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High-affinity ouabain binding by yeast cells expressing Na^+,K^+-ATPase α subunits and the gastric H^+,K^+-ATPase β subunit

(heterologous expression/subunit isoforms/cardiac glycosides)

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ABSTRACT **Recently, a β subunit for the rat gastric H^+,K^+-ATPase (HKβ), which is structurally similar to the β subunit of Na^+,K^+-ATPase, has been cloned and characterized. Using heterologous expression in yeast, we have tested the specificity of β subunit assembly with different isoforms of the α subunit of Na^+,K^+-ATPase. Coexpression in yeast cells of the HKβ with both the sheep α1 subunit and the rat α3 subunit isoforms of Na^+,K^+-ATPase (α1 and α3, respectively) leads to the appearance of high-affinity ouabain-binding sites in yeast membranes. These ouabain-binding sites (α1 plus HKβ, α3 plus HKβ) have a high affinity for ouabain (K_d, 5–10 nM) and are expressed at levels similar to those formed with the rat β1 subunit of Na^+,K^+-ATPase (β1) (α1 plus β1 or α3 plus β1). Potassium acts as a specific antagonist of ouabain binding by α1 plus HKβ and α3 plus HKβ just like sodium pumps formed with β1. Sodium pumps formed with the HKβ, however, show quantitative differences in their affinity for ouabain and in the antagonism of K^+ for ouabain binding. These data suggest that the structure of the β subunit may play a role in sodium pump function.**

The Na^+,K^+-ATPase, also known as the sodium pump, is a member of the extended family of P-type ion-motive ATPases that includes the mammalian gastric H^+,K^+-ATPase, mammalian Ca^{2+}-ATPases from plasma membranes and sarcoplasmic reticulum, the plasma membrane H^+-ATPase from yeast, and the K^+-ATPase from *Escherichia coli* (1). These enzymes couple ATP hydrolysis to the transport of cations across cell membranes. The catalytic subunits (α) of these enzymes share both primary amino acid sequence homology and structural features, such as multiple membrane-spanning domains and a large cytoplasmic domain that binds ATP. Structural isoforms of the Na^+,K^+-ATPase and Ca^{2+}-ATPase enzymes have also been identified. All functional properties of the P-type ATPases, including ATP binding and hydrolysis, cation-binding sites, and binding sites for enzyme inhibitors, appear to reside with the α subunit.

Until recently, the Na^+,K^+-ATPase was thought to be unique in this class of enzymes in its requirement for a second subunit (β). The Na^+,K^+-ATPase β subunit is a 60-kDa glycoprotein (core protein ≈29 kDa), which remains tightly associated with the α subunit even after detergent extraction. Assembly of the β subunit with the Na^+,K^+-ATPase α subunit is required to form an active enzyme complex (2, 3). Using expression of α and β complementary RNAs by injection into *Xenopus* oocytes, Geering and coworker (4, 5) have observed that assembly of α and β subunits takes place in the endoplasmic reticulum of the cell and is accompanied by the maturation of the α subunit to a trypsin-resistant conformation. Assembly and maturation are thought to be necessary for enzyme transport to the cell surface (6). Currently there is no evidence, however, that the β subunit plays a role in the enzymatic functions of the enzyme.

The first Na^+,K^+-ATPase β subunit genes (β1) cloned from a number of species showed a remarkable degree of sequence conservation—>90% identical residues for six different mammalian species. Recently, a second isoform of the Na^+,K^+-ATPase β subunit (β2), which shows only 35% sequence identity to the β1 genes previously characterized, has been cloned from three mammalian species (7, 8). A β subunit for the H^+,K^+-ATPase has also been identified (9, 10) and cloned (11, 12). The H^+,K^+-ATPase β subunit shows limited homology to both β1 (≈30% identity) and β2 (≈35% identity). This diversity suggests that other isoforms of the β subunit may exist, which remain to be discovered. All of the β subunits share the same basic structure of a glycoprotein with a single transmembrane domain and have many conserved structural features, such as the location of glycosylation sites and disulfide-bonded residues.

Previous work has shown that expression of functional Na^+,K^+-ATPase molecules in yeast cells requires the synthesis of both α and β subunits of Na^+,K^+-ATPase (2). Yeast cells are an ideal expression system for these experiments because they lack endogenous Na^+,K^+-ATPase molecules. The expressed Na^+,K^+-ATPase is characterized by high-affinity ouabain binding, ouabain-sensitive ATPase and *p*-nitrophenylphosphatase activities, and ouabain-sensitive ^{86}Rb uptake into the cells (2, 13). Expression of either α or β subunits alone in yeast cells does not lead to the appearance of any of these activities. The expression of Na^+,K^+-ATPase in yeast cells has been used in the experiments described below to examine the assembly of the rat gastric H^+,K^+-ATPase β subunit (HKβ) with different isoforms of Na^+,K^+-ATPase α subunits. Like expression of β1, coexpression of HKβ with Na^+,K^+-ATPase α subunits in yeast cells leads to the appearance of high-affinity ouabain-binding sites in yeast membranes. The β subunit of the H^+,K^+-ATPase, therefore, appears capable of interactions with the Na^+,K^+-ATPase α subunit similar to those of the Na^+,K^+-ATPase β subunit. Ouabain-binding sites formed by HKβ and Na^+,K^+-ATPase α subunits have properties characteristic of functional Na^+,K^+-ATPase, including the antagonism of ouabain binding by millimolar concentrations of K^+. There appear to be quantitative differences, however, between ouabain-binding sites formed by HKβ and Na^+,K^+-ATPase α subunits and ouabain-binding sites formed from β1 and Na^+,K^+-ATPase α subunits in the affinity for ouabain and in the antagonism of ouabain binding by K^+. These differences suggest that the

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Abbreviations: α1, sheep α1 subunit isoform of Na^+,K^+-ATPase; α3, rat α3 subunit isoform of Na^+,K^+-ATPase; β1, β1 subunit isoform of Na^+,K^+-ATPase; HKβ, rat β subunit of gastric H^+,K^+-ATPase.

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structure of the β subunit may affect the enzymatic properties of the Na^+,K^+-ATPase. The cross-assembly of subunits of different types of ion-motive ATPases raises interesting questions about how they might interact—or be prevented from interacting—in mammalian cells that express both types of pumps.

MATERIALS AND METHODS

Materials. The yeast strain 30-4 (*MAT* α, *trp1*, *ura3*, *Vn2*, *GAL*$^+$) was obtained from R. Hitzeman (Genentech) and used for all heterologous expression studies. [^{3}H]Ouabain (specific activity, 15–30 Ci/mmol; 1 Ci = 37 GBq) was purchased from New England Nuclear. Polyclonal rabbit antisera against Na^+,K^+-ATPase (JK594) were obtained from J. Kyte (University of California at San Diego) and monoclonal antibodies against HKβ (2/2E6) were obtained from J. Forte (University of California, Berkeley).

Expression Plasmid Constructions. Clones of the sheep Na^+,K^+-ATPase α1 (α1) cDNA in the vector YEp1PT (YEpαNKA) and the rat β1 cDNA in the vector pG1T (pG1T-Rβ1) have been described (14, 15). A plasmid with the rat Na^+,K^+-ATPase α3 cDNA was the gift of E. Benz (Yale University). A 3.4-kilobase-pair (kbp) *Sac* II–*Nhe* I fragment of this clone, which contained 11 base pairs (bp) of 5′ untranslated sequence and ≈370 bp of 3′ untranslated sequence was isolated. The fragment was treated with T4 DNA polymerase to create blunt ends and was ligated to YEp1PT (16), which had been digested with *Eco*RI, filled in with Klenow fragment, and treated with calf intestinal phosphatase to prevent self-ligation. This plasmid was designated YEpRα3. A plasmid (pRG4) with the HKβ cDNA was obtained from R. Levenson (Yale University) (12). The plasmid was digested with *Apa* I and *Bal* I, and the 900-bp fragment containing the gene was isolated. This fragment was treated with mung bean nuclease to digest the *Apa* I site to a blunt end and ligated to the vector pG1T (15), which was opened with *Bam*HI, blunt-ended with mung bean nuclease, and treated with calf intestinal phosphatase to prevent self-ligation.

Yeast Methods. Standard yeast media were used throughout this study (17). Yeast were transformed using the lithium acetate procedure of Ito *et al.* (18). Passage of transformants on plates for long periods of time was found to decrease expression levels of heterologous proteins, particularly for cells transformed with multiple plasmids. After selection on minimal medium, frozen glycerol stocks were made of transformants and stored at −80°C.

[^{3}H]Ouabain-Binding Assays. A microsomal fraction of yeast cell membranes was isolated as described (2) with the following modifications. After removal of cell debris at 1000 × *g*, yeast lysates were further centrifuged at 6000 × *g* for 20 min at 4°C. The supernatants were loaded on step gradients of 50% sucrose/20% sucrose in 25 mM imidazole·HCl/1 mM Na_2EDTA, pH 7.4, and centrifuged at 100,000 × *g* for 90 min. The membranes at the 20/50 sucrose interface were collected, diluted ≈2-fold with cold buffer, and repelleted. Pellets were suspended in a small volume of 25 mM imidazole·HCl/1 mM Na_2EDTA, pH 7.4. Protein concentrations were assayed by the method of Lowry *et al.* (19).

Assays for [^{3}H]ouabain binding were as described (2) with the following modifications. For comparison of membranes from cultures grown in galactose or membranes from cultures shifted to glucose for 18–24 hr, [^{3}H]ouabain was added to a final concentration of 20 nM. For determination of ouabain affinity and the effect of K^+ on ouabain binding, [^{3}H]ouabain was added to a final concentration of 1 nM, and various concentrations of unlabeled ouabain (1 nM–1 μM) or KCl (1 mM–100 mM) were added to the assays. One milligram of membrane protein was used per assay. Membrane pellets were washed by resuspending in 0.5 ml of ice-cold buffer and recentrifuging (12,000 × *g* for 15 min at 4°C) to decrease background in the assay. Nonspecific binding was determined by adding 1 mM unlabeled ouabain in all assays and was indistinguishable from [^{3}H]ouabain bound to yeast membranes not expressing heterologous proteins.

SDS/PAGE and Immunoblots. Yeast membrane samples were suspended in 2× SDS/PAGE sample buffer and heated at 65°C for 5 min just before loading. Two hundred microgram of protein was loaded per lane onto standard SDS/PAGE gels (4% stacking gel, 8% separating gel) (20). After electrophoresis, proteins were transferred to Immobilon-P membranes (DuPont) (21). Blots were probed with either JK594 antiserum (1:1000 primary antibody dilution) or 2/2E6 ascites fluid (1:5000 primary antibody dilution) and a secondary antibody (1:2000) conjugated to alkaline phosphatase and visualized with 5-bromo-4-chloro-3-indolylphospate and nitroblue tetrazolium.

RESULTS

cDNAs encoding the sheep Na^+,K^+-ATPase α1 (α1) and rat Na^+,K^+-ATPase α3 (α3) isoforms of Na^+,K^+-ATPase α subunit were cloned into the yeast heterologous expression vector YEp1PT (16). cDNAs encoding the rat Na^+,K^+-ATPase β1 subunit (β1 abbreviation hereafter refers to rat) and the HKβ were cloned into the yeast heterologous expression vector pG1T (15). These vectors have different selectable markers for transformation into auxotrophic yeast strains, TRP1 in the case of YEp1PT and URA3 in the case of pG1T. A yeast strain that has multiple auxotrophic defects was simultaneously transformed with different combinations of the α and β expression plasmids. In the pG1T vector, the synthesis of heterologous mRNA is under the control of the *GAL1* promoter. The *GAL1* promoter in yeast is strongly repressed when cells are grown in the presence of glucose and is induced when galactose is used as a carbon source in yeast medium. Thus, β subunit expression in this system depends on growth of transformed cells in galactose medium.

Fig. 1 shows an immunoblot of yeast membranes from cells transformed with different combinations of α and β subunits. Fig. 1*A* was probed with a polyclonal rabbit antiserum to Na^+,K^+-ATPase holoenzyme that reacts with α1, α3, and β1 subunits. Fig. 1*B* was probed with a monoclonal antibody specific for the HKβ. The first two lanes of Fig. 1 *A* and *B* show membranes from cells transformed with the α1 and β1 expression plasmids. These cells were grown either in galac-

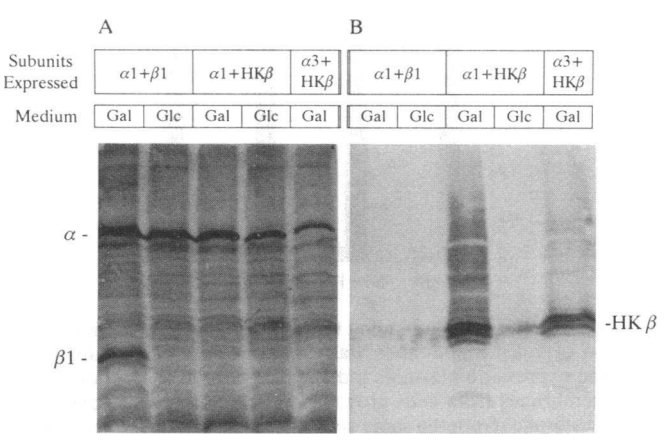

FIG. 1. Immunoblot of ATPase subunits produced in yeast. ATPase expression plasmids are indicated at top. Identical cultures were either grown in galactose (Gal) or shifted to glucose (Glc) for 18 hr before membrane isolation. (*A*) ATPase subunits were probed with anti-Na^+,K^+-ATPase polyclonal antiserum. (*B*) ATPase subunits were probed with an anti-HKβ monoclonal antibody.

tose for the entire incubation time or were shifted from galactose to glucose medium for the final 18 hr of growth to shut off expression of the β subunit. The dark band at ≈110 kDa represents the α1, which is present in comparable amounts in both growth conditions. The band at ≈40 kDa represents core-glycosylated β1 (2). The steady-state level of the β1 protein drops to background levels in membranes from cells shifted to glucose medium. The next two lanes of Fig. 1 *A* and *B* show membranes from cells transformed with the α1 and HKβ expression plasmids, again grown entirely in galactose or shifted to glucose for 18 hr. Although these lanes in Fig. 1*A* show only the presence of α1, Fig. 1*B* demonstrates that a 50-kDa protein corresponding to the core-glycosylated HKβ is produced in cells grown in galactose and repressed when cells are shifted to glucose. The increased size of the HKβ glycoprotein, as compared to the β1, is expected because the predicted amino acid sequence of HKβ indicates that HKβ has additional sites for N-linked glycosylation not present on β1. The diffuse smear seen in lanes with HKβ suggests that at least some of the protein is undergoing hyperglycosylation in yeast. The final lanes of Fig. 1 *A* and *B* show membranes from cells transformed with the α3 and HKβ expression plasmids grown in galactose. In this case Fig. 1*A* shows the presence of a band for α3, whereas Fig. 1*B* confirms the production of HKβ in these cells. These results demonstrate that reasonable steady-state levels of α1, α3, β1, and HKβ proteins are being achieved by heterologous expression in yeast and that β subunit expression depends on growth in galactose medium. In comparison to purified dog kidney Na^+,K^+-ATPase standards, we estimate that these proteins represent ≈0.1% of the total yeast membrane protein.

The membrane samples shown in Fig. 1 were tested for binding of [^{3}H]ouabain. Ouabain binds with high affinity to the phosphoenzyme intermediates of Na^+,K^+-ATPase, and previous work (2) has shown that high-affinity ouabain binding to yeast membranes requires the synthesis of both α and β subunits of Na^+,K^+-ATPase. Coexpression of α1 plus β1, α3 plus β1, α1 plus HKβ, or α3 plus HKβ resulted in the appearance of high-affinity [^{3}H]ouabain-binding sites in yeast membranes (Fig. 2). Membranes of the same transformed yeast cells shifted to glucose medium for 18 hr showed ≈95% decrease in specific [^{3}H]ouabain binding, in parallel with the decrease in β subunit protein levels seen in Fig. 1. The small amount of residual [^{3}H]ouabain binding may represent either ouabain-binding sites in cells before the shift to glucose medium or a very low level of β subunit expression that may continue in glucose medium because the *GAL1* promoter is not always completely repressed for genes present in multiple copies, such as on a 2-μm plasmid (22). In all cases, ouabain binding was detected only in membranes from cells expressing a β subunit, either β1 or HKβ. Similar levels of [^{3}H]ouabain binding were seen with all four combinations, in agreement with the similar levels of subunit protein expression observed on immunoblots (Fig. 1). A titration of [^{3}H]ouabain binding by the different α/β combinations was analyzed by Scatchard plots. The Scatchard plots for all combinations were linear, showing the presence of a single class of high-affinity ouabain-binding sites in each sample under these binding conditions. The results are summarized in Table 1. The α subunit of Na^+,K^+-ATPase is generally thought to be the primary locus for ouabain binding because mutation of as few as two amino acids in the first extracellular loop of α subunit have been shown to convert a high-affinity form of Na^+,K^+-ATPase (IC_{50} = 1–5 × 10^{-8} M) to a low-affinity form (IC_{50} = 1–8 × 10^{-5} M) (23). Comparison of the affinities observed when the same α subunit is expressed with different β subunits suggests that the β subunit may also influence affinity of the complex for ouabain, although to a much lesser extent. In both cases, combination of HKβ with a Na^+,K^+-ATPase α subunit causes a slightly increased affinity for ouabain (Table 1). The differences between β1 and HKβ glycosylation in yeast seen in Fig. 1 could play a role in differences in ouabain affinity.

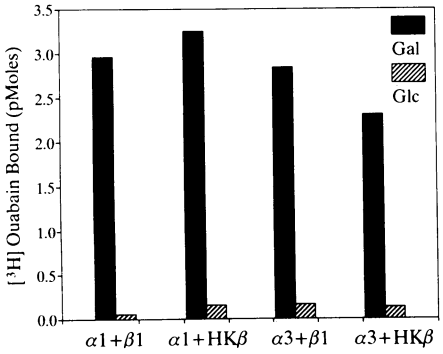

FIG. 2. [^{3}H]Ouabain binding to yeast membranes. A microsomal fraction of yeast membranes was prepared from cells transformed with the expression plasmids indicated at bottom. Solid bars represent membranes from cells grown in galactose; hatched bars represent membranes from the same cells after shift to glucose medium for 18 hr before membrane isolation. Bars represent the average of duplicate points; variation between duplicates was <5% in all cases. Nonspecific binding, determined by addition of 1 mM unlabeled ouabain, ranged from 0.01 to 0.02 pmol and was subtracted from values. Binding to membranes from untransformed yeast cells or to membranes from yeast cells transformed with only one expression plasmid was indistinguishable from nonspecific binding (2).

Binding of ouabain to Na^+,K^+-ATPase is antagonized by millimolar concentrations of extracellular K^+ (24). Ouabain binding to yeast membranes expressing the HKβ subunit in combination with Na^+,K^+-ATPase α subunits is less sensitive to competition with K^+ than membranes expressing the β1 subunit (Fig. 3). In the experiments shown in Fig. 3*A*, [^{3}H]ouabain binding to membranes with α1 plus β1 or α1 plus HKβ was titrated with KCl. Fig. 3*B* shows a similar titration comparing the α3 plus β1 and α3 plus HKβ combinations. In both cases, membranes containing the HKβ required higher concentrations of K^+ to inhibit ouabain binding, and the maximal level of K^+ inhibition is decreased. These results demonstrate that the HKβ subunit decreases the apparent affinity of the enzyme for K^+. Decrease in the maximal level of K^+ inhibition also suggests that the HKβ may also affect conformational transitions of the enzyme. Although these effects require further investigation, these results indicate that the β subunit may play a role in ligand binding and enzymatic activities of the sodium pump. A comparison of Fig. 3 *A* and *B* also reveals that pumps formed with the α1 are more sensitive to K^+ antagonism of ouabain binding than pumps formed with α3.

DISCUSSION

Our results demonstrate that coexpression in yeast of the HKβ with either of two isoforms of the α subunit of Na^+,K^+-ATPase leads to the appearance of high-affinity ouabain-

Table 1. Ouabain binding by yeast membranes expressing different subunit combinations

Subunits expressed	Ouabain binding	
	K_d, nM	B_{max}, pmol/mg of protein
α1+β1	10.1	2.10
α3+β1	10.2	4.25
α1+HKβ	5.8	2.91
α3+HKβ	8.3	3.62

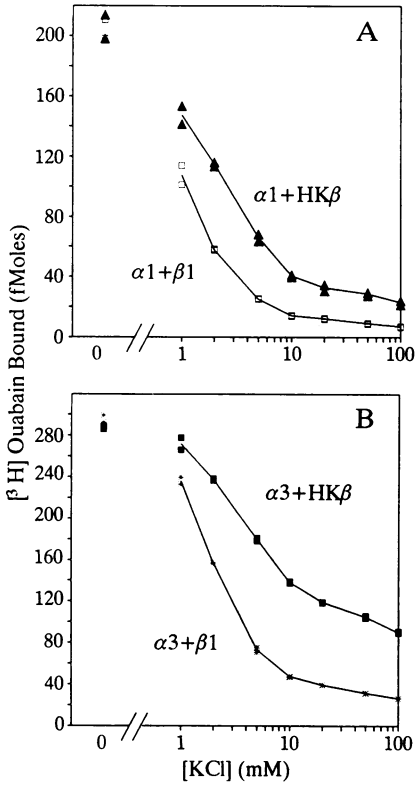

FIG. 3. Effects of KCl on [^{3}H]ouabain binding. Membranes were isolated from yeast cells transformed with indicated subunit combinations and assayed for [^{3}H]ouabain binding in the presence of increased KCl concentrations. Nonspecific binding, determined by addition of 1 mM unlabeled ouabain, was subtracted from values.

binding sites in yeast membranes. High-affinity ouabain binding is a characteristic of the Na^+,K^+-ATPase holoenzyme and requires assembly of the α and β subunits. In the experiments described here, the requirement of HKβ expression for the appearance of ouabain-binding sites is consistent with an interaction between HKβ and α1 or α3, although direct evidence for a physical interaction between the two subunits has not been obtained. The affinity of ouabain-binding sites formed with HKβ is at least as high as the affinity of Na^+,K^+-ATPase itself. The binding of ouabain to Na^+,K^+-ATPase is also known to be antagonized by millimolar concentrations of K^+, which are thought to cause a conformational shift in the enzyme, resulting in increased rate of ouabain dissociation (24). Like sodium pumps formed with β1, ouabain binding by yeast membranes containing HKβ and α subunits of Na^+,K^+-ATPase is also antagonized by millimolar concentrations of K^+. This observation indicates that both β subunits facilitate similar interactions of Na^+,K^+-ATPase α subunits with K^+, an ion transported by H^+,K^+-ATPase and Na^+,K^+-ATPase. Previous work (2, 13) has shown that ouabain binding by yeast membranes indicates an active enzyme complex capable of ATP hydrolysis and ion transport. Although further work is necessary to characterize the ATPase and ion-transport properties of molecules formed in yeast cells with HKβ, the data in this report are consistent with known characteristics of active enzyme complexes. During review of this paper, results were reported showing that injection of mRNA for HKβ and Na^+,K^+-ATPase α subunit into *Xenopus* oocytes yields a small ouabain-sensitive ion current, suggesting that these molecules are functional (25).

The data presented here show quantitative differences both in ouabain affinity and in antagonism of ouabain binding by K^+ when sites formed from Na^+,K^+-ATPase α and β subunits are compared with sites formed from Na^+,K^+-ATPase α and HKβ. These differences may reflect a previously unrecognized role for the β subunit in the enzymatic activity of Na^+,K^+-ATPase and H^+,K^+-ATPase. Previous work has had difficulty in assigning a functional role to the β subunit for primarily two reasons. (*i*) The Na^+,K^+-ATPase is an essential enzyme in all higher eukaryotes. Thus, expression and mutagenesis studies with mammalian cell lines or even *Xenopus* oocytes as host must contend with a background of endogenous sodium pumps. Under these circumstances, orders of magnitude differences are often necessary to detect functional differences in heterologously expressed pumps, such as the difference between ouabain-sensitive and ouabain-resistant pumps (23). The ability to express and assemble pumps in yeast cells, which have no background of endogenous Na^+,K^+-ATPase activity, offers the opportunity to detect more subtle changes in function. (*ii*) The Na^+,K^+-ATPase β1 genes cloned from a number of mammalian species are extremely homologous (>90% identical with one another). This high degree of homology may mean that these clones lack significant structural differences to give rise to observable differences in function. Identification and cloning of other isoforms of β subunits for P-type ATPases that are only 30–35% identical with each other allow a more diverse population of β subunit structures to be examined for effects on sodium pump activity. Besides the HKβ, a second isoform of the Na^+,K^+-ATPase β subunit (β2) has been identified, based on its sequence similarity to the β1 protein (7, 8) and its association with Na^+,K^+-ATPase α subunit (8, 26). Coexpression of the β2 protein in yeast with either α1 or α3 also leads to the appearance of ouabain-binding sites in yeast membranes; however, low levels of β2 protein expression have made characterization of these complexes difficult (K.E., unpublished observations).

Assembly of the α and β subunits of Na^+,K^+-ATPase is a crucial step in formation of an active enzyme complex (4, 5). The observation that HKβ will substitute for β subunit of Na^+,K^+-ATPase in the expression of ouabain-binding sites by yeast cells suggests that both of these polypeptides can assemble with α subunit of Na^+,K^+-ATPase. Although little is known about how the two subunits of Na^+,K^+-ATPase interact, the assembly of oligomeric membrane complexes is generally thought to be highly specific. Thus, upon first inspection, the cross-assembly of an α subunit from one enzyme with the β subunit of another, albeit related, enzyme might seem both surprising and problematic. Examination of the structures of both the α and β subunits suggests that this cross-assembly should not be so surprising after all. Although the HKβ subunit is only ≈30% identical to β1 genes, the Na^+,K^+-ATPase β2 gene also shows this degree of sequence similarity. Moreover, the regions most highly conserved between the β1 and Na^+,K^+-ATPase β2 sequences are also conserved in the HKβ sequence. Likewise, the α subunits of Na^+,K^+-ATPase and H^+,K^+-ATPase are also very similar. Three isoforms of the α subunit of Na^+,K^+-ATPase have been characterized that show ≈85% sequence homology when compared with one another. The α subunit of H^+,K^+-ATPase is ≈65% homologous to Na^+,K^+-ATPase α isoforms—much more closely related than the next most homologous member of the family of P-type ATPases, the Ca^{2+}-ATPase, which is only 25% homologous (1).

Assembly of the HKβ with Na^+,K^+-ATPase α subunits raises several interesting questions. (*i*) How general is this phenomenon? For example, are the Na^+,K^+-ATPase β subunits also capable of assembly with the H^+,K^+-ATPase α subunit? The β subunit of Na^+,K^+-ATPase, once thought to be the only β subunit in the family of P-type ATPases, is part of an emerging family of β subunit proteins (7, 8, 11, 12). These β polypeptides are much more structurally diverse than previously suspected, sharing only 30–40% sequence

identity with one another, and interact not only with Na^+,K^+-ATPase α subunits but also with the H^+,K^+-ATPase α subunit. As other members of the β subunit family are identified, it will be interesting to see whether they are also capable of combinatorial assembly with the α subunits of various P-type ATPases. A ouabain-sensitive H^+,K^+-ATPase has been described in the apical membrane of guinea pig colon (27), and determining the subunit composition of this enzyme will also be of interest. (*ii*) Does the assembly of HKβ with Na^+,K^+-ATPase α subunits occur in mammalian tissues, or are there mechanisms to prevent this assembly? Both Na^+,K^+-ATPase and H^+,K^+-ATPase enzymes are expressed in gastric mucosal cells, where they are specifically localized to different membranes—the H^+,K^+-ATPase to the apical surface and the Na^+,K^+-ATPase to the basolateral surface of the cell. It is not known whether the cross-assembly of subunits of the two enzymes observed by expression in yeast also occurs in these cells. If so, this cross-assembly would preclude models where the β subunit functions as a localization signal to direct enzymes to the appropriate membrane (9). If not, then there must be mechanisms to prevent such cross-assembly. These mechanisms could include a role for chaperoning proteins to prevent incorrect assembly or the spatial or temporal segregation of synthesis and assembly of these two enzymes. (*iii*) Does regulation of the assembly of subunits of ion-motive ATPases play any significant role in physiology and/or pathology? Recent results indicate that regulation of the expression of the β1 subunit by thyroid hormone is tissue-specific (28) and that increases in Na^+,K^+-ATPase activity in regenerating liver are accompanied by increases in β subunit but not in α subunit expression (29). It will be interesting to learn whether the expression of other β subunit isoforms and/or their assembly with different α subunits plays a role in regulating ion transport in response to physiological challenges.

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1. Jorgensen, P. L. & Andersen, J. P. (1988) *J. Membr. Biol.* **103,** 95–120.
2. Horowitz, B., Eakle, K. A., Scheiner-Bobis, G., Randolph, G. R., Chen, C. Y., Hitzeman, R. A. & Farley, R. A. (1990) *J. Biol. Chem.* **265,** 4189–4192.
3. Noguchi, S., Mishina, M., Kawamura, M. & Numa, S. (1987) *FEBS Lett.* **225,** 27–32.
4. Ackermann, U. & Geering, K. (1990) *FEBS Lett.* **269,** 105–108.
5. Geering, K. (1990) *J. Membr. Biol.* **115,** 109–121.
6. McDonough, A. A., Geering, K. & Farley, R. A. (1990) *FASEB J.* **4,** 1598–1605.
7. Martin-Vasallo, P., Dackowski, W., Emanuel, J. R. & Levenson, R. (1989) *J. Biol. Chem.* **264,** 4613–4618.
8. Gloor, S., Antonicek, H., Sweadner, K. J., Pagliusi, S., Frank, R., Moos, M. & Schachner, M. (1990) *J. Cell Biol.* **110,** 165–174.
9. Okamoto, C. T., Karpilow, J. M., Smolka, A. & Forte, J. G. (1990) *Biochim. Biophys. Acta* **1037,** 360–372.
10. Hall, K., Perez, G., Anderson, D., Gutierrez, C., Munson, K., Hersey, S. J., Kaplan, J. H. & Sachs, G. (1990) *Biochemistry* **29,** 701–706.
11. Shull, G. E. (1990) *J. Biol. Chem.* **265,** 12123–12126.
12. Canfield, V. A., Okamoto, C. T., Chow, D., Dorfman, J., Gros, P., Forte, J. G. & Levenson, R. (1990) *J. Biol. Chem.* **265,** 19878–19884.
13. Scheiner-Bobis, G., Eakle, K. A. & Farley, R. A. (1991) in *The Sodium Pump: Recent Developments*, eds. Kaplan, J. H. & De Weer, P. (Rockefeller University Press, New York), pp. 43–47.
14. Horowitz, B. & Farley, R. A. (1988) in *The Na^+,K^+-Pump, Part B: Cellular Aspects*, eds. Skou, J. C., Norby, J. G., Maunsbach, A. B. & Esmann, M. (Liss, New York), pp. 85–90.
15. Eakle, K. A., Horowitz, B., Kim, K. S., Levenson, R. & Farley, R. A. (1991) in *The Sodium Pump: Recent Developments*, eds. Kaplan, J. H. & De Weer, P. (Rockefeller University Press, New York), pp. 125–129.
16. Hitzeman, R. A., Leung, D. W., Perry, L. J., Kohr, W. J., Levine, H. L. & Goeddel, D. V. (1983) *Science* **219,** 620–625.
17. Sherman, F., Fink, G. R. & Lawrence, C. W. (1979) *Methods in Yeast Genetics: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
18. Ito, H., Fukuda, Y., Murata, K. & Kimura, A. (1983) *J. Bacteriol.* **153,** 163–168.
19. Lowry, O. H., Rosebrough, N. J., Farr, A. S. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–273.
20. Laemmli, U. K. (1970) *Nature (London)* **227,** 680–685.
21. Small, G. M., Imanaka, T. & Lazaro, P. B. (1988) *Anal. Biochem.* **169,** 405–409.
22. Fuller, R. S., Brake, A. & Thorner, J. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 1434–1438.
23. Price, E. M. & Lingrell, J. B. (1988) *Biochemistry* **27,** 8400–8408.
24. Forbush, B. (1983) *Curr. Topics Membr. Transport* **19,** 167–201.
25. Horisberger, J.-D., Jaunin, P., Reuben, M. A., Lasater, L. S., Chow, D. C., Forte, J. G., Sachs, G., Rossier, B. C. & Geering, K. (1991) *J. Biol. Chem.* **266,** 19131–19134.
26. Shyjan, A. W., Gottardi, C. & Levenson, R. (1990) *J. Biol. Chem.* **265,** 5166–5169.
27. Suzuki, Y. & Kaneko, K. (1989) *Am. J. Physiol.* **256,** G979–G988.
28. Horowitz, B., Hensley, C. B., Quintero, M., Azuma, K. K., Putman, D. & McDonough, A. A. (1990) *J. Biol. Chem.* **265,** 14308–14314.
29. Lu, X. P. & Leffert, H. L. (1991) *J. Biol. Chem.* **266,** 9276–9284.