Role of β-Subunit Domains in the Assembly, Stable Expression, Intracellular Routing, and Functional Properties of Na,K-ATPase*

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The β-subunit of Na,K-ATPase (βNK) interacts with the catalytic α-subunit (αNK) in the ectodomain, the transmembrane, and the cytoplasmic domain. The functional significance of these different interactions was studied by expressing αNK in Xenopus oocytes along with N-terminally modified βNK or with chimeric βNK/βHK-ATPase (βHK). Complete truncation of the βNK N terminus allows for cell surface-expressed, functional Na,K-pumps that exhibit, however, reduced apparent K⁺ affinities as assessed by electrophysiological measurements. A mutational analysis suggests that these functional effects are not related to a direct interaction of the β N terminus with the αNK but rather that N-terminal truncation induces a conformational change in another functionally relevant β domain. Comparison of the functional properties of αNK/βNK, αNK/βHK, or αNK/βNK/βHK complexes shows that the effect of the βNK on K⁺ binding is mainly mediated by its ectodomain. Finally, βHK/αNK containing the transmembrane domain of βHK produces stable but endoplasmic reticulum-retained αNK/β complexes, while αNK/βHK complexes can leave the ER but exhibit reduced ouabain binding capacity and transport function. Thus, interactions of both the transmembrane and the ectodomain of βNK with αNK are necessary to form correctly folded Na,K-ATPase complexes that can be targeted to the plasma membrane and/or become functionally competent. Furthermore, the β N terminus plays a role in the β-subunit’s folding necessary for correct interactions with the α-subunit.

Still, little is known of the molecular determinants in oligomeric proteins that are involved in subunit assembly and of the particular structural and/or functional importance of different interaction sites. In this study, we have addressed these questions for the oligomeric Na,K-ATPase. Na,K-ATPase is a ubiquitous plasma membrane enzyme that is responsible for the K⁺ and Na⁺ homeostasis of animal cells (for a review, see Ref. 4). The functionally active enzyme is a heterodimeric protein that transports K⁺ and Na⁺ against their electrochemical gradients by using the energy of the hydrolysis of ATP. The catalytic α-subunit is a polytopic membrane protein with 10 transmembrane segments that contains the binding sites for the cations, for ATP and phosphate, and for drugs such as cardiac glycosides. The β-subunit is a type II membrane protein with a short cytoplasmic N terminus, one transmembrane segment, and an ectodomain that carries three disulfide bridges and several sugar chains. The β-subunit plays an essential role in the maturation of the catalytic α-subunit. Synthesized alone, the α-subunit is rapidly degraded in or close to the ER, and only assembly with β-subunits permits a structural maturation of the α-subunit, which protects it from cellular degradation and renders it functionally active (5). In addition to this important structural function, the β-subunit also influences the transport properties of mature Na,K-pumps expressed at the cell surface. Several studies have reported that the β-subunit is involved in the apparent K⁺ affinity of Na,K-ATPase (5–12).

Experimental evidence suggests that the β-subunit interacts with the α-subunit at multiple sites, which are located in the ectodomain, the transmembrane, and the cytoplasmic domain. By using the yeast two-hybrid system, it was shown that in the ectodomain, the extracytoplasmic loop between transmembrane segment M7 and M8 of the α-subunit interacts with a β domain located within 63 amino acids adjacent to the transmembrane segment (13). Another putative interaction site is located in the 10 most C-terminal amino acids of the β-subunit (14). Cross-linking studies suggest that the two subunits interact in the membrane domain, with the β intramembrane helix being in contact with portions of the M8–M10 membrane domain of the α-subunit (15). Finally, proteolysis protection assays suggest that the cytoplasmic N terminus of the β-subunit interacts with the α-subunit (5). The functional roles of the various interactions have not yet been clearly defined. Experimental evidence indicates that β assembly with the extracytoplasmic M7/M8 loop favors the correct packing of the C-terminal membrane domain and in consequence influences the stability of the α-subunit against cellular degradation (16). Other data also suggest that interaction of the transmembrane domain of the β-subunit may be important for efficient and stable assembly with the α-subunit (8, 10). On the other hand, both ectodomain (8–10) and cytoplasmic (5, 12) interactions of the β-subunit were proposed to play a role in the K⁺ binding process of the enzyme.

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We have previously shown that truncation of the cytoplasmic N terminus of the β-subunit does not influence the stabilization of the α-subunit but significantly reduces the apparent affinity for K⁺ of the Na,K-ATPase (5). This result suggested an implication of cytoplasmic α-β interactions in the control of K⁺ binding. In this study, we intended to further characterize the functional role of the N terminus and to identify the functionally relevant molecular determinants. We show that N-terminal truncation not only influences the apparent affinity for K⁺ but also that of Na⁺ of the enzyme. However, our mutational analysis suggests that the β N terminus is not involved in K⁺ and Na⁺ binding through direct interaction with the α-subunit but rather by the effect of this domain on the correct folding of the whole β-subunit. Together with results obtained concerning the functional properties of chimeric Na,K-β/H,K-ATPase β-subunits, our data indicate that neither the cytoplasmic nor the transmembrane domain but rather the ectodomain of the β-subunit is involved in the β-subunit's effects on the functional properties of Na,K-ATPase.

MATERIALS AND METHODS

Site-directed Mutagenesis, Construction of Chimeric β-Subunits, and cRNA Preparation—Truncation and deletion and point mutants were prepared using a XenoNa, Na,K-ATPase β (βNK) cDNA contained in a pSD5 vector (pSD5βNK) by the PCR method described by Nelson and Long (17). The mutants t34, t26, t29, and t32 (for description of mutants, see Fig. 1) were prepared as described previously (5). The sense oligonucleotides for the preparation of the t26 (GTACCATGGGCAGAA- CAGGT), the t29 (GTACCATGGGTGGTAGCTGG), and the t32 mutant (GTACCATGGCCCTGGTTTAAA) contained a Ncol restriction site covering the sequence coding for the initiator methionine, which permitted us to subclone the mutant cDNA into a Ncol site-containing wild type pSD5βNK vector with Ncol and BamHI.

For the preparation of multiple point mutants, fragments of pSD5βNK were first amplified by PCR using an antisense oligonucleotide containing part of the pSD5 vector and primer D of Nelson and Long (17) and sense oligonucleotides containing the mutated sequences replacing amino acids 26–34. The amplified mutated DNA fragments were then used as primers to elongate the inverse DNA strand and finally were amplified using a sense oligonucleotide encoding part of the pSD5 vector and primer D of Nelson and Long (17) and sense oligonucleotides containing the mutated sequences replacing amino acids 26–34. The amplified mutated DNA fragments were then introduced into wild type pSD5βNK using NheI and HpaI restriction sites and served as templates for mutants replacing amino acids 3–5, 9–12, or 21–24.

For mutants in which four alanine (t26/G31A(4)), valine (t26/G31V(4)), asparagine (t26/G31D(4)), or arginine (t26/G31R(4)) residues replaced amino acids Gly31–Gly35, and sequences coding for four alanine, valine, aspar- tate, or arginine residues followed by the sequence coding for Lys36 Leu37 of the βNK. The same antisense oligonucleotide was used as described above for the preparation of multiple point mutants. t26/G31A(4) and t26/G31V(4) cDNAs were used as templates for the preparation of mutants in which eight alanine or valine residues, respectively, replaced amino acids 27–34.

Chimera NK TM/HK (see Fig. 1), in which the cytoplasmic and transmembrane domains derive from XenoNa, Na,K-ATPase β-subunits (βNK) and the ectodomain from rabbit gastric H,K-ATPase subunits (βHK) and HK TM/NK with the inverse orientation were prepared as described previously (8). For construction of the chimera Nkcyt/HK, containing the cytoplasmic domain of βNK and the transmembrane and ectodomain of βHK, one fragment encoded the first 38 amino acid residues of βHK and was PCR-amplified using a sense oligonucleotide encoding part of the pSD5 vector and an antisense oligonucleotide encoding Leu116–Val19 of βHK and Lys26–Phe39 of βNK. Using pSD5βNK as a template, another fragment encoding the transmembrane domain of βNK was also amplified using an antisense oligonucleotide encompassing nucleotides 628–648 (Glu154–Ser160) of βNK and a sense oligonucleotide Leu141–Val149 of βHK and Lys26–Phe39 of βNK. The two amplified fragments were then joined by recombinant PCR. NheI and HpaI restriction sites were used to subclone the β chimera into pSD5βNK.

The nucleotide sequences of all constructs were confirmed by dideoxysequencing.

cRNAs coding for Bufo a1 Na,K-ATPase (6), XenoNa β1 Na,K-ATPase (19), rabbit, gastric β H,K-ATPase (20), and β mutants were obtained in vitro transcription (21).

Expression in Xenopus Oocytes and Immunoprecipitation of α- and β-Subunits—Oocytes were obtained from Xenopus females (Noordhoek, Republic of South Africa) as described (22). Routinely, 7 ng of α and/or 0.2–1 ng of β RNA were injected into oocytes. Oocytes were incubated in modified Barth’s medium containing 0.5 mM [35S]methionine (Hartmann Analytic) for 24 h and then subjected to a chase period of 48 h in the presence of 10 mM cold methionine. Microsomes were prepared as described (5), and the α- and β-subunits of Na,K-ATPase and the β-subunit of H,K-ATPase were immunoprecipitated under denaturing conditions as described (8) with a Xenopus β subunit antibody (23) or a Bufo α-subunit (24). The dissociated immune complexes were separated by SDS-polyacrylamide gel electrophoresis, and labeled proteins were detected by fluorography. Quantification of immunoprecipitated bands was performed with a laser densitometer (LKB Ultrascan 2202).

Pump Current Measurements and Determination of Apparent K⁺- and Na⁺ Affinities—Na,K-pump activity was measured as the K⁺-induced outward current using the two-electrode voltage clamp method as described earlier (11). Current measurements were performed 3 days after injection of oocytes with Bufo α cRNA together with different β cRNAs. To determine the apparent K⁺ affinity of Na,K-pumps, oocytes were loaded with Na⁺ in a K⁺-free solution containing of 200 mM one of the endogenous Na,K-pumps but not the ouabain-resistant exogenous Bufo Na,K-pumps (25). The kinetics of Na,K-pump current activation by K⁺ was determined in a Na⁺-containing solution (80 mM sodium gluconate, 0.82 mM MgCl₂, 0.41 mM CaCl₂, 10 mM N-methyl-d-glucamine (NMDG)-HEPES, 5 mM BaCl₂, 10 mM tetraethylammonium chloride, pH 7.4) or in a Na⁺-free solution (sodium gluconate was replaced by 140 mM sucrose). The current in-duced by increased concentrations of K⁺ (with Na⁺: 0.3, 1.0, 3.3, and 10 mM K⁺; without Na⁺: 0.02, 0.1, 0.5, and 5.0 mM K⁺) was measured at −50 mV. To determine the kinetic parameters (maximal current (I₅₀,Kₐ) and half-activation constant (Kₐ,h)), the Hill equation was fitted to the data of the current (I) induced by various K⁺ concentrations (K) using a least square method.

\[ I = I_{\text{max}}(1 + (K_{\text{Hill}}[K])^n) \]  

(Eq. 1)

According to previously published data (11), the Hill coefficient (n_Hill) was set to a value of 1.6 for experiments in the presence of external Na⁺ and 1.0 for experiments in the absence of external Na⁺.

Measurements of the half-activation constant for internal Na⁺ were performed by a recently developed method.² The principle of this method is to co-express the amiloride-sensitive Na⁺ channel with the Na,K-pump and to use the induced amiloride-sensitive conductance, first, to repeatedly measure the Na⁺/K⁺-subunits (0.3 ng/subunit/oocyte) of the rat renal epithelial Na⁺ channel (26). Injected oocytes were incubated in modified Barth’s medium containing and 0.1 mM amiloride for 24 h and then washed for 15 h after the night before the measurements, the oocytes were incubated in a Na⁺-free solution (50 mM NMDG-Cl, 40 mM KCl, 1 mM CaCl₂, 1 mM MgCl₂, 10 mM NMDG-Hepes, pH 7.4) in order to maximally reduce the internal Na⁺ concentration.

5–8 pairs of measurements of [Na], and of the Na,K-pump K⁺-activated current were performed successively on each oocyte. Between each pair of measurements, the oocytes were allowed to increase their

² P. Béguin, X. Wang, K. Geering, and J-H. Horisberger, manuscript in preparation.
intracellular Na\(^+\) concentration by exposure to a 100 mM Na\(^+\) solution (100 mM sodium gluconate, 1 mM MgCl\(_2\), 0.5 mM CaCl\(_2\), 10 mM Na-Hepes, pH 7.4) in the absence of amiloride and at a holding potential of −50 to −100 mV.

Intracellular sodium concentrations were calculated from the reversal potential of the amiloride-sensitive current obtained from a pair of I-V curves recorded with and without amiloride in a solution containing 5 mM Na\(^+\) (5 mM sodium gluconate, 0.5 mM MgCl\(_2\), 2.5 mM BaCl\(_2\), 95 mM NMDG-Cl, 10 mM NMDG-Hepes, pH 7.4). The Na,K-pump K\(^-\)-activated current was measured in the Na\(^-\)-free solution by the addition of increasing concentrations of external K\(^+\) (see above) in the presence of 20 mM amiloride.

From each oocyte, we obtained a series of 5–8 quasimultaneous measurements of [Na\(^\text{m}\)] and of the maximal K\(^-\)-activated Na,K-pump current. [Na\(^\text{m}\)] was between 1 and 5 mS for the first measurement and increased progressively to above 50 mS for the last measurements. To avoid errors due to a small signal/noise ratio, only oocytes with a maximal Na,K-pump current of at least 200 nA and for which a final [Na\(^\text{m}\)], of at least 50 mS was reached were analyzed.

Then the kinetic parameters of the Na,K-pump activation by internal Na\(^-\) were determined by fitting the Hill equation to the measured I\(_{\text{max}}\) and [Na\(^\text{m}\)], values.

\[
I_{\text{max}} = I_{\text{max}0}(1 + (K_{\text{dNa}}/[Na])^n)^H
\]

Parameter fitting was first performed with the Hill coefficient (n\(_H\)) as a parameter to fit, yielding values of n\(_H\) close to 3. Thereafter, the fitting procedure was repeated using fixed n\(_H\) values of 2, 2.5, 3, and 4. The I\(_{\text{max}0}\) and K\(_{\text{dNa}}\), values obtained with these different n\(_H\), values were slightly different, but the same amplitude of difference between experimental groups was observed with all n\(_H\), values. The values reported are those obtained with a fixed value of 5 for n\(_H\).

\(^{3}H\)Ouabain Binding on Microsomes—rRNA injected oocytes were incubated in modified Barth’s medium for 3 days before preparation of microsomes as described (5) and resuspended in a medium containing 30 mM DL-histidine HCl (pH 7.4), 5 mM EDTA, and 200 mM sucrose. Aliquots of 5 \(\mu\)g of protein were first incubated for 30 min at 19 \(^\circ\)C with 0.15 \(\mu\)g of SDS/protein of protein before the addition (total volume, 500 \(\mu\)l) of a solution containing 100 mM NaCl, 4 mM ATP, 4 mM MgCl\(_2\), and 4 mM imidazole/HCl, pH 7.4, and varying concentrations (1–50 \(\mu\)M) of 21,22-\(^{3}H\)Ouabain (Amersham Pharmacal Biotech). After 90 min incubation at 37 \(^\circ\)C, 450-\(\mu\)l aliquots were rapidly filtered under vacuum on glass fiber filters (Whatman GF/C). Filters were rinsed three times with 4 ml of an ice-cold buffer containing 100 mM NaCl and 30 mM imidazole/HCl, pH 7.4, and counted by liquid scintillation counting (Packard model 2250CA) after the addition of 4 ml of Emulsifier Scintillator Plus scintillation liquid (Packard). Nonspecific binding was measured in the presence of a 1,000-fold excess of cold ouabain and amount to about 10% of the total binding.

RESULTS

Truncation of the Cytoplasmic, N-terminal Domain of the Xenopus \(\beta\)-Subunit Leads to a Decrease in the Apparent K\(^+\) Affinity of Na,K-ATPase—Previous results have shown that the cytoplasmic N terminus of the \(\beta\)-subunit interacts with the \(\alpha\)-subunit (5). This interaction is not necessary for the structural maturation of the \(\alpha\)-subunit. On the other hand, truncation of the \(\beta\) N terminus produces Na,K-pumps at the cell surface that exhibit a decreased apparent K\(^+\) affinity compared to wild type Na,K-pumps (5), suggesting that the N-terminal interaction with the \(\alpha\)-subunit may have a role in the functional properties of the \(\alpha\)-subunit. In order to identify the amino acids in the cytoplasmic N terminus of the \(\beta\)-subunit that might be important for this functional effect, we prepared a series of Xenopus Na,K-ATPase \(\beta\)-mutants with various truncations or point mutations in the N terminus. The \(\beta\) mutants were expressed in Xenopus oocytes together with Bufo Na,K-ATPase \(\alpha\)-subunits and the cellular expression and the transport properties of the exogenous \(\alpha\)-\(\beta\) complexes were tested by immunoprecipitation of metabolically labeled proteins and by electrophysiological measurements, respectively.

As previously observed for Xenopus Na,K-ATPase \(\alpha\)-subunits (5), the t34 \(\beta\) mutant, in which 34 amino acids of the N terminus of Xenopus \(\beta\)\(_1\)-subunits were truncated and the initiator methionine directly preceded the lysine residue adjacent to the transmembrane segment (for descriptions of mutants, see Fig. 1), was able to assemble with and stabilize co-expressed Bufo Na,K-ATPase \(\alpha\)-subunits during a 48-h chase similar to wild type \(\beta\)-subunits (Fig. 2A, lanes 1–4, 11, and 12). The \(\alpha\)-\(\beta\) complexes containing either the wild type \(\beta\)-subunit or the t34 mutant became fully glycosylated (Fig. 2, A and B, lanes 3, 4, 11, and 12), indicating that they are targeted to the plasma membrane. Similar results with respect to assembly, stabilization of the \(\alpha\)-subunit, and ER exit of the \(\alpha\)-\(\beta\) complexes were obtained with \(\beta\) truncation mutants t26, t29, and t32 as well as with a deletion mutant (dG27-F34) in which Gly\(^{27}\) up to Phe\(^{34}\) in the \(\beta\) N terminus were deleted (Fig. 1, A and B, lanes 3–10, 13, and 14).

All \(\beta\) mutants formed active Na,K-pumps at the cell surface with Bufo \(\alpha\)-subunits as shown by pump currents ranging between 200 and 600 nA (Fig. 2, C and D). The maximal pump currents (I\(_{\text{max}}\)) measured were variable but not consistently different from those of wild type \(\alpha\)-\(\beta\) complexes when measured in different batches of oocytes. The apparent affinities for external K\(^+\) of the wild type and mutant Na,K-pumps were compared in the presence or absence of external Na\(^+\) (Fig. 2, C and D). In both conditions, the K\(^+\) activation constant (K\(_{\text{I}}\)) of Na,K-pumps containing the wild type \(\beta\)-subunit or the t26 \(\beta\) mutant was similar. The K\(_{\text{I}}\) slightly but significantly increased in Na,K-pumps containing the t29 (lane 3) or the t32 \(\beta\) mutant (lane 4). A most pronounced increase in the K\(_{\text{I}}\) compared with wild type Na,K-pumps, was observed in Na,K-pumps containing the t34 \(\beta\) mutant (lane 5), in particular in the absence of external Na\(^+\). These results suggested that amino acids between Leu\(^{26}\) and Phe\(^{34}\) in the \(\beta\) N terminus could be implicated in the apparent affinity for external K\(^+\) of the \(\alpha\)-subunit.

The Cytoplasmic N Terminus of the Xenopus \(\beta\)-Subunit Is Not Directly Involved in the Apparent Affinity for Extracellular K\(^+\) of the Na,K-ATPase—To verify the functional role of amino acids between Leu\(^{26}\) and Phe\(^{34}\) in the \(\beta\) N terminus, we measured the K\(^+\) activation kinetics of Na,K-pumps containing the dG27-F34 \(\beta\) mutant in which this domain was deleted (see Fig. 1) and found that the K\(_{\text{I}}\) of the \(\alpha\)-\(\beta\)dG27-F34 complexes was not significantly different from that of the wild type \(\alpha\)-\(\beta\) complexes both in the presence (Fig. 2C, compare lanes 1 and 6) and absence (Fig. 2D, compare lanes 1 and 6) of extracellular Na\(^+\). One possible explanation for this result is that amino acid residues upstream of Gly\(^{27}\) may compensate for the loss of amino acids in the dG27-F34 \(\beta\) deletion mutant. To test this hypothesis, we prepared a series of \(\beta\) mutants containing double or triple alanine substitutions in the Gly\(^{27}\)–Phe\(^{34}\) region (mutants 7–12 in Fig. 1) and measured the expression and the apparent K\(^+\) affinity of the Na,K-pumps formed after association of these \(\beta\) mutants with Bufo Na,K-ATPase \(\alpha\)-subunits. All \(\beta\) mutants associated with and stabilized co-expressed \(\alpha\)-subunits similar to wild type \(\beta\)-subunits, and the \(\alpha\)-\(\beta\) complexes left the ER as indicated by the acquisition of complex type sugars of the \(\beta\)-subunits after a 48-h chase (Fig. 3A, compare lanes 1 and 2 with lanes 3–14). All mutant \(\alpha\)-\(\beta\) complexes were well expressed at the plasma membrane and produced high pump currents (Fig. 3B, inset). In contrast to the about 6-fold increase in the K\(^+\) affinity constant measured in the absence of Na\(^+\) for Na,K-pumps containing the t34 \(\beta\) mutant (Fig. 3B, lane 8), no significant differences in the K\(_{\text{I}}\) values were observed between wild type Na,K-pumps and any of the Na,K-pumps containing \(\beta\) mutants with various alanine substitutions in the full-length N terminus (Fig. 3B, lanes 1–7).

The absence of a functional effect of amino acid substitutions
and segment deletion in the β N terminus suggests that the large effects on the apparent Kᵢ₁ affinity observed in Na,K-pumps containing the truncated t34 β mutant may not be due to a loss of a direct interaction between the α-subunit and a specific sequence in the cytoplasmic β N terminus. To further analyze this possibility, we performed a mutagenic analysis of the t26 truncated β-subunit, which exhibits a wild type phenotype (Fig. 2C, lane 2). Four or all eight amino acids in the Gly27-Phe34 stretch of the t26 truncated β-subunit were replaced by various amino acids (alanine, valine, aspartate, or arginine) (mutants 13–18 in Fig. 1), and the effects of these mutations on the formation and the functional properties of αβ complexes were studied. t26 β mutants in which amino acid residues 31–34 were replaced by either negatively charged aspartate (t26/G31D(4)) or positively charged arginine residues (t26/G31R(4)) were also able to form stable αβ complexes. However, these latter complexes were largely retained in the ER in their core-glycosylated form even after a 48-h chase period (Fig. 4A, lanes 13–16; Fig. 3C), which may explain why a significantly lower Na,K-pump activity could be measured at the cell surface compared with oocytes expressing wild type αβ complexes (Fig. 4B, inset, lanes 6 and 7). Except for the t26/G31D(4) β mutant, all β mutants of this series produced Na,K-pumps with currents large enough to allow the study of the potassium activation kinetics. No significant difference in the apparent Kᵢ₁ affinity was found between Na,K-pumps containing wild type β-subunits or the t26/G31A(4), the t26/G27A(8), or the t26/G31R(4) β mutants (Fig. 4B, lanes 1, 2, 4, and 7). Na,K-pumps containing the t26/G31V(4) (lane 3) or the t26/G27V(8) (lane 5) β mutants showed a slight but significant increase in the Kᵢ₁ when com-
current measurements were performed in the presence of 200 nM ouabain, which inhibited the endogenous, that the
amino acids between Gly27 and Phe34 (GRTGGSWF) are different amino acids produces a similar effect, it is unlikely
fully restore the wild type phenotype. Since the addition of
pumps but that a short amino acid extension can partially or
involved in a specific interaction between the
Bufo were injected with
Xenopus
were not injected (lanes 15 and 16) or injected with 7 ng of Bufo αNK crNA alone (lanes 1 and 2) or together with 0.5 ng of wild type βNK cRNA (lanes 3 and 4) or 1 ng of mutant βNK crNA (lanes 5–14) (for descriptions of mutants, see Fig. 1). After a 24-h pulse (odd-numbered lanes) and a 48-h chase (even-numbered lanes) with [35S]methionin, microsomes were prepared, and immunoprecipitations were performed with an α antibody under nondenaturing conditions (A) or a β antibody under denaturing conditions (B). α- and β-subunits were revealed by SDS-polyacrylamide gel electrophoresis and fluorography (one out of two similar experiments is shown). The expression of exogenous α-subunits (A, lanes 1 and 3–14) significantly increases the α signal compared with that observed in noninjected oocytes (A, lanes 15 and 16). Wild type and mutant β-subunits co-immunoprecipitate with α-subunits, indicating their association. In contrast to α-subunits expressed alone (A, lanes 1 and 2), co-expressed β-subunits stabilize the α-subunits (A, lanes 3–14). All β-subunits are processed from an ER core-glycosylated form (cg) visible after a 24-h pulse to a fully glycosylated form (fg) after a 48-h chase (A and B), indicating that the αβ complexes have been routed though a distal Golgi compartment. C and D, K+ activation of wild type and mutant αβ complexes. Oocytes were injected with Bufo α and wild type (WT, lane 1) or mutant β (lanes 2–6) cRNA as described above. Three days after injection, the
max extrapolated from the K
activation curves. *, p < 0.05 compared with oocytes expressing wild type αβ complexes. Inset, Imax extrapolated from the K+ activation curves.

Fig. 2. Assembly and functional properties of Na,K-ATPase αβ complexes containing N-terminally truncated β-subunits. A and B, stabilization and intracellular transport of αβ complexes. Xenopus oocytes were not injected (lanes 15 and 16) or injected with 7 ng of Bufo αNK crNA alone (lanes 1 and 2) or together with 0.5 ng of wild type βNK cRNA (lanes 3 and 4) or 1 ng of mutant βNK crNA (lanes 5–14) (for descriptions of mutants, see Fig. 1). After a 24-h pulse (odd-numbered lanes) and a 48-h chase (even-numbered lanes) with [35S]methionin, microsomes were prepared, and immunoprecipitations were performed with an α antibody under nondenaturing conditions (A) or a β antibody under denaturing conditions (B). α- and β-subunits were revealed by SDS-polyacrylamide gel electrophoresis and fluorography (one out of two similar experiments is shown). The expression of exogenous α-subunits (A, lanes 1 and 3–14) significantly increases the α signal compared with that observed in noninjected oocytes (A, lanes 15 and 16). Wild type and mutant β-subunits co-immunoprecipitate with α-subunits, indicating their association. In contrast to α-subunits expressed alone (A, lanes 1 and 2), co-expressed β-subunits stabilize the α-subunits (A, lanes 3–14). All β-subunits are processed from an ER core-glycosylated form (cg) visible after a 24-h pulse to a fully glycosylated form (fg) after a 48-h chase (A and B), indicating that the αβ complexes have been routed though a distal Golgi compartment. C and D, K+ activation of wild type and mutant αβ complexes. Oocytes were injected with Bufo α and wild type (WT, lane 1) or mutant β (lanes 2–6) cRNA as described above. Three days after injection, the
max extrapolated from the K
activation curves. *, p < 0.05 compared with oocytes expressing wild type αβ complexes. Inset, Imax extrapolated from the K+ activation curves.

pared with wild type Na,K-pumps, but this change was clearly smaller than that observed with Na,K-pumps containing the t34 β mutant (lane 8).

Altogether, these results show that complete truncation of the β N terminus perturbs the apparent K+ affinity of Na,K-pumps but that a short amino acid extension can partially or fully restore the wild type phenotype. Since the addition of different amino acids produces a similar effect, it is unlikely that amino acids between Gly27 and Phe34 (GRTGGSWF) are involved in a specific interaction between the β N terminus and the α-subunit that would mediate the effects of the β-subunit on the apparent K+ affinity of Na,K-pumps. Our results rather suggest that complete removal of the β N terminus may modify the conformation of the ectodomain and/or the transmembrane domain of the β-subunit, which in turn may perturb other specific, functionally important interactions between the β- and the α-subunit.

Truncation of the β N Termi

n Influences the Affinity of Na,K-pumps for Internal Na+—Since truncation of the β N terminus permitted us to demonstrate that the β-subunit determines to some extent the apparent K+ affinity of Na,K-ATPase, we wondered whether other functional effects of the β-subunit, for instance on the Na+ affinity, might be revealed with the t34 β mutant.

The apparent affinity for intracellular Na+ of Na,K-pumps containing wild type or mutant β-subunits was tested in oo-
cytes co-expressing the renal epithelial Na\(^+\) channel, which permitted us to measure and control the intracellular Na\(^+\) concentrations. Fig. 5 shows that the \(K_{1/2}^\text{Na}\) for Na\(^+\) was significantly higher for \(\alpha\beta\)34 than for wild type \(\alpha\beta\) complexes (\(K_{1/2}^\text{Na}\) for wild type \(\alpha\beta\), 16 ± 0.98 mM, \(n = 21\); \(K_{1/2}^\text{Na}\) for \(\alpha\beta\)34, 25.2 ± 1.9 mM, \(n = 12, p < 0.1\)). As was the case for \(K^+\), the observed decrease in the apparent affinity for Na\(^+\) of the \(\alpha\beta\)34 complex does not appear to be due to a loss of a specific interaction of the cytoplasmic \(\beta\) N terminus with the \(\alpha\)-subunit. The apparent affinity for intracellular Na\(^+\) was instead similar in wild type Na\(\text{K}\)-pumps and Na\(\text{K}\)-pumps containing the t26/G27A(8) \(\beta\) mutant in which the N terminus consists of only eight alanine residues (\(K_{1/2}^\text{Na} = 15.7 \pm 0.58, n = 10\)) (Fig. 5, A and B).

**Functional Role of \(\alpha\)-\(\beta\) Interactions in the Transmembrane Domain and Ectodomain**—The present study indicates that the effects of the removal of the \(\beta\) N terminus on the \(K^+\) and Na\(^+\) affinity of Na\(\text{K}\)-pumps may be due to a change in the conformation of the \(\beta\) ectodomain and/or transmembrane domain. Previous studies have shown that Na\(\text{K}\)-pumps composed of \(\text{Bufo}\) \(\alpha\)NK and rabbit \(\beta\)HK subunits have a lower apparent \(K^+\) affinity than Na\(\text{K}\)-pumps composed of \(\text{Bufo}\) \(\alpha\)NK and \(\text{Bufo}\) \(\beta\)HK subunits (11). To determine which domain(s) of the \(\beta\)-subunit participate(s) in the Na\(\text{K}\)-ATPase affinity for extracellular \(K^+\), we tested the expression and the functional properties of Na\(\text{K}\)-pumps containing chimaera between the \(\text{Xenopus}\) \(\beta\)NK and rabbit \(\beta\)HK subunits with the following composition (see Fig. 1): 1) the cytoplasmic and transmembrane domain from \(\text{Xenopus}\) \(\beta\)NK and the ectodomain from rabbit gastric \(\beta\)HK (\(\text{NK}\) TM/\(\text{HK}\)); 2) the cytoplasmic domain from \(\beta\)NK and the transmembrane and ectodomain from \(\beta\)HK (\(\text{NK}\) TM/\(\text{HK}\)); 3) the cytoplasmic and transmembrane domain from \(\beta\)HK and the ectodomain from \(\beta\)NK (\(\text{HK}\) TM/\(\text{NK}\)); and 4) the cytoplasmic domain from \(\beta\)HK and the transmembrane and ectodomain from \(\beta\)NK (\(\text{HK}\) cyt/\(\text{HK}\)).

**\(\alpha\beta\) NK TM/\(\text{HK}\)** (Fig. 6, inset, lane 3) and \(\alpha\beta\) HK cyt/\(\text{HK}\) (inset, lane 6) complexes produced similar Na\(\text{K}\)-pump currents and \(\alpha\beta\)HK, \(\alpha\beta\) HK cyt/\(\text{HK}\) (inset, lane 4), and \(\alpha\beta\) HK TM/\(\text{NK}\) (inset, lane 5) complexes produced significantly lower Na\(\text{K}\)-pump currents than \(\alpha\beta\)NK complexes (inset, lane 1). Despite the low Na\(\text{K}\)-pump activity measured for some of these \(\alpha\beta\) complexes, we could measure the \(K^+\) activation kinetics for all Na\(\text{K}\)-ATPase complexes and could confirm previous observations (8) that \(\alpha\)NK/\(\beta\)HK exhibits a more than 2-fold increase in the \(K_{1/2}^\text{HK}\) compared with that of \(\alpha\)NK/\(\beta\)NK complexes (Fig. 6B, compare lanes 2 and 1, respectively). Furthermore, our results show that the \(\text{Bufo}\) \(\alpha\)NK/\(\beta\)HK chimaera complexes that contain the ectodomain of the \(\beta\)HK (\(\text{NK}\) TM/\(\text{HK}\), \(\text{HK}\) cyt/\(\text{NK}\)) have \(K_{1/2}^\text{HK}\) values similar to that of \(\alpha\)NK/\(\beta\)HK complexes (Fig. 6B, compare lanes 3 and 4 with lane 1), while \(\alpha\)NK/\(\beta\) chimera complexes containing the ectodomain of the \(\beta\)NK (\(\text{HK}\) TM/\(\text{NK}\), \(\text{HK}\) cyt/\(\text{NK}\)) have \(K_{1/2}^\text{HK}\) values similar to that of \(\alpha\)NK/\(\beta\)NK complexes (compare lanes 5 and 6 with lane 1). These results differ from our previous observation made with \(\text{Xenopus}\) \(\alpha\)NK/\(\beta\) chimera complexes expressed in \(\text{Xenopus}\) oocytes, which exhibited an
apparent $K^+$ affinity that was intermediate between that of $\alpha$NK-$\beta$HK and $\alpha$NK-$\beta$HK complexes (8). It is likely that in this previous study, the measurements of the apparent $K^+$ affinity were influenced by the presence of endogenous oocyte Na,K-pumps. The results of the present study in which the endogenous Na,K-pumps were inhibited indeed suggest that the difference in the apparent affinity for $K^+$ imparted by different $\beta$-subunits is likely to be due to a difference in the ectodomain and not in the transmembrane domain of the $\beta$-subunits.

A last question concerning the role of $\beta$-subunit interaction with the $\alpha$-subunit was why $\alpha$NK TM/HK and $\alpha$NK HKcyt/NK complexes produced similar Na,K-pump activity as $\alpha$NK complexes but a much lower Na,K-pump activity was observed for $\alpha$NK-$\beta$ HK, $\alpha$NK-$\beta$ HKcyt, and $\alpha$HK TM/NK. To answer this question, we again studied the assembly, the stability, and the cellular routing of these $\alpha$NK complexes. As shown in Fig. 6A, similar to Xenopus $\beta$NK (lanes 1 and 2), rabbit gastric $\beta$HK (lanes 3 and 4) and all $\beta$ chimera were able to stabilize co-expressed Bufo $\alpha$NK during a 48-h chase. $\alpha$NK-$\beta$HK, $\alpha$NK-$\beta$HKcyt/HK, and $\alpha$HK TM/NK could leave the ER and become fully glycosylated in agreement with their efficient cell surface expression as reflected by the high Na,K-pump activity. On the other hand, $\alpha$NK-$\beta$HK, $\alpha$NK-$\beta$HKcyt/HK, and $\alpha$HK TM/NK could leave the ER and become fully glycosylated in agreement with their efficient cell surface expression as reflected by the high Na,K-pump activity.
that contained the transmembrane domain of the βHK (lanes 7–10) mainly remained in the ER in their core-glycosylated form, a result that may explain the low Na,K-pump current measured at the cell surface. These results are particularly relevant in the light of the observations that when expressed alone in the oocyte, the chimera NK TM/HK (8) and NKcyt/HK (data not shown) can leave the ER as the wild type βHK, but the chimera HK TM/NK (8) and HKcyt/NK (data not shown) remain in the ER as the wild type βNK. Altogether, our results indicate that NKβ and NKα interact in the transmembrane domain and that this interaction is important to form NKA αβ complexes that can efficiently exit the ER.

In contrast to the αNKβ chimera complexes, the small Na,K-pump current measured in oocytes expressing αβHK complexes (Fig. 6A, inset, lane 2) could not be explained by ER retention of these complexes. Indeed, αNKβHK complexes became fully glycosylated during the chase period (Fig. 6A, lanes 3 and 4) showing that they have passed a trans-Golgi compartment. This result indicated that the αNKβHK complexes cannot reach the plasma membrane and/or that the complexes are functionally inactive. Since previous results have already shown that ouabain binding to intact oocytes is proportional to pump current measurements (8), we tested in this study the ouabain binding to microsomes of oocytes expressing Xenopus αNKβNK and Xenopus αNKβHK complexes and compared the ouabain binding data with the expression level of the two different Na,K-ATPase complexes. Fig. 6C shows that ouabain binding to microsomes of αNKβHK-expressing oocytes was
about 3 times higher than that of noninjected oocytes but represented less than 20% of that measured in microsomes of oocytes expressing αNKβNK complexes. On the other hand, the protein expression level of the αNKβHK complexes represented about 70% of the expression of the αNKβNK complexes (Fig. 6, inset). The Kd values for ouabain calculated were similar for αNKβSNK (4.9 nM) and αNKβHK (6.5 nM) complexes. These data indicate that a large proportion of the αNKβHK complexes formed are blocked in a conformation with low ouabain binding capacity and Na,K-pump function.

**DISCUSSION**

In this study, we first confirm that the β-subunit of Na,K-ATPase influences the transport properties of Na,K-ATPase. Our data indicate that the β-subunit has an effect not only on the apparent affinity for K⁺ but also on that for Na⁺. We then show that direct interaction with the α-subunit of the cytoplasmic β tail or the β transmembrane domain is not involved in the K⁺ and Na⁺ activation of Na,K-ATPase but that the ectodomain of the β-subunit plays an important role in the definition of the functional properties of Na,K-pumps.

Previous studies have shown that the cytoplasmic N terminus of the β-subunit of Na,K-ATPase is susceptible to proteolysis when the β-subunit is expressed alone in Xenopus oocytes, but it becomes protected when the α-subunit is co-expressed with the α-subunit and proteolysis is performed in the presence of K⁺ but not in the presence of Na⁺. Furthermore, we showed that complete removal of the β N terminus decreased significantly the apparent affinity for K⁺ of the Na,K-pumps formed (5). These results suggested that a specific interaction occurs between the β N terminus and the α-subunit, which may be responsible for the functional effect. However, in this study we show that deletions and multiple point mutations in the β N terminus do not change the apparent K⁺ affinity of the αβ complexes and that the addition of a small number of different amino acids to the truncated β N terminus abolishes the functional effect of the truncation on the apparent affinity of K⁺ and Na⁺ for their binding sites on the Na,K-ATPase. Although we cannot definitively exclude the possibility that a small, nonspecific amino acid extension at the N terminus may be sufficient for the β-subunit to interact with the α-subunit and to produce similar effects on the Na,K-ATPase activity as an intact β N terminus, it is more likely that complete truncation of the β N terminus leads to some conformational changes in other domains of the β-subunit that may be involved in Na,K-ATPase functions. A similar explanation may account for the results obtained by Shainskaya and Karlish (12), who showed that progressive proteolytic cleavage of the N terminus of a 16-kDa β fragment contained in a 19-kDa α membrane preparation produced by extensive proteolytic digestion influenced Rb⁻ occlusion. Due to the lack of the ectodomain in these preparations, the conformation of the 16-kDa β fragment may be more susceptible to truncations of the N terminus.

Our hypothesis that N-terminal truncations may affect the conformation of the β-subunit is supported by observations made about two other type II proteins, the asialoglycoprotein receptor (27) and the invariant chain of class II histocompatibility antigen (28). Truncations of the N terminus of these proteins allow the transmembrane segment to shift its position within the membrane, making a putative signal peptidase cleavage site accessible on the luminal side of the ER membrane. For the β-subunit of Na,K-ATPase, structural alterations after N-terminal truncation remain to be identified as well as the differential protection against these alterations by small extension with different amino acids. In our truncated β construct, Lys35 immediately preceding the hydrophobic domain was preserved in order to respect the “inside positive rule” which is mainly responsible for the Nα-Cα orientation of the transmembrane segments (29). The presence of this lysine residue may be a reason that structural changes in the truncated β-subunit are subtle as reflected by the fact that the truncated β-subunit still associates with the α-subunit, stabilizes it, and produces functional Na,K-pumps at the cell surface (5). Lys35 is conserved in all β1-subunits but not in β2- or β3-subunits of Na,K-ATPase nor in β-subunits of H,K-ATPase. To learn more about the structural requirements for correct membrane integration of type II proteins, it may be interesting to study the biosynthesis and functional consequences of N-terminal truncations in mutant β1-subunits or other β-subunits lacking this lysine residue.

Our study provides evidence that the functional effects observed with N-terminally truncated β-subunits may be mediated by possible structural alterations in the ectodomain of the β-subunit, since the study of the functional properties of chimeric Na,K- and H,K-ATPase β-subunits shows that it is this domain that is mainly implicated in the apparent K⁺ affinity. Indeed, the presence of the ectodomain of βSNK or of βHK in the βHK/βSNK or βNK/βHK chimeras is necessary and sufficient to produce Na,K-pumps that exhibit apparent K⁺ affinities similar to those containing wild type βSNK or βHK subunits, respectively. The role of the β ectodomain in the K⁺ binding properties of the Na,K-ATPase demonstrated in this study confirms and extends previous observations made by several groups using different approaches (8–10). Similar conclusions for a role of the β ectodomain were drawn for H,K-ATPase (for a review, see Ref. 30).

The precise sites of interaction in the α- and the β-subunits and the mechanism of action involved in the β-subunit’s effects on the K⁺ binding remain to be determined. Based on proteolysis assays in the presence of different ligands, Lutsenko and Kaplan (9) have proposed that Rb⁺ (K⁺) binding provokes a conformational change in the loop between the first disulfide bond in the β-subunit and more tight interactions with the α-subunit, which closes access of Rb to its binding site from the extracytoplasmic side. In view of the firm coupling between the α- and the β-subunit, it is likely that the K⁺ transport function is mediated by a concerted conformational change of both subunits rather than the β-subunit having a functional effect on a reaction step, independent of the α-subunit (30).

In this study, we provide evidence that the β ectodomain also plays a role in the Na⁺ binding function of Na,K-ATPase. This is consistent with data showing that the strong reducing conditions needed for disulfide bond reduction in the β-subunit of purified Na,K-ATPase preparations can be attenuated not only by K⁺ but also by Na⁺ (31, 32) and with the observation that a NK TM/HK β chimera associated with αNK subunits produces Na,K-pumps with a reduced Na⁺ requirement for phosphozyme formation from ATP (33). In this context, it is interesting that a 584DDRW387 motif located in the extracytoplasmic loop between M7 and M8 may be part of a membrane invaginated structure that is possibly involved in the control of acceptance and/or release of Na⁺ coming from the cytoplasmic side (34). Since this motif is in close proximity to the β interaction site 884SYQG397 (13), it may be possible that the β-subunit influences the flexibility of this domain.

The analysis of the Na,K/H,K-ATPase β chimera indicates that interaction of the transmembrane domain does not play a role in the β-subunit’s effect on the K⁺ binding function of Na,K-ATPase. However, our data confirm that membrane interactions between the α- and the β-subunits must exist, and they show that these interactions may be particularly important for β-subunit-specific interactions. The transmembrane domain plus nine adjacent amino acids of the Na,K-ATPase
β-subunit cannot be replaced by the corresponding domain of the H,K-ATPase β-subunit without important consequences on the intracellular targeting of the αβ complexes formed. Although these chimeric β-subunits (β HK TM/NK) stabilize α-subunits and produce a limited number of functional Na,K-pumps at the cell surface as assessed by pump current measurements, most of the αβ complexes are retained in the ER, which probably reflects partial misfolding of the protein. This observation may also explain why similar HK TM/NK β chimera expressed in yeast together with Na,K-ATPase αγ-subunits produce less than 5% of ouabain binding sites in a cell membrane preparation than wild type Na,K-ATPase β-subunits and why ouabain binding of these NKαβHK TM/NK complexes is lost after SDS extraction (10). Ueno et al. (35) were unable to detect functional activity of similar αNKβHK TM/NK complexes expressed in oocytes, probably due to the less sensitive Na,K-ATPase assay used.

Significantly, the defect in ER exit of αNKβHK TM/NK complexes is overcome in αβ complexes that contain the wild type H,K-ATPase β-subunit. These latter complexes indeed become fully glycosylated after a chase period, indicating that they have passed a trans-Golgi compartment. This result indicates that the presence of the βHK ectodomain and/or cytoplasmic domain can at least partially compensate for the lack of the presence of the βNK transmembrane segment. This could also explain why αNKβHK complexes are more resistant to trypsin digestion than αNKβHK TM/NK complexes (35). We have previously shown that, compared with oocytes expressing αNKβNK complexes, oocytes expressing αNKβHK complexes exhibited a parallel reduction of the Na,K-pump current and the cell surface ouabain binding (8), indicating that the turnover of the heterologous Na,K-pumps expressed at the cell surface ouabain binding (8), indicating that the turnover of the heterologous Na,K-pumps expressed at the cell surface is identical to that of homologous Na,K-pumps. In this study, we show that this lower activity of the Xenopus αNK-rabbit gastric βHK complexes occurs despite stable association, normal ER exit, and a cellular expression level similar to that of αNKβNK complexes. Since the total ouabain binding capacity of αNKβHK complexes was much reduced compared with that of αNKβNK and since the affinity for ouabain of these two complexes is probably not significantly different (Ref. 36 and this study), these results indicate that most of the αNKβHK behave as inactive enzymes. Ueno et al. (35), who expressed Torpedo αNK with pig gastric βHK in oocytes, were not able to demonstrate Na,K-ATPase activity or pump currents at all. On the other hand, our results and those of Ueno et al. (35) contrast with observations made with sheep αNKrat βHK complexes expressed in yeast, which produced a similar number of ouabain binding sites as αNKβNK complexes (36, 37). However, in these latter studies, ouabain binding assays were performed in the presence of Mg$^{2+}$/P$^{-}$, instead of ATP and Na$^{+}$. It is possible that under these conditions ouabain binds also to Na,K-ATPase with conformational defects. It was later shown that αNK indeed forms less stable complexes with βHK than with βNK, since extraction with SDS abolishes the ouabain binding capacity of αNKβHK complexes (10). The fact that αNKβHK complexes are structurally and probably also functionally impaired proteins compared with αNKβNK complexes may be functionally relevant in tissues such as the stomach, where both βNK and βHK subunits are expressed, and may prevent the formation of functional, heterologous αNKβHK pumps.

In conclusion, the data presented in this study show that correct assembly of αNK with βNK is mediated by a concerted action of transmembrane and ectodomain interactions. Interaction of one domain but lack of efficient interaction with the other domain leads to structural and functional defects of Na,K-ATPase. Our data also indicate that the ectodomain and not the transmembrane domain of the β-subunit is directly involved in the β-subunit’s functional effects on both the $K^{+}$ and $Na^{+}$ affinities of the Na,K-pump. On the other hand, lack of correct interaction in the transmembrane domain has structural consequences that are reflected in ER retention (this study) or thermal destabilization (18). Finally, the functional role of the interaction of the β N terminus with the α-subunit remains to be determined, but our data clearly show that the β N terminus is essential for a correct conformation of the β-subunit and thus indirectly for the functional role of the β-subunit in the cation transport of the Na,K-pump.

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