Determinants of Topogenesis and Glycosylation of Type II Membrane Proteins

ANALYSIS OF Na,K-ATPase β1 AND β3 SUBUNITS BY GLYCOSYLATION MAPPING*

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The structural and molecular determinants that govern the correct membrane insertion and folding of membrane proteins are still ill-defined. By following the addition of sugar chains to engineered glycosylation sites (glycosylation mapping) in Na,K-ATPase β isoforms expressed in vitro and in Xenopus oocytes, in combination with biochemical techniques, we have defined the C-terminal end of the transmembrane domain of these type II proteins. N-terminal truncation and the removal of a single charged residue at the N-terminal start of the putative transmembrane domain influence the proper positioning of the transmembrane domain in the membrane as reflected by a repositioning of the transmembrane domain, the exposure of a putative cryptic signal peptidase cleavage site, and the production of protein species unable to insert into the membrane. Glycosylation mapping in vivo revealed that the degree of glycosylation at acceptor sites located close to the membrane increases with the time proteins spend in the endoplasmic reticulum. Furthermore, core sugars added to such acceptor sites cannot be processed to fully glycosylated species even when the protein is transported to the cell surface. Thus, the glycosylation mapping strategy applied in intact cells is a useful tool for the study of determinants for the correct membrane insertion of type II and probably other membrane proteins, as well as for the processing of sugar chains in glycoproteins.

Subunit assembly of oligomeric membrane proteins often involves multiple but poorly understood interactions between the different subunits (for review see Ref. 1). Na,K-ATPase and H,K-ATPases are interesting model proteins for the study of functional roles of different subunit interaction sites, because these two enzymes are the only members of the cation-transporting P-type ATPase that are oligomeric and contain, in addition to the catalytic α subunit, a second subunit, the β subunit in the functionally active enzyme. Similar to most other P-type ATPases, the α subunits of Na,K- and H,K-ATPases are polytopic membrane proteins with 10 transmembrane segments that carry the main functional properties. The β subunits associated with Na,K- and H,K-ATPase α subunits are type II membrane proteins with a short cytoplasmic N terminus, a single transmembrane domain, and a large glycosylated ectodomain. To date, three Na,K-ATPase and one gastric H,K-ATPase β isoforms have been identified, which exhibit a similar domain structure but a low degree of sequence identity of 20–35%. At present, we know that β subunits have several functions that may be finely regulated by different isoforms. A primary role of the β subunit is to support the maturation of the Na,K- and H,K-ATPase α subunits, which, in contrast to other P-type ATPases, are stably expressed and become functionally active only when properly associated with a β subunit (2). In addition to this chaperone function, the β subunit has also been shown to influence the transport properties of the mature Na,K-ATPase, e.g. its apparent affinities for K+ and Na+ (3–7).

Interaction sites that have been identified in Na,K- and H,K-ATPase α and β subunits involve the extracellular, transmembrane, and cytoplasmic domains. By using the two-hybrid system, an extracellular β domain adjacent to the transmembrane segment has been shown to interact with the extracellular loop between transmembrane segments M7 and M8 of the α subunit of Na,K-ATPase (8) and H,K-ATPase (9). A β sheet-like structure formed by the 10 most C-terminal amino acids most likely represents another α-interaction site in the ectodomain of the β subunit (10). Interactions in the ectodomains of α and β subunits are important for the correct folding and the stabilization of the α subunit of oligomeric P-type ATPases (11, 12). Furthermore, studies performed on chimeras formed between Na,K-ATPase β1 and gastric H,K-ATPase β subunits suggest that interactions with the β-ectodomain are responsible for the differences observed in the transport properties of the Na,K-ATPase associated with different β isoforms (5, 7, 13). Evidence for interactions between transmembrane segments of the Na,K-ATPase α and β subunits has been obtained by cross-linking experiments (14, 15), but the functional role of these interactions is not yet defined. Analysis of chimeric Na,K-ATPase/β subunits suggests that only transmembrane interactions of Na,K-ATPase β but not that of H,K-ATPase β subunits permit the correct folding and ER1 exit of the Na,K-ATPase α subunit (7, 13). Finally, the functional implications of subunit interactions in the cytoplasmic domains, which are supported by proteolysis protection assays (2, 16), are the least well understood. Indeed, truncation of the N terminus of Na,K-ATPase β1 subunit does not impede α interaction and stabilization but significantly decreases the appar-

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‡ The abbreviations used are: ER, endoplasmic reticulum; EndoH, endoglycosidase H; PAGE, polyacrylamide gel electrophoresis; BIP, binding protein; Lep, leader peptidease; TPCK, 1-tosylamido-2-phenyl-ethyl chloromethyl ketone.
ent K⁺ and/or Na⁺ affinity of the Na,K-ATPase (2, 6, 7). However, results of a detailed mutational analysis indicated that the β N terminus may not be directly involved in the functional effects observed after complete N-terminal truncation (7), rather that N-terminal truncation could indirectly affect another domain of the β subunit.

To better understand the structural and functional roles of α-interaction domains in Na,K-ATPase β subunits, we aimed in this study to 1) define the membrane-spanning domain of the β subunit by identifying the amino acids that actually make up the transmembrane α-helix of Na,K-ATPase β subunits and 2) probe potential changes in the transmembrane domain after N-terminal truncation. To address these questions, we have applied a glycosylation mapping technique (17). This assay is based on the observation that an engineered consensus glycosylation acceptor site can be modified by oligosaachytransf erase only if this site is placed at a precise “minimal glycosylation distance” from a transmembrane segment (17, 18). Therefore, the active site of oligosaachytransferase can be used as a reference point against which the position of membrane helices can be determined (18). In this study, we apply for the first time the glycosylation mapping assay to proteins synthesized in intact cells, and our results show that the minimal glycosylation distance in intact cells is shorter than that of proteins synthesized in an in vitro translation system. Our studies also suggest that the C-terminal ends of the transmembrane helices of Na,K-ATPase β1, and β2 subunits are located near Leu58 and Met64, respectively. N-terminal truncation of β1 and β2 subunits results in a repositioning of the transmembrane helices relative to the membrane.

Although these results do not resolve the question of the functional role of cytosolic α–β interactions, they clearly show that the N terminus of Na,K-ATPase β subunits is crucial for a correct β subunit topology that is compatible with proper assembly and, in consequence, the acquisition of the correct structural and functional properties of the Na,K-ATPase α subunit. The results also support our hypothesis that structural changes in the ectodomain and/or the transmembrane domain are responsible for the K⁺ effect observed in Na,K-ATPase associated with N-terminally truncated β subunits. Finally, our results validate the glycosylation assay applied in intact cells as a general tool to identify determinants of correct membrane insertion and, in addition, of the glycosylation processing of membrane proteins.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis of Na,K-ATPase β1 and β3 Isoforms, Construction of Lep/β1 Chimera, and cRNA Preparation—Truncation and point mutants of Xenopus Na,K-ATPase β1 and β3 isoforms contained in the psD5 vector (19) were prepared by using the polymerase chain reaction method of Nelson and Long (20). β1 subunits lacking 33 amino acids after the initiator methionine (β1t3, see Fig. 1) were prepared as described previously (2). For the preparation of β1t37, lacking 36 amino acids after the initiator methionine, a β1 cDNA fragment was amplified using an antisense oligonucleotide consisting of nucleotides 301–320 and a sense oligonucleotide comprising part of the noncoding sequence, the ATG coding for the first methionine, and the sequence coding for the amino acids Leu36 to Tyr43 of the β1 isoform. The amplified DNA fragment was then used as a primer to elongate the inverse DNA strand and was finally amplified using a sense oligonucleotide encoding part of the psD5 vector and primer D of Nelson and Long. The mutated DNA fragment was introduced into the psD5 vector using NheI and Stul restriction sites.

N-Linked glycosylation acceptor sites were introduced in the β1 and β3 isoforms at various positions after the putative end of the transmembrane domain predicted by Kyte-Doolittle hydrophathy analysis (21) (see Fig. 1). For this purpose, β1 fragments were amplified between an antisense oligonucleotide covering nucleotides 628–648 and tagged by primer D of Nelson and Long and sense oligonucleotides containing codons for the glycosylation acceptor site Asn-Ser-Thr. In these replace-
some by centrifugation of the yolk-depleted homogenate at 20,000 \( \times g \) for 30 min at 4 °C. Denaturing immunoprecipitations were performed with a β3 antibody on the total volume of the collected media and on microsomes corresponding to six oocytes.

**Protease Assays**—To test the protease sensitivity of the 28-kDa protein species produced in β3,137-mutant oocytes, we used two assays. In the first assay, oocytes were injected with wild type β1, or β3,137 mutant cRNA together with 4 μCi/oocyte of Easy Tag Express (NEN Life Science Products). After a 4-h pulse period, oocytes were homogenized with a plastic pestle in an Eppendorf tube in a solution containing 50 mM Tris-HCl (pH 7.5), 0.25 mM sucrose, 50 mM potassium acetate, 5 mM MgCl\(_2\), and 1 mM dithiotreitol. Aliquots were incubated with 10 μM CaCl\(_2\) in the absence or presence of 0.2 mg/ml proteinase K (Merck) for 1 h at 4 °C before addition of 1 mg/ml phenylmethylsulfonyl fluoride. Immunoprecipitations were performed under denaturing conditions with a β3 antibody. In the second protease assay, oocytes were injected with wild type β1, or β3,137 mutant cRNA or with BigP cRNA and labeled for 24 h. Oocytes were then injected with 30 nl of H\(_2\)O or 30 nl of a solution containing 25 mg/ml trypsin (TPCK-treated, Fluka). Oocytes were left for 1 h at 19 °C before preparation of digoxigenin extracts and immunoprecipitation with a β3, or a BigP antibody (27).

**Pump Current Measurements**—Na,K-pump activity was measured as the K+-induced outward current using the two-electrode voltage clamp method as described earlier (4). Current measurements were performed 3 days after injection of oocytes with Bufo c cRNA together with different comRNAs. To determine the maximal Na,K-pump current \( I_{\text{max}} \), oocytes were loaded with Na\(^+\) in a nominally K+-free solution containing 200 mM ouabain, a concentration that inhibits endogenous Na,K-pumps but not the moderately ouabain-resistant exogenous Bufo Na,K-pumps (32). The activation of the Na,K-pump current by K+ was determined in a Na+-free solution (140 mM sucrose, 0.82 mM MgCl\(_2\), 0.41 mM CaCl\(_2\), 10 mM N-methyl-D-glucamine-HEPES, 5 mM BaCl\(_2\), and 0.1 mM tetrathylammonium chloride, pH 7.4), and the current induced by increasing concentrations of K+ \((0.02, 0.1, 0.5,\) and 5.0 mM K\(^+\)) was measured at ~50 mV. To determine \( I_{\text{max}} \) values, the Hill equation was fitted to the data of the current \( I \) induced by various K+ concentrations \((\text{IK}^+)\) using a least square method, \( I = I_{\text{max}}(1 + (\text{IK}^+)^{nH}) \), where \( nH \) is the half-activation constant. According to previously published data (4), the Hill coefficient was set to a value of 1.0.

**RESULTS**

To define the transmembrane domains of Na,K-ATPase β1 and β3 isoforms and the possible changes in the transmembrane domain topology after N-terminal truncation, we have used a glycosylation mapping assay together with other biochemical techniques. For the glycosylation mapping assay, we have introduced Asn-Ser-Thr glycosylation acceptor sites at various positions around the predicted C-terminal ends of β1 and β3 subunit transmembrane domains and have used the concept of minimal glycosylation distance as defined as the number of amino acids separating the C-terminal end of the transmembrane domain from the first Asn residue that is half-maximally glycosylated (17).

**Delineation of the Membrane-spanning Domain of Na,K-ATPase β1 and β3 Subunits by Glycosylation Mapping**—Transmembrane domain predictions of β1 subunits by various computer programs are shown in Fig. 1A. All programs predict that β1 contains a transmembrane domain α-helix, but predictions of its N- and C-terminal ends vary between programs. Kyte-Doolittle hydropathy analysis predicts that the β1 transmembrane domain begins at Trp\(^33\) and ends at Ser\(^35\) with the exception of the HMMTOP program, predictions of the N-terminal end of the β1 transmembrane domain was similar with all programs and was located downstream of that predicted by Kyte-Doolittle analysis. Predictions of the C-terminal end of the β1 transmembrane domain varied among the different programs, ranging from Leu\(^66\) to Leu\(^65\).

For the glycosylation mapping assay of β3, we left the four natural glycosylation sites intact and introduced new glycosylation acceptor sites at position -1, +3, +5, +7, and +10 with respect to Leu\(^66\); i.e., the C-terminal end of the transmembrane domain predicted by Kyte-Doolittle hydropathy analysis (see Fig. 1B). Glycosylation analysis of proteins synthesized in vitro showed that the wild type β3 subunit and all β3 glycosylation mutants were EndoH-sensitive and, according to the shift in the molecular mass, were glycosylated on the four natural glycosylation sites (Fig. 2C). Engineered glycosylation sites at positions -1, +3, or +5 (lanes 3, 5, and 7) were not significantly glycosylated, whereas those at positions +7 and +10 were nearly 100% glycosylated (lanes 9 and 11). Extrapolation of these data predicts that half-maximal glycosylation occurs at position +6 (Fig. 2D). Taking 10 amino acids as a reference for minimal glycosylation distance (see above), our
data suggest that the $\beta_3$ transmembrane domain, like the $\beta_1$ transmembrane domain, is shorter than predicted by Kyte-Doolittle hydropathy and that it ends around Met 61.

The reasons for the different shapes of the glycosylation curves observed with $\beta_1$ and $\beta_3$ isoforms (Fig. 2D) are not known. One possible explanation for the observed all-or-nothing glycosylation of $\beta_3$ subunits compared with the nearly linear relationship between the percentage of glycosylation and the Asn position in $\beta_1$ subunits may be that the presence of natural sugars decreases the flexibility of the protein and thus the probability of post-translational glycosylation of engineered glycosylation sites situated close to the membrane.

The Glycosylation Efficiency of Proteins Increases with the Time Spent in the ER—Because glycosylation in translation systems in vitro appears to be somewhat inefficient as reflected by the incomplete glycosylation of wild type $\beta_1$ subunits (Fig. 2A, lane 1), we compared glycosylation mapping results obtained by in vitro translations with results obtained after expression of glycosylation mutants in intact cells. Upon expression in Xenopus oocytes and labeling during a 6-h pulse, the total population of wild type $\beta_1$ subunits (Fig. 3A, lanes 1 and 2) as well as of the $\beta_1$ glycosylation mutant +11 (lanes 15 and 16) was glycosylated. Furthermore, the glycosylation mutant +2 was more efficiently glycosylated compared with the in vitro translated protein (compare Fig. 3A, lane 9, to Fig. 2A, lane 4; Fig. 3B). With the exception of the glycosylation mutant –5, which was never glycosylated (data not shown), the proportion of glycosylated species for all other glycosylation mutants further increased after a 24- or a 72-h chase period (Fig. 3A, lanes 5–8 and lanes 11–14; Fig. 3B). Xenopus $\beta_1$ subunits expressed in oocytes in the absence of $\alpha$ subunits are retained in the ER and are slowly degraded (2). Our results therefore suggest that the efficiency of glycosylation increases with the time proteins spend in the ER and that the actual minimal glycosylation distance is shorter in proteins synthesized in intact cells than in those translated in vitro, especially if the rate of exit from the ER is low.

Role of Gln$^{56}$ in Defining the C-terminal End of the $\beta_1$ Transmembrane Domain—Because glycosylation mapping suggests that the C-terminal end of the $\beta_1$ transmembrane domain is more proximal than that predicted by Kyte-Doolittle hydropathy analysis, we considered the possibility that Gln$^{56}$ plays a
Fig. 2. In vitro glycosylation mapping of the Na,K-ATPase \( \beta_1 \) and \( \beta_3 \) subunits and of Lep/\( \beta \) chimera. In vitro translations were performed as described under “Experimental Procedures,” and the labeled proteins were treated or not treated with endoglycosidase H (EndoH) before revelation by SDS-PAGE and fluorography. A, in vitro glycosylation of wild type (lane 1) and \( \beta_1 \)-glycosylation mutants (lanes 2–8). The position of migration of proteins of known molecular mass is shown. Introduced glycosylation acceptor sites are depicted as black dots in a schematic representation of the \( \beta_1 \) subunit (for a more detailed description of mutants, see Fig. 1). B, in vitro glycosylation of Lep/\( \beta_3 \) chimera. The second Lep transmembrane domain was replaced by the putative \( \beta_3 \) transmembrane domain ending at Thr\(^{61} \) and preceded by part of the \( \beta_3 \) cytoplasmic domain. The last five C-terminal amino acids of the \( \beta_1 \) transmembrane domain and the first five N-terminal amino acids of Lep in Lep/\( \beta_3 \) chimera are shown. Glycosylation acceptor sites were introduced at positions +3 and +16 in the Lep protein. cg, core-glycosylated protein species; ng, non-glycosylated protein species. C, in vitro glycosylation of wild type and \( \beta_3 \)-glycosylation mutants. D, quantification of data shown in A and C (mean of two experiments). The percentage glycosylation of \( \beta_1 \) (closed squares) or \( \beta_3 \) (closed triangles) is shown as a function of the position of engineered asparagine (Asn) residues from the putative C-terminal transmembrane domain end.

Role in defining the C-terminal end of the \( \beta_1 \) transmembrane domain. Consistent with this idea, a \( \beta_1 \), Q56L mutant containing a glycosylation site at +2 (+2 Q56L) was significantly less glycosylated than the glycosylation mutant +2 (compare Fig. 3A, lanes 21-26 to lanes 9–14). This suggests that introduction of a hydrophobic residue at this position “pulls” the C-terminal end of the transmembrane domain into the membrane.

Engineered Glycosylation Sites Adjacent to the \( \beta_1 \) and \( \beta_3 \) Transmembrane Domain Impede Efficient \( \alpha \)-\( \beta \) Interactions and Correct Glycosylation Processing—Because an \( \alpha \)-assembly domain is possibly located within the 68 amino acids succeeding the \( \beta \) transmembrane domain, according to the two-hybrid assay (8), we wondered whether the glycosylation mapping assay could also provide information on the assembly process of \( \beta \) with \( \alpha \) subunits. In other words, does introduction of glycosylation sites close to the \( \beta \) transmembrane domain interfere with subunit oligomerization and maturation and/or does subunit oligomerization influence glycosylation at these sites?

After a 6-h pulse, wild type \( \beta_1 \) subunits co-expressed with \( \alpha \) subunits in Xenopus oocytes co-immunoprecipitated and were thus assembled with \( \alpha \) subunits, mainly in their core-glycosylated ER form (Fig. 4A, lane 1). In contrast to unassembled \( \alpha \) subunits, which are rapidly degraded (lanes 22–24), formation of \( \alpha \)-\( \beta \) complexes in the ER led to the stable expression of \( \alpha \) subunits and permitted ER exit of \( \alpha \)-\( \beta \) complexes as reflected by the acquisition of complex sugars by the \( \beta \) subunit (lanes 1–3) and the expression of functional Na,K-pumps at the cell surface as assessed by pump current measurements (Fig. 4B, lane 1). Significantly, non-glycosylated \( \beta_1 \) subunits (Fig. 4A, lanes 4–6) and in particular \( \beta_3 \)-glycosylation mutants containing glycosylation sites at positions +1, +2, and +4 (lanes 7–15) co-immunoprecipitated efficiently, and thus associated with \( \alpha \) subunits, but only after a 24- and a 72-h chase and not after a 6-h pulse. The delayed assembly of the \( \beta \)-glycosylation mutants was reflected in the partial degradation of the \( \alpha \) subunits after the 24-h chase period. Oocytes co-expressing \( \alpha \) subunits with \( \beta_1 \)-glycosylation mutants −1, +2, and +4 (lanes 7–15) co-immunoprecipitated efficiently, and thus associated with \( \alpha \) subunits, but only after a 24- and a 72-h chase. These results indicate that the sugar moieties and/or the mutations introduced at positions close to the transmembrane domain significantly impede correct \( \alpha \)-\( \beta \) interactions and reduce cell surface expression. Despite the partial degradation of the \( \alpha \) subunit, non-glycosylated \( \beta_1 \) subunits (Fig. 4B, lane 2) and the \( \beta_1 \)-glycosylation mutants +7 (lane 6) or +11 (lane 7) produced a similar number of functional \( \alpha \)-\( \beta \) complexes at the cell surface as wild type \( \beta_1 \) subunits (lane 1) probably due to the regulated cell surface expression in Xenopus oocytes. Previous studies (22) have shown that Xenopus oocytes only express a limited number of exogenous Na,K-pumps at the cell surface, which does not exceed six to eight times the number of endogenous Na,K-pumps and which is therefore to a certain extent independent of the number of stable \( \alpha \)-\( \beta \) complexes formed.

Interestingly, association of the \( \beta_1 \)-glycosylation mutant −1 with the \( \alpha \) subunit reduced its glycosylation compared with that of the glycosylation mutant −1 expressed without \( \alpha \) subunits from 78 ± 5% (n = 3) to 47 ± 4% (n = 4) (p < 0.01) (compare Fig. 4A, lane 8 to Fig. 3A, lane 5). Furthermore, the extent of glycosylation remained stable over time in the \( \alpha \)-associated \( \beta_1 \) −1 mutant in contrast to the non-associated \( \beta_1 \) −1 mutant in which glycosylation further increased after a 72-h chase (compare Fig. 4A, lanes 8 and 9 with Fig. 3A, lanes 5 and 7). These results may reflect that, after delayed assembly of the \( \beta_1 \) −1 mutant, \( \alpha \)-\( \beta \) complexes leave the ER in their non-glycosylated or core-glycosylated form. Finally, despite expression at the cell surface, all \( \alpha \)-associated \( \beta_1 \) subunits with engineered glycosylation sites up to position +11 did not, in contrast to wild type \( \beta_1 \) subunits (Fig. 4A, lanes 2 and 3), become fully glycosylated after 3 days of expression but remained in their core-glycosylated form (lanes 9, 12, 15, 18, and 21).

In contrast to \( \beta_1 \) isoforms devoid of natural sugars or containing engineered glycosylation sites, \( \beta_3 \) isoforms containing natural and engineered glycosylation sites associated efficiently with co-expressed \( \alpha \) subunits after a 6-h pulse (Fig. 4C, lanes 4, 7, and 10) similar to wild type \( \beta_3 \) subunits (lane 1). This is perhaps due to the presence of natural sugar chains, which may favor a more efficient folding of the \( \beta_3 \) subunit compared with the non-glycosylated \( \beta_3 \) subunit. Nevertheless, the presence of engineered glycosylation sites in \( \beta_3 \) subunits prevented complete stabilization of the \( \alpha \) subunit and reduced the proc-
methionine and a 24- or a 72-h chase period, microsomes were prepared and immunoprecipitated with a truncated sylation acceptor sites were introduced into the N-terminally shown in Figs. 3A and 2B conditions. Immunoprecipitates were not treated or treated with EndoH and subjected to SDS-PAGE and fluorography.

Digitonin extracts were prepared after a 6-h pulse, a 24-h chase, and a 72-h chase period, and immunoprecipitations were performed using an closed triangles in intact cells during a 6-h pulse (open squares) or a 72-h chase (open circles), 1 ng of non-glycosylated 22–24 glycogen processing of b under "Experimental Procedures." Measurements were performed in the presence of 200 nM ouabain, which inhibits endogenous 

Transmembrane Domain in the Membrane—Previous observations (7) have led to the assumption that N-terminal truncation may indirectly affect the ectodomain and/or the transmembrane (lanes 4–12) further supporting the notion (lanes 1–3) or glycosylated, whereas the corresponding proteins lacking the natural glycosylation sites were glycosylated (Fig. 5, A and B, lanes 3 and 4). On the other hand, the glycosylation pattern was different in β1t34 mutants and full-length β1 subunits containing engineered glycosylation sites at the same positions (compare Fig. 5, A and B, lanes 5–12; Fig. 5C). Apart from the glycosylation site at position −5, which was glycosylated to a

![Image](http://www.jbc.org/)

**Fig. 3.** Glycosylation of engineered glycosylation sites in β subunits in vivo. A, in vivo glycosylation mapping of the β1 subunit. Xenopus oocytes were injected with 0.5 ng of wild type (lanes 1 and 2) or 0.5–1 ng of β3,4-glycosylation mutant cRNA (lanes 3–26). After a 6-h pulse with [35S]methionine and a 24- or a 72-h chase period, microsomes were prepared and immunoprecipitated with a β1 antibody under denaturing conditions. Immunoprecipitates were not treated or treated with EndoH and subjected to SDS-PAGE and fluorography. B, quantification of data shown in Figs. 3A and 2A (mean of two to three experiments). The percentage glycosylation of β1 subunits synthesized in vitro (open squares) or in intact cells during a 6-h pulse (open circles), a 24-h chase (closed circles) or a 72-h chase (closed triangles) is shown as a function of the position of engineered asparagine (Asn) residues from the putative C-terminal transmembrane domain end.

![Image](http://www.jbc.org/)

**Fig. 4.** Engineered glycosylation sites in β subunits impede efficient α-β interactions, and the core sugars are not correctly processed. A, α-subunit assembly and glycosylation processing of β3,4-glycosylation mutants. Oocytes were injected with 7 ng of Bufo α, and wild type (lane 1) or mutant (lanes 2–7) β1-cRNA. 3 days after injection, maximal pump currents (I_pmax) of α-β complexes were determined by extrapolation of K^+_activation curves as described under “Experimental Procedures.” Measurements were performed in the presence of 200 nM ouabain, which inhibits endogenous Xenopus Na,K-pumps but not the moderately ouabain-resistant Bufo Na,K-pumps. Shown are means ± S.E. of data from 15–30 oocytes obtained from four different Xenopus females. *, p < 0.05; **, p < 0.01 compared with oocytes injected with wild type α-β complexes. C, α-subunit assembly and glycosylation processing of β3,4-glycosylation mutants. Oocytes were injected with 7 ng of Bufo α1 and 1 ng of wild type (lanes 1–3) or β3,4-glycosylation mutant (lanes 4–12) cRNA. Digitonin extracts were prepared after a 6-h pulse, a 24-h chase, and a 72-h chase period, and immunoprecipitations were performed with an α-antibody under non-denaturing conditions. One out of two representative experiments is shown. ng, non-glycosylated; cg, core-glycosylated; fg, fully glycosylated β subunits.
higher extent in β1t34 than in full-length β1, glycosylation sites at more distal positions were less glycosylated in β1t34 than in full-length β1 subunits.

These results clearly indicate that N-terminal truncation results in a repositioning of the β1 transmembrane domain in the membrane. Our results suggest that the C-terminal end of the β1t34 transmembrane domain is located about 4 residues downstream of that of the β1 transmembrane domain, i.e. around Ile62. This would also be consistent with the increased glycosylation efficiency seen at position −5 in β1t34. Although this Leu to Asn mutation lies right after the end of the 1t34 transmembrane domain and could be expected to “push” the transmembrane domain out of the membrane, possibly far enough to allow some degree of glycosylation as is indeed observed.

We have previously shown that co-expression of α subunits with β1t34 containing the three natural glycosylation sites leads to the formation of stable and functional Na,K-pumps (7). β1t34 glycosylation mutants devoid of natural but containing engineered glycosylation sites showed qualitatively similar effects in assembly and processing as full-length β1 glycosylation mutants (Fig. 4). However, the consequences of inefficient assembly on cell surface expression of functional Na,K-pumps were even more pronounced (data not shown).

In conclusion, the results obtained with the glycosylation mapping assay suggest that the position in the membrane of the β1t34 transmembrane domain and the conformation of adjacent C-terminal domains differ from those of the wild type β1 subunit and that the cytoplasmic N terminus is therefore an important determinant for the correct membrane insertion and folding of this type II protein.

Effects of N-terminal Truncation on the Positioning of the β1 Transmembrane Domain in the Membrane—To confirm the general relevance of our observation we also investigated the effects of N-terminal truncations on β3 isoforms. For this purpose, we prepared a N-terminally truncated β3t37 mutant, which placed the initiator methionine at the same position with respect to the putative N-terminal end of the transmembrane domain as in the β1t34 mutant (see Fig. 1B) but which left the four natural glycosylation sites intact. Compared with full-length β3 subunits, β3t37 was less well expressed after a 24-h pulse and a 48-h chase both in the absence (data not shown) or in the presence of co-expressed α subunits (Fig. 6A, compare lanes 1 and 3 to lanes 5 and 7) indicating that β3t37 is more susceptible to degradation. Also, N-terminally truncated β3t37 did not become fully glycosylated, was not able to efficiently stabilize co-expressed α subunits during a 48-h chase (Fig. 6C, compare lanes 1–4 to lanes 5–8), and produced low levels of functional α-β complexes at the plasma membrane (Fig. 6D, lane 2). This contrasts with wild type β3 subunits (Fig. 6, A–C, lanes 1–4, Fig. 6D, lane 1), β3 subunits (Fig. 4A, lanes 1–3, Fig. 6D, lane 4), or N-terminally truncated β3t34 (Fig. 6, A–C, lanes 17–20, Fig. 6D, lane 5), which, after co-expression with α subunits, could produce stable, functional Na,K-pumps at the cell surface and were themselves stably expressed and correctly processed to the fully glycosylated form.

Immunoprecipitations of β3t37 revealed the presence of two major protein species in addition to the core-glycosylated protein both in the presence (Fig. 6A, lane 5) or absence (data not shown) of co-expressed α subunits. These protein species were not apparent after expression of the wild type β3 subunit (lane 1) or the β3t34 mutant (lane 17). The first of these products (asterisk) with a molecular mass of about 35 kDa migrated slightly faster than the core-glycosylated β3t37 and was itself core-glycosylated as suggested by its cleavage by EndoH to a 24-kDa species (compare lanes 5 and 6). This product was also observed in microsomal preparations (Fig. 6B, lanes 5 and 6) that permit enrichment of membrane proteins as well as of soluble proteins contained within right-side-out ER vesicles (27). The 35-kDa protein species persisted after a 48-h chase period (lanes 7 and 8) and was able to transiently associate with the α subunit (data not shown). In contrast to the wild type β3 subunit (Fig. 6, A and B, lanes 3 and 4), neither the authentic β3t37 nor the glycosylated 35-kDa product became fully glycosylated during a 48-h chase period (Fig. 6, A and B, lanes 7 and 8). The second protein species (Fig 6A, solid dot) of about 28 kDa, which was revealed in digitonin extracts (Fig. 6A, lane 5) but not in microsomal preparations (Fig. 6B, lane 5) after a 24-h pulse, migrated with the EndoH-treated β3t37 (Fig. 6A, lane 6). This protein species disappeared after a 48-h chase period (lane 7) and was not able to associate with the α subunit (data not shown). The identity of the 35- and 28-kDa products is discussed below.

Effects of N-terminal Charged Residues on the β1 and β3 Transmembrane Domains—One possibly important difference between β3 and β1 N-terminally truncated mutants is the presence, after the initiator methionine, of a positively charged lysine residue in β3t34 as opposed to a leucine residue in β1t37 (see Fig. 1). We tested whether the presence or absence of a single, positively charged residue could account for some of the differences observed between β1t34 and β3t37 expression by replacing the leucine by lysine in β3t37 (β3t37L38K) and the lysine by leucine in β1t34 (β1t34K38L). Replacing the leucine
Fig. 6. Alterations in the membrane topology of β subunits after N-terminal truncation and influence of the presence of a positively charged amino acid residue. Oocytes were injected with α and β, βt34, βt37, βt37L38K, βt34K35L, or βt34 cRNA and subjected to a 24-h pulse and a 48-h chase before digitonin extracts (A) or microsomes (B) were prepared. A and B, denaturing immunoprecipitations of β3 and β subunits with a β3 (lanes 1–12) or a β3 antibody (lanes 13–20), respectively. *, glycosylated 35-kDa protein species of βt34 mutants, which is EndoH sensitive and only apparent in digitonin extracts (A). C, denaturing immunoprecipitations from digitonin extracts of α subunits with an α antibody. D, Na,K-pump current measurements of cell surface-expressed α-β complexes. Oocytes were injected with Bufo α1 and wild type or mutant β3 or β3 cRNA. 3 days after injection, maximal pump currents (Imax) of α-β complexes were determined. Shown are means ± S.E. of data from 16 oocytes obtained from two different Xenopus females. *, p < 0.01 compared with oocytes injected with wild type α-β complexes.

by a lysine residue in β3t37 did indeed significantly improve its stability and led to the disappearance of the 28-kDa band and to a reduction in the 35-kDa protein species (Fig. 6A, lanes 9 and 10). Furthermore, β3t37L38K could stabilize co-expressed α subunits (Fig. 6C, lanes 9–12), became fully glycosylated (Fig. 6, A and C, lanes 11 and 12), and increased, although only slightly, the pump current measured at the cell surface compared with that measured in oocytes expressing β3t37 (Fig. 6D, lanes 2 and 3). In contrast, replacing the lysine residue by a leucine residue in β3t34 led to the appearance of a non-glycosylated product (35 kDa) that was mainly observed in digitonin extracts after a 24-h pulse period (Fig. 6A, lanes 13 and 14) analogous to the 28-kDa product of β3t37 (lanes 5 and 6). β3t34K35L was processed to a fully glycosylated, EndoH-resistant form (Fig. 6A, lanes 15 and 16) but was not able to stabilize the α subunit as efficiently as β3t34 (Fig. 6C, compare lanes 13–16 to lanes 17–20), which probably explains the significant decrease in the cell surface expression of functional pumps compared with oocytes expressing β3t34 (Fig. 6D, lanes 5 and 6). These results confirm previous observations (34) that a single positive charge close to the N-terminal end of the transmembrane domain segment can indeed play an important role in the correct membrane integration of type II membrane proteins.

A final set of experiments were aimed at the identification of the nature of the additional protein species observed in βt37- or βt34K35L-expressing oocytes to better understand the molecular basis of the perturbations in the biosynthesis and stability of these two proteins. The lack of glycosylation, the nearly complete absence in microsomal preparations, and the rapid degradation indicated that the 28-kDa protein species may be a cytosolic form of the βt37 protein. To test this possibility, we followed the appearance of the 28-kDa species in the medium of oocytes permeabilized with saponin. Saponin treatment produced some nonspecific detergent effects as reflected by the decrease in immunoprecipitable wild type β3 subunits from microsomal preparations (Fig. 7A, lanes 1 and 2) and the appearance of about 1% of core-glycosylated β3 subunits in the medium (lanes 3 and 4). However, under similar experimental conditions, saponin removed preferentially the contaminating 28-kDa protein species from microsomes (lanes 5 and 6) and, in particular, produced a much more significant release of the 28-kDa protein species than of the β3t37 protein to the medium (lanes 7 and 8) indicating that the 28-kDa protein species might indeed be cytosolic. This result was further supported by the observation that proteinase K treatment of homogenates from oocytes expressing wild type β3 subunits or βt37 mutants only removed the cytoplasmic N-terminal tail of the wild type β3 subunit (Fig. 7B, lanes 1 and 2) and had no effect on the N-terminally truncated βt37, but completely digested the 28-kDa protein species as well as some additional protein species of lower molecular mass occasionally observed in βt37-expressing oocytes (lanes 3 and 4). Finally, a similar result was obtained when trypsin was injected into intact oocytes. Injected trypsin only removed the cytosolic tail of wild type β3 subunits (Fig. 7C, lanes 1 and 2) and did not digest β3t37 or the glycosylated 35-kDa protein species but almost completely digested the 28-kDa and lower molecular mass species. Trypsin had no access to intraluminal ER proteins as reflected by the resistance to trypsinolysis of the molecular chaperone BiP (binding protein) (lanes 5 and 6). Altogether, these results support the hypothesis that the 28-kDa protein species is a cytosolic, non-glycosylated form of βt37, which, due to the severe topological effects of N-terminal truncation, cannot be integrated into the membrane.

In contrast to the 28-kDa species, the 35-kDa species of the βt37 mutant is glycosylated (Fig. 6A, lanes 5 and 6) and resistant to trypsinolysis performed on oocyte homogenates (Fig. 7C, lanes 3 and 4). Furthermore, the 35-kDa species shows a slightly lower molecular mass than the βt34 mutant.
**Fig. 7.** N-terminal truncation of β subunits produces soluble, cytosolic, and ER luminal protein species. A, a 28-kDa protein species is preferentially released to the medium after cell permeabilization. Oocytes were injected with 3 ng of wild type β3, or β3t37 cRNA, labeled during a 24-h pulse and incubated without or with 0.1% saponin for 2 h as described under “Experimental Procedures.” Microsomes (M) prepared from the oocytes and the collected medium (Med) were immunoprecipitated with a β3 antibody. B and C, the 28-kDa but not the 35-kDa protein species is sensitive to trypsin digestion. B, oocytes were injected with 3 ng of wild type β3 or β3t37 cRNA and 4 μCi/oocyte of [35S]methionine. After a 4-h incubation, homogenates were prepared and subjected to trypsinolysis as described under “Experimental Procedures.” Note the high and low proportion of the 28- and 35-kDa protein species, respectively. After this short pulse compared with that observed after a 24-h pulse (A and C) indicating that the 28-kDa species is an early synthesis product that is rapidly degraded and completely digested by trypsin (lane 4) and that the production of the 35-kDa species involves a slower process. C, oocytes were injected with 3 ng of wild type β3, β3t37, or BiP cRNA, labeled for 24 h and then injected or not with 0.75 μg of trypsin per oocyte as described under “Experimental Procedures.” After 1 h at 19 °C, digitonin extracts were prepared and immunoprecipitated with a β3 antibody (lanes 1–4) or a BiP antibody (lanes 5 and 6). D, oocytes were injected with 3 ng of β3t37, β3t37L62A/V64G, or β3t37L62V/T64A cRNA and subjected to a 24-h pulse before preparation of microsomes and immunoprecipitation with a β3 antibody. Samples were treated or not treated with EndoH. One out of three representative experiments is shown. *, glycosylated 35-kDa protein species; solid dot, non-glycosylated 28-kDa protein species.

These characteristics suggest that this protein species has been translocated across the ER membrane, has become glycosylated, and has been cleaved by about 2 kDa. The difference in the molecular mass between the EndoH-treated β3t37 mutant (28 kDa) and the EndoH-treated cleaved product (24 kDa) would be roughly compatible with the removal of the transmembrane domain, e.g. by signal peptidase cleavage. The computer software program SignalP (35) was used to evaluate the possibility that a signal peptidase cleavage site is present in the β3 transmembrane domain, which, due to α helix perturbations of the transmembrane domain after N-terminal truncation of the β3 subunit, may become accessible. Signal peptidase cleavage sites characteristically contain small neutral residues at position −1 (1 amino acid residue upstream of the site of cleavage) and small neutral and uncharged residues at position −3 (for review see Ref. 36). The SignalP program predicted a probability of 50% for signal peptidase cleavage between amino acids Thr64 and Leu65, which according to the glycosylation mapping assay are located adjacent to the C-terminal end of the β3 transmembrane domain. In an attempt to verify the hypothesis of the exposure of this putative signal peptidase cleavage site in the β3t37 mutant, we replaced Leu62 (position −4) by alanine and Thr64 (position −1) by glycine residues (mutant β3t37L62A/V64G) or by valine and alanine residues (mutant β3t37L62V/T64A), respectively. These mutations should increase the cleavage probability by signal peptidase to 100%, according to the SignalP program. When expressed in Xenopus oocytes, the β3t37L62A/V64G mutant no longer produced the 35-kDa protein species (Fig. 7D, lanes 3 and 4) contrary to the SignalP prediction, whereas the β3t37L62V/T64A mutant (lanes 5 and 6) produced a higher proportion of 35-kDa protein species than β3t37 (lanes 1 and 2).

In EndoH-treated samples, the 24-kDa product derived from the 35-kDa species represented 20% and 60% of the total β population in oocytes expressing the β3t37 (lane 2) and the β3t37L62V/T64A (lane 6) mutants, respectively. These results suggest that in Na,K-ATPase β3 subunits, N-terminal truncation and consequent changes in transmembrane domain topology lead to the exposure of a cryptic signal peptidase cleavage site in a certain population of the newly synthesized proteins.

**DISCUSSION**

The glycosylation mapping and biochemical techniques used in this study have provided new information on the position of the C-terminal ends of the transmembrane domains of Na,K-ATPase β subunits and on the role of the N terminus and of specific amino acids adjacent to the transmembrane domain in the definition of these domains. Furthermore, glycosylation mapping has allowed the identification of regions in the β subunit that are important for α-interaction and have revealed novel features in the processing of glycoproteins in Xenopus oocytes. Delineation of the Transmembrane Domains of Na,K-ATPase β and ρ Subunits and the Role of the Cytoplasmic N-terminal Tail and of Single Positive Charges for Correct Membrane Insertion—Our previous studies have suggested that the cytoplasmic N terminus of the Na,K-ATPase β subunit interacts with the catalytic α subunit (2), that its truncation changes the K+ and Na+ affinities of Na,K-ATPase (2, 7), but also that N-terminal interactions might not be directly involved in the observed functional effects (7). One of the aims of this study was to check whether N-terminal truncations may have consequences for the structural integrity of the β subunit.

To assess this question, we have made use of a glycosylation...
mapping technique that has permitted us to delineate the C-terminal end of the transmembrane domain of wild type and mutant \( \beta \) subunits. In previous studies using the glycosylation mapping technique in translation systems in vitro, the minimal glycosylation distance, e.g. the number of residues separating the end of a transmembrane domain and the active center of the oligosaccharyltransferase, was calibrated against transmembrane domains with known positions in the membrane (18). From these studies, a reference minimal glycosylation distance value of about 10–11 residues was determined. Glycosylation mapping assays performed on Na,K-ATPase \( \beta_1 \) and \( \beta_3 \) subunits show that their transmembrane domains are significantly shorter than predicted by Kyte Doolittle hydropathy analysis and end around Leu\(^{58} \) and Met\(^{61} \), respectively (Fig. 8), close to the C-terminal transmembrane domain ends predicted by several other prediction programs (see Fig. 1).

In \( \beta_1 \) subunits, N-terminal truncation significantly decreased the minimal glycosylation distance compared with wild type \( \beta_1 \) subunits (see Fig. 8), indicating that removal of the N-terminal cytoplasmic tail leads to a repositioning of the \( \beta_1 \) transmembrane domain that may be transmitted to the ectodomain. This result lends support to our previously raised hypothesis that the effects on the cation affinities of Na,K-ATPase \( \beta_1 \) subunits are not due to structural changes in the extracytoplasmic domain, but rather to interactions with the \( \alpha \) subunit.

The observed importance of the \( \beta \) N terminus for the correct positioning of the \( \beta \) transmembrane domain in the membrane is likely to be of general relevance for type II proteins. Indeed, N-terminal truncations of other type II proteins such as invariant chain (37) and the asialoglycoprotein receptor (38) also lead to a shift in the position of the proteins in the membrane as reflected by the exposure of a cryptic signal peptidase cleavage site present in the transmembrane domain. We have observed a similar phenomenon after N-terminal truncation of Na,K-ATPase \( \beta_3 \) subunits. The SignalIP program predicts a higher probability for the existence of a signal peptidase cleavage site at the C-terminal end of the \( \beta_3 \) transmembrane domain than of the \( \beta_1 \) transmembrane domain. Accordingly, after N-terminal truncations of \( \beta_3 \), but not of \( \beta_1 \) subunits, we observe a small population of glycosylated protein species with a molecular mass that is compatible with the removal of the transmembrane domain by signal peptidase acting at the predicted cryptic signal peptidase cleavage site. In addition, these protein species increase after certain mutations, which should increase the probability of signal peptidase cleavage, according to the SignalIP program.

Beyond the effects on the membrane position of the transmembrane domain, N-terminally truncated \( \beta_3 \) subunits produce a population of apparently cytosolic protein species, which are not glycosylated and are highly sensitive to cellular and tryptic degradation. This result suggests that in \( \beta_3 \) subunits, in contrast to \( \beta_1 \) subunits, N-terminal truncation changes the topology of the transmembrane \( \alpha \) helix to an extent that impedes stable membrane insertion of a detectable fraction of the molecules. \( \beta_1 \) and \( \beta_3 \) isoforms display about 39% and 62% sequence identity in the cytoplasmic N-terminal tail and the transmembrane domain, respectively. An apparently important sequence difference which may explain the less deleterious effects of the \( \beta_3 \) than of the \( \beta_1 \) N-terminal truncations, is the presence after the initiator methionine of a lysine residue in \( \beta_1 \) subunits instead of a leucine residue in \( \beta_3 \) subunits. Replacement of the lysine residue by a leucine residue in N-terminally truncated \( \beta_3 \) subunits produces a population of non-glycosylated, unstable \( \beta_3 \) species similar to those observed in N-terminally truncated \( \beta_1 \) subunits. On the other hand, a leucine residue replacing the analogous leucine residue in N-terminally truncated \( \beta_3 \) subunits reduces the proportion of these putatively non-membrane-inserted protein species.

The predominance of positively charged residues at the cytoplasmic side of the transmembrane domain ("positive inside" rule (39); "charge difference" rule (40)) is a major determinant for the orientation of transmembrane domains in membrane proteins.
proteins, although the length of the transmembrane domain and the presence or absence of hydrophilic N-terminal tails may also be important (41). In Na,K-ATPase β subunits, the absence of positively charged residues at the cytoplasmic side in combination with the lack of the cytoplasmic N-terminal tail produces a population of non-membrane-inserted, cytoplasmic proteins. At present, it is not known whether topology changes in the transmembrane α helix of these proteins diminish the interaction with the signal recognition particle responsible for the targeting of nascent membrane proteins to the ER membrane (for review see Ref. 42). Alternatively, it is also possible that part of the mutant proteins initially insert into the membrane in an unstable, inverted Ccyt/Nout orientation and, consequently, are eventually released into the cytoplasm.

**The Glycosylation Mapping Assay Reveals New Features of Protein Glycosylation and Glycosylation Processing**—The glycosylation mapping assay used in this study has also provided new information on protein glycosylation and glycosylation processing in *Xenopus* oocytes. For the first time, the glycosylation mapping assay has been carried out in intact cells where the time allowed for folding of newly synthesized proteins can be considerably increased compared with *in vitro* translation systems. Our results suggest that this time factor is an important determinant for the actual efficiency of glycosylation at sites close to the transmembrane domain. Indeed, translation of Na,K-ATPase β subunits in *Xenopus* oocytes rather than in translation systems *in vitro* significantly reduces the minimal glycosylation distance by 5–6 residues depending on the chase time (Fig. 8). Because the distance to the active site of the oligosaccharyltransferase is likely to be the same in *in vivo* and *in vitro* translation systems, this result suggests that the domain between the end of the transmembrane domain and the glycosylation acceptor sites is not a rigid structure and permits post-translational glycosylation of glycosylation acceptor sites while the protein is retained in the ER. Our results differ from observations made in HeLa cells that the major histructocompatibility complex class II-associated invariant chain, a type II glycoprotein, cannot be glycosylated post-translationally on its natural glycosylation sites (43). Additional experiments are needed to decide whether the glycosylation process differs in *Xenopus* oocytes compared with mammalian cells; possibly, the slow synthesis, processing, and intracellular routing of proteins in oocytes caused by the low incubation temperature (19 °C) may be one reason for the observed differences. Alternatively, the observed post-translational glycosylation may be characteristic for glycosylation sites close to the membrane. In any case, our results indicate that the minimal glycosylation distance is not a constant value, but is nevertheless, a valuable tool for defining the extracytoplasmic end of transmembrane domains of type II and probably other membrane proteins if assayed under controlled experimental conditions.

In addition to the observation that the glycosylation efficiency increases with the time proteins spend in the ER and thus that glycosylation can be post-translational, the *in vivo* glycosylation mapping assay also provides information on the requirements for glycosylation processing. Our results show that β subunits, with certain engineered glycosylation sites close to the C-terminal end of the transmembrane domain, acquire core sugars and can associate with α subunits and produce functional α-β complexes at the cell surface but never become fully glycosylated. This result is inconsistent with the dogma that, during intracellular transport in animal cells, high mannose core sugars are necessarily trimmed in the ER and proximal Golgi compartments and complex type sugars are added to glycoproteins in a distal Golgi compartment (for recent review see Ref. 44). A possible explanation of our results is that glycosylation processing enzymes have no access to the core sugars added to glycosylation sites situated close to the membrane. Because the core-glycosylated β species, which escape ER retention, do not show any decrease in their molecular mass during long chases, it is possible that ER mannosidase I, which, as the oligosaccharyltransferase, is a type II membrane protein (45), is not able to remove the first five mannose residues that are necessary for the further trimming of glycoproteins. At present, the nature of the conformational constraints, which may impede mannose trimming of core sugars located as far as 16 amino acids from the C-terminal end of the transmembrane domain, is not known and the glycosylation mapping assay could become a valuable tool to determine the minimal distance from the membrane that permits mannose processing.

**Introduction of Novel Glycosylation Sites Close to the β Transmembrane Domain Impedes Efficient α-β Interaction and/or Intracellular Routing**—Finally, our studies on the Na,K-ATPase β subunits have also provided information on the location of putative sites of interaction with the partner α subunit. According to the two-hybrid assay, an α-assembly domain is located within the 68 amino acids succeeding the β transmembrane domain (8). The results of the present study show that introduction of new glycosylation sites within a region of about 10 amino acids from the C-terminal end of the β transmembrane domain has a significant effect on the α-β interaction efficiency as reflected in delayed α-β assembly, decreased stability of the α subunit, and reduced cell surface expression of functional Na,K-pumps. Similar defects have previously been observed in vesicular stomatitis virus G proteins (46, 47) and influenza virus hemagglutinin (48) containing sugar chains at new glycosylation sites, and it was concluded from these studies that the primary role of natural sugars is to promote proper protein folding. However, neither in those nor in the present study, can it be definitively decided whether the alterations in the folding of the glycosylation mutants is due to the presence of a new sugar chain or to the amino acid substitutions introduced to create a new glycosylation site. β subunits, which contain a new, additional glycosylation site at apparent positions +3 and +5 from the C-terminal end of the transmembrane domain, are not or are only poorly glycosylated, but their ER exit is also significantly impeded as reflected by the absence of full glycosylation of the natural sugar chains. This result indicates that, at least in this case, the mutation itself rather than the presence of a sugar chain in this region is responsible for the folding defect of the protein. Further mutational analysis is needed to determine whether the decreased α-interaction efficiency of the β-glycosylation mutants reflects a discrete disruption of an assembly domain or is due to more general conformational perturbations introduced by the mutation and/or the sugar chain.

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Determinants of Topogenesis and Glycosylation of Type II Membrane Proteins: ANALYSIS OF Na,K-ATPase β1 AND β3 SUBUNITs BY GLYCOSYLATION MAPPING

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