Regions of Association between the \(\alpha\) and the \(\beta\) Subunit of the Gastric H,K-ATPase*

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A binding and a yeast two-hybrid analysis were carried out on the gastric H,K-ATPase to determine interactive regions of the extracytoplasmic domains of the \(\alpha\) and \(\beta\) subunits of this P type ATPase. Wheat germ agglutinin fractionation of fluorescein 5-maleimide-labeled tryptic fragments of detergent-solubilized H,K-ATPase showed that a fragment Leu\(^{855}\) to Arg\(^{922}\) of the \(\alpha\) subunit was bound to the \(\beta\) subunit. The yeast two-hybrid system showed that the region containing only a part of the seventh transmembrane segment, the loop, and part of the eighth transmembrane segment was capable of giving positive interaction signals with the ectodomain of the \(\beta\) subunit. The sequence in the extracytoplasmic loop close to the eighth transmembrane segment, namely Arg\(^{898}\) to Thr\(^{928}\), was identified as being the site of interaction using this method. We deduced that the sequence Arg\(^{898}\) to Arg\(^{922}\) in the \(\alpha\) subunit has strong interaction with the extracytoplasmic domain of the \(\beta\) subunit. Again, using yeast two-hybrid analysis, two different sequences in the \(\beta\) subunit Gin\(^{134}\) to Asn\(^{139}\) and Ala\(^{156}\) to Arg\(^{188}\) were identified as association domains in the extracytoplasmic sequence of the \(\beta\) subunit. These data enable identification of major associative regions of the \(\alpha\)-\(\beta\) subunits of the H,K-ATPase.

The gastric H,K-ATPase is a polytopic integral membrane protein belonging to the P type ATPase ion transport family that exchanges H\(_2\)O\(^+\) for K\(^+\) to generate gastric acid secretion. This ATPase consists of two noncovalently associated subunits. There is, in the rabbit, a large \(\alpha\) subunit of 1035 amino acids with 10 transmembrane segments and a \(\beta\) subunit of 290 amino acids with a single transmembrane segment and with seven N-linked glycosylation consensus sequences (1–3). The \(\alpha\) subunit is required for correct assembly of the enzyme. It has been shown for the Na,K-ATPase that the \(\beta\) subunit stabilizes the nascent \(\alpha\) subunit in the endoplasmic reticulum and plays a role in targeting the \(\alpha\)-\(\beta\) complex to the plasma membrane (7–10). Prevention of glycosylation of the \(\beta\) subunit also results in inadequate targeting and loss of catalytic function (11). There are also data showing that modification of the \(\beta\) subunit of either enzyme has an effect on the catalytic function. For example, reduction of the disulfide bonds appears to alter K\(^+\) affinity in the case of the Na,K-ATPase (12). The subunits can be separated using SDS but not with nonionic detergents. Hence there are regions of strong interaction between the two subunits that have both assembly and structural effects on the holoenzyme.

The specific regions of interaction between the \(\alpha\) and the \(\beta\) subunits of the Na,K-ATPase have been investigated by the expression of chimeras of the Na,K-ATPase and the sarcoplasmic Ca-ATPase (13). Twenty-six residues in the extracellular loop between the seventh and eighth transmembrane domains have been implicated as providing the interactive region in the \(\alpha\) subunit. The region of the \(\beta\) subunit of the Na,K-ATPase near the first S–S bridge in the extracytoplasmic domain is thought to interact with the \(\alpha\) subunit (14). Some hydrophobic C-terminus amino acids as well as a conserved proline residue in the loop between the third disulfide bond of the \(\beta\) subunit ectodomain have also been implicated in subunit assembly (15, 16). Recently, the use of the yeast two-hybrid system has indicated that 63 amino acids of the \(\beta\) subunit extracytoplasmic sequence may be a region of interaction with the \(\alpha\) subunit (17). A specific sequence containing SYGQ was identified as important using alanine scanning mutagenesis. These data suggest that several regions of one or both subunits of the Na,K-ATPase might be involved in \(\alpha\)-\(\beta\) interaction.

The \(\beta\) subunit of H,K-ATPase can act as a surrogate for the \(\beta\) subunit of Na,K-ATPase in the formation of functional Na,K-pumps in Xenopus oocytes (18). This observation suggests some common regions of association in the two enzymes. However, the efficiency of assembly is much lower and surrogate activity has not been found in a mammalian cell expression system (19). Trypsinization of cytoplasmic side out hog gastric vesicles and wheat germ agglutinin (WGA)\(^1\) column retention of fragments associated with the bound \(\beta\) subunit have provided direct evidence for binding of a region of the H,K-ATPase \(\alpha\) subunit, the TM7/loop/TM8 sector, to the \(\beta\) subunit (20). Since a monoclonal antibody Abl46 raised against rat parietal cells recognized both an extracytoplasmic epitope on the \(\beta\) subunit, between Cys\(^{162}\) and Cys\(^{178}\), and an epitope at the putative extracytoplasmic surface of TM7 on the \(\alpha\) subunit, between Ala\(^{177}\) and Asp\(^{179}\), it was suggested that those two regions may

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1 The abbreviations used are: WGA, wheat germ agglutinin; PCR, polymerase chain reaction; TM, transmembrane; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.
Association between α and β Subunits of Gastric H,K-ATPase

**Experimental Procedures**

**Trypsin Digestion of Solubilized H,K-ATPase—**The H,K-ATPase was derived from hog gastric mucosa by previously published methods (23). All manipulations were carried out at 4 °C.

Gastric vesicles (0.5 mg) enriched in gastric H,K-ATPase were solubilized at 4 °C in a buffer composed of 1% Nonidet P-40, 50 mM Tris/ HCl, pH 8.0, at a protein concentration of 2 mg/ml. The mixture was spun at 100,000 g for 10 min. The supernatant containing the solubilized protein (about 1.4 mg/ml) was digested for 20 min at room temperature with trypsin/protein. The digestion was stopped by adding 10-fold excess of soybean trypsin inhibitor against trypsin, and the mixture was kept on ice before WGA column chromatography.

**WGA Fractionation of Solubilized H,K-ATPase Digest—**WGA fractionation was carried out as described previously (20). Tryptic digest was loaded on a WGA-Sepharose 6MB column (1 cm2 of column volume). After equilibration for 20 min at 4 °C, a fraction not retained on the column was eluted using 1 ml of a buffer composed of 1% Nonidet P-40, 50 mM Tris/HCl, pH 7.0, and collected for analysis. This fraction was concentrated in vacuo and precipitated with 0.7 ml of cold acetone for removing Nonidet P-40. The mixture was then washed with a buffer (20 ml) composed of 50 mM Tris/HCl, pH 7.0, and 1% Nonidet P-40. This washing removed all peptide fragments not bound to WGA, including soybean trypsin inhibitor and trypsin. Elution of the WGA-retained components was carried out using 0.5 N acetic acid. The acetic acid eluate was collected and dried in vacuo. The WGA-binding fraction was resuspended in 100 μl of 50 mM Tris/HCl, pH 7.8, 0.3% SDS, and 0.1 μl fluorescein 5-maleimide. This allowed fluorescent labeling of peptide fragments enabling localization on SDS-polyacrylamide gel electrophoresis.

The labeled, SDS-solubilized peptide fragments were combined with a 20% volume of sample buffer (0.3 M Tris, 10% SDS, 5% glycerol, and 0.025% bromphenol blue), and the solution was placed on top of a 10% polyacrylamide gel. The gel was run for 20–24 h at 48-mA constant current, along with a lane for prestained molecular mass (Bio-Rad, 16–106 kDa) standards and CNBr fragments of horse myoglobin (Sigma; 2.5–17 kDa). The gel was transferred to polyvinylidene difluoride membranes as described previously (20).

**Protein Sequencing—**Peptide bands were sequenced with a gas phase sequencer at the UCLA Protein Microsequencing facility using the Applied Biosystems 475 A system composed of a 470 A Sequencer, a 120 A HandiPrep, and 70 μl of anhydride gel electrophoresis.

The labeled, SDS-solubilized peptide fragments were combined with a 20% volume of sample buffer (0.3 M Tris, 10% SDS, 5% glycerol, and 0.025% bromphenol blue), and the solution was placed on top of a 10% polyacrylamide gel. The gel was run for 20–24 h at 48-mA constant current, along with a lane for prestained molecular mass (Bio-Rad, 16–106 kDa) standards and CNBr fragments of horse myoglobin (Sigma; 2.5–17 kDa). The gel was transferred to polyvinylidene difluoride membranes as described previously (20).

**Yeast Strains and Media—**The Saccharomyces cerevisiae yeast strain Y187 (MATa, ura3-52, his3-200, ade2-21, trpl1-901, leu2-3, 112, gal4/4, met-5, gal80D, URA3-GAL1::GAL1–GAL7, GAL1–lacZ) (CLONTECH Matchmaker System II) was grown at 30 °C in either YPD medium or synthetic defined dropout yeast medium lacking the appropriate amino acids, i.e. tryptophan and leucine (CLONTECH). All the media contain 2% glucose as a source of carbon.

**Construction of Fusion cDNAs—**Fusion proteins of different fragments of the rabbit H,K-ATPase α and β subunits with either the DNA binding domain or the activation domain of the GAL4 transcription factor GAL4 were generated by cloning the corresponding rabbit cDNA fragments into the multiple cloning site of pAS2–1 and pACT2 vectors respectively (Matchmaker yeast two-hybrid system 2, CLONTECH). For clarity, these two vectors will be referred to as pAS and pAC, respectively. The cDNA fragments were generated by polymerase chain reaction (PCR) with useful restriction sites incorporated into the primers. The primers and the restriction sites are listed in Table I. The PCR reactions were carried out for 30 cycles using 1 unit AmpliTaq DNA polymerase (Perkin-Elmer) and as follows: 30 s at 94 °C, 30 s at a temperature ranging from 52 and 60 °C depending on the pair of primers used, and 72 °C for 1 min. An additional 5-min cycle at 72 °C ended each program. The PCR product was then cleaved according to the conditions recommended by the commercial supplier of the restriction enzymes (Promega). It was purified using PCR Clean-up

**Table I**

| Plasmid | PCR primers | Plasmid | PCR primers |
|---------|-------------|---------|-------------|
| pASa1–2 | 5′-ACATGCAGGTTGAAAGTGGCCGGA-3′ | pACh64–291 | 5′-GCTTGCGTTGTCACACTGACCCTACAC-3′ |
| pASa3–4 | 5′-TACCTACTGCGCACTTACGAGCCGGAACAC-3′ | pACh64–291 | 5′-ATTACCGACCATGACACCTGAC-3′ |
| pASa5–6 | 5′-GCTAGAATTGAGCTGCTGACGGAATGGA-3′ | pACh64–81 | 5′-GCTTGCGTTGTCACACTGACCCTACAC-3′ |
| pASa7–8 | 5′-ATTACGATGCGGACGTTGCTGCTGACGGA-3′ | pACh64–130 | 5′-GCTTGCGTTGTCACACTGACCCTACAC-3′ |
| pASa9–10 | 5′-GCTGACGGCTCCCTACTGTGTTAAGGC-3′ |
| pASa989–928 | 5′-GCTGAGGCGCTCGCACTTACGAGCCGGAACAC-3′ | pACa156–188 | 5′-GCTTGCGTTGTCACACTGACCCTACAC-3′ |
| pAs860–903 | 5′-GCTGACGGCTCCCTACTGTGTTAAGGC-3′ | pACb1566–250 | 5′-GCTTGCGTTGTCACACTGACCCTACAC-3′ |
| pAc860–925 | 5′-GCTGACGGCTCCCTACTGTGTTAAGGC-3′ | pACb197–291 | 5′-GCTTGCGTTGTCACACTGACCCTACAC-3′ |
| pAc898–928 | 5′-GCTGACGGCTCCCTACTGTGTTAAGGC-3′ | pACb197–291 | 5′-GCTTGCGTTGTCACACTGACCCTACAC-3′ |

**Restriction sites within the PCR primers are underlined.**
Promega columns and then ligated for 1 h at room temperature into the pAS and pAC vectors. The nucleotide sequence and the reading frame of each construct were checked by automated DNA sequencing. The plasmids were then amplified in Escherichia coli and purified using Qiagen purification columns.

Transformation of S. cerevisiae—Transformation of yeast was carried out by using the lithium acetate method described by Gietz et al. (25).

β-Galactosidase Assays—For qualitative evaluation of regions of contact, β-galactosidase filter lift assays were carried out. After 2–4 days of growth at 30 °C, yeast transformants were transferred onto sterile VWR Scientific grade 410 paper filters, which were then submerged in growth at 30 °C, yeast transformants were transferred onto sterile VWR Scientific grade 410 paper filters, which were then submerged in liquid nitrogen for permeabilizing the cells. Filters were then placed onto filter paper presoaked in Z buffer (100 mM sodium phosphate (pH 7.0) 10 mM KCl, 1 mM MgSO4) supplemented with 50 mM β-mercaptoethanol and 0.07 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside. Filters were then incubated at 30 °C and checked periodically for the appearance of blue colonies.

For quantitative studies, yeast strains were grown to stationary phase in synthetic medium lacking leucine and tryptophan, diluted to 5 × 10^6 cells/ml, and then incubated at 30 °C for 3–4 h. β-Galactosidase activity was determined using the chemiluminescent Galacton-Star detection kit according to the manufacturer’s instructions (CLONTECH). Values reported are the average of assays of three independent transformants.

Materials—The materials were of the highest grade purity available. Trypsin type XIII was obtained from Sigma, poly(vinylidene difluoride) membranes were from Millipore, and Nonidet P-40 was from Sigma. Fluorescein 5-maleimide was obtained from Molecular Probes. WGA-Sepharose 6MB was obtained from Pharmacia.

RESULTS

The TM7/Loop Domain from Leu869 to Arg922 of the α Subunit Associates with the β Subunit as Shown by WGA Fractionation of Trypsin-digested Solubilized H,K-ATPase—In a previous study, by using WGA fractionation of trypsin-digested H,K-ATPase, we have demonstrated that the region TM7/loop/TM8 domain of the α subunit interacts with the β subunit (20). In this study, we applied the same technique, but on previously solubilized enzyme. When solubilized H,K-ATPase was digested with trypsin, several peptide fragments were observed in the range from 3 to 60 kDa, including trypsin, auto-digested trypsin, and trypsin inhibitor. Among these, six peptide fragments found at 20, 11, 8, 7.5, 6.2, and 5 kDa from the SDS-gel were identified. The 20-kDa peptide fragment as well as the other peptide fragments found at 11 and 8 kDa had an N-terminal sequence LVNEPLAA corresponding to the TM7/loop domain and the 11-kDa peptide contains the TM7/loop/TM8 domain and the 8-kDa peptide contains the TM7/loop/TM8 domain. The first band, at 20 kDa, represents the TM7 domain and the second band at 11 kDa represents the TM7/loop/TM8 domain, respectively, having the same N-terminal sequence, LVNEPLAA. Band b was shown at 20, 11, and 8 kDa respectively, having the same N-terminal sequence, LVNEPLAA. Band d shown at 5 kDa provided one N-terminal sequence NIPELTPY, shown (20). A peptide of 5 kDa with an N-terminal sequence QLAGGLQ, contained the TM1/loop/TM2 domain as previously shown (20). A peptide of 5 kDa with an N-terminal sequence TPIAIEI, defined the domain TM3/loop/TM4, and a peptide of 6.2 kDa with the N-terminal sequence, QLAGGLQ, contained the TM1/loop/TM2 domain as previously shown (20). A peptide of 5 kDa with an N-terminal sequence NIPELTPY represented the TM5/loop/TM6 domain.

When the trypsin digest of solubilized H,K-ATPase was applied to the WGA column, nonbinding fragments were very widely distributed below 60 kDa, including trypsin and trypsin inhibitor (Fig. 1). However, the WGA-retained portion provided four strong fluorescent bands at 20, 11, 8, and 5 kDa. The first three of these fragments had the same N-terminal sequence, LVNEPLAA. Based on molecular weight and N-terminal sequence, the 20-kDa peptide contains the TM7/TM8/TM9/TM10 domain and the 11-kDa peptide contains the TM7/loop/TM8 domain as described previously (20). The 8-kDa peptide fragment corresponds to the sequence beginning at Leu855 and ending at Arg922, representing the TM7 and part of the extracellular loop. The 5-kDa peptide fragment provided only one N-terminal sequence, NIPELTPY, corresponding to a fragment containing the TM5/loop/TM6 domain.

The three fragments of 20, 11, and 8 kDa are associated with the β subunit, which enabled them to be retained by the WGA column. The 5-kDa peptide fragment corresponding to TM5/loop/TM6 seems to be associated with the C terminus containing the 20-kDa fragment, since the 5-kDa fragment disappears when the 20-kDa fragment is eliminated after longer digestion (Fig. 1). These data suggest an association of the β subunit with the region extending from TM7 through Arg922.

The Luminal Domain of the Rabbit Gastric H,K-ATPase β Subunit Interacts Only with the TM7/Loop/TM8 Region of the α Subunit When Assayed with the Two-hybrid System—Five cDNA fragments corresponding to the five putative extracellular loops of the rabbit gastric H,K-ATPase α subunit were fused to the binding domain of the transcription factor GAL4 (pASa124–161TM2, pASa319–337TM3–4, pASa803–833TM5–6, pASa869–933TM7–8, and pASa972–1001TM8–10). Fig. 2 shows the putative structure of the α subunit of the rabbit H,K-ATPase and Fig. 3 a diagram of the same subunit along with the different regions used in yeast two-hybrid assays. Each construct was co-transformed in yeast together with a β construct corresponding to the whole extracytoplasmic region of the β subunit fused to the activation domain of GAL4 (Fig. 4B, pAC694–291). The resulting activation of the β-galactosidase reporter gene was first checked for each co-transformation using the β-galactosidase filter lift assay. Then the more sensitive and quantitative β-galactosidase luminescent assay was performed on those clones showing a blue coloration, i.e. β-galactosidase expression, in the first 8 h. Several negative white clones, of some of them reported in this study (cf. Tables II–IV) have been checked by the luminescent assay, and each always showed an activity not significantly different from background. Results are presented in Table II, together with the control experiments corresponding to the transformation of each plasmid with the plasmids pAS and pAC. We found that pAC694–291 was slightly auto-activating, giving rise to a weak activation of the reporter gene in the absence of an α insert. However,
a strong β-galactosidase activity was observed when the TM7/loop/TM8 domain of the α subunit was fused to the binding domain (pASo689–933Tm7-8) and co-transformed with the ectodomain of the β subunit fused to the activation domain (pAC64–291). The time of appearance of the blue color was 7 and 3 h for the former and the latter transformation, respectively. Consistent with this blue lift assay, the luminescent assay showed that the activation of the reporter gene is about 5 times higher when the β construct fused to the activation domain is expressed together with the α construct fused to the binding domain than when expressed with pAS. The same combination of α and β fragments in the opposite orientation was not informative. The same α subunit region fused to the GAL4 activation domain (pACo860–928Tm7-8) gave rise to a very strong auto-activation of the β-galactosidase reporter gene when transformed with the nonrecombinant pAS2–1 vector (Table III). The β ectodomain was not found to interact with any other extracellular loop of the α subunit, namely those loops between TM1 and TM2, TM3 and TM4, TM5 and TM6, and TM9 and TM10 in the yeast two-hybrid analysis.

**A Unique Region of the TM7/Loop/TM8 Domain of the α Subunit Interacts with the β Subunit**—To define more specifically which region or regions of the α subunit are involved in the α-β interaction, shorter fragments of the region were fused to both the binding domain (Fig. 4a, pASo689–903Tm7-8 and pASo898–928Tm7-8) and the activation domain of the GAL4 transcription factor (Fig. 4a, pACo898–928Tm7-8 and pACo869–903Tm7-8). Each construct was co-transformed in the yeast with pAS64–291, pAC64–291, or the corresponding control plasmid. The results are presented in Table III. The region close to the seventh transmembrane segment could not be analyzed when linked to the binding domain of GAL4 because of auto-activation found in the construct pASo680–903Tm7-8. However, when linked to the activation domain of GAL4 this region of the α subunit was not auto-activating and showed no interaction with the construct pAC64–291 (pACo689–903Tm7-8). On the other hand, the downstream region of the TM7/loop/TM8 domain (pASo898–928Tm7-8) showed interaction with the β subunit ectodomain when fused to the binding domain. This sequence was auto-activating when fused to activating domain (pACo898–928Tm7-8). These results showed that the region of the α subunit closer to the eighth transmembrane segment of the H,K-ATPase, 898–928, was responsible for α-β association in the yeast two-hybrid system.

**Two Independent Domains of the β Subunit of the Rabbit H,K-ATPase Interact with the Same Region of the α Subunit**—Six cDNA fragments of the ectodomain of the β subunit of the rabbit H,K-ATPase were fused to the activating domain of GAL4 (pACβ64–81, pACβ64–130, pACβ126–155, pACβ156–188, pACβ186–250, and pACβ197–291). A diagram of the β subunit is presented on Fig. 4b together with the different β constructs used in the two-hybrid system. Each of the six clones was transformed with either the whole TM7/loop/TM8 domain (pASo689–933Tm7-8), the downstream region of the TM7/loop/TM8 domain (pASo898–928Tm7-8) or the vector pAC. Results are presented in Table IV. Two independent sequences of the β subunit ectodomain, one containing 66 amino acids and positioned between the putative transmembrane domain and the first disulfide bond (Glu64 to Asn130), and the second consisting of 32 amino acids and spanning the second disulfide bond (Ala156 to Arg188) were found to interact with both α constructs. No interaction was detected with either a small fragment of 17 amino acids directly adjacent to the transmembrane domain (pAC64–81) or the fragment of β spanning the first disulfide bond (pACβ126–155). No analyzable results were possible for the C-terminal region of the β subunit due to strong auto-activation by sequences from this region. Two of these are represented in Fig. 4b (pACβ186–250 and pACβ197–291) that gave rise to auto-activation of the β-galactosidase reporter gene when co-transformed with pAS. From these results, it can be concluded that at least two regions of the β subunit interact with the same region of the α subunit close to the eighth transmembrane domain.

**DISCUSSION**

It is now well established that strong interactions between the α and the β subunits of the H,K-ATPase are necessary for the stabilization and targeting of a functional pump to the plasma membrane of transfected cells or *Xenopus* oocytes and hence presumably to the tubulovesicles or secretory canaliculus of the parietal cell. Identification of domains involved in assembly of this membrane protein can therefore lead to a better understanding of not only the general structure of the pump but also the mechanisms controlling its biosynthesis and trafficking in the parietal cell.

Several assembly domains have been partially characterized in both the α and the β subunit of the Na,K-ATPase as outlined earlier. There is direct and indirect evidence for the involvement of the TM7/loop/TM8 extracellular α subunit region in α-β assembly of the H,K-ATPase. No specific region of the β subunit of this enzyme has been identified heretofore.

In the present study, we extended our previous work using WGA fractionation of tryptic digested solubilized H,K-ATPase. The minimal fragment of α which was able to associate with the β subunit was 8 kDa in length from Lue145 to Arg222. The sequence from Arg222 to Arg245 which includes the TM8 region did not interact significantly with the β subunit by this biochemical assay. Since the association is known to occur in the lumen, the region on the α subunit between TM7 and Arg222 was deduced as being involved in α-β interaction. Although direct, this technique does not allow much flexibility in the size of the fragments that can be analyzed given the few tryptic cleavage sites in the loop between TM7 and TM8. Moreover, it can not provide any information on the regions of the β subunit that are important for α-β association, since there is little quantitative cleavage by trypsin of this region. Further, it relies on stability of the interaction in the nonionic detergents used to solubilize the heterodimer.

Therefore, we took advantage of the sensitivity of the yeast two-hybrid system for looking for possible interactions between any extracellular fragments of the α and the β subunit of the H,K-ATPase. Several regions were analyzed by this method for auto-activation, but usually it was possible to eliminate or minimize this effect by interchanging the insertion between the activating or the binding domain of the yeast expression vector.

The five extracellular domains of the α subunit, as defined with little variation in most structural models proposed today for this subunit, were assayed for their interaction with the entire luminal domain of the β subunit by using this yeast
two-hybrid system. An interaction between the ectodomain of the β subunit and only the TM7/loop/TM8 region of α was detected.

Since the yeast two-hybrid system detects interaction in the probable absence of glycosylation, these data show that glycosylation of the β subunit is not required for the association with the α subunit. This confirms previous observations (11).

Furthermore, the absence of the cytoplasmic and transmembrane domains in the β subunit construct showed that neither of those two domains is critical for interaction of the two subunits as assayed here. It has been shown in the case of the Na,K-ATPase, that deletion of the cytoplasmic and most of the

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**Fig. 3.** Schematic representation of the rabbit H,K-ATPase α subunit shows the different extracytoplasmic regions analyzed by the yeast two-hybrid system. TM, transmembrane domain. Boundaries for each construct are indicated by the position of the first and last amino acid, as well as by short segments of sequence corresponding to these positions.

**Fig. 4.** Schematic representation of (A) the TM7/loop/TM8 region of the α subunit and (B) the β subunit of the rabbit gastric H,K-ATPase shows the different constructs used in the two-hybrid assay. TM, transmembrane domain. The different constructs were cloned either as a fusion cDNA with the binding domain (BD) or the activation (AD) of the GAL4 transcription factor. Boundaries for each construct are indicated by the position of the first and last amino acid, as well as by short segments of sequence corresponding to these positions. Arrowheads on the β subunit point out the seven N-linked glycosylation consensus sequences. The three disulfide bonds are indicated by the filled circles linked by pair.
transmembrane domain of the chicken β subunit allowed assembly with the α subunit (26). On the other hand, analysis of chimeric proteins between subunits of Xenopus Na,K-ATPase and rabbit H,K-ATPase that were constructed by exchanging their N-terminal plus transmembrane domain and their cytoplasmic COOH-terminal domain have been interpreted as showing that the transmembrane domain of the β subunit plays an important role for efficient association with α subunits (27). It has therefore been proposed that assembly between the α and β ectodomains occurs first and then there is stabilization of the complex by other interactions. Because of its high sensitivity, the yeast two-hybrid system is able to detect weak interactions.

No interaction of the β subunit with any of the other extra

**TABLE II**

Interaction of the ectodomain of the β subunit with the extracellular loops of the α subunit checked by the yeast two-hybrid assay

| Plasmid coding for a fusion protein with the DNA binding domain of GAL4 | Plasmid coding for a fusion protein with the activation domain of GAL4 | β-Galactosidase filter assay | Luminescent β-galactosidase assay |
|-------------------------------------------------|-------------------------------------------------|-----------------------------|-------------------------------|
| pASa124–161TM1–2                                 | pAC                                            | White                       | NDa                          |
| pASa124–161TM1–2                                 | pAC\(64–291\)                                   | White                       | ND                           |
| pAS319–337TM3–4                                 | pAC                                            | White                       | ND                           |
| pAS319–337TM3–4                                 | pAC\(64–291\)                                   | White                       | ND                           |
| pAS803–833TM5–6                                 | pAC                                            | White                       | ND                           |
| pAS803–833TM5–6                                 | pAC\(64–291\)                                   | White                       | ND                           |
| pAS869–933TM7–8                                 | pAC                                            | White                       | 31.3 ± 2.3                   |
| pAS869–933TM7–8                                 | pAC\(64–291\)                                   | Blue after 3 h              | 179 ± 27.4                   |
| pAS972–1001TM9–10                               | pAC                                            | White                       | ND                           |
| pAS972–1001TM9–10                               | pAC\(64–291\)                                   | Blue after 7 h              | 39.6 ± 3.8                   |
| pAS                                            | pAC                                            | White                       | 22.1 ± 4.5                   |

*a ND, not determined.

**TABLE III**

Interaction of the ectodomain of the β subunit with different parts of the TM7/loop/TM8 region of the α subunit checked by the yeast two-hybrid assay

| Plasmid coding for a fusion protein with the DNA binding domain of GAL4 | Plasmid coding for a fusion protein with the activation domain of GAL4 | β-Galactosidase filter assay | Luminescent β-galactosidase assay |
|-------------------------------------------------|-------------------------------------------------|-----------------------------|-------------------------------|
| pAS898–928TM7–8                                 | pAC                                            | White                       | 24.9 ± 1.9                    |
| pAS898–928TM7–8                                 | pAC\(64–291\)                                   | Blue after 3 h              | 160 ± 20.0                    |
| pAS860–907TM7–8                                 | pAC\(64–291\)                                   | Blue after 0.5 h            | 2290 ± 93                     |
| pAS                                            | pAC\(860–928\) \(\text{TM}7\)–\(\text{TM}8\) | Blue after 1 h              | 2340 ± 138                    |
| pAS                                            | pAC\(860–928\) \(\text{TM}7\)–\(\text{TM}8\) | Blue after 1 h              | ND                           |
| pAS                                            | pAC\(860–928\) \(\text{TM}7\)–\(\text{TM}8\) | Blue after 1 h              | ND                           |
| pAS                                            | pAC\(860–928\) \(\text{TM}7\)–\(\text{TM}8\) | Blue after 1 h              | ND                           |
| pAS                                            | pAC\(64–291\)                                   | White                       | 23.9 ± 3.7                    |
| pAS                                            | pAC\(64–291\)                                   | White                       | 14.5 ± 0.6                    |

*a ND, not determined.

**TABLE IV**

Interaction of the extracellular TM7/loop/TM8 region of the α subunit with different parts of the ectodomain of the β subunit checked by the yeast two-hybrid assay

| Plasmid coding for a fusion protein with the DNA binding domain of GAL4 | Plasmid coding for a fusion protein with the activation domain of GAL4 | β-Galactosidase filter assay | Luminescent β-galactosidase assay |
|-------------------------------------------------|-------------------------------------------------|-----------------------------|-------------------------------|
| pAS                                            | pAC\(64–291\)                                   | White                       | 19.6 ± 2.0                     |
| pAS                                            | pAC\(64–291\)                                   | White                       | 24.2 ± 1.9                     |
| pAS                                            | pAC\(64–291\)                                   | White                       | 23.2 ± 4.9                     |
| pAS                                            | pAC\(64–291\)                                   | Blue after 3 h              | 16.8 ± 1.5                     |
| pAS                                            | pAC\(64–291\)                                   | Blue after 3 h              | 50.1 ± 1.9                     |
| pAS                                            | pAC\(64–291\)                                   | Blue after 3 h              | 66.0 ± 10.7                    |
| pAS                                            | pAC\(64–291\)                                   | White                       | 19.6 ± 0.6                     |
| pAS                                            | pAC\(64–291\)                                   | White                       | 25.8 ± 2.2                     |
| pAS                                            | pAC\(64–291\)                                   | White                       | 24.3 ± 1.1                     |
| pAS                                            | pAC\(64–291\)                                   | White                       | 13.9 ± 4.5                     |
| pAS                                            | pAC\(64–291\)                                   | Blue after 3 h              | 54.4 ± 3.6                     |
| pAS                                            | pAC\(64–291\)                                   | Blue after 3 h              | 59.9 ± 10.0                    |
| pAS                                            | pAC\(64–291\)                                   | Blue after 1 h              | ND                           |
| pAS                                            | pAC\(64–291\)                                   | Blue after 1 h              | ND                           |
| pAS                                            | pAC\(64–291\)                                   | Blue after 1 h              | ND                           |
| pAS                                            | pAC\(64–291\)                                   | Blue after 1 h              | ND                           |
| pAS                                            | pAC\(64–291\)                                   | Blue after 1 h              | ND                           |

*a ND, not determined.
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cellular loops of the α subunit was detected other than that contained within the TM7/loop/TM8 region. Although negative results should be viewed with caution since problems may exist in the expression, targeting, and stability of the fusion protein, this result suggests that the two subunits have strong interaction only in the region of the TM7/loop/TM8 domain. This finding confirms our previous biochemical data (20).

Shorter fragments of the α subunit used in the yeast two-hybrid system allowed us to define one unique region from Arg908 to Thr929, as being involved in the interaction with the β subunit. A combination of these results with the biochemical data reported earlier in this study, shows that a region of only 25 amino acids on the α subunit, from Arg908 to Arg929, is the likely region of strong interaction with β. This region contains the four amino acids, SYGQ, shown to be essential for the interaction between the α and the β subunits of the Na,K-ATPase by yeast two-hybrid system using alanine scanning mutagenesis and assumed to be similarly important for the H,K-ATPase based on sequence similarity (17). On the other hand, previous work, using resistance to trypptic digestion and ouabain binding, showed that a chimera of the Na,K-ATPase α subunit containing the region from Glu908 to Val929 of the rat gastric H,K-ATPase preferentially assembled with β subunit of H,K-ATPase (22). Therefore, a homologous 17 amino acid sequence of the α subunit of the two enzymes, from position 907 to position 924 in the H,K-ATPase and position 894 to 911 in the Na,K-ATPase, might be a point of stable contact with the β subunit while differences in the sequence in this region can account for selective assembly of the β subunits with their α counterparts. The alignment of this region of the H,K- and Na,K-ATPases as shown in Scheme 1 suggests that differences in this region, such as the smaller number of charged amino acids in the H,K-ATPase sequence may account for the selective association of the two subunits.

No experiment on the interactive regions of the β subunit of the H,K-ATPase has been reported. In the present study, we sought direct evidence for assembly of specific H,K-ATPase β fragments with α fragments. Six adjacent fragments of β were analyzed. In the presence of the yeast two-hybrid system and two domains were found to interact with both the whole TM7/loop/TM8 domain and the shorter Arg908 to Thr928 fragment defined above on the α subunit. The first fragment Glu94 to Asn130 is the extracellular region directly adjacent to the transmembrane domain ending before the first disulfide bond. The second fragment is a 32-amino acid fragment spanning the entire sequence between the second disulfide bond Ala136 to Arg168. This region of interaction was deduced from reactivity of a monoclonal antibody, mAb 146 with both the α and β subunits of the gastric H,K-ATPase (21).

The location of these two fragments showed that the disulfide bonds in the β subunit are nonessential for the assembly of the heterodimer complex. This was also found to be true in the case of the Na,K-ATPase when using the yeast two-hybrid system (17). However, the expression in Xenopus oocytes of different mutants with a substitution of one or both cysteine residues involved in the three disulfide bonds of the β-subunit of Torpedo californica Na,K-ATPase has shown that disruption of either the second or the third disulfide bond prevented α-β association (28). It might be that the interactions detected by the yeast two-hybrid system are weak interactions, otherwise not detected by other techniques because of a lower sensitivity. This is consistent with the finding that deletions of up to 146 extracellular amino acids from the carboxyl terminus of the β subunit of the Na,K-ATPase result in less efficient assembly with the α subunit (29).

Some of the C-terminal hydrophobic amino acids have been shown to be important in correct assembly of the α and β subunits of the Na,K-ATPase in Xenopus oocytes (18). Unfortunately, the yeast two-hybrid system used here was unable to confirm these data since all the variations tried in the C-terminal region resulted in auto-activation.

In summary, both biochemical and yeast two-hybrid analysis have shown that a single region of the α subunit and two regions of the β subunit of the H,K-ATPase are able to associate in the absence of other regions. From the data above, the deduced associative region of the α subunit is contained within the 22 amino acids between Arg908 and Arg929. The selective assembly of chimeras narrows this region down to and the two regions of the extracytoplasmic surface of the β subunit are found between Gln964 and Asn130 and between Ala136 and Arg168. The latter sequence is between the second disulfide pair hence disulfide formation is not important for this interaction. Since there is little or no glycosylation of the nuclear transciption factors, the yeast two hybrid system shows that glycosylation is not required for the initial phase of α-β association.

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