lateral diffusion into the ER membrane.

Schematic generated by H. Malaby.
Post-translational translocation

Recently, a number of protein classes have been shown to not utilize a co-translational translocation insertion mechanism [8]. Signal sequences that are only slightly hydrophobic might not be recognized by the SRP, as well as peptides where the secondary structure of the signal sequence is inadequate for binding. Secretory proteins that are shorter than the length of the ribosomal exit tunnel (40-50 amino acids) will be skipped by the SRP because translation will have finished by the time the SRP can direct the ER targeting of these peptides [44]. Type II transmembrane proteins with short C-termini (termed tail-anchored (TA) proteins) have also been shown to be post-translational translocation substrates for similar reasons [45].

For all substrates of SRP-independent mechanisms of translocation, the first proteins to bind the peptides after leaving the ribosomal exit tunnel are chaperones (Figure I-6). Hsp70, Hsp40, calmodulin, Sgt2, and Bag6 have all been identified as “first responder” chaperones for a variety of peptide substrates [44]. The Bag6 chaperone has been found to actually bind to translating ribosomes, deftly positioned to bind a nascent peptide chain [46]. These chaperones then pass off the peptide substrate to a protein targeting complex. A specific chaperone for TA proteins has been identified as the transmembrane domain recognition complex (TRC), composed of proteins Get3 and/or TRC40 [45]. The pass off between chaperone and TRC is accomplished through a mediator complex composed of TRC35 and Ubl4A [44]. Structural studies of
Figure I-6. **Post-translational translocation.** Post-translational translocation occurs when the pathway for co-translational translocation via the SRP is unavailable due to an inability to bind the ER signal sequence. When the peptide exits the ribosome, a chaperone protein binds the hydrophobic signal sequence (ex. Hsp70, Hsp40, calmodulin, Sgt2, Bag6). Protein targeting complexes (ex. Get3, TRC40) can bind to or replace the chaperones on the peptide substrate and relocate the complex to the ER membrane. The translocation of the peptide occurs through either the Sec61 translocon in complex with Sec62 and Sec63 for small peptides or peptides with mild hydrophobic signal sequences or through the WRB/Get1 and CAML/Get2 complex for TA proteins. It is not known if WRB/Get1 or CAML/Get2 forms a translocon pore.

Schematic generated by H. Malaby.
Get3 have shown that it functions as a dimer that fluctuates between “open” and “closed” states. The open state exposes a hydrophobic groove that could bind the transmembrane domain of a TA protein [47,48].

The protein targeting machinery then relocates the complex to one of two possible post-translational translocational channels in the ER membrane: Sec61 with the Sec62-Sec63 complex or Get1/WRB complexed with Get2/CAML [44]. Sec61 is the most common translocon in the ER membrane and is usually responsible for co-translational translocation. However, Sec61 has been shown to form a unique complex (termed the Sec complex) with Sec62 and Sec63 proteins that convert this translocon for post-translational translocation tasks (Sec66 and Sec72 also have nonessential roles in the complex in yeast) [49]. Small, less hydrophobic substrates are often targeted to the Sec complex. Here, the substrates diffuse through the translocon until one end can be bound and ratcheted through (or presumably into) the membrane by Kar2, which associates with Sec63.

The Get1/WRB complexed with Get2/CAML translocon is unique in that an actual pore for translocation has yet to be identified. The mechanism of translocation is still not well understood although it does appear to be specific for TA proteins. It is thought that the TMs of this complex could arrange to allow the TM of a TA protein to wedge into the membrane through local membrane distortion, or even create a temporary gap for proteins to diffuse through into the lumen [44].
It is unknown how N-glycosylation occurs for post-translationally translocated proteins, but it is known that they can be glycosylated in mammalian cells when a glycosylation consensus site is introduced [50]. By definition these proteins would be post-translationally N-glycosylated, but it is not known which enzymatic isoform of the OST would attach the N-glycan. It is possible that the co-translational N-glycosylation machinery (OST STT3A) cannot assemble with Sec61 when it is complexed as a post-translational translocon or with the Get1/WRB complexed with Get2/CAML translocon, which would indicate that STT3A mediated N-glycosylation would not be possible for these protein classes. A protein that was inserted post-translationally could then only be recognized by the OST isoform STT3B for N-glycosylation of these consensus sites. Thus, post-translational N-glycosylation could occur either due to missed N-linked consensus sites during co-translational N-glycosylation, or due to post-translational ER membrane insertion.

**Formation of the LLO and Congenital Disorders of Glycosylation**

Regardless of its utilization by OST STT3A or STT3B isoforms, the lipid-linked oligosaccharide (LLO) goes through its own extensive synthesis before peptide attachment. Building from a dolichol (Dol) lipid base, monosaccharides (for eukaryotes, this is a combination of GlcNAc, mannose, and glucose) are subsequently added by formation of glycosidic bonds performed by an ordered series of saccharide specific enzymes ultimately creating a final branched chain
of oligosaccharides (Figure I-7) [1]. The first seven saccharides are added in the cytosol. After a fifth mannose addition to create Man$_5$GlcNAc$_2$-PP-Dol, the enzyme Rft1 flips the LLO precursor into the ER lumen (classified as a flippase). Once inside, an additional four mannoses are added followed by three glucoses to create the completed LLO donor Glc$_3$Man$_9$GlcNAc$_2$-PP-Dol [51].

In the LLO synthesis pathway there are many identified mutations (42 currently known) in the genes encoding enzymes required for both LLO synthesis and LLO transfer to the peptide that lead to congenital disorders of glycosylation (CDGs) type I (Figure I-7) [52]. Recently, the first CDG causing mutations have been identified in the STT3A and STT3B catalytic subunits of the mammalian OST [53]. Patients with a homozygous point mutant and a homozygous intronic mutant resulted in decreased levels of STT3A or STT3B respectively, and presented with hypoglycosylation of a number of substrates. Additionally, defects in glycan trimming and processing enzymes, monosaccharide transporters, or essential structural proteins to the pathway are also associated with CDGs (CDG type II) [52]. Most of these are found farther along the secretory pathway in the Golgi apparatus, but a few have been identified in ER processing steps (see next section).
Figure I-7. Formation of the lipid-linked oligosaccharide (LLO). LLO formation begins in the cytosol by the addition of two N-acetylglucosamine (GlcNac) monosaccharides to a dolichol precursor. UDP (or UMP for the first GlcNac) is released in the process. Five mannose (Man) monosaccharides are then added in a branching formation. The integral protein Rft1 flips this complex into the ER lumen, where more mannose monosaccharides are added followed by three terminal glucose (Glu) monosaccharides. The monosaccharides are brought into the ER lumen by dolichol flipping. Enzymes catalyzing the addition of each monosaccharide attachment step are indicated. Those in red have known mutations that lead to CDGs.

Schematic generated by H. Malaby.
**N-glycosylation in protein folding and trafficking**

Once an N-glycan is attached to a peptide, a series of glycan truncations are performed to direct chaperone activity and to insure that properly folded and assembled glycoproteins traffic outside of the ER while off pathway proteins are relegated to ER associated degradation (ERAD) (Figure I-8) [3,54]. First, the glucose trimming enzymes glucosidase I and II sequentially remove the two terminal glucoses, resulting in a single glucose remaining on the oligosaccharide. This specific glycan complex, Glc$_1$Man$_9$GlcNAc$_2$, is a substrate for the ER resident protein folding chaperones calnexin (membrane bound) and calreticulin (soluble) and is recognized by their carbohydrate-binding globular domains [55]. A disulfide isomerase, ERp57, is also known to catalyze disulfide bond formation and associate with calnexin and calreticulin [56]. Once the glycoprotein is released from the protein folding chaperone, glucosidase II then trims off the last glucose, resulting in a Man$_9$GlcNAc$_2$ glycan. If this protein is folded properly, ER mannosidase I removes several mannoses from the glycan, allowing the glycoprotein to be recognized for transport in COPII coated vesicles to the Golgi apparatus [54]. How a glycoprotein is recognized as being properly folded and the exact mechanism of mannose-trimmed glycoprotein recognition for forward trafficking is not fully understood.

Some glycoproteins, however, are not fully folded after a single session with the chaperones and require further assistance. These proteins are recognized by UDP-glucose:glycoprotein glucosyltransferase (UGGT); the UGGT
Figure I-8. N-linked glycosylation and protein folding in the ER. After a glycan is attached to a peptide, glucosidase I and II trim the first and second glucose residues, respectively. This glycan structure (Glc\textsubscript{1}Man\textsubscript{9}GlcNAC\textsubscript{2}) is a specific substrate for the ER folding chaperones calnexin (pictured) and calreticulin. ERP57 aids in disulfide bond formation. Once the peptide is released, glucosidase II trims the remaining glucose creating Man\textsubscript{9}GlcNAC\textsubscript{2}. If the protein is folded correctly, several mannose residues are trimmed by mannosidase I and are recognized for forward trafficking and ER exit. If the peptide is not folded correctly, UGGT reattaches a glucose residue making the peptide a substrate for ER folding chaperones once again. If a peptide remains stuck in this...
folding cycle, it is sequestered by ERAD macherinery (OS-9) and transported outside the ER via Hrd1 and Sel1 for degradation by the proteasome. Glucosidase I and mannosidase I both have known mutations that lead to CDGs.

Schematic generated by H. Malaby.
N-terminal domain binds the unfolded peptide and the UGGT C-terminal domain catalyzes the readdition of glucose to the terminal position on the glycan [57,58]. This once again allows the glycoprotein to be recognized as a substrate by calnexin and calreticulin. This cycle will continue until either 1) the glycoprotein is properly folded and forward trafficking can occur, or 2) the glycoprotein is sequestered by ERAD. It has been proposed that trimming of terminal mannose residues acts as a molecular timer limiting glycoprotein presence in the folding cycle, although this has not been fully elucidated [3]. It is known that ER resident lectins OS-9 and XTP3-B recognize trimmed ERAD substrates by their mannose-6-phosphate receptor homology (MRH) domains [59]. OS-9 and XTP3-B then target these substrates to retrotranslocation machinery in the ER membrane (Sel1 in the Hrd1 complex), where peptides are moved into the cytosol for ubiquitination and proteasome degradation [3].

Interestingly, the OST enzymatic isoform STT3B has been implicated in ERAD by marking unfolded proteins for degradation through glycosylation of consensus sites that normally are buried in a natively folded protein [43]. This finding resonates well with the known role of post-translational N-glycosylation, which serves to find and glycosylate unoccupied, recognizable consensus sites after translation is completed [30].

While there are exceptions to protein targeting for ERAD (not all proteins targeted for destruction are glycoproteins and thus there must be other recognition mechanisms for degradation that do not involve lectins [60]), all
proteins that have an attached N-glycan are recognized by the folding and assembly machinery and are required to undergo correct folding regulated by glycan trimming for forward trafficking to occur. Thus, it is essential for glycoproteins to not only have an intact N-linked consensus site, but also to have a high rate of N-glycan occupancy [61].

**Previous primary sequence trends in N-linked consensus sites**

Whether an N-glycan is attached by co-translational or post-translational N-glycosylation, the consensus sequence for attachment is the same: N-(X)-T/S-(Y), where X and Y can be any amino acid other than proline. Using computational and *in vitro* approaches, several trends in N-linked glycosylation consensus sites have been noted.

The first study on the N-glycosylation consensus sequence examined a number of known glycoproteins and determined that a tripeptide sequence of NXT/S is necessary for N-glycosylation [62]. Several *in vitro* studies have since found that threonine residues in the consensus site result in more glycosylated protein than serine residues in peptides translated for a similar time period [63,64]. By adding short peptides containing NXT, NXS, or NXC sites to calf liver microsomal membranes with $^{14}$C radiolabeled GlcNAc and tracking the incorporation of the radioactive N-glycan over 30 minutes, it was found that threonine consensus sites incorporated glycans about 40 times faster than serine consensus sites [63]. Cysteine consensus sites were 2.5 times slower than
serine; so slow that at steady state NXC sites are mostly unglycosylated and are found so infrequently that they are often, including here, left out of the NXT/S consensus sequence. These results were repeated two decades later, this time including non-physiological analogues of threonine. Again, threonine produced the fastest kinetics by a large margin. Threonine analogues were not supported in the hydroxyl residue consensus site position [64].

The N-glycosylation efficiencies of consensus sites with varying amino acids in the X position has also been studied using a similar 14C radiolabeled LLO. Using short peptides containing NXT consensus sites, it was discovered that proline is not tolerated in the X position [65], or in the amino acid immediately following the hydroxyl residue (the Y position) [66]. This proline intolerance can now be explained by the structure of the peptide binding site in the OST homolog PglB since proline would kink the peptide to such an extent that favorable confirmation is impossible for both hydroxyl residue coordination and asparagine activation [25].

Other studies have also observed the role the X residue plays on the efficient production of glycoprotein [67,68]. Using the rabbit reticulocyte in vitro translational approach, a peptide consensus sequence library was translated to determine N-glycan attachment efficiency. From experiments given one hour for equilibrated translation, it was found that some X residues are much better than others in NXS consensus sites. Serine, cysteine, histidine, and threonine all produced robust glycopeptides, where as tryptophan, aspartate, glutamate, and
leucine were all poorly N-glycosylated. Interestingly, these X residue effects on N-glycosylation efficiency were diminished in an NXT site, where all amino acids (except proline) in the consensus site glycosylated proficiently [67].

Several studies have branched out of the immediate consensus site area, looking for more long-range effects on N-glycosylation [69,70,71]. Bacterial glycopeptides were found to require a negatively charged residue in the -2 position upstream of the consensus site [70], and this has been attributed to a predicted salt bridge formation between the negatively charged residue in the peptide and an arginine in the PglB structure [25]. A computational study looking at 617 glycoproteins found a few trends in flanking consensus site residues, such as a slightly increased likelihood of a positively charged residue 5 residues upstream or hydrophobic residues 10-20 residues downstream of NXS consensus sites [71].

While many of these biochemical and computational findings have been immensely helpful and predictive, characterizing the N-glycosylation efficiencies for native consensus sites of physiologically relevant glycoproteins, and the roles of co- and post-translational N-glycosylation in the sequential context of these consensus sites, has yet to be fully elucidated.
The KCNE family of type I transmembrane peptides

Type I transmembrane glycopeptides can be a particularly powerful tool for studying the kinetics of N-glycosylation, as many of the peptides are small and expected to translate rapidly [72]. This rapid translation puts a significant time restraint on co-translational N-glycosylation, making these glycopeptides sensitive to factors that shift the balance of co- and post-translational N-glycosylation. This thesis focuses on the heavily N-glycosylated KCNE family of type I transmembrane peptides as an ideal model to study the kinetics of N-glycosylation.

Discovery

The KCNE family consists of five members (KCNE1-KCNE5) that act as β accessory subunits essential for the modulation of voltage-gated potassium (K⁺) channels [73]. KCNE1 (E1) (also known as MinK, minimal K⁺ channel protein) was discovered in 1988 when fractionated mRNA was injected into *Xenopus* oocytes and was initially thought to be the pore forming K⁺ channel [74]. Almost a decade later, it was established that E1 is not in fact the K⁺ channel responsible for conductance, but rather an accessory peptide β subunit that preferentially assembles with a *Xenopus* oocyte native voltage-gated K⁺ channel, KCNQ1 (Q1) [75,76]. Through BLAST homology searches of EST databases, four other potential peptides were identified by sequence similarity. These MiRPs (MinK related protein) were designated MiRP1-4, but are now most commonly referred
to as their gene names along with E1: KCNE2 (E2), KCNE3 (E3), and KCNE4 (E4) and KCNE5 (E5) [77].

**Structure and N-linked glycosylation consensus sites**

As type I transmembrane peptides, the KCNE family is composed of an extracellular N-terminus, transmembrane domain, and cytosolic C-terminus. The KCNEs range in length from 103 (E3) to 177 (E4) residues, with variable length of the N- and C-termini (Figure I-9) [73]. All of the KCNEs contain an ER retention signal of three to four arginine and lysine residues immediately following the transmembrane domain, explaining the type I orientation and lack of forward trafficking unless assembled with a channel [78]. All of the KCNEs also contain at least one, and up to three, N-linked glycosylation consensus sites: E1 contains two consensus sites starting at residues 5 (NTT) and 26 (NMS); E2 has two consensus sites located at residues 6 (NFT) and 29 (NTT); E3 has three consensus sites found at residues 5 (NGT), 22 (NAT), and 41(NQT); E4 has one consensus site at residue 8 (NST); and E5 has two consensus sites at residues 2 (NCS) and 25 (NAS) [40].

The N-terminal, transmembrane, and C-terminal domains of KCNEs are thought to form α-helical sections based on circular dichroism (CD), nuclear magnetic resonance (NMR), and alanine/leucine scanning experiments [79,80,81,82]. However, only the transmembrane and C-terminal domains are implicated in K⁺ channel modulation [73,83,84,85].
Figure I-9. The KCNE family. Cartoon depictions of the KCNE (E1-E5) family. Number of glycosylation sites are designated and consensus site sequences are shown. Blue residues indicate the beginning of the TM domain; red residues mark the last amino acid of the peptide.

Schematic generated by H. Malaby.
**KCNE Physiology**

Members of the KCNE family have been shown to modulate a large number of voltage-gated K⁺ channels [73]; however, the only known K⁺ channel to readily assemble with all five KCNE members is KCNQ1 (Q1). Each Q1 subunit contains six transmembrane domains (S1-S6) and cytosolic N- and C-termini (Figure I-10A) [86]. Four homomeric Q1 subunits assemble to form a functional channel [87]. S1-S4 form the voltage sensing domain, and S4 contains several arginine residues responsible for outward movement that causes channeling opening upon depolarization [88]. S5-S6 form the pore region, through which a string of highly conserved residues among voltage-gated K⁺ channels, TVGYG, align such that the carbonyl backbones mimic the hydration shell of K⁺ ions to form the K⁺ selectivity filter [89,90,91]. Q1 contains a short N-terminus with little known function, but a lengthy C-terminus with four α-helices containing multiple calmodulin binding motifs, phosphorylation sites for many kinases including PKC and Src Kinase, a binding site for the regulatory protein Yotiao, and several putative dimerization and tetramerization domains [86,92].

For both two-electrode voltage clamp (TEVC) utilizing *Xenopus* oocytes and whole-cell patch clamp performed on mammalian cells at physiological pH, Q1 expressed alone elicits rapid activation (equilibration reached within 1 sec), and undergoes inactivation upon depolarization (Figure I-10A) [86]. When E1 is expressed with Q1, a drastic change is observed that results in significant slowing of activation (over 4 sec for equilibration), increased current amplitude,
Figure I-10. KCNE modulation of the K⁺ channel KCNQ1. (A) One KCNQ1 (Q1) subunit is composed of six transmembrane domains (S1-S6), where S4 is the voltage-sensing domain containing several Arg residues and S5 and S6 form the pore. Four subunits assemble into a complete channel. Q1 expressed alone opens rapidly as shown by a schematic of an electrophysiological trace where the channel is opened at various voltages ranging from -100 mV to +60mV for 2 sec. (B) Electrophysiological traces of the KCNEs with Q1 under the same conditions as (A). All cartoons are approximations of well-documented, reproducible channel complex phenotypes [74].

Schematic generated by H. Malaby.
and loss of inactivation (Figure I-10B) [75]. In contrast, E2 expressed with Q1 results in what is basically a leak channel – a linear voltage-current relationship, with instantaneous activation and deactivation (meaning the channel does not fully close at physiological equilibrium) [93]. Q1/E3 complexes are constitutively conducting leading to a linear voltage-current relationship (meaning the channel fully opens at all voltages, but remains closed in the absence of a charge differential) [94]. When Q1 coassembles with E4, current is severely reduced [95]. Q1/E5 complexes have similar biophysical properties as Q1/E1 with an activation threshold shifted to about +40mV and increased kinetics of channel closing [73,96]. Since physiological membrane potential of most cells is about -80 mV, the Q1/E5 channel complex would only be functional after depolarization has occurred for sometime, otherwise E5 complexes would mostly be non-functional.

There is also evidence that the KCNEs can form mixed heteromeric channel complexes [97]. E1 and E4 have been found to assemble with Q1 as one complex and present a conductance pattern that is a hybrid of Q1/E1 and Q1/E4 complexes [98]. Likewise, Q1/E1/E3 complexes have been detected, also presenting a blending of channel modulatory effects [99]. The possibility remains that other KCNE heteromeric complex combinations can occur and may play an instrumental role for in vivo electrochemical homeostasis.

A number of studies have been conducted utilizing cysteine cross-linking and chimeric constructs to determine KCNE residues important in modulation and to identify the location of the β subunit in the channel complex.
[73,83,84,85,100]. From these findings, it is thought that the KCNEs could reside between the S5/S6 linker region and S4 in Q1, and that most if not all interaction with the channel is occurring in the transmembrane and C-terminal domains of the KCNE peptides [101].

Tissue expression

mRNA for all of the KCNEs has been detected readily throughout a number of mammalian tissues by RT-PCR [102]; however, KCNE protein detection has not been as efficiently cataloged. mRNA for all five KCNEs have been found expressed in cardiac tissue, however protein has only been detected for E1 (from human, horse, and guinea pig), E2 (human), and E3 (horse) [73,103,104]. Likewise, KCNE mRNA is often found throughout smooth muscle and epithelial tissues, including: small intestine (E1, E3, E4), stomach (E1, E2, E3), pancreas (E1, E3), kidney (E1-E4), lung (E1-E4), uterus (E1, E4), testis and ovaries (E1, E3), leukocytes (E1, E3), inner ear (E1), bladder (E2, E3), eye (E2), colon (E3), and trachea (E3) [102]. KCNE protein for smooth muscle and epithelial tissues has only been identified in the stomach (E1, E2), inner ear (E1), colon (E3) and small intestine (E3) [105,106,107,108]. Finally, KCNE mRNA in skeletal muscle has been found for E2, E4, and E5 and all KCNE mRNA are found in the central nervous system [102], but only E1 and E3 proteins have been detected in rat brain [109].
While much work has yet to be done on further identifying KCNE expression and particularly identifying KCNE protein in channel complexes in mammalian tissues, it is clear that the KCNEs are widely diverse and potentially fulfill a plethora of homeostatic roles. Amongst the tissues that the KCNEs are found, the system arguably the most sensitive to electrochemical fluctuations is cardiac tissue and it is here that the most well understood KCNE complex, Q1/E1, contributes to an essential function – the cardiac action potential [73].

The KCNE family and the cardiac action potential

The cardiac action potential consists of five distinct phases, numerically designated 0-4 (Figure I-11A) [110]. Phase 0 is the depolarization at the cell membrane caused in response to the rapid opening of voltage-sensitive Na⁺ channels. The repolarization phases 1-4 are contributed by distinctly identified currents whose timing is crucial for proper cardiac function. Phase 1 is associated with $I_{to}$ K⁺ current, a rapidly activating current that is formed by Kv1.4, Kv4.2, or Kv4.3 (depending on the species) [111]. The plateau of phase 2 in the action potential is maintained by Ca²⁺ current and several minor K⁺ currents ($I_{Kp}$, $I_{Ksus}$, or $I_{Kur}$) possibly carried out by Kv1.5 or K2P [110]. Phase 3 is composed of delayed outward rectifying K⁺ currents ($I_{Kr}$ and $I_{Ks}$) formed by hERG (human Ether-à-go-go Related Gene) and Q1/E1 respectively [75,76,112]. Lastly, phase 4 is the initiation of the cardiac action potential and is mediated by inward rectifying K⁺ currents: $I_{K1}$ (Kir 2.1-2.4), $I_{Kach}$ (Kir 3.1 and 3.4), and $I_{KATP}$ (KATP...
Figure I-11. Conductance patterns of cardiac muscle. (A) A localized schematic of the cardiac action potential. Amount of current is shown as a function of time. Phases are indicated above and shaded for distinction. The predominant K$^+$ currents associated with each phase are shown. (B) top Schematic of a normal electrocardiogram (ECG) for a healthy individual. Peak waves are labeled appropriately. bottom Schematic of an ECG for an individual with Long QT Syndrome (LQTS).

Schematic generated by H. Malaby.
channels) [110]. Depolarization (phase 0) will occur again once a threshold potential is reached after the electrochemical gradient is restored. This restoration is assisted by a number of exchange pumps (Na⁺-K⁺ and Na⁺-Ca⁺) in addition to the inward rectifiers [113,114].

While Q1/E1 is the only KCNE channel complex that has been identified in the cardiac action potential, the mRNA presence of the other KCNEs in cardiac tissue hints at larger roles for the KCNE family. Specifically, there has been debate and speculation over whether Q1/E2 also is part of I_{Ks}, and if hERG, which controls I_{Kr}, might be in complex with E2 as well [115,116]. Moreover, the existence of cardiac diseases that arise from mutation in the KCNEs [117,118,119] has lent stronger evidence for E1, E2, and E3 roles in the cardiac action potential.

The KCNE family and disease

Many mutations have been identified in genes encoding cardiac ion channels that drastically alter the conductance pattern of the heart and lead to disease [117,119,120]. Inherited arrhythmias are most often found amongst the channels responsible for the essential timing of phase 3 of the cardiac action potential – the delayed rectifier channels hERG and Q1/E1 [117]. On a surface electrocardiogram (ECG), these mutants result in a prolongation of the QT interval (Figure I-11B), and thus their associated disease was named Long QT
 Syndrome (LQTS) [120]. Patients with LQTS are predisposed to ventricular fibrillation, torsades de pointes arrhythmia, and sudden death [121].

A large number of point mutations have been identified across all domains of Q1, hERG, E1, and E2 that result in LQTS [117]. In the KCNEs, most of these mutations are found in the transmembrane and C-terminal domains, regions already known to effect channel modulation and much work has been done to identify the exact biophysical cause of these disease mutants. Interestingly, there have also been several identified mutants in the N-terminus of E1 (T7I) [122] and E2 (T8A, Q9E) [77,123] that have no explainable cause since the N-terminus of KCNEs has not been found to effect modulation. In addition, a mutant in the N-terminus of E3 (T4A) has recently been linked to Burgada Syndrome, another ECG irregularity appearing as malformed QRS peaks [124]. Interestingly, all of these mutations occur in or directly adjacent to N-glycosylation consensus sites. However, since all of these KCNEs have at least one additional consensus site that is potentially unaffected, it was unclear why these mutations were leading to disease.

The E1 T7I mutant was previously characterized and found that the ensuing LQTS is indeed due to an N-glycosylation deficiency [2]. It was discovered that the first consensus site in E1 (NTT) is a co-translationally N-glycosylated site, while the second consensus site (NMS) is actually a post-translationally N-glycosylated site (Figure I-12A). The N-glycosylation kinetics of E1 were not affected by co-expression with Q1 [2]. The LQTS mutant T7I
Figure I-12. Characterization of the E1 T7I mutation. (A) The first wtE1 N-glycosylation consensus site (NTT) is co-translationally N-glycosylated while the second consensus site (NMS) is post-translationally N-glycosylated. (B) The E1 T7I mutation destroys the first consensus site, leaving only the NMS site which is inefficiently post-translationally N-glycosylated and does not traffic to the cell surface. (C) A point mutant (E1 T7I S28T) converting the NMS site into an NMT site shifts the N-glycosylation kinetics of this peptide back to co-translational N-glycosylation and forward trafficking is restored.

eliminates the first consensus site such that the second post-translational N-glycosylation site is the only site remaining (Figure I-12B). Although wild-type (wt) E1 efficiently traffics to the plasma membrane in the presence of Q1 [78], E1 T7I does not traffic despite co-expression of the channel. Surprisingly, it was discovered that if the second consensus site was converted from a NMS to a NMT site (E1 T7I S28T, Figure I-12C), this consensus site was switched from post- to co-translational N-glycosylation, and was again efficiently trafficked to the plasma membrane with Q1 [2].

Identifying primary sequence factors that can cause subtle shifts in N-glycan attachment rates and have drastic effects on protein expression and function is a crucial topic of study which has yet to be fully explored. This thesis focuses on defining the N-glycosylation kinetics for the consensus sites in the KCNE family, and uncovering several new determinants that affect the distribution of co- and post-translational N-glycosylation for type I transmembrane peptides.
CHAPTER II: CHARACTERIZATION OF N-GLYCOSYLATION CONSENSUS SITES IN THE KCNE FAMILY

Abstract

Type I transmembrane peptides acquire N-linked glycans during and after protein synthesis to facilitate forward 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: 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.
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 [14,15]. 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 [30,36] and membrane-embedded proteins [2,41,42]. Although either threonine or serine is required at the (n + 2) position [64], previous studies have shown that serine-containing consensus (NXS) sites are less efficiently N-glycosylated than the analogous threonine-containing consensus (NXT) sites [63,67,68]. This difference in N-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 [25]. 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 I-9). These N-terminally glycosylated membrane proteins co-assemble with and modulate several different voltage-gated K⁺ channels [73]. Mutations that destroy N-linked glycosylation sites in E1 and E2 give rise to Long QT syndrome (LQTS) [122,123], a disorder of the cardiac rhythm that is sometimes accompanied with neural deafness [125]. 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 [2]. 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.
Materials and Methods

Cloning and plasmids: Human KCNE1 – KCNE5 were cloned into pcDNA™3.1 vector such that five additional methionine residues and an HA tag (YPYDVPDYA) were added to the C-terminus of each KCNE peptide [2]. 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 µg KCNE DNA and 80 µL 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 x 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 100µCi/mL EasyTag EXPRESS [35S] Protein Labeling Mix (Perkin Elmer). The radioactive media was removed and the cells washed with PBS (2 x 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 x 4 mL), and lysed with 750 µL 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 fluoride (PMSF) and 1µg/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 x 750 µL) in low salt lysis buffer. After pelleting the cell debris, the supernatant was precleared with 50 µL 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 µL beads pre-incubated with
1 µL 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 x 750 µL); high salt buffer (1 x 750 µL): 50 mM 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 x 750 µL). For enzymatic deglycosylation assays, 1 µL Endo Hf (New England Biolabs) was added to beads in 50 µL low salt lysis buffer and incubated at 37°C for 1 hour. Peptides were eluted from the beads with (50 µL 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 [2,30,126]. 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 [2]. To observe these rapid kinetics in radioactive pulse-chase experiments requires extremely short (2 min) pulses of [\(^{35}\text{S}\)] methionine and cysteine. 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 metazoans except *Caenorhabditis* [14], 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 [2]. In addition, the subsequent Golgi modifications in CHO cells mirrors both the N- and O-glycosylation of E1 observed in cardiomyocytes [127] where all five KCNE peptides are expressed [102,103]. 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 (Figure II-1).

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 II-2). Cells were metabolically labeled with $^{[35}S]$, 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 II-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 II-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 II-2B) showed that the amount of glycosylated material slowly increased over the entire time course. As expected, we observed similar results in other cell lines (Figure II-3).

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 I-5). Although both modes of N-glycan attachment are rapid, post-translational N-glycosylation is substantially slower than co-translational [2,30]. 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
Figure II-1. Experimental design for pulse-chase assay with the KCNE family. Upper left: A KCNE construct (with 5 extra methionines and an HA tag) is expressed in CHO cells. On the pulse-chase day, cells are starved for 30 minutes to deplete cellular methionine and cysteine which stops translation, pulsed for 2 minutes in media containing $^{35}$S methionine and cysteine, and chased from 0-12 minutes at 3 minute intervals. Several purification steps are performed as outlined in the Material and Methods section. A pulse-chase result is shown for wtE1. Each band per lane shows a different glycan species. To graph the data, the maximally N-glycosylated species is quantified and graphed as a percentage of total protein per time point. The N-glycosylation kinetics can further be divided by co- (0 minute glycosylation) and post (12 minute - 0 minute glycosylation) contributions.
Figure II-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 ± s.e. from 3 – 4 experiments. Co- and post-translational glycosylation percentages are shown for wtE4.
Figure II-3. N-glycosylation kinetics of threonine and serine sites are similar in different mammalian cell lines. (A) Representative gels of E4 T10 and T10S constructs expressed in CHO (top), HEK (middle), and HeLa (bottom) cells. (B) Plots of the maximally glycosylated species (1-Gly): E4 T10 (top); E4 T10S (bottom). Error bars are ± s.e. from 3-4 experiments; no error bars indicate n = 1.
chase periods as post-translational (see Experimental). Using this convention, only 16% of the N-glycosylated E4 protein is post-translationally glycosylated (Figure II-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 II-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 II-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 II-4A and II-4C). 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 II-4B and II-4D). The N-glycosylation kinetics at N6 was
<table>
<thead>
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<th></th>
<th>% Total</th>
<th>% Co-trans.</th>
<th>% Post-trans.</th>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td>83.6 ± 7.0</td>
<td>16.4 ± 7.7</td>
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<tr>
<td>T10S</td>
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<td>49.9 ± 4.5*</td>
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<tr>
<td>E4 (XS)</td>
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<td>50.1 ± 1.3*</td>
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<td><strong>E2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wt</td>
<td>92.9 ± 2.0</td>
<td>82.1 ± 5.9</td>
<td>17.9 ± 6.0</td>
</tr>
<tr>
<td>T8S</td>
<td>69.5 ± 2.7*</td>
<td>64.1 ± 3.6*</td>
<td>35.9 ± 4.9*</td>
</tr>
<tr>
<td>T31S</td>
<td>38.4 ± 1.1*</td>
<td>65.3 ± 3.7*</td>
<td>34.7 ± 4.3*</td>
</tr>
<tr>
<td>N6, T8</td>
<td>92.5 ± 2.5</td>
<td>80.5 ± 3.7</td>
<td>19.5 ± 4.1</td>
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<tr>
<td>N6, T8S</td>
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<td>52.5 ± 5.4*</td>
<td>47.5 ± 5.7*</td>
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<tr>
<td>N29, T31</td>
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<td>71.0 ± 1.8</td>
<td>29.0 ± 2.3</td>
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<td>N29, T31S</td>
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<td>ND</td>
</tr>
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<td><strong>E5</strong></td>
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<td>79.0 ± 2.8</td>
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<tr>
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<td>72.5 ± 2.3</td>
<td>27.5 ± 2.9</td>
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<td>77.4 ± 3.7</td>
<td>22.6 ± 3.7</td>
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<td>N25, S27</td>
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<td>67.0 ± 1.8</td>
<td>33.0 ± 2.0</td>
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<td><strong>E3</strong></td>
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<td></td>
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<td>63.4 ± 7.6</td>
<td>36.6 ± 8.8</td>
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<tr>
<td>wt (XL)#</td>
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<td>12.6 ± 5.1</td>
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<tr>
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<tr>
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<td>14.2 ± 6.0</td>
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<td>N41 (XL)</td>
<td>30.6 ± 0.2</td>
<td>71.4 ± 4.6*</td>
<td>28.6 ± 4.6*</td>
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Table II-1. KCNE N-glycosylation. Total 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 ± 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.
Figure II-4. 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 ± s.e. from 3 experiments.
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 II-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 II-4B) 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 II-5A). 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 II-5B and II-5C). 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
**Figure II-5. 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 ± s.e. from 3 – 5 experiments.
single consensus site mutants (Table II-1), with the N25, S27 site (Figure II-5C) 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 I-9, NXT: N5, N22 and N41). Despite having three NXT sites, wtE3 is a poor substrate for N-glycosylation (Figure II-6A), with over half of the metabolically-labeled protein remaining unglycosylated after 12 min. Of the N-glycoforms (confirmed by Endo H, Figure II-7), 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 II-6A and II-6B: 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-
Figure II-6. 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 ± s.e. from 3 – 4 experiments. Cartoon of the E3 N-terminus depicting the three consensus sites.
Figure II-7. Identification of unglycosylated and glycosylated E3 bands. For wtE3, endoglycosidase H treatment of wtE3 identified the unglycosylated band, which confirmed that the four bands were 0 – 3-Gly. For wtE3 (XL), a singly glycosylated XL construct (N22 (XL)) was run alongside the wtE3 protein to identify the different glycoforms.
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 [8]. 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 II-6A and II-6B: closed symbols). The unglycosylated and differently glycosylated bands were identified by comparing gel mobilities to a singly-glycosylated construct (Figure II-7). 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 II-6C). 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 II-6D). 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 II-2 and II-4). 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 [63]. Although we observed a threonine preference for co-translational N-glycosylation with type I transmembrane peptides, a similar preference has not been observed with water soluble proteins [30]. This discrepancy can be attributed to the insertion mechanism of type I transmembrane peptides into the ER translocon (Sec 61) [8]. For type I transmembrane peptides, the N-terminal N-linked consensus sites are inserted into the ER lumen en masse after the transmembrane domain exits the ribosomal tunnel (Figure I-2). 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 I-9). 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 [30] (Figure I-5). Recently, a
hydroxyl residue preference was also discovered for type II transmembrane
proteins [42], 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 II-5) and the E1 N-terminal
consensus site (Figure II-8) 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 II-4). 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 II-5A), 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
Figure II-8. Examination of the E1 N5 consensus site shows no hydroxyl preference. (Left) Cartoon of the E1 N-terminus. (Center) Pulse-chase results for the first E1 glycosylation consensus site, N5, which lacks the second site at N26: NXT, solid squares; NXS, open squares. (Right) The maximally glycosylated species (1-Gly) of the T7 and T7S constructs was plotted for each time point. Error bars are ± s.e. from 3 experiments.
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 I-1A) 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 SRP and delivered and inserted into the ER translocon before protein translation is complete [8]. 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 may instead be inserted by a post-translational ER translocation mechanism [44]. 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 (Materials and Methods), the C-terminus of all constructs were actually 18 aa longer; therefore, for true wild type E3—which is N-glycosylated [128]- 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 [78], mutations can derail the process, leading to un- and hypoglycosylated 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 [2]. 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 II-4B). 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 II-4B).

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 II-2 and II-4) may be sufficient to result in a disease state. Consistent with this mild hypoglycosylation/haploinsufficiency hypothesis are the Brugada syndrome (E3: T4A) [124] and antibiotic-induced long QT syndrome mutations (E2: T8A, Q9E) [77,123] 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.
CHAPTER III: CONSENSUS SITE MIDDLE RESIDUE AFFECTS THE DISTRIBUTION OF CO- AND POST-TRANSLATIONAL N-GLYCOSYLATION FOR TYPE I TRANSMEMBRANE PEPTIDES

Abstract

Asparagine (N)-linked glycosylation is essential for efficient protein folding in the endoplasmic reticulum (ER) and anterograde trafficking along the secretory pathway. N-glycans are attached to a nascent polypeptide at a consensus site of N-X-T/S-Y (X,Y ≠ P) by one of two enzymatic isoforms of the oligosaccharyltransferase (OST), STT3A or STT3B. We show that these two isoforms are linked to co- and post-translational N-glycosylation respectively for type I transmembrane peptides, in agreement with previous findings for other peptide topologies. We also examined the effect of the consensus site X residue on the distributions of co- and post-translational N-glycosylation on a type I transmembrane scaffold peptide (KCNE2). For NXS sites, large hydrophobic and negatively charged residues significantly hinder co-translational N-glycosylation. This deficiency in co-translational N-glycosylation can be recovered by conversion to an NXT site. The post-translational rates of N-glycosylation were also examined and a correlation was found between the amount of co-translational N-glycosylation and the rate of post-translational N-glycosylation, implicating similar enzymatic mechanisms for the OST STT3 isoforms.
Introduction

Asparagine (N)-linked glycosylation occurs for the vast majority of secretory and membrane bound proteins that require an N-glycan in order to ensure efficient trafficking out of the endoplasmic reticulum (ER). The covalent attachment of the 14-sugar oligosaccharide to a nascent chain at a consensus site of N-X-T/S-Y, where X and Y can be any amino acid except proline, is catalyzed by the oligosaccharyltransferase (OST) [14,15]. This ER luminal membrane protein complex is composed of seven or eight individual subunits in eukaryotes and undergoes a vast array of diverse functions, including positioning the lipid-linked oligosaccharide donor and scanning and positioning a peptide chain for N-glycosylation. This process can occur co-translationally along side the Sec61 complex, which transfers growing peptides across or into the ER membrane [8], or post-translationally after the peptide is fully synthesized [8,36].

The OST catalytic subunit STT3 is the only domain of the complex that is conserved from eukaryotes to eubacteria [17]. For vertebrates, insects, and plants, there are two known eukaryotic isoforms of the OST catalytic subunit designated STT3A and STT3B, and these have recently been linked to two, temporally distinct pathways of N-glycosylation for water-soluble, type II, and multi-spanning transmembrane glycoproteins [30,41,42]. Utilizing kinetic assays, the OST STT3A isoform has been shown to predominantly perform co-translational N-glycosylation while the OST STT3B isoform preferentially N-
glycosylates peptides post-translationally. Intriguingly, STT3B was found to perform co-translational N-glycosylation if STT3A is depleted, but STT3A does not perform post-translational N-glycosylation in the absence of STT3B [30], implicating at least affinity differences between these different OST isoform complexes. No structure of either STT3 isoform has been solved; however, crystal structures for the OST homologs of bacterial *C. lari* PglB [25] and archaeal *A. fulgidus* AglB [26] have been determined. Despite having only 20% sequence identity, these structures were remarkably similar and were concluded to function by a mutually conserved catalytic mechanism.

Primary sequence context of an N-linked glycosylation consensus site has been known to effect the efficiency of N-glycan attachment, including the hydroxyl residue in the consensus site [63,64], the middle residue in the consensus site [67,68], specific residues upstream or downstream of the consensus site [69,71] including the residue immediately following the hydroxyl position [66], and the proximity of the consensus site to other consensus sites [39] and the C-terminus [42]. Although many factors are known that affect the efficiency of N-glycosylation, particularly for NXS consensus sites, a plausible mechanism for this disparity has not been determined, nor have the distributions of co- and post-translational N-glycosylation efficiencies and the biophysical ramifications of these differences in the context of the OST STT3A and STT3B isoforms been characterized.
Here, we use a type I transmembrane scaffold glycopeptide (KCNE2) to determine the co- and post-translational N-glycosylation distributions for all amino acids in the middle residue of the consensus site. We have found that bulky hydrophobic residues and negatively charged residues hinder co- and post-translational N-glycosylation, while small hydrophobics, positively-charged, and polar residues are efficiently N-glycosylated through co-translational N-glycosylation. We determine the post-translational rates of N-glycosylation for X residues in NXS sites and report a correlation between the post-rate and the amount of co-translational N-glycosylation. Finally, we identify the enzymatic isoforms associated with N-glycosylation mechanisms for NXS and NXT type I transmembrane glycopeptide consensus sites: STT3A predominantly performs co-translational N-glycosylation and STT3B predominantly performs post-translational N-glycosylation. This is in agreement with previously determined roles for these OST subunits on water-soluble and type II transmembrane proteins [30,42]. Our finding that a correlation exists for co- and post-translational N-glycosylation of NXS sites provides evidence that STT3A and STT3B glycosylate at similar efficiencies, and implies that these isoforms likely function by a conserved catalytic mechanism.
Materials and Methods

Cloning and plasmids: Human KCNE2 and KCNE4 were cloned into pcDNA™3.1(−) vector such that five additional methionine residues and an HA tag (YPYDVPDYA) were added to the C-terminus of each KCNE peptide [2,40]. The second N-glycosylation consensus site was removed in KCNE2 by mutation to glutamine (N29Q). Mutations for creating different X residues in NXS or NXT sites were introduced using traditional PCR cassette mutagenesis (Syzygy Biotech Taq 2x MeanGreen Master Mix) between the 5′ KpnI and 3′ BglII sites. All constructs were confirmed by DNA sequencing the entire gene.

Cell culture and plasmid transfection for western blot: Chinese hamster ovary-K1 (CHO) cells were maintained 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 35 mm dishes at 80% confluency. After 24 h, the cells were transiently transfected with a mixture of 1.5 µg KCNE DNA and 8 µL Lipofectamine in 1 mL OptiMem. After 6 h at 37°C, the transfection cocktail was removed and 2 mL of F12K media with supplements was added and the cells were incubated for 40-45 h.
**Plasmid transfection for pulse-chase:** Cells were passaged using 0.5% Trypsin-EDTA and plated onto 100 mm dishes at 80% confluency. After 24 h, the cells were transiently transfected with a mixture of 3 µg KCNE DNA and 80 µL Lipofectamine in 2 mL OptiMem. After 6 h at 37°C, the transfection cocktail was removed and 10 mL of F12K media with supplements was added and the cells were incubated for 15-20 h.

**siRNA transfection for pulse-chase and western blot:** Cells were passaged using 0.5% Trypsin-EDTA and plated onto 100 mm dishes at 80% confluency. After 24 h, the cells were transiently transfected with a mixture of small interfering RNA (siRNA):

- *(scr. A+B)*: 100 nmol scrambled A (GAGAACUAACGCAAGAGAAdTdT) + 150 nmol scrambled B (GAAGGCACCGGUAAUAGUdTdT);
- *(A)*: 100 nmol A (GAAGACACAUCAAGGAGAAdTdT);
- *(B)*: 150 nmol B (GGACUACUCUGGUGGAUAAdTdT); or
- *(A+B)*: 100 nmol A + 150 nmol B.

Incubated 5 min individually before combining with 80 µL Lipofectamine 2000 in OptiMem for 30 min, then diluted into 2 mL OptiMem. After 6 h at 37°C, the transfection cocktail was removed and 2 mL of F12K media with supplements was added and the cells were incubated for 40-45 h. Another round of siRNA transfection was then performed as above, this time adding 3 µg E2 scaffold DNA along with the siRNAs. After another 6 h at 37°C, the transfection cocktail
was removed and 2 mL of F12K media with supplements was added and the cells were again incubated for 40-45 h.

**Pulse-chase assays and cell lysis:** Pulse-chase experiments and cell lysis were performed as previously described [40]. Briefly, transfected cells were washed with PBS 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 DMEM High Glucose Media containing 100µCi/mL EasyTag EXPRESS [35S] Protein Labeling Mix (Perkin Elmer). The radioactive media was removed and the cells were washed with PBS and chased with F12K media for 3, 6, 9, or 12 min at 37°C. The cells were then washed with PBS and lysed with 750 µL 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 fluoride (PMSF) and 1µg/mL each of leupeptin, pepstatin, and aprotinin (LPA). Cells were lysed for 30 min with vigorous shaking a 4°C, and the cell debris scraped and pelleted at 14,000 rpm for 10 min at room temperature.

**Radioimmunoprecipitation and electrophoresis for pulse-chase:** Radioimmunoprecipitation and electrophoresis for pulse-chase were performed
as previously described [40]. Briefly, Protein G agarose beads (Pierce) were prepared by washing in low salt lysis buffer. After pelleting the cell debris, the supernatant was precleared with 50 µL beads for 2 hours at 4°C. The beads were then spun down, and the supernatant transferred to new tubes containing 25 µL beads pre-incubated with 1 µL 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 3 washes of low salt lysis buffer, 1 wash of high salt buffer: 50 mM 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. For enzymatic deglycosylation assays, 1 µL Endo Hᵢ (New England Biolabs) was added to beads in 50 µL low salt lysis buffer and incubated at 37°C for 1 hour. Peptides were eluted from the beads with 100 mM DTT and 2x SDS gel loading buffer at 55°C for 15 min. Samples were analyzed by 15% SDS-PAGE 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 14 – 42 d.

**Cell lysis and electrophoresis for western blots:** Cells were washed in PBS (3 x 750 µL) and treated with RIPA cell lysis buffer: 10 mM TRIS-HCl, pH 7.4, 140 mM NaCl, 1 mM EDTA pH 7.4, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, supplemented with protease inhibitors (PMSF and LPA, see pulse chase). 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. Total protein in each sample was determined by bicinchonimic acid (BCA) assay and 60 µg protein utilized for electrophoresis. Samples were prepared by adding 100 mM DTT and 2x SDS gel loading buffer to the lysis sample to a total volume of 50 µL. Samples were analyzed by 8% SDS-PAGE, transferred to nitrocellulose membrane, and blocked for 30 min in milk (5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween-20 (Western wash buffer)). Primary antibody (STT3A 1:2,500 or STT3B 1:6,000) was obtained from R. Glimore’s lab [30]. The primary antibody was added overnight by diluting into Western blocking buffer. Membranes were then washed in Western wash buffer (3 x 6 mL) for 5 min each, and secondary antibody (STT3A 1:4,000 or STT3B 1:10,000 [30] was added in Western blocking buffer for 45 min at room temperature. More washes were performed (3 x 6 mL western wash buffer) and the membrane was incubated with SuperSignal West Dura Extended Duration Substrate (Pierce) for 5 min and the chemiluminescence captured using a LAS-3000 CCD camera and quantified by Multi Gauge software (Fujifilm).

Determination of co- and post-translational N-glycosylation: All signals were quantified using Image Gauge software (Fujifilm) [40]. For all constructs, percentage of glycosylation was determined by dividing the signal of the glycosylated species by the total amount of signal (0-Gly + 1-Gly) per time point. Percent total N-glycosylation is the amount present at the end of the time course
(12 min) and the error in total N-glycosylation graphs is that associated with this time point. To determine the co-translational N-glycosylation contribution for each construct, we divided the percent N-glycosylated at 0 min by the percent N-glycosylated at 12 min. Our simplified calculation for co-translational N-glycosylation assumes that the peptide substrates are fully synthesized before the 3 min time point [72] and that the observable rates for co- and post-translational N-glycosylation are substantially different for this class of peptides [2]. For the post-translational N-glycosylation contribution, we subtracted out the 0 min contribution (12 - 0 min percent N-glycosylation) and divided this by the total N-glycosylation (12 min). For percent total N-glycosylation graphs, co- (solid bars) and post- (slashed bars) contributions are shown, with the errors between this interface shown for both co- (up error) and post- (down error). Post-translational N-glycosylation kinetic rates ($k$) were determined by fitting the data to a one-phase association equation: $Y = Y_0 + (\text{Plateau} - Y_0)(1-e^{-kx})$. 
Results

In order to determine the consensus site X residue effects on co- and post-translational N-glycosylation for type I transmembrane peptides, we first needed to identify an ideal, kinetically sensitive peptide candidate that would allow for the highest resolution of N-glycan attachment mechanistic shifts. We previously determined that the N-glycosylation kinetics of the KCNE family (E1-E5) are sensitive to primary sequence changes and the resulting shifts in co- and post-translational N-glycosylation readily detected by metabolic pulse-chase [40]. We chose E2 and E4 as the most probable candidates for determining middle residue effects as their N-glycosylation kinetics are well understood and have robust radiolabel incorporation for amplified detection of glycoforms. wtE2 has two N-glycosylation consensus sites, NFT and NTT, and wtE4 has one consensus site, NST. Since it is known that NXS consensus sites have higher sensitivity to the X residue for N-glycosylation efficiency [67], we decided to first look at X residues in the presence of NXS sites for E2 and E4. The second N-glycosylation consensus site in E2 was removed by asparagine mutation to glutamine for simplified tracking of the N-glycosylation kinetics of this peptide.

Based on previous consensus site X residue efficiency rankings [67], we first chose to observe the N-glycosylation kinetics of E2 and E4 peptides using three ranging residues: NSS, NFS, and NWS (Figure III-1A). The unglycosylated (0-Gly) and singly glycosylated (1-Gly) species were readily observable to
Figure III-1. E2 and E4 N-glycosylation kinetics of NXS sites alter co- and post-translational distributions. (A) Cells were pulsed for 2 min and chased for indicated times. Immunoprecipitated E2 (one NXS site at N6) and E4 (one NXS site at N8) proteins were separated by electrophoresis and detected by autoradiography. Glycosylated (1-Gly) and unglycosylated (0-Gly) species are labeled for NSS, NFS, and NWS consensus sites. 1-Gly species not detected for E4 NWS. (B) EndoH treatment for E2 and E4 NXS consensus sites. All experiments were performed at the same time. Glycosylation species are labeled. (C) Left graph: Plots of maximally glycosylated (1-Gly) species for NSS sites in E2 (black triangles) and E4 (gray triangles). Co- and post-translational N-glycosylation divisions are shown. Right graph: Quantification for maximally glycosylated (1-Gly) species for all E2 and E4 sites: E2 (black), E4 (gray): NSS (triangles), NFS (squares), NWS (circles). (D) Co-, post-, and total N-
glycosylation for E2 (black) and E4 (gray) consensus sites. Total N-glycosylation divisions show co- (solid) and post- (slashed) translational contributions with errors for co- (middle bar, up), post- (middle bar, down), and total (top bar, up) N-glycosylation. Error bars are ± s.e. from 3 experiments. n.d., not detected.
varying intensities for all consensus sites except for E4 NWS where no 1-Gly species was observable. The 0-Gly species was confirmed by endoglycosidase H digestion for peptides where both species were not readily detected (Figure III-1B). We first quantified our signal as was previously reported [40]; for each peptide and construct, the 1-Gly intensity was normalized to the total glycosylation for each time point, and the resulting percentage of maximal glycosylation graphed (Figure III-1C). The co- and post- contributions can be determined from the total glycosylation by exploiting the temporal distinctions between these two mechanisms. Because post-translational N-glycosylation is significantly slower than co-translational N-glycosylation [30], a simplified convention can be utilized where the co-translational contribution is defined as what is present at the beginning of the chase period (0 min) and the post-translational contribution is defined as the increase in glycosylation seen over the time course (12 min – 0 min; Figure III-1C left). Because these X residue consensus site kinetic differences can be slight and difficult to observe directly on a linear plot (Figure III-1C right), we chose to individually graph the co-translational, post-translational, and total N-glycosylation for each peptide’s consensus site (Figure III-1D). Now it is readily observable that while E2 and E4 both show varying N-glycosylation kinetic rates from varying consensus site X residues, E2 appears to be more sensitive to these changes, ranging from about 90% to 20% N-glycosylation between S and W in total N-glycosylation (Table III-1).
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<td>S</td>
<td>22.0 ± 1.9</td>
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<tr>
<td>F</td>
<td>31.0 ± 3.1</td>
<td>28.0 ± 3.3</td>
<td>59.0 ± 1.2</td>
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<td>I</td>
<td>38.2 ± 3.1</td>
<td>23.1 ± 3.4</td>
<td>61.3 ± 1.2</td>
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<td>E</td>
<td>40.2 ± 4.2</td>
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<td>63.4 ± 2.6</td>
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<td>M</td>
<td>46.2 ± 0.4</td>
<td>26.2 ± 0.7</td>
<td>72.4 ± 0.6</td>
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<td>16.2 ± 5.2</td>
<td>90.7 ± 1.1</td>
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<td><strong>E2 NXT</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
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<td>41.9 ± 1.8*</td>
<td>30.5 ± 2.6*</td>
<td>72.4 ± 1.9*</td>
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<tr>
<td>L</td>
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<td>17.9 ± 2.3</td>
<td>86.2 ± 2.2*</td>
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<tr>
<td>E</td>
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<td>17.3 ± 5.7</td>
<td>88.1 ± 5.6*</td>
</tr>
<tr>
<td>F</td>
<td>74.4 ± 2.8*</td>
<td>18.0 ± 3.7</td>
<td>92.5 ± 2.5*</td>
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Table III-1. Consensus site middle residue N-glycosylation. Co-translational, post-translational, and total N-glycosylation shown for each E2 or E4 NXS or NXT middle residue consensus site. The percentages of co- and post-translational N-glycosylation were determined as described in the methods section. Values are mean ± s.e.m. for 3 pulse-chase experiments. n.d., not determined. *, p < 0.05 (one-way ANOVA for NXS sites, two-way ANOVA for NXS vs. NXT sites) * next to values indicates significance by Tukey’s multiple comparison test (E2 NXT values compared to equivalent E2 NXS sites; siRNA knockdown values compared against scr. A+B control).

<table>
<thead>
<tr>
<th></th>
<th>E2 NFT (OST STT3 knockdown)</th>
<th>E2 NFS (OST STT3 knockdown)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>scr. A+B</td>
<td>A</td>
</tr>
<tr>
<td>scr. A+B</td>
<td>63.8 ± 2.0</td>
<td>24.0 ± 4.1</td>
</tr>
<tr>
<td>A</td>
<td>63.8 ± 2.3</td>
<td>24.3 ± 3.4</td>
</tr>
<tr>
<td>B</td>
<td>51.1 ± 3.7*</td>
<td>14.6 ± 4.0*</td>
</tr>
<tr>
<td>A+B</td>
<td>22.0 ± 5.0*</td>
<td>n.d.</td>
</tr>
</tbody>
</table>
We next determined the N-glycosylation kinetics for all consensus site middle amino acids (except proline) using E2 as a scaffold type I transmembrane peptide (Figure III-2A). The co-translational, post-translational, and total N-glycosylation quantifications are shown in Figure III-2B. The co-translational N-glycosylation of consensus sites is most affected by large hydrophobic (left graph, green bars) and negatively charged residues (coral bars). Polar and small hydrophobics (purple bars) as well as positively charged residues (navy bars) are efficiently co-translationally N-glycosylated NXS sites. In agreement with the temporal divisions between co- and post-translational N-glycosylation, the largest post-translational contributors are the less efficiently co-translationally N-glycosylated NXS sites (Figure III-2B, middle graph). The availability of a larger pool of unglycosylated substrates most likely enhances this contribution for post-translational N-glycosylation. However, this slight increase is not enough to compensate for the poor co-translational contributions for the large hydrophobic middle residues, keeping the total N-glycosylation efficiency for these consensus sites significantly below the other sites (Figure III-2B, right graph).

We next wanted to see if our poorest N-glycosylation NXS sites could be recovered to higher N-glycosylation efficiencies by mutation to NXT sites as has been previously observed [40,42], and determine which N-glycosylation contributions would be greatest effected for each middle residue assayed. We observed the N-glycosylation kinetics for NWT, NLT, NET, and NFT and found that all consensus sites were significantly improved (Figure III-2C). NWT and
Figure III-2. N-glycosylation of an E2 NXS site is most hindered by large hydrophobic and negatively charged residues at the X position. (A) Representative fluorographs from pulse-chase experiments for X residues in an E2 NXS consensus site. Glycosylated (1-Gly) and unglycosylated (0-Gly) species are labeled. Side: E2 NCS glycospecies confirmation by EndoH. (B) Graphs for co-, post-, and total N-glycosylation contributions for the consensus site X residues. Coloring by the biophysical properties of each residue: polar and small hydrophobics (purple), positively charged (navy), negatively charged (coral), large hydrophobics (green). Total N-glycosylation divisions show co- (solid) and post- (slashed) translational contributions with errors for co- (middle bar, up), post- (middle bar, down), and total (top bar, up) N-glycosylation. (C) Left: Representative fluorographs from pulse-chase experiments for several NXT sites. Glycosylated (1-Gly) and unglycosylated (0-Gly) species are labeled. Right: Graph showing total N-glycosylation with co- (solid bars) and post- (slashed bars) translational N-glycosylation divisions for NXS (black) and NXT (purple) sites with common X residues. Errors are shown for co- (middle bar, up), post- (middle bar, down), and total (top bar, up) N-glycosylation. Error bars are ± s.e. from 3 experiments. n.d., not detected.
NLT were more efficient for both co- and post-translational N-glycosylation than their NXS counter parts. NET and NFT saw improvement in only co-translational N-glycosylation, again implicating the temporal divide of post-translational N-glycosylation with a finite pool of peptide substrates and indicating that the highest level of N-glycan occupancy has been reached for these sites.

We next wanted to quantify the kinetic rates for post-translational N-glycosylation of the middle X residue consensus sites to see how these findings compared to the measured co-translational N-glycosylation. While a precise, physiological rate of N-glycosylation from our pulse-chase assay can not be determined due to an inability to control substrate, enzyme, and competitor concentrations as well as the rate of protein degradation in living cells, we can compare our observed N-glycosylation kinetic rates for each middle X residue as a controlled approximation. We first scaled our pulse-chase kinetics to zero to remove the co-translational N-glycosylation contribution and fit the data to a one-phase association equation (Figure III-3A). We observed a significant difference among post-translational N-glycosylation rates for different middle residues, where once again large hydrophobic residues had the slowest rates of N-glycosylation (Figure III-3B, upper graph and Table III-2). We next compared the post-translational N-glycosylation kinetic rate of each middle X residue to its measured amount of co-translational N-glycosylation (Figure III-3B, lower graph), and found a significant correlation ($R^2 = 0.76$). This is the first evidence by detection of N-glycan attachment that co- and post-translational N-glycosylation...
Figure III-3. **Post-translational N-glycosylation rates reveal a similar efficiency towards consensus site X residues as co-translational N-glycosylation.** (A) Kinetics of post-translational N-glycosylation for each X residue with measurable post- contributions. Scaled to zero for direct comparison. (B) Post-translational N-glycosylation rates for consensus site X residues. Coloring by the biophysical properties of each residue: polar and small hydrophobics (purple), positively charged (navy), negatively charged (coral), large hydrophobics (green). One-way ANOVA performed showing p < 0.05. (C) Correlation of co- and post-translational N-glycosylation efficiencies for varying X residues. Coloring is the same as in (B). $R^2 = 0.76$, p < 0.05. Error bars are ± s.e. from 3 experiments. n.d., not detected.
<table>
<thead>
<tr>
<th>Residue</th>
<th>Post-rate (min⁻¹)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>S</td>
<td>0.284 ± 0.050</td>
</tr>
<tr>
<td>R</td>
<td>0.253 ± 0.022</td>
</tr>
<tr>
<td>N</td>
<td>0.247 ± 0.066</td>
</tr>
<tr>
<td>H</td>
<td>0.226 ± 0.053</td>
</tr>
<tr>
<td>C</td>
<td>0.221 ± 0.112</td>
</tr>
<tr>
<td>Y</td>
<td>0.205 ± 0.073</td>
</tr>
<tr>
<td>G</td>
<td>0.194 ± 0.062</td>
</tr>
<tr>
<td>Q</td>
<td>0.189 ± 0.033</td>
</tr>
<tr>
<td>V</td>
<td>0.187 ± 0.015</td>
</tr>
<tr>
<td>E</td>
<td>0.183 ± 0.036</td>
</tr>
<tr>
<td>D</td>
<td>0.176 ± 0.077</td>
</tr>
<tr>
<td>T</td>
<td>0.194 ± 0.034</td>
</tr>
<tr>
<td>A</td>
<td>0.146 ± 0.048</td>
</tr>
<tr>
<td>K</td>
<td>0.123 ± 0.020</td>
</tr>
<tr>
<td>M</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>L</td>
<td>0.002 ± 0.002</td>
</tr>
<tr>
<td>W</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

**Table III-2. NXS middle residue post-translational N-glycosylation rates.**

Post-translational N-glycosylation rates for all X residues in the E2 NXS consensus site. Rates were averaged from independent pulse-chase experiments where the association rate (k) was determined by fitting the data to a one-phase association equation: \( Y = Y_0 + \text{Plateau} - Y_0 (1 - e^{-kx}) \). Values are mean ± s.e.m. for 3 experiments, except for NKS which is 2 experiments. n.d., not detected. *, p < 0.05 (one-way ANOVA).
work at similar efficiencies, and therefore the OST STT3 isoforms associated
with each pathway most likely work by a similarly conserved mechanism.

To definitively link the two N-glycosylation pathways to specific STT3
enzymatic isoforms for type I transmembrane peptides, we adapted an STT3
small interfering RNA (siRNA) knockdown approach [30,42] for CHO cells as
described in the Methods section. Using two siRNA transfections, we were able
to knockdown STT3A 73.8 ± 3.3% alone and 57.3 ± 4.3% in combination with
STT3B. STT3B was knocked down 62.8 ± 6.0% alone or 69.7 ± 2.3% in
combination with STT3A. Scrambled sequences or siRNA for the opposite
isoform had no significant effect on STT3 protein levels (Figure III-4).

For these experiments we chose the native E2 phenylalanine middle
residue as our N-glycosylation substrate. Pulse-chases were performed with
transfection of NFT or NFS DNA along with an siRNA treatment of scrambled
A+B, A, B, or A+B (Figure III-5A). For the NFS site, we saw a significant
reduction of co-translational N-glycosylation when treated with A+B siRNA
(Figure III-5B). Our observation that knocking down STT3A alone does not affect
co-translational N-glycosylation is in agreement with previous findings that
STT3B can fulfill a co-translational role when STT3A is not present [30]. We are
confident that this is occurring because while knocking down STT3B alone had
no effect on co-translational N-glycosylation, adding STT3A with STT3B
produces significant reduction (Figure III-5B, NFS co-contribution double
knockdown). Post-translational N-glycosylation is significantly reduced for STT3B
Figure III-4. OST STT3A and STT3B isoforms are specifically and efficiently knocked down upon siRNA treatment. Representative western blots for STT3A (top) and STT3B (bottom). Graphs for each are shown directly below each image, each knockdown is normalized to the scrambled A+B treatment. Error bars are ± s.e. from 3 experiments. M.M., microsomal membranes. Scr. A, Scr. B, A, and B lanes depict treatment with that siRNA. •, non-specific band. *, p < 0.05.
Figure III-5. OST catalytic isoforms STT3A and STT3B perform co- and post-translational N-glycosylation, respectively, for type I transmembrane peptides in NXS and NXT sites. (A) Representative fluorographs from pulse-chased siRNA treated CHO cells expressing either E2 NFS or E2 NFT. The specificity of each siRNA is shown on the left. Glycosylated (1-Gly) and unglycosylated (0-Gly) species are labeled. (B) Graphs for co- (top), post- (middle), and total (bottom) N-glycosylation contributions for NFS (black) and NFT (gray) consensus sites. Total N-glycosylation divisions show co- (solid) and post- (slashed) translational contributions with errors for co- (middle bar, up), post- (middle bar, down), and total (top bar, up) N-glycosylation. (C) Post-translational N-glycosylation rates shown for E2 NFT and NFS sites under scrambled double knockdown conditions. The fits are extrapolated back to -2 minutes. Vertical dashed line marks 0 minute. Horizontal dashed line marks y-intercept. The percentage on each graph is the difference between the 0 minute and -2 minute intercepts. Error bars are ± s.e. from 3 experiments. n.d., not detected. *, p < 0.05.
and double knockdowns to the extent that these are no longer detectable. This also is in agreement with the previous finding that STT3A does not compensate for post-translational N-glycosylation when STT3B levels are reduced [30].

STT3 knockdown for the NFT site proceeded similarly; co-translational N-glycosylation for STT3A knockdown alone showed no effect, but a significant reduction was seen for the double knockdown, and post-translational N-glycosylation was reduced for both knockdown of STT3B alone and in combination with STT3A. Surprisingly, we did see a slightly significant reduction in co-translational N-glycosylation upon knockdown of STT3B alone for the NFT site. Because this reduction is only present in the NFT site, we believe that this disparity arises from high efficiency of NXT sites and the limitations of our co- and post-translational N-glycosylation kinetic definitions. Figure III-5C shows the post-translational N-glycosylation kinetics for NFT and NFS peptides under the control knockdown (scr. A+B). When these fits are extrapolated back to -2 min, the beginning of the pulse, it is evident that the NFT site is twice as likely to contain post-translational events during this 2 min pulse period due to its higher kinetic rate. This indicates that some highly efficient N-glycosylation consensus sites might be slightly misrepresented in our co- and post- definitions and will be addressed in the discussion.
Discussion

We have identified the contributions of co- and post-translational N-glycosylation for NXS sites by systematically screening the middle residue in an E2 scaffold peptide. We found that large hydrophobic middle residues and negatively charged residues receive the least co-translational N-glycosylation. While these residues have a slightly higher rate of post-translational N-glycosylation, it is not sufficient to recapitulate the total N-glycosylation of more efficient N-glycosylation sites containing polar and positively charged residues.

We also examined the rates of post-translational N-glycosylation and found that when we compared these rates to the percent of co-translational N-glycosylation contribution there was a significant correlation between efficiencies of co-amount and post-rate implicating similar mechanisms for the OST isoforms. This implies that the amount of time a peptide spends adjacent to the OST is a key factor in consensus site recognition, especially for co-translational N-glycosylation where the peptide only remains in the associated translocon until translation is complete [8]. Thus, the kinetic differences we observe between co- and post-translational N-glycosylation are purely spatiotemporal in nature and not dependent on the mechanism of N-glycosylation.

We then identified the OST STT3 isoforms behind co- and post-translational N-glycosylation for NXS and NXT consensus sites, and found that STT3A predominantly performs co-translational N-glycosylation while STT3B
predominantly performs post-translational N-glycosylation, in agreement with the roles of these isoforms on other topological proteins [30,42]. While the NFS consensus site displayed high sensitivity to disruption of either OST STT3 isoform, STT3B knockdown of the NFT consensus site reduced co-translational N-glycosylation and did not completely inhibit post-translational N-glycosylation. This implicates our co- and post-translational N-glycosylation definitions are slightly misrepresented for highly efficient consensus sites.

While a 2 min pulse period is necessary to obtain enough metabolically labeled peptide, most likely there are a number of peptides that complete translation and exit the translocon during this period. Hemagglutinin, which is more than twice the size of the KCNEs, only takes ~ 1 min for detectable radiolabel incorporation in a CHO cell pulse-chase assay [72]. Using this as a standard, we would then expect KCNE peptides with highly efficient N-glycosylation sites, like NXT sites, to obtain some post-translational N-glycans during the pulse period. The post-translational N-glycosylation kinetics for NFT and NFS peptides extrapolated back to -2 min show that the NFT site is twice as likely to contain post-translational events during this 2 min window (Figure III-5C). This also explains why we don’t see a similar decrease in % co- for the NFS site upon STT3B knockdown (Figure III-5B, NFS B knockdown top graph). Therefore, a reduction of STT3B for NFT sites could be detected as a slight lost of co-translational as well as the expected reduction in post-translational N-glycosylation (Figure III-5B, NFT B knockdowns). Some post-translational N-
glycosylation would be expected in a B knockdown from the ~35% STT3B remaining in these knockdowns and the higher rate of N-glycosylation for these efficient NFT consensus sites.

Co-translational N-glycosylation is less likely to be present in our post-definition because of the rapid (< 1 min) depletion of metabolic radiolabel that has been observed during chase times using proteins with evenly distributed methionines [72]. The KCNEs are heavily back loaded with 5 extra methionines engineered onto the C-terminus for enhanced signal, which would further increase the rate of radiolabel depletion. Therefore, we would expect to observe only post-translation N-glycosylation in our co-definition for highly efficient N-glycosylation consensus sites. This would be represented in our data by slightly higher than expected co-translational N-glycosylation and lower than expected post-translational rates, which is exactly what is seen in the % co- and post-rate correlation plot of NXS sites (Figure III-3B lower graph). The highly efficient polar X residues are clustered slightly above and to the left of the fit. This finding also holds true for other KCNE consensus sites (Figure III-6). For an E1, E2, E4, and E5 single consensus site comparison, the more efficiently N-glycosylated consensus sites (all E5 sites, and E2 and E4 NXT sites) clustered slightly higher and to the left of an expected linear trend. Overall, these findings, while important for precise comparisons, do not affect the general determinants of co- and post-translational N-glycosylation for any KCNE consensus sites studied to date.
Figure III-6. A correlation between KCNE co-translational and post-translational N-glycosylation from several consensus sites shows co- and post-definition accuracy. Plot of % co- and post-translational rate for several KCNE consensus sites. N-glycosylation rates are from previously published findings [2,41]. All peptides are single consensus site KCNE constructs for a similar rate comparison. Number in each data point reflects the consensus site position (1\textsuperscript{st} or 2\textsuperscript{nd}) in that peptide. Black data points are the E2 NXS consensus site data shown in Figure III-3. $R^2 = 0.67$, $p < 0.05$. Error bars are ± s.e. from 3 experiments.
When we previously characterized the N-glycosylation kinetics of KCNE5 (E5), which contains two NXS sites, it was discovered that both sites were efficiently N-glycosylated despite a strong hydroxyl preference in the rest of the family [40]. Upon further examination, we now see that this likely is due to the role of the middle residue in these sites, which are cysteine and alanine, both efficiently N-glycosylated NXS consensus sites. This implies that unlike the other wtKCNEs that mostly contain NXT sites, E5 likely evolved an efficient middle residue over a more efficient hydroxyl residue, and implicates multiple avenues of evolution to ensure high N-glycan occupancy rate. In evidence, a recent computational study by R. Gilmore showed that amongst mouse glycoprotein NXS consensus sites determined to be occupied in vivo [129], cysteine in particular is overrepresented (unpublished data by personal communication).

While most of the observable rates of N-glycosylation for the KCNEs have been explained by the determinants identified to date (hydroxyl preference, number of consensus sites, length of the C-terminus, and type of middle residue) [2,40], several standing questions from the KCNE family remain. For instance, the second consensus site in E2 is not glycosylated as an NTS site when expressed alone, despite the efficient threonine middle residue. This hints at remaining determinants still to be characterized that effect N-glycosylation for type I transmembrane peptides. Further, defining how the dependency of these determinants on peptide length and peptides of other topologies would affect the
distribution of co- and post-translational N-glycosylation will be necessary for a broader understanding of N-glycan site occupancy.
CHAPTER IV: DISCUSSION AND FUTURE DIRECTIONS

High N-glycosylation consensus site occupancy is essential for efficient trafficking along the secretory pathway. Genetic mutations in any of the many proteins and enzymes that regulate this pathway can lead to catastrophic consequences and often present as diseases that classify under CDGs [52]. What is less clear is how a hypoglycosylated peptide substrate with an intact secretory pathway can also lead to disease, like the E1 T7I mutation leading to LQTS [122], especially when there are two opportunities for N-glycosylation to occur – i.e., co- and post-translational N-glycosylation – and there is evidence that some glycopeptides do not need to be N-glycosylated for ER exit and forward trafficking and some peptides that traffic through the secretory pathway do not contain N-glycosylation consensus sites [60].

Here, we approached these questions by identifying determinants in the KCNE type I transmembrane peptide family that altered the kinetics of N-glycan attachment. In Chapter II, we showed that threonine residues in the consensus site and coupled consensus sites are more efficiently co-translationally N-glycosylated and by the end of our time course have a higher rate of N-glycan site occupancy. We also showed that short C-termini hinder co-translational N-glycosylation, and as a result these glycopeptides have low N-glycan site occupancy. In Chapter III, we further characterized determinants by observing the N-glycosylation kinetics of the middle X residue in a consensus site and
found that large hydrophobic and negatively charged residues hinder both co- and post-translational N-glycosylation, resulting in lower N-glycan site occupancy.

**Structural modeling of X residue consensus sites**

While these findings will be helpful for defining N-glycan occupancy of a peptide simply through primary sequence analysis, several questions still remain to be answered. Although large hydrophobic X residues hinder co- and post-translational N-glycosylation kinetics, there is currently no structural explanation why these consensus sites hinder N-glycan attachment. To provide a plausible mechanism for OST STT3 N-glycosylation variation of diverse consensus sites, molecular modeling can be performed simulating different peptide consensus sites in the crystal structure of bacterial *C. lari* PglB [25]. Since the peptide crystalized in the recognition site of PglB contains a large angle bend centered around the middle residue of the consensus site (Figure I-4), we expect varying this residue would effect the time needed for equilibrium of the peptide into the recognition site. From our findings in Chapter III, we would predict that bulky hydrophobic residues may need significantly more time to equilibrate than small polar residues. We would also expect these findings to be applicable beyond PglB. While STT3A and STT3B share just 60% sequence similarity with each other and only 16% with PglB, the crystal structures for bacterial *C. lari* PglB and aracheal *A. fulgidus* AglB, despite 20% sequence homology, were found to be
remarkably similar [26]. This indicates that structural modeling in PglB would most likely be relevant for other OST homologs as well.

Identifying remaining molecular determinants

While working on characterizing the N-glycosylation kinetics of the first E5 and E1 consensus sites, we thought that perhaps the distance from the N-terminus might be aiding in the strong co-translational N-glycosylation of these peptides because they are at the second and fifth residues of the peptide respectively. We started by making an insertion mutation in E1 (adding ASA residues immediately following the methionine) and an E1 chimeric construct with E2 (residues leading up to the first site in E1 are the E2 sequence) (Figure IV-1A). We used E1 constructs that only contained the first consensus site for simplicity, and used an NTS site (as opposed to the native NTT site) for added N-glycosylation kinetic sensitivity. While the E1 add ASA construct has similar N-glycosylation kinetics to both the wild type N-terminal NTT or NTS sites, surprisingly there was a significant decrease in co-translational N-glycosylation in the E2/E1 chimera.

In a reciprocal experiment, in which the equivalent N-terminal sequence in E2 was swapped for that of E1 (E2 del S2, Figure IV-1B), there was also a significant shift in the N-glycosylation kinetics through an improvement in co-translational N-glycosylation. These findings indicate that either the length or primary sequence of the proximal N-terminus also influences N-glycosylation
Figure IV-1. Mutations at the proximal N-terminus of E1 can affect N-glycosylation kinetics. (A) *Left:* Cartoon depictions of E1 N-terminal constructs assayed. *Middle:* Representative fluorographs for E1 add ASA and E2/E1 chimera constructs. Glycosylated (1-Gly) and unglycosylated (0-Gly) species are labeled. *Right:* Plots of maximally glycosylated (1-Gly) species for: wild type N-terminus E1 sequence with an NTT site (black), wild type N-terminus E1 sequence with an NTS site (blue), E1 add ASA sequence with an NTS site (purple), and E2/E1 chimera with an NTS site (red). Error bars are ± s.e. from 3 experiments for all constructs except E1 add ASA, which is from 1 experiment. *p* < 0.05 for E2/E1 chimera against all other E1 constructs by two-way ANOVA. (B) *Left:* Cartoon depictions of E2 N-terminal construct assayed. *Middle:* Representative fluorographs for E2 del S2 construct. Glycosylated (1-Gly) and unglycosylated (0-Gly) species are labeled. *Right:* Plots of maximally glycosylated (1-Gly) species for: wild type N-terminal E2 sequence with an NFT site (black), wild type N-terminal E2 sequence with an NFS site (blue), and E2 del S2 sequence with an NFS site (red). Error bars are ± s.e. from 3 experiments for all constructs. *p* < 0.05 for E2 del S2 against both NFT/S wild type N-terminal sequences by two-way ANOVA.
kinetics. Since at least the E1 N-terminus is expected to form an α-helix [80], it is also possible that these data reflect a preferred orientation for the consensus site on the helix. Further experiments should utilize our established pulse-chase assay to identify the precise determinants that affect these shifts in N-glycan attachment rates.

**Characterizing an E3 insertion mechanism**

Another question still left unanswered is an explanation for the large unglycosylated pool of wtE3 in our kinetic assays. As discussed in Chapter II, it is possible that E3 is inserted into the ER membrane via a post-translational translocation system (detailed in Chapter I). To test this, we performed ultracentrifugation assays at 10,000 g to separate membrane bound and membrane free protein (Figure IV-2). Membrane free protein will remain soluble in these spins (S) while membrane bound proteins will pellet with the membrane (P). E1 is thought to be inserted by the traditional SRP/co-translational translocation system, and this is further evidenced by an absence of E1 in the S fraction, while all glycopeptide forms are found in the P fraction (Figure IV-2, lanes 1 and 2). However, when E3 is assayed, unglycosylated protein is found in the S fraction (lanes 4 and 6) with all glycopeptide forms still detected in the P fraction (lane 5 and 7). This membrane-free population of E3 implicates a post-translational translocation mechanism yet to be defined.
E1, E3, and E3/4 peptides were subjected to ultracentrifugation as described below. E1 is only found in the pelleted fraction (P, lane 2), and not found in the soluble fraction (S, lane 1). E3 was pulse-chased with a standard protocol to use as a marker for the different glycoforms ((-), lane 3). In two side-by-side experiments (lanes 4-5 and 6-7) E3 unglycosylated peptide is found in the S fraction. E3/4 unglycosylated peptide ((-) marker, lane 8) is also found in the S fraction, though less unglycosylated peptide appears in the P fraction than for wtE3 (lane 10 compared to lanes 5 and 7). All glycoforms are labeled for each construct. E1 and E3, n=2; E3/4, n=1.

Methods: Pulse-chase assay proceeded as described in Chapter II, but plates for ultracentrifugation were pulsed for 3 min with $^{35}$S and chased for 10 min only. Cells were washed once with PBS and once with 100 mM NaCl. Plates were scraped and homogenized in 1 mL 0.1 M NaCO$_3$ pH 11.5 with protease inhibitors using a 25.5 gauge syringe. Samples spun at 80,000 rpm for 30 min with a TLA 100.4 rotor. Pellets were then solubilized with 720 µL low salt lysis buffer with 50 µM Tris HCl pH 7.4. The supernatant fractions were pH adjusted to 7.4 by adding 0.1% HCl, then low salt lysis buffer and 50 µM Tris HCl pH 7.4 was added to a volume of 750 µL. The samples then underwent IP through film development steps as described in Chapter II.
We also assayed the E3/4 chimera used in Chapter II (E3 XL), which we found shifts E3 towards co-translational N-glycosylation. Interestingly, this peptide also has a large soluble unglycosylated presence in the S fraction (lane 9), but significantly reduced levels of unglycosylated peptide in the P fraction (lane 10) compared to wtE3 (20% 0-Gly for E3/4 as compared to 63.5% 0-Gly for wtE3 in P fraction). The fact that this long C-terminal peptide also has a membrane-free population of unglycosylated peptide needs to be further explored. One intriguing possibility is that these assays are not implicating post-translational translocation, but rather peptide topology. The large pool of unglycosylated E3 peptide in the P fraction could be due to peptides inserted in a type II orientation, possibly recognized by the TA protein machinery, blocking the peptide’s consensus sites from N-glycosylation. E3/4 chimeric peptides perhaps then could insert correctly more often, explaining the different ratios observed in the P fraction. More experiments to test these hypotheses will be necessary, particularly proteolytic assays to determine peptide topology.

**Elucidating KCNE N-glycosylation deficiency mutations in disease**

Lastly, a number of disease mutants in the N-terminal domains of the KCNE family still have unexplained etiologies. While E1 T7I was previously characterized as an N-glycosylation deficiency mutation [2], the molecular mechanisms of two mutations in E2 (T8A and Q9E) [77,123] and one mutation in E3 (T4A) [124] have yet to be elucidated. We assayed the E3 T4A mutant for
obvious deficiencies in N-glycosylation kinetics, and found this mutant to behave almost identically to wtE3 (Figure IV-3A). Other possible etiologies, including topological questions and the potential role of O-linked glycosylation, need to be explored to further describe this mutant.

E2 T8A and Q9E are unique in that they cause drug-induced LQTS. Macrolide antibiotics, including clarithromycin and sulfamethoxazole (SMX), are administered as treatment for a number of bacterial infections [130]. It was found however, that only persons carrying E2 T8A or Q9E mutations developed LQTS after treatment with these drugs. Since T8A eliminates the first N-glycosylation consensus site, it is possible that these antibiotics are affecting the N-glycosylation kinetics by an unknown mechanism. We began to test this hypothesis by expressing the E2 mutants and pretreating cells with 50 µM SMX from transfection through the pulse-chase. We found preliminary evidence that SMX might have an effect on the kinetics of N-glycosylation by a slight but significant decrease in co-translational N-glycosylation (Figure IV-3B); however, further drug optimization is still needed. Specific effects of macrolide antibiotics resulting in an N-glycosylation deficiency would be a novel mechanism of N-glycosylation inhibition, and this mechanism would need to be elucidated in future studies.

Overall, this thesis defines molecular determinants that effect the distributions of co- and post-translational N-glycosylation for type I transmembrane peptides. Additional characterization of these and other
Figure IV-3. N-terminal LQTS mutants may affect N-glycosylation kinetics. (A) Left: Pulse-chase fluorograph of the LQTS mutant E3 T4A. All glycoforms are labeled. Right: Plots of total N-glycosylation (1+2+3-Gly) for wtE3 (black) and E3 T4A (red). wtE3 error bars are ± s.e. from 3 experiments. E3 T4A, n=1. (B) Left: Pulse-chase fluorograph of the LQTS mutant E2 T8A incubated with SMX (50 µM dissolved in ethanol) from transfection through completion of pulse-chase. Glycoforms are labeled. Right: Plots of maximally glycosylated (1-Gly) species for the equivalent glycosylation mutation E2 N6Q (black) and E2 T8A + SMX (purple). p < 0.05 by two-way ANOVA. E2 N6Q error bars are ± s.e. from 3 experiments. E2 T8A, n=1.
determinants for other peptide topologies – type II, water-soluble, multi-spanning TM – will allow for a broader, generalized understanding of N-glycosylation site occupancy for all TM and secretory proteins. The ability to predict success of a consensus site by primary sequence alone would be immensely helpful in the study of all glycoproteins and the identification and characterization of N-glycosylation deficiency diseases.
APPENDICES
CHAPTER A1: DETERMINATION OF THE ASSEMBLY ORDER FOR KCNQ1 AND KCNE1 CHANNEL COMPLEXES

Abstract

The KCNQ1 (Q1) voltage-gated potassium (K⁺) channel is composed of four α subunits that assemble with the family of KCNE β subunits for proper physiological function. Q1 co-assembly with individual members of the KCNE family drastically alters the channel’s properties (Figure I-10), allowing Q1 to perform different tasks in a wide variety of tissues. Currently, little is known about the mechanism of assembly between Q1 and KCNE members. In particular, an outstanding issue to be addressed is the unusual architecture of the Q1/KCNE complex. It has been shown through electrophysiological studies that homotetrameric Q1 channels only associate with two β subunits despite the potential to bind in a 1:1 stoichiometry based on the four-fold symmetry of the ion conducting pore. To directly examine the process of Q1 channel and β subunit assembly, we performed cysteine-crosslinking experiments with an in vitro translation system to observe early assembly events and determine the order of KCNE1 assembly with KCNQ1. While this project was ultimately unsuccessful as designed, several findings could be used for future work in other systems.
Materials and Methods

Cloning and plasmids: Human KCNQ1 and KCNE1 were cloned into pcDNA™3.1(1) vector and a Kozak sequence (GCCGCCACC) was added immediately preceding the start codon for in vitro translation. E1 contains a C-terminal HA tag. Cysteine mutations were created using either Quikchange Site Directed Mutagenesis Kit or by traditional PCR cassette mutagenesis (Agilent Pfu Turbo) between the 5’ BamHI and 3’ XbaI sites for Q1 and between the 5’ KpnI and 3’ BgIII sites for E1. All constructs were confirmed by DNA sequencing the entire gene.

mRNA transcription: Plasmid DNA for Q1 and E1 was linearized with MluI for 16 h. DNA was then extracted using phenol:chloroform by phase lock gel. The DNA was then precipitated with 1/10 volume 3 M NaOAc pH 5 and 1 volume ethanol at -80°C for 30 min, and pelleted at 14,000 rpm for 20 min at 4°C. The supernatant was removed and 100 µL 70% ethanol was added and again pelleted at 14,000 rpm for 5 min at 4°C. The supernatant was again removed and the pellet allowed to air dry for 10 min to evaporate the remaining alcohol. The pellet was dissolved in water and the transcription reaction components added (all Promega): 1X transcription buffer, 10 mM DTT, 1.1 U/µL RNase Inhibitor, 1mM NTP mix, 1mM 5’ G cap, and 0.3 U/µL T7 RNA Polymerase. Reaction was incubated for 1 h at 37°C, another 0.3 U/µL T7 RNA Polymerase was added, and
reaction was incubated at 37°C for another 1h. The DNA was destroyed using 0.2 U/µL DNase I (NEB) with IX reaction buffer and incubated 37°C for 10 min. The remaining RNA was then extracted by phenol:chloroform phase lock gel, and precipitated using 1/10 volume 3 M NaOAc pH 5 and 1 volume isopropanol incubated on ice for 20 min, and pelleted at 14,000 rpm for 15 min at 4°C. The supernatant was removed and 100 µL 70% ethanol was added and again pelleted at 14,000 rpm for 5 min at 4°C. The supernatant was again removed and the pellet spun under a vacuum for 5 min, resuspended in in 25 µL water, the concentration determined (Abs260/280), and the mRNA was stored at -80°C.

**In vitro translation assay:** mRNA was denatured by heating at 65°C for 3 min and plunged into ice. The in vitro components were then added to a fresh tube: 5 µg/mL - 20 µg/mL mRNA, 1/50 to 1/5 reaction volume of microsomal membranes (Promega) or permeabilized CHO cells (see below), 40 U/µL RNAse Inhibitor (Promega), 1 mM amino acid mix without methionine and cysteine (Promega), 1 volume rabbit reticulocyte lysate (Promega), and 1,200 Ci/mmol at 10 mCi/mL EasyTag EXPRESS [35S] Protein Labeling Mix (MP Biomedicals). Samples were incubated at 30°C for 1 h, and prepared for electrophoresis by adding 2x SDS gel loading buffer to the sample, and 100 mM DTT (if no cross-linking was to be observed). Samples were analyzed by 15% SDS-PAGE 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 1-3 d.

**Permeabilized CHO cells:** CHO cells were washed with PBS, and removed from plates using 0.5% Trypsin-EDTA. Cells were put on ice and 8 mL KHM buffer added: 100 mM KC$_2$H$_3$O$_2$, 20 mM HEPES pH 7.2, 2 mM MgC$_2$H$_3$O$_2$ supplemented with 100 µg/mL soybean trypsin inhibitor (Sigma). After 5 min, cells were pelleted at 12,000 rpm for 3 min, and resuspended in 6 mL KHM with 40 µg/mL digitonin and incubated on ice for 5 min. 8 mL KHM was added to quench, and samples again pelleted at 12,000 rpm for 3 min. Pellets were resuspended in 50 mM HEPES pH 7.2 and 90 mM KC$_2$H$_3$O$_2$, and incubated on ice 10 min. Samples were again pelleted at 12,000 rpm for 3 min and resuspended in 100 µL KHM with 1 mM CaCl$_2$ and 10 µg/mL staphylococcal nuclease and incubated at room temperature for 12 min. Samples were quenched with 4 mM EGTA for 2 min at room temperature, pelleted at 12,000 rpm for 3 min, resuspended in 100 µL KHM, and stored -80°C.

**Transfection, cell lysis, and electrophoresis for western blots:** Cells were passaged using 0.5% Trypsin-EDTA and plated onto 35 mm dishes at 80% confluency. After 24 h, CHO cells were transiently transfected with 1.5 µg E1 and/or 0.75 µg Q1 construct DNA and 8 µL Lipofectamine in 1 mL OptiMem. After 6 h at 37°C, the transfection cocktail was removed and 2 mL of F12K media
with supplements was added and the cells were incubated for 40-45 h. Cells were washed in PBS (3 x 750 µL) and treated with 2.5 mg/mL N-ethylmaleimide for 10 min and treated with SDS cell lysis buffer: 10 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8.0, and 1% SDS supplemented with protease inhibitors (PMSF and 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. Samples were prepared by adding 100 mM DTT and 2x SDS gel loading buffer to the lysis sample (with DTT), or 2x SDS gel loading buffer only (no DTT) to a total volume of 50 µL. Samples were analyzed by SDS-PAGE (10 or 15% gel) transferred to a nitrocellulose membrane, and blocked for 30 min in milk (5% nonfat dry milk in Tris-buffered saline containing 0.2% Tween-20 (Western wash buffer)). Primary antibody (Q1 1:1,000 (Sigma) or HA (E1) 1:1,000 (Roche)) was added overnight by diluting into Western blocking buffer. Membranes were then washed in Western wash buffer (3 x 6mL) for 5 min each, and secondary antibody (Q1 1:4,000 or HA (E1) 1:2,000) was added in Western blocking buffer for 45 min at room temperature. More washes were performed (3 x 6 mL western wash buffer) and the membrane was incubated with SuperSignal West Dura Extended Duration Substrate (Pierce) for 5 min and the chemiluminescence captured using a LAS-3000 CCD camera and quantified by Multi Gauge software (Fujifilm).

**Ultracentrifugation of in vitro translation assay with sucrose cushion:** In vitro translation performed as described above, with or without the presence of
microsomes. 16.5 µL 200 mM Na₂CO₃ was added to the 20 µL sample and incubated on ice for 10 min, and put over 50 µL of 100 mM Na₂CO₃ + 200 mM sucrose, pH 7.4. Samples were centrifuged at 80,000 rpm for 5 min with a TLA 100.4 rotor. Pellets (P) were then solubilized with 25 µL TRIS/SDS (5 mL 1M Tris, 3.13 mL 20% SDS, water to 10 mL) at 55°C for 30 min, then adjusted to final concentrations of 100 mM DTT and 2x SDS gel loading buffer for 50 µL loading volume. The supernatant fractions (S) were treated with 50 µL saturated (NH₄)SO₄ for 15 min on ice, then pelleted at 14,000 rpm for 10 min at room temperature. This pellet was then redissolved in 50 µL 5% trichloroacetic acid for 5 min on ice, followed by a 10 min spin, and this pellet was then treated with same TRIS/SDS, DTT, and SDS gel loading buffer as described for the P fraction. Samples were analyzed by 15% SDS-PAGE and prepared for film as described in the in vitro section.
Results and Discussion

The voltage-gated potassium channel, Kv1.3, has been shown to assemble via a dimerization of dimers pathway [131] and this mechanism has been speculated to be the pathway of thermodynamic choice for all tetrameric proteins, membrane embedded or cytosolic [132]. This preference was shown through in silico simulations, where it was found that assembly involving a trimer intermediate could result in aggregation prone intermediates if the monomeric population became depleted. It is thought that other potassium channels, including Q1 homotetramers, assemble in a similar manner to Kv1.3 given the homology among voltage-gated potassium channels [86]. However, it is not understood how the β subunits fit into the assembly mechanism, or if they alter the assembly pathway of the α subunits.

KCNE peptides could be associating prior to (pre) or after (post) tetramerization of the Q1 α subunits. A pre-tetramerization mechanism would allow for co-assembly of Q1 and KCNE peptides in such a way as to explain the complex stoichiometry of four α subunits to two β subunits. Likewise, if the β subunits are incorporated by complementing a dimerization of dimers pathway of the α subunits, it should be possible to identify intermediate species along the pathway.

To differentiate between E1 addition pre- or post-tetramerization of the Q1 channel, we utilized a well documented in vitro translation system [133,134],
which simulates ER translation by combining rabbit reticulocyte lysate with canine microsomal membranes. Translated protein can be detected using radiolabeled $^{35}$S cysteine and methionine amino acids. By examining assembly *in vitro*, we can have temporal control over translation of individual species that cannot be achieved *in vivo* by adding mRNA for each construct at different times, as well as the ability to slow assembly events to easily detectable levels. Assembly could be detected by specific cross-linking between cysteine mutants of Q1 (I145C in cysteine-less Q1 background) and E1 (G40C/K41C), which have been shown to be functionally unchanged by a cysteine substitution and cross-link to each other when both are present [135]. These mutants were confirmed to cross-link when expressed in CHO cells as detected by western blot (Figure A1-1).

We were able to detect both Q1 and E1 in the *in vitro* system when Kozak sequences were added immediately prior to the start codon to improve translation (Figure A1-2A). We were also able to see E1 glycosylation was occurring (Figure A1-2B), and that Q1 and E1 were being incorporated into microsomes (Figure A1-2C). However, we were never able to detect a cross-linked species. This could be because Q1 and E1 proteins were not being expressed in the same microsomes at concentrations high enough to be detected, or the *in vitro* system was chemically incompatible for cross-link formation (despite attempts to make a favorable cross-link environment by treatments with oxidized glutathione and hydrogen peroxide). We also believe
Figure Al-1. Q1 I145C and E1 G40C or E1 K41C can cross-link in vivo. **Left:** E1 blots for E1 constructs transfected with Q1 I145C in the presence or absence of 100 mM DTT. Maturely glycosylated E1/Q1 and imm maturely glycosylated E1/Q1 cross-linked complexes were detected when lysates are not treated with DTT. Glycosylation states of E1 were confirmed by PNGaseF and EndoH treatments (not shown). These bands were absent in the presence of DTT. **Right:** Q1 blots for E1 constructs transfected with Q1 I145C in the presence or absence of 100 mM DTT. Q1 dimer is also detected readily along with the cross-linked constructs without DTT. Q1 dimer confirmed with the use of a Q1 tandem dimer marker (not shown). Representative blots from 3-5 experiments.
Figure Al-2. Q1 and E1 proteins can be detected with *in vitro* translation and incorporated into microsomal membranes, but Q1 membrane incorporation is limited. (A) Q1 and E1 protein can be synthesized *in vitro* when a Kozak sequence is present. (B) E1 expressed *in vitro* with varying concentrations of canine microsomal membranes (CMM). Glycoforms of E1 are detectable in the presence of microsomes (volume in µL). (C) Supernatants (S) and pellets (P) from ultracentrifugation of *in vitro* translated Q1 (left) and E1 (right) proteins with and without CMMs. (D) Ultracentrifugation of increasing concentrations of Q1 mRNA translated *in vitro* with 2 µL permeabilized CHO cells.
that while the *in vitro* system works well for small peptides like E1, it does not incorporate significantly larger Q1 proteins very well, despite attempts to optimize Q1 mRNA with an excess of the recommended microsomal concentration or freshly prepared permeabilized CHO cells (Figure AI-2D) [136]. While much optimization of microsomes and mRNA concentrations was attempted, it is possible that further optimization may produce the right mix necessary for efficient cross-linking; however, a more relevant avenue for addressing assembly would be to develop an assay to temporally differentiate translation of Q1 and E1 *in vivo*. This could plausibly be accomplished by engineering Q1 and E1 inducible plasmids and detecting cross-linking by utilizing radiolabeled kinetic assays described in Chapters II and III.
KCNO1 (Q1) is a voltage-gated potassium (K+) channel composed of four homomeric subunits that assemble with a KCNE (E1-E5) β subunit for proper physiological function. While the number of KCNE peptides in a Q1 channel complex is still debated, there is strong evidence that only two KCNEs assemble into the completed channel. It is not known where these peptides would preferentially reside because each Q1 channel contains four identical KCNE binding sites. Here, electrophysiological assays utilizing cysteine cross-linking pairs and small molecule modifications were employed to determine the orientation of E1 in the Q1 channel complex. These assays are ideal for the high sensitivity and detailed conductance information that can be extracted from slight perturbances to channel complex structure. However, the difficulties in working with heteromeric populations of homomeric complexes dependent on E1 cross-linking or chemical modification produced ambiguous results that could not be overcome with these experimental design strategies.
Material and Methods

**Cloning and plasmids:** Human KCNQ1 and KCNE1 were cloned into pcDNA™3.1(+) vector. E1 contains a C-terminal HA tag. Cysteine mutations and tandem constructs (no linker between subunits) were created using either Quickchange Site Directed Mutagenesis Kit or by traditional PCR cassette mutagenesis (Agilent Pfu Turbo) between the 5' BamHI and 3’ XbaI sites for Q1 and between the 5' KpnI and 3’ BglII sites for E1. All constructs were confirmed by DNA sequencing the entire gene.

**mRNA transcription:** As described in Appendix A1.

**Electrophysiology:** Oocytes were surgically removed from Xenopus laevis frogs and defolliculated for 60 min using 2 mg/mL collagenase dissolved in OR2 solution (in mM): 82.5 NaCl, 2.5 KCl, 1 MgCl₂, and 5 HEPES, pH7.4. Oocytes were then rinsed and stored at 16°C in ND96 storage solution (in mM): 96 NaCl, 2KCl, 1.8 CaCl₂, 1 MgCl₂, and 5 HEPES and supplemented with antibiotics: 50 µg/mL gentamicin and tetracycline pH 7.4 and 3 mM glutathione (for TYP assays). mRNA microinjection of Q1 (12.6 ng) and E1 (6.4 ng) was performed after 24 h, and incubated 16°C for 40-48 h. Current was measured using Warner Instrument OC-725 two-electrode voltage clamp and data recorded with Digidata 1322A and pClamp9 software (Axon Instruments). Currents were measured in
ND96 recording buffer (in mM): 96 NaCl, 2 KOH, 0.3 CaCl$_2$, 1 MgCl$_2$, 10 HEPES, pH 7.6, and a high K$^+$ electrode solution (3 M KCl, 5 mM EGTA, 10 mM HEPES, pH 7.6). Oocytes were held at -80 mV and pulsed to +40 mV. For the TPY time course experiments, the cells were continually pulsed for 2 s every 30 s. For the TPY experiments, oocytes were (all in ND96 recording buffer): pre-incubated with 1 mM TCEP, pH 7.6 for 5 min, washed three times, incubated with 100 µM TPY for 5 min, put on rig and began recording, washed in 500 µM FeSO$_4$, washed with ND96 buffer, washed in 120 nM toxin, and washed with ND96 buffer.

**Data Analysis:** Maximal current was determined by using Clampfit9 (Aon Instruments) software to find the amplitude measured at the very beginning of (for cross-linking study) and the very end of the 2 sec pulse. These measurements where then presented as a ratio for the cross-linking study, or the 2 sec amplitude was plotted on a time course for the TPY study.

**Terpyridine (TPY) linker synthesis:** TPY linker synthesis strategy was developed by Karen Mruk and outlined in her thesis, Chapter AII [137]. Her TPY linker precursors were used as material to attach the pyridyl disulfide group. These reactions were dissolved in dichloromethane with excess pyridyl disulfide and catalyzed by N,N$'$-Dicyclohexylcarbodiimide (DCC) and N-hydroxysuccinimide (NHS) (Sigma). The full TPY-linker-pyridyl disulfide was purified by silica column with 4:1 chloroform to methanol, pumped down and
weighed to determine concentration, and dissolved in chloroform to aliquot. The samples were then lyophilized and stored at -20°C.

**Charybdotoxin (CTX) protein preparation:** BL21 (DE3) cells were transformed with 150 ng CTX DNA and grown at 37°C for 16 h on LB agar with 50 µg/mL ampicillin. The entire plate was scraped for a started culture in LB with 50 µg/mL ampicillin and grown at 37°C until turbid, then distributed into 4 L TB (12 g/L bacto-tryptone, 24 g/L bacto-yeast extract, 4 mL glycerol) with 50 µg/mL ampicillin and grown until OD600 = 1.5. 0.5 mM IPTG (isopropyl-beta-D-thiogalactopyranoside) was added and cells grown for 3 h. Cells were centrifuged at 5,000 rpm for 15 min at 4°C and pellet resuspended in 200 mL lysozyme buffer: 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 2 mM Na-EDTA, pH 8.0. Cells were incubated on ice 30 min. 50 µL 2-mercaptoethanol (BME), 50 µL pepstatin-leupeptin (0.5 mg/mL in methanol), and 1.2 mL PMSF (4.5 mg/mL in methanol) was added and cells lysed by cell disruption. Lysate was spun at 14,000 rpm for 15 min at 4°C, and 1/10 volume of 30% streptomycin sulfate added slowly while stirring on ice. Lysate was spun at 12,000 rpm for 20 min at 4°C, and 31 g/100 mL solid ammonium sulfate added while stirring for 30 min on ice. Lysate was spun at 12,000 rpm for 20 min at 4°C. The pellets were rinsed with water and dissolved overnight in 400 mL Buffer A: 50 mM Tris-HCl pH 7.0, and 50 mM NaCl with 5 mM BME. A DE-52 column was preequilibrated with Buffer A and protein loaded at 5-6 mL/min. Protein was eluted by gradient application of Buffer B: 50
mM Tris-HCl pH 7.0, and 500 mM NaCl. Protein fractions were combined and
dialyzed 20 h in dialysis buffer: 100 mM NaCl and 20 mM HEPES, pH 8.0.
Protein was further purified through a SPS column at 3 mL/min with SPS buffer
C: 10 mM KCl and 10 mM KP, pH 7.1 and eluted with SPS buffer D: 1 M KCl and
10 mM KP, pH 7.1. Protein was again dialyzed 16 h in dialysis buffer. Protein was
then subjected to HPLC purification and confirmed by mass spectrometry.
Aliquots were made by addition of 100 mM KP, pH 7.0 to 5 mL and the OD$_{280}$
was measured and the concentration of CTX was calculated. Samples aliquoted
appropriately and lyophilized down. Dried protein was stored on desiccant at
-80°C.
Results and Discussion

The number of β subunits present in the Q1 channel complex has been hotly debated for a number of years [138,139,140,141,142]. Previous findings from our lab have demonstrated through sensitive electrophysiological recordings that only two E1 peptides associate in a Q1/E1 channel complex [141]. This raises an intriguing area of study because two β subunits assembling into a channel complex would break the four-fold symmetry of the channel and only utilize two of four possible identical binding sites. Presumably, these β subunits could be either adjacent or opposite each other in the channel complex. It is thought that β subunits orienting opposite each other across the pore would be more likely for KCNE placement in the complex because this retains a two-fold symmetry, and could assemble in combination with a Q1 dimerization of dimers assembly model outlined in Chapter A1, but this has never been directly tested. In addition, the question of channel complex structure is further complicated by evidence suggesting that one E1 peptide could make contacts with up to three of the four Q1 subunits [101].

To determine whether E1 assembles on adjacent or opposite sides of the channel complex, we chose to utilized several electrophysiological approaches. Electrophysiology is both a more sensitive assay than biochemical counterparts and more quantitative [143]. First, we attempted to determine E1 subunit orientation using an established cysteine cross-linking pair on channel
complexes (Q1 V324C and E1 L42C) [101]. These constructs produce a conductance pattern where the channel pore is constitutively open, and this effect is not seen when Q1 V324C is expressed with wtE1 (Figure AII-1A). Because a conductance ratio can be measured from the current at the beginning of the voltage pulse (0 sec) and the end of the pulse (2 sec), the ratios between cross-linked (~1.0) and non-cross-linked channel complexes (~0.0) can be quantified. The placement of E1 could be determined by expressing Q1 V324C-Q1 V324C tandem dimers with E1 L42C and comparing the measured conductance ratio to a generated binomial curve (where E1 would be either fully cross-linked or not cross-linked) created by expressing Q1 V324C monomers at varying ratios of wtE1/E1 L42C.

Unfortunately, when this binomial curve was generated (Figure AII-1B), it would not fit to a binomial distribution, but instead fit nicely to a polynomial distribution. This implies that cross-linking in these Q1/E1 pairs fluctuates between intermediate states, and therefore is not suitable to answer an E1 orientation question that requires only either full cross-linking or no cross-linking in this system for a definitive answer.

We next attempted to determine E1 subunit orientation utilizing an engineered 40.3 Å chemical linker that would specifically disulfide bond with an external E1 cysteine (E1 T14C). A cysteine-less Q1 construct was used with this system. This terpyridine (TPY) based linker was designed to dimerize in the presence of iron [144], and would inhibit charybdotoxin (CTX) block of the
Figure AII-1. Q1 V324C and E1 L42C form a multi-state cross-link that cannot be utilized in E1 subunit orientation studies. (A) Family current traces for Q1 V324C expressed with wtE1 and Q1 V324C expressed with E1 L42C in *Xenopus* oocytes. Scale bars indicate 0.5 sec and 1 µA. Oocytes were held at -80 mV and pulsed from -100 mV to 40 mV for 4 sec every 30 sec. Dotted line indicates 0 µA. Quantifications for (B) were taken as a ratio of the currents measured at each arrow indication from the 40 mV (top) trace. (B) Conductance ratio (0 sec / 2 sec) of Q1 V324C expressed with varying ratios of wtE1 (0) to E1 L42C (1). Binomial ($R^2 = 0.97$) and Polynomial (3rd degree, $R^2 = 0.99$) fits are shown.
channel [145] if the linker went over the channel pore from an E1 opposite orientation, but would allow toxin block if E1 peptides were located adjacently (Figure AII-2A). The Q1 channel is predicted to be ~ 80 Å across at its longest from opposite subunits and ~ 60 Å across at its longest from adjacent subunits [146]. This chemical linker designed by K. Mruk [137] was composed of three distinct regions: a 2,2′:6′,2″-terpyridine (TPY) tridentate ligand that forms a stable dimer with a transition metal ion [144], a peptide linker that can vary in length as needed, and a pyridyl disulfide leaving group which can react transiently with a reduced cysteine on the cell surface (Figure AII-2B). We initially chose to test our longest 40.3 Å linker, which is predicted to reach across any part of the channel complex. Unfortunately, our results were difficult to interpret because iron treatment to dimerize TPY caused inconsistent fluctuations in current, and toxin washes also showed substantial fluctuations that made definitive conclusions difficult (Figure AII-3). Many experiments were performed to optimize each concentration, solvent, and treatment order but the system continued to behave erratically. It is possible that further optimization of these conditions could eventually result in an ideal assay, but we decided to discontinue this particular project for the time being.

Determining β subunit orientation in Q1 channel complexes remains an interesting question to address; however, the difficulties in experimental design to differentiate between apparent symmetries present for a homomeric channel in an unknown homo- or heterogeneous channel population are challenging to
Figure All-2. Schematic of TPY labeling design strategy used to determine E1 orientation. (A) Oocytes are pretreated with TPY that can form a disulfide bond with an available cysteine on E1 (T14C). At this point CTX can block the channel and inhibit current. Once FeSO₄ is added to the bath solution, TPY dimerizes. If E1 is located in opposite subunits, TPY dimerization would block CTX access and current would be measured uninhibited. If E1 is located in adjacent subunits, some CTX block would be expected. (B) Schematic of TPY linker construct. This TPY linker can extend up to 40.3 Å.
Figure All-3. Addition of iron and toxin cause erratic fluctuations in current after TPY oocyte labeling. Top: Two assay results showing inconsistent current fluctuations upon FeSO₄ treatments after TPY labeling. FeSO₄ also affected currents of oocytes not treated with TPY (not shown). Maximal current is measured from a 40 mV 2 sec pulse taken every 30 sec. All measurements normalized to the current measured after treatment with FeSO₄. Bottom: One representative assay showing CTX treatments before and after treatment with FeSO₄. Note that current does not recover fully after CTX treatments and behaves erratically after the second CTX treatment for unknown reasons, and FeSO₄ treatment strangely reduces and then increases the current amounts. All graphs shown maintained less than -0.1 µA oocyte leak throughout the assays.
overcome. It is possible that biochemical assays using chemical cross-linkers of specific lengths engineered to attach to tandem Q1 constructs and E1 peptides could be used to address this question, especially if native gels could be employed. However, given that the number of subunits in Q1/KCNE complexes remains an unanswered question in the field at large, a clear analysis of KCNE subunit orientation will be difficult to address until the number of KCNE peptides in the channel complex is definitively established.


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