Oligomerization and Maturation of Na,K-ATPase: Functional Interaction of the Cytoplasmic NH₂ Terminus of the β Subunit with the α Subunit

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Abstract. Subunit assembly plays an essential role in the maturation of oligomeric proteins. In this study, we have characterized the main structural and functional consequences of the assembly of α and β subunits of Na,K-ATPase. Xenopus oocytes injected with α and/or β cRNA were treated with brefeldin A, which permitted the accumulation of individual subunits or α-β complexes in the ER. Only α subunits that are associated with β subunits become resistant to trypsin digestion and cellular degradation. Similarly, assembly with β subunits is necessary and probably sufficient for the catalytic α subunit to acquire its main functional properties at the level of the ER, namely the ability to adopt different ligand-dependent conformations and to hydrolyze ATP in an Na⁺- and K⁺-dependent, ouabain-inhibitable fashion. Not only the α but also the β subunit undergoes a structural change after assembly, which results in a global increase in its protease resistance. Furthermore, extensive and controlled proteolysis assays on wild-type and NH₂-terminally modified β subunits revealed a K⁺-dependent interaction of the cytoplasmic NH₂ terminus of the β subunit with the α subunit, which is likely to be involved in the modulation of the K⁺-activation of the Na,K-pump transport activity. Thus, we conclude that the ER assembly process not only establishes the basic structural interactions between individual subunits, which are required for the maturation of oligomeric proteins, but also distinct, functional interactions, which are involved in the regulation of functional properties of mature proteins.

Many plasma membrane and secretory proteins are oligomeric. The subunits of these proteins are synthesized independently of each other and are inserted into the ER membrane or the lumen during their synthesis. They are subjected to cotranslational modifications, fold, and then oligomerize. Once correctly assembled, the proteins leave the ER and are targeted to their final cellular site of action. Misfolded or unassembled subunits are retained in the ER and are degraded (15). Although it is increasingly clear that the oligomeric state controls the proper function of the protein, little is known about the nature of interactions that are involved in subunit assembly and about the structural and functional consequences of subunit oligomerization. In this study, we investigate several aspects of this question by analyzing the oligomerization and the functional maturation of Na,K-ATPase.

The ubiquitous Na,K-ATPase is responsible for the maintenance of the sodium and potassium gradients between the intra- and extracellular milieu. The enzyme is composed of two heterologous subunits. The α subunit is a large multimembrane-spanning protein, while the β subunit is a smaller type II membrane glycoprotein with one transmembrane domain, a short cytoplasmic NH₂-terminal tail, and a large COOH-terminal ectodomain. Both subunits are cotranslationally inserted into ER membranes, and the β subunit acquires three to seven core sugars and forms three disulphide bridges (for review see 9). In the β subunit, a 16-amino acid stretch in the membrane domain, but not the hydrophilic NH₂ terminus is needed for membrane insertion (24), while in the α subunit, alternating membrane insertion and stop-transfer sequences are included in the first four transmembrane segments (16).

The α subunit possesses the enzymatic properties and the binding sites for cardiac glycosides, and probably forms the pore for cation transport. However, studies in cRNA-injected oocytes or in transfected cells have revealed that β subunits are needed for the expression of functional Na,K-pumps at the cell surface (for review see 8). Association of the β subunit with the newly synthesized, catalytic α subunit at the level of the ER is necessary for its structural maturation, which is characterized by an increase in the trypsin resistance (10) and/or the half-life as well as the ER exit of the α subunit (1, 19). Finally, in addition to its function in the early maturation, the β subunit plays a role in the mature enzyme and affects the...
transport activity, in particular the \( K^+ \)-activation of \( Na,K-\) pumps expressed in the plasma membrane of \( Xenopus \) oocytes (18, 19, 34) or of yeast (6).

Little is known about the nature of the interaction sites that mediate the various functions of the \( \beta \) subunit. Several lines of evidence indeed suggest that there might be different types of interaction sites in the \( \beta \) subunit, some of which are needed to stabilize a correct \( \alpha \)-subunit configuration and others which are implicated in an indirect control of the \( \alpha \) subunit's functional properties. Likely candidates for the first category of interaction sites, which we would tentatively call "structural" interaction sites, are a hydrophobic domain in the most COOH-terminal part of the \( \beta \) subunit (2), the transmembrane region (20), and a region between amino acids 126 and 170 (7), while the cytoplasmic \( N_H \) terminus of the \( \beta \) subunit does not appear to play a role in the cell surface expression of \( Na,K-\) pumps (31). On the other hand, studies with chimera, in which the \( N_H \)-terminal part including the transmembrane domain and the COOH-terminal ectodomain of different \( \beta \) subunits were exchanged, suggest that both domains participate in the modulation of the transport properties of the \( Na,K-ATPase \) (6, 20) and thus might contain so-called "functional" interaction sites.

In this study, we have investigated the functional consequences of subunit assembly of \( Na,K-ATPase \) and have explored whether the short cytoplasmic \( N_H \)-terminal tail of the \( \beta \) subunit interacts with the \( \alpha \) subunit and plays a role in the early maturation (structural interaction) and/or in the modifying effect on the \( K^+ \)-activation of \( Na,K-\) pump transport (functional interaction). Our data show that assembly with the \( \beta \) subunit is necessary and sufficient for the catalytic \( \alpha \) subunit to acquire its main functional properties at the level of the ER. Furthermore, expression of wild-type or \( N_H \)-terminally modified \( \beta \) subunits alone or together with \( \alpha \) subunits in \( Xenopus \) oocytes confirmed that the \( N_H \) terminus is not involved in the maturation and the cell surface expression of the \( Na,K-ATPase \). However, a follow-up of the protease sensitivity of individual or \( \alpha \)-associated ER-retained \( \beta \) subunits expressed in brefeldin A (BFA)-treated oocytes permitted us to reveal that oligomerization of the subunits provokes a conformational change in the \( \beta \) subunit that reflects an interaction with the \( \alpha \) subunit of the \( N_H \)-terminal, cytoplasmic \( \beta \) domain likely to be implicated in the \( K^+ \) modulation of \( Na,K-\) pump transport.

**Materials and Methods**

**cDNA Constructs**

The mutant \( \betaA \Delta N \) was constructed by deletion of 33 amino acids after the first methionine of the \( Xenopus \) \( \beta \) subunit (37) using the PCR method described by Nelson and Long (29). In a first step, a DNA fragment of the linearized pGEM1 vector containing the \( \beta \) cDNA was amplified between the sense oligonucleotide \( GAAAGGACAACATGAAAATITYATTGT-TCTATG \) comprising part of the noncoding sequence, the ATG coding for the first methionine and the sequence coding for the amino acids Lys35 to Leu40 of the \( \beta \) subunit, and the antisense oligonucleotide consisting of the primer D of Nelson and Long (1989) followed by the \( \beta \) sequence \( G_{517}C_{503} \). In a second step, the amplified fragments were used to elongate the inverse DNA strand. In this step, a long annealing time (15 min) and a low temperature (40°C) were chosen to favor the hybridization between the amplified fragment and the template, leading to the formation of a loop. In a third step, the elongated DNA strands were amplified between a sense oligonucleotide corresponding to the SP3 promoter and the antisense oligonucleotide D. Finally, the amplified mutated fragment was isolated and introduced into a wild-type pGEM2 \( \betaa \) plasmid by using EcoRI and BamHI restriction sites.

For the introduction of a \( N_H \)-terminal myc epitope EQLKISEELD (25), \( \beta1 \) (37), and \( \beta3 \) (13) cDNAs were recombined into the vector pSDM1, a derivative of pS65 that contains a single copy of the myc epitope followed by an Ncol site that encodes an in-frame AUG codon. For the \( \beta3 \) isoform, the Ncol site at the initiator AUG and a Seal site in the AMP gene were used to recombine the \( \beta3 \) cDNA from pSD5/\( \beta3 \) into pSDM1. For the \( \beta1 \) isoform, the sequence surrounding the initiator AUG was converted to an Ncol site by PCR mutagenesis, and the altered \( \beta1 \) cDNA was recombined into the pSDM1 vector. For a hemagglutinin (HA) (YPYDVPDYA (25) epitope-tagged \( \beta3 \) isoform, the \( \beta3 \) cDNA insert was recombined as described above into the vector p64T-HA, a derivative of pSP64T with an amino-terminal HA epitope followed by an Ncol site that encodes an in-frame AUG codon. All inserts and mutants generated by PCR amplification were sequenced by dideoxy sequencing (32).

**Expression in Xenopus Oocytes and Immunoprecipitation of \( \alpha \) and \( \beta \) Subunits of \( Na,K-ATPase \)**

cDNAs encoding \( Xenopus \) \( \alpha1 \) (37), \( \beta1 \), \( \beta3 \), \( \betaN \), \( \beta-myc \), \( \beta-myec \), and HA subunits were obtained by in vitro transcription (28) of linearized cDNAs recloned into the plasmid pSD5 (12). Stage V-VI oocytes were obtained from \( Xenopus \) females (South African Xenopus Facility, Noordhoek, Republic of South Africa) as described (19). Routinely, 5-7 ng of \( \alpha \) and/or 0.2-0.5 ng of \( \beta \) cDNA was injected into oocytes. Oocytes were incubated in modified Barth's medium containing 0.7-1.5 mMCo (II) of \( \alpha \)-[\( \beta \)-methyl-\( \mathrm{H} \)]methionine (Amersham Corp., Arlington Heights, IL) in the absence or presence of 5 \( \mu \)g/ml of BFA (kindly provided by Sandoz Pharma Ltd., Basel, Switzerland) for times indicated in figure legends. In preliminary experiments, it was determined that the effect of BFA on ER retention of proteins was attained within 1-2 h and was completely reversible after washing of the drug. After the labeling period or after an additional chase period in the presence of 10 mM cold methionine and in the absence or presence of BFA, microsomes were prepared as described below, and the \( \alpha \) and \( \beta \) subunits were immunoprecipitated under denaturing or nondenaturing conditions, as described (20), before revelation by SDs-PAGE and fluorography. Denaturing immunoprecipitations were performed with antibodies against the \( N_H \) terminus of the \( Xenopus \) \( \alpha1 \) subunit (A7) (1) or the purified BfD\( \alpha1 \) (RN) (11) or with antibodies against the extracytoplasmic domain of the \( Xenopus \) \( \beta1 \) (1) or the \( \beta3 \) subunit (13) to reveal the \( \alpha \), the \( \beta1 \), or \( \beta3 \) subunits, respectively. In non-denaturing immunoprecipitations, the \( \alpha \) antibody coprecipitated \( \beta \) subunits associated with \( \alpha \) subunits. The myc-tagged \( \beta-myc \) or the hemagglutinin-tagged \( \beta \)HA were immunoprecipitated under non-denaturing conditions with mAbs 9E10 or 12CA5 (Boehringer Mannheim), respectively, which permitted a \( \alpha \) subunit associated with \( \beta \) subunits to coprecipitate. Antigen–antibody complexes were absorbed on protein G–Sepharose-CL-4 (Pharmacia Fine Chemicals, Piscataway, NJ) when 9E10 or 12CA5 antibodies were used and on protein A–Sepharose when \( \alpha \), \( \beta1 \), or \( \beta3 \) antibodies were used. In some instances, immunoprecipitated \( \beta \) subunits were subjected to endoglycosidase H (Endo H) (Calbiochem-Novabiochem Corp., La Jolla, CA) treatment as described (19). The dissociated immune complexes were separated by SDs-PAGE, and the labeled proteins were detected by fluorography. Quantifications and determinations of the molecular mass of the immunoprecipitated bands were performed with an analytic program for electrophoretic images (Bio-1D) from Vilber Lourmat (Marne La Vallée, France).

**Cell Extraction, Preparation of Microsomes, and Cellular Fractionation of Xenopus Oocytes**

For the preparation of microsomes, oocytes were homogenized by 15 strokes in a glass Teflon homogenizer in a buffer (25 \( \mu \)l per oocyte) containing (in mM) 83 NaCl, 1 MgCl\(_2\), 10 Heps, pH 7.9, 0.5 PMSF, and 5 \( \mu \)g/ml (each) of antipain, pepstatin, and leupeptin (Sigma Chemical Co., St. Louis, MO). Homogenates were first centrifuged twice at 1,000 \( \times \)g for 10 min at 4°C to remove yolk granules and then at 10,000 \( \times \)g for 20 min to yield
a microsomal pellet (P1) in which 2-3 μg protein per oocyte were recovered. Preliminary experiments revealed that >90% of the total α and β subunit population from cRNA-injected oocytes were recovered in P1. Less than 1% of the subunits were found in a secondary microsomal fraction (P2) produced by high speed (165,000 g, 90 min) centrifugation of the supernatant of P1, although ~5.5 μg protein per oocyte was recovered. Finally, 0.5% of the total subunit population was recovered in the high speed supernatant (~10 μg protein per oocyte). Na,K-ATPase activity measurements (see below) and residence of Bip protein (data not shown) indicate that P1 is enriched in plasma membranes as well as in ER, and therefore this microsomal fraction was used for most studies. In some instances, oocyte homogenates were fractionated by density centrifugation on 12-50% (wt/vol) linear sucrose gradients as previously described (19, 30). 12 fractions of sucrose densities between 1.05 and 1.17 were collected from the top of the gradient and were either recentrifuged at 228,000 g for 3 h or directly subjected to immunoprecipitations (see above).

Proteolysis of α and β Subunits

To test the trypsin sensitivity of newly synthesized α subunits, fractions 8 to 11 of sucrose density gradients that are enriched in ER membranes (19) were subjected to control trypsinolysis. Fractions were incubated for 5 min on ice with trypsin (type XI, Sigma Chemical Co.) at a trypsin/protein ratio of ~0.5 and 150 mM K-acetate before addition of a fivefold (wt/wt) excess over trypsin of soybean trypsin inhibitor (Sigma Chemical Co.). The reaction mixture was left on ice for 10 min before addition of SDS to a final concentration of 3.7% and immunoprecipitation with α antibodies. A control trypsinolysis assay was also used to follow the ability of newly synthesized α subunits to perform ligand-dependent conformational changes. In this case, P1 fractions were prepared from oocytes that were injected with α or a plus β cRNA and labeled for 24 h in the presence of BFA. Aliquots (5-12 μg protein) of P1 fractions resuspended in buffer A, containing (in mM) 30 DL-histidine, 5 EDTA, and 18 Tris base, pH 7.4, were incubated either with 15 mM NaCl and 15 mM KCl or with 7.5 mM MgCl2, 7.5 mM Tris-Pi, and 1 mM ouabain for 20 min at 25°C before addition of different concentrations of trypsin (total volume, 50 μl). Trypsinolysis proceeded for 60 min at 25°C for β-associated α subunits and for 5 min on ice for unassembled α subunits, and then was stopped with a fivefold (wt/wt) excess of soybean inhibitor over trypsin. After 10 min at 25°C, SDS was added and the sample was immunoprecipitated with the RN antibody. For proteolysis assays on α subunits, we verified in preliminary experiments that the β subunit was not limiting for the formation of α-β complexes.

To follow conformational changes of newly synthesized β subunits upon assembly with α subunits, aliquots (5-12 μg protein) of microsomal fractions were subjected to proteolysis under the following conditions: (a) trypsin or α-chymotrypsin (type II; Sigma Chemical Co.) at protease/protein ratios between 0.01 and 3.5 for 2 h at 25°C in the presence of different ligands; and (b) chymotrypsin at a chymotrypsin/protein ratio of 50 for 60 min at 25°C. To stop proteolysis, a fivefold (wt/wt) excess of soybean inhibitor or 5 mM PMSF was added to samples treated with trypsin or chymotrypsin, respectively, and the samples were left on ice or at 25°C for 10 min before addition of SDS to a final concentration of 3.7%. Samples were then immunoprecipitated with β antibodies (see above) and subjected to SDS-PAGE in the presence or absence of 2% β-mercaptoethanol. Determinations of the molecular mass of the immunoprecipitated β subunits were performed on samples loaded in different arrangements on the gel to exclude artifacts due to gel "smiling." For proteolysis assays of the β subunit, we verified in preliminary experiments that the α subunit was not limiting for the formation of α-β complexes.

Na,K-ATPase Measurements

The Na,K-ATPase activity was measured in P1 fractions obtained from oocytes injected with α cRNA alone or together with different β cRNAs and incubated for 24 h in the presence of BFA or for 3 d in the absence of BFA. Na,K-ATPase activity was measured in triplicate by an enzyme-linked assay, according to Schoner et al. (33), in which the reconstitution of ATP consumed by the ATPase is coupled by the pyruvate and lactate dehydrogenase reactions to NADH oxidation. The oxidation rate of NADH was recorded at 340 nm wavelength in the automated enzyme kinetic accessory of a DU-64 spectrophotometer (Beckman Instruments, Inc., Palo Alto, CA). Activity measurements were done in the presence of 0.15 mg SDS per mg of protein, a detergent concentration that was determined to give maximal activation of latent pumps. Specific Na,K-ATPase activity was calculated from the difference between samples incubated in the absence or presence of 1 mM ouabain. Specific Na,K-ATPase activity accounted for 2-14% of the total ATPase activity. Statistical analysis was done by unpaired t test.

Ouabain Binding and 86Rb Uptake Measurements

Ouabain binding to oocytes and 86Rb uptake measurements were essentially done as previously described (20) in oocytes injected with α and β cRNA and incubated for 3 d. For both measurements, oocytes were preincubated for 1 h in a nominally K⁺-free solution to load the oocytes with Na⁺. Ouabain binding was performed in solution A containing (in mM) 90 NaCl, 1 CaCl2, 1 MgCl2, and 10 Hepes, pH 7.4, and supplemented with 0.4 μM [21, 22-3H] ouabain (Amersham Corp.) for 20 min at room temperature. Non-specific binding, measured in the presence of a 1,000-fold excess of cold ouabain, amounted to 3-7% of the total binding. Statistical analysis was done by unpaired t test. 86Rb uptake was performed in oocytes injected with Bufo α (17) and Xenopus β wild-type β or β1AN cRNA in solution A without NaCl but supplemented with 5 mM BaCl2, 5 μCi/ml of 86RbCl (Amersham Corp.) and varying concentrations of KCl for 12 min. During the preincubation of the oocytes and the 86Rb uptake, 1 μM ouabain was added, which permitted the complete inhibition of the endogenous oocyte Na,K-pumps (17). Individual oocytes were solubilized with 5% SDS before counting. The parameters of the Hill equation V = Vmax/[1 + (K1/2/CK) nH] were fitted to the data of the 86Rb uptake (V) induced by various concentrations of K⁺ (CK) and yielded least-square estimates of the maximal uptake (Vmax), the half-maximal activation constant (K1/2), and the Hill coefficient (H).

Results

ER Assembly and Functional Maturation of the α-Subunit

A first aim of this study was to determine whether the structural change in the α subunit, which occurs upon assembly with the β subunit and which is reflected by an increased trypsin resistance (10) and an increased half-life (1), coincides with the acquisition of its functional properties. To assess this question, we made use of BFA, which, in most somatic cells, blocks the exit of proteins out of the ER, causes vesiculation of the Golgi cisternae, and leads to redistribution of resident Golgi membrane proteins to the ER (27).

To test the effect of BFA in Xenopus oocytes, we injected oocytes with Xenopus α1 and β1 cRNA, labeled the oocytes in the absence or presence of BFA, and subjected them to cell fractionation by sucrose density centrifugation. The α and the β subunits synthesized in the absence of BFA during a continuous pulse of 17 h distributed, as expected, in both ER- and plasma membrane-enriched fractions (Fig. 1, A and C). For assembly with the β subunit and which is reflected by an increased trypsin resistance (10) and an increased half-life (1), coincides with the acquisition of its functional properties. To assess this question, we made use of BFA, which, in most somatic cells, blocks the exit of proteins out of the ER, causes vesiculation of the Golgi cisternae, and leads to redistribution of resident Golgi membrane proteins to the ER (27).

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BFA. In any case, the results indicate that BFA inhibits ER exit of proteins in Xenopus oocytes and justify the use of this drug to study the direct consequences of the assembly process in the ER on the properties of Na,K-ATPase subunits.

Fig. 1 E shows that individual α subunits from ER membranes of BFA-treated oocytes were highly trypsin sensitive, while β-assembled α subunits became trypsin resistant (Fig. 1 F). This result confirms that the structural maturation of the α subunit is a direct consequence of the assembly with the β subunit in the ER. To monitor the functional maturation of the catalytic α subunit after subunit assembly, we first assessed the ability of individual and β-assembled α subunits to undergo ligand-dependent conformational changes, which is a characteristic feature of functional Na,K-ATPases (for review see 21). As a biochemical tool to follow the conformational transitions, we adapted a controlled trypsinolysis assay, which has previously been used for the same purpose in purified enzyme preparations (22). The assay is based on the prediction that proteins in different conformations exhibit distinct tryptic sites and that digestion with trypsin would produce distinct tryptic patterns according to the conformation adopted by the protein.

To analyze the ability of the α subunits to perform ligand-dependent conformational changes, we performed trypsinolysis of individual or β-associated α subunits in the presence of either NaCl, KCl, or Mg/Tris-Pi/ouabain. Individual α subunits were digested up to 80% after incubation of microsomes of BFA-treated oocytes for 5 min at 4°C at a trypsin/protein ratio of 0.01. Although some tryptic fragments could be revealed, there were no striking dif-
measurements. (A) Immunoprecipitations of a-13 complexes synthesized or injected with 7 ng alone (P < 0.001). Injections of oocytes expressing the various a-B complexes are significantly increased by three- to sevenfold after a 24-h incubation (Fig. 3 A, lanes 1-12). Nondenaturing immunoprecipitations were performed with an antibody (lanes 7-11, 63-kD fragment). Mg/Tris-Pi/ouabain also produced characteristic tryptic fragments (lanes 12-16, 41-kD fragment) that were distinct from those produced in the presence of each of these ligands alone (data not shown). Finally, the ligand-dependent tryptic patterns of the a subunit were similar when trypsinolysis was performed on microsomes of oocytes that were not treated with BFA (data not shown). Thus, altogether the trypsinolysis data indicate that in contrast to individual a subunits, which cannot adopt different conformations necessary for ion transport, a subunits that associate with B subunits acquire these functional properties at the level of the ER.

This result was also supported by our second functional test in which we assessed the Na+- and K+-dependent ouabain-inhibitable ATPase activity in microsomes of BFA-treated oocytes expressing a subunits alone or a plus B subunits. Despite a significant accumulation of newly synthesized, exogenous a subunits in the ER (Fig. 3 A, compare lanes 1 and 2), a low and similar Na,K-ATPase activity was measured in microsomes prepared from noninjected and a cRNA-injected oocytes treated with BFA (Fig. 3 B, lanes 1 and 2). This result confirms that a subunits alone have no Na+- and K+-dependent hydrolytic activity. Significantly, in BFA-treated oocytes that expressed a-13 complexes containing exclusively core-glycosylated B subunits (Fig. 3 A, lane 3), the Na,K-ATPase activity increased by three- to sevenfold after a 24-h incubation (Fig. 3 B, lane 3). In nontreated oocytes that expressed a-13 complexes containing mainly fully glycosylated B subunits (Fig. 3 A, lane 5), the Na,K-ATPase activity increased up to 12-fold after a 3-d incubation (Fig. 3 B, lanes 7-9). Since we have previously shown that glycosylation of the a subunit does not influence the enzyme activity of Na,K-ATPase (38), these data indicate that a subunits become functionally active, e.g., acquire Na,K-ATPase activity upon assembly with B subunits at the level of or close to the ER.

The NH2 Terminus of the a Subunit Is Not Involved in the Structural and Functional Maturation of the a Subunit

To elucidate whether the NH2 terminus of the a subunit plays a role in the early functional maturation of the Na,K-ATPase, we compared the ability of Xenopus wild-type a1 and a3 subunits and of two NH2-terminally modified a subunits to form a-13 complexes with Na,K-ATPase activity. All B subunits including a NH2-terminally deleted a1 (a1ΔN) and a myc-tagged a3 (a3myc) subunit, synthesized in the presence of BFA, associated efficiently with a subunits as shown by the coprecipitation with an a antibody.

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Figure 3. The NH2 terminus of the B subunit is not involved in the functional maturation of the a subunit. Oocytes were not injected or injected with 7 ng Xenopus a1 cRNA alone, or with 7 ng a1 plus 0.2 ng Xenopus B1, 0.5 ng B2ΔN, 0.5 ng B3, or 0.5 ng B3myc cRNA and incubated for 24 h or 3 d with or without [35S]methionine in the presence or absence of BFA. Microsomes were prepared and were either subjected to immunoprecipitation under nondenaturing conditions or used for Na,K-ATPase activity measurements. (A) Immunoprecipitations of a-13 complexes synthesized during a 24-h pulse-labeling of oocytes in the presence of BFA (lanes 1-4 and 7-9) or during a 3-d pulse in the absence of BFA (lanes 5, 6, and 10-12). Nondenaturing immunoprecipitations were performed with an antibody (lanes 7-11, 63-kD fragment) or of a myc antibody (9E10) (lanes 9 and 12). Nondenaturing immunoprecipitations were performed with an antibody (lanes 7-11, 63-kD fragment) or of a myc antibody (9E10) (lanes 9 and 12). (B) Na,K-ATPase activity of a subunits and a-13 complexes synthesized during a 24-h incubation of oocytes in the presence of BFA (lanes 1-6) or during a 3-d incubation in the absence of BFA (lanes 7-10). Numbers of experiments performed on different batches of oocytes are given in the bars. Na,K-ATPase activities measured in nontreated and a cRNA-injected oocytes are statistically not different (P > 0.1). On the other hand, Na,K-ATPase activities measured in oocytes expressing the various a-13 complexes are significantly different from those measured in oocytes expressing a subunits alone (P < 0.001).
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Interaction of the NH2 Terminus of the β Subunit with the α Subunit and Its Functional Consequences

Since the cytoplasmic NH2 terminus of the β subunit does not play a role in the early maturation and the cell surface expression of the Na,K-ATPase, the question remains whether this domain indeed interacts with the α subunit and might mediate the regulatory function of the β subunit in the transport activity of mature pumps. To test this hypothesis, we expressed α subunits together with wild-type or NH2-terminally deleted β subunits in oocytes and measured the transport properties of the α–β complexes expressed at the cell surface, in particular the K+–activation of the 86Rb uptake mediated by the Na,K-pumps. Fig. 5 shows that the apparent K+-affinity of the Na,K-pump–mediated 86Rb uptake is about four times lower for α–βΔN complexes (apparent K1/2 for K+: 1.6 mM) than for α–β wild-type complexes (apparent K1/2 for K+: 0.4 mM). Thus, indeed, the cytoplasmic NH2 terminus of the β subunit appears to be involved in the modulation of the K+-activation of the Na,K-ATPase transport activity.

The question arose whether the predicted functional in-
Figure 5. NH₂-terminally deleted β subunits affect the K⁺-activation of Na,K-pumps. Oocytes were injected with 9 ng ufo α₁ cRNA plus 0.3 ng Xenopus β₁, (β₁wt, ○) or 0.5 ng β₁,ΔN cRNA (●) and incubated for 3 d. ^86Rb uptake into individual oocytes was measured as described in Materials and Methods at different K⁺ concentrations in the external medium. Each experimental point represents the mean ± SE of 15-19 oocytes. At close to Vmax conditions, at 5 mM external K⁺, ^86Rb uptake was 119 ± 15 pmol per min per oocyte in oocytes expressing α–βwt complexes and 94.5 ± 7.6 pmol per min per oocyte in oocytes expressing α–β,ΔN complexes. One out of two similar experiments is shown. In comparative studies performed on oocytes expressing ouabain-sensitive Xenopus α–β complexes, we have found a good correlation between ouabain binding and transport function for both wild-type and mutant α–β complexes, indicating that there is no significant difference in the molecular activity of wild-type and mutant pumps.

interaction of the NH₂ terminus with the α subunit could be revealed as a discrete conformational change occurring in the β subunit after association with the α subunit. To assess this question, we checked for differences in the protease sensitivity and in the digestion patterns of individual and α-associated β subunits synthesized in BFA-treated oocytes. β subunits synthesized in vivo alone or together with α subunits turned out to be highly resistant to trypsin or chymotrypsin treatment. Only extreme digestion conditions at a chymotrypsin/protein ratio of 50 permitted the production of a significant fragmentation of the individual or α-associated β subunits (Fig. 6, lanes 1–4). Most likely, the digestion of the β subunit under these conditions is not due to a specific action of chymotrypsin but rather to the activity of a nonidentified proteolytic contaminant. Nevertheless, we consistently observed a difference in the production of two small proteolytic fragments in individual and α-associated Xenopus β₁ subunits, indicating that, indeed, the β subunit undergoes a structural change upon assembly with the α subunit. Protein digestion produced a fragment of ~14.4-kD in individual β subunits (lane 2) and a fragment of ~16.5-kD in assembled β subunits (lane 4). In an attempt to locate the proteolytic sites responsible for the production of these fragments, we subjected the digested immunoprecipitated sample to nonreducing gel electrophoresis. In the absence of β-mercaptoethanol, the two small molecular mass fragments disappeared (lanes 5–8), indicating that the cleavage site is located within a region held together by a disulphide bridge. According to the molecular mass of the fragments, they might be derived from the NH₂-terminal part of the β subunit by cleavage between Cys126 and Cys149 known to form the first of three disulphide bridges in the ectodomain of β subunits (for references see 8) (see Fig. 10). In agreement with this prediction is the fact that, in contrast to the other proteolytic fragments of 29 and 21 kD, the 14.4- and 16.5-kD fragments are not affected by Endo H treatment (Fig. 6, lanes 11, 12, 15, and 16) and thus do not contain sugar chains, as expected from the Xenopus β₁ model (see Fig. 10). Furthermore, none of the proteolytic fragments produced from an NH₂-terminally myc-tagged β₁ subunit, which was subjected to similar proteolytic digestion, were recognized by a myc antibody (data not shown), indicating that the NH₂ terminus was missing in the cleaved products.

Although these data argue that the 14.4- and 16.5-kD fragments are produced from the NH₂ terminus of the β subunit, they did not permit us to decide whether the increase in the molecular mass of the fragment from α-associated β subunits was due to a protection of the NH₂-terminal or the COOH-terminal part in the 16.5-kD fragment. In this context, it is interesting to note that Lutsenko and Kaplan (27) have recently suggested that a trypsic cleavage site at Arg¹³⁴–Gly¹³⁵ in the β subunit of microsomal Na,K-ATPase is hidden in the presence of MgCl₂ but exposed in the presence of Mg/P. To identify the proteolytic cleavage sites that become protected in the β subunit after association with the α subunit, milder and thus more specific proteolysis conditions were applied. Significantly, when trypsinolysis was performed for 2 h at 25°C at a trypsin/protein ratio of 3.5 in the presence of MgCl₂, the individual β subunits were nearly completely digested (Fig. 7 A, lane 3) while the α-associated β subunits were partly resistant to proteolytic attack (lane 6). Similarly, α-associated β subunits resisted better to proteinase K digestion (protease/protein ratio of 1, 1 h, 4°C) in the presence of 1% Triton X-100 than individual β subunits (compare lanes 9 and 12). These data argue that the β subunit undergoes an important structural change upon assembly...
Figure 7. Controlled proteolysis reveals a global conformational change in α-associated β subunits and a specific cleavage of the cytoplasmic NH₂ terminus. Oocytes were injected with 0.2 ng Xenopus β₁ cRNA, with 0.5 ng β₁ΔAN, with 0.5 ng β₁myc, with 7 ng α₁ plus 0.2 ng β₁ cRNA, or with 7 ng α₁ plus 0.5 ng β₁myc and incubated for 24 h (A and B) or 48 h (C) with [³⁵S]methionine in the presence (A) or absence of BFA (B and C) before preparation of microsomes. (A) Digestion of individual and α-associated β subunits with trypsin and proteinase K. Aliquots of microsomes from BFA-treated oocytes injected with β₁ cRNA alone (lanes 1–3, and 7–9) or with α₁ plus β₁ cRNA (lanes 4–6, and 10–12) were incubated either without or with trypsin (lanes 1–6) or proteinase K (lanes 7–12) before denaturing immunoprecipitation with a β antibody. For trypsin digestion, samples were preincubated for 3 h at 25°C with 15 mM KCl (lanes 1, 2, 4, and 5) or 7.5 mM MgCl₂ (lanes 3 and 6) before addition of trypsin at a protease/protein ratio of 3.5 and deoxycholate at a detergent/protein ratio of 0.3 for 2 h at 25°C. Proteinase K digestion was performed at a protease/protein ratio of 1 for 1 h at 4°C in the presence of 10 mM CaCl₂ and in the absence (lanes 8, 10, and 11) or presence (lanes 9 and 12) of 1% Triton X-100. (B) Trypsinolysis of individual, NH₂-terminally modified β subunits. Aliquots of microsomes from oocytes injected with β₁ (lanes 1 and 2), β₁ΔAN (lanes 3 and 4), or β₁myc (lanes 5–10) cRNA were incubated without or with trypsin at a protease/protein ratio of 3.5 for 2 h at 25°C in the presence of 7.5 mM MgCl₂ (lanes 1–4, 7, 8, and 10) or 15 mM KCl (lanes 5, 6, and 9). Immunoprecipitations were performed under denaturing conditions with a β antibody (lanes 1–7) or under nondenaturing conditions with the myc antibody 9E10 (lanes 8–10). (C) Trypsinolysis of individual and α-associated fully glycosylated β₁myc. Aliquots of microsomes from oocytes injected with β₁myc (lanes 1–3) or with α₁ plus β₁myc (lanes 4–9) cRNA were incubated without or with trypsin at a protease to trypsin ratio of 0.1 for 1 h at 25°C in the presence of 15 mM KCl (lanes 1, 2, 4, 5, 7, and 8) or 7.5 mM MgCl₂ (lanes 3, 6, and 9). Immunoprecipitations were performed under denaturing immunoprecipitations with a β antibody (lanes 1–6) or under nondenaturing conditions with the myc antibody 9E10 (lanes 7–9).
The cytoplasmic NH$_2$-terminal tail of the $\beta$ subunit becomes protected against proteolysis after assembly with the $\alpha$ subunit. Oocytes were injected with 7 ng $\alpha$ plus 0.2 ng $\beta$ cRNA (lanes 1-6) or with 0.2 ng $\beta$ cRNA alone (lanes 7-12) and incubated for 24 h with $[^{35}S]$methionine in the presence of BFA. Microsomes were prepared and aliquots were incubated with trypsin (A) or chymotrypsin (B) at protease/protein ratios between 0.01 and 3.5 for 2 h at 25°C in the presence of 15 mM KCl before immunoprecipitation under denaturing conditions with a $\beta$ antibody. $C$ and $D$ show the relative changes of the molecular mass of the $\beta$ subunits shown in A and B respectively, as a function of the protease/protein ratio used. The molecular mass of the nondigested $\beta$ subunit has arbitrarily been set to 1. One out of three similar experiments is shown.

Since softer proteolysis conditions permitted us to selectively cleave the $\beta$ subunit from the NH$_2$-terminal side, we performed in a last set of experiments a detailed analysis on the protease sensitivity of individual and $\alpha$-assembled $\beta$ subunits to demonstrate the putative functional interaction site in the cytoplasmic tail of the $\beta$ subunit. When microsomes from BFA-treated oocytes expressing individual or $\alpha$-assembled $\beta$ subunits were incubated with increasing concentrations of trypsin or chymotrypsin for 2 h at 25°C in the presence of KCl, the NH$_2$ termini of individual and $\alpha$-associated $\beta$ subunits were progressively cleaved to fragments of lower molecular mass with increasing protease concentrations (Fig. 8). However, for a similar shift in the molecular mass, $\alpha$-assembled $\beta$ subunits required ~10 times more protease than individual $\beta$ subunits (Fig. 8, $A$-$D$). The results were particularly striking with chymotrypsin. While the individual $\beta$ subunits were nearly maximally cleaved by 3 kD at the lowest protease concentration used (Fig. 8, $B$ [lanes 7-12] and $D$), the molecular mass of the $\alpha$-associated $\beta$ subunits only progressively decreased with increasing protease concentrations (Fig. 8, $B$ [lanes 1-6] and $D$). Interestingly, the NH$_2$ terminus of the $\alpha$-assembled $\beta$ subunit was only efficiently protected when trypsinolysis was performed in the presence of KCl but not when it was performed in the presence of NaCl (Fig. 9, lanes 4-13). KCl but not NaCl protects the NH$_2$ terminus of the $\beta$ subunit from digestion with trypsin. (A) Oocytes were injected with 0.2 ng $\beta$ cRNA alone (lanes 1-3) or with 7 ng $\alpha$ plus 0.2 ng $\beta$ cRNA (lanes 4-13) and incubated for 24 h with $[^{35}S]$methionine in the presence of BFA. Microsomes were prepared, and aliquots were incubated without trypsin (lanes 1, 4, and 9) or with trypsin at indicated trypsin/protein ratios in the presence of 15 mM NaCl (lanes 2 and 5-8), or 15 mM KCl (lanes 3, 6, and 10-13) for 2 h at 25°C before immunoprecipitation with a $\beta$ antibody. (B) Relative changes of the molecular mass of the $\beta$ subunits shown in A as a function of the protease/protein ratio used.

**Discussion**

In this study, we document for the first time the multiple effects of subunit assembly on a house-keeping protein, the Na,K-ATPase. In particular, we show that association of the $\beta$ subunit in or close to the ER is necessary and sufficient for the catalytic $\alpha$ subunit to acquire its structural stability and its main functional properties. Furthermore, our studies reveal that the $\beta$ subunit itself is subjected to a structural change upon assembly. Finally, we document that the cytoplasmic NH$_2$ terminus of the $\beta$ subunit interacts with the $\alpha$ subunit and modulates the K$^+$-activation of the Na,K-pumps.

**Subunit Assembly is Necessary and Sufficient for the Functional Maturation of the Catalytic $\alpha$ Subunit**

Individual subunits generally assemble into oligomers at
the level of the ER after extensive folding. Oligomerization is, however, likely to induce an additional structural change that is necessary for the protein to attain its native state and to be transported to its cellular site of action. For many viral and eukaryotic membrane proteins, it has been shown that subunit assembly is involved in their structural maturation, which permits the proteins to escape from the ER quality control. Only assembled subunits can leave the ER and avoid pre-Golgi degradation (for review see 5, 15). On the other hand, only a few examples document how oligomerization influences the functional maturation of proteins. For the pentameric acetylcholine receptor, it was shown that the efficiency of the binding of the specific ligand bungarotoxin is related to the sequential association of the four different yet homologous subunits (14 and references therein). In the case of Na,K-ATPase, we have studied the functional consequences of subunit assembly on the hydrolytic activity, and the ligand-dependent conformational changes, as well as the ouabain binding. The results show that the Na,K-ATPase becomes functionally active at the level of the ER, and this is, to our knowledge, the first demonstration that subunit assembly might be sufficient for the acquisition of the main functional properties of an oligomeric protein.

The basic prerequisite for the cation transport of P-type ATPases is the ligand-dependent adoption of two main conformations, an E₁ and an E₂ conformation (for review see 21). In the case of Na,K-ATPase, the E₁ conformation exposes binding sites for Na⁺ to the cytoplasmic face and becomes immediately phosphorylated from ATP. The change to the E₂ conformation permits the release of Na⁺ to the extracellular milieu and the binding of K⁺, as well as the interaction with the specific inhibitor ouabain. The transport cycle is closed by an E₂ to E₁ transition. It has previously been shown on transport-competent, purified enzyme preparations that ligand-dependent conformational changes can be monitored by controlled trypsinolysis, which produces different tryptic fragments of the α subunit in the presence of different ligands and characteristic functional effects (22). In the presence of Na⁺, which induces the E₁ conformation, trypsin preferentially cleaves in the first cytoplasmic loop between the transmembrane regions M2 and M3, and in the presence of K⁺, which induces the E₂ conformation, trypsin cleaves in the big cytoplasmic loop between M4 and M5.

In this study, we have developed a similar biochemical approach to probe for the ability of newly synthesized α subunits, which were expressed either alone or together with β subunits in BFA-treated oocytes, to perform ligand-dependent conformations. Our data indicate that individual α subunits are devoid of Na⁺- and K⁺-dependent ATPase activity and cannot undergo conformational transitions permitting ion transport. More data are needed to reveal whether unassembled α subunits might have certain other functional properties. It has indeed been reported in a recent study that individual α subunits exhibit an ATPase activity that occurs in the absence of Na⁺ and K⁺ but in the presence of Mg²⁺ and that is not inhibited by ouabain (3). In any case, our data show that association with the β subunit at the level of the ER is required for the acquisition of the normal activity of the enzyme. Only β-associated α subunits are able to hydrolyze ATP in an Na⁺-, K⁺-dependent and ouabain-inhibitable fashion and to perform Na⁺-, K⁺-, or ouabain-dependent conformational transitions as reflected by the production of specific tryptic α fragments in the presence of the different ligands. With respect to this latter property, it should be noted that the tryptic fragments produced from our native α subunits (68 and 63 kD) exhibit different molecular masses than those described for purified enzyme preparations obtained by SDS purification (77 and 58 kD in reference 22). The reason for these discrepancies is not clear, but they could result from discrete local differences in the compactness of α subunits in native membranes and of SDS-purified α subunits. A similar deviation from the "classical" splits has been described for α subunits in proteoliposomes (23 and references therein).

Altogether, these results indicate that the newly formed α-β complexes in the ER exhibit similar structural and functional characteristics than the transport-competent α-β complexes expressed at the cell surface. The question may arise whether these potentially transport-competent Na,K-ATPases are active in the ER in intact cells. Although the ionic conditions of the ER lumen are not defined, one might speculate that they would not favor the transport activity of Na,K-pumps. Furthermore, active α-β complexes, once formed in intact cells, do not accumulate but rapidly leave the ER, which might prevent any functional effect in the ER.

According to our results, the β subunit could be considered as a specific molecular chaperone that is necessary for the correct folding of the newly synthesized α subunit. A major challenge for future studies will be to identify the nature of the interaction sites in the two subunits and to reveal the structural changes in the α subunit that permit its functional maturation. In view of the temporal coincidence of the structural maturation characterized by a global increase in the trypsin sensitivity, and the functional maturation of the α subunit, it is possible that both processes are mechanically linked and might depend on a primary interaction between extracytoplasmic and transmembrane domains of the two subunits. Likely candidates for these interaction domains that we would tentatively call structural interaction sites are the most COOH-terminal part (2), the transmembrane (6, 20) and adjacent regions (7) in the β subunit, and the extracellular loop between M7 and M8 (7) in the α subunit.

The NH₂ Terminus of the β Subunit Interacts with the α Subunit and Modulates its Apparent K⁺ Affinity

Interactions in the cytoplasmic domains of subunits of oligomeric membrane proteins have been described, but, as suggested by mutational studies on viral proteins, these interactions generally have little effect on oligomer stability (for review see 5). As suggested by the present study, interactions in the cytoplasmic domains might instead have consequences on functional properties of the completed oligomer.

Increasing experimental evidence suggests that besides their role in the maturation of the α subunit, β subunits have an additional function. Several studies document that β subunits of Na,K-, as well as of H,K-ATPase, might act as indirect modulators of the transport activity and in par-
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The nature of interaction of the β NH₂ terminus with the α subunit is definitely different from that of the ectodomain. Although it occurs soon after synthesis of the subunits at the level of the ER, similar to the interactions implicated in the structural and functional maturation of the α subunit, the interaction of the cytoplasmic NH₂ terminus with the α subunit is not implicated in these processes. Indeed, as previously suggested by Renaud et al. (31), we confirm in this study that NH₂-terminally deleted β subunits produce functional Na,K-pumps at the cell surface. Altogether, our data indicate that the cytoplasmic NH₂ terminus of the β subunit represents a functional interaction site in opposition to the structural interaction sites mainly needed for the early maturation of the catalytic α subunit.

Figure 10. Linear model of the β subunit and putative proteolytic cleavage sites in the cytoplasmic NH₂-terminal tail. (A) Linear model of the Xenopus β₁ subunit with the cytoplasmic NH₂ terminus, the transmembrane domain (M), and the COOH-terminal ectodomain. Indicated are the positions of the cysteine (C) residues that form disulphide (S-S) bridges and the asparagine (N) residues that are glycosylated. (B) Amino acid sequence of the NH₂ terminus of the Xenopus wild-type β₁ subunit (I) and the β₁ΔN mutant (2). In B₁, the putative proteolytic sites for chymotrypsin or trypsin (arrowheads) and the approximate molecular mass of the fragments that are removed in the maximally digested β₁ subunits (㎏) are indicated. Molecular masses above and below arrowheads refer to chymotryptic and tryptic fragments that are produced from splits between the cytoplasmic domain and Arg^{42} in 19-kD membranes (35). In B₂, the expected change in the molecular mass of β₁ΔN compared to wild-type β₁ is indicated. For more details see text.

The involvement of the β NH₂ terminus in the K⁺-activation is supported by our recent observation that NH₂-terminally deleted mutants expressed in Xenopus oocytes together with α subunits influence the apparent K⁺-affinity of the Na,K-pump current (Jaisser, F., X. Wang, K. Geering, and J.D. Horisberger, manuscript in preparation). In this study, we were able to show that a stretch of eight amino acids close to the transmembrane domain might be important for the functional effect. An implication of the cytoplasmic NH₂ terminus of the β subunit was also proposed recently in Rb⁺ occlusion (36). These studies were performed on 19-kD membranes that are produced by extensive tryptic digestion of purified Na,K-ATPase preparations and that contain a 19-kD COOH-terminal fragment of the α subunit and an NH₂-terminal 16-kD and a COOH-terminal 50-kD fragment of the β subunit. Rb⁺ and Na⁺ occlusion as well as ouabain binding are preserved in these preparations. It was shown that at 37°C, but not at 20°C, cleavage of the β NH₂ terminus by chymotrypsin from a 16- to a 15-kD fragment (Fig. 10) reduced Rb⁺ affinity and increased deocclusion rates.

How could the cytoplasmic NH₂ terminus affect the binding of extracellular K⁺? One possibility is that the cytoplasmic NH₂ terminus is part of the intramembranous K⁺ binding site. Our observation that interaction of the β NH₂ terminus with the α subunit is favored in the presence of K⁺ but not in the presence of Na⁺ would be in agreement with a direct sensing of K⁺ by the NH₂ terminus. An indirect conformational effect cannot, however, be excluded since, as discussed above, it is indeed likely that several regions determine the functional interaction in the β subunit. It might be envisioned that the binding of K⁺ either to another site in the β subunit or to the α subunit might indirectly influence a “gating” property of the β NH₂ terminus.

In conclusion, our studies demonstrate that the NH₂ terminus of the β subunit interacts with the α subunit in a K⁺-dependent fashion and that this functional interaction is implicated in the modulation of the K⁺-activation of Na,K-pumps at the cell surface rather than in their maturation. Further studies are needed to define more precisely the identity of the amino acids in the β and the α subunits involved in this interaction and the nature of the functional effects.

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References

1. Ackermann, U., and K. Geering. 1990. Mutual dependence of Na,K-ATPase α-subunits and β-subunits for correct posttranslational processing and intracellular transport. FEBS Lett. 269:105–108.

2. Beggah, A.T., P. Beguin, P. Jaunin, M.C. Peitsch, and K. Geering. 1993. Hydrophobic C-terminal amino acids in the β-subunit are involved in assembly with the α-subunit of Na,K-ATPase. Biochemistry. 32:14177–14174.

3. Blanco, G., A.W. Detomaso, J. Koster, J.Z. Xie, and R.W. Mercer. 1994. The α-subunit of the Na,K-ATPase has catalytic activity independent of the β-subunit. J. Biol. Chem. 269:23420–23425.

4. Chow, D.C., and J.G. Forte. 1993. Characterization of the β-subunit of the H1,K-ATPase using an inhibitory monoclonal antibody. Am. J. Physiol. 265:C1562–C1570.

5. Eakle, K.A., M.A. Kabalin, S.G. Wang, and R.A. Farley. 1994. The influence of β subunit structure on the stability of Na+/K+-ATPase complexes and interaction with K+. J. Biol. Chem. 269:6553–6557.

6. Fambrough, D.M., V.M. Lema, M. Hamrick, M. Emerick, K.J. Renaud, E.M. Inman, B. Hwang, and K. Takeyasu. 1994. Analysis of subunit assembly of the Na-K-ATPase. Am. J. Physiol. 266:C579–C588.

7. Geering, K. 1990. Subunit assembly and functional maturation of Na,K-ATPase. J. Membr. Biol. 115:109–121.

8. Geering, K. 1991. The functional role of the β-subunit in the maturation and intracellular transport of Na,K-ATPase. FEBS Lett. 285:189–193.

9. Geering, K., I. Thelaz, F. Verrey, M.T. Häuptle, and B.C. Rossier. 1989. A role for the β-subunit in the expression of functional Na+–K+–ATPase in Xenopus oocytes. Am. J. Physiol. 257:C301–C358.

10. Girardet, M., K. Geering, J.M. Frantes, D. Geser, B.C. Rossier, J.-P. Kraehenbuhl, and C. Bron. 1981. Immunochemical evidence for a transmembrane orientation of both the Na+–K+–ATPase subunits. Biochem. J. 200:6684–6691.

11. Good, P.J., R.C. Welch, A. Barkan, M.B. Somasekhar, and J.E. Mertz. 1988. Both VP2 and VP3 are synthesized from each of the alternatively spliced 198 RNA species of simian virus 40. J. Virol. 62:944–953.

12. Green, P.J., K. Richter, and I.B. Dawid. 1990. A nervous system-specific isoform of the β-subunit of Na+–K+–ATPase expressed during early development of Xenopus laevis. Proc. Natl. Acad. Sci. USA. 87:9888–9892.

13. Green, W.N., and T. Claudio. 1993. Acetylcholine receptor assembly: subunit folding and oligomerization occur sequentially. Cell. 74:57–69.

14. Helenius, A., T. Marquardt, and L. Braakman. 1992. The endoplasmic reticulum as a protein-folding compartment. Trends Cell Biol. 2:227–231.

15. Holmari, H., K. Kawai, K. Nagano, and H. Matsum. 1989. Localization of signal sequences for membrane insertion of the Na+, K+-ATPase α subunit. Mol. Cell. Biol. 9:5742–5745.

16. Jaisser, F., C.M. Canessa, J.D. Horisberger, and B.C. Rossier. 1992. Primary sequence and functional expression of a novel ouabain-resistant Na,K-ATPase: The β-subunit modulates potassium activation of the Na,K-pump. J. Biol. Chem. 267:10895–10903.

17. Jaisser, F., P. Jaunin, K. Geering, B.C. Rossier, and J.D. Horisberger. 1994. Modulation of the Na,K-pump function by β subunit isoforms. J. Gen. Physiol. 103:605–623.

18. Jaunin, P., J.D. Horisberger, K. Richter, P.J. Good, B.C. Rossier, and K. Geering. 1992. Processing, intracellular transport, and functional expression of endogenous and exogenous α-β Na,K-ATPase complexes in Xenopus oocytes. J. Biol. Chem. 267:577–585.

19. Jaunin, P., F. Jaisser, A.T. Beggah, K. Takeyasu, P. Mangeat, B.C. Rossier, J.D. Horisberger, and K. Geering. 1993. Role of the transmembrane and extracytoplasmic domain of β-subunits in subunit assembly, intracellular transport, and functional expression of Na,K-pumps. J. Cell Biol. 123:1751–1759.

20. Jørgensen, P.L., and J.P. Mertz. 1986. Structural basis for E2–E1 conformational transition in Na,K-pump and Ca-pump protein. J. Membr. Biol. 103:95–120.

21. Jørgensen, P.L., and R.A. Farley. 1988. Proteolytic cleavage as a tool for studying structure and conformation of pure membrane-bound Na+,K+-ATPase. Methods Enzymol. 156:291–301.

22. Karlish, S.J., D. Goldschleger, and P.L. Jorgensen. 1983. Location of the α subunit of Na,K-ATPase at the cytoplasmic surface. Implication for topological models. J. Biol. Chem. 268:3471–3478.

23. Kawakami, K., and K. Nagano. 1988. The transmembrane segment of the human Na,K-ATPase β-subunit acts as the membrane incorporation signal. J. Biol. Chem. 263:1054–1060.

24. Kolodziej, P.A., and R.A. Young. 1991. Epitope tagging and protein surveillance. Methods Enzymol. 194:508–519.

25. Lippincott-Schwartz, J., J.G. Donaldson, A. Schweizer, E.G. Berger, H.-P. Hauri, L.C. Yuan, and R.D. Klausner. 1989. Microtubule-dependent retrograde transport of proteins into the ER in presence of brefeldin A suggests an ER recycling pathway. Cell. 60:821–836.

26. Lutsenko, S, and J.H. Kaplan. 1994. Molecular events in close proximity to the membrane associated with the binding of ligands to the Na,K-ATPase. J. Biol. Chem. 269:4555–4564.

27. Melton, D.A., P.A. Krieg, M.R. Rebagliati, T. Maniatis, K. Zinn, and M.R. Green. 1984. Efficient in vitro synthesis of biologically active RNA and RNA hybridization probes from plasmids containing a bacteriophage SP6 promoter. Nucleic Acids Res. 12:7035–7056.

28. Nelson, R.M., and G.L. Long. 1989. A general method of site-specific mutagenesis using a modification of the Thermus aquaticus polymerase chain reaction. Anal. Biochem. 180:147–151.

29. Prakong-Zamofing, D., Q.H. Yi, G. Schmalzing, P. Good, and K. Geering. 1992. Regulation of α1-β3 Na+,K+-ATPase isoforme during maternal maturation of Xenopus laevis oocytes. Am. J. Physiol. 262:C1520–C1530.

30. Renaud, K.J., E.M. Inman, and D.M. Fambrough. 1991. Cytoplasmic and transmembrane domain deletions of Na,K-ATPase β-subunit: effects on subunit assembly and intracellular transport. J. Biol. Chem. 266:20491–20497.

31. Sanger, F., S. Nicklen, and A.R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA. 74:5463–5467.

32. Schoner, W., C. Von Ilberg, K. Nagano, and W. Seubert. 1967. On the mechanism of Na+– and K+–stimulated hydrolysis of adenosine triphosphate. I. Purification and properties of a Na+- and K+-activated ATPase from ox brain. Eur. J. Biochem. 3:343–345.

33. Schmalzing, G., S. Kroner, M. Schachner, and S. Gloo. 1992. The adhesion molecule on glia (AMOG82) and α1 subunits assemble to functional sodium pumps in Xenopus oocytes. J. Biol. Chem. 267:20212–20216.

34. Stainska, A., and S.J.D. Karlish. 1994. Evidence that the cation occlusion domain of Na,K-ATPase consists of a complex of membrane-spanning segments: analysis of limit membrane-embedded tryptic fragments. J. Biol. Chem. 269:10780–10789.

35. Stainska, A., and S.J.D. Karlish. 1996. Modulation of cation occlusion on Na,K-ATPase by the cytoplasmic domain of the β subunit. J. Biol. Chem. 271:10309–10316.

36. Verrey, F., P. Kairouz, E. Schaerer, P. Fuentes, K. Geering, B.C. Rossier, and J.P. Kraehenbuhl. 1989. Primary sequence of Xenopus laevis Na+,K+-ATPase and its localization in A6 kidney cells. Am. J. Physiol. 256:F1034–F1043.

37. Zamofing, D., B.C. Rossier, and K. Geering. 1989. Inhibition of N-glycosylation affects transepithelial Na+ but not Na+-K+-ATPase transport. Am. J. Physiol. 256:C985–C996.