Subunit Interactions in the Na,K-ATPase Explored with the Yeast Two-hybrid System*

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Subunit interactions of the α- and β-subunits of the chicken Na,K-ATPase were explored with the yeast two-hybrid system. Gal4-fusion proteins containing domains of the α- and β-subunits were designed for examining both intersubunit and intrasubunit protein-protein interactions. Regions of the α- and β-subunits known to be involved in α-β-subunit assembly were positive in two-hybrid assay, supporting the validity of the assays. A library of β-subunit ectodomains with C-terminal truncations was screened to find the maximal truncation retaining an interaction with the α-subunit extracellular H7H8 loop (where H7 refers to the seventh membrane span, and so on). The maximal truncation removed all the cysteines involved in disulfide bridges, leaving only 63 amino acids of the β-subunit ectodomain. Scanning alanine mutagenesis led to identification of an evolutionarily conserved sequence of four amino acids (SYGQ) in the extracellular H7H8 loop of the α-subunit that is crucial to α-β-intersubunit interactions. Oligomerization studies with single domains failed to detect self-association of either of the two large cytosolic loops (H2H3 and H4H5) within the α-subunit. However, evidence was found for an interaction between these two cytoplasmic loops.

The Na,K-ATPase is an integral membrane protein that plays a central role in ionic homeostasis in animals by mediating the translocation of Na⁺ and K⁺ ions across the plasma membrane against their electrochemical gradients. The active Na,K-ATPase is a heterodimer comprised of a 100-kDa α-subunit that spans the plasma membrane 10 times, and a 40–60-kDa glycoprotein β-subunit that has a short cytoplasmic N-terminal domain, a single transmembrane domain, and a large extracellular domain. Both subunits are required for Na⁺ and K⁺ ion transport (1–3). The α-subunit contains the cation binding sites and the sites of ATP binding and phosphorylation, and it is therefore sometimes referred to as the catalytic subunit. The β-subunit is involved in the structural and functional maturation of the holoenzyme (4, 5) and transport to the plasma membrane (2, 6), and it appears to influence K⁺ sensitivity (7, 8). The α- and β-subunits assemble in a noncovalent, glycosylation-independent manner during or soon after biosynthesis (9, 10), and assembly is required for exit from the endoplasmic reticulum (11).

Identification of domains involved in assembly of Na,K-ATPase subunits has been approached in previous studies by immune precipitation experiments that have involved co-expression of truncated β-subunits (12, 13) and chimeras between the Na,K-ATPase α-subunit and either sarcoplasmic/endo-plasmic Ca-ATPase (14–16) or the gastric H,K-ATPase catalytic subunit (17–19). Expression of Na,K/Ca-ATPase chimeric catalytic subunits together with the avian β-subunit in mammalian cells, usually in the T7 RNA polymerase-based expression system (20), has allowed us to define a 26-amino acid segment within an extracellular loop of the Na,K-ATPase α-subunit that is necessary and sufficient for assembly of the chimeras with the Na,K-ATPase β-subunit. To define further the specific amino acids involved in Na,K-ATPase subunit interactions, we have employed the yeast two-hybrid assay system (21, 22).

There is evidence that the Na,K-ATPase exists as an (α-β)₂ heterotetramer in cell membranes, at least during some portion of the transport cycle (23–25). Thus, one would expect that, in addition to sites of α-β-subunit assembly, there must be sites at which α-β-subunit heterodimers interact to form the native tetramers. Blanco et al. (26) demonstrated that α-β oligomers containing two different isoforms of the α-subunit could be purified by immune precipitation from detergent-solubilized rat brain membranes and that α-α dimers formed when two α-subunit isoforms were co-expressed in SF-9 insect cells. In studies reported here, we used the yeast two-hybrid system to screen for intrasubunit interactions in the major cytosolic domains of the α-subunit and in the extracellular domain of the β-subunit.

In the two-hybrid system assay, plasmids are constructed that encode two hybrid proteins: one consists of the DNA-binding domain of the transcription factor Gal4 fused to one test protein, X, and the other consists of the Gal4 activation domain fused to another test protein, Y. These plasmids are transformed into a Saccharomyces cerevisiae strain that contains reporter genes whose regulatory region contains Gal4 binding sites. Either hybrid protein alone must be unable to activate transcription of the reporter genes. The DNA-binding domain hybrid should not activate transcription because it does not provide the activation function, whereas the activation domain hybrid also should not activate transcription because it cannot localize to the Gal4 binding sites. Interaction of the two test proteins reconstitutes the function of the Gal4 transcription factor and results in expression of the reporter genes, which are detected by assays for the reporter gene products. For our studies, a set of plasmids encoding Gal4-fusion proteins that contained elements of the Na,K-ATPase α- and β-subunits were constructed and used in the two-hybrid system to explore intersubunit interactions and to look for intrasubunit interactions. The results of these experiments are presented in this report.

EXPERIMENTAL PROCEDURES

Construction of Plasmids Encoding Hybrid Proteins—All of the hybrid constructs were created using amplification by polymerase chain

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reaction (PCR). The PCR reactions contained 10 ng of template pBlueScript SK+ plasmid (Stratagene) containing a cDNA encoding either the chicken Na,K-ATPase α1- or β1-subunit, 100 ng of each primer (see below), 1 unit of Perkin-Elmer Taq DNA polymerase, 50 mM KCl, 20 mM Tris-HCl, pH 8.3, 1.5 mM MgCl2, 0.001% gelatin, and 0.2 mM of each of the four deoxynucleotide triphosphates (Pharmacia Ultrapure), in a reaction volume of 0.1 ml, overlaid with 50 μl of mineral oil (Sigma). Amplification was performed for 30 cycles with a temperature profile of 1 min at 95 °C, 1 min at 42 or 50 °C, and 1 min at 72 °C.

All of the PCR fragments were digested with the appropriate restriction enzymes (BamHI, BglII, NcoI, and/or Smal) overnight at room temperature or for 16–24 h at 37 °C for the other restriction enzymes. The digested PCR products were purified by agarose gel electrophoresis, electroeluted, ligated overnight at 15 °C into both the pAS2 and pACT2 vectors for use in the yeast two-hybrid system (27), and transformed into Escherichia coli DH5α competent cells (Boehringer Mannheim). Ampicillin-resistant colonies were screened for the presence of the PCR fragment by restriction analysis of their plasmids.

The nucleotide sequences of candidate plasmids were determined, and the desired plasmids were used in the transformations described below.

DNA encoding the α-subunit cytoplasmic loop 1 (H2H3) was constructed using the PCR primers TC126 (5′-GATCCCGGGCAAGAAGG-GAAAGATTGCAAG-3′) and TC121 (5′-GATTCGATCTCCCTATG-GCGGACTAATGAC-3′). This loop contains amino acids Glu63 to Ser304 of the chicken Na,K-ATPase α1-subunit, numbering residues beginning with N-terminal glycine of the mature protein as residue 1. The DNA encoding the α-subunit cytoplasmic loop 2 (H4H5) was constructed using the PCR primers TC128 (5′-GATCCCGGGGTAACTGATGTCT-GACACTA-3′) and TC119 (5′-GATCCGCTCTCAGAATGTTGGTGAATCC-3′) and TC120 (5′-GATCGA TCTGTTGCTATGCGAATGGATACCCTCC-3′). This loop contains amino acids Val335 to Leu765 of the β1-subunit, numbering residues beginning with N-terminal glycine of the mature protein. The DNA encoding the extracellular β1X94 domain was constructed using PCR primers TC105 (5′-GATCCGGATCCGCTGGATTTGAAAGCAAC-3′) and TC110 (5′-ATCCGGGATCCGGAGAATGGTTCAGCTCGGAT-3′). This domain contains amino acids Glu63 to Ser304 of the chicken Na,K-ATPase α1-subunit, numbering residues beginning with N-terminal glycine of the mature protein.

DNA encoding the β-subunit ectodomain was constructed using the PCR primers TC115 (5′-GCGGATCCGGCTGCTTTTTATGTCAAATT-TACGTC-3′) and TC116 (5′-ATCGGGAATCCGGTATGCTGACACTA-3′). This domain contains amino acids Met866 to Ala914 of the chicken Na,K-ATPase β1-subunit. The DNA encoding the EC49 loop was constructed using the PCR primers TC117 (5′-GATCCGGATCCGCTGGATTTGAAAGCAAC-3′) and TC118 (5′-ATCGGGAATCCGGTATGCTGACACTA-3′). This loop contains amino acids Val335 to Leu765 of the β1-subunit.

For alanine scanning mutagenesis, both strands of DNA were synthesized and hybridized together to form double-stranded DNA fragments for cloning into the pAS2 and pACT2 vectors. The DNA encoding the EC49 loop was digested with two restriction enzymes, cleaving the DNA just 3′ of the desired restriction site and leaving a 5′ overhang in the direction of the mature protein. The 5′ overhang is resistant to exonuclease III digestion. The 3′ overhang is required for β-galactosidase activity. Transformant cells were then plated directly onto sterile Whatman number 1 filters that had been layered onto selective growth media. After colonies had grown, the filters were assayed for β-galactosidase activity. The cells were permeabilized by a freeze of the filters in liquid nitrogen and thawing to room temperature. Each filter is then soaked with 2 ml of β-galactosidase assay solution (0.2 M NaCl, 5 mM EDTA, pH 8.0), and incubated at 70 °C for 5 min to inactivate the exonuclease III enzyme. The digestion was then ethanol-precipitated and redissolved in 20 μl of μg proteinase K buffer (New England Biolabs) (66 mM Tris-HCl, pH 8.0, and 0.66 mM MgCl2), 100 units of exonuclease III enzyme (New England Biolabs) were added, and the digestion mixture was incubated at 37 °C. The exonuclease III digestion rate under these conditions is approximately 3000 bases per min. At 2-min intervals, 5 μl of aliquots were removed, mixed with STOP solution (0.2 M NaCl, 5 mM EDTA, pH 8.0), and incubated at 70 °C for 10 min to inactive the exonuclease III enzyme. The digested DNA was then ethanol-precipitated and redissolved in 20 μl of μg of bovine testicle deoxyribonuclease (Boehringer Mannheim) buffer (10 mM Tris-HCl, pH 8.0, 1 mM ZnSO4), 10 units of μg of bovine testicle deoxyribonuclease (Boehringer Mannheim) were added, and the reactions quenched after 2 min. Plasmid DNA was digested with two restriction enzymes, cleaving the DNA just 3′ of the desired restriction site and leaving a 5′ overhang in the direction of the mature protein. The 3′ overhang is required for β-galactosidase activity. Transformant cells were then plated directly onto sterile Whatman number 1 filters that had been layered onto selective growth media. After colonies had grown, the filters were assayed for β-galactosidase activity. The cells were permeabilized by a freeze of the filters in liquid nitrogen and thawing to room temperature. Each filter is then soaked with 2 ml of β-galactosidase assay solution (0.2 M NaCl, 5 mM EDTA, pH 8.0), and incubated at 70 °C for 5 min to inactivate the exonuclease III enzyme. The digestion was then ethanol-precipitated and redissolved in 20 μl of μg of bovine testicle deoxyribonuclease (Boehringer Mannheim) buffer (10 mM Tris-HCl, pH 8.0, 1 mM ZnSO4), 10 units of μg of bovine testicle deoxyribonuclease (Boehringer Mannheim) were added, and the reactions quenched after 2 min. Plasmid DNA was digested with two restriction enzymes, cleaving the DNA just 3′ of the desired restriction site and leaving a 5′ overhang in the direction of the mature protein. The digestion was then ethanol-precipitated and redissolved in 20 μl of μg of bovine testicle deoxyribonuclease (Boehringer Mannheim) buffer (10 mM Tris-HCl, pH 8.0, 1 mM ZnSO4), 10 units of μg of bovine testicle deoxyribonuclease (Boehringer Mannheim) were added, and the reactions quenched after 2 min. Plasmid DNA was digested with two restriction enzymes, cleaving the DNA just 3′ of the desired restriction site and leaving a 5′ overhang in the direction of the mature protein.
**Na,K-ATPase Subunit Interactions**

### Fig. 1. Diagram of the sodium pump, denoting the regions of the α- and β-subunits that were used in yeast two-hybrid assays.

The line representing the polypeptide chain of each subunit is thickened in regions used in making Ga4 fusion proteins for this study, and the lengths of line segments are proportional to the number of aminoacyl residues they contain. The N and C termini are indicated, and membrane spanning regions are indicated by boxes and numbered 1-10 for the α-subunit. The positions of the three disulfide bridges in the β-subunit are indicated by close approximations along the line representing the β-subunit polypeptide chain, and the positions of the N-glycosylation sites are symbolized by shaded ovals. In the Ga4 fusion proteins, the sodium pump component was always at the C terminus.

β-subunit showed that these fusion proteins form protein-protein interactions resulting in activation of transcription of the β-galactosidase reporter gene. Fig. 1 is a diagram of the Na,K-ATPase, indicating the regions of the α- and β-subunits that were analyzed in two-hybrid assays. Table I shows the results indicating that neither pAS αEC49 nor pACTβ alone activates transcription. Table II shows that when both the pAS αEC49 and pACTβ are co-expressed, β-galactosidase transcription is activated. These results demonstrate that these α and β peptides as fusion proteins in yeast retain their ability to assemble and therefore are compatible for use in the two-hybrid system.

**aEC49 (H7H8) Interacts with the Extracellular βX149 and βX96 Truncated Domains—Hamrick et al. (13) showed that the avian Na,K-ATPase β-subunit, truncated by 92 or 146 residues from the C terminus, remained competent to form αβ complexes when expressed in mammalian cells. As an extension of these experiments, we constructed GAL4-fusion proteins containing deletions in the extracellular β domain that were the same as those of Hamrick et al. (13), that is deletions of 92 and 146 amino acids from the C terminus. These β deletions were named βX149 and βX96 because they retain 149 and 96 aminoacyl residues of the β-subunit ectodomain, respectively (see Fig. 2). These deletion constructs co-expressed with the αEC49 (H7H8) fusion protein yielded positive results in the two-hybrid assay (Table II), suggesting that the N-terminal 96 amino acids of the extracellular domain of the β-subunit (Glu^{63}-Asn^{158}) are sufficient to form a protein-protein interface with the EC49 extracellular loop (H7H8) of the α-subunit.

**Further Truncations of the β-Subunit Extracellular Domain—**To determine the minimal C-terminal extent of the β-subunit involved in protein-protein interactions with the αEC49 (H7H8) α-domain, we performed a series of exonuclease III digests on the pACTβ construct from the 3’ end. The pools of pACTβ deletions were transformed into yeast together with the pAS αEC49 (H7H8) construct. The co-transformants were plated onto media lacking histidine. If the co-expressed fusion proteins interacted, they would cause transcription of a histidine reporter gene, allowing those co-transformants to grow on histidine-deficient medium. Two separate experiments were performed, resulting in a total of 25 transformant colonies after 12 min of exonuclease III digestion. The DNA from each of these 25 colonies was isolated and used as a template in PCR reactions primed by the primers for the βX96 construct (TC150 and TC190). This PCR screen was used to look for any deletion that encoded less than the 96 amino acids of the extracellular β domain already identified. The results of this screening indicated that three of the β deletions contained fewer than 96 amino acids of the extracellular β domain. Then, a set of nested oligonucleotide primers was used to estimate the extent of coding region remaining in the three β deletion clones. From this experiment, it appeared that the shortest β truncation encoded no more than 63 amino acids and possibly as few as 61. Finally, to test this conclusion, site-directed mutagenesis was performed on the pACTβX96 plasmid, introducing a stop codon after the codon for Asp^{125} of the β-subunit ectodomain. This construct, pACTβX63, co-expressed with pAS αEC49, yielded positive results in the yeast two-hybrid system, confirming that Glu^{63} to Asp^{125} is a sufficient extent of the extracellular β-subunit domain for interaction with the α-subunit EC49 loop (H7H8). These results suggest that the three disulfide loops of the β-subunit are not required for interaction with αEC49 (H7H8) and that no more than the 63 amino acids adjacent to the transmembrane domain are necessary for this interaction. This region is shaded in the diagram of the β-subunit in Fig. 2.

**Scanning Alanine Mutagenesis of the αEC49 Domain—**To identify individual amino acids involved in the α-β intersubunit interaction, we constructed Ga4-fusions with altered forms of the EC49 α-subunit domain (H7H8) that contained

| Construct | β-Galactosidase activity |
|-----------|-------------------------|
| pAS β     | +                       |
| pAS βX149 | −                       |
| pAS βX96  | −                       |
| pAS cytoplasmic α-loop 1 | − |
| pAS cytoplasmic α-loop 2 | − |
| pACT αEC49 | −                       |
| pACT αEC49 Ala 1 | − |
| pACT αEC49 Ala 2 | − |
| pACT αEC49 Ala 3 | − |
| pACT αEC49 Ala 4 | − |

### Table I

**Two-hybrid constructs tested individually**
of the charides. Disulfide bridges (50, 51) are also shown. The initial segment C-terminal residues of truncated and b cluster of residues well-conserved among all the known a found in this study to interact with the H7H8 loop of the (see Fig. 3), and these constructs were co-expressed with alanine scanning variants of the EC49 region were constructed (see Fig. 3), and these constructs were co-expressed with the Gal4-β-subunit constructs pACTβ, pACTβX149, and pACTβX96 in yeast two-hybrid assays. Table III shows the results of the two-hybrid assays. The fusion proteins EC49 Ala1, EC49 Ala3, and EC49 Ala4 all demonstrate positive protein-protein interactions with all three β fusion proteins. These results suggest that the 12 mutated aminoacyl residues may not be crucial to protein-protein interactions between the α- and β-subunits. However, the fusion protein EC49 Ala2 did not show positive protein-protein interactions with any of the three Gal4-β-subunit fusion proteins. These results suggest that the group of four highly conserved amino acids (SYGQ) is directly involved in protein-protein interactions between the α- and β-subunits.

A Search for Homotypic α-Subunit or β-Subunit Interactions—There is substantial evidence that the Na,K-ATPase exists largely as (α-β)2 dimers in cell membranes. The yeast two-hybrid system was used to seek elements of the α-subunit that might be involved in α-α interactions; similarly, we sought evidence for β-β interactions. The two largest individual cytosolic domains of the α-subunit (see Fig. 1) were tested for self-association: α cytoplasmic loop 1 (H2H3) with itself, and α cytoplasmic loop 2 (H4H5) with itself. The results in Table IV demonstrate that in two-hybrid assays no α-α self-associations occurred. Likewise, tests for oligomerization involving the ectodomain of the β-subunit were mostly negative (Table IV). Only the βX96 fusion protein demonstrated any oligomerization interactions with itself. These results are inconclusive as to whether biologically significant β-β-subunit interactions occur. The lack of positive results with the larger ectodomain suggests that β-β interactions are not biologically meaningful. The full-length β-subunit ectodomain could not be tested for β-β interactions because the β-subunit ectodomain fused to the Gal4 DNA-binding domain was positive when expressed in yeast by itself.

| Construct | DNA-binding domain | Activation domain | β-Galactosidase activity |
|-----------|--------------------|-------------------|--------------------------|
| pAS αEC49 | pACT β             | +                 |
| pAS αEC49 | pACT βX149        | +                 |
| pAS αEC49 | pACT βX96         | +                 |
| pAS β    | pACT αEC49        | +                 |
| pAS βX149| pACT αEC49        | +                 |
| pAS βX96 | pACT αEC49        | +                 |

**DISCUSSION**

While the major application of the two-hybrid system has been for screening cDNA libraries to find clones encoding proteins that bind some target protein, the same methodology is useful for identifying domains or amino acids involved in interactions between proteins that are known to interact. Many combinations of proteins have been used successfully in the two-hybrid assay. These combinations include many nuclear,
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... and membrane-spanning domains, although these are not ab-
membrane protein. It is important to note some caveats about
domains of subunit interaction in a multi-subunit plasma
study is the first to utilize the two-hybrid system to define
cytoplasmic, mitochondrial, and viral proteins but only a few
membrane-associated proteins (22). To our knowledge, this
study is the first to utilize the two-hybrid system to define
domains of subunit interaction in a multi-subunit plasma
membrane protein. It is important to note some caveats about
the studies reported here. First, α- and β-subunit interactions
that are essential to the processes involved in assembly of the
Na,K-ATPase in the endoplasmic reticulum need not be enti-
tirely the same as sites of subunit interaction in the mature,
active enzyme. Second, while studies on assembly identify do-
main and residues that are important to the α and β contacts,
they do not distinguish those elements that indirectly regulate
the α-β interface from those that are the interface. This same
caveat, of course, applies to virtually all studies that involve
analysis of perturbations in protein structure. Third, while
these experiments reveal some domains and residues that are
at interfaces, they do not identify all relevant interfaces. We
know, for example, that subunit assembly of the Na,K-ATPase
is more efficient when the β-subunit includes its cytoplasmic
and membrane-spanning domains, although these are not ab-
olutely necessary for assembly (13, 30).

β-Subunit Interactions with the α-Subunit H7H8 Loop—To
define features of the β-subunit necessary and sufficient for
assembly with the α-subunit, Renaud et al. (12) began by mak-
ning mutations in the avian β1-subunit and assaying for the
ability of mutant β-subunits to assemble with mouse α-sub-
units in transfected mouse L-cells. These experiments showed
that the cytosolic 33 amino acids at the N terminus of the
β-subunit were not required for assembly. A set of small dele-
tions in the membrane-spanning region of the β-subunit
were not required for assembly. However, subunit assembly
was not detected between avian α-subunits and a chimera
consisting of the cytosolic and membrane spanning domains of

**TABLE III**

| DNA-binding domain | Activation domain | β-Galactosidase activity |
|--------------------|------------------|-------------------------|
| pAS αEC49         | pACT β           | +                       |
| pAS αEC49 Ala 1   | pACT β           | +                       |
| pAS αEC49 Ala 2   | pACT β           | -                       |
| pAS αEC49 Ala 3   | pACT β           | +                       |
| pAS αEC49 Ala 4   | pACT β           | -                       |
| pAS αEC49         | pACT βX149       | +                       |
| pAS αEC49 Ala 1   | pACT βX149       | +                       |
| pAS αEC49 Ala 2   | pACT βX149       | -                       |
| pAS αEC49 Ala 3   | pACT βX149       | +                       |
| pAS αEC49 Ala 4   | pACT βX149       | +                       |
| pAS βX149         | pACT αEC49       | +                       |
| pAS βX96          | pACT αEC49       | -                       |
| pAS βX96          | pACT αEC49 Ala 1 | -                       |
| pAS βX96          | pACT αEC49 Ala 2 | -                       |
| pAS βX96          | pACT αEC49 Ala 3 | +                       |
| pAS βX96          | pACT αEC49 Ala 4 | +                       |

**TABLE IV**

| DNA-binding domain | Activation domain | β-Galactosidase activity |
|--------------------|------------------|-------------------------|
| pAS cytoplasmic α-loop 1 | pACT cytoplasmic α-loop 1 | -                       |
| pAS cytoplasmic α-loop 2 | pACT cytoplasmic α-loop 2 | -                       |
| pAS α-loop 2       | pACT β           | +                       |
| pAS α-loop 1       | pACT cytoplasmic α-loop 2 | -                       |
| pAS α-loop 1       | pACT cytoplasmic α-loop 1 | +                       |

Fig. 3. A, diagram of the chicken Na,K-ATPase α-subunit H7H8
region. Bold circles indicate the 26 amino acids previously shown
to include the essential β-subunit-binding domain. B, sequence align-
ments of the aminoacyl residues in the β-subunit-binding region of the
α-subunit H7H8 domain. Sequences of the avian Na,K-ATPase α1, α2,
and α3 isoforms (6, 52) and the homologous region of the pig gastric
H,K-ATPase (53) are shown. C, positions of the alanine-scanning mu-
tations in the 26-amino acid region of the H7H8 loop.
Protein-protein interfaces are made up of a mixture of hydrophobic and hydrophilic residues. They are usually well paired so that hydrogen bond donors and acceptors are matched along with the hydrophobic groups (43). Alanine is chosen as a generic replacement residue because it is the most common amino acid in proteins, and it is found within buried and exposed positions and in all manner of secondary structures (43). Alanine does not supply new hydrogen bonding, sterically bulky, or unusually hydrophobic side chains (43). Alanine substitutions reduce the functional comparisons among the mutants to a common standard state. To increase the efficiency of analysis one can mutate amino acid groups to alanine in clusters ranging from 2 to 5 residues within segments of 10 to 15 residues (43). This allows one to determine quickly which clustered mutants are most disruptive and subsequently dissect them to identify the important residues. The scanning mutational approach directly tests only the importance of side chains; information about main chain interactions remains unknown. Main chain interactions are common among protein-inhibitor complexes but less so in subunit-subunit and antibody-antigen interactions (43).

With this alanine-scanning strategy, the fusion proteins EC49 Ala1, EC49 Ala3, and EC49 Ala4 all demonstrate positive protein-protein interactions with the full β-subunit ectodomain and the two truncated β fusion proteins. However, the fusion protein EC49 Ala2 did not evidence interaction with any of the three β fusion proteins. This result suggests that the group of four highly conserved amino acids, SYGQ, in the α-subunit H7H8 loop includes residues directly involved in protein-protein interactions between the α- and β-subunits.

A search for a region involved in dimerization of αβ complexes—Although structural studies of the sodium pump support a subunit stoichiometry of one α-subunit to one β-subunit, the exact quaternary structure is still in debate. The formation of a higher order enzyme complex is supported by studies of α-α interactions among the Na,K-ATPase isoforms in rat brain and among rat α-subunits expressed in virally infected SF9 insect cells (26). Expression of a truncated α1 isoform with the full-length α-subunit demonstrated that the C-terminal half of the α-subunit is required for α-α oligomerization in insect SF9 cells. Through the use of chimeras between the catalytic α-subunits of the Na,K-ATPase and H,K-ATPase, the region involved in α-α interaction was further defined as lying between residues Gly554 and Pro785 in the central, cytosolic loop (44). Cross-linking studies by Sarvazyan et al. (45) indicate α-α associations between the N-terminal H1-H2 and C-terminal H8-H10 segments of the Na,K-ATPase α-subunit, with the most probable interacting helices being the H1-H10 pair and the H2-H8 pair. It is not known whether these contacts involve intra- or inter-α-subunit interactions.

The results of α-α oligomerization experiments performed with the two-hybrid system did not reveal any self-association of either large cytoplasmic loop of the α-subunit, even though α cytoplasmic loop 2 contained the residues Gly554 to Pro785 implicated in α-α oligomerization by Koster et al. (44). One possible rationalization of the differing results is that α-α dimerization involves elements of α-subunit structure that are not intrinsic to an isolated α-subunit cytosolic loop, such as would be present in Gal4 fusion proteins. There is evidence to support this interpretation in recent experiments of Froehlich and colleagues (46), experiments that suggest that dimerization of αβ complexes to form (αβ)2 complexes may involve conformational states that occur transiently during the transport cycle.

α-Subunit Cytoplasmic Loop 1 (H2H3) and Cytoplasmic Loop 2 (H4H5) Interactions—The cytoplasmic loop 2 of the α-subunit...
(H4H5) contains the phosphorylation and nucleotide binding sites. However, some mutations within cytoplasmic loop 1 (H2H3) influence the ATPase activity and vanadate sensitivity of the Na,K-ATPase, suggesting that loop 1 may interact with loop 2 (H4H5). The possibility of loop 1-loop 2 interaction was tested in two-hybrid assays. The assays were positive when cytoplasmic loop 1 (H2H3) was fused to the activation domain of Ga4 transcription factor, and cytoplasmic loop 2 (H4H5) was fused to the DNA-binding domain but not vice versa. In two-hybrid assays of defined protein combinations, one orientation of the hybrids (i.e., protein X fused to the DNA-binding domain and protein Y to the activation domain) often activates transcription much more efficiently than the reverse hybrids (29). This may reflect differences between the levels of expression or stability of hybrids containing X and those containing Y. Transcription is optimal when the activation domain hybrid is in excess over the DNA-binding domain hybrid (29). When the reverse is true, DNA-binding domain hybrids bound to the reporter gene promoters are less likely to be engaged in the X-Y protein-protein interaction and therefore may not give positive results. Since β-subunit loop 1 and loop 2 interactions were seen with only one orientation, this constitutes positive but weak evidence for inter-loop interaction.

**Future Studies**—There are additional protein-protein interactions within the Na,K-ATPase that can be approached with two-hybrid studies. For example, the present studies do not include a search for interactions involving the cytosolic domain of the β-subunit nor the N-terminal, C-terminal, and H6H7 and H8H9 cytosolic loops of the α-subunit. In addition, the Na,K-ATPase is known to interact with ankyrin (47–49), and there is evidence that the β2 isoform β-subunit expressed by glial cells may interact with “receptors” on some central nervous system neurons (34). The yeast two-hybrid system appears to be a promising approach not only for defining the subunit assembly domains more completely but also for observing other protein-protein interactions that involve the Na,K-ATPase.

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