Domain Swapping between Na,K- and H,K-ATPase Identifies Regions That Specify Na,K-ATPase Activity†

Victor A. Canfield and Robert Levenson*

Department of Pharmacology, Penn State College of Medicine, Milton S. Hershey Medical Center, P.O. Box 850, Hershey, Pennsylvania 17033

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ABSTRACT: We have used expression of chimeras between the structurally related Na,K- and H,K-ATPase α subunits to localize regions that determine Na,K-ATPase activity. Segments of the rat Na,K-ATPase α1 subunit were replaced by the corresponding portions of the rat gastric H,K-ATPase α subunit, and the constructs were transfected into ouabain-sensitive human HEK 293 cells. Using the ability to transfer ouabain resistance as a measure of sodium pump activity, we identified segments within the sodium pump that could be replaced with proton pump sequences without the loss of biological activity. These functionally interchangeable segments encompassed approximately 75% of the amino acid differences between the two transporters. Segments that could not be exchanged mapped to three discrete regions. One region spans residues 63−117 and includes the first transmembrane (TM) segment and a portion of the amino-terminal cytoplasmic domain. The second, from residue 320 to residue 413, encompasses TM 4 and a portion of the third cytoplasmic domain, while the third region (encompassing residues 735−861 and 898−953) includes several TM domains in the carboxyl-terminal portion of the ATPase. Our results suggest that functional differences between Na,K- and H,K-ATPase, including differences in ion transport specificity, are likely to reside within these noninterchangeable segments.

The P-type ATPases are a family of transporters which utilize energy derived from the hydrolysis of ATP to transport cations across cellular membranes. These ion pumps (which include Ca^{2+}-ATPases, heavy-metal ATPases, and the H^+-ATPases of fungi and plants) contain structurally similar catalytic (α) subunits that possess binding sites for ATP and the transported cations (*1*). Within the P-type ATPase family, Na,K- and H,K-ATPases comprise a subfamily characterized by similarities in sequence and gene organization. In addition to the α subunit, Na,K- and H,K-ATPases also possess a glycosylated *β* subunit, a polypeptide whose exact cellular function has not been established (*2, 3*). The mammalian gastric H,K-ATPase α subunit exhibits ∼60% sequence identity to each of the Na,K-ATPase α subunit isoforms (*4*). In view of the high degree of sequence and predicted secondary structure similarity, the sequences responsible for functional differences between Na,K- and H,K-ATPase, including their ion-binding and ion-transport properties, may reside within discrete segments of the α subunit polypeptides.

Na,K-ATPase and the gastric H,K-ATPase utilize the energy derived from the hydrolysis of ATP to transport cations across the plasma membrane against their concentration gradients (*5*). Na,K-ATPase promotes the active exchange of Na^+ for K^+, whereas the H,K-ATPase catalyzes the exchange of H^+ for K^+ ions. For both Na,K- and H,K-ATPase, ion transport is a function of the α subunit. However, the regions within the α subunit polypeptide responsible for ion transport selectivity have not been identified. Several different strategies have been employed in an attempt to identify sites resposible for ion binding and transport. Experiments involving extensive proteolysis suggest that ion binding sites are located within transmembrane segments of the Na,K-ATPase (*6*). Chemical modification experiments have implicated specific carboxylate residues within the α subunit that appear to contribute to ion binding (*7, 8*). In general, however, site-directed mutagenesis experiments have failed to substantiate a direct role for these residues in ion binding or transport (*9*).

We have made chimeric transporters in an attempt to identify regions that are responsible for the differences in ion transport between the Na,K-ATPase and H,K-ATPase α subunits. We previously developed a biological assay for analyzing Na,K-ATPase cDNAs based upon the ability of a transfected Na,K-ATPase α subunit to substitute for the endogenous Na,K-ATPase and rescue ouabain-sensitive cells from ouabain cytotoxicity (*10, 11*). We have now used this assay to determine whether segments of the Na,K-ATPase can be replaced by H,K-ATPase sequences without compromising Na,K-ATPase function. Using this approach, we have found that regions constituting approximately 75% of the sequence differences between Na,K-ATPase and H,K-ATPase α subunits are functionally interchangeable. Nonexchangeable regions localized to three discrete segments within the α subunit. These segments are likely to contain sequences that specify functional differences, such as ion transport properties, between Na,K- and H,K-ATPase.

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* Corresponding author. Telephone: (717) 531−4545. Fax: (717) 531−5013. Email: Levenson @neuro.hmc.psu.edu.

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EXPERIMENTAL PROCEDURES

DNA Constructs. Chimeric constructs were generated by replacement of segments of rat Na,K-ATPase α1 subunit cDNA with the corresponding regions of rat H,K-ATPase α subunit cDNA. Chimeras are depicted schematically in Figure 1, and junctions between Na,K- and H,K-ATPase segments are described in Table 1. Chimeras HN and NH were generated by ligating Na,K-ATPase and H,K-ATPase α subunit cDNAs at a conserved *Nar*I restriction site. Chimera HN contains residues 1−520 of the H,K-ATPase fused to residues 511−1023 of the Na,K-ATPase α subunit, while chimera NH contains residues 1−510 of the Na,K-ATPase fused to residues 521−1034 of the H,K-ATPase α subunit. Chimeras I, II, III, IV, VI, and VII were generated by engineering restriction sites present in the Na,K-ATPase α1 subunit into the corresponding region of H,K-ATPase α subunit cDNA by the polymerase chain reaction (PCR) method. Chimera V was constructed by replacing an *Nar*I−*Nco*I restriction fragment of the Na,K-ATPase α subunit (aa 511−734) with the corresponding segment of the H,K-ATPase α subunit (aa 521−744). Chimera VIII was generated by replacing residues 954−1023 in the Na,K-ATPase with a cDNA fragment encoding residues 965−1034 of the H,K-ATPase α subunit. Segments of constructs derived by PCR mutagenesis were verified by DNA sequencing. Full-length chimeric cDNAs and a full-length H,K-ATPase β subunit cDNA (*3*) were subcloned into the eukaryotic expression vector pCB6 (*12*). Junctions in additional chimeras are described in Table 2. Epitope-tagged chimeras were generated by replacing the C-terminal *Eco*RI restriction fragment of a chimeric Na,K-/H,K-ATPase cDNA with the corresponding segment of an Na,K-ATPase construct containing the influenza virus hemagglutinin (HA) tag at residue 1013 (*13*).

Cell Culture, DNA Transfection, and Immunolocalization. Human embryonic kidney (HEK) 293 cells were used as recipients for transfection. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (FCS). Transfections were performed by the calcium phosphate coprecipitation method described previously (*14*). Chimeric α subunit cDNAs were transfected alone (10 μg) or in combination with the H,K-ATPase β subunit (5 μg of each plasmid). For stable transfectants, cultures were split 1:4 48 h after transfection, and medium containing 0.5 μM ouabain was added 4 h later. Colonies of transfected cells were picked in 2−3 weeks and expanded into cell lines that were maintained in medium containing 0.5 μM ouabain. Immunofluorescence microscopy was performed on transiently transfected cells as described previously (*14*). The HA tag was detected using the HA-specific monoclonal antibody 12CA5 (Boehringer Mannheim) and visualized with Cy-3 conjugated second antibody (Jackson Immunoresearch). Confocal laser scanning microscopy was performed using a Zeiss LSM 210 confocal microscope (Rh filter block, 543-nm excitation wavelength) as previously described (*14*).

RESULTS

Biological Assay for Na,K-ATPase Function. We have used expression of chimeric Na,K-/H,K-ATPase cDNAs as a strategy for localizing sequences that specify functional differences between Na,K- and gastric H,K-ATPase. The feasibility of this approach relies on the fact that the Na,K- and H,K-ATPase α subunits exhibit a high degree of structural similarity, thus making it likely that the overall structural integrity of chimeric α subunits will not be compromised. To localize segments that distinguish Na,K- from H,K-ATPase activity, we have generated a panel of chimeric ATPases in which segments of the ouabain-resistant rat Na,K-ATPase α1 subunit were replaced by the corresponding segments of the ouabain-insensitive H,K-ATPase α subunit. The biological activity of a chimera is measured by an assay of its ability to confer ouabain resistance upon ouabain-sensitive human fibroblasts. We have previously used this approach to carry out structure−function analysis of the Na,K-ATPase α subunit (*11, 13*−*15*). Transfer of ouabain resistance indicates that the transfected α subunit can substitute for the endogenous α subunit of recipient cells. Because endogenous sodium pumps are poisoned in the presence of ouabain, rescue from ouabain cytotoxicity provides a clear demonstration that the transfected α subunit contributes to a functional Na,K-ATPase. Conversely, failure of a cDNA to transfer ouabain resistance indicates that the chimera is unable to substitute for the endogenous Na,K-ATPase.

Table 1: Na,K-/H,K-ATPase α Subunit Chimeras

chimera	H,K-ATPase segment[a]	junction sites	Na,K-ATPase segment(s)*
HN	1−510	*Nar*I	511−1023
NH	511−1023	*Nar*I	1−510
I	1−62	*Stu*I	63−1023
II	63−173	*Stu*I; *Asu*II	1−62; 174−1023
III	175−318	*Asu*II; *Xho*I	1−174; 319−1023
IV	320−510	*Xho*I; *Nar*I	1−319; 511−1023
V	511−734	*Nar*I; *Nco*I	1−510; 735−1023
VI	685−861	*Kpn*I; *Pfl*MI	1−684; 862−1023
VII	863−953	*Pfl*MI; *Bam*HI/*Bgl*II	1−862; 954−1023
VIII	954−1023	*Bam*HI/*Bgl*II	1−953

[a] Amino acid numbers refer to the position of residues within the rat Na,K-ATPase α1 subunit (numbered from initiating methionine).

Identification of Regions That Specify Na,K-ATPase Function. A panel of 10 chimeric Na,K-/H,K-ATPase α subunits was constructed (Table 1 and Figure 1) and transfected into HEK 293 cells. In each chimera, a single segment of the rat H,K-ATPase α subunit has been substituted for the corresponding region of the rat Na,K-ATPase α1 subunit. Together, these substituted H,K-ATPase sequences span the entire length of the Na,K-ATPase α1 subunit (Figure 1). As previously demonstrated (*13*), wild-type rat Na,K-ATPase α1 subunit cDNA confers ouabain resistance upon HEK 293 cells at high efficiency (∼250 colonies/10 μg of DNA/10^6 cells). Transfection of the rat gastric H,K-ATPase α subunit into HEK 293 cells never produced ouabain-resistant colonies (>15 separate experiments), indicating that the H,K-ATPase α subunit cannot substitute for the Na,K-ATPase α subunit in this assay. Chimeras in which the amino-terminal half of the H,K-ATPase α subunit was fused to the carboxyl-terminal half of the NaK-ATPase α1 subunit (chimera HN), or the amino-terminal half of the Na,K-ATPase α1 subunit was fused to the carboxyl-terminal half of the H,K-ATPase α subunit (chimera NH), failed to transfer the ouabain-resistant phenotype. These results suggest that sequences responsible for functional differences between Na,K-ATPase

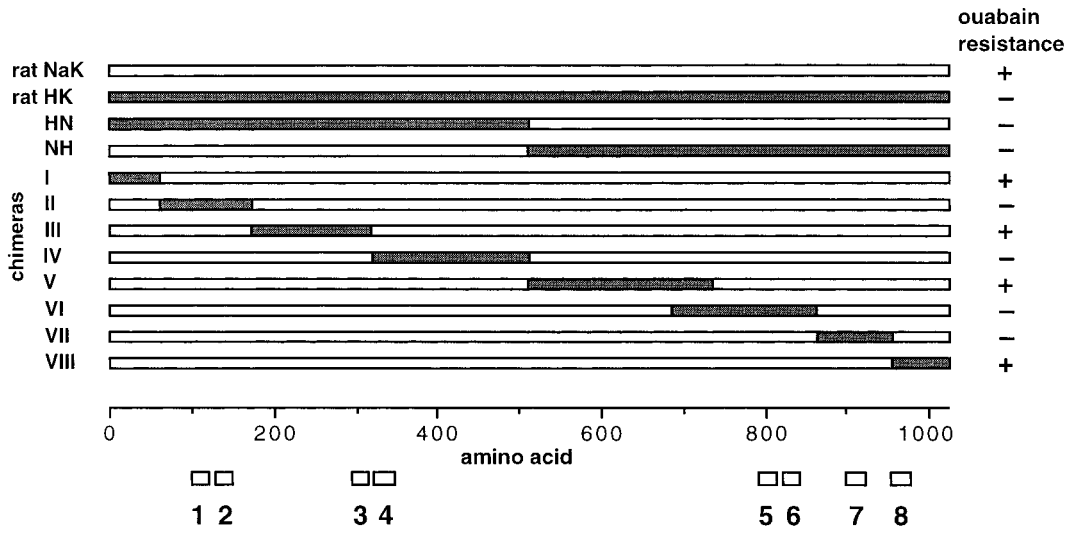

FIGURE 1: Schematic representation of Na,K-/H,K-ATPase α subunit chimeras. Rat Na,K-ATPase α1 subunit sequences are shown as open boxes, while rat gastric H,K-ATPase α subunit sequences are shown as closed boxes. Scale (every 200 amino acids) is shown below. Predicted transmembrane domains (*14*) are indicated by numbered open boxes at bottom. The ability (+) or failure (−) of a construct to confer ouabain resistance upon HEK293 cells is indicated at the right.

and H,K-ATPase are likely to reside within both the amino- and carboxyl-terminal halves of the α subunit polypeptide.

Chimeras I, III, V, and VIII were found to confer ouabain resistance upon HEK 293 cells. In these chimeras (Figure 1), H,K-ATPase α subunit sequences were substituted for Na,K-ATPase α subunit sequences in the regions spanning residues 1−62 (I), 175−318 (III), 511−734 (V), and 954−1023 (VIII). These segments include the amino and carboxyl termini, a large portion of the cytoplasmic loop between TM 2 and TM 3, and most of the large cytoplasmic domain between TM 4 and TM 5. The ability of these chimeras to transfer ouabain resistance suggests that the substituted segments are functionally equivalent in the Na,K- and H,K-ATPase α subunits. In contrast, chimeras II, IV, VI, and VII were unable to transfer ouabain resistance, either alone, or when cotransfected with the H,K-ATPase β subunit. The failure of these chimeras to transfer ouabain resistance indicates that several segments within the Na,K- and H,K-ATPase are not interchangeable. These segments, spanning residues 63−173 (domain A), 320−510 (domain B), and 735−953 (domain C) of the Na,K-ATPase α subunit, are likely to contain sequences that distinguish Na,K-ATPase from H,K-ATPase activity.

The noninterchangeable regions defined by chimeras II, IV, VI, and VII were more precisely defined by subdividing domains A, B, and C into smaller regions containing H,K-ATPase sequences (Table 2). Chimeric constructs that subdivide domains B and C are shown schematically in Figure 2. Based on the ability of chimera IVB to confer ouabain resistance upon HEK 293 cells, residues 425−520 of the H,K-ATPase are functionally interchangeable with residues 415−510 of the Na,K-ATPase. In contrast, chimera IVA failed to transfer ouabain resistance (Figure 2A), indicating that the segment spanned by residues 320−413 contains sequences that are essential for Na,K-ATPase function. Chimera VIIA, containing H,K-ATPase sequences at positions 863−897 of domain C, was capable of transferring ouabain resistance (Figure 2B). Substitution of Na,K-ATPase sequences with H,K-ATPase sequences at positions 735−785 (chimera VIA), 786−861 (chimera VIB), 899−941 (chimera VIIB), and 942−953 (chimera VIIC) produced α subunits that failed to confer ouabain resistance upon HEK 293 cells. These chimeras define four subsegments of domain C that cannot be exchanged between Na,K- and H,K-ATPase α subunits. Cotransfection of chimeric α subunit and H,K-ATPase β subunit cDNAs failed to produce ouabain-resistant colonies. It is likely that each of these nonexchangeable segments contains residues that contribute to functional differences between the Na,K- and H,K-ATPase.

Table 2: Chimeric Na,K-/H,K-ATPase α Subunit Constructs: Subdivision of Domains A, B, and C

chimera	HK segment	junction sites[a]
IIA	63−117	*Stu*I; PCR
IIB	119−128	PCR
IIC	130−173	PCR; *Asu*II
IID	118−129	PCR
IVA	320−413	*Xho*I; *Aat*II
IVB	415−510	*Aat*II; *Nar*I
VIA	735−785	*Nco*I; *Bsp*EI
VIB	786−861	*Bsp*EI; *Pfl*MI
VIIA	863−897	*Pfl*MI; *Bgl*II
VIIB	898−941	*Bgl*II; *Eco*RI
VIIC	942−953	*Eco*RI; *Bam*HI/*Bgl*II

[a] Junctions labeled 'PCR' did not incorporate restriction sites.

Segments within domain A (residues 63−173) that may contribute to the specificity of Na,K-ATPase activity were localized by expression of a panel of Na,K-/H,K-ATPase chimeras that subdivide this region of the α subunit (Figure 3). This segment includes the first extracellular loop of the α subunit, a domain that contributes to the ouabain sensitivity of Na,K-ATPase (*15*, *16*). Charged residues (R118 and D129) at the borders of this extracellular loop have been shown to be responsible for the natural ouabain resistance of the rodent α1 subunit, while neutral residues at these positions (L118, Q118, and N129) are present in ouabain-sensitive α subunit isoforms. Although H,K-ATPase is insensitive to ouabain, it also possesses neutral residues (Q128 and N139) at the TM 1−TM 2 borders. The failure of chimera II to transfer ouabain resistance may result from the inability of residues 73−183 of H,K-ATPase to substitute

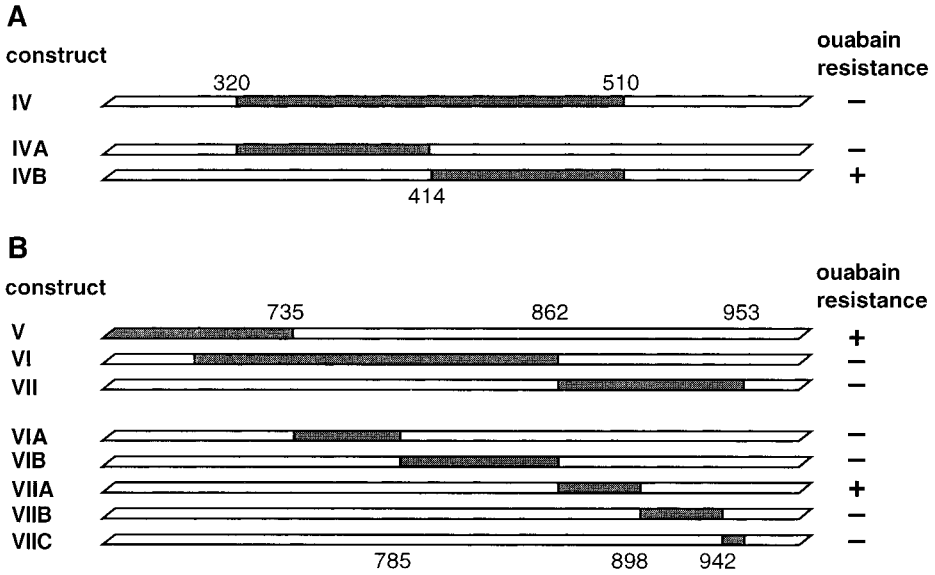

FIGURE 2: Subdivision of chimeric constructs. (A) Chimera IV, in which residues 320−509 of Na,K-ATPase (domain B) were replaced with the corresponding residues of H,K-ATPase, was further subdivided to produce chimeras IVA and IVB, containing H,K-ATPase segments from residues 320−413 and 415−510, respectively. (B) Domain C, defined by chimeras V, VI, and VII, was subdivided to produce chimeras VIA, VIB, VIIA, VIIB, and VIIC. These chimeras were produced by replacing residues 735−785 (VIA), 786−861 (VIB), 863−897 (VIIA), 898−941 (VIIB), and 942−953 of the Na,K-ATPase α1 subunit with the corresponding sequences from the H,K-ATPase α subunit. Rat Na,K-ATPase segments are represented by open boxes, while H,K-ATPase sequences are closed boxes. Positions of junction sites are designated by amino acid number. The ability (+) or failure (−) of a construct to confer ouabain resistance is shown at the right.

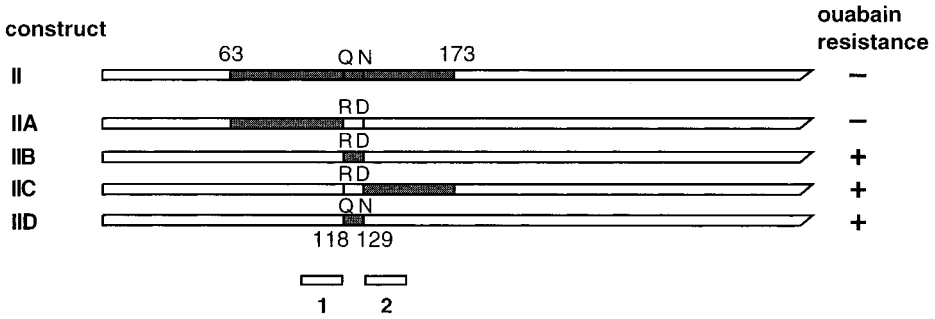

FIGURE 3: Subdivision of domain A. Chimera II, in which residues 63−173 of the Na,K-ATPase α1 subunit (domain A) were replaced with the corresponding H,K-ATPase α subunit sequences, was further subdivided. Chimeras IIA, IIB, IIC, and IID contain H,K-ATPase segments from residues 63−117, 119−129, 130−173, and 118−129, respectively. Open boxes are Na,K-ATPase segments, and closed boxes are H,K-ATPase segments. Positions of junctions are indicated by the amino acid sequence number. Amino acids at boundaries of the first extracellular loop are indicated (single-letter amino acid code). Positions of TM 1 and TM 2 are indicated by open boxes at the bottom. Ouabain resistance (+) or the sensitivity (−) of constructs is shown at the right.

for the corresponding Na,K-ATPase sequences (residues 63−173). Alternatively, this chimera may produce a ouabain-sensitive α subunit due to the presence of neutral residues at the TM 1−TM 2 border positions. To distinguish between these possibilities, we constructed and expressed chimeras IIA, IIB, and IIC, containing H,K-ATPase segments at positions 63−117, 119−128, and 130−173, respectively (Figure 3). Each of these constructs retains the charged border residues (R118 and D129) that are responsible for the natural ouabain resistance of the rat Na,K-ATPase α1 subunit. A fourth chimera (IID), containing the entire first extracellular loop of the H,K-ATPase, and including neutral residues Q118 and N129, was also tested for ouabain resistance. As shown in Figure 3, chimeras IIB and IIC were able to confer ouabain resistance upon HEK 293 cells, whereas chimera IIA failed to transfer the ouabain-resistant phenotype, either alone or in combination with the H,K-ATPase β subunit. Surprisingly, chimera IID also proved capable of producing ouabain-resistant colonies. Taken together, these results suggest that residues 118−173 are interchangeable between Na,K- and H,K-ATPase. In contrast, residues 63−117 are not exchangeable but instead appear to contain sequences that distinguish Na,K- from H,K-ATPase activity. Our data also show that replacement of the entire first extracellular loop of the Na,K-ATPase α subunit with H,K-ATPase sequences produces a ouabain-resistant ATPase. These results therefore demonstrate that sequences within the first extracellular domain, in addition to the border residues, can contribute to ouabain resistance.

Subcellular Localization of Na,K-/H,K-ATPase Chimeras. The inability of a chimeric Na,K-/H,K-ATPase construct to transfer ouabain resistance implies that the region within the Na,K-ATPase that has been replaced by H,K-ATPase sequences is essential for sodium pump function. However, it is also possible that some chimeras fail to transfer ouabain resistance because the proteins are rapidly degraded or fail to reach the plasma membrane. To determine whether chimeric ouabain-sensitive Na,K-/H,K-ATPase α subunits were targeted to the plasma membrane, we examined the cellular distribution of chimeras II, IV, VI, and VII by

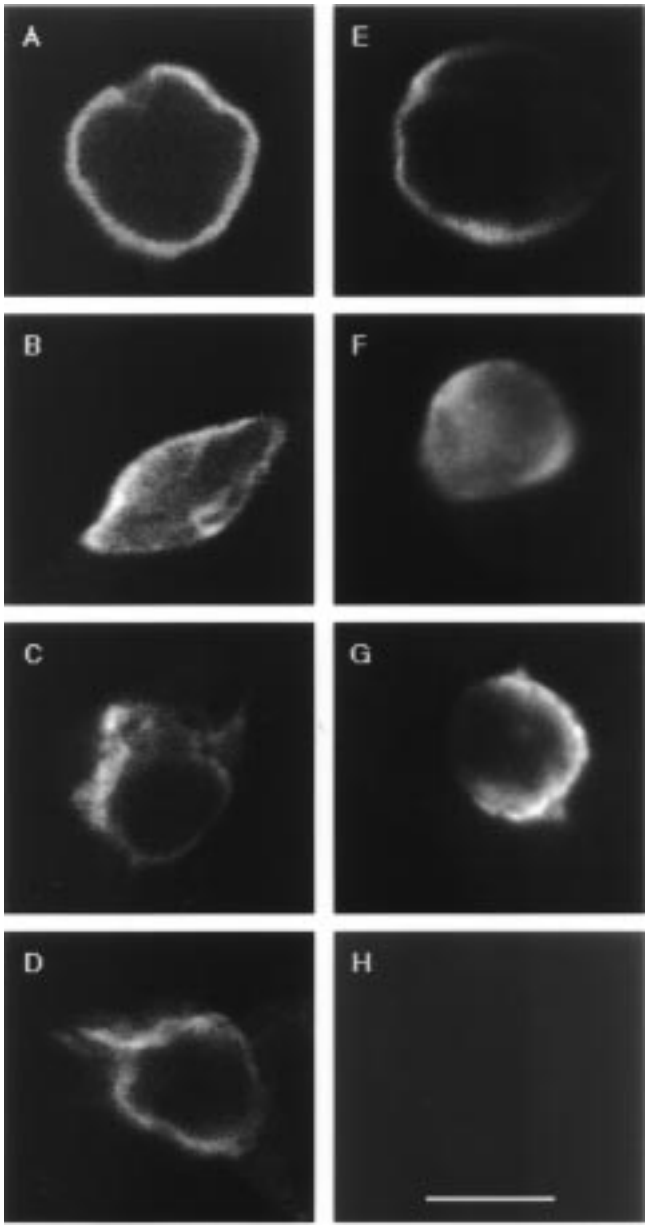

FIGURE 4: Visualization of epitope-tagged constructs by confocal microscopy. HEK 293 cells expressing epitope-tagged chimeras were fixed and permeabilized. The HA epitope was detected using the 12CA5 antibody and Cy3-conjugated anti-mouse antibodies. (A) I−HA; (B) II−HA; (C) III−HA; (D) IV−HA; (E) V−HA; (F) VI−HA; (G) VII−HA; (H) untagged chimera VIII. Optical sections were scanned through cells ~2 μm above the coverslip. Scale bar = 10 μm.

immunofluorescence and confocal microscopy. To visualize chimeric α subunit polypeptides, we generated chimeric cDNAs containing an HA tag at position 1013 near the carboxyl terminus of the α subunit. We have previously shown that wild-type rat Na,K-ATPase α1 cDNA carrying an HA tag at this position was capable of conferring ouabain resistance upon HEK 293 cells (*13*). Each of the epitope-tagged chimeras was transiently transfected into HEK 293 cells and visualized using the HA epitope-specific monoclonal antibody 12CA5 (*17*). Representative cells expressing each epitope-tagged chimeric α subunit are shown in Figure 4. As positive controls, we monitored immunoreactivity of the tagged versions of chimeras I, III, and V (Figure 4A,C,E, respectively). Examination of HEK 293 cells expressing each of these constructs revealed bright staining at cell margins. These results are consistent with a predominantly plasma membrane localization of the expressed proteins. HA-tagged chimeras IV and VII (Figure 4D,G, respectively) gave a similar pattern of membrane-associated staining. Chimeras II and VI (Figure 4B,F) produced α subunits that were detectable in the plasma membrane, although a significant amount of cytoplasmic staining was observed as well. These results suggest that both ouabain-resistant and ouabain-sensitive chimeric α subunits are properly targeted to, and resident within, the plasma membrane of transfected cells. The ability to detect an epitope tag situated at the carboxyl terminus of the chimeras provides assurance that the chimeric polypeptides are full length. The failure of chimeras II, IV, VI, and VII to confer ouabain resistance therefore does not appear to be due to the instability or mislocalization of the chimeric α subunit polypeptides.

DISCUSSION

Expression of chimeric Na,K-/H,K-ATPase cDNAs has been used to identify segments of the α subunit that specify Na,K-ATPase, rather than H,K-ATPase, activity. We identified five discrete segments within the rat Na,K-ATPase α1 subunit that can be replaced by the corresponding segments of the gastric H,K-ATPase without compromising Na,K-ATPase function. These segments span residues 1−62, 118−318, 415−735, 863−898, and 953−1023, and comprise 73% of all amino acid differences between the Na,K-ATPase and H,K-ATPase. Although these functionally interchangeable segments are likely to encode properties that are common to the Na,K- and H,K-ATPases, some of the segments exhibit a surprisingly high degree of amino acid sequence divergence. For example, the regions spanning residues 1−62 and 863−897 exhibit only 31−33% amino acid sequence identity between Na,K- and H,K-ATPase, while the region located at residues 954−1023 exhibits 43% amino acid identity. The other interchangeable segments show 64%−68% amino acid identity, slightly greater than the overall 62% identity between these transporters. The ability to replace segments of the Na,K-ATPase with highly divergent H,K-ATPase sequences suggests that these non-conserved segments are likely to have a structural rather than a catalytic role in ATPase function.

The regions within the Na,K-ATPase that can be exchanged with H,K-ATPase sequences encompass ~70% of the α subunit. In this context, it is interesting to consider whether it would be possible to construct a biologically active α subunit that contains all the exchangeable H,K-ATPase segments. Expression of a chimera incorporating the substitutions of chimeras IVB and V (aa 415−510 and 511−735) produced oubain-resistant colonies at high efficiency (Canfield and Levenson, unpublished data). However, a chimera containing a combination of substitutions from constructs V and VIII (aa 511−735 and 954−1023) failed to give ouabain resistance. These results suggest that replacement of all of the exchangeable Na,K-ATPase domains with H,K-ATPase segments within a single chimera will not produce a functional ATPase. It is possible that chimeras have reduced rates of transport compared to the wild-type Na,K-ATPase, and that introduction of multiple H,K-ATPase segments reduces Na^+ transport below the level necessary to support cellular viability. Alternatively, sub-

FIGURE 5: Amino acid sequence alignments between nonexchangeable regions of Na,K- and H,K-ATPase α subunits. For each of the nonexchangeable regions, the Na,K-ATPase sequence is shown above the corresponding H,K-ATPase sequence. Amino acid numbers are shown at the left and right of each segment. Bullets indicate amino acid differences between the sequences. The open triangle indicates the position of a single amino acid insertion in the H,K-ATPase sequence relative to the Na,K-ATPase sequence.

stitution of multiple H,K-ATPase segments may compromise the structural integrity of the chimeric protein.

Our experiments define four regions within the Na,K-ATPase α subunit that cannot tolerate substitution with H,K-ATPase sequences without loss of Na,K-ATPase function (Figure 5). These regions include the segments spanning residues 63−117, 320−413, 736−861, and 899−952. The regions spanning residues 63−117 and 899−952 are each 56% identical between Na,K- and H,K-ATPase, while the regions spanning residues 320−413 and 736−861 are 79% and 71% identical, respectively, between the two transporters. Residues that specify functional differences between Na,K-ATPase and H,K-ATPase, including ion binding selectivity, are likely to be contained within these nonexchangeable segments. Identification of ion binding sites within the Na,K-ATPase α subunit has been difficult to achieve. For example, chemical modification experiments using *N*,*N*′-dicyclohexylcarbodiimide (DCCD) and 4-(diazomethyl)-7-(diethylamino)coumarin (DEAC) have suggested that E960 (*7*) and E786 (*8*), respectively, are involved in cation binding. However, site-directed mutagenesis experiments appear to exclude these residues (as well as E334, D933, and E961) from direct roles in cation binding (*9*). In contrast, site-directed mutation of residues D811 and D815 abrogates competition between K^+ and ouabain binding (*9, 18*). It is possible that D811 and D815 may be directly involved in K^+ binding. Because D815 is conserved between Na,K- and H,K-ATPase, it is unlikely that this residue contributes to the specificity of Na^+ vs H^+ binding. In contrast, D811 is replaced by E in H,K-ATPase. Introduction of the D811→E mutation into the Na,K-ATPase completely abolishes ATPase activity, K^+-dependent competition with ATP and ouabain binding, and Na^+-dependent phosphorylation (*9, 18*). Thus, it is possible that D811 may contribute to the specificity of Na^+ vs H^+ binding. The inability of chimera VIB, containing H,K-ATPase sequences at position 811, to transfer ouabain resistance is consistent with the view that this segment may contain residues that constitute part of the Na^+ binding site.

Two of the nonexchangeable segments that we have identified contain only a few amino acid differences between Na,K- and H,K-ATPase. For example, the segment 735−785, which extends from a position near the FSBA [5′-(*p*-fluorosulfonyl)benzoyladenosine]-reactive lysine through the putative fifth TM segment, is very highly conserved, containing only 6 substitutions over a stretch of 51 amino acids (Figure 5). This high degree of conservation suggests a functionally important role for this segment in ATP binding or in energizing ion transport. Five of the six differences in this region have been found in all Na,K- and H,K-ATPases α subunits that have been sequenced to date. One of the five residues is an invariant serine (S782) in Na,K-ATPases, and a lysine in the H,K-ATPase. A single amino acid substitution at this serine (S782→A) has been shown to completely inactivate the Na,K-ATPase (*19*). It is therefore possible that this residue may play an important role in distinguishing Na,K- from H,K-ATPase function. Within the 10 amino acid segment spanned by residues 943−952, the H,K-ATPase contains 3 conservative substitutions and an amino acid insertion (M949→FF) relative to Na,K-ATPase (Figure 5). Each of the three conservative substitutions occurs at amino acids that are variable among Na,K-ATPase isoforms or between Na,K- and H,K-ATPases of different species. These substitutions are therefore unlikely to be responsible for differences between Na,K- and H,K-

ATPase function. In contrast, the M949→FF insertion is found in all H,K-ATPases that have been examined, and represents the only insertion/deletion in the entire alignment between Na,K-ATPase and H,K-ATPase α subunits with the exception of the amino terminus. It is therefore likely that in chimera VIIB, it is the M949→FF insertion that leads to inactivation of Na,K-ATPase activity. Using site-directed mutagenesis, it should now be possible to test whether amino acid substitutions or insertions at this position alter the ion binding properties of the Na,K-ATPase.

The expression system we have used provides an assay only for Na,K-ATPase function. H,K-ATPase does not confer ouabain resistance upon transfected cells. Therefore, chimeras that possess H,K-ATPase activity will score as negatives in this assay. Because the ability to measure H,K-ATPase transport activity in transfected cells has not yet been achieved (*20*), we cannot distinguish chimeras that are nonfunctional from those that possess H,K-ATPase activity. However, our subcellular localization experiments indicate that chimeras containing nonexchangeable segments are targeted to the plasma membrane, suggesting that these chimeras are not grossly misfolded or mislocalized. Although it is possible that chimeras which fail to transfer ouabain resistance may encode ouabain-inhibitable Na,K-ATPases, we view this possibility as unlikely, since all of the chimeras were constructed from the naturally ouabain-resistant rat Na,K-ATPase α1 subunit and the ouabain-insensitive H,K-ATPase. Further characterization of chimeras that fail to transfer ouabain resistance will require generation of stable cell lines that express these constructs. Blostein and co-workers (*21*) have generated a Na,K-/H,K-ATPase chimera (H519N) essentially identical to our HN chimera, and shown that this chimeric α subunit is capable of catalyzing Rb uptake. However, we have found that chimera HN is unable to transfer ouabain resistance, suggesting that neither chimera HN nor H519N is likely to encode functional sodium pumps.

Na,K-ATPase and H,K-ATPase exhibit an absolute requirement for association with their respective β subunits for activity. Reconstitution experiments suggest that the Na,K-ATPase α subunit can associate with the H,K-ATPase β subunit (*22*). In contrast, the H,K-ATPase α subunit does not appear capable of assembly with the Na,K-ATPase β subunit (*23*). Failure of a chimeric Na,K-/H,K-ATPase to confer ouabain resistance may reflect the fact that the chimera requires the H,K-ATPase β subunit for assembly. In particular, chimera VIIB contains H,K-ATPase sequences (aa 895−920) within a segment of the Na,K-ATPase that has previously been shown to promote α−β subunit interaction (*24*, *25*). However, coexpression of chimera VIIB (or any of the other chimeras that failed to transfer ouabain resistance) with the H,K-ATPase β subunit never produced ouabain-resistant colonies. These results therefore rule out the possibility that any of the chimeric constructs failed to transfer ouabain resistance due to lack of an appropriate β subunit.

Expression of chimeric Na,K-/H,K-ATPase α subunits has revealed a previously unrecognized aspect of Na,K-ATPase structure/function. Na,K-ATPase is the cellular receptor for ouabain and other cardiac glycosides (*26*). Previous experiments have shown that residues located at the borders of the first extracellular loop are major determinants of the ouabain sensitivity of the Na,K-ATPase. Replacement of the entire first extracellular loop of the rat Na,K-ATPase α1 subunit (RSATEEEPPNDD) with the corresponding region of the H,K-ATPase (QASEGDLTTDDN) in chimera IID produced an α subunit capable of transferring ouabain resistance. This result is somewhat surprising in view of the fact that the substituted loop now contains the neutral border residues (Q118 and N129) that have been shown to account for the natural ouabain sensitivity of the sheep α1 subunit (*16*). Our data therefore indicate that residues within the TM 1−TM 2 loop, in addition to the border residues, can contribute to ouabain resistance. This conclusion is supported by the observation that replacement of the first extracellular loop of the rat α1 subunit with a modified HA epitope tag (QYPYDVPDYADN) also produces ouabain resistance (Canfield and Levenson, unpublished data). This substituted loop also contains neutral residues (Q and N) at its boundaries. Site-directed mutagenesis experiments have revealed that residues P125 and D128 of the α subunit appear to contribute to the ouabain sensitivity of Na,K-ATPase (*27*, *28*). Replacement of the TM 1−TM 2 bridge with either H,K-ATPase or HA sequences retains D128, but introduces the substitutions P125→T and P125→D, respectively. Because previously described mutations at P125 produced only weak ouabain resistance, our results suggest that multiple residues within the first extracellular loop contribute to the ouabain-resistant phenotype. This interpretation is consistent with the view that the overall conformation of the first extracellular loop, in addition to its charge, is important for the interaction of the Na,K-ATPase with cardiac glycosides.

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