Membrane Integration of Na,K-ATPase α-Subunits and β-Subunit Assembly*

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Pascal Béguin, Udo Hasler, Ahmed Beggah, Jean-Daniel Horisberger, and Käthi Geering‡

From the Institut de Pharmacologie et de Toxicologie de l'Université, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland

The control of membrane insertion of polytopic proteins is still poorly understood. We carried out in vivo translation/insertion experiments in Xenopus oocytes with combined wild type or mutant membrane segments of the α-subunit of the heterodimeric Na,K-ATPase linked to a glycosylation reporter sequence. We confirm that the four N-terminal hydrophobic segments of the α-subunit behave as alternating signal anchor/stop transfer motifs necessary for two lipid-inserted membrane pairs. For the six C-terminal membrane segments, however, proper packing depends on specific sequence information and association with the β-subunit. M5 is a very inefficient signal anchor sequence due to the presence of prolines and polar amino acids. Its correct membrane insertion is probably mediated by posttranslational hairpin formation with M6, which is favored by a proline hairpin in the connecting loop. M7 has partial signal anchor function, which may be mediated by the presence of glycine and glutamine residues. The formation of a transmembrane M7/M8 pair requires the association of the β-subunit, which induces a conformational change in the connecting extracytoplasmic loop that favors M7/M8 packing. The formation of the M9/M10 pair appears to be predominantly mediated by the efficient stop transfer function of M10. Mutations that provide signal anchor function to M5, M7, and M9 abolish or impede the transport activity of the enzyme. These data illustrate the importance of specific amino acids near or within hydrophobic regions as well as of subunit oligomerization for correct topographical alignment that is necessary for proper folding and/or activity of oligomeric membrane proteins.

Many of the important details of the membrane insertion and the maturation of polytopic, oligomeric membrane proteins are still obscure (for reviews, see Refs. 1–3). During the membrane integration, these membrane proteins are subjected to an initial folding process, which is mediated by specific α-helical packing (for a review see Ref. 4), interaction with molecular chaperones, and co-translational modifications (for a review, see Ref. 5). Finally, in the case of oligomeric proteins, the maturation process is completed by the assembly of different subunits (for a review, see Ref. 6). Each maturation step is necessary to ultimately result in the correct tertiary structure of oligomeric proteins that is compatible with intracellular transport and function. Analysis of many polytopic membrane proteins is consistent with a model according to which these proteins are produced by the sequential insertion into the endoplasmic reticulum (ER) membrane of signal anchor and stop transfer motifs that generally contain a sequence of about 23 amino acids favoring an α-helical conformation and are interrupted by a run of hydrophilic amino acids. The insertion of the first ER-targeting sequence is mediated by the signal recognition particle, which interrupts translation until docked with the signal recognition particle receptor on the ER membrane. Subsequent transmembrane regions are thought to insert without involvement of signal recognition particle. During the integration process, polytopic proteins are found adjacent to the Sec61 complex and TRAM, which together form a polar, transmembrane channel as part of the translocon. The Sec61-TRAM complex permits the insertion of the signal anchor sequences and passage of succeeding hydrophobic regions of membrane proteins until the insertion of stop transfer sequences into the ER membrane. The complex also mediates the transfer of the transmembrane domains, perhaps during or even after completion of synthesis of the entire protein, from the aqueous translocation channel into the lipid bilayer. This general model of membrane insertion may have to be modified based on a requirement for specific sequences upstream, downstream, or within membrane-targeted, hydrophobic sequences. Other subunits of an oligomeric protein may also assist in correct membrane insertion. These ideas have not been investigated in detail for polytopic membrane proteins.

Na,K-ATPase, an oligomeric member of the P-type ATPase family, was used as a model protein to study these questions. In addition to the catalytic α-subunit, which is a polytopic membrane protein, Na,K-ATPases contains a β-subunit, which is a type II membrane protein. The number and position of transmembrane segments in the α-subunit of Na,K-ATPase and in H,K- and the ER Ca-ATPase have been determined by biochemical, immunological, and mutational analysis (for review and references, see Refs. 7 and 8). A 10-transmembrane segment topological model with the N and the C termini located in the cytoplasm is now widely accepted for the α-subunit in the functional Na,K- and H,K-ATPase, e.g. when it is associated with the β-subunit (for a review, see Ref. 9). There is clear prediction, using a variety of algorithms, of four N-terminal transmembrane sequences (M1 to M4) in α-subunits of P-type ATPases. These are followed by a long cytoplasmic region containing about 400 amino acids, which contains the phosphorylation and the ATP (fluorescein 5'-isothiocyanate) binding sites. After this, there is a relatively long hydrophobic sequence (M5-M6) that does not appear to contain the hydrophilic sequences necessary for separation of two

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‡ To whom correspondence should be addressed: Institute of Pharmacology and Toxicology, University of Lausanne, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland. Tel.: 41 21 692 54 10; Fax: 41 21 692 53 55; E-mail: kathi.geering@ipharm.unil.ch.

1 The abbreviations used are: ER, endoplasmic reticulum; SR, sarcoplasmic reticulum; PCR, polymerase chain reaction; Endo H, endoglycosidase H; MOPS, 4-morpholinopropanesulfonic acid; SA, signal anchor; ST, stop transfer.
membrane insertion signals. This sequence is followed by four domains of varying hydrophobicity (M7–M10) that make up the C-terminal part of the protein that are usually predicted as being membrane-spanning.

Low resolution crystal structure of the membrane sector of SR Ca-ATPase has been interpreted as showing the presence of a core and a peripheral domain (10). In this structure, there are therefore segments that strongly interact with the lipid bilayer and segments that interact more with other transmembrane segments. These latter segments may show membrane insertion properties that are distinct and may require additional sequence information to be insertion-competent. Results of in vitro translation of M5 and M6 of the Na,K-(11), H,K-(12), or SR Ca-ATPases (13) have been consistent in unable to show the signal anchor and the stop transfer properties expected of a membrane pair interacting solely with membrane lipids. In addition, in the heterodimeric P-type ATPases, the association of the $\beta$-subunit with the extracytoplasmic region between M7 and M8 may influence the stability and folding of the C-terminal part of the $\alpha$-subunit during translation, since the $\beta$-subunit has to be present for the $\alpha$-subunit to become trypsin-resistant, to be protected from cellular degradation, and to become functionally active (14).

After expression in intact Xenopus oocytes, we have analyzed in this study the intrinsic and extrinsic sequence dependence of putative transmembrane sequences of $\alpha$-subunits of Na,K-ATPase for behavior as signal anchor or stop transfer sequences as well as the effect of $\beta$-assembly on the topographical features of the $\alpha$-subunit. As a test for membrane insertion, we followed the presence or absence of glycosylation of chimera containing single or multiple transmembrane segments of the $\alpha$-subunit and the ectodomain of the $\beta$-subunit, which contains several glycosylation sites. Our results indicate that intrinsic structural information other than hydrophobicity provides quantitatively variable signal anchor or stop transfer function to each transmembrane segment of the $\alpha$-subunit of Na,K-ATPases. The intrinsic membrane insertion properties of C-terminal membrane sequences are shown to be essential for the transport function of the enzyme. Finally, assembly with the $\beta$-subunit through interaction with the extracytoplasmic loop between M7 and M8 is important for the correct membrane insertion of the M7 and M8 pair.

**EXPERIMENTAL PROCEDURES**

**Constructs of Chimeras and Site-directed Mutagenesis—**Chimeras constructed different regions of the N terminus of the $\alpha_{1}$-subunit of Xenopus laevis Na,K-ATPase (15) (see Table I) and 223 amino acids (M81-L303) of the ectodomain of the $\beta_{1}$-subunit of Buffalo marinus Na,K-ATPase (16) containing four glycosylation sites. To create the chimera, KpnI and HindIII restriction sites were introduced at different sites into the $\alpha$ cDNA in a pSD5 vector by the polymerase chain reaction (PCR) method of Nelson and Long (17), and the $\beta$-subunit previously digested by KpnI and HindIII, was added. The introduction of single or double point mutations into the cDNA of chimera or the full-length $\alpha$-subunit was also performed by PCR.

The transmembrane segments are indicated by M1 through M10, and "el" represents the 32-amino acid-long extracytoplasmic loop between M7 and M8. The chimeras M1–2/Met1, M1–2/Met7M8, M1–2/Met6Q, Met6Q/Q686L, and M1–2/7el Q855L/Q869L, and M1–2/7el Q855L/Q869L (for description of mutants, see Table I) were prepared by 1) PCR amplification of the C termini of the constructs M7el, Met7elM8, Met6Q/Q686L, and Met7el Q855L/Q869L with a sense oligonucleotide containing a HpaI site and an antisense oligonucleotide containing a HpaI site and an antisense oligonucleotide containing KpnI, HindIII, and XhoI restriction sites, 2) digestion of the amplified fragments, and 3) subcloning by using a HpaI site and a XhoI site present in the pSD5 vector. The $\beta$ fragment was then introduced between the KpnI and the HindIII sites.

To investigate the role of the interaction of the extracytoplasmic loop domain with the $\beta$-subunit, chimeras M1–3/el and M1–3/el were prepared by recombinant PCR. Briefly, one fragment from the M1–3 region was amplified with an antisense oligonucleotide containing a short overlap of the extracytoplasmic loop region (for description see Table I), whereas another fragment was amplified from the C termini of the extracytoplasmic loop or el8 constructs with a sense oligonucleotide containing a short overlap of the M1–3 region and an antisense oligonucleotide containing KpnI, HindIII, and XhoI restriction sites. The two amplified fragments were joined by recombinant PCR.

The chimeras M1–3/el and M1–3/el4 were prepared by 1) introduction of a EcoRI site into the wild type alpha or M1–3/el8 cDNA at nt1010; 2) amplification of the C termini of the construct M8 or M4, respectively, with a sense oligonucleotide containing an EcoRI site and the antisense oligonucleotide containing KpnI, HindIII, and XhoI restriction sites; and 3) subcloning of the amplified fragment into the wild type alpha or the M1–3/el8 cDNA by using a EcoRI site and a XhoI site present in the pSD5 vector. In the M1–3/el4, alanine 322 was changed to phenylalanine. All constructs generated by PCR amplification were sequenced by dideoxy sequencing.

The Xenopus $\beta_{1}$ was epitope-tagged for the use of radiolabeling binding assays (see below) as described earlier (18). The Flag sequence replaced the sequence 14-NSLPPFM, thereby deleting the second glycosylation site. The flags are recognized by the anti-Flag M2 (Eastman Kodak Co.).

**Expression of the Na,K-ATPase in Xenopus Oocytes and Immunoprecipitation of $\alpha$- and $\beta$-Subunits—**Stage V I-VI oocytes were obtained from X. laevis females (Noordhoek, Republic of South Africa) as described previously (14). In vitro synthesized RNA (cRNA) was prepared according to Melton et al. (19). Routinely, 8–10 ng of chimeric alpha cRNA of Na,K-ATPase and/or 0.5 ng of beta cRNA of Xenopus Na,K-Pump was injected into oocytes. Oocytes were metabolically labeled with 0.6 mCi/ml of [35S]methionine (Hartmann Analytic) for 24 h and in some instances subjected to a 48-h chase period. Microsomal fractions were prepared, and the pellets were taken up in 0.5% digitonin and subjected to immunoprecipitation under denaturing or non-denaturing conditions (14) with polyclonal antibodies against alpha- or beta-subunits of Na,K-ATPase (20). Endoglycosidase H (Endo H) (Calbiochem) treatment was performed as before (21) in order to determine the location of the C terminus of the newly synthesized alpha-chimera. SDS-polyacrylamide gel electrophoresis was performed on the immunoprecipitated proteins without or with Endo H treatment, and the protein pattern was quantified by densitometry with a LKB 2202 Ultrascan.

**Immunoradiolabeling of $\beta$-subunits of Xenopus Na,K-ATPase in Xenopus Oocytes—**The binding assay was performed as described (18), 3 days after Xenopus alpha and beta cRNA injection. Twelve oocytes were incubated on ice for 30 min in modified Barth's solution supplemented with 10% heat-inactivated calf serum. The addition of a 70 nM concentration of the iodinated antibody, in a final volume of 100 ml, initiated binding. After a 1-h incubation on ice, the oocytes were washed and subjected individually to gamma-counting. Nonspecific binding was determined on oocytes expressing untagged beta-subunits and accounted for ~10% of total binding. Immunoradiolabeling with untagged alpha-subunits in oocytes with alpha-subunits provides a direct measurement of the number of Na,K-pumps expressed at the cell surface, since it was previously shown that tagged beta-subunits cannot reach the plasma membrane alone and do not influence the number of Na,K-pumps expressed (18).

**[3H]Ouabain Binding—**Ouabain binding to oocyte was essentially done as described previously (21). Oocytes were preincubated for 2 h in a nominally K-free solution to load the oocytes with Na+. Ouabain binding was performed in a solution containing 90 mM NaCl, 2 mM CaCl$_2$, 5 mM BaCl$_2$, 20 mM tetraethylammonium chloride, 5 mM MOPS, pH 7.4, and 1 mM [21,22-3H]ouabain (Amersham Pharmacia Biotech) for 20 min at room temperature. Nonspecific ouabain binding determined in the presence of a 1000-fold excess of cold ouabain accounted to 3–7% of the total binding.

**Na,K-Pump Current Measurements—**The transport activity of wild-type and mutant Na,K-ATPase complexes was measured as the potassium-activated Na,K-pump current in Na$^{-}$-loaded oocytes by using the two-electrode voltage clamp technique as described previously (22). Functional expression of Na,K-pumps and kinetics of activation by K$^{+}$ were studied in the presence of external Na$^{-}$ (80 mM sodium glutonate, 0.82 mM MgCl$_2$, 0.41 mM CaCl$_2$, 10 mM N-methyl-D-glucamine-HEPES, pH 7.4) and in the absence of external Na$^{-}$ (sodium glutonate replaced by 140 mM sucrose). The current induced by increasing concentrations of K$^{+}$ (with Na$^{-}$: 3.3, 10, and 3.3 10 mM K$^{+}$; without Na$: 0.02, 0.10, 0.50, and 5.0 mM K$^{+}$) was measured at $-50$ mV. The maximal current ($I_{max}$) and the half-activation constant ($K_{1/2}$) of exogenous pumps were obtained after subtraction of the pump current of endogenous pumps at each K$^{+}$ concentration. The corrected data were fitted to the Hill equation using
Membrane Insertion Mechanisms of Na,K-ATPase

N-Glycosylation of newly synthesized proteins only occurs in the ER lumen. Therefore, the presence or absence of glycosylation of a C-terminal reporter sequence containing N-glycosylation sites can be used to determine whether a translated sequence ends on the luminal or the cytoplasmic side of the membrane if one assumes that all membrane-integrated protein is glycosylated. We used this approach 1) to define the topographic properties of transmembrane segments of the Na,K-ATPase α-subunit, e.g. their ability to act as signal anchor (SA) or stop transfer (ST) sequences and 2) to identify topographic changes in the α-subunit that are induced by the assembly with the β-subunit. Glycosylation of α deletion mutants containing at their C terminus the glycosylation-competent ectodomain of a β-subunit was followed after expression in Xenopus oocytes by cRNA injection, metabolic labeling, and immunoprecipitation. Endo H, which specifically cleaves N-linked high mannose core sugars, was used to distinguish glycosylated from nonglycosylated protein species after SDS-polyacrylamide gel electrophoresis.

Insertion Competence of N- and C-terminal Transmembrane Segments of Na,K-ATPase α-Subunits Synthesized in Intact Cells and Topogenic Effects of β-Assembly—As previously reported using in vitro translation (23, 24), the four N-terminal membrane segments of the Na,K-ATPase α-subunit are efficiently membrane-integrated during synthesis in vivo (Fig. 1). Transmembrane segment M1 was co-translationally inserted into the membrane (not shown), and the addition of M2, acting as a ST sequence, prevented glycosylation of the M1–2 protein (Fig. 1A, lanes 1 and 2; for description of constructs see Table I), which reflects the formation of the first transmembrane pair. The M1–3 protein again became glycosylated (lanes 3 and 4), confirming the SA function of M3. Finally, the M1–4 protein was not well expressed in oocytes, but M4 consistently prevented glycosylation (lanes 5 and 6), indicating that, in its natural context, M4 acts as a ST sequence.

In contrast to the complete membrane insertion of M1 and M3, only partial membrane insertion was observed for M5, M7, and M9 of the Na,K-ATPase α-subunit. 5–10% of the M1–5 protein was Endo H-sensitive and glycosylated, whether the construct extended up to Gly248 (Fig. 1B, lanes 1–4) or up to Leu294 (data not shown). 40–50% of the M1–7 protein (Fig. 1B, lanes 5 and 6) or of the M1–7el protein containing the extracytoplasmic loop between M7 and M8 (data not shown) became glycosylated, and finally between 40 and 60% of M9 in the M1–9 protein was Endo H-sensitive (lanes 9 and 10). We consistently observed that the nonglycosylated species of M5, M7, and M9 in the M1–5 (Fig. 1B, lanes 3 and 4), M1–7 (lanes 7 and 8), and M1–9 (lanes 11 and 12) proteins, respectively, were completely degraded after a chase period, but the glycosylated, Endo H-sensitive species were stable. This observation provided an additional means to distinguish between the closely migrating glycosylated and nonglycosylated species of high molecular mass α constructs. The degree of membrane integration was distinct and consistent for each of these membrane segments and did not change after longer pulse periods (data not shown), indicating that M5, M7, and M9 contain distinct structural information, which only permits partial and membrane segment-specific SA function in the case where these segments are not followed by other transmembrane segments.

M6, M8, and M10 in the M1–6 (Fig. 1A, lanes 7 and 8), M1–8 (lanes 9 and 10), and M1–10 (lanes 11 and 12) proteins, respectively, were Endo H-resistant and not glycosylated, indicating that they can all form a pair with the preceding segment, which may or may not be membrane-inserted in α-subunits synthesized without a β-subunit.

An interactive domain with the β-subunit has been identified in the extracytoplasmic loop between M7 and M8 in the α-subunit of the Na,K-ATPase (25). To test whether β-association with this domain may influence the topographic features of the α-subunit, we co-expressed the β-subunit with the M1–7el protein containing the β-assembly (extracytoplasmic loop) domain and with M1–8, M1–9, or M1–10 proteins. Non-denaturing immunoprecipitations with an α-antibody showed co-immunoprecipitation and assembly of the β-subunit with all of these constructs (Fig. 1C, lanes 1–4). In M1–7, M1–8, and M1–10 proteins, the glycosylation pattern did not change in the presence of β-subunits (compare Fig. 1C, lane 1, with Fig. 1B, lane 5; compare Fig. 1C, lanes 2 and 4, with Fig. 1A, lanes 9 and 11). On the other hand, in the M1–9 protein, the proportion of
glycosylated species decreased from about 50% in the absence of β-subunits (Fig. 1B, lane 9) to 20% in the presence of β-subunits (Fig. 1C, lane 3), indicating that association with the β-subunit impedes the SA function of M9 in the M1–9 protein. Thus, β-subunit assembly with the interactive extracytoplasmic loop region in the extracytoplasmic loop between M7 and M8 can influence membrane insertion at least of partial constructs of the Na,K-ATPase α-subunit. The results of Fig. 1 are summarized in Table I.

**TABLE I**

| No Fig. name | Glycosylation |
|--------------|---------------|
| 1 2 3 4 5 6 7 8 9 10 | -β + β | -β + β |
| 28 4 M1-10 Q8SSL Q863L | Y1025 0 0 |
| 29 4 M1-8 R928N V930N | Q949 45-50 45-50 |
| 30 4 M1-8 R928A V930A | Q949 40-50 |
| 31 4 M1-8 N/V/N Q/L-Q/L | Q949 100 |
| 32 4 M1-8 I/A-I/A Q/L-Q/L | Q949 0 0 |
| 33 5 M1-5 P801L P803L | G815 100 |
| 34 5 M1-6 F801L P803L | P841 100 |
| 35 5 M1-7 P801L P803L | S923 100 |
| 36 5 M1-8 P801L P803L | Q949 100-40-45 |
| 37 5 M1-9 P801L P803L | Y1025 80-90 10-20 |

**Structural Determinants for Membrane Insertion of M7 and M9 of Na,K-ATPase α-Subunits**—We studied in more detail the importance of some of the amino acids present in the sequences for the observed membrane insertion properties of M7 and M9 in unassembled α-subunits and also evaluated effects of changing specific upstream sequences.

M7 but not M9 retained SA function when they were added to the rigid membrane pair M1–2. Similar to M1–7el, 40–50% of the M1–27el (for description, see Table I) population became Endo H-sensitive and thus glycosylated after expression in oocytes (Fig. 2A, lanes 1 and 2), confirming that M7 has an intrinsic, although partial, SA function that is independent of specific interaction with preceding or succeeding transmembrane segments. In contrast, M1–29 (in which M9 was next to M1–2) was completely Endo H-resistant (lanes 3 and 4), indicating that SA function of M9 is impeded by a preceding stable membrane pair.

M9 contains two adjacent negatively charged residues, Glu862 and Glu863, and a polar residue Thr864. These residues were changed to Leu to test whether they influence the membrane insertion of M9 in the M1–9 protein. Replacement of all three residues led to complete membrane insertion of M9 in the M1–9 protein as reflected by the formation of 100% Endo H-sensitive, glycosylated forms (Fig. 2B, lanes 3 and 4), compared with about 60% in the wild type M1–9 (lanes 1 and 2). The addition of M10 (M1–10 E962L/E963L/T964L) completely inhibited glycosylation (lanes 5 and 6), indicating that M10 acts as an efficient and independent ST sequence. Thus, the limited membrane insertion properties of M9 are in part determined by the presence of negatively charged residues.

In M7, replacement with Pro of a positively charged residue (Arg850) adjacent to the N-terminal, cytoplasmic part of the membrane segment did not change the partial membrane insertion of M7 in the M1–7el protein (Fig. 2C, lanes 1 and 2). On the other hand, substitution with Leu of Gly867 (lanes 5 and 6) significantly increased glycosylation of M1–7el and substitution of Gly860 and Gly867 (lanes 7 and 8) or Gln858 and Gln863 (lanes 9 and 10) rendered the total M1–7el population Endo H-sensitive and glycosylated. When either helix-breaking...
Characterization of Intrinsic Properties of the M7/M8 Extracytoplasmic Loop and Effects of β-Assembly on the Membrane Insertion of Extracytoplasmic Loop Constructs—The intrinsic properties of the extracytoplasmic loop region and the effects of β association were investigated in more detail by analyzing the membrane insertion behavior of various extracytoplasmic loop-containing constructs.

The addition of M7 including the extracytoplasmic loop domain to M1–2 (M1–2/7el8) did not alter the SA function of M7 (compare Fig. 3A, lanes 1 and 2, with Fig. 1B, lanes 5 and 6). M8 in a M1–2/7el8 protein (lanes 3 and 4) showed more glycosylation than the M1–8 protein (Fig. 1A, lanes 9 and 10), indicating a reduction in the ST properties of M8 by the presence of the loop between M7 and M8. The ST function of M8 was also reduced when the extracytoplasmic loop domain was preceded by a strong SA sequence such as M3 in the M1–3/el8 protein (Fig. 3A, lanes 9 and 10) or by the stably membrane-inserted M7 mutant (lanes 13 and 14) in the M1–2/7el8 Q858L/Q863L protein (lanes 15 and 16). Furthermore, in a M1–3/8 protein lacking the extracytoplasmic loop domain, glycosylation of M8 was less pronounced (lanes 11 and 12) than in the M1–3/el8 protein (lanes 9 and 10). Finally, the ST properties of M4, expressed in its natural context of the M1–4 protein (Fig. 1A, lanes 5 and 6) was inhibited when it was preceded by the extracytoplasmic loop domain in a M1–3/el4 protein (Fig. 3A, lanes 7 and 8). These data indicate that the presence of the extracytoplasmic loop domain upstream of different membrane segments (M8, M4) can impair their inherent ST function depending on the topographic context.

Co-expression with β-subunits had no effect on the glycosylation pattern of the M1–2/7el, M1–3el, and M1–2/7el Q858L/Q863L proteins, in which the extracytoplasmic loop domain is directly followed by the reporter sequence, or of the M1–3/8 protein, in which the extracytoplasmic loop domain is missing (compare corresponding lanes in Fig. 3, A and B). Co-expression with β-subunits improved or completely reestablished the Endo H resistance and thus the ST function of M8 in the M1–2/7el8, M1–3/el8, or M1–2/7el8 Q858L/Q863L proteins and that of M4 in the M1–3/el4 protein (compare corresponding lanes in Fig. 3, A and B). Thus, these data indicate that the β-subunit is able to associate with the extracytoplasmic loop domain present in an unusual context. This β-association helps to correctly fold the extracytoplasmic loop domain, thereby enabling membrane retention of the following membrane segments (M8, M4). The results of Fig. 3 are summarized in Table I.

β-Assembly with the extracytoplasmic loop domain can also affect the M7/M8 and the M9/M10 pair topography in the natural topographic context. We analyzed the M1–8, M1–9, and M1–10 proteins containing the Q858L/Q863L mutations, which stabilize membrane insertion of M7 and M1–8 proteins in which the M8 sequence was modified. Although to a lesser extent than in the context of the M1–2/7el8 protein (Fig. 3A, lanes 15 and 16), a membrane-inserted M7 impaired membrane retention of M8 also when it was present in its natural topographic context. About 50% of the M1–8 Q858L/Q863L mutant population was glycosylated (Fig. 4A, lanes 3 and 4). In a M1–9 Q858L/Q863L protein, M9 did not increase the proportion of Endo H-sensitive, glycosylated forms compared with that of the M1–8 Q858L/Q863L protein (Fig. 4A, compare lanes 5 and 6 with lanes 3 and 4), which is consistent with the finding that a stable preceding membrane pair impedes the SA function of M9. M10 in a M1–10 Q858L/Q863L protein acted again as an efficient ST sequence, which rendered the total protein population nonglycosylated (lanes 7 and 8). Inefficient membrane retention of M8 is not only induced when M8 follows a membrane-fixed M7 but also when some amino acids in M8 are mutated. Replacement in M8 of Ile928 and Val930 with polar asparagines (lanes 9 and 10) or less hydrophobic alanines (lanes 11 and 12) produced about 50% Endo H-sensitive, glycosylated forms of the M1–8 proteins. When the mutations in M8 were combined with the Q858L/Q863L mutations in M7 and M8 in the M1–8 (I/N)(V/N)(Q/L)(Q/L) (lanes 13 and 14) and M1–8 (I/A)(V/A)(Q/L)(Q/L) (lanes 15 and 16), proteins remained completely exposed in the ER lumen in their Endo H-sensitive, glycosylated form.

Assembly with the β-subunit permitted membrane retention of M8 in most of these proteins as reflected by the abolition of Endo H-sensitive glycosylation in the M1–8 Q858L/Q863L, M1–9 Q858L/Q863L, M1–8 I928A/V930A, and M1–8 (I/A)(V/A) proteins after co-expression with β-subunits (compare corresponding lanes in Fig. 4A and B). The only proteins in which M8 could not be retained in the membrane by β-assembly were those that had substitutions of Ile928 and Val930 by polar asparagines (compare lanes 9 and 10 and lanes 13 and 14 in Fig. 4, A and B). These results clearly establish that assembly of the β-subunit with the extracellular loop between M7 and M8 in the α-subunit is involved in retention of M8 in the membrane and thus the formation of a transmembrane M7/M8 pair. The results of Fig. 4 are summarized in Table I.
**Forced Membrane Insertion of M5 Perturbs Membrane Topography of the α-Subunit**—On the basis of results obtained in an *in vitro* translation system with Na,K-ATPase α deletion mutants starting at Met729 and containing a P784L mutation in M5, it was suggested that M5 initiates membrane integration of the C-terminal part of the α-subunit. Then translocation of all following hydrophobic segments into the ER lumen was found until M10 acts as an ST segment (11). These data generated a six-segment model of unassembled α-subunits, which contrasts with our observation that M5 is a very poor SA sequence but that M7 and M9 clearly have membrane insertion properties.

We changed some of the amino acids that could possibly change the degree of insertion of M5, such as the prolines of which there are four in the M5 region. Mutations of prolines had important effects on the insertion properties of M5. Substitution by leucine of Pro787 increased membrane insertion of M1–5 by about 40% compared with that of wild type M1–5 (data not shown). P791L (which corresponds to the P784L mutant in Ref. 11) (Fig. 5B, lanes 1 and 2), P801L, P803L, and P801L/P803L (Fig. 5A, lanes 1 and 2) mutations permitted nearly complete glycosylation and membrane insertion of the M1–5 protein even of those sequences that extended only up to Leu804 (data not shown). Not only prolines but polar amino acids also reduce the insertion efficiency of M5, since a N799L mutation in the M1–5 protein permitted complete glycosylation and membrane insertion of M5 (Fig. 5C, lanes 1 and 2).

If the properties of M5 are changed so that now this segment is able to interact with the lipid or the hydrophobic surface of the translocon, the assembly of other membrane segments may be altered, particularly if they require interaction with a face of M5 that is no longer available due to its altered membrane binding characteristics. The changes in the topogenic properties of membrane segments following a mutated, membrane-inserted M5 as well as the influence of β-assembly on this topography were studied in M1–5 up to M1–10 that contained the P801L/P803L double mutations, the P791L, or the N799L mutation. As found with the double proline-mutated M1–5 mutant in Ref. 11 (Fig. 5B, lanes 1 and 2), P801L, P803L, and P801L/P803L (Fig. 5A, lanes 1 and 2) mutations permitted nearly complete glycosylation and membrane insertion of the M1–5 protein even of those sequences that extended only up to Leu804 (data not shown). Not only prolines but polar amino acids also reduce the insertion efficiency of M5, since a N799L mutation in the M1–5 protein permitted complete glycosylation and membrane insertion of M5 (Fig. 5C, lanes 1 and 2).

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protein (Fig. 5A, lanes 1 and 2), the total M1–6 P801L/P803L population was Endo H-sensitive and glycosylated (lanes 3 and 4), indicating that M6 is translocated to the ER lumen when M5 is fixed. The addition of M7 (lanes 5 and 6) or M8 (lanes 9 and 10) did not change the glycosylation pattern, indicating that from M6 on, with M5 fully membrane inserted, all of these segments were transferred to the ER lumen and could not be retained in the membrane as SA/ST sequences (see model in Fig. 6). Only M9 in the M1–9 P801L/P803L protein (Fig. 5A, lanes 13 and 14) and M10 in the M1–10 P801L/P803L protein (lanes 17 and 18) decreased glycosylation by 20% and 90%, respectively. These results confirm that M10 is an efficient ST assembly with the extracytoplasmic loop domain leads to partial membrane retention of M8 in M1–8 P801L/P803L proteins and finally to the incorrect membrane topology shown for M1–10 P801L/P803L. This latter model with M6 and M7 still in the ER lumen is favored over a model in which β-assembly would permit their membrane retention, since full length α-subunits containing the P801L/P803L are completely misfolded (see Fig. 7). In B, the putative membrane topology of the unassembled (−β) and β-assembled (+β) α-subunit mutated in Pro791 is shown. In unassembled P791L mutants with a fixed M5, half of the M6 population is found to be released to the ER, and half is retained in the membrane, which leads to the proposed membrane topography of the full-length α-subunit. β-Assembly with the extracytoplasmic loop domain permits membrane retention of the C-terminal domain. P indicates the position of Pro791. Dark squares, fully inserted membrane segments; white squares, ER lumen released membrane segments; gray squares, topologically labile membrane segments. For details, see text and legend to Fig. 8.

Similar experiments performed with P791L and N799L mutants not only confirmed the role of β-assembly in the formation of a transmembrane M7/M8 pair but also provided evidence for a potential role of proline residues in the formation of the M5/M6 pair. Similar to the P801L/P803L mutations, the P791L (Fig. 5B, lanes 1 and 2) or the N799L (Fig. 5C, lanes 1 and 2) mutation permitted nearly complete glycosylation and membrane insertion of M5 in M1–5 proteins. In contrast to the situation in the P801L/P803L mutant, a succeeding M6 was able to act as a partial ST sequence in the M1–6 P791L (Fig. 5B, lanes 3 and 4) and the M1–6 N799L proteins (Fig. 5C, lanes 3 and 4), producing 50–60% nonglycosylated forms in their correct membrane orientation (see model in Fig. 6). This result could indicate that the presence of Pro791 and/or Pro803 prevents the release of M6 into the ER lumen. These prolines might induce a kink in the α-helix that favors the retention of M6 in the membrane in a Nout-Cyt ST orientation. M7 in the M1–7el P791L protein (Fig. 5B, lanes 5 and 6) or M8 in the M1–8 P791L protein (lanes 9 and 10) did not significantly decrease glycosylation compared with the M1–6 P791L protein (lanes 3 and 4), but the addition of M9 (lanes 13 and 14) and M10 (lanes 17 and 18) decreased and abolished, respectively, glycosylation. This result is compatible with the topographic model shown in Fig. 6. It is likely that a dynamic equilibrium exists between a P791L mutant population in which M6 adopts a correct membrane orientation permitting...
the following membrane segments to fold into pairs and a population that exposes M6 up to M8 to the ER lumen and is anchored in the membrane by M9 and M10. Co-expression with β-subunits had little effect on the extent of glycosylation of the M1–7εl P791L protein (Fig. 5B, compare lanes 7 and 8 with lanes 5 and 6) but clearly abolished glycosylation of M8 in the M1–8 P791L protein (compare lanes 11 and 12 with lanes 9 and 10), indicating that β-assembly is able to restore a correct membrane topology by forming a transmembrane M7/M8 pair (see model in Fig. 6).

The results obtained with the M5, M7, and M9 mutants show that proline, glycine, and polar amino acid residues provide distinct properties to these segments, which permit their correct membrane insertion. Any change in such residues leads to fixation of these segments in the membrane and in consequence may result in a perturbed topography of the following membrane segments. In many cases, however, the assembly of the β-subunit with the extracytoplasmic loop domain is able to restore the defect through formation and stabilization of the M7/M8 pair.

Functional Implications of Forced Membrane Insertion of M5, M7, and M9 of Na,K-ATPase α-Subunits—Site-directed mutagenesis has implicated M4, M5, M6, and M8 as being involved in ion transport and conformational changes of the Na,K- and H,K-ATPase (for a review and references, see Ref. 7) or the SR Ca-ATPase (for a review, see Ref. 26). We analyzed whether forced membrane insertion of M5, M7, and M9 might affect the ion transport function of the Na,K-ATPase. For this purpose, we introduced mutations that were shown to stabilize membrane insertion of M5, M7, and M9 into the full-length α-subunit and tested the consequences on the bio-synthesis, assembly, cell surface expression, and functional properties of these α mutants after co-expression with β-subunits in oocytes. α-Subunits containing P787L, P791L, P801L/P803L, N799L, Q535L/Q636L, G860L/G867L, or E962L/E963L/T964L mutations associated with β-subunits during a pulse period similar to that of the wild type α-subunit (Fig. 7A, odd lanes), but all mutant αβ complexes were partially or completely retained in the ER as reflected by incomplete or absent processing of the β-subunit to its fully glycosylated form during a chase period (Fig. 7, A and B, even lanes). As a consequence of ER retention or complete degradation as in the case of the P801L/P803L mutant (Fig. 7A, lanes 9 and 10), the cell surface expression of P787L αβ, Q535L/Q636L αβ, G867L αβ, and E962L/E963L/T964L αβ complexes was reduced and that of P801L/P803L αβ and G860L/G867L αβ complexes was completely abolished as measured by anti-Flag binding to the epitope-tagged β-subunit on intact oocytes (Fig. 7C, compare lanes 4–11 with lane 3).

The functional properties of cell surface-expressed Na,K-pumps could be determined only for P787L αβ, P791L αβ, N799L αβ, G867L αβ, and E962L/E963L/T964L αβ complexes. In oocytes expressing P787L αβ (Fig. 7, C and D, lane 4) and G867L αβ (Fig. 7, C and D, lane 10) complexes, anti-Flag and ouabain binding increased 3–4-fold over that observed in oocytes expressing β-subunits alone (Fig. 7, C and D, lane 3), but no Na,K-pump current could be revealed for either mutant αβ complex (Fig. 7E, compare lanes 4 and 10 with lane 2). These results indicate that P787L αβ and G867L αβ complexes have lost their transport function and may be blocked in the E2 conformation, which permits ouabain binding. The P791L αβ complexes exhibited another functional defect. The P791L αβ complexes were expressed at the same level as wild type αβ complexes as measured by anti-Flag binding (Fig. 7C, compare lane 5 with lane 3), but ouabain binding (Fig. 7D, compare lane 5 with lane 3) and Na,K-pump currents (Fig. 7E, compare lane 5 with lane 3) were decreased by 50%. Furthermore, the activation constant for K+ increased from about 1 mM in the wild type αβ complexes to 5 mM in the P791L αβ complexes when measured in the presence of external Na+ and from 0.23 to 2.3 mM when measured in the absence of external Na+ (Fig. 7F). These mutants have a decreased apparent K+ affinity and a reduced turnover, probably due to a retardation of conformational transitions. The N799L αβ and E962L/E963L/T964L αβ complexes did not show a change in the I_{max} (Fig. 7F) compared with the wild type αβ complexes, but their K+ activation constant measured in the presence or absence of external Na+ increased by about 2-fold (Fig. 7F), indicating that the affinity for both external Na+ and K+ binding sites is changed. These data show that introduction of mutations in the α-subunit that force membrane insertion of M5, M7, and M9 have deleterious effects on the maturation and/or the transport properties of the Na,K-pumps.

**DISCUSSION**

An analysis of the membrane topography of constructs of wild type and mutated membrane segments of the Na,K-ATPase α-subunit has shown that there is a variety of structural determinants that govern the insertion of the transmembrane segments of polytopic proteins. In the case of the heterodimeric Na,K-ATPase, this includes effects of the assembly with the β-subunit. Furthermore, evidence is provided that the intrinsic membrane insertion properties of C-terminal membrane segments are essential for the correct folding and the transport function of this protein.

Our glycosylation scanning assay of α deletion mutants shows that formation of the two N-terminal and the three C-terminal membrane pairs of the Na,K-ATPase α-subunit is governed by different mechanisms. M1 and M3 of the Na,K-ATPase α-subunit exhibit full signal anchor activity, and M2 and M4 exhibit efficient stop transfer function when synthesized in vivo, leading to the formation of membrane-integrated M1/2 and M3/4 membrane pairs. These data are consistent with previous results obtained by in vitro translation of membrane segments of Na,K-ATPase (23, 24) as well as of H,K-ATPase (12). Since each of the four N-terminal segments of H,K-ATPase α-subunits can adopt Ncyt or Nout orientations in the membrane (12), it is likely that the native orientation is determined by other factors than sequence information within these topogenic segments. The cytoplasmic anchoring function of the N-terminal segment and the absence of positive charges between M1 and M2 may determine the orientation of this pair, and the cytoplasmic anchoring properties of the sequence between M2 and M3 may determine the orientation of the M3/M4 pair as suggested by the analysis of model proteins (for reviews, see Refs. 27 and 28).

According to the 10-transmembrane segment model of P-type ATPase α-subunits, M5, M7, and M9 should act as SA sequences. However, by using an in vitro transcription-translation assay, it had been shown that although the putative transmembrane segments M9 of the gastric H,K-ATPase α-subunit can act both as SA (Ncyt-Cout configuration) and ST (Nout-Cyt configuration) sequence, M5, M6, and M7 have no membrane insertion properties (12). As shown in this study, M5, M7, and M9 of the Na,K-ATPase α-subunit produced a mixture of glycosylated as well as nonglycosylated species in M1–5, M1–7, and M1–9 α proteins (see model in Fig. 8), indicating that these membrane segments have partial SA function. Since the proportion of glycosylated species produced is constant and specific for each segment, it is likely that the limited membrane insertion observed reflects a distinct property of each of these topogenic sequences necessary for their correct membrane insertion and the packing of the C-terminal membrane domain.
The membrane region of P-type ATPases appears to be a compact structure. For example, Na,K-ATPase and H,K-ATPase retain cation occlusion even after cytoplasmic cleavage with trypsin (for a review, see Ref. 9). Analysis of two-dimensional crystals of the SR Ca-ATPase (10) would place four membrane segments in a structure where their surfaces interact largely with other transmembrane segments and the others would interact with both other segments and the hydrophobic core of the membrane. The parameters that determine membrane insertion based on hydrophobic core interaction might be distinct from those that determine membrane insertion based on protein-protein interaction. There might be variations in translocon regions that enable both processes to occur with equal efficiency, or the translocon may permit only hydrophobic core structures to insert fully. Hence, the limited insertion of M1–5, M1–7, and M1–9 suggests that these three putative signal anchor sequences require more than hydrophobic interaction for full membrane insertion.

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The limited insertion found for M5 of the Na,K-ATPase a-subunit is probably determined by the presence of proline and polar residues. Prolines produce kinks in transmembrane a-helices (for a review, see Ref. 29), and studies by von Heijne (30) suggest that this structural effect could be important for specific packing with neighboring transmembrane segments. Based on the observation that the formation of a transmembrane M1–2 loop in CFTR may be initiated by M2 (31), it is possible that the low insertion efficiency of M5 in an M1–5 protein of the Na,K-ATPase a-subunit is due to the absence of
M6, which might be needed for the formation of a transmembrane pair. Membrane insertion of the M5/M6 pair could indeed occur by a helical hairpin mechanism, which was recently proposed for membrane insertion of closely spaced hydrophobic segments (for a review, see Ref. 32). Rescue of membrane integration of M5 in an M1–5 protein in which proline residues were mutated might be due to a general improvement of helicity of M5. Significantly, mutations of proline residues in the C-terminal part, but not of those located in the N-terminal part, of M5 completely prevented retention of M6 in the membrane. This result suggests that Pro<sup>802</sup>/Pro<sup>803</sup> are located in the short M5/M6 loop and might form a kink that assists in the formation of the closely spaced M5/M6 pair. This would favor a model (Table 1) for the Na,K-ATPase α-subunit similar to that proposed by Moller et al. (7) for H,K-ATPase α-subunits, which predicts a conformationally flexible M5/M6 pair connected by two proline residues.

In view of the partial membrane insertion of M7 and M9 of the Na,K-ATPase α-subunit, posttranslational hairpin formation with subsequent membrane segments might also be necessary for membrane insertion, as suggested for M5. In M7, mutations of conserved glycine and glutamine residues permit complete membrane insertion, presumably by allowing efficient interaction with the translocon and/or lipid bilayer. Glycines in M7 might be important for interactions with M8, since glycines in TM segments appear to play a role in helix-helix interaction and packing (for a review, see Ref. 1). As discussed below, hairpin formation of M7 and M8 is not the only factor that must determine membrane insertion, but association with the β-subunit is likely to be the determining factor that stabilizes the M7/M8 pair in the membrane. In M9, the presence of negative amino acids is at least partially responsible for the poor membrane insertion as an independent β sequence.

Added to M1–5, M1–7, or M1–9, M6, M8, and M10, respectively, abolish glycosylation, indicating that they can form pairs with their preceding segments. The glycosylation assay does not permit us to distinguish whether these pairs are membrane-inserted or released to the cytoplasm in α-subunits that are not associated with β-subunits. A likely model for the membrane topography of unassembled α-subunits that is compatible with the present data and the observation that unassembled α-subunits are highly sensitive to degradation (for a review, see Ref. 6) is that the C-terminal membrane pairs are in a dynamic equilibrium between the cytoplasm and the membrane or more precisely the membrane pore formed by the Sec61 complex (see model in Fig. 8). Unless M5 is fixed in the membrane by mutations of prolines (see model in Fig. 7), there is no evidence that M6 to M9 are released to the extracytoplasmic side as suggested by Xie et al. (11).

Association of the β-subunit with the extracytoplasmic loop between M7 and M8 in the α-subunit is necessary to stabilize the C-terminal membrane domain of Na,K-ATPase α-subunits by promoting the formation of a transmembrane pair M7/M8. β-Association not only enables membrane insertion of this pair, but our data indicate that it might also be implicated in its correct packing. M8 is not a good ST sequence per se. A small reduction of its hydrophobicity, by changing Ile<sup>926</sup> and Val<sup>1500</sup> to alanine, reduces significantly its membrane retention. Furthermore, when M8, preceded by the extracytoplasmic loop domain, is added to a membrane-fixed segment or when M5 is rendered inflexible by proline mutations, M8 is released to the ER lumen. Association of the β-subunit with the extracytoplasmic loop domain renders M8 an efficient ST sequence (see models in Figs. 6 and 8). Our results indicate that the extracytoplasmic loop domain is a flexible structure that can interact with the β-subunit and respond with a conformational change that favors M8 membrane retention and in consequence M7/M8 transmembrane pair formation. Previous observations, which suggest that reduction of disulfide bonds and/or heating that is thought to disrupt interaction of β-subunits with the α-subunit
may release M8 from the membrane in purified enzyme preparations (33, 34), are consistent with these translational observations.

Important information on the membrane topology and dynamics of the α-subunits of heterooligomeric P-type ATPases such as Na,K- and H,K-ATPase have been obtained by biochemical analysis of purified enzyme preparations (for reviews, see Refs. 7 and 9). Our results also indicate that protein-protein interactions within the α-subunit are necessary to stabilize the C-terminal transmembrane domain. It is possible that assembly with the β-subunit is the triggering factor that enables at least some of these stabilizing intermolecular interactions to occur. Furthermore in the literature, the selective release of M5/M6 following removal of potassium ions has been interpreted as having a mechanistic significance in the context of potassium binding and transport, and it is suggested that M5/M6 was surrounded by and interacted with protein segments and not lipid (35). This proposal is also supported by the present studies.

Mutations in M5, M7, and M9 that were shown to immobilize these segments in the membrane had two main effects on Na,K-ATPase αβ complexes. Double proline or glycine mutations in M5 or M7, respectively, led to retention of αβ complexes in the ER, probably due to important misfolding of the α-subunit despite β-association (see model in Fig. 6A). All other mutations that permitted partial or complete cell surface expression had severe functional consequences. P787L and G867L mutations in M5 and M7, respectively, completely abolished transport function, while P791L and N799L mutations in M5, G867L mutations in M7, and E962L/E963L/T964L mutations in M9 produced distinct functional defects. The most prominent feature of all of these mutations is a fixed membrane insertion of the respective segment, and this may impede the dynamic interactions between membrane segments that are important for the transport function of the enzyme.

In conclusion, the study of membrane insertion of Na,K-ATPase α-subunits shows that there are several factors that govern membrane integration of polytopic, oligomeric membrane proteins. N-terminal membrane segments of the α-subunits are sequentially and fully inserted into the membrane during synthesis and conform to the SA-ST sequential mechanism of membrane folding. On the other hand, C-terminal membrane segments in unassembled α-subunits exhibit variable membrane insertion, which is determined by specific sequence information as well as the topographic context of the segments. β-Assembly is the most important factor for the correct packing and the membrane stabilization of the C-terminal domain of the α-subunit by changing the conformation of an extracytoplasmic α domain and thereby favoring the formation of a transmembrane M7/M8 pair. Finally, perturbations of intrinsic molecular properties of C-terminal membrane segments that lead to forced membrane insertion affect the correct folding and/or the ion transport function of the Na,K-ATPase α-subunit.

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