Intersubunit Interactions in Human X,K-ATPases: Role of Membrane Domains M9 and M10 in the Assembly Process and Association Efficiency of Human, Nongastric H,K-ATPase α Subunits (ATP1al1) with Known β Subunits†

Käthi Geering,*,‡,§ Gilles Crambert,‡,§ Chuliang Yu,|| Tatyana V. Korneenko,⊥ Nikolay B. Pestov,||,⊥ and Nikolai N. Modyanov||

Institut de Pharmacologie et de Toxicologie de l'Université, rue du Bugnon 27, CH-1005 Lausanne, Switzerland, Department of Pharmacology, Medical College of Ohio, Toledo, Ohio 43614-5804, and Shemyakin and Ovchinnikov Institute of Bioorganic Chemistry, Russian Academy of Sciences, Moscow 117871, Russia

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ABSTRACT: Na,K- and H,K-ATPase (X,K-ATPase) α subunits need association with a β subunit for their maturation, but the authentic β subunit of nongastric H,K-ATPase α subunits has not been identified. To better define α−β interactions in these ATPases, we coexpressed human, nongastric H,K-ATPase α (AL1) and Na,K-ATPase α1 (α1NK) as well as AL1−α1 and α1−AL1 chimeras, which contain exchanged M9 and M10 membrane domains, together with each of the known β subunits in *Xenopus* oocytes and followed their resistance to cellular and proteolytic degradation and their ER exit. We show that all β subunits (gastric βHK, β1NK, β2NK, β3NK, or *Bufo* bladder β) can associate efficiently with α1NK, but only gastric βHK, β2NK, and *Bufo* bladder β can form stably expressed AL1−β complexes that can leave the ER. The trypsin resistance and the forces of subunit interaction, probed by detergent resistance, are lower for AL1−β complexes than for α1NK−β complexes. Furthermore, chimeric α1−AL1 can be stabilized by β subunits, but α1-AL1−gastric βHK complexes are retained in the ER. On the other hand, chimeric AL1−α1 cannot be stabilized by any β subunit. In conclusion, these results indicate that (1) none of the known β subunits is the real partner subunit of AL1 but an as yet unidentified, authentic β should have structural features resembling gastric βHK, β2NK, or *Bufo* bladder β and (2) β-mediated maturation of α subunits is a multistep process which depends on the membrane insertion properties of α subunits as well as on several discrete events of intersubunit interactions.

The X,K-ATPases represent a family of closely related potassium-dependent P-type ATPases which function as cation exchangers that pump K^+ into the cell and Na^+ (Na,K-ATPase) or H^+ (H,K-ATPase) out of the cell. All X,K-ATPases consist of a catalytic α subunit which is a large polytopic protein with 10 transmembrane segments and a glycosylated β subunit, a type II membrane protein which is absent in other P-type ATPases. The X,K-ATPase family combines three distinct groups of ion pumps which can be distinguished on the basis of their structure−function relationship. The first group consists of the Na,K-ATPase isozymes (four α and three β isoforms) (*1*) and the second group of the gastric H,K-ATPase (*2*). Finally, the third group is represented by the recently discovered catalytic α subunits of nongastric H,K-ATPases (*3*, *4*). This latter group is clearly distinct from the gastric H,K-ATPase, showing significant differences in primary structure and sensitivity to inhibitors. For instance, the human ATP1AL1 gene product, ATP1al1 (AL1)[1] (*5*−*7*), and its animal analogues (*8*, *9*) are catalytic α subunits of nongastric H,K-ATPases which, in contrast to gastric H,K-ATPase α subunits, are moderately sensitive to ouabain (*8*, *10*−*12*), may transport H^+ or Na^+ in exchange for K^+ (*13*, *14*), and are structurally equally distant (63−64%) from the Na,K-ATPase α subunit (αNK) isoforms and the gastric H,K-ATPase α subunit (g αHK) (*15*).

β subunits are indispensable for the structural and functional maturation of X,K-ATPases (*16*, *17*). However, none of the nongastric H,K-ATPases have been isolated, and therefore, their intrinsic subunit composition remains un-

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* To whom correspondence should be addressed: Institut de Pharmacologie et Toxicologie de l'Université, rue du Bugnon 27, CH-1005 Lausanne, Switzerland. Telephone: 41 (21) 692 54 10. Fax: 41 (21) 692 53 55. E-mail: kaethi.geering@ipharm.unil.ch.

‡ Institut de Pharmacologie et de Toxicologie de l'Université.

§ Käthi Geering and Gilles Crambert have contributed equally to this work.

|| Medical College of Ohio.

⊥ Russian Academy of Sciences.

[1] Abbreviations: AL1, human, nongastric H,K-ATPase α subunit; α1NK, human Na,K-ATPase α1 subunit; g αHK, rabbit, gastric H,K-ATPase α subunit; g βHK, rabbit, gastric H,K-ATPase β subunit; βNK, Na,K-ATPase β subunit; h β1NK, human Na,K-ATPase β1 subunit; h β2NK, human Na,K-ATPase β2 subunit; h β3NK, human Na,K-ATPase β3 subunit; B bl β, *Bufo* bladder β subunit; X β3NK, *Xenopus* Na,K-ATPase β3 subunit; β NK−HK and β HK−NK, chimeras between *Xenopus* Na,K-ATPase β1 and rabbit gastric H,K-ATPase β subunits; AL1−α1, chimera between AL1 and human Na,K-ATPase α1 subunits containing membrane domains M9 and M10 of α1; α1−AL1, chimera between human Na,K-ATPase α1 subunits and AL1 containing M9 and M10 of AL1; EndoH, endoglycosidase H.

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known. The known β subunits (the three Na,K-ATPase β isoforms, β1NK, β2NK, and β3NK, the β2-like *Bufo* bladder β, B bl β, and the gastric H,K-ATPase β subunit, g βHK) share a common domain structure, but exhibit a much lower degree of sequence similarity (~20−35% identical) than X,K-ATPase α subunits. So far, no authentic β subunit for nongastric H,K-ATPase has been described. Previously, we have shown that AL1, like other X,K-ATPase α subunits, is degraded when expressed in *Xenopus* oocytes without a β subunit (*10*). Coexpression with g βHK stabilizes AL1 and produces functional pumps at the cell surface (*10, 11*). However, g βHK is probably not the real partner subunit for AL1 in vivo since the presence of g βHK has only been demonstrated in the stomach and the kidney (*18*), whereas RT-PCR revealed significant expression of ATP1AL1 not only in the kidney but also in human skin and lower expression in colon, brain, placenta, and lung (*19*). Furthermore, it was recently reported that not only g βHK but also β1NK can form functional ATPase complexes with nongastric αHK expressed in *Xenopus* oocytes (*20*). Two recent studies also suggest that β1NK could be the real partner subunit of nongastric H,K-ATPases in the kidney and the distal colon because β1NK antibodies recognized the protein that co-immunoprecipitated with a nongastric αHK antibody (*21, 22*). On the other hand, Sangan et al. (*23*) reported that β3NK like colonic αHK is upregulated in apical membranes of distal colon after K^+ depletion and is physically associated with colonic αHK, suggesting that β3NK may be the putative β subunit of colonic H,K-ATPase.

Clearly, the current state of knowledge does not enable us to decide whether the nongastric αHK associates in situ with one of the five known β subunits or with a hitherto unidentified β subunit. In the study presented here, we have aimed to define the type of β subunit that best fulfils the criteria as a candidate for a β subunit of a nongastric H,K-ATPase. For this purpose, we have analyzed the effects of different β subunits on the correct folding and the maturation of newly synthesized AL1, which determine the expression of functional pumps (*16*). The efficiency of assembly of each of the known β subunits with AL1 was determined, by coexpression in *Xenopus* oocytes, as the ability of each β subunit to protect AL1 from cellular and tryptic degradation and to produce detergent-resistant AL1−β complexes that can leave the ER. Our results suggest that none of the known β subunits may be the authentic β subunit of AL1 but that an as yet unidentified β subunit of nongastric H,K-ATPase should have structural features resembling those of g βHK, β2NK, and/or β_2-like B bl β.

To further define the characteristics of β interaction with nongastric αHK, we also carried out domain swapping between human AL1 and αNK and compared the role of transmembrane segments M9 and M10 in the interaction with β subunits and the stabilization of the two α proteins. The M9−M10 loop was selected for exchange since, from a structural point of view, this domain is the most divergent in all X,K-ATPases α subunits (*15*). Furthermore, evidence exists that intramembraneous interactions occur between β subunits and the M7−M10 domains of αNK (*24, 25*). Finally, M9 and M10 in g αHK (*17*) and αNK (*26*) play a distinct role in the membrane insertion of the M7−M8 membrane pair and thus in the exposure of the principle β interaction site in the extracytoplasmic M7−M8 loop which is common to all X,K-ATPases α subunits (*27, 28*). Our results show that like the g αHK (*17*) but in contrast to αNK, the correct packing of the C-terminal membrane domain of AL1 and its interaction with β subunits strongly depend on the presence of its own M9−M10 membrane pair. While αNK in which the M9−M10 membrane pair is replaced with that of AL1 can associate with various β subunits and form stable α−β complexes, AL1 in which the M9−M10 membrane pair is replaced with that of the αNK produces misfolded α subunits that cannot associate with β subunits and which are degraded.

MATERIALS AND METHODS

Preparation of Expression Constructs and cRNAs. Generation of the ATP1AL1 expression construct in the pSD3 vector (*29*) from the original pHAS 34.1 clone (*15*) has been described previously (*10*). The 5′ untranslated (5′UT) region of ATP1AL1 was replaced with that of a truncated *Xenopus* Na,K-ATPase α1 cDNA (*30*). This modification was found to significantly improve the translation of human cRNAs in *Xenopus* oocytes (*10*). In addition to the protein coding sequence, the ATP1AL1 construct contains 118 nucleotides of the 3′UT region. The translation product of this construct is called AL1.

The full-length cDNA encoding the human α1 isoform (α1NK) was reconstructed as described previously (*31*) from two overlapping clones, pSNa100 and pSN54 (*32*), which were kindly provided by K. Kawakami. The α1 cDNA was transferred into the pNKS2 vector (kindly provided by G. Schmalzing).

Chimeric cDNA constructs were generated by exchanging the C-terminal transmembrane domains M9 and M10 between AL1 and human α1NK cDNAs using the common *Eco*RI restriction site as an exchange point for recombination. This *Eco*RI site is located within the sequence corresponding to the highly conserved cytoplasmic loop between M8 and M9. To prepare the chimera AL1 (M^1−R^{956})−αNK (N^{942}−Y^{1021}), the *Eco*RI (polylinker)−*Eco*RI (2928 nucleotides) fragment from the AL1 construct was isolated and was used to replace the corresponding region in the α1NK construct. The translation product of this construct is called AL1−α1. To prepare the inverse chimera α1NK (M^1−R^{941})−AL1 (N^{95}−Y^{1039}), the *Eco*RI (polylinker)−*Eco*RI (3142 nucleotides) fragment was isolated from partial digests of αNK cDNA and was used to replace the corresponding region in the ATP1AL1 construct in the pSD3 vector. The translation product of this construct is called α1-AL1.

IMAGE Consortium clone 133072 (Research Genetics, Huntsville, AL) containing the full-length human β3 cDNA was identified by restriction mapping and sequencing as described previously (*31, 33*). All constructs were verified by sequencing.

Human β1NK (*34*) (kindly provided by K. Kawakami), human β2NK and β3NK (*31, 33*), *Xenopus* β1NK (*35*), *Xenopus* β3NK (*36*), rabbit, gastric H,K-ATPase α (g αHK) and β (g βHK) subunits (kindly provided by G. Sachs), and *Bufo* bladder β subunits (B bl β) (*8*) (kindly provided by J. Jaisser) were subcloned into the pSD5 vector containing the Xe 5′UT region. The β NK−HK chimera in which cytoplasmic and transmembrane domains (M^1−D^{71}) are derived from *Xenopus* β1NK and the ectodomain (Q^{76}−K^{291}) from

rabbit g βHK and the β HK−NK chimera with the inverse orientation (M^{1}−L^{77} from rabbit g βHK and A^{74}−S^{304} from *Xenopus* β1NK) were prepared as described previously (*37*).

In vitro-transcribed RNAs were synthesized from linearized cDNA templates according to the method of Melton et al. (*38*).

Assembly and Test of Stabilization of α *Subunits Coexpressed in Xenopus Oocytes with Different* β *Subunits.* Oocytes were obtained from *Xenopus* females (Noerhoek) as described in ref *16*. Routinely, 12 ng of α and 1.5 ng of β cRNA were injected into oocytes. To investigate the ability of different β subunits to associate with and stabilize different α subunits, oocytes were incubated in modified Barth's medium containing 0.6 mCi/mL of [^{35}S]Easytag Express Protein Labeling Mix (New England Nuclear) for 24 h and then subjected to a chase period of 24 and/or 48 h in the presence of 10 mM cold methionine. Digitonin extracts were prepared as previously described (*16*), and the α−β complexes were immunoprecipitated under nondenaturing conditions in the presence of 1% digitonin which preserves α−β interactions (*37*) or under denaturing conditions after heating the samples for 7 min at 56 °C in the presence of 3% SDS. αNK and α1-AL1 were immunoprecipitated with a *Bufo* α1NK antibody (*39*). AL1 and AL1−α1 were immunoprecipitated with rabbit, polyclonal AL1 antibodies which were raised against a recombinant N-terminal fragment of AL1 (S^{14}−I^{104}) prepared as described previously (*40*). g αHK and g βHK were immunoprecipitated with autoimmune antibodies produced in mice after neonatal injection of H,K-ATPase-enriched gastric membranes (*41*) (kindly provided by D. Claeys). Finally, immunoprecipitations were performed with a *Xenopus* β1NK antibody (*42*), a *Xenopus* β3NK antibody (*43*) (kindly provided by P. Good), or a human β3NK antibody. Rabbit polyclonal β3 antibodies were raised against a recombinant extracellular fragment of human β3 (A^{153}−A^{279}) expressed in *Escherichia coli* and purified by metal affinity chromatography as described previously (*40*). The dissociated immune complexes were separated by SDS−polyacrylamide gel electrophoresis, and labeled proteins were detected by fluorography.

Controlled Proteolysis of α *Subunits.* The ability of different β subunits to promote the structural maturation of α subunits was probed by a controlled proteolysis assay. Oocytes injected with different combinations of α and β cRNAs were metabolically labeled for 24 h and then homogenized in an Eppendorf tube with a Teflon pestle in a buffer containing 250 mM sucrose, 50 mM Tris-HCl (pH 7.5), 50 mM potassium acetate, 5 mM $MgCl_2$, and 1 mM dithiothreitol. Aliquots of homogenates were incubated in the presence of diphenyl carbamyl cloride-treated trypsin (Sigma Chemical Co.) at a final concentration of 100 μg/mL for 1 h at 4 °C before addition of a 5-fold excess (w/w) of soybean trypsin inhibitor (Sigma Chemical Co.). After 15 min on ice, the samples were subjected to denaturing immunoprecipitation with an appropriate α antibody.

Resistance of α−β *Complexes to Detergent Treatment.* As a measure of the forces of interaction between different α and β subunits, we tested the resistance of α−β complexes to treatment with digitonin and Triton X-100 (*44*). Oocytes were injected with different combinations of α and β cRNA, metabolically labeled for 24 h in the presence of brefeldin A (final concentration of 5 μg/mL) (Calbiochem) as previously described (*16*), and then subjected to detergent extraction using an extraction buffer (*16*) containing either 1% digitonin or 0.5% Triton X-100. α−β complexes were immunoprecipitated with an appropriate α antibody, absorbed onto Sepharose A beads (Amersham), and washed three times with a buffer containing 1% digitonin or 0.5% Triton X-100. Immunoprecipitated samples were subjected to EndoH (Biolab) treatment (*37*) before gel electrophoresis. Brefeldin A, which prevents ER exit of newly synthesized α−β complexes and therefore full glycosylation of β subunits and EndoH, which specifically cleaves core sugars from ER-resident proteins, was used for quantification of nonglycosylated β subunits associated with α subunits. Quantification of immunoprecipitated α and β subunits was performed with a laser densitometer (LKB Ultroscan 2202) or by phosphorimager analysis (Molecular Imager, Bio-Rad).

RESULTS

Protection against Cellular Degradation of Human AL1 by Different β *Subunits and Chimeric* β *Variants.* Like Na,K-ATPase α subunits (*16*), AL1, the α subunit of a human, nongastric H,K-ATPase (*5*−*7*), needs association with a β subunit for its structural maturation and cell surface expression (*10*). Since, so far, no specific nongastric H,K-ATPase β subunit has been identified, we tested the efficiency of known β subunits to associate with AL1 and to protect it from cellular degradation after expression in *Xenopus* oocytes.

As previously observed (*10*), AL1 expressed in oocytes in the absence of a β subunit was completely degraded during a chase period (Figure 1A, lanes 1 and 2). Coexpressed h β1NK co-immunopreciptated and thus associated with AL1 during the pulse but stabilized AL1 only partially during the chase period (lanes 6 and 7). More complete stabilization of AL1 was achieved after coexpression with g βHK (Figure 1A, lanes 3−5, and Figure 1C, lanes 1 and 2), h β2NK (Figure 1A, lanes 8 and 9), or the β2-like B bl β (Figure 1A, lanes 10 and 11). The association of h β2NK and the partial stabilization of AL1 were specific for nongastric αHK since gastric αHK was stabilized by g βHK (Figure 1B, lanes 1−3) but not by h β2NK (Figure 1B, lanes 4−6).

Partial stabilization of AL1 was also observed with a β NK−HK chimera (containing the cytoplasmic and transmembrane domain of X β1NK subunits and the ectodomain of g βHK; see Materials and Methods) (Figure 1A, lanes 16 and 17, and Figure 1C, lanes 5 and 6) but not with an inverse β HK−NK chimera (Figure 1A, lanes 18 and 19, and Figure 1C, lanes 7 and 8). This result indicates that the ectodomain and not the transmembrane domain of g βHK is important for the stabilizing effect on AL1.

Immunopreciptation with an AL1 antibody under nondenaturing conditions clearly revealed association of AL1 with B bl β in its core-glycosylated form after a pulse (Figure 1A, lane 10) and in its fully glycosylated form after a chase period (Figure 1A, lane 11), indicating that the AL1−B bl β complexes can leave the ER during the chase period and are routed to the plasma membrane. Co-immunoprecipitation of g βHK (lanes 1−3), h β2NK (lanes 8 and 9), or β NK−HK (lanes 12 and 13) with an AL1 antibody was less obvious. Here, the presence of g βHK and β NK−HK and their association with AL1 in the core-glycosylated form after

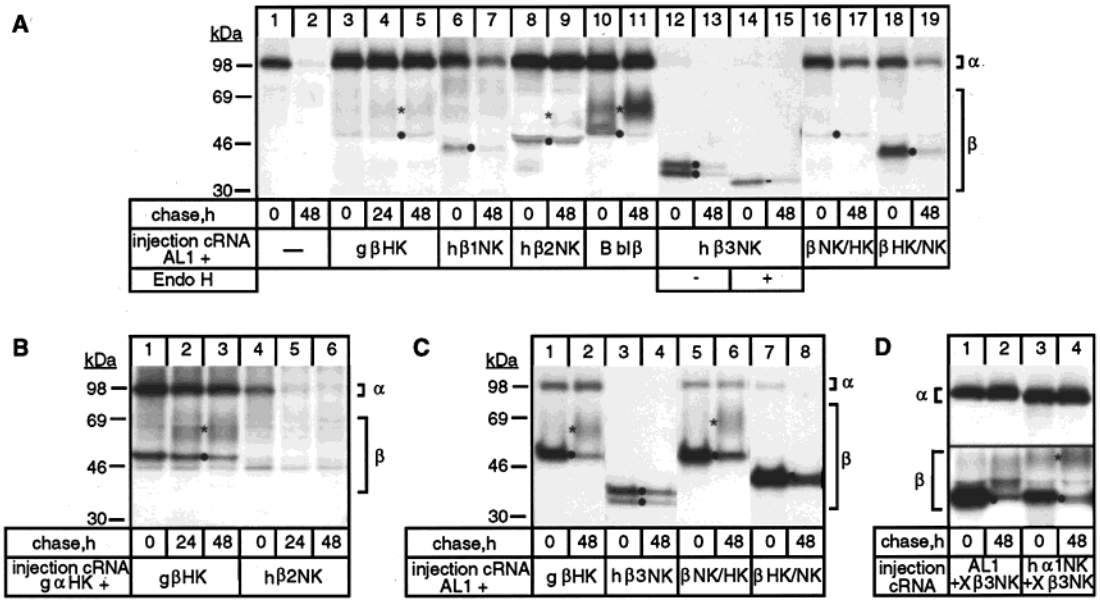

FIGURE 1: Protection against cellular degradation of AL1 by different β subunits. *Xenopus* oocytes were injected with AL1 (A, C, and D), rabbit g αHK (B), or human α1NK (D) and different β cRNAs as indicated, metabolically labeled for 24 h and subjected to chase periods of 24 and/or 48 h as described in Materials and Methods. Digitonin extracts were prepared and subjected to immunoprecipitation. Shown are fluorograms of immunoprecipitates after SDS−polyacrylamide gel electrophoresis. (A) Immunoprecipitations with an AL1 antibody under nondenaturing conditions. Samples shown in lanes 14 and 15 were treated with EndoH after immunoprecipitation. (B and C) Immunoprecipitations with β antibodies. Immunoprecipitations were performed with a gastric HK antibody (B, lanes 1−6; C, lanes 1, 2, 5, and 6) under nondenaturing conditions or with a human β3NK antibody (C, lanes 3 and 4) or a *Xenopus* β1 antibody (C, lanes 7 and 8) under denaturing conditions. (D) Immunoprecipitations with AL1 (upper panel, lanes 1 and 2), αNK (upper panel, lanes 3 and 4), or with *Xenopus* β3NK antibodies (lower panel, lanes 1−4) under denaturing conditions. Indicated are the positions of the α and β subunits which are co-immunoprecipitated under nondenaturing conditions: (−) nonglycosylated β subunits, (·) core-glycosylated β subunits, and (∗) fully glycosylated β subunits. The migration of proteins of known molecular mass is indicated at the left. One out of two to five representative experiments is shown.

the pulse period (Figure 1C, lanes 1 and 5) or in the fully glycosylated form after the chase period (Figure 1C, lanes 2 and 6) could be confirmed by nondenaturing immunoprecipitations with an antibody recognizing g βHK.

A curious phenomenon was observed in oocytes expressing AL1 together with h β3NK (Figure 1A, lanes 12−15). Immunoprecipitations with an AL1 antibody under nondenaturing conditions revealed minimal amounts of AL1 proteins after the pulse or the chase period but a significant amount of co-immunoprecipitated h β3NK which was glycosylated on one or two of the putative glycosylation sites (Figure 1A, lanes 12 and 13, and Figure 1C, lanes 3 and 4) as suggested by EndoH treatment which cleaved both species into one faster migrating, nonglycosylated h β3NK band (Figure 1A, lanes 14 and 15). The reason why low levels of AL1 should be associated with large amounts of h β3NK is not known, but it may be suggested that h β3NK, expressed in *Xenopus* oocytes, forms aggregates which associate with and degrade AL1. A similar phenomenon was observed with *Xenopus* gastric βHK which provoked degradation of αNK or αHK subunits coexpressed in *Xenopus* oocytes (*45*). To test whether the observed degradation of AL1 is limited to the particular combination of human AL1 with h β3NK or rather represents a general characteristic of all β3NK, we compared the stabilization of AL1 and h α1NK after coexpression with h β3NK and X β3NK. In contrast to AL1, α1NK was stabilized by h β3NK (ref *31* and see Figure 4). On the other hand, X β3NK protected both α subunits from degradation (Figure 1D, lanes 1−4, upper panel). However, in contrast to X β3NK associated with h α1NK (lanes 3 and 4, lower panel), X β3NK associated with AL1 (lanes 1 and 2, lower panel) was not correctly glycosylated during the chase period but produced glycosylated species that were intermediate between core-glycosylated and fully glycosylated forms. This result indicates a defect in intracellular transport competence and/or in the global structural integrity of AL1−X β3NK complexes. Altogether, our data indicate that g βHK, h β2NK, and the β2-like B bl β preferentially promote the structural maturation of AL1 which is necessary for its protection against cellular degradation and its expression at the cell surface. β1 isoforms of Na,K-ATPase are less efficient in this process, while β3NK may have intermediate properties.

Influence of M9 and M10 of AL1 and α1NK on the β Assembly Process. The overall amino acid sequences of human AL1 and Na,K-ATPase α subunits are 64.2% identical except for transmembrane domains M9 and M10 which are very dissimilar (32.7% identical). The majority of differences are concentrated in M9 where only 7 out of 26 amino acids are identical in AL1 and α1NK (26.9% identical) and in the connecting loop between M9 and M10 which consists of 4 different residues in the two α subunits. To investigate the possible importance of the highly variable M9 and M10 domains in the β assembly process, we produced AL1−α1 chimeras (containing domains M1−M8 of AL1 and M9 and M10 of α1NK) or α1−AL1 chimeras (containing domains M1−M8 of α1NK and M9 and M10 of AL1) and tested their ability to become stabilized by different β subunits.

The replacement of the two last transmembrane domains of AL1 with M9 and M10 of h α1NK in an AL1−α1 chimera had a drastic, deleterious effect on the efficiency of

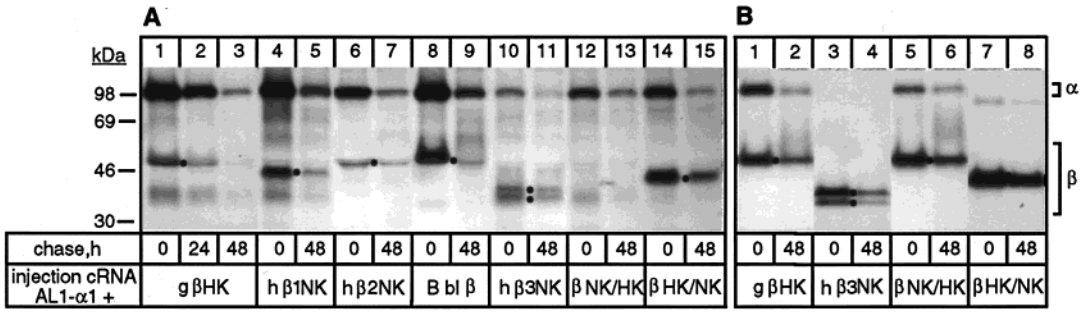

FIGURE 2: Protection against cellular degradation of AL1−α1 by different β subunits. Oocytes were injected with AL1−α1 cRNA and different β cRNAs as indicated, metabolically labeled for 24 h, and subjected to chase periods of 24 and/or 48 h. Digitonin extracts were prepared and subjected to immunoprecipitations. (A) Immunoprecipitations with an AL1 antibody under nondenaturing conditions. (B) Immunoprecipitations with β antibodies. Immunoprecipitations were performed with a gastric HK antibody (lanes 1, 2, 5, and 6) under nondenaturing conditions or with a human β3 (lanes 3 and 4) or a *Xenopus* β3 antibody (lanes 7 and 8) under denaturing conditions. One out of three representative experiments is shown. Abbreviations and symbols are like those in Figure 1.

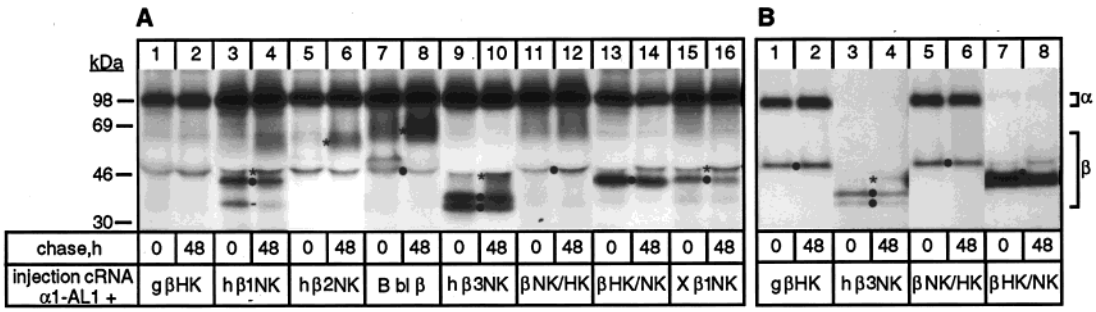

FIGURE 3: Protection against cellular degradation of α1−AL1 by different β subunits. Oocytes were injected with α1−AL1 cRNA and different β cRNAs as indicated, metabolically labeled for 24 h, and subjected to a chase period of 48 h. Digitonin extracts were prepared and subjected to immunoprecipitations. (A) Immunoprecipitations with an αNK antibody under nondenaturing conditions. (B) Immunoprecipitations with β antibodies. Immunoprecipitations were performed with a gastric HK (lanes 1, 2, 5, and 6) or with a human β3NK antibody (lanes 3 and 4) under nondenaturing conditions or with a *Xenopus* β1 antibody (lanes 7 and 8) under denaturing conditions (lanes 7 and 8). One out of three representative experiments is shown. Abbreviations and symbols are like those in Figure 1.

association of all β subunits or chimeric β variants and as a consequence on the stabilization of this α chimera (Figure 2A,B). Compared to AL1 (Figure 1), AL1−α1 proteins were much less stabilized during a chase period when coexpressed with g βHK (Figure 2A, lanes 1−3, and Figure 2B, lanes 1 and 2), h β2NK (Figure 2A, lanes 6 and 7), or B bl β (Figure 2A, lanes 8 and 9). Consistent with these results, all β subunits coexpressed with AL1−α1 remained in their core-glycosylated form and were degraded during the chase period (Figure 2A,B). Like that of AL1, coexpression of AL1−α1 with h β3NK reduced the magnitude of the signal of immunoprecipitated AL1−α1 observed after the pulse period, but the ratio of α over β subunits significantly increased compared to that observed with AL1 (Figure 2A, lanes 10 and 11). The data obtained with AL1 and AL1−α1 proteins reveal that M9 and M10 play an important role in the acquisition of a folding state of AL1 which is compatible with interaction with the β subunit.

In contrast to AL1−α1, the inverse chimera α1−AL1, in which M9 and M10 of h α1NK subunits were replaced with M9 and M10 of AL1, was efficiently stabilized by all the β subunits that were tested, including g βHK, h β1NK, X β1NK, h β2NK and h β3NK, B bl β, and the β NK−HK and β HK−NK chimeras (Figure 3A,B). Interestingly, however, differences existed between the different α−β complexes in their ability to leave the ER. The total h β2NK (Figure 3A, lanes 5 and 6) and B bl β population (Figure 3A, lanes 7 and 8) and part of the of h β1NK (Figure 3A, lanes 3 and 4), X β1NK (Figure 3A, lanes 15 and 16), and h β3NK population (Figure 3A, lanes 5 and 6, and Figure 3B, lanes 3 and 4) which were associated with α1−AL1 became fully glycosylated after the chase period, indicating that all or part of the complexes had left the ER and had passed a trans Golgi compartment. On the other hand, g βHK (Figure 3A,B, lanes 1 and 2) and the two β NK−HK (Figure 3A, lanes 11 and 12, and Figure 3B, lanes 5 and 6) and β HK−NK (Figure 3A, lanes 13 and 14, and Figure 3B, lanes 7 and 8) chimera which were associated with α1−AL1 remained core-glycosylated during the chase period, showing that the complexes were retained in the ER.

In contrast to AL1−α1 proteins which markedly differed from wild-type AL1 proteins, the stabilization properties of α1−AL1 proteins closely ressembled those of wild-type α1NK. As for α1−AL1 proteins, α1NK could permanently associate with all βNK isoforms as well as with g βHK and with chimeric β subunits and became stabilized (Figure 4A,B). However, contrary to α1−AL1−β complexes (Figure 4A,B), α1NK−β complexes containing h β1NK (Figure 4A, lanes 3, 4, 13, and 14), g βHK (Figure 4A,B, lanes 1 and 2), or chimeric β variants (Figure 4A, lanes 9−12, and Figure 4B, lanes 5−8) were partially fully glycosylated during the chase period, indicating that they are routed to the plasma membrane. These results suggest that, compared to AL1, M9 and M10 in αNK are not as important for adopting a conformational state which allows interaction with the β subunit and as a consequence the protection of the α subunit against cellular degradation. However, the integrity of the αNK protein containing its proper M9 and M10 is necessary for the ultimate, correct packing of the αNK protein which permits ER exit.

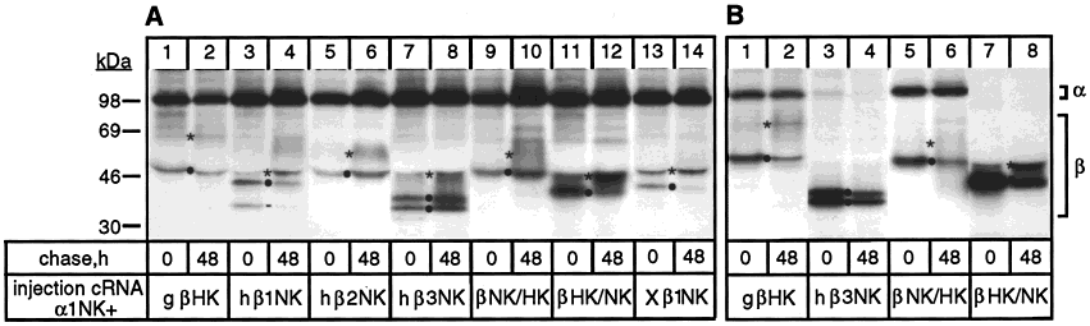

FIGURE 4: Protection against cellular degradation of human Na,K-ATPase α1 subunits by different β subunits. Oocytes were injected with human α1 cRNA and different β cRNAs as indicated, metabolically labeled for 24 h, and subjected to a chase period of 48 h. Digitonin extracts were prepared and subjected to immunoprecipitations. (A) Immunoprecipitations with an αNK antibody under nondenaturing conditions. (B) Immunoprecipitations with β antibodies. Immunoprecipitations were performed with a gastric HK antibody (lanes 1, 2, 5, and 6) under nondenaturing conditions or with a human β3 (lanes 3 and 4) or a *Xenopus* β1 antibody (lanes 7 and 8) under denaturing conditions. One out of three representative experiments is shown. Abbreviations and symbols are like those in Figure 1.

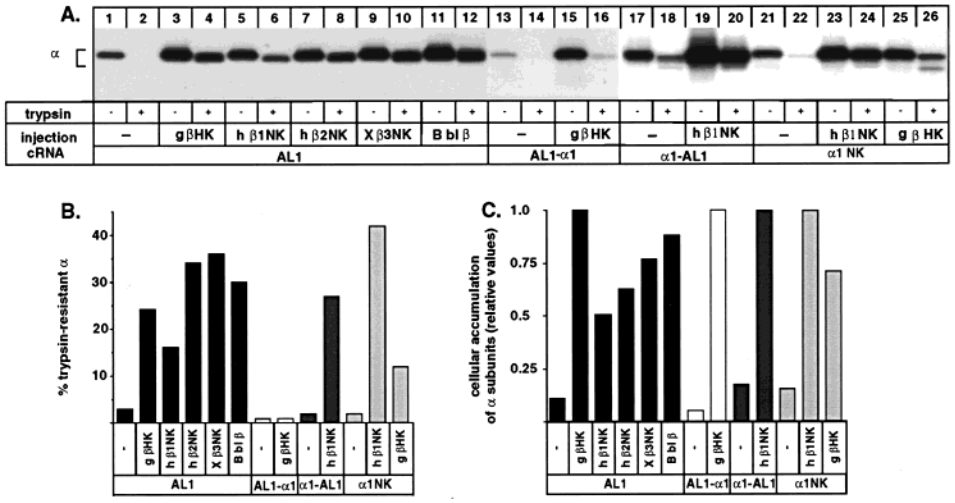

FIGURE 5: Controlled proteolysis of human AL1−β, α1−β, and chimeric α−β complexes. Oocytes were injected with AL1, AL1−α1, α1−AL1, or α1 together with different β cRNAs as indicated and metabolically labeled for 24 h. Homogenates were prepared, and aliquots were subjected to controlled trypsinolysis for 1 h at 4 °C at a trypsin concentration of 0.1 mg/mL before immunoprecipitation of the α subunit under denaturing conditions. (A) Representative examples of immunoprecipitations of α−β complexes subjected to controlled proteolysis. Immunoprecipitations were performed with an AL1 or a αNK antibody. (B) Trypsin resistance of α subunits. Represented is the percentage of α subunits which resist controlled trypsinolysis expressed in the absence or presence of different β subunits. Quantification of the data shown in panel A and of additional experiments (mean of two experiments). (C) Cellular accumulation of α subunits after a 24 h pulse period. Represented is the relative amount of α subunits expressed in the absence or presence of different β subunits. Nontrypsinized AL1, AL1−α1, α1−AL1, and α1 which showed the highest level of expression after a 24 h pulse were arbitrarily set to 1. Quantification of the data shown in panel A and of additional experiments (mean of two experiments) is shown.

Probing of the β-Interaction by Controlled Proteolysis of the α Subunit. To further characterize the specificity of the β interaction with AL1 and αNK subunits and the role played by M9 and M10 in the β assembly process, we probed the resistance of AL1, α1NK, and the chimeric α proteins to controlled proteolysis. This assay has previously been shown to provide information about the β-mediated structural maturation of the α subunit in addition to the cellular stabilization assay (*16*). When expressed in *Xenopus* oocytes in the absence of β subunits, AL1 (Figure 5A, lanes 1 and 2, and Figure 5B), AL1−α1 (Figure 5A, lanes 13 and 14, and Figure 5B), α1−AL1 (Figure 5A, lanes 1 and 2, and Figure 5B), and α1NK (Figure 5A, lanes 5 and 6, and Figure 5B) are digested by more than 97% after incubation with trypsin at a concentration of 0.1 mg/mL. AL1 coexpressed with g βHK (Figure 5A, lanes 3 and 4), h β2NK (Figure 5A, lanes 7 and 8), X β3NK (Figure 5A, lanes 9 and 10), and B bl β (Figure 5A, lanes 11 and 12) was less sensitive to trypsinolysis, leaving a trypsin-resistant fraction of 24−36% (Figure 5B). On the other hand, AL1−α1 coexpressed with g βHK (Figure 5A, lanes 15 and 16) was completely digested by trypsin like AL1−α1 expressed alone (Figure 5A). Coexpression with h β1NK produced a trypsin-resistant α population of about 16% for AL1 (Figure 5A, lanes 5 and 6, and Figure 5B), 27% for α1−AL1 (Figure 5A, lanes 19 and 20, and Figure 5B), and 42% for α1NK (Figure 5A, lanes 23 and 24, and Figure 5B). Finally, a specific cleavage product of α1NK was observed when coexpressed with g βHK (Figure 5A, lanes 25 and 26) and the trypsin-resistant fraction was about 12% (Figure 5B).

The results shown in Figure 6 permit direct comparison between the cellular degradation and the trypsin sensitivity of α subunits expressed alone or together with different β subunits. Indeed, it is likely that the efficiency of synthesis of one particular α subunit expressed with different β subunits is similar. In this case, the expression level of α subunits after a pulse period is a reflection of their acquisition of resistance to cellular degradation depending on the

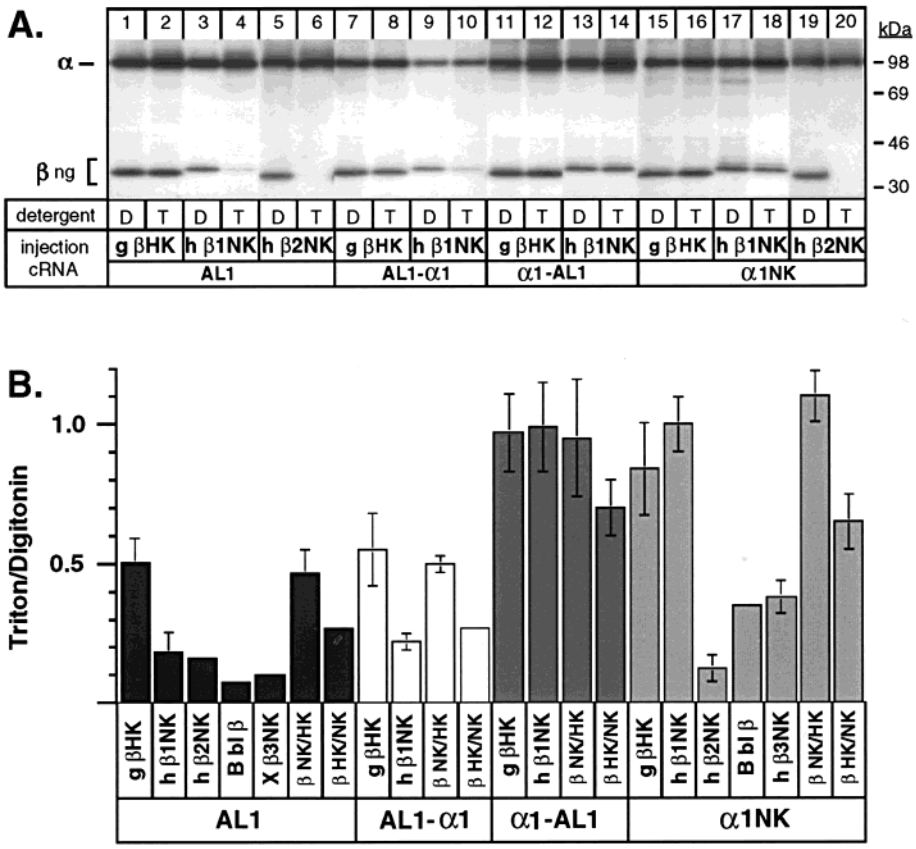

FIGURE 6: Resistance to detergent treatment of AL1−β, α1−β, and chimeric α−β complexes. Oocytes were injected with AL1, AL1−α1, α1−AL1, or α1 together with different β cRNAs as indicated and metabolically labeled for 24 h in the presence of brefeldin A before preparation of digitonin or Triton X-100 extracts and immunoprecipitation as described in Materials and Methods. (A) Representative examples of immunoprecipitations of α−β complexes. Aliquots of digitonin (D) or Triton X-100 (T) extracts were immunoprecipitated with an AL1 antibody (lanes 1−10) or an αNK antibody (lanes 11−20) under nondenaturing concentrations. To facilitate quantification, samples were deglycosylated with EndoH before gel electrophoresis. The positions of the α subunits and the co-immunoprecipitated, nonglycosylated (ng) β subunits are shown. (B) Quantification of the data shown in panel A and additional experiments is shown (means ± the standard error of three to six experiments). Bars without errors represent one experiment. Ratios between β and α subunits were separetely calculated in samples treated with digitonin or Triton X-100, and a final β/α ratio between Triton- and digitonin-treated samples was deduced.

coexpressed β subunit. Compared to the expression levels of AL1−g βHK, AL1−α1−g β HK, α1−AL1−h β1NK, and α1NK−h β1NK complexes which were arbitrarily set to 100%, the expression levels of the respective, individual α subunits only represented about 5−15% (Figure 5C), which is consistent with their rapid cellular degradation. This high sensitivity to cellular degradation (Figure 5B) correlated with the high sensitivity to trypsin digestion (Figure 5B) and confirms the extensive misfolding of individual α subunits. On the other hand, as illustrated for AL1, the effect on the cellular stabilization of the α subunit, the extent of which progressively increased after coexpression with h β2NK, X β3NK, B bl β, or g βHK (Figure 5C), was not paralleled by a similar increase in trypsin resistance (Figure 6B). A similar observation was made with g βHK-associated α1NK which was quite resistant to cellular degradation (Figure 5C) but which was extensively digested by trypsin (Figure 5B). Finally, among AL1, α1−AL1, or α1NK proteins coexpressed with h β1NK, AL1 exhibited the lowest level of cellular expression and the highest trypsin sensitivity. This result may reflect the low association efficiency of h β1NK, resulting in a high proportion of nonassembled AL1 and/or an inefficient folding of AL1 by h β1NK. In conclusion, the results obtained by the controlled trypsinolysis assay support the hypothesis that h β1NK is an inefficient partner subunit of AL1. Furthermore, the data confirm that the presence of M9 and M10 of α1 in AL1 impedes association with β subunits. Finally, the difference between the cellular stabilization and the trypsin resistance of α subunits expressed with certain β subunits indicates that ultimate and correct folding of α subunits is a finely tuned, multistep process which is strongly dependent on specific β association.

Probing the Force of α−β Interactions. To further characterize β association with AL1 and αNK subunits, we probed the strength of interaction between different α and β subunits by using as a criterion the resistance of dissociation of α−β complexes to treatment with a "mild" detergent, digitonin (1%), or with a "harsh" detergent, Triton X-100 (0.5%). This approach has been used by Schmalzing et al. (*44*) to define the specificity of the interaction between α and β isoforms of Na,K-ATPase. Figure 6A shows representative examples of immunoprecipitations performed under nondenaturing conditions on digitonin or Triton X-100 extracts of oocytes expressing AL1 (lanes 1−6), AL1−α1 (lanes 7−10), α1−AL1 (lanes 11−14), or α1NK (lanes 15−20) together with different β subunits. The ratios of β/α signals were determined in samples treated with digitonin or Triton X-100, and a final Triton/digitonin ratio was deduced for each α−β complex (Figure 6B). A low Triton/digitonin ratio reflects a high sensitivity to detergent dis-

sociation of α−β complexes and therefore weak α−β interactions. The main results of this analysis were as follows. First, with the exception of the AL1−h β2NK and α1NK−h β2NK complexes, which both exhibited weak subunit interactions, the Triton/digitonin ratios were consistently lower for all other AL1−β complexes than for the corresponding α1NK−β complexes. Second, with the exception of h β1NK, which exhibited a very weak interaction with AL1 and a strong interaction with α1NK, the relative changes in the forces of interaction of AL1 or α1NK with different β subunits were similar. For instance, h β2NK, h β3NK, and B bl β interact weakly, the β HK−NK chimera interacts more strongly, and g βHK and the β NK−HK chimera interact strongly with both AL1 and h α1. Finally, AL1 and AL1−α1 associated with different β subunits exhibit a similar pattern of Triton/digitonin ratios and thus similar interactive characteristics despite the important effect of replacement of the M9 and M10 in AL1 on the stabilization efficiency by β subunits. Overall, the results on the detergent resistance of α−β complexes confirm the intrinsic differences of β assembly with AL1 and α1NK, respectively, and they also reveal features of the β interaction that are independent of the α stabilization process.

DISCUSSION

A comparative analysis of the efficiency of assembly of different β subunits with human, nongastric H,K-ATPase α subunits (AL1) and with Na,K-ATPase α subunits revealed a high complexity of the functional role of β assembly in the maturation of oligomeric P-type ATPases. Our results show that protection against cellular degradation and controlled trypsinolysis of the α subunit as well as of the forces of interaction and the ER exit of α−β complexes can be differentially influenced by different β subunits. Furthermore, our studies suggest that nongastric H,K-ATPase α subunits exhibit more stringent requirements for β assembly than Na,K-ATPase α subunits both with respect to the nature of the β subunit that can efficiently assemble and with respect to the exposure of the β assembly domain during synthesis of the α subunit.

Assembly Efficiency of AL1 with Different β Subunits. Na,K-ATPase and the gastric and nongastric H,K-ATPases are the only members of the P-type ATPase family which need a β subunit for the structural and functional maturation of their catalytic α subunits (*10*, *16*, *17*). The authentic β counterpart of nongastric αHK subunits has so far not been identified. Indeed, several β subunits, e.g., g βHK (*10*, *20*), B bl β (*8*), and β1NK (*20*), coexpressed with nongastric αHK in *Xenopus* oocytes, were shown to produce functional, ouabain-sensitive H,K-ATPases. On the other hand, Caplan and co-workers (personal communication) observed that in MDCK or LLCPK-1 cells transfected with AL1 alone, the α subunit accumulated intracellularly, indicating that it cannot efficiently associate with endogenous β1NK. The persisting uncertainty of the authentic nature of the β subunit associated with nongastric αHK is further illustrated by recent studies which identified either β1NK (*21*, *22*) or β3NK (*23*) as the possibly authentic β subunit of rat, nongastric αHK because β1NK or β3NK antibodies, respectively, recognized the protein which co-immunoprecipitated with nongastric αHK from microsomes of colon or kidney. These contradictory data clearly show that, unless the immunochemical data are not confirmed by amino acid sequencing of the α-associated β subunit, the possibility that the antibodies used to detect the β subunit cross-react with a hitherto unidentified β subunit cannot be excluded.

One of the goals of this study was to identify among all known β subunits the best candidate which could act as an authentic β subunit of human, nongastric αHK (AL1). A fundamental criterion for the relevance of a particular β subunit to act as a natural counterpart for a given α subunit is its ability to associate tightly and to support the correct folding of newly synthesized α subunits. Efficient association should be reflected by a certain resistance of the α−β complex to dissociation by detergents (*44*), whereas the correct folding can be probed by the protection of the α subunit from cellular degradation and controlled proteolysis, and by the ability of the α−β complex to leave the ER (*16*). Testing of these parameters indicates that certain β subunits tend to associate preferentially with AL1 but that probably none of the known β subunits fulfils all requirements to function as a real partner subunit of nongastric αHK.

AL1 is indeed well protected against cellular degradation by g βHK, β2NK, and β2-like B bl β, and the α−β complexes can leave the ER (Figure 1). On the other hand, β1NK is much less efficient in this respect and produces only a few stable enzyme complexes that can leave the ER (Figure 1). This particular β selectivity of AL1 stabilization differs from that of g αHK or αNK. Gastric αHK is efficiently stabilized by only g βHK but not by β1NK (*46*) or β2NK (Figure 1), and αNK can be stabilized by all known β subunits (Figure 4). Despite the better protection of AL1 against cellular degradation by gastric βHK, β2NK, and β2-like B bl β than by β1NK, these former β subunits produce AL1−β complexes that are less resistant to controlled proteolysis than α−β complexes known to be authentic, e.g., α1NK−β1NK complexes (Figure 5). In this context, it is interesting to note that nonauthentic g βHK can also stabilize αNK (Figure 4) but it produces αNK−βHK complexes that are sensitive to trypsinolysis (Figure 5) and, as previously shown (*47*), are poorly functional. These results indicate that (1) protection against cellular degradation and acquisition of trypsin resistance of α subunits go in parallel in authentic α−β complexes but are two independent effects of β association and (2) a link may exist between trypsin sensitivity and functionality of α−β complexes. Taking these considerations into account, we could argue that the greater trypsin sensitivity of AL1−β complexes compared to that of authentic αNK−βNK complexes could be a reflection of impaired function. Of course, the possibility that AL1 when associated with β subunits has an intrinsically higher trypsin sensitivity than α1NK associated with β1NK cannot be entirely excluded.

β3NK exhibited still another phenotype when expressed with AL1 in *Xenopus* oocytes. *Xenopus* β3NK produced stable but relatively trypsin-sensitive AL1−β complexes that were impaired in their ability to leave the ER. Human β3NK even promoted degradation of AL1 coexpressed in *Xenopus* oocytes which may reflect a more severe consequence of the same defect.

The low stabilization effect of β1NK, the high trypsin sensitivity of AL1 complexes containing g βHK, β2NK, and B bl β, and the impaired ER exit of AL1−β3NK complexes are all consistent with the hypothesis that none of the known

β subunits is the real β subunit of AL1. This hypothesis is further supported by another characteristic of the AL1−β complexes, namely, the relatively high sensitivity to dissociation of AL1−β complexes by Triton X-100 which reflects weak interactions between the two subunits. Schmalzing et al. (*44*) have shown that all six possible isozymes can be formed after expression of three αNK and two βNK isoforms in *Xenopus* oocytes, but that only α1NK−β1NK and α2NK−β2NK complexes and not α1NK−β2NK complexes are resistant to Triton X-100 dissociation. From these results, it was deduced that some isozymes are preferentially formed which may permit a specific pattern of isozyme expression in cells in which different α and β isoforms are colocalized. We have recently shown that all nine possible isozymes of human Na,K-ATPase can be produced with three αNK and three βNK isoforms (*31*), and in the study presented here, we confirm that α1NK−β1NK but not α1NK−β2NK complexes exhibit strong interactions (Figure 6). With the exception of β2NK, which is readily dissociated from both AL1 and αNK, all other β subunits produce αNK−β complexes that are more resistant to detergent dissociation than AL1−β complexes (Figure 6). This observation may indicate that in cells which coexpress AL1 and αNK with β1NK, β3NK, and/or g βHK, these β subunits would associate preferentially with αNK due to the stronger forces of interaction. Our results, however, also show that the force of interaction may not be the only determinant that governs preferential association of specific α and β subunits in a given cell. Indeed, α1NK−β1NK and α1NK−g βHK complexes exhibit similar high resistance to Triton X-100 dissociation (Figure 6) which excludes the possibility that this characteristic represents a discriminating factor for preventing the formation of heterologous α1NK−g β1HK complexes, e.g., in parietal cells of the stomach that express both these β subunits. In this particular α−β combination, it is not the assembly process itself but the resulting incorrect folding of the αNK, reflected by its trypsin sensitivity (Figure 5) and its poor functionality (*47*), or other stomach-specific factors, which may be responsible for the lack of expression of functional α1NK−g βHK complexes in the stomach.

Our results for the intrinsically low efficiency of association of β1NK with AL1 apparently contrast with observations made by Codina et al. (*20*), who reported that colonic αHK produces a similar increase in ouabain-resistant and Sch-28080-insensitive ^{86}Rb uptake in *Xenopus* oocytes when expressed with either β1NK or g βHK. Even though AL1 is structurally more closely related to other nongastric αHKs than to αNK, we cannot definitively exclude the possibility that human AL1 has β assembly properties different from those of known nongastric αHK of other species. However, it is more likely that the discrepancies in the results may be due to the different parameters that were measured. As mentioned above, we chose criteria for α−β assembly which most truly reflect the intrinsic association efficiency of a β subunit with an α subunit. This is not the case in a unique analysis of the functional expression of pumps at the cell surface. Indeed, in *Xenopus* oocytes and probably also in other cells, overexpression can cause a certain number of slightly misfolded proteins to escape the ER quality control system which normally retains these proteins in the ER and convey them to degradation. Furthermore, *Xenopus* oocytes have an inherent, regulatory system which limits the total number of pumps at the cell surface. Previous studies (*48*) have shown that, for instance, the number of exogenous Na,K pumps at the cell surface does not exceed 6−8 times the number of endogenous Na,K pumps and is therefore, to a certain extent, independent of the number of stable α−β complexes that are formed. As a consequence, inefficiently assembled α−β complexes may indeed produce a similar number of pumps at the cell surface of *Xenopus* oocytes compared to efficiently associated α−β complexes.

In conclusion, our comparative analysis on the effects of different β subunits on the maturation of AL1 and αNK suggests that none of the known β subunits may be the authentic β subunit of AL1 in situ. For all the parameters that were measured, β1NK is the least efficient partner subunit of AL1. With respect to β3NK, it exhibits a particular defect which impedes intracellular transport of α−β complexes and, in the case of human β3NK, even provokes degradation of AL1. Although these results need confirmation in other cells, it is likely that our observations made in the *Xenopus* oocyte indeed reflect a certain incompatibilty of association between AL1 and these two β subunits. On the other hand, g βHK, h β2NK, and β2-like B bl β are able to stabilize newly synthesized AL1 and to form AL1−β complexes that can leave the ER and in the case of nongastric αHK−g βHK (*10*, *11*) and αHK−B bl β (*12*) complexes were shown to be functionally active. Thus, even though these complexes exhibit weak interactions and are relatively trypsin-sensitive, it is likely that authentic β subunits of nongastric αHK resemble these β subunits. At present, we do not know what determines the preferential association of AL1 with these β subunits, but in view of their particular location in the stomach, in the case of g βHK, and in the urinary bladder, in the case of B bl β, it may be suggested that β subunits associated with nongastric αHK must have characteristics which render them particularly apt to resist to harsh environmental conditions. It remains to be shown whether the heavy glycosylation (seven or eight glycosylation sites) common to g βHK, β2NK, and B bl β is an essential feature for the stable cell surface expression of nongastric αHK−β complexes. Of course, even though our results suggest that none of the known β subunits is the authentic β subunit of nongastric αHK, we cannot entirely exclude the possibility that other subunits or proteins which may be missing in the *Xenopus* oocyte system could be necessary and/or facilitate interaction of nongastric αHK with one of the known β subunits.

β-Mediated Maturation of the α Subunit Is a Multistep Process Which Depends on the Membrane Insertion Properties of the α Subunit and on Discrete, Coordinated β-Interaction Events. So far, only little is known about the nature of β interaction sites and their role played in the specificity of β assembly or in the various functions necessary for the maturation of Na,K- and H,K-ATPases. Several domains in the β subunit participate in the association with the α subunit, including the ectodomain (*27*, *49*) and the transmembrane domain (*37*). The results obtained in this study with β chimera indicate that the ectodomain of βHK is more important than the transmembrane domain for efficient assembly with AL1 both for stabilizing the α subunit and for providing a strong interaction as reflected by the detergent resistance of the α−β complexes. This result is consistent with previous observations made with g αHK (*37*).

Concerning determinants in the α subunit that may specify β interaction, Lemas et al. (*50*) have identified a 26-amino acid stretch encompassing the C-terminal end of the extracellular M7−M8 loop and part of M8 in αNK which is essential for association with β subunits. Alignments of these 26 amino acids from αNK and gastric and nongastric αHK show that nearly half of the residues are identical with a strict conservation of a four-amino acid stretch, SYGQ, which, according to results obtained by the two-hybrid assay, are involved in α−β interaction (*27*). It is likely that the SYGQ motif is one of the primary β interaction sites which mediates stabilization of the α subunit (*17*, *26*, *51*), but obviously, it cannot be responsible for the selectivity of β interaction. Whether the sequence surrounding the SYGQ motif plays a role in the access and the selectivity of β assembly remains to be determined.

Significantly, our results obtained with the AL1 chimera containing M9 and M10 of αNK (AL1−α1) indicate that in AL1, interaction of the β subunit and the stabilization of AL1 are not only determined by the existence of the SYGQ motif. Indeed, in contrast to the αNK chimera which contain M9 and M10 of AL1 (α1−AL1), the AL1−α1 chimera loses the ability to associate with and become stabilized by any of the β subunits (Figure 2). This result points to a particular role of M9 and M10 of AL1 in the efficient association with the β subunit, a phenomenon which is consistent with previous observations made with g αHK (*17*). Results obtained with truncated α subunits containing transmembrane segments M1−M8 showed that only αNK M1−M8 (*26*) but not g αHK M1−M8 (*17*) proteins can permanently associate with β subunits and become stabilized. In g αHK, stabilization by the β subunit is only possible after completion of synthesis including M9 and M10. Together with the observation that M7 of g αHK has no signal anchor properties (*17*) while M7 of αNK has partial signal anchor function (*26*), our results suggest that formation of the M9−M10 membrane pair in g αHK, but not in αNK, is necessary for the correct membrane insertion of the M7−M8 pair and in consequence the exposure of the SYGQ assembly motif to the extracytoplasmic side. In view of the similar hydrophobicity of M9 of AL1 and g αHK, which is higher than that of M9 of αNK, it may be suggested that the membrane insertion properties of this domain is similar in AL1 and g αHK. In this case, the lack of assembly of β subunits with the AL1−α1 chimera may not reflect a need of a direct interaction of β subunits with M9 and/or M10 to stabilize AL1 but rather the inability of αNK M9 and M10 to support proper packing of the AL1 protein, which is compatible with assembly in the ectodomains of the two subunits.

Of course, these conclusions do not exclude the existence of intramembrane interactions between α and β subunits which may be important for β selectivity and/or a fine regulation of protein folding. Indeed, our results obtained with the αNK chimera which contains M9 and M10 of AL1 reveal subtle differences in the β-mediated maturation process of the AL−α1 chimera and wild-type αNK. Even though the α1−AL1 chimera can associate with all β subunits and become stabilized (Figure 3) which reflects the correct accessibility of the SYGQ motif to β interaction, only α−β complexes containing βNK isoforms can leave the ER, while α−β complexes containing g βHK or the β NK−HK or β HK−NK chimera are retained in the ER. This result supports the hypothesis that the ultimate correct folding of the α subunit, which is mediated by β assembly, may involve interactions of the two subunits not only in the ectodomains but also in the transmembrane domains. The properties of association of g βHK with AL1, αNK, or the chimeric α subunits illustrate especially well the multiple discrete steps of β assembly that are necessary to ultimately define the overall maturation of the catalytic α subunit. Gastric βHK can stabilize g αHK and AL1, as well as αNK, indicating a correct interaction with the SYGQ motif in these proteins. Gastric βHK can also stabilize the AL1−α1 chimera, but the AL1−α1−g βHK complexes cannot leave the ER. This result illustrates that stabilization through interaction with the SYGQ motif is not the only step in the β-mediated maturation of the α subunit.

In conclusion, our data obtained with the AL1−α1 chimera suggest that transmembrane segments M9 and M10 play a critical role in the permenant association of β subunits with the AL1 protein. Apparently, M9 and/or M10 does not favor interaction with a particular β subunit but rather influences the probabilty of interaction with any of the β subunits tested. On the other hand, the results obtained with the α1−AL1 chimera suggest that M9 and/or M10 in α1NK is not important for β interactions that lead to stabilization of α subunits but nevertheless may be involved in discrete β-mediated folding steps of the α subunit that are necessary for ER exit of α−β complexes. Future studies should also reveal whether M9−M10 domain exchange between AL1 and αNK has any consequences on the functional properties of αNK.

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