Robust post-translocational N-glycosylation at the extreme C-terminus of membrane and secreted proteins in *Xenopus laevis* oocytes and HEK293 cells

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N-Glycosylation is normally a co-translational process that occurs as soon as a nascent and unfolded polypeptide chain has emerged ~12 residues into the lumen of the endoplasmic reticulum (ER). Here, we describe the efficient utilization of an N-glycosylation site engineered within the luminal extreme C-terminal residues of distinct integral membrane glycoproteins, a native ER resident protein and an engineered secreted protein. This N-glycan addition required that the acceptor asparagine within an Asn-Trp-Ser sequon be located at least four residues away from the C-terminus of the polypeptide and, in the case of membrane proteins, at least 13 residues away from the luminal side of the transmembrane segment. Pulse-chase assays revealed that the natural N-glycans of the proteins studied were attached co-translationally, whereas C-terminal N-glycosylation occurred post-translocationally within a time frame of hours in *Xenopus laevis* oocytes and minutes in human embryonic kidney 293 (HEK293) cells. In oocyte and HEK cell expression systems, affinity tag-driven C-terminal N-glycosylation may facilitate the determination of orientation of the C-terminal tail of membrane proteins relative to the membrane.

Keywords: blue native PAGE / C-terminal N-glycosylation / Cys-loop receptors / Ni-NTA affinity chromatography / Strep-Tactin affinity chromatography

Introduction

N-Glycosylation largely occurs co-translationally as polypeptides are translocated across or integrated into the endoplasmic reticulum (ER) membrane. A close coupling of protein synthesis, translocation and N-glycosylation is achieved by the organization of the ribosome, the hetero-trimeric Sec61 αβγ complex and the hetero-octameric oligosaccharyltransferase (OST) complex in a ternary supramolecular assembly (Harada et al. 2009). The OST is strategically positioned at the exit of the Sec61 translocation channel on the luminal side of the ER membrane to co-translationally scan the emerging unfolded polypeptide for the presence of a glycosylatable NXS/T tripeptide motif (also designated as a sequon).

Both sequon scanning and catalysis of the N-glycosidic linkage between the asparagine acceptor and the preassembled high-mannose core oligosaccharide chain (Glc3-Man9GlcNAc2) from the ER membrane lipid donor dolichyl pyrophosphate are mediated by the OST subunit STT3 (Iyengar and Lennarz 2002; Nilsson et al. 2003; Kelleher and Gilmore 2006). The STT3 subunit occurs in vertebrates in two widely expressed isoforms, STT3A and STT3B, that assemble into OST isoforms with distinct substrate specificities (Kelleher et al. 2003; Kelleher and Gilmore 2006). The transfer occurs of the core oligosaccharide chain when the sequon has emerged 12–14 amino acids deep into the ER lumen, corresponding to a distance of 75 residues between the ribosomal peptidyl transferase site (P-site) on the cytoplasmic side of the ER membrane and the active site of the OST on the luminal side (Whitley et al. 1996). Sequons that are located <12–14 residues from a transmembrane segment are not glycosylated, indicating that the OST active site is located 30–40 Å above the luminal membrane surface of the ER (Nilsson and von Heijne 1993).

The NXS/T sequence frequently occurs in proteins, but not all sequons of secretory and membrane proteins are glycosylated. In sets of non-redundant polypeptides with experimentally verified glycosylated and non-glycosylated sequons, the rate of occupancy was ~60% (Shakin-Eshleman et al. 1996; Apweiler et al. 1999; Ben-Dor et al. 2004). Factors identified to impair N-glycosylation at NX(S/T)Y motifs (for review, see Jones et al. 2005) include the particular amino acids present in the +2 (X) or +4 (Y) positions, with proline being particularly inhibitory. Additionally, a serine instead of a threonine in the +3 position, rapid folding of the nascent chain and the location of the sequon in proximity to the C-terminal end (Gavel and von Heijne 1990; Nilsson and von Heijne 2000) are inhibitory. However, a recent analysis of 617 well-defined, non-redundant N- and O-glycoproteins listed in the SWISS-PROT database...
identified 18 proteins with glycosylated sequons in the last 10 C-terminal residues, suggesting that C-terminal N-glycosylation may be more common than expected (Ben-Dor et al. 2004). Here, we characterized C-terminal N-glycosylation driven by a sequon inserted within the last nine amino acids of several membrane proteins, a secreted protein and an ER resident protein in *Xenopus laevis* oocytes and human embryonic kidney 293 (HEK293) cells.

### Results

**An NWS sequence in a C-terminal Strep(III) tag can serve as an N-glycan acceptor**

Glycine receptors (GlyRs) belong to the superfamily of Cys-loop receptors and mediate fast inhibitory synaptic transmission by opening an intrinsic transmembrane anion pore in response to the binding of the neurotransmitter glycine. Like other Cys-loop receptors, GlyRs function as obligate pentamers of identical or homologous subunits, which comprise an extracellular neurotransmitter-binding domain together with a transmembrane region characterized by four membrane-spanning helical segments, TM1–TM4 (Figure 1A). The GlyR α1 polypeptide contains four potential N-glycosylation sites with the canonical N-X-T/S sequence. We previously characterized sequons in the TM3 (43NVT). The markedly larger PNGase F-induced mass shift observed with the C-terminally Strep(II)-tagged GlyR α1 subunit (Figure 1D, lanes 1–9) at the plasma membrane provided evidence for the presence of a C-terminal N-glycan. GlyRs in *X. laevis* oocytes and homopentameric GlyR (α1) or only slightly increased (α1 and α3 GlyR, 2.7-fold and 1.4-fold, respectively) by Strep(II)-tagging. The most plausible explanation for the higher molecular mass is that the NWS sequence present in the NWSHPQFEK Strep(II) tag but not in the Strep(I) tag (AWHRPQFGG) is efficiently used as an N-glycosylation site despite its close proximity to the C terminus of the subunits. To address this issue, we used Endo H and peptide-N-glycosidase F (PNGase F) to probe for high-mannose and complex-type N-glycans, respectively. The GlyR α1 subunit (Figure 1E, lanes 1–9) and α3 subunit (Figure 1F, lanes 1–9) at the plasma membrane were exclusively in the complex-glycosylated form with ~3 or ~9 kDa of releasable oligosaccharide depending on the presence of a Strep(I) or a Strep(II) tag, respectively. The ~3 kDa release fully coincides with the usage of the single N-sequence found in the ectodomain of both GlyR α1 (i.e. 38NVS) and GlyR α3 (44NVT). The markedly larger PNGase F-induced mass shift observed with the C-terminally Strep(II)-tagged GlyR subunits is consistent with hyperglycosylation.

In contrast to GlyR α1 and α3, the GlyR α2 subunit without a Strep(II) tag exists only in the core-glycosylated form at the plasma membrane (Figure 1E, lanes 10–15), suggesting that its N-glycans are inaccessible to modification by Golgi enzymes. This behavior allowed the Strep(II) tag-induced hyperglycosylation to be directly visualized by deglycosylation analysis because the plasma membrane-bound GlyR α2 subunit acquired a complex-type N-glycan in the Strep(II)-fused version in addition to the natural high-mannose type N-glycan (Figure 1E, compare lanes 17 and 18 with lanes 14 and 15). Altogether, these findings provide robust evidence that C-terminal N-glycosylation underlies the Strep(II)-tag-induced increase in molecular mass.

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**Fig. 1.** Hyperglycosylation of α GlyRs in *X. laevis* oocytes mediated by a C-terminal Strep(II) affinity tag. (A) Diagram showing the topology of the mature GlyR α1-His-Strep(II) subunit, including the C-terminal locations of the His tag (green), the consecutive Strep(II) tag (blue) and the positions of the two N-glycans (γ) (one in the single native position; orange, 38NVS) and the second in the C-terminal sequon introduced by the Strep(II) tag (sky blue, 529NWS). The positions of the extracellular cysteine residues (red) are also shown; disulfide-bonded cysteines are linked by red lines, with 13C and 15C forming the so-called Cys-loop. (B–D) Oocytes expressing the indicated proteins were metabolically labeled overnight with [35S]methionine, chased for 24 h, and then surface-labeled with membrane-impermeable fluorescent Cy5 NHS ester, followed by Ni-NTA purification. Purified proteins were resolved by reducing SDS–urea–PAGE to display (B) their Cy5-labeled surface form (in green) by Typhoon fluorescence scanning and (C) their total [35S]methionine-labeled form (in gray) by phosphorimaging. The same protein samples were also resolved by BN-PAGE followed by 35S-phosphorimaging to display their oligomeric state (D). Blue ovals schematically illustrate the oligomeric states of the non-denatured and denatured α1 GlyR. (E and F) The same samples as in B–D were treated with the indicated N-glycosidases following by reducing SDS–urea–PAGE and fluorescence scanning. The N-glycan acceptor site (44NWS) introduced as part of the Strep(I) tag after the C-terminal hexahistidine sequence was used in all the three α GlyRs, as indicated by the increases in molecular mass that could be reversed by enzymatic deglycosylation.
The Trp and Ser residues of the NWS sequon are not critical for C-terminal N-glycosylation
To further verify the presence of a second N-glycan on Strep(II)-tagged GlyR α1 and to assess the possible roles of the X and S/T positions in C-terminal N-glycosylation, we subjected the NWS sequon introduced by the Strep(II) tag (NWSStrep(II)) to mutational analysis. Replacement of W or S by A or T, respectively, did not affect hyperglycosylation (Figure 2A and B, lanes 1, 3 and 4, and C, lanes 1–3 and 7–12). The low recovery of the NAS mutant by Strep-Tactin purification (Figure 2B, lane 3) may be due to a decrease in the affinity of the mutated Strep(II) tag for the Strep-Tactin resin. As expected from the above data, elimination of the N-glycan acceptor site by mutation to glutamine (QWSStrep(II)) prevented hyperglycosylation (Figure 2A and B, compare lanes 1 and 2). The PNGase F-induced mass reduction in the QWS mutant (Figure 2C, lanes 4–6) corresponds to the release of the single natural N-glycan from 38N of the GlyR α1 ectodomain (cf. Figure 1A).

C-terminal N-glycosylation requires a minimum distance of 13 residues from the membrane
Next, we examined the distance from the plasma membrane at which C-terminal N-glycosylation occurs. The luminaly oriented OST active site is positioned at a distance of 12–14 amino acids above the surface of the ER membrane (Nilsson and von Heijne 1993). Two topology programs, MEMSAT3 (Jones 2007) and TMHMM 2.0 (Krogh et al. 2001), predict that the TM4 of the GlyR α1 subunit extends from residues 393 to 410Y of the 421 amino acid-long mature polypeptide. The numbering refers to the mature α1 subunit [i.e. after the removal of the N-terminal signal peptide between positions 28 and 29 (AEA-AR)] according to SignalP 3.0 prediction (Bendtsen et al. 2004). The additional hexahistidine tag present between the extreme C-terminal residue (421Q) of the parental wt GlyR α1 subunit and the fused NWSHGPFEK Strep(II) sequence places the N-glycan acceptor site at a distance of 17 residues from the luminal membrane surface (cf. Figure 1A). To make the distance between the acceptor asparagine and residue 410Y at the TM4 exit site vary from 11 to 16 residues, we inserted one to five histidine residues

Table I. Effects of Strep(II)-tagging of GlyR α subunits on glycine-induced current responses

<table>
<thead>
<tr>
<th>GlyR</th>
<th>I_{max} (µA)</th>
<th>EC_{50} (µM)</th>
<th>n_{H}</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>α1-His</td>
<td>6.0 ± 1.4</td>
<td>175 ± 22</td>
<td>3.0 ± 0.3</td>
<td>11</td>
</tr>
<tr>
<td>α1-His-Strep(II)</td>
<td>6.2 ± 1.2</td>
<td>467 ± 58</td>
<td>1.8 ± 0.2</td>
<td>4</td>
</tr>
<tr>
<td>α2-His</td>
<td>9.9 ± 1.5</td>
<td>231 ± 57</td>
<td>2.0 ± 0.4</td>
<td>5</td>
</tr>
<tr>
<td>α2-Strep(II)</td>
<td>8.1 ± 2.0</td>
<td>334 ± 61</td>
<td>1.5 ± 0.2</td>
<td>5</td>
</tr>
<tr>
<td>α3-His</td>
<td>5.8 ± 1.7</td>
<td>85 ± 8</td>
<td>1.9 ± 0.3</td>
<td>5</td>
</tr>
<tr>
<td>α3-His-Strep(II)</td>
<td>6.3 ± 0.6</td>
<td>89 ± 23</td>
<td>1.8 ± 0.2</td>
<td>4</td>
</tr>
</tbody>
</table>

Glycine responses were recorded by two-electrode voltage-clamp; data are presented as means ± SD from N oocytes. n_{H}, Hill coefficient; I_{max}, maximal glycine-inducible current.
between the C-terminal end of the wt α1 subunit and the Strep(II) tag (Figure 3A). Due to the complete or partial lack of the hexahistidine affinity tag, we purified the GlyR α1 variants by Ni-NTA chromatography (Figure 3B) and by their Strep(II) tags via Strep-Tactin chromatography (Figure 3C). GlyR α1-Strep(II) variants with zero or one histidine residue inserted (Figure 3A) acquired no additional N-glycan chain (Figure 3C, lanes 1 and 2), indicating that a distance of <13 residues from the membrane is too short for the NWS-Strep(II) acceptor asparagine to be accessible to the OST. However, increasing the distance to 13 residues by inserting two histidine residues resulted in efficient C-terminal N-glycosylation (Figure 3C, lane 3, see also A). This result was further verified by deglycosylation analysis, demonstrating that the mass increase at a distance of ≥13 residues resulted entirely from bi-glycosylation due to N-glycan addition to the C-terminal NWS sequence (Figure 3D). Obviously, the presence of the C-terminal N-glycan did not impair the accessibility of the Strep(II) tag to the Strep-Tactin resin, as inferred from the equally efficient capture of the mono- and di-glycosylated forms of α1-Strep(II) GlyR (Figure 3C). Thus, the minimal distance between the acceptor asparagine and the luminal membrane surface required for C-terminal N-glycosylation (13 residues) is the same as that required for sequons located at a more N-terminal position (Nilsson and von Heijne 1993).

A collateral finding was that efficient binding of α1 GlyR to the Ni-NTA resin required only a tri-histidine sequence (Figure 3B), despite the low binding affinity of tri-histidine peptides for Ni-NTA matrices (Knecht et al. 2009). A likely explanation is that the binding affinity is increased by the multimeric nature of GlyR, which results in a C-terminal accumulation of 15 histidine residues per pentameric complex. Moreover, there are two additional histidine residues in close proximity to the His tag that may also increase the affinity for the Ni-NTA resin; one in the natural C-terminal tail and the other at position +4 of the Strep(II) tag (cf. Figure 3A).

Two residues C-terminal to the NWS sequon are sufficient for efficient N-glycosylation

In the experiments described thus far, the acceptor asparagine was located eight amino acids away from the very C-terminal end of the respective polypeptide. To determine the minimum number of residues C-terminal to the acceptor asparagine required for N-glycan addition, we fused the NWS sequence directly to the C-terminal end of the α1-His subunit and then extended its C-terminal end by adding one Strep(II)-equivalent residue at a time until C-terminal N-glycosylation occurred (Figure 4). Addition of the NWS...
sequon alone did not result in hyperglycosylation, as evident from the unchanged migration compared with the parent α1-His GlyR (Figure 4A and B, lanes 1 and 2) and a ~4 kDa shift upon deglycosylation (Figure 4C and D, lanes 1–3). Further, addition of a single histidine residue at the +4 position relative to the acceptor asparagine also failed to promote C-terminal N-glycosylation. Instead, it impaired cell surface exposure and total expression for unknown reasons (Figure 4A and B, lane 3). However, when a second residue was inserted corresponding to the +5 position, a ~7 kDa increase occurred (Figure 4A and B, lane 4), indicating hyperglycosylation of most of the isolated protein (see also Figure 4E). Only a minor fraction of the α1-His-NWSP mutant migrated in a mono-glycosylated form (i.e. at the same position as the parent α1-His subunit). This conclusion was further verified by deglycosylation analysis (Figure 4C and D, lanes 4–6). The addition of a residue at the +6 position resulted in complete di-glycosylation (Figure 4A and B, lane 5, and C and D, lanes 7–9), which was also supported by the complete C-terminal Strep(II) sequence (Figure 4A and B, lane 6). Thus, we conclude that C-terminal N-glycosylation can occur unexpectedly closely to the extreme C-terminal end of a protein.

C-terminal N-glycosylation occurs post-translocationally

Co-translational N-glycosylation at the OST active site occurs at a distance of 75 amino acids from the ribosomal P-site (Whitney et al. 1996). Therefore, any N-glycosylation within the last 75 C-terminal residues is (a priori) a post-translational process because it takes place after the chain has been released from the ER-attached ribosome, though the chain may still move through the translocon. Accordingly, post-translational and post-pulse N-glycosylation are more precise terms. To assess the time course of C-terminal N-glycosylation, we labeled oocytes expressing the GlyR α1-Strep(II) subunit and several mutants containing variations of the C-terminal NWS motif with a 3 h [35S]methionine pulse. The cells were then chased in the presence of the protein synthesis inhibitor cycloheximide to inhibit further translation (Braakman et al. 1991). Blue native PAGE (BN-PAGE) after the 3 h [35S]methionine pulse revealed that most of the GlyR α1 subunits migrated as diffuse aggregates with an overall mobility slower than that of the homopentamer (Figure 5A, lanes 1, 3, 5 and 7). Partial denaturation with 0.1% SDS revealed that the aggregates included defined pentamers (Figure 5A, lanes 2, 4, 6 and 8) that were potentially in a chaperone-bound conformation of low mobility in the BN-PAGE gel. After a 12 h chase period, all GlyR variants migrated as well-defined homopentamers (Figure 5A, lanes 9, 11, 13 and 15).

On SDS-PAGE, the GlyR α1-NWS-Strep(II) subunit isolated directly after the 3 h [35S]methionine pulse resolved primarily as a mono-glycosylated peptide (Figure 5B, lane 5) that migrated at the same position as the α1-His-QWS-Strep(II) mutant with the NWS sequon knocked out by an N to Q mutation (Figure 5B, lane 2). In addition, a weaker band indicative of C-terminal N-glycan occupancy was visible with all of the mutants harboring a glycosylatable C-terminal NXS/
T motif (Figure 5B, lanes 3–5) but not with those containing the non-glycosylatable QWS sequence (Figure 5B, lane 2). During the subsequent chase periods, the di-glycosylated polypeptide form increased at the expense of the mono-glycosylated form (Figure 5B, lanes 8–10) and was eventually processed into the complex-glycosylated form (Figure 5B, lanes 13–15). Quantification revealed that the relative amount of the di-glycosylated form increased up to 4-fold, and the various NXS/T sequons supported C-terminal N-glycosylation with similar efficiency (Figure 5C).

Fig. 5. Time course of C-terminal N-glycosylation in X. laevis oocytes. The filled blue dot and blue arrowhead indicate the migration positions of GlyR α1 subunits mono-glycosylated at $^{38}$N or additionally glycosylated at the $^{428}$NWS sequence of the Strep(II) tag, respectively. The open blue dot denotes the non-glycosylatable $^{38}$Q-α1-His-QWSStrep(II) double mutant that lacks the natural N-glycan acceptor site $^{38}$N (also indicated by $^{38}$Q in the figure legend). The blue rhombus indicates complex-type double-glycosylated GlyR α1. GlyR α1-His-Strep(II) subunits with the indicated mutational variation of the Strep(II) tag-inherent NWS sequence (mutated residues indicated by red letters) were isolated by Ni-NTA chromatography immediately after a 3 h $[^{35}$S]methionine pulse or after the indicated additional chase intervals and resolved by (A) BN-PAGE or (B) reducing SDS–urea–PAGE. (C) Quantification of time- and sequon-dependent N-glycan addition. The radioactivity of the mono- and di-glycosylated bands in (B) was quantified with ImageQuant software. Bars indicate the mean ± SD of the relative fraction of di-glycosylated polypeptides. Note that no non-glycosylated polypeptides are visible. (D) C-terminal N-glycosylation of assembly incompetent GlyR α1-His-Strep(II) TM4 mutants. $^{395}$F, $^{399}$F and $^{402}$F belong to a series of membrane-imbedded aromatic residues of the GlyR α1 subunit, each of which is crucial for functional homopentameric assembly (Haeger et al. 2010).
The α1-His-QWSStrep(II) mutant lacking the C-terminal N-glycosylation site due to the N to Q mutation persisted in the mono-glycosylated form (Figure 5B, lanes 2, 7 and 12). Direct evidence that the α1-His-QWSStrep(II) mutant co-translationally acquires a single N-glycan at the natural 38NVS sequon comes from a comparison with the 38Q-α1-QWS-Strep(II) double mutant. This double mutant, which lacks any glycosylatable sequon due to additional mutational knockout of the natural 38N acceptor site, migrated with a ~3 kDa lower mass (corresponding to one high-mannose N-glycan) than the mono-glycosylated α1-His-QWSStrep(II) mutant (Figure 5B, compare lanes 1, 6 and 11 with lanes 2, 7 and 12) and was subjected to rapid degradation. We conclude that glycosylation of the natural 38NVS sequon occurs co-translationally, whereas C-terminal N-glycosylation at the NWSStrep(II) sequence is a post-translational process that is not impeded by the bulky W in the +2 position.

Like C-terminal N-glycosylation (Figure 5B and C), the assembly of the GlyR α1 protomers into a defined homopentamer (Figure 5A) also occurred with a delayed time course. To determine whether this slow C-terminal N-glycosylation coincides with slow assembly, we took advantage of a series of GlyR α1 mutants that are entirely assembly incompetent due to single alanine substitutions of defined aromatic residues in the fourth transmembrane segment, TM4 (Haeger et al. 2010). Like the assembly competent parental GlyR α1-His-Strep(II) polypeptide (Figure 5D, lanes 1–3), all of the three examined assembly incompetent Strep(II)-tagged TM4 mutants acquired a second N-glycan during the chase period (Figure 5D, lanes 4–12). An expected difference from the assembly competent α1-His-Strep(II) polypeptide was that the TM4 mutants were subjected to rapid degradation, which is consistent with their inability to assemble as stable homopentamers (Haeger et al. 2010). We conclude from this data that post-translational N-glycosylation is an event that occurs independently of the proper assembly of a multi-subunit protein complex.

**C-terminal N-glycosylation occurs in a variety of proteins that enter the secretory pathway**

Next, we examined whether efficient C-terminal glycosylation is related to either the polytopic or multimeric structure of the GlyR. We fused the Strep(II) tag to the C-terminal end of the mouse Na,K pump β1 subunit (Na,K-β1), a 304 amino acid membrane protein with type II topology. Na,K-β1 consists of one membrane-spanning segment, which serves as a signal sequence, and a long extracellular domain harboring three N-glycans (Figure 6A, left panel). Na,K-β1 was synthesized as a core-glycosylated 44 kDa polypeptide (Figure 6B, lane 1) and oligomerized into higher-order species (Figure 6C, lane 2). It appeared in an Endo H-resistant, complex-glycosylated form at the plasma membrane (Figure 6B, lane 3), as confirmed by deglycosylation analysis (Figure 6D, lanes 1–3). Because of its diffuse migration, the complex-type Na,K-β1 glycoform is only weakly visible (at the chosen image gain setting) within the total Na,K-β1 pool, which also contains the readily visible distinctly migrating core-glycosylated Na,K-β1 (Figure 6B, lane 1).

C-terminal fusion with a Strep(II) tag resulted in the appearance of a double band migrating with molecular masses ~1 and ~4 kDa higher than the parental Strep(II) tag-less Na,K-β1 (Figure 6B, lane 2). Although ~1 kDa of the mass shift can again be assigned to the fused 1.1 kDa Strep(II) tag itself, the additional ~3 kDa increase is typical for the addition of one core N-glycan and, therefore, again indicative of Strep(II) tag-mediated C-terminal N-glycosylation (Figure 6B, compare lane 2 with lane 1). Hyperglycosylation of the Strep(II)-tagged Na,K-β1 compared with the parental Na,K-β1 was verified by deglycosylation analysis (Figure 6E, lanes 7–12). With respect to the complex-glycosylated Na,K-β1 form, Strep(II)-dependent hyperglycosylation was even more clearly pronounced and accounted for an ~8 kDa increase (62 vs. 54 kDa; Figure 6B, compare lane 4 with lane 3) that could be reversed by PNGase F treatment (Figure 6D, lanes 4–6).

As an example of a secreted glycoprotein, we studied the ectodomain of Na,K-β1 (designated Na,K-eβ1) encoded by an engineered construct in which the intracellular N-terminal tail and the single transmembrane domain of Na,K-β1 were replaced by a cleavable signal peptide (Figure 6A, middle panel). Na,K-eβ1 was synthesized as a core-glycosylated 40 kDa polypeptide (Figure 6B, lane 5) that oligomerized weakly into homodimers (Figure 6C, lane 4). As expected for a secreted protein, Na,K-eβ1 could not be detected at the cell surface (data not shown) but could be isolated as a complex-glycosylated secreted protein from the extracellular medium (Figure 6B, lane 7). As observed with Na,K-β1 (Figure 6B,
lanes 1 and 2), fusion with a C-terminal Strep(II) tag resulted in an ~4 kDa mass increase corresponding to the ~1 kDa Strep(II) tag and the attachment of one additional core glycan of ~3 kDa (Figure 6B, compare lanes 5 and 6), and this was verified by deglycosylation analysis (Figure 6E, lanes 1–6). As with Na,K-β1, the Strep(II)-dependent mass increase in the complex-type Na,K-β1 amounted to ~8 kDa (Figure 6B, compare lanes 7 and 8) and observed with the ~4 kDa increase observed with the core-glycosylated glycoform (Figure 6B, lanes 5 and 6). The complex-type nature of the secreted Na,K-β1 implied by its diffuse migration in the SDS-PAGE gel was confirmed by deglycosylation analysis (Figure 6D, lanes 7–9).

Finally, we studied an ER-resident non-glycosylated soluble protein, the molecular chaperone BiP, which is also directed to the ER lumen by a cleavable signal peptide (Figure 6A, right panel). BiP is the most abundant ER protein. A His tag or a His-Strep(II) dual tag were inserted immediately N-terminal to the C-terminal “KDEL” ER retention motif of human BiP, which is a common motif in soluble ER-localized proteins (Munro and Pelham 1987). Strep(II) tagging of BiP resulted in a 4–5 kDa increase in mass (Figure 6B, compare lanes 9 and 10), but it did not impair the ER retention of BiP as judged by the absence of secreted BiP in the medium (Figure 6B, lanes 11 and 12). The observed existence of BiP as a mixture of monomers and dimers (Figure 6C, lane 6) is consistent with previous results (Carlino et al. 1992) and was not affected by the Strep(II) tag (Figure 6C, lane 7).

Deglycosylation analysis confirmed the presence of one Endo H-sensitive N-glycan resulting from Strep(II) tagging (Figure 6E, lanes 16–18) when compared with the expected non-glycosylated state of the parental Strep(II) tag-less BiP-His (Figure 6E, lanes 13–15).

To examine whether the Strep(II) tag-mediated C-terminal N-glycosylation of the three proteins shown in Figure 6A occurs with a delayed time course, we used the same pulse-chase protocol as for Strep(II)-tagged GlyR α1 (cf. Figure 5). Parental Na,K-β1 (Figure 6F, lanes 1–3), Na,K-β1 (lanes 7–9) and BiP (lanes 13–15) existed as single bands during the chase, with the sole exception of an apparently non-glycosylated ~35 kDa byproduct of Na,K-β1 (lane 7, blue open dot) that disappeared during the chase (lanes 8 and 9). In contrast, Strep(II)-tagged Na,K-β1 (Figure 6F, lanes 4–6), Na,K-β1 (lanes 10–12) and BiP (lanes 16–18) existed as double bands that differed from the parental polypeptides in the content of the fused 1.1 kDa tag and one ~3 kDa core N-glycan. Quantification revealed that during the chase, the relative amount of the hyperglycosylated upper band increased relative to the lower band for each of the three proteins (Figure 6G). We conclude from this data that proteins with very distinct structures can undergo post-translational N-glycosylation.

C-terminal N-glycosylation also occurs in HEK293 cells.

To determine if C-terminal N-glycosylation also occurs in cells other than X. laevis oocytes, we generated two tetracycline-inducible HEK293 cell lines that stably express GlyR α1-His variants C-terminally fused with either a glycosylatable NWSStrepl(II) sequence or a non-glycosylatable QWSStrep(II) sequence. Selective fluorescence visualization of the surface GlyR pool with cyanine 5 (Cy5) combined with deglycosylation analysis revealed that both plasma membrane-bound GlyR variants existed entirely in the Endo H-resistant complex-glycosylated form (Figure 7A, lanes 1, 2, 4 and 5). The C-terminal NWSStrepl(II) tag resulted in a mass ~5-kDa greater than the QWSStrep(II) tag (Figure 7A, compare lanes 1 and 2 with lanes 4 and 5), whereas the masses of the fully PNGase F-deglycosylated polypeptides were identical (Figure 7A, compare lanes 3 and 6). These results provide clear evidence that C-terminal N-glycosylation also occurs in HEK293 cells.

Fig. 7. Post-translational N-glycosylation in HEK293 cells. (A) Flp-In HEK293 cells stably expressing the indicated GlyR variants were surface-labeled with Cy5 NHS ester and extracted with dodecyl maltoside, followed by Strep-Tacin affinity purification. The purified proteins were treated with the indicated endoglycosidases and resolved by SDS–urea–PAGE. Note that the NWSStrepl(II) tag mediated efficient C-terminal N-glycosylation of the GlyR α1 subunit. (B) GlyR-expressing Flp-In HEK293 cells were pulse-labeled with [35S]methionine for 5 min and then chased for 10 or 90 min, followed by Strep-Tacin affinity purification and SDS–urea–PAGE. The filled blue dot and blue arrowhead indicate the migration positions of GlyR α1 subunits mono-glycosylated at 35S or additionally glycosylated at the NWSStrepl(II) tag, respectively.
Finally, we examined the time course of C-terminal N-glycosylation in HEK293 cells using a similar pulse-chase protocol used in *X. laevis* oocytes. NWSStrep(II)-tagged α1 GlyR isolated directly after a 5 min [35S]methionine pulse migrated as two bands that differed in mass by ~3 kDa, i.e. the mass of a core N-glycan (Figure 7B, lane 1). The lower band (Figure 7B, lanes 1 and 2) migrated at exactly the same position as the non-hyperglycosylatable QWSStrep(II) GlyR variant (Figure 7B, lane 4) and disappeared entirely during the subsequent chase in a manner consistent with post-translational N-glycosylation of the NWSStrep(II) sequence (Figure 7B, lanes 2 and 3). In contrast (but as expected), no such post-translational mass shift was observed with the QWSStrep(II) GlyR variant that contains only the natural co-translational glycosylated 38NVS sequon (Figure 7B, lanes 4–6). We conclude that C-terminal N-glycosylation occurs in HEK293 cells with a delayed time-course that is nonetheless significantly faster than that in *X. laevis* oocytes.

**Discussion**

We demonstrated in this study that an engineered C-terminal NWS sequon is slowly but nearly completely N-glycosylated in the *X. laevis* oocyte expression system, even if only two residues are present C-terminal to the NWS sequence. Delayed but quantitative C-terminal N-glycosylation close to the very C-terminus was also observed with HEK293 cells, indicating that this phenomenon is not species-specific. This observation is surprising in view of the low glycosylation efficiency of sequons close to the C-terminus in a microsome-containing in vitro translation system (Nilsson and von Heijne 2000). In *X. laevis* oocytes and HEK293 cells, Strep(II) tagged-mediated C-terminal glycosylation was an extremely robust effect because it was observed in each experiment without exception.

Full glycosylation was observed previously only when the N-glycan acceptor site and the C-terminus were >60 residues apart (Shakin-Eshleman et al. 1993; Nilsson and von Heijne 2000; Walmsley and Hooper 2003a). Because this is approximately the number of residues needed to span the distance between the ribosomal P-site and the OST catalytic site (Whitely et al. 1996), it was concluded that glycosylation efficiency near the C-terminus decreases because the sequon passes the OST active site faster after the nascent chain has been released from the ribosome. These findings are in line with previous statistical studies showing that glycosylated sites are rare near the C-terminus of secreted glycoproteins (Gavel and von Heijne 1990). However, efficient N-glycosylation at a distance of 26 residues from the C-terminus has been previously observed with a GPI-anchored membrane dipeptidase in human neuroblastoma SH-SY5Y cells (Walmsley and Hooper 2003b). Additionally, several proteins with glycosylated sequons in the last 10 C-terminal residues have been found in the SWISS-PROT database, suggesting that C-terminal N-glycosylation may be more common than expected (Ben-Dor et al. 2004). Our findings provide strong experimental support for the notion that C-terminal N-glycosylation is feasible regardless of the distance to the next membrane-spanning domain. However, it should be noted that, thus far, this conclusion is only valid for small sequence tags, and the usage of a natural C-terminal sequon may be significantly impeded by prior folding of the C-terminal domain.

Position relative to a membrane anchoring sequence has also been observed to strongly affect N-glycosylation site usage (Nilsson and von Heijne 2000; Walmsley et al. 2001). In the present experiments, however, efficient C-terminal N-glycosylation occurred in *X. laevis* oocytes with both a single-spanning membrane protein (the Na,K-pump β1 subunit), with its C-terminal NWS sequon quite distant from the membrane-spanning segment and with polytopic membrane proteins (GlyR α subunits), with NWS sequons quite close to a membrane-spanning segment. Quantitative or nearly quantitative C-terminal N-glycosylation was observed even with two unrelated membrane anchor-less proteins, the ER-resident chaperone BiP and the engineered ectodomain of the Na,K-pump β1 subunit, which were directed into the ER by a natural and a heterologous signal sequence, respectively. However, we observed kinetic differences that may be inherent to specific proteins: NWSStrep(II)-tagged Na,K-β1 and Na,K-eβ1, as closely related examples of a membrane-bound and a secreted protein, acquired the C-terminal N-glycan with almost the same kinetics. Over 50% of these two polypeptides were in the hyperglycosylated form at the end of the 3 h pulse period. A further increase in the hyperglycosylated form occurred during the chase, but this plateaued to a maximum of ~70% by 3 h of chase. In contrast, the soluble ER-resident protein BiP acquired the NWSStrep(II)-dependent N-glycan more slowly, with a time course similar to that of Strep(II)-tagged α1 GlyR, which is a tightly assembled integral membrane protein.

From their rough temporal correlation with GlyR receptor subunit homopentamer formation, it may be inferred that folding and oligomerization are determinants of C-terminal glycosylation. However, our observation that assembly incompetent α1 GlyR TM4 mutants were efficiently glycosylated at the NWSStrep(II) tag implies that post-translational N-glycosylation occurs independently of proper assembly. Moreover, BiP and the secreted Na,K-pump β1 ectodomain, which exist mostly as monomers rather than oligomers, were efficiently C-terminally N-glycosylated. Accordingly, aside from access to the ER lumen, the sole requirement for robust C-terminal N-glycosylation appears to be sufficient ER residence time. Given the rather slow rate at which C-terminal glycosylation occurred, it was surprising to find that only the fully C-terminal glycosylated forms of the membrane proteins examined in this study were at the cell surface. A possible explanation may be that the same subset of proteins that fail to acquire a C-terminal N-glycan are, for other reasons, unable to bypass a quality-control step.

Comparably slow post-pulse N-glycosylation has previously been demonstrated for recombinant human coagulation factor VII in CHO-K1 and HEK293A cells (Bolt et al. 2005). Similar to the present study, the more proximal acceptor site was co-translationally occupied in these cells by an N-glycan, whereas the more distal acceptor site acquired the N-glycan only after the pulse. A notable difference is that the distal acceptor site was skipped by the OST during translocation,
despite being located >80 residues from the C-terminal end (i.e. at a position where the chain is still attached to the ribosome; Bolt et al. 2005).

The post-translational glycosylation of factor VII was greatly reduced in STT3B-depleted HeLa cells, indicating that post-translational glycosylation of skipped glycosylation sites is mediated by the STT3B OST isoform (Ruiz-Canada et al. 2009). Unlike STT3B, the STT3A OST isoform does not mediate post-translational glycosylation; it is responsible for co-translational glycosylation as soon as the nascent chain enters the ER lumen. By analogy, we assume that the same OST isoforms are also present in \( X.\) laevis oocytes and act in sequence to co- and post-translocationally fully glycosylate the polypeptides studied here. \( X.\) laevis STT3A shares 99.6% (97.4%) protein sequence similarity (identity) to its human ortholog, as determined using ClustalW (Larkin et al. 2007). In addition, the STT3B ortholog is highly conserved (~81% DNA sequence identity), but the exact protein sequence similarity cannot be calculated because of frame-shift errors in the published \( X.\) laevis STT3B sequence (accession number BC044321).

The findings that a bulky W in the +2 position of the NWS sequence of the Na,K-\( \beta \)1 construct to yield His-\( \beta \)-1 and His-\( \epsilon \)-1, respectively.

Full-length cDNAs for the human GlyR \( \alpha \)2 subunit (Grenningloh et al. 1990, GenBank accession NM_001118885) and the human GlyR \( \alpha \)3 subunit (Nikolic et al. 1998, GenBank accession NM_001042543) were cloned (from mammalian vectors used in previous studies) into the oocyte expression vector pNKS2 (Gloor et al. 1995) and C-terminally fused with the sequence for six histidines. The full-length cDNA of human BiP (GenBank accession NM_005347) was purchased from ImaGenes (Berlin, Germany), and after subcloning into pNKS2, it was tagged by inserting the hexahistidine sequence at the C-terminal end immediately before the ER retention signal KDEL to yield BiP-His.

Dual-tagged versions of the above constructs were generated by adding the sequences for the affinity tags Strep(I) (AWRHPQFGG) or Strep(II) (NWSHPQFEK; Schmidt et al. 1996) to the C-terminal end of the C-terminal histidine affinity tag, as indicated by the respective extension to the construct names: -His-Strep(I) or -His-Strep(II).

Oocyte expression and two-electrode voltage-clamp electrophysiology

Collagenase-defolliculated \( X.\) laevis oocytes (stage V or VI) were isolated and injected with capped cRNAs, as described previously (Schmalzing et al. 1991). Oocytes were maintained at 19°C in sterile oocyte Ringer’s solution (ORi: 90 mM NaCl, 1 mM KCl, 1 mM CaCl\(_2\), 1 mM MgCl\(_2\) and 10 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), pH 7.4) supplemented with 50 mg L\(^{-1}\) of gentamicin. One to three days after cRNA injection, glycine responses were measured by two-electrode voltage-clamp at a holding potential of ~70 mV, as described previously (Laube et al. 1995).

Protein labeling, purification and PAGE

cRNA-injected oocytes were metabolically labeled overnight by incubation with \( l-\left[ ^{35}\text{S}\right]\text{methionine} (>40 \text{TBq mmol}^{-1}\) Amersham, Braunschweig, Germany) at ~100 MBq mL\(^{-1}\) (0.1 MBq per oocyte) in ORi at 19°C and then chased with unlabeled medium for 24 h. Immediately prior to protein extraction, the intact oocytes were additionally surface-labeled by Cy5 NHS ester (GE Healthcare Biosciences, Freiburg, Germany), an amine-reactive, membrane-impermeant fluorescent dye (Southwick et al. 1990). Oocytes were labeled for 30 min at ambient temperature, as described previously (Becker et al. 2008; Haeger et al. 2010). In some experiments, oocytes were \( ^{35}\text{S}\)methionine-labeled with a shorter pulse of
only 3 h and then chased with unlabeled medium containing 1 mM cycloheximide for the times indicated in the figure legends. Affinity-tagged proteins were purified by Ni-NTA chromatography (Qiagen, Hilden, Germany) or Strep-Tactin chromatography (IBA, Göttingen, Germany) from digitonin (1%, w/v) extracts of oocytes, as detailed previously (Nica et al. 1998; Becker et al. 2008), in the presence of iodoacetamide to prevent artificial cross-linking by inter-subunit disulfide bonds (Sadtlerr et al. 2003). To increase the recovery of proteins extracted directly after a 3 h [35S]methionine pulse interval, the digitonin homogenization buffer was supplemented with 2 mM puromycin (PAA, Colbe, Germany). Bound proteins were released from Ni-NTA sepharose or Strep-Tactin sepharose with non-denaturing elution buffer consisting, respectively, of 1% (w/v) digitonin and 2.5 mM desthiobiotin in 100 mM Tris–HCl (pH 7.6) or 1% (w/v) digitonin and 2.5 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0. BN-PAGE (Schägger et al. 1994), tricine SDS–PAGE (Schägger and von Jagow 1987) and SDS–urea–PAGE were performed as described (Nica et al. 1998; Sadtler et al. 2003; Haeger et al. 2010). Where indicated, the samples were treated prior to SDS–PAGE for 2 h at 37°C with either Endo H or PNGase F (New England Biolabs, Frankfurt, Germany) in the presence of 1% NP-40 to decrease inactivation of PNGase F. The wet SDS–PAGE gels were first scanned in a fluorescence scanner (Typhoon, GE Healthcare Biosciences) to visualize the fluorescently labeled plasma membrane-bound proteins and then dried for subsequent [35S]-phosphorimaging. Individual bands were quantified with ImageQuant software (Amersham, GE Healthcare).

Generation of HEK293 cell lines stably expressing GlyR α1-His-Strep(II) variants

The GlyR α1-His subunit coding sequence was subcloned into the pcDNA5/FRT/TO inducible expression vector using the Gateway cloning system (Invitrogen, Karlsruhe, Germany) and fused in-frame with a C-terminal Strep(II) sequence (NWSHPQFEK) or a non-glycosylatable variant of the Strep (II) sequence, QWS(II)(Strep) (QWSHPQFEK). The N is not part of the Strep-Tactin recognition motif (Schmidt et al. 1996). Stable Flp-In HEK293 cell lines were generated as recommended by the manufacturer (Invitrogen). Expression was induced by the addition of 1 μg mL⁻¹ of tetracycline for 36 h. The cells were then surface labeled with Cy5-NHS ester for 30 min at 21–23°C, quenched with 20 mM glycine for 5 min, and proteins were extracted using dodecyl maltoside (0.1%, w/v) in phosphate-buffered saline, pH 8.0. The GlyR variants were purified by Strep-Tactin™ Sepharose (IBA) affinity chromatography, eluted with 2.5 mM desthiobiotin in 100 mM Tris–HCl, pH 8.0, supplemented with 0.02% dodecyl maltoside (w/v) and resolved by SDS–urea–PAGE. Bound Cy5 fluorescence was visualized by scanning the wet SDS–PAGE gels in a Typhoon fluorescence scanner, as described in the section protein labeling, purification and PAGE.

Alternatively, the cells were induced with 1 μg mL⁻¹ of tetracycline for 18 h, pulse-labeled with ~100 MBq mL⁻¹ L-[35S]methionine and then chased in the presence of 1 mM cycloheximide and 1 mM unlabeled methionine. The tagged GlyRs were purified by Strep-Tactin chromatography and subjected to SDS–urea–PAGE followed by [35S]-phosphorimaging, as described in the section protein labeling, purification and PAGE.

Data presentation

The intensity of the protein bands was quantified using ImageQuant TL software version 7.0 (GE Healthcare Biosciences). Figures with halftone images were prepared using ImageQuant TL for contrast adjustment, Adobe Photoshop CS 8.0 for level adjustment and cropping and Microsoft PowerPoint 2000 for lettering. The topology maps were drawn with T2Xtogo (Beitz 2000). Bar graphs were prepared with SigmaPlot 8.0 (Systat Software Inc., San Jose, CA).

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Conflict of interest statement

None declared.

Abbreviations

BN, blue native; Endo H, endoglycosidase H; ER, endoplasmic reticulum; GlyR, glycine receptor; HEK, human embryonic kidney; Na,K-β1, Na,K pump β1 subunit; Ni-NTA, nickel-nitriloacetic acid; ORi, oocyte Ringer’s solution; OST, oligosaccharyltransferase; PNGase F, peptide:N-glycosidase F; SDS–PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; wt, wild type.

References


