Role of the Transmembrane and Extracytoplasmic Domain of β subunits in Subunit Assembly, Intracellular Transport, and Functional Expression of Na,K-pumps

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Abstract. The ubiquitous Na,K- and the gastric H,K-pumps are heterodimeric plasma membrane proteins composed of an α and a β subunit. The H,K-ATPase β subunit (βHK) can partially act as a surrogate for the Na,K-ATPase β subunit (βNK) in the formation of functional Na,K-pumps (Horisberger et al., 1991. *J. Biol. Chem.* 257:10338-10343). We have examined the role of the transmembrane and/or the ectodomain of βNK in (*a*) its ER retention in the absence of concomitant synthesis of Na,K-ATPase α subunits (αNK) and (*b*) the functional expression of Na,K-pumps at the cell surface and their activation by external K^+. We have constructed chimeric proteins between *Xenopus* βNK and rabbit βHK by exchanging their NH_2-terminal plus transmembrane domain with their COOH-terminal ectodomain (βNK/HK, βHK/NK). We have expressed these constructs with or without coexpression of αNK in the *Xenopus* oocyte. In the absence of αNK, *Xenopus* βNK and all chimera that contained the ectodomain of βNK were retained in the ER while βHK and all chimera with the ectodomain of βHK could leave the ER suggesting that ER retention of unassembled *Xenopus* βNK is mediated by a retention signal in the ectodomain.

When coexpressed with αNK, only βNK and βNK/HK chimera assembled efficiently with αNK leading to similar high expression of functional Na,K-pumps at the cell surface that exhibited, however, a different apparent K^+ affinity. βHK or chimera with the transmembrane domain of βHK assembled less efficiently with αNK leading to lower expression of functional Na,K-pumps with a different apparent K^+ affinity. The data indicate that the transmembrane domain of βNK is important for efficient assembly with αNK and that both the transmembrane and the ectodomain of β subunits play a role in modulating the transport activity of Na,K-pumps.

Na,K-ATPase is the molecular equivalent of the Na,K-pump that is responsible for the maintenance of the Na^+ and K^+ gradients existing between the intra- and extracellular milieu of animal cells (for review see Jørgensen and Andersen, 1988; Skou and Esmann, 1992). The minimal active enzyme unit is an α–β complex in which the catalytic properties and the binding domains for ATP and cations are associated with the α subunit. The catalytic α subunit is a large polypeptide that spans the membrane 8 to 10 times while the β subunit is a type II glycoprotein with a short cytoplasmic NH_2 terminus, a single transmembrane, and a large extracytoplasmic domain. Na,K-ATPase shares these structural and functional features with the gastric H,K-ATPase (for review see Wallmark et al., 1990), another member of the P-type ATPases that form an aspartyl phosphate intermediate during the catalytic cycle.

It is now well established that β subunits play a critical role in the posttranslational processing and the intracellular transport of the catalytic α subunits of Na,K-ATPase. Assembly of β subunits indeed permits the stabilization of newly synthesized α subunits (for review see Geering, 1991). In addition, as in other multimeric proteins, subunit oligomerization is needed for the ER exit of α subunits as well as of *Xenopus* β_1- and β_3-isoforms expressed in the *Xenopus* oocyte (Jaunin et al., 1992). Finally, recent experimental evidence suggests that β subunits might be modulators of the transport activity of Na,K-ATPase expressed at the plasma membrane (Eakle et al., 1992; Jaisser et al., 1992; Lutsenko and Kaplan, 1992; Schmalzing et al., 1992).

The structural determinants that govern the various properties of the β subunit are still poorly understood. In this study, we attempt to determine the importance of the trans-

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membrane and/or the ectodomain of β subunits for subunit assembly, for the ER retention of unassembled Na,K-ATPase β subunits, and for its participation in the pump function.

Recently, it has been reported that deletions of the COOH-terminus of β subunits abolish oligomerization with α-subunits (Renaud and Fambrough, 1991), whereas deletions of the cytoplasmic tail or important portions of the transmembrane domain do not (Renaud et al., 1991). Though these data suggest that the specific signals for subunit assembly might be associated with the ectodomain of the β subunit, the results do not definitely exclude a role of the transmembrane region in the oligomerization process. Indeed, the deletion approach has a low resolving power due to the possible perturbations of the correct folding of the resultant deletion mutant. To avoid this potential problem, we have chosen a chimera approach and have examined chimera between β subunits of *Xenopus* Na,K-ATPase, and rabbit H,K-ATPase. These two β-subunits are structurally and functionally very similar but sufficiently dissimilar to define large domains which could be of significance in subunit oligomerization. Indeed, β subunits of the two ATPases have a similar type II membrane topology, but the overall homology in the β subunits is only in the order of 35% (Reuben et al., 1990). Nevertheless, the β subunit of H,K-ATPase can act as a surrogate for the β subunit of Na,K-ATPase in the formation of functional Na,K-pumps though the efficiency of assembly with α subunits of Na,K-ATPase is much lower (Horisberger et al., 1991*b*).

In addition to the identification of certain assembly domains, the study of these oligomeric proteins was expected to also give some information on the domains that are implicated in ER retention of individual β subunits. Indeed, in contrast to *Xenopus* Na,K-ATPase β_1 and β_3 subunits, H,K-ATPase β subunits apparently do not need association with α subunits to leave the ER (Horisberger et al., 1991*b*).

The analysis of chimeric proteins between β subunits of *Xenopus* Na,K-ATPase and rabbit H,K-ATPase that were constructed by exchanging their NH_2-terminal plus transmembrane domain and their extracytoplasmic COOH-terminal domain, as well as the analysis of chimeric proteins with the transmembrane domain of the transferrin receptor (another type II glycoprotein) and the COOH-terminal domain of the β subunits of Na,K- or H,K-ATPase permitted us to draw several conclusions concerning the structure–function relationship in β subunits. Thus, the transmembrane domain of the β subunit plays an important role for efficient association with α subunits. In addition, the transmembrane and the ectodomain cooperate for the formation of functional Na,K-pumps with characteristic K^+ affinities. Finally, a signal for ER retention of unassembled *Xenopus* Na,K-ATPase β_1 subunits is localized in the extracytoplasmic domain of the polypeptide.

Materials and Methods

Construction of Chimeric β Subunits

To construct the chimera between the β subunit of *Xenopus* Na,K-ATPase (βNK)[1] and the β subunit of the rabbit gastric H,K-ATPase (βHK), we introduced a PvuII (384) restriction site in the cDNA of β_1NK (Verrey et al., 1989), at a position corresponding to an inherent PvuII (282) site in the cDNA of βHK (Reuben et al., 1990). This site-specific mutagenesis was done according to the PCR method described by Nelson and Long (1989). Mutagenic deoxynucleotides were synthesized with a DNA synthesizer (PCR-Mate; Applied Biosystems, Inc., Foster City, CA). First, using a linearized pSD5β_1NK (Jaunin et al., 1992) as a template, a DNA fragment was amplified between the sense oligonucleotide βNK (G_{372}-G_{398}), in which the sequence C_{382}GTGTT was mutated into C_{382}AGCTG (PvuII site), and the antisense deoxynucleotide consisting of primer D of Nelson and Long (1989) and βNK G_{647}-C_{628}. Second, the amplified fragments were used as primers to elongate the inverse DNA strand. Third, the elongated DNA strands that bear the design mutation were selectively amplified between the sense oligonucleotide pSD5 (T_{2709}-C_{2729}) and the antisense oligonucleotide D-Nelson (=GGGGTACTAGTAACCCGGGC). Finally, the amplified mutated DNA fragments were introduced into a wild-type pSD5βNK plasmid by using NheI and BamHI restriction sites. At the protein level, this mutation leads to the replacement of the amino acids Arg72 and Val73 by the amino acids Gln72 and Leu73, which are the corresponding amino acids of βHK. By using the created PvuII (384) site and a NheI (in the case of βNK/HK) or another PvuII site (in the case of βHK/NK) located on the pSD5 vector, we could excise DNA fragments corresponding to the NH_2-terminal and the transmembrane segments or to the extracytoplasmic COOH-terminal segment of βHK and replace them with the corresponding segments of the βNK (Fig. 1). The chimera βNK/HK is composed of the NH_2-terminal and the transmembrane segment of βNK and of the extracytoplasmic segment of βHK. The chimera βHK/NK is the inverse construction. The amino acid sequence of the wild-type βNK and βHK as well as of the chimera βNK/HK and βHK/NK in the mutated segment is shown

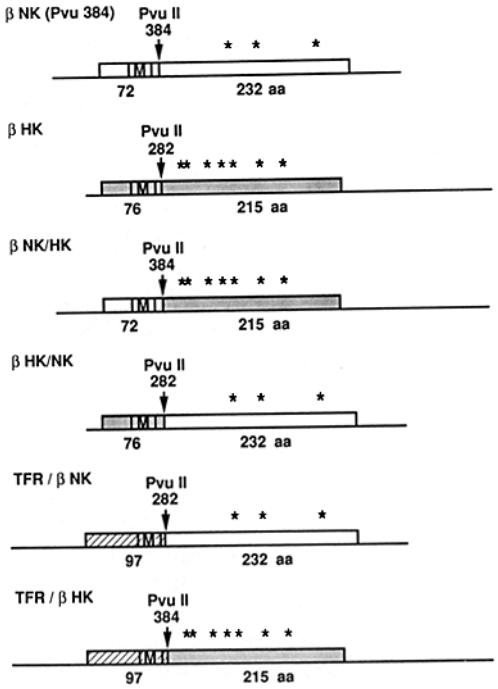

Figure 1. Linear models of the chimera βNK/HK, βHK/NK, TFR/βNK, and TFR/βHK. The coding regions are represented by segments, and the noncoding regions are represented by lines. White segments originate from *Xenopus* Na,K-ATPase β_1 subunits (βNK), grey segments from gastric H,K-ATPase β subunits (βHK), and hatched segments from transferrin receptors (TFR). *M* refers to the transmembrane segment and asterices refer to putative glycosylation sites in the extracytoplasmic domain. The localization of the PvuII restriction site used to construct the different chimera is indicated by an *arrow*. Below each figure, the number of amino acids from the NH_2-terminal to the PvuII site, and the number of amino acids from the PvuII site to the COOH-terminal are indicated.

1. *Abbreviations used in this paper*: αNK, Na,K-ATPase α subunit; βHK, H,K-ATPase β subunit; βNK, Na,K-ATPase β subunit; DWB, digitonin-washing buffer; TFR, transferrin receptor.

in Fig. 2. To construct the chimera between the human transferrin receptor (TFR, obtained from American Type Culture Collection, Rockville, MD) and the β subunit of the rabbit H,K-ATPase (TFR/βHK), a PvuII restriction site was created by PCR at the encoding position Thr95-Gln96 in the TFR. The nucleotide sequence encoding the Met 1 to Gln 96 of the TFR was amplified by PCR using 50 ng each of oligonucleotide primers 5'-CCAAGCTTGGCCGCGGGTCGACGGA3' (for polylinker portion) and 5'-CTGAGTTTTTGGTTCTACCCC-3' (a part of newly created Pvu II site is underlined) and TFR cDNA (50 ng) in pSVDF (Takeyasu et al., 1987). The chimeric TFR/βHK cDNA was constructed by substituting the 5'-region of the wild-type βHK (inserted in pSD5 vector) by the TFR fragment amplified, using the PvuII site as an exchange junction. To construct the chimera between the human TFR and the β_1 subunit of *Xenopus* Na,K-ATPase (TFR/ βNK), the TFR DNA fragment of the chimera TFR/βHK was excised, using NheI (in pSD5 DNA) and PvuII (junction site), and exchanged against the βHK fragment of the chimera βHK/NK. The PCR fragment sequences were confirmed for all chimera by dideoxysequencing (Sanger et al., 1977).

In Vitro Translation, Expression in Xenopus Oocytes and Immunoprecipitation of Chimera

cDNAs from α_1 and β_1 subunits of the *Xenopus* Na,K-ATPase (Verrey et al., 1989) and from β subunits of the rabbit gastric H,K-ATPase (Reuben et al., 1990) of the H,K-ATPase were recloned into the plasmid pSD5 which allows for synthesis of capped, full-length, poly(A)$^+$ cRNA (Good et al., 1988). cRNAs were obtained by in vitro transcription of linearized templates with SP6-RNA-polymerase according to Melton et al. (1984). In vitro translation in a reticulocyte lysate was done as previously described (Geering et al., 1985). All cRNAs were efficiently translated in vitro, and the core proteins exhibited the expected molecular mass (Fig. 3). To test their immunoreactivity, the various proteins were immunoprecipitated with specific antibodies as previously described (Geering et al., 1982, 1985). αNK were immunoprecipitated with a polyclonal antiserum prepared against the purified α subunit from *Bufo marinus* (Girardet et al., 1981) which cross-reacts with the *Xenopus* α subunit (Geering et al., 1985, 1989). This antibody also detects the endogenous oocyte α subunit. Immunoprecipitation of βNK was performed with an antibody prepared against the purified β subunit from *Xenopus* kidney (Paccolat et al., 1987) that does not cross-react with βHK. βHK was immunoprecipitated by mAb that do not cross-react with βNK, either by a clone 2/2 E6 (kindly provided by J. G. Forte, University of California, Berkeley, CA) or 146.14 (Mercier et al., 1989). This latter antibody was used in immunoprecipitations under nondenaturing conditions (see below), and protein G–Sepharose-CL-4B (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ), rather than protein A–Sepharose, was used for absorption of the antigen–antibody complex. The chimera TFR/βNK or TFR/βHK were immunoprecipitated with anti-

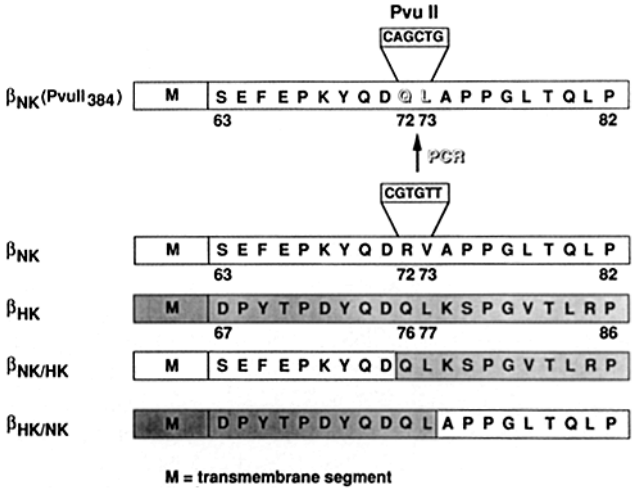

Figure 2. Amino acid sequence of βNK (PvuII 384), wild-type βNK, βHK, and the chimera βNK/HK and βHK/NK in the mutated segment. Introduction of a PvuII site into βNK did not change but shifted the amino acid sequence in the chimera. *Open frame*, amino acids originating from βNK; *shaded frame*, amino acids originating from βHK; *M*, transmembrane segment.

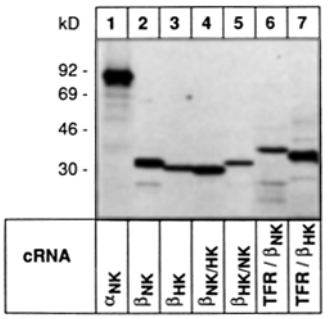

Figure 3. Expression of αNK, and of the chimera βNK/HK, βHK/NK, TFR/βNK, and TFR/βHK in a reticulocyte lysate. 200 ng of cRNA coding for αNK (lane *1*), βNK (lane *2*), βHK (lane *3*), and for the chimera βNK/HK (lane *4*), βHK/NK (lane *5*), TFR/βNK (lane *6*), and TFR/βHK (lane *7*), were translated in a reticulocyte lysate in the presence of [^{35}S]methionine as described in Materials and Methods. The samples were then submitted to SDS-PAGE and the translated proteins were revealed by fluorography (see Materials and Methods).

βNK- or anti-βHK-antibodies, respectively. The immunoprecipitations revealed that the chimera βNK/HK reacts with the anti-βHK antibodies, but not with the anti-βNK antibodies. On the contrary, the chimera βHK/NK was immunoprecipitated by the anti-βNK antibodies, but not by the anti-βHK antibody. Moreover, we observed that the mAb 2/2 E6 against the βHK showed a decreased efficiency of immunoprecipitation compared to the polyclonal antibodies against the Na,K-ATPase subunits (data not shown). No immunoreactivity was observed with different preimmune sera. None of the β antibodies cross-reacts with the endogenous oocyte β_3 subunits (Jaunin et al., 1992).

Stage V-VI oocytes were obtained from *Xenopus* females (Noordhoek, Republic of South Africa) as described (Geering et al., 1989). In preliminary experiments, we determined the amount of cRNA to be injected into oocytes in order to obtain a similar expression and/or a similar signal in immunoprecipitations of the different β-subunits. Because of the poor immunoreactivity of the mAb 2/2 E6 (see above), we routinely injected 3–5 times more βHK or βNK/HK cRNA than βNK or βHK/NK cRNA. It was confirmed that the different amounts of cRNA were not responsible for the difference in the cellular accumulation of α subunits observed with the different β subunits. Noninjected or cRNA-injected *Xenopus* oocytes were incubated in modified Barth's medium containing 2–3 mCi/ml of [^{35}S]methionine (Amersham Corp., Arlington Heights, IL) for 4 or 16 h at 19°C, and subjected to 16–72-h chase periods in modified Barth's medium plus 10 mM cold methionine. After the chase period, oocytes were either extracted with Triton X-100 or digitonin. Triton extracts were obtained as described (Jaunin et al., 1992). Digitonin extracts were essentially prepared as described by Schmalzing et al. (1992). 30 μl/oocyte of a solution containing 100 mM NaCl, 20 mM Tris-HCl (pH 7.6), 10 mM methionine, 0.5% (wt/vol) digitonin (water soluble; Fluka Chemie AG, Buchs, Switzerland), 1 mM PMSF, and 5 μg/ml of each leupeptin, pepstatin, and antipain were added. After vortexing with intermittent cooling, the extracts were incubated 10 min on ice and further processed as for Triton extracts.

Immunoprecipitations of Triton extracts denatured with SDS (final concentration 3.7%), SDS-PAGE fluorography and laser densitometry were performed as previously described (Geering et al., 1982, 1985). Immunoprecipitations of digitonin extracts in nondenaturating conditions were performed as follows: the samples were diluted to a volume of 400 μl with DWB buffer (digitonin-washing buffer: 100 mM NaCl, 1 mM EDTA, 20 mM Tris-HCl, pH 7.4, 0,2% digitonin), then 2 mM (final concentration) phenylmethylsulfonyl fluoride and 2% (final concentration) BSA were added. After overnight incubation at 4°C with the antibody, the immunocomplexes were recovered on protein-A or protein-G Sepharose beads and washed six times with DWB and once with DWB without digitonin. In some instances, immunoprecipitated β subunits were subjected to endoglycosidase H (Endo H; Calbiochem-Novabiochem Corp., La Jolla, CA) treatment as described (Jaunin et al., 1992).

As previously established, *Xenopus* oocytes express excess endogenous α subunits over endogenous β_3 subunits (Jaunin et al., 1992). Expression of exogenous Na,K-ATPase subunits does not influence the biosynthesis of the endogenous subunits. In addition, endogenous subunits only minimally contribute to the formation of newly synthesized α–β complexes since expression of exogenous subunits is in a large excess.

Cell Fractionation of Xenopus Oocytes on Nondenaturing Sucrose Gradients

Yolk-depleted digitonin extracts (see above) were loaded on a 9.8-ml linear

sucrose gradient (5–30%, wt/vol), in 100 mM NaCl, 0.25% (wt/vol) digitonin, and 20 mM Tris–HCl, pH 7.6, and centrifuged in a SW50Ti rotor for 18 h at 40,000 rpm at 20°C. 18 fractions of 600 μl were collected from the bottom of the tubes, and aliquots were subjected to immunoprecipitations under nondenaturing conditions (see above).

Ouabain Binding, Pump Current Measurements and Determination of Apparent K^+ Affinities

Ouabain binding was essentially done as previously described (Jaunin et al., 1992). Na,K-pump activity was measured in Na^+-loaded oocytes as the outward current activated by addition of K^+ (Horisberger et al., 1991*a*). The activation of the Na,K-pump by external K^+ was studied in a Na^+ free medium as described by Rakowski et al. (1991). We measured the current induced by a stepwise increase of the K^+ concentration from 0 to 0.02, 0.1, 0.5, and 5 mM in a solution containing (mM): Ca^{2+}, 0.41; Mg^{2+}, 0.82; Ba^{2+}, 5; TEA^+, 10; Cl^-, 22.5; and sucrose, 140. Maximal currents (I_{max}) and the half-activation constants of K^+ ($K_{1/2}$) were determined as described (Horisberger et al., 1991*a*).

Results

Involvement of the Transmembrane and/or Ectodomain of the β Subunit in the Assembly with Na,K-ATPase α Subunits

We have previously shown that *Xenopus* βNK and, to a lesser extent, rabbit gastric βHK, synthesized in the *Xenopus* oocyte from injected cRNA, can assemble with and stabilize coexpressed *Xenopus* αNK leading to a proportional increase in the cellular accumulation of αNK (Horisberger et al., 1991*b*). As illustrated in Fig. 4 *A*, αNK expressed alone in the *Xenopus* oocyte is indeed degraded within a 72-h chase (Fig. 4 *A*, lane *1*) but can be stabilized by coexpression of βNK (Fig. 4 *A*, lane 2) and to a lesser extent by βHK (Fig. 4, lanes *3*). The difference in the association competence of the two β subunits prompted us to produce chimeric proteins between the two β subunits (Fig. 1) to assess the respective importance of the transmembrane and/or the ectodomain in the efficient assembly of βNK with αNK. Expression in oocytes of the βNK/HK with the transmembrane domain of βNK and the ectodomain of βHK (Fig. 1) led to a higher accumulation of α subunits in the oocyte than βHK (Fig. 4 *A*, compare lanes *4* and *5* to lane *3*) and had a similar stabilizing effect on the α subunit than βNK wild type (Fig. 4 *A*, compare lane *4* to lane 2). On the other hand, the chimera βHK/NK with the transmembrane domain of βHK and the ectodomain of βNK showed a lower association efficiency similar to βHK (Fig. 4 *A*, compare lane 5 to lane 2). The level of expression of the α subunit obtained with the different β subunits at the protein level was closely paralleled by the number of functional pumps assessed at the cell surface by ouabain binding and pump current measurements (see below and Table I).

Direct proof for association of the different β subunits with αNK could be obtained by immunoprecipitations performed under nondenaturing conditions (Fig. 4 *B*). From digitonin extracts of pulse-chase-labeled oocytes, previously injected with αNK and different β cRNAs, an anti αNK-serum coprecipitated βNK wild type (Fig. 4 *B*, lane 2) or βHK/NK (Fig. 4 β, lane *5*) with α subunits, and a monoclonal βHK-antibody coprecipitated α subunits with βHK (Fig. 4 *B*, lane *3*) or βNK/HK (Fig. 4 *B*, lane *4*). Since the gel migration of β subunits is mainly determined by the number of sugar chains, the chimera βHK/NK with the extracytoplasmic domain of βNK exhibits a similar molecular mass to βNK, while the chimera βNK/HK with the extracytoplasmic domain of βHK (see Fig. 1) exhibits a similar molecular mass to βHK. In contrast to αNK–βNK, αNK–βHK, or αNK–βNK/HK complexes, which are mainly found in their fully glycosylated form (Fig. 4 *B*, lanes *2–4*), a significant fraction of αNK–βHK/NK complexes are still in their coreglycosylated ER form after a 24-h chase (Fig. 4 *B*, lane *5*), indicating that the transport of these latter complexes from the ER to a distal Golgi compartment, where full glycosylation occurs, is slowed down.

In conclusion, the data obtained with the chimera between βNK and βHK indicate that the integrity of the NH_2-

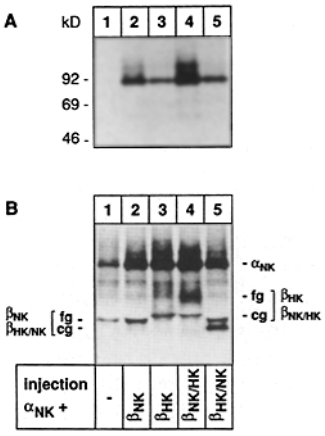

Figure 4. Cellular accumulation of αNK after assembly with βNK, βHK, or with chimera βNK/HK and βHK/NK. (*A*) Cellular accumulation of αNK subunits. Oocytes were injected with 5 ng of αNK cRNA alone (lane *1*); 5 ng of αNK cRNA and 0.3 ng of βNK cRNA (lane *2*); 1 ng of βHK cRNA (lane *3*); 1 ng of βNK/HK cRNA (lane *4*); or 0.3 ng of βHK/NK cRNA (lane *5*). After a 16-h pulse with [^{35}S]*methionine* (2 mCi/ml) and a chase period of 72 h, Triton extracts were prepared and αNK subunits were immunoprecipitated from aliquots containing 10^6 cpm with a polyclonal anti-αNK serum as described in Materials and Methods. Similar results were obtained when equal amounts of cRNA for the different β subunits were injected (see Materials and Methods). Compared to βHK, 3.4 ± 0.6, 3.8 ± 0.6, and 1.3 ± 0.2 times more α-subunits accumulated with βNK, βNK/HK, and βHK/NK, respectively (means ± SE, n = 6). (*B*) Assembly of αNK subunits with βHK, βNK/HK, and βHK/NK subunits. Oocytes were injected with 5 ng of αNK cRNA alone (lane *1*); 5 ng of αNK cRNA and 0.2 ng of βNK cRNA (lane *2*); 1 ng of βHK cRNA (lane *3*); 1 ng of βNK/HK cRNA (lane *4*); or 0.2 ng of βHK/NK cRNA (lane *5*). After a 5-h pulse with [^{35}S]methionine (2 mCi/ml) and a chase period of 24 h, digitonin extracts were prepared and the α–β heterodimers were immunoprecipitated under nondenaturing conditions, either with a polyclonal anti-αNK serum (lanes *1*, *2*, and *5*) or with a monoclonal anti-βHK antibody (146.14) (lanes *3* and *4*), as described in Materials and Methods. *cg*, coreglycosylated and *fg*, fully glycosylated forms of β subunits. The estimated molecular mass of the coreglycosylated forms (Endo H sensitive) is ~42 kD for βNK and βHK/NK and ~49 kD for βHK and βNK/HK. The molecular mass of the fully glycosylated forms (Endo H resistant) is ~46 kD for βNK and βHK/NK and 56–64 kD for βHK and βNK/HK. The intensities of the α-subunits cannot be compared in this experiment because of the use of different antibodies. The origin of the band seen in lane *1* at the βNK position is not clearly identified. We have previously shown that β_1-subunit expression is not detectable in *Xenopus* oocytes (Jaunin et al., 1992). On the other hand, a band with similar migration is obtained with preimmune serum and with an anti-action serum (data not shown) suggesting that this band might represent artifactual immunoprecipitation of actin or of actin associated with α subunits. Most likely it represents an artifact produced at the migration front of the light chains of the antibodies.

terminal transmembrane of βNK are important for efficient assembly with and stabilization of αNK.

Involvement of the Transmembrane and/or Ectodomain in Intracellular Transport of β Subunits

We have previously shown that in *Xenopus* oocytes, exogenous *Xenopus* βNK are retained in the ER if they are not associated with α subunits (Ackermann and Geering, 1990, Jaunin et al., 1992). In contrast, βHK becomes fully glycosylated even in the absence of concomitant α subunit synthesis (Horisberger et al., 1991*b*) suggesting that ER exit and transport to a distal Golgi compartment do not depend on assembly with α subunits. To substantiate this finding, we fractionated *Xenopus* oocytes expressing βHK alone or together with αNK on sucrose gradients under nondenaturing conditions and analyzed the association of βHK with αNK and/or with other proteins. When oocytes coexpressed αNK and βHK, antibodies against βHK immunoprecipitated αNK–βHK complexes from heavy sucrose fractions (Fig. 5 *A*, lanes *1* and *2*). The βHK in these complexes was in its coreglycosylated form, indicating that the complexes are mainly derived from the ER. The ratio of the intensities between the α and β bands was close to 3 as expected from the number of methionines available for biosynthetic labeling with [^{35}S]methionine in the *Xenopus* αNK (26 methionines) and the rabbit βHK (8 methionines) indicating that all coreglycosylated βHK detected in these fractions are associated with αNK. The same stoichiometry was not obtained in immunoprecipitations of lighter fractions (Fig. 5 *A*, lanes *3–5*). Only a minor proportion of the mainly fully glycosylated βHK species was associated with αNK in these fractions. In addition, no other prominent protein coprecipitated with βHK in the same fractions. These data indeed support the idea that full glycosylation and thus ER exit of βHK does not necessarily imply association with another protein. This finding is further supported by the fact that βHK expressed alone in the oocyte was also mainly found in its fully glycosylated form though only minor amounts of endogenous oocyte α subunits and no other proteins coprecipitated with the βHK (Fig. 5 *B*, lanes *1–6*).

To identify the structural domains that might be responsible for the differential behavior of βNK and βHK, we analyzed the transport competence of chimeric βNK–βHK constructs. When expressed together with αNK, βNK, βHK, and the chimera βNK/HK and βHK/NK were, as expected, mainly found in their corresponding fully glycosylated form after a 72-h chase (Fig. 6, lanes *1–4*). Significantly, when these β subunits were expressed alone, the chimera βHK/NK behaved like βNK and was recovered predominantly in the coreglycosylated ER form after a 24-h or longer chase, while the chimera βNK/HK was processed at least in part to the fully glycosylated form indicating that ER retention of unassembled *Xenopus* βNK in oocytes is primarily determined by the extracytoplasmic domain.

To further substantiate our two main findings, namely that ER retention of unassembled *Xenopus* βNK is governed by the ectodomain, and that efficient assembly of βNK with αNK is significantly influenced by the transmembrane domain of βNK, we tested, in a last set of experiments, the assembly and transport competence of chimeric constructs composed of the NH_2-terminal and the transmembrane segment of the transferrin receptor and the extracytoplasmic domain of either βNK (TFR/βNK) or βHK (TFR/βHK) (Fig. 1). In comparison to βNK, TFR/βNK only weakly assembled with αNK and did not lead to a significant increase in the cellular accumulation of coexpressed αNK (Fig. 7 *A*, lanes *1–3*). In addition, the chimera TFR/βHK, in which the transmembrane domain of βHK is replaced by the one of the transferrin receptor, stabilized the α subunit even less than βHK (Fig. 7 *B*, lanes *1–3*). No coprecipitation of αNK with TFR/βNK or TFR/βHK could be observed in nondenaturing immunoprecipitations (data not shown). These results further emphasize the importance of the transmembrane segment in βNK for proper assembly with αNK.

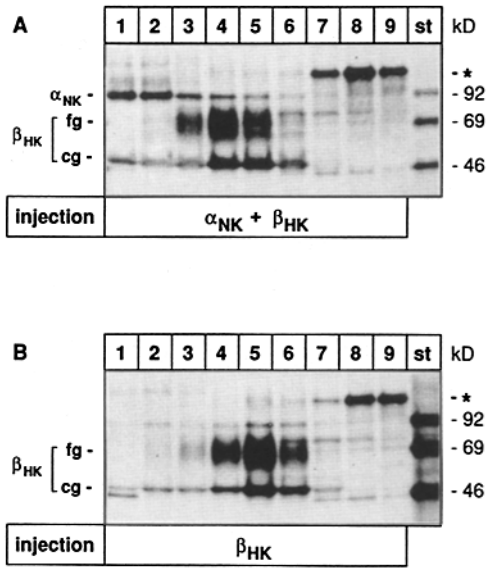

Figure 5. Intracellular transport of βHK is independent on the association with α subunits. *Xenopus* oocytes were injected with 10 ng of αNK cRNA and 3 ng of βHK cRNA (*A*) or with 3 ng of βHK cRNA alone (*B*). After a 5-h pulse with [^{35}S]methionine (2.5 mCi/ml) and a 24-h chase, digitonin extracts were prepared as described in Materials and Methods. Aliquots were loaded on linear sucrose gradients containing 0.25% digitonin and centrifuged as described in Materials and Methods. Immunoprecipitations under nondenaturing conditions were performed on 18 fractions collected from the bottom of the gradient with the βHK-antibody 146.14. Shown are immunoprecipitations of nine fractions with sucrose densities ranging from 1.127 to 1.068. No signal was obtained in the other fractions of lower densities. *cg*, coreglycosylated; *fg*, fully glycosylated forms of βHK. In fractions 7 to 9 a high molecular mass band (asterisk) was consistently immunoprecipitated. The origin of this band is unknown but could represent trimers of coreglycosylated βHK that cannot be dissociated by SDS treatment.

The study of these chimeric proteins also confirms that ER retention of unassembled *Xenopus* βNK is likely to be mediated by a signal located in the extracytoplasmic domain. Compared to βNK that becomes fully glycosylated in the presence (Fig. 7 *A*, lane *7*) and remains coreglycosylated in the absence (Fig. 7 *A*, lane *9*) of αNK, TFR/βNK is exclusively found in its coreglycosylated ER form both in the presence (Fig. 7 *A*, lane *8*) and absence (Fig. 7 *A*, lane *10*) of αNK. On the other hand, similar to βHK (Fig. 7 *B*, lanes *7* and *9*), TFR/βHK appears in its fully glycosylated form both in the presence (Fig. 7 *B*, lane *8*) or absence (Fig. 7 *B*, lane *10*) of αNK. Clearly, the presence of the extracytoplasmic domain of *Xenopus* βNK is responsible for the inability

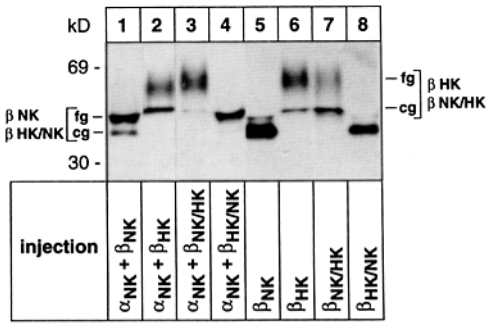

Figure 6. Posttranslational processing of βHK and of the chimera βNK/HK and βHK/NK in the presence or absence of αNK. Oocytes were injected with 0.3 ng of βNK cRNA (lanes *1* and *5*); 1 ng of βHK cRNA (lanes *2* and *6*); 1 ng of βNK/HK cRNA (lanes *3* and *7*); or 0.3 ng of βHK/NK cRNA (lanes *4* and *8*); either alone (lanes *5–8*) or together with 5 ng of αNK cRNA (lanes *1–4*). After a 16-h pulse (lanes *1–4*) or a 4-h pulse (lanes *5–8*) with [^{35}S]methionine (2 or 3 mCi/ml, respectively), and a chase period of 72 h (lanes *1–4*) or 24 h (lanes *5–8*), Triton extracts were prepared, and the β subunits were immunoprecipitated with a polyclonal anti-βNK serum (lanes *1*, *4*, *5*, and *8*) or with the monoclonal anti-βHK antibody 2/2 E6 (lanes *2*, *3*, *6*, and *7*) as described in Materials and Methods. The β subunits synthesized in the presence of α subunits (lanes *1–4*), and α subunits shown in Fig. 4 *A* (lanes *2–4*) were immunoprecipitated from Triton extracts of the same batch of oocytes.

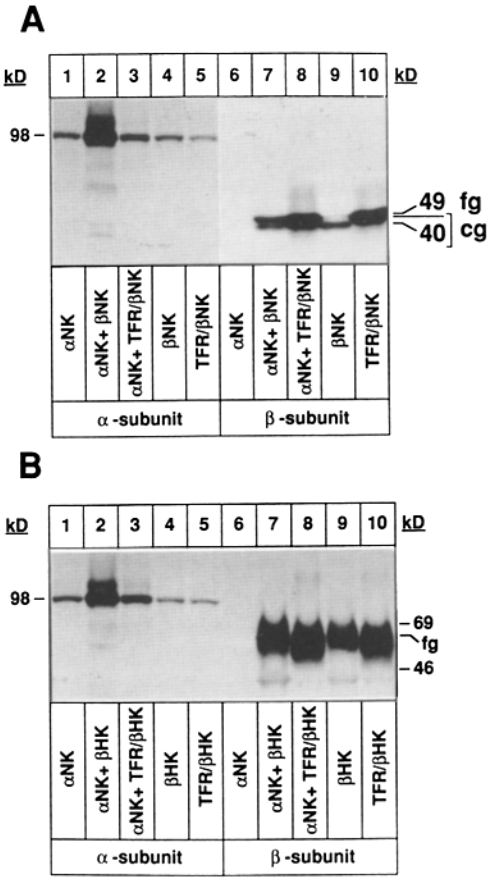

Figure 7. Intracellular transport and assembly of chimera TFR/βNK and TFR/βHK with αNK. (*A*) *Xenopus* oocytes were injected with 5 ng of αNK cRNA alone (lanes *1* and *6*); 5 ng of αNK cRNA and 0.1 ng of βNK cRNA (lanes *2* and *7*); 5 ng of αNK cRNA and 3 ng of TFR/βNK cRNA (lanes *3* and *8*); 0.1 ng of βNK cRNA (lanes *4* and *9*); or 3 ng TFR/βNK cRNA (lanes *5* and *10*) alone. After a 5-h pulse with [^{35}S]methionine (2.5 mCi/ml) and a chase of 24 h, Triton extracts were prepared and immunoprecipitations of α subunits (lanes *1–5*) or β subunits (lanes *6–10*) were performed under denaturing conditions on aliquots containing 5×10^5 cpm as described in Materials and Methods. βNK and TFR/βNK were immunoprecipitated with anti-βNK antibodies. TFR/βNK synthesized in the presence (lane *8*) or absence (lane *10*) of αNK is sensitive to Endo H treatment and thus represent the coreglycosylated ER form. (*B*) Same experiment as *A* but 5 ng of βHK cRNA (lanes *2*, *4*, *7*, and *9*) or 3 ng of TFR/βHK (lanes *3*, *5*, *8*, and *10*) cRNA were injected into oocytes either alone or in the presence of 5 ng of αNK cRNA. *cg*, Coreglycosylated; *fg*, fully glycosylated forms of β-subunits. βHK and TFR/βHK were immunoprecipitated with the mAb 2/2 E6. βHK or TFR/βHK synthesized in the presence (lanes *7* and *8*) or absence (lanes *9* and *10*) of αNK are resistant to Endo H treatment and thus represent the fully glycosylated forms.

of the β subunits to leave the ER of *Xenopus* oocytes if they are not assembled with α subunits.

Functional Expression of αNK-chimeric β Complexes

To further substantiate our findings on the assembly competence of chimeric β subunits, we finally analyzed the expression and the Na,K-pump activity of the different α–β complexes at the cell surface. As assessed by ouabain binding, the number of different α–β complexes increased in proportion to the previously established association efficiency of the various β subunits (compare Fig. 4 *A* with Fig. 8 *A*). Compared to noninjected controls, the highest increase in Na,K-pump activity is indeed observed in oocytes expressing αNK and βNK or βNK/HK and the lowest increase in oocytes expressing αNK and βHK (Fig. 8 *A*). When Na,K-pump current was measured in the same batch of oocytes under V_{max} conditions (5 mM K^+), the relative increase in the number of the different α–β complexes was closely paralleled by a similar increase in the maximal Na,K-pump current (Fig. 8 *A*). The data fall on a straight line with a slope close to 1 indicating that under the experimental conditions used, all α–β complexes have similar maximal transport rates.

Recent electrophysiological studies performed on *Xenopus* oocytes expressing exogenous α–β complexes have shown that Na,K-pumps composed of *Bufo* αNK and rabbit gastric βHK have a lower apparent affinity for K^+ than Na,K-pumps composed of Bufo αNK and Bufo βNK (Jaisser, F., P. Jaunin, K. Geering, B. C. Rossier, and J. D. Horisberger; manuscript submitted for publication). These data indicate that β subunits have a significant influence on the ion transport activity of Na,K-pumps. In a preliminary attempt to identify structural domains that might define the inherent characteristics of βNK and βHK, we co-expressed *Xenopus* αNK with βNK, βHK, or the chimeric β subunits in *Xenopus* oocytes and measured the apparent K^+ affinity of the different α–β complexes.

In agreement with previous observations (Jaisser, F., P. Jaunin, K. Geering, B. C. Rossier, and J. D. Horisberger; manuscript submitted for publication), endogenous oocyte Na,K-pumps and exogenous αNK–βHK complexes have a lower apparent affinity for K^+ than exogenous αNK–βNK

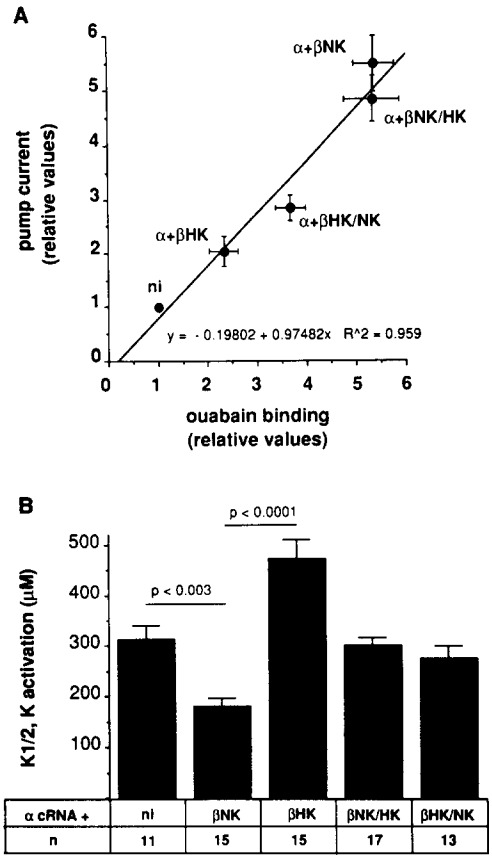

Figure 8. Cell surface expression and activation of Na,K-pump current by external K^+ in oocytes expressing αNK-βNK, αNK-βHK, αNK-βNK/HK, or αNK/βHK/NK complexes. Oocytes were injected with 5 ng of αNK cRNA and 0.3 ng of βNK cRNA; 1 ng of βHK cRNA, 0.3 ng of βNK/HK cRNA; 1 ng of βHK/NK cRNA; or were not injected (*ni*) and incubated for 5 d at 19°C. (*A*) Ouabain binding and pump current. Ouabain binding was determined as described in Materials and Methods on oocytes of two different animals (n = 14–20). On the same batches of oocytes, K^+-induced pump current was determined as described in Materials and Methods (n = 6–12). The graph represents the pump current as a function of ouabain binding. Ouabain binding and pump currents measured in noninjected oocytes were arbitrarily set to one. Noninjected oocytes bound 14.9 ± 0.6 fmol of ouabain per oocyte (n = 20) and produced a pump current of 71.8 ± 6.0 nA per oocyte (n = 6). (*B*) Measurements of the half maximal activation constant. The half-maximal activation constant ($K_{1/2}$) was obtained as described in Materials and Methods. Values are the mean ± S.E. and n is the number of observations. Unpaired Student's t test: all different α–β complexes show significantly different apparent K^+ affinities except α-βNK/HK and α-βHK/NK complexes.

complexes (Fig. 8 *B*). Significantly, the α-βNK/HK or the α-βHK/NK complexes exhibited neither the low nor the high apparent $K_{1/2}$ of K^+ stimulation of α-βNK or α-βHK complexes, respectively, but showed an intermediate K^+ activation (Fig. 8 *B*).

Thus these data demonstrate that β subunits influence the K^+ activation of Na,K-pumps. In addition, the study of the K^+ activation of α–β complexes composed of αNK and chimera between βNK and βHK reveals that structural changes both in the transmembrane and ectodomain of the β subunits can significantly influence the K^+ activation of Na,K-pumps.

Table I. Summary of the Properties of βNK, βHK, and Chimera βNK/HK and βHK/NK

	I Assembly efficiency	II Na,K-pumps (cell surface)	III K1/2	IV ER retention
			μM	
βNK	+++	+++	180	Yes
βHK	+	+	480	No
βNK/HK	+++	+++	300	No
βHK/NK	+	++	280	Yes

I: Efficiency of assembly with α subunits of Na,K-ATPase; II: Number of Na,K-pumps expressed at the cell surface as assessed by ouabain binding; III: Apparent K^+ affinity ($K_{1/2}$) of α–β complexes expressed at the cell surface; IV: Retention of unassembled β subunits in the ER. For further details see text.

Discussion

In the present study we have used a chimera approach to characterize the importance of the transmembrane and/or the ectodomain of the β subunit of Na,K-ATPase for (*a*) the assembly with α subunits; (*b*) the formation of functional Na,K-pumps at the cell surface; (*c*) the β subunit defined apparent K^+ affinity of cell surface expressed α–β complexes; and (*d*) the ER retention of unassembled β subunits. The data are summarized in Table I.

The Transmembrane Domain of the Na,K-ATPase β Subunit Is Important for Efficient Assembly with α Subunits

Previous studies have shown that heterologous assembly of αNK with βHK is possible (Horisberger et al., 1991*b*; Eakle et al., 1992; Noguchi et al., 1992), but that assembly is less efficient than with βNK (Horisberger et al., 1991*b*). This observation prompted us to produce chimera between the two β subunits in the hope of identifying structural domains that are important for subunit assembly. Our data show that the exchange of the NH_2-terminal transmembrane domain and the COOH-terminal extracytoplasmic domain between the two β subunits influences the assembly efficiency with αNK (Table I).

Indeed, according to our results obtained with the chimeric β subunits, assembly of βNK with αNK seems to be significantly affected by the transmembrane domain. In addition, the results obtained with chimeric proteins in which the transmembrane region of βNK and βHK is replaced by the transmembrane region of another type II glycoprotein, the transferrin receptor, are also in favor of an involvement of the transmembrane domain in assembly of β subunits with α subunits.

Apparently, these data are in contradiction with recently published data that show that deletions of the cytoplasmic NH_2-terminal and of up to 11 amino acids of the transmembrane region of βNK still permit the formation of α–β complexes (Renaud et al., 1991). In addition, recent observations indeed point to an important role of the extracytoplasmic domain of the β subunit in assembly. First, mutation of a conserved proline residue in the ectodomain of βNK abolishes subunit assembly, probably by preventing a proper assembly competent folding of the extracytoplasmic domain (Geering et al., manuscript in press). Second, short deletions of the ectoplasmic COOH terminus of βNK abolishes subunit as-

sembly (Renaud and Fambrough, 1991). More generally, many oligomeric proteins appear to assemble via their ectodomains (for review see Hurtley and Helenius, 1989). Verrall and Hall (1992) conclude from this finding that interactions between hydrophobic transmembrane regions might be too weak and not specific enough to permit highly selective subunit assembly. However, Cosson and Bonifacino (1992) recently provided evidence that α and β chains of class II major histocompatibility complexes interact via glycine residues in the putative α helices of the transmembrane domains.

The most likely explanation to reconcile the observations made in this and other studies is that a certain cross talk exists between the transmembrane domains and the ectodomains of subunits during oligomerization. It is possible that the transmembrane and/or a closely adjacent region participate in the adoption of a correct assembly competent configuration of the ectodomain. On the basis of results obtained on the oligomerization of type II glycoproteins, Kundu et al. (1991) also suggested that the formation of stable oligomers might only be possible after initial assembly of the ectodomains of the subunits and a further interaction among the transmembrane regions. Alternatively, an initial interaction between the transmembrane regions could be needed to bring the two subunits close to each other and to permit stable interaction of the ectodomains.

The Ectodomain of the β_1 Subunits of Xenopus Na,K-ATPase Contains a Signal for ER Retention in the Xenopus Oocyte

ER retention of unassembled subunits of oligomeric proteins is a common mechanism and part of the cellular quality control. Many misfolded or unassembled proteins have been found to be degraded, to aggregate, or to be bound to heavy chain binding protein (Bip) (for review see Pelham, 1989). The α subunit of Na,K-ATPase is subjected to a similar control in that newly synthesized α subunits that are not associated with β subunits accumulate in the ER (Takeyasu et al., 1988; Jaunin et al., 1992) where they are eventually degraded (Ackermann and Geering, 1990). In this study, we provide evidence that the β subunits may or may not follow this rule and that ER retention appears to be determined by specific structural characteristics of the protein. In *Xenopus* oocytes, exogenous β_1 and β_3 subunits of *Xenopus* Na,K-ATPase indeed cannot leave the ER in the absence of concomitant synthesis of α subunits (Ackermann et al., 1990; Jaunin et al., 1992) while β subunits of the mammalian gastric H,K-ATPase are transported through the secretory pathway without association with α subunits or with another protein (Table I). The βHK in *Xenopus* oocytes behaves in this respect similar to overexpressed rat βHK in insect cells (Martin and Mangeat, unpublished data) or overexpressed chicken βNK that were found at the cell surface of transfected mouse L cells (Takeyasu et al., 1987).

In view of these results, several questions arise. First, it remains to be determined whether assembly with α-subunits permits the *Xenopus* βNK to adopt a transport-competent configuration or rather to release the protein from an ER retention factor, e.g., a chaperone such as Bip. With respect to βHK that leave the ER in an unassembled state, the questions arise whether βHK possess a particular transport signal, miss a retention signal, or whether they cannot recognize the retention factor of *Xenopus* oocytes since they are derived from a mammalian species. This latter possibility would indicate that the ER retention signal is highly cell-type and species specific.

Another important question is what is the nature of the structural information that determines ER retention of unassembled proteins. It is unlikely that a specific sequence identical in all proteins is responsible for ER retention. It rather appears that each protein is a special case (for review see Rose and Doms, 1988).

In this study we show that in *Xenopus* oocytes, ER retention of unassembled *Xenopus* βNK is mediated by the extracytoplasmic domain of the β subunit (Table I). We do not yet know whether these β subunits are associated with Bip proteins but we do know that they are not severely misfolded. Indeed, we could demonstrate that unassembled β subunits maintain a configuration that is compatible with posttranslational association with α subunits and with release from the ER constraint (Ackermann and Geering, 1992). These data are clearly distinct from recent work on the ER retention and assembly of chimeric human-chicken NKA β subunits expressed in mouse cells. Renaud et al. (1991) show that deletions of five amino acids from the transmembrane domain results in ER retention of this β subunit despite its association with α subunits. These results pointed to the possibility that these β-subunits might have a transport signal in the transmembrane region that is abolished in the mutant or else a retention signal in the α-subunit that would be masked by assembly with wild type but not with mutated β-subunits. The data did however not exclude that the mutations in the transmembrane domain could have affected the correct conformation of the ectodomain resulting in the ER retention of the misfolded protein. To understand the molecular mechanisms underlying the differential transport properties of β-subunits, it will be interesting to investigate the behavior of other β-isoforms either expressed in homologous or heterologous systems. A comparison of the sequences of the different β subunits might ultimately permit us to identify the ER retention signal in the ectodomain of *Xenopus* βNK.

Both the Transmembrane and the Ectodomain of β Subunits Are Involved in the Modulation of the Transport Activity of Na,K-pump

In this study we were mainly concerned with characterizing structural determinants in the β subunit of Na,K-ATPase that are implicated in its posttranslational fate, namely in its assembly with α subunits and its ER retention as an unassembled protein. However, the use of chimeric β-proteins permitted us also to substantiate the recent observation that β subunits might not only have a primary role in the structural and functional maturation of newly synthesized α subunits, but could as well be modulators of the transport activities of mature Na,K-pumps expressed at the plasma membrane (Eakle et al., 1992; Jaisser et al., 1992; Lutsenko and Kaplan, 1992; Schmalzing et al., 1992; Jaisser, F., P. Jaunin, K. Geering, B. C. Rossier and J. H. Horisberger; manuscript submitted for publication). On the one hand, we show that Na,K-pumps composed of αNK and βHK exhibit a lower apparent affinity for K^+ than αNK-βNK complexes and on the other hand, we provide evidence that the exchange of the NH_2- and the COOH-terminal in chimeric proteins of βNK and βHK is not sufficient to produce Na,K-pumps with an apparent K^+ affinity of either α-βNK or α-βHK complexes

(Table I). The results obtained with the chimeric β-proteins indeed indicate that the integrity of both the NH_2-terminal transmembrane and the extracytoplasmic domain must be retained to produce the specific effect of βNK or βHK on the apparent K^+ affinity of Na,K-pumps. A finer molecular analysis is needed to delineate more precisely the structural domains that are responsible for the observed functional differences of β subunits.

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