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Subunit requirements for expression of functional sodium pumps in yeast cells

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Abstract

Na^+/K^+-ATPase from animal cell membranes is known to consist of an α-subunit and a β-subunit. Amino acids within the α-subunit have been shown to participate in the catalytic functions of the enzyme and in the binding of cardioactive steroids. Although the function of the β-subunit is not known, expression of both α- and β-subunits is required for the functional enzyme. A putative third subunit, the γ-subunit, has been suggested to be a part of the functional Na^+/K^+-ATPase complex, based on experiments showing that both the catalytic α-subunit and a small peptide of $M_r = 11000$ can be labeled by a photoreactive ouabain analog. Although the primary structure for the putative γ-subunit from rat and sheep was recently deduced from cDNA clones, participation of this small protein in the catalytic activity of the Na^+/K^+-ATPase has not been demonstrated. In experiments described here, the heterologous expression of Na^+/K^+-ATPase in yeast cells was used to investigate whether the γ-subunit is an essential component of the Na^+/K^+-ATPase. Yeast cells do not contain an endogenous Na^+/K^+-ATPase. The α- and β-subunits or the α-, β- and the putative γ-subunits of Na^+/K^+-ATPase were expressed in the yeast *Saccharomyces cerevisiae* and ouabain-sensitive ATPase, *p*-nitrophenylphosphatase, and ^{86}Rb uptake activities were measured either in membranes prepared from transformed yeast cells, or in intact yeast cells. Nontransformed yeast cells or yeast cells transformed with the γ-subunit alone served as controls. Northern analysis and Western blots demonstrated that yeast cells do not contain an endogenous peptide with significant sequence homology to the putative γ-subunit. Yeast samples containing only Na^+/K^+-ATPase α and β subunits were capable of ouabain-inhibitable enzymatic activity and ^{86}Rb transport. No γ-subunit-dependent differences in the measured enzymatic activities or transport properties were detected in the different samples. These observations establish that the $\alpha\beta$-subunit complex is the minimum structural unit required for all the ouabain-sensitive reactions of Na^+/K^+-ATPase.

Key words: ATPase, Na^+/K^+-; Heterologous expression; Gamma subunit; Ion transport; (Yeast)

1. Introduction

Na^+/K^+-ATPase is an oligomeric membrane protein found in animal cells. The enzyme binds and hydrolyzes ATP, and couples energy released from ATP hydrolysis to the transport of Na^+ ions out of the cells and of K^+ ions into the cells [1–4]. Na^+/K^+-ATPase consists of an α-subunit polypeptide of $M_r = 112000$ and a β-subunit polypeptide of $M_r = 56000$ [1–6]. All catalytic properties of the enzyme are associated with the α-subunit and are inhibited by cardioactive steroids, which induce a positive inotropic response in heart muscle [7–11]. The role of the β-subunit is not well understood. Data suggest, however, that the β-subunit is involved in the stabilization and transport of the α-subunit to the plasma membrane [12–14], and may be important in the interactions between the pump and the transported cations [15–17]. The requirement for these two subunits in the formation of the catalytically active Na^+/K^+-ATPase has been demonstrated [13,14].

Abbreviations: Na^+/K^+-ATPase, sodium and potassium activated adenosine 5'-triphosphatase, EC 3.6.1.37; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate; ATP, adenosine 5'-triphosphate.

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Radiolabeled derivatives of cardioactive steroids have been used to localize the binding site for these molecules to the α-subunit of the Na^+/K^+-ATPase [8,18–23]. Experiments in which Na^+/K^+-ATPase was photolabeled with 2-nitro-5-azidobenzoyl-[^{3}H]ouabain (NAB-[^{3}H]ouabain), however, showed that a large amount of radioactivity was also incorporated into a small acidic proteolipid with a relative molecular weight of 11 000–12 000 on polyacrylamide gels [20,21]. Since the incorporation of the radioactive label could be prevented by excess nonradioactive ouabain, it was concluded that this peptide may be involved in specific binding of cardioactive steroids to Na^+/K^+-ATPase. This observation, together with the earlier observation that a peptide of $M_r = 11\,000–12\,000$ is also labeled with covalent radioactive ATP analogs [24], is consistent with the existence of a third subunit of Na^+/K^+-ATPase, called γ. This γ-peptide is present in preparations of Na^+/K^+-ATPase from different tissues and has a highly conserved amino acid composition [25–27].

Although the putative γ-subunit is specifically labeled by NAB-ouabain, it has been separated from the catalytically active α/β-subunit complex under nondenaturing conditions without loss of ATPase activity, indicating that the ouabain-sensitive ATPase activity is independent of the smaller γ-proteolipid [25]. It is possible that the γ-subunit is required for some function of Na^+/K^+-ATPase other than the hydrolysis of ATP, or participates in the regulation of Na^+/K^+-ATPase activity in cells. One possibility, first suggested by Racker [28], is the formation of the ionophoric part of the Na^+/K^+-ATPase. The hydrophobic character and the size of the γ-polypeptide are consistent with insertion of this peptide into the cell membrane [29,30].

The complete primary structure of a putative γ-subunit from rat and sheep was recently deduced from cDNA clones [30]. In the experiments reported here, the cDNA for rat γ-subunit was expressed in yeast cells in order to investigate the possibility that the γ-peptide might participate in Na^+/K^+-ATPase substrate hydrolysis, cardiac glycoside binding, or in the formation of the ion channel of the enzyme. Yeast cells do not have an endogenous Na^+/K^+-ATPase and do not bind cardioactive steroids. The presence of functional Na^+/K^+-ATPase in the yeast can be easily detected by the binding of [^{3}H]ouabain to the heterologously expressed enzyme [14]. α-, β- and γ-subunits of Na^+/K^+-ATPase were expressed in the yeast cells, and ouabain-sensitive hydrolysis of ATP and *p*-nitrophenyl phosphate, and ouabain-sensitive ^{86}Rb uptake, were measured. Nontransformed yeast cells or cells expressing either the γ-subunit alone or the α- and β-subunits simultaneously served as controls. No differences in enzymatic or transport activities were detected in samples containing either α-and β-subunits or α-, β-, and γ-subunits. Thus, these results suggest that the the putative γ-subunit of Na^+/K^+-ATPase is not directly involved in the mechanism of the Na^+/K^+-ATPase. The results do not exclude, however, the modulation of Na^+/K^+-ATPase function by γ in mammalian cells.

2. Experimental procedures

2.1. Materials

[^{3}H]Ouabain (15.4–28.9 Ci/mmol), ^{86}RbCl (0.12–4.23 Ci/mmol), and [γ-^{32}P]ATP (3000 Ci/mmol) were from Du Pont-New England Nuclear. Electrophoresis chemicals were purchased from Bio-Rad or Sigma. The ECL Western blotting system is a product of Amersham. Yeast growth media were from J.T. Baker. Enzymes for the coupled spectrophotometric assay were from Boehringer-Mannheim. Restriction enzymes or modifying enzymes for molecular biology were purchased from Boehringer-Mannheim or Stratagene. All other chemicals or biochemicals of the highest available degree of purity were from Sigma or J.T. Baker.

2.2. Methods

Construction of yeast expression plasmids

The plasmid pCGY1406$\alpha\beta$ coding for the α- and β-subunits of the Na^+/K^+-ATPase has been described earlier [14]. A pGEM-7Z plasmid containing cDNA coding for the rat γ-subunit [30] was provided by R. Mercer (Washington University). The plasmid was digested with *Nco*I to isolate a fragment beginning at the initiation ATG for the γ-subunit and ending 340 bp after the termination codon. The ends of the fragment were filled using the Klenow fragment of DNA polymerase. The cDNA was ligated into a unique *Hin*dIII site of the plasmid pAAH5 ([35]; gift from J. Lingrel, University Cincinnati) which had been filled using the Klenow fragment and had subsequently been treated with bovine intestinal alkaline phosphatase. The new plasmid containing the cDNA coding for the γ-subunit is designated pA-RG1. The plasmid carries the yeast LEU2 gene and the yeast 2 μm origin of replication. The transcription of the γ-cDNA is controlled by an ADH promoter. Yeast cells were transformed with each plasmid separately or with both simultaneously according to Ito et al. [31].

Yeast strains and media

The yeast strain RF44 (Mat α, ade 2–101, his$^-$, ura 3–52, leu 2,3–112, trp1-Δ901, trk1$^-$, gal$^-$, LYS$^+$) was used in these experiments. RF44 was developed by crossing strain DKY6224 (Mat a, suc 2-Δ9, ura 3–52, his 3-Δ200, trp 1-Δ901, ade 2–201, leu 2,3–112, Δ(pep4)::LEU2) and R1155 (Mat α, his 4–15, ura 3–52, lys 9, trk1$^-$). R1155 was a gift from R. Gaber

(Northwestern University). The trk1$^-$ mutation in R1155 and RF44 inactivates the high-affinity potassium transporter of these cells [42].

Ouabain sensitive uptake of ^{86}Rb into yeast cells

Yeast cells, grown overnight at 30°C with agitation at 250 rpm, were pelleted at 5000 × *g* for 10 min, were washed with cold water, and were pelleted again under the same conditions. The pelleted cells were suspended in 2% glucose at A_{600}/ml = 200 and were stored on ice. 500 μl of the cell suspension were incubated for 30 min at 37°C in in 10 mM Tris-HCl (pH 7.5), 5 mM PO_4(Tris) (pH 7.0), and 5 mM $MgCl_2$, in the presence or absence of 0.1 mM ouabain. Thereafter 2.5 mM ^{86}RbCl was added to each sample. The time-dependent accumulation of $^{86}Rb^+$ into the cells was followed at 22°C. The reaction was interrupted by centrifugation for 3 min in an Eppendorf centrifuge. Non-specifically trapped ^{86}RbCl was removed by resuspending the cells in cold water, followed by an additional centrifugation. Cells were dissolved in TS-1 tissue solubilizer prior counting for radioactivity. An internal standard for the specific radioactivity of $^{86}Rb^+$ was counted in parallel.

Isolation of mRNA and Northern analysis of α, β and γ transcription

mRNA isolation from yeast cells and Northern transfer and hybridization of the RNA with [^{32}P]DNA probes were performed using standard protocols [32,33]. The hybridization probe for the α-subunit was the full length sheep kidney α-subunit cDNA, for the β-subunit was the full length dog kidney β-subunit cDNA, and for the γ-subunit was the full length rat kidney γ-subunit cDNA

Isolation of yeast membranes enriched in Na^+/K^+-ATPase

Yeast membranes enriched in Na^+/K^+-ATPase were prepared following the method of Jørgensen [36] for purification of the enzyme from renal microsomal membranes. 12.3 mg of microsomal protein isolated from yeast cells [14] were incubated with 4.8 ml 100 mM imidazole/HCl, 4 mM Na_2EDTA (pH 7.5) and 0.29 ml 100 mM Na_2ATP at room temperature. While the solution was stirred, 2.53 ml of 1.9 mg SDS/ml were added within a time of 15 min. The final volume of the solution was 9.6 ml. The final concentrations of the solution were: 1.28 mg protein/ml, 0.5 mg SDS/ml, 25 mM imidazole/HCl, 1 mM Na_2EDTA (pH 7.5) and 3.0 mM Na_2ATP. After an additional 5 min of stirring at room temperature, the microsomal solution was layered on a discontinuous gradient formed by 8 ml of 40% sucrose, 5.5 ml of 30% sucrose and 3.5 ml of 25% sucrose in 25 mM imidazole/1 mM EDTA (pH 7.5). After centrifugation for 1.5 h at 140000 × g_{av} and 4°C, a fraction enriched in Na^+/K^+-ATPase was recovered from the interface between 30% and 40% sucrose. This fraction was diluted 1:3 with 25 mM imidazole/HCl, 1 mM Na_2EDTA (pH 7.4), and was pelleted for 30 min at 140000 × *g* and 4°C. The pellet was suspended in 0.2 ml of the imidazole/EDTA buffer and stored at −80°C.

Na^+/K^+-ATPase and p-nitrophenylphosphatase activities in membranes isolated after SDS treatment

50 μl of the protein suspension isolated after the SDS treatment were incubated for 30 min at 37°C in 10 mM Tris-HCl (pH 7.5), with or without 10 μM ouabain, 5 mM PO_4(Tris) (pH 7.0), and 5 mM $MgCl_2$. The total volume of the mixture was 100 μl. ATPase activities were determined in a coupled spectrophotometric assay [14]. The *p*-nitrophenylphosphatase activities were determined by incubating 25 μl of the protein suspensions with or without 100 μM ouabain in 5 mM *p*-nitrophenyl-phosphate, 10 mM KCl and 10 mM Tris-HCl (pH 7.5). The total volume was 1 ml. At different times 100 μl of the reaction mixtures were transferred to 1 ml 1 N NaOH in ice. The phosphatase activity was determined from the absorbance of the samples at 578 nm, with an ε = 18500 l/mol per cm [14]. Data were analyzed by analysis of variance using the Instat program (GraphPad Software).

Binding of [^{3}H]ouabain to Na^+/K^+-ATPase expressed in yeast

Microsomal membranes prepared from transformed or untransformed yeast cells [14] were incubated for 10 min at 37°C in 10 mM Tris-HCl (pH 7.5), 5 mM PO_4(Tris) (pH 7.0), 5 mM $MgCl_2$ and 162 nM [^{3}H]ouabain. The membranes were pelleted in an Eppendorf centrifuge for 5 min, were washed with cold distilled water, and were pelleted again. The pellets were dissolved in 0.5 ml 1 M NaOH at 55°C within 15 min, and radioactivity was measured after subsequent neutralization with HCl and addition of scintillation fluid. Non-specifically bound [^{3}H]ouabain was measured in the presence of 1 mM non-radioactive ouabain or in the presence of 50 mM KCl.

[^{3}H]ouabain incorporation as a function of [Na^+] in the presence of ATP and Mg^{2+} was measured by incubating microsomal membranes for 5 min at 37°C with different concentrations of NaCl in the presence of 100 μM ATP, 0.3 μM [^{3}H]ouabain and 10 mM Tris-HCl (pH 7.5). Non-specifically bound [^{3}H]ouabain was determined in the absence of ATP.

The K_d for ouabain binding to the Na^+/K^+-ATPase was determined according to Scatchard, as described earlier [14].

Detection of the γ-subunit in Western blots

40 μg of microsomal protein from yeast cells transformed with either the pCGY1406αβ plasmid, with

the pA-RG1 plasmid, or with both plasmids simultaneously, was separated on an SDS-polyacrylamide gel containing 12% polyacrylamide and 0.3% *N,N'*-methylene-bis-acrylamide [34]. Proteins of known relative molecular mass were run in parallel as molecular weight markers. After electrophoresis, the proteins were electroblotted to a nitrocellulose membrane. A rabbit polyclonal antiserum (GAMBLE) which was raised against the rat γ-subunit (gift of B. Forbush, Yale University) was used as a primary antibody. A commercially-available Enhanced Chemiluminescence (ECL) Western blotting system (Amersham) was used for the detection of the γ-subunit.

3. Results

An obligatory role for the putative γ-subunit in the catalytic functions of Na^+/K^+-ATPase has been investigated by the heterologous expression of the enzyme subunits in yeast cells. Yeast do not contain endogenous ouabain-sensitive Na^+/K^+-ATPase activity, and it has previously been shown that the presence of ouabain-sensitive ATPase activity in yeast requires the heterologous expression of both α- and β-subunits of the enzyme [14]. Although this observation indicates that the γ-subunit is not required for ATP hydrolysis by Na^+/K^+-ATPase, the γ-subunit may be required for ion transport, or may modulate the apparent affinity of the pump for transported cations or ATP.

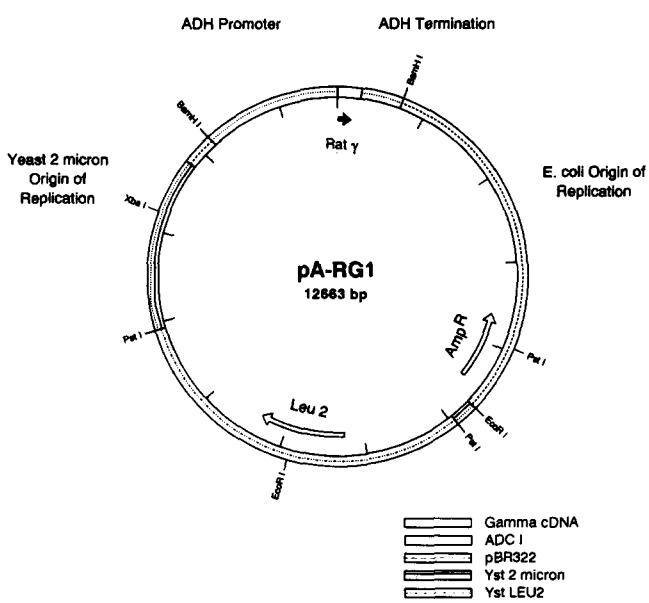

Fig. 1. Yeast expression plasmid for the putative γ-subunit. cDNA coding for the rat γ-subunit was digested with *Nco*I to remove a 25 bp fragment from the 5' untranslated region of the cDNA. The ends were filled using the Klenow fragment. The cDNA was ligated into a unique *Hin*dIII site of the plasmid pAAH5, which prior to the ligation reaction, was filled using the Klenow fragment and subsequently treated with calf intestinal alkaline phosphatase. The new plasmid containing the cDNA coding for the γ-subunit is designated pA-RG1. Yeast cells were transformed with pA-RG1 and with pCGY1406αβ [14].

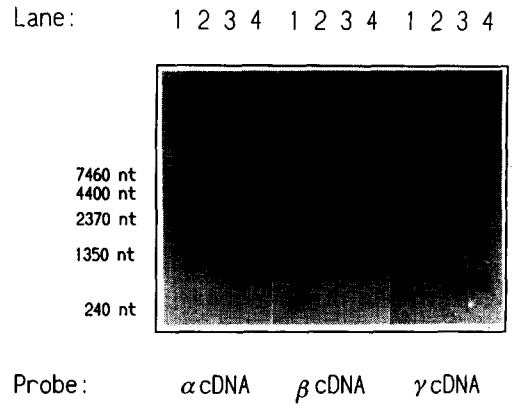

Fig. 2. Northern analysis of α, β and γ transcription. RNA isolation from yeast cells and Northern transfer and hybridization of the RNA with [^{32}P]DNA, were performed using standard protocols. In all panels lane 1 contains RNA from non-transformed cells, lane 2 contains RNA from cells transformed with pCGY1406αβ, lane 3 contains RNA from cells transformed with pA-RG1, and lane 4 contains RNA from cells transformed with both pCGY1406αβ and pA-RG1. The left lanes 1–4 show the hybridization of the isolated RNA with cDNA coding for the α-subunit. The sheep kidney α-mRNA is 4050 nt long. The middle lanes 1–4 show the hybridization with cDNA coding for the β-subunit. The dog kidney β-mRNA is 1680 nt long. The right lanes 1–4 show the hybridization between isolated RNA and cDNA coding for the γ-subunit. The rat kidney γ-mRNA is 680 nt long. The results demonstrate that the cells transformed with both plasmids (lane 4) synthesize full length mRNA for all α-, β- and γ-subunits.

cDNA coding for the γ-subunit was ligated into the yeast expression vector pAAH5 [35]. The new vector, designated pA-RG1, is shown in Fig. 1. In this plasmid, the transcription of γ-subunit mRNA is under the control of the yeast alcohol dehydrogenase promoter, and the yeast LEU2 gene is used as an auxotrophic marker. pA-RG1 was used together with the previously described plasmid pCGY1406αβ [14], coding for both α- and β-subunits of Na^+/K^+-ATPase, to transform yeast strain RF44. Control cells contained either one of the above plasmids, or neither of them.

mRNA extracted from yeast cells transformed with pCGY1406αβ, pA-RG1, or with both plasmids, was probed by Northern analysis using probes for the α-, β-, and γ-subunits of Na^+/K^+-ATPase. The results of this analysis (Fig. 2), indicate that mRNA for each subunit is transcribed in the yeast cells, and that the size of the mRNA molecules that hybridize to each subunit probe is sufficient to encode full-length polypeptides. None of the probes hybridize to RNA from non-transformed cells (lane 1), indicating that non-transformed yeast cells do not have mRNA with significant sequence homology to the α-, β-, or γ-subunits of Na^+/K^+-ATPase. Cells containing expression plasmids encoding either the α- and β-subunits (lane 2), or the

γ-subunit alone (lane 3), contain mRNA that hybridizes with probes for those subunits only. In rat tissues, there are two RNA transcripts of 1.5 and 0.8 kb for γ [30], but only a transcript of about 0.8 kb is found after heterologous expression of γ in yeast. Hybridization of the mRNA to all three radiolabeled cDNA probes is seen only in lane 4 of Fig. 2, indicating that the mRNA for all of the Na^+/K^+-ATPase subunits is transcribed in the doubly-transformed cells.

A Western blot of membranes prepared from yeast cells and incubated with anti-γ subunit antiserum shows that the γ-subunit mRNA is translated into protein in yeast cells transformed with pA-RG1 alone, or pA-RG1 and pCGY1406αβ (Fig. 3). Translation of the α- and β-subunits of Na^+/K^+-ATPase has been demonstrated previously [14]. The polyclonal antiserum used in these experiments binds to several proteins from yeast cells, as do many polyclonal antisera raised in rabbits against either α- or β-subunits (data not shown). Despite its low specificity, however, the antiserum recognizes a small protein at $M_r = 11000$ that is present only in membranes from yeast cells transformed with both pA-RG1 and pCGY1406αβ (lane 1) or with pA-RG1 alone (lane 3). Membranes from cells transformed only with the pCGY1406αβ do not contain this protein (lane 2). The electrophoretic mobility of this protein is identical to the mobility of a $M_r = 11000$ protein present in purified Na^+/K^+-ATPase from pig kidney (lane 4), and which is also recognized by the antiserum. This small protein is presumably the γ-subunit, and this experiment indicates that the γ-subunit encoded by the plasmid pA-RG1 is translated into protein in the yeast cells.

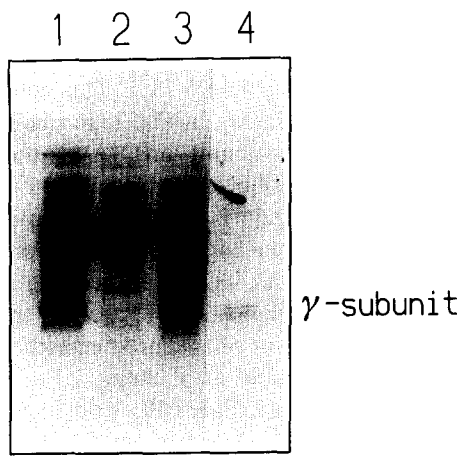

Fig. 3. Immunodetection of the γ-subunit expressed in yeast cells. 40 μg of microsomal protein isolated from yeast cells which had been transformed with the pA-RG1 and the pCGY1406αβ simultaneously (lane 1), or with either the pCGY1406αβ (lane 2) or the pA-RG1 (lane 3), was separated on SDS polyacrylamide gels (36). 1.5 μg Na^+/K^+-ATPase from pig kidney served as a positive control (lane 4). After electroblotting, the nitrocellulose was incubated with a rabbit polyclonal antiserum (GAMBLE) raised against rat γ-subunit. The secondary antibody was anti-rabbit immunoglobulin G, coupled to horse radish peroxidase. Proteins were detected by a commercially available system (Amersham) which uses chemiluminescence for the detection of the bound horse radish peroxidase-conjugated antibodies. A doublet of immuno-positive bands at $M_r = 11000$ in lane 4 indicates the position of the γ-subunit.

Table 1
Ouabain-sensitive ATPase and *p*-nitrophenylphosphatase activity in yeast membranes containing the indicated subunits of Na^+/K^+-ATPase, after extraction with SDS

Subunits	ATPase activity (mU/mg)			
	total activity	+ouabain	difference	%
None	17.1±0.3	17.5±0.3	−0.4±0.4	(−2.3)
γ	17.6±0.4	18.2±0.3	−0.6±0.5	(−3.4)
α+β	21.3±1.0	17.5±0.9	3.8±1.3	17.7
α+β+γ	19.9±0.7	16.3±0.5	3.7±0.9	18.4
Subunits	**Phosphatase activity (mU/mg)**			
	total activity	+ouabain	difference	%
None	2.17±0.03	2.17±0.13	0.0	0
γ	2.17±0.03	2.17±0.13	0.0	0
α+β	2.60±0.25	2.13±0.19	0.47±0.31	17.9
α+β+γ	2.70±0.10	2.17±0.03	0.49±0.10	18.0

Yeast cells were transformed with expression plasmids encoding the Na^+/K^+-ATPase subunits indicated, or non-transformed cells were used. Microsomal membranes were prepared from the cells as described in Methods, and the membranes were extracted with SDS [36]. Ouabain sensitive Na^+/K^+-ATPase (top) and *p*-nitrophenylphosphatase (bottom) activities were measured as described in Methods. Activities are shown as means±S.E. of different preparations ($n = 3$). 1 mU is defined as the hydrolysis of 1 nmol substrate per min.

In order to determine whether the γ-subunit has an effect on the Na^+/K^+-ATPase enzymatic activities, membranes from yeast cells expressing α+β or α+β+γ subunits were isolated after SDS treatment and their ATPase or *p*-nitrophenylphosphatase activities were measured [37,38] in the presence or absence of ouabain. The results of these measurements are presented in Table 1. The absence of ouabain-sensitive ATPase and *p*-nitrophenylphosphatase activities in membranes from non-transformed yeast cells and from cells expressing only γ confirms previous results in which expression of α- and β-subunits were required for Na^+/K^+-ATPase activity [14]. In the membranes containing α- and β-subunits, approximately 18% of the total ATPase activity measured under these conditions was inhibited by ouabain, and this fraction is not different when the γ-subunit is co-expressed with the α- and β-subunits. Differences in specific ATPase activity between samples are significant ($P < 0.001$) only for α+β vs. γ or α+β vs. none, and α+β+γ vs. γ or α+β+γ vs. none. Differences are not significant ($P > 0.05$) for both α+β vs. α+β+γ and for γ vs. none. Fractional inhibition of hydrolysis of *p*-nitrophenyl phosphate by membranes from cells expressing either Na^+/K^+-ATPase α+β subunits or

$\alpha+\beta+\gamma$ subunits was similar to the inhibition of ATPase activity. Activity in membranes from cells expressing $\alpha+\beta$ and $\alpha+\beta+\gamma$ subunits was significantly different from zero ($P<0.05$), but was not significantly different from each other ($P>0.05$). The ratio of the ATPase activity to the phosphatase activity for membranes expressing Na^+/K^+-ATPase activities is in the same range as for the kidney Na^+/K^+-ATPase [39]. These data indicate that ouabain-sensitive enzymatic activities in the yeast membranes are due to the presence of Na^+/K^+-ATPase, and confirm that the α and β subunits are sufficient for these activities [25].

Since a role for the γ-subunit in Na^+/K^+-ATPase was first suggested by experiments using cardiac glycoside derivatives, the interaction between Na^+/K^+-ATPase and ouabain was examined to determine whether the presence of the γ-peptide had any effect on cardiac glycoside binding. Scatchard analysis indicated that [^{3}H]ouabain binds to a single class of sites on yeast cell membranes expressing either α- and β-subunits [14,15] or α-, β-, and γ-subunits (data not shown). K_d values of 12.0 nM and 12.3 nM were calculated for ouabain binding to membranes from cells expressing either α- and β-subunits or α-, β-, and γ-subunits, respectively. Ouabain binding to Na^+/K^+-ATPase is antagonized by K^+ ions, which decrease the amount of the (Na^+/K^+-ATPase · ouabain) complex formed in the presence of phosphate by increasing the dephosphorylation rate of the enzyme [40,41]. K^+ ions have previously been shown to antagonize the binding of ouabain to Na^+/K^+-ATPase expressed in yeast cells [15]. The displacement of the bound [^{3}H]ouabain from the yeast membranes by K^+ verifies that ouabain is specifically bound to the Na^+/K^+-ATPase expressed in these cells. Binding of [^{3}H]ouabain to membranes from yeast cells expressing $\alpha+\beta$ or $\alpha+\beta+\gamma$ subunits was measured in the presence or absence of 50 mM KCl (data not shown). The maximum amount of bound [^{3}H]ouabain was 4.8 pmol/mg yeast membrane protein in the absence of the γ-peptide, and 4.6 pmol/mg yeast membrane protein in the presence of the γ-peptide. 50 mM KCl reduced the amount of ouabain bound to membranes expressing $\alpha+\beta$ subunits by 85% and reduced the amount of ouabain bound to membranes expressing $\alpha+\beta+\gamma$ subunits by 93%. Membranes from non-transformed cells or from cells expressing only the γ-oligopeptide did not bind [^{3}H]ouabain (not shown). These results demonstrate that the γ-peptide is not required for high-affinity ouabain binding by Na^+/K^+-ATPase, and that the sensitivity of the enzyme to antagonism of ouabain binding by potassium is not affected by the γ-subunit.

In order to determine whether the γ-polypeptide is necessary for the formation of the ionophoric part of the Na^+/K^+-ATPase, the uptake of ^{86}Rb into yeast cells was measured. Rb^+ is known to be transported by Na^+/K^+-ATPase as an analog of K^+ [43,44]. Measurements of ouabain-sensitive $^{86}Rb^+$ uptake into several transformed yeast strains used for enzymatic assays were unsuccessful due to the presence of a high-affinity potassium transporter encoded by the TRK1 gene [42]. The ouabain-sensitive component of $^{86}Rb^+$ uptake was too small to measure against the background uptake due to this transporter. A strain of yeast in which the TRK1 gene had been deleted (R1155) was crossed with a yeast strain containing appropriate auxotrophic markers (DKY 6224) to generate strain RF44. RF44 was transformed with pCGY1406$\alpha\beta$ alone or with pA-RG1 and pCGY1406$\alpha\beta$, and was used in the experiments described below. Non-transformed RF44 cells were used as a control.

Fig. 4 compares the uptake of $^{86}Rb^+$ in RF44 cells containing the different expression plasmids. While ouabain sensitive $^{86}Rb^+$ uptake was not detected in the non-transformed cells (Fig. 4a), $^{86}Rb^+$ uptake into cells expressing the α- and β-subunits or the α-, β- and γ-subunits was inhibited approximately 30% by ouabain (Fig. 4b and c). Since the uptake of $^{86}Rb^+$ in the presence of ouabain is indistinguishable in all cells, the ouabain-sensitive increase in the transport of $^{86}Rb^+$ into the cells is attributed to the activity of the expressed Na^+/K^+-ATPase. The cells expressing all three subunits (Fig. 4c) show no difference in the ouabain-sensitive transport activity compared to the cells expressing only α- and β-subunits (Fig. 4b). This observation indicates that the γ-subunit is not essential for the transport of Rb^+ ions by Na^+/K^+-ATPase.

Using yeast strain RF44 it was not possible to detect a ouabain-sensitive efflux of $^{22}Na^+$ from non-transformed cells or from cells expressing the $\alpha+\beta$ or the $\alpha+\beta+\gamma$ subunits. A very efficient endogenous Na^+ efflux pathway located in the yeast plasma membrane [45] obscures the part of the transport that is catalyzed by the heterologously-expressed sodium pump. As a measure of the apparent affinity of the enzyme for Na^+, therefore, the binding of [^{3}H]ouabain was measured in the presence of ATP and Mg^{2+} and different concentrations of NaCl.

The scheme for the reaction is [1,2,46]:

$$E_1 + ATP + (3Na^+)_{in} \longleftrightarrow (Na)_3 \cdot E_1\text{–}P \cdot ADP$$

$$\Updownarrow$$

$$(Na)_3 \cdot E_1\text{–}P + ADP$$

$$\Updownarrow$$

$$E^*P \cdot [^3H]\text{ouabain} \longleftrightarrow E_1\text{–}P + (3Na^+)_{out} + [^3H]\text{ouabain}$$

The high-affinity binding of ouabain to Na^+/K^+-ATPase depends on the formation of the phosphoenzyme (E_1-P), which, in this reaction, requires Na^+. In

the presence of ATP and Mg^{2+}, Na^+ is known to stimulate ouabain binding with an apparent affinity of 0.6 mM [47]. Fig. 5 shows the Na^+-concentration dependence of ouabain binding to yeast membranes. The $K_{0.5}$ value calculated for the effect of Na^+ on ouabain binding to the membranes from cells expressing $\alpha+\beta$ subunits is 2.7 mM, and is 2.1 mM for cells expressing $\alpha+\beta+\gamma$ subunits. These values are similar to a $K_{0.5}$ of 1.6 mM for the stimulation of phosphorylation of the enzyme by Na^+ acting at cytosolic sites [48]. Assuming that the $K_{0.5}$ value for the Na^+-dependence of ouabain binding is an indication of the affinity of the expressed enzymes for Na^+, these data indicate that the γ-subunit does not influence Na^+ binding or transport by Na^+/K^+-ATPase.

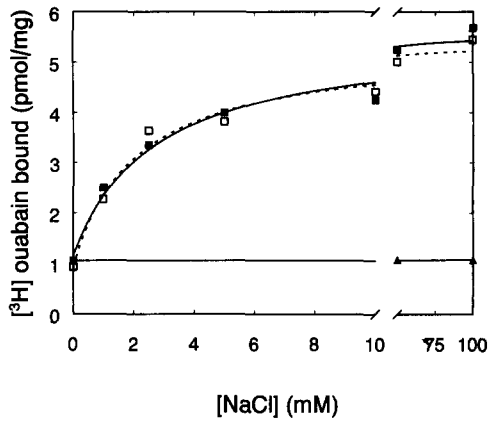

Fig. 5. Na^+, Mg^{2+} and ATP dependent binding of [^{3}H]ouabain. [^{3}H]Ouabain incorporation was measured as a function of Na^+ concentration in the presence of 0.1 mM ATP and 5 mM Mg^{2+}. Microsomal membranes from cells expressing $\alpha+\beta$ subunits (□) or $\alpha+\beta+\gamma$ subunits (■) were incubated for 5 min at 37°C with varying concentration of NaCl in the presence of 100 μM ATP, 0.3 μM [^{3}H]ouabain, 5 mM $MgCl_2$, and 10 mM Tris-HCl (pH 7.5). The solid curve and the dashed curve are fits of the Michaelis-Menten equation to the data from membranes from cells expressing the $\alpha+\beta$ subunits and the $\alpha+\beta+\gamma$ subunits, respectively. Non-specifically bound [^{3}H]ouabain was determined in the absence of ATP (▲), and was subtracted from the total binding observed in membranes from cells expressing the $\alpha+\beta$ subunits and the $\alpha+\beta+\gamma$ subunits for analysis. The data are fit with a straight line. Data are shown from a single representative experiment.

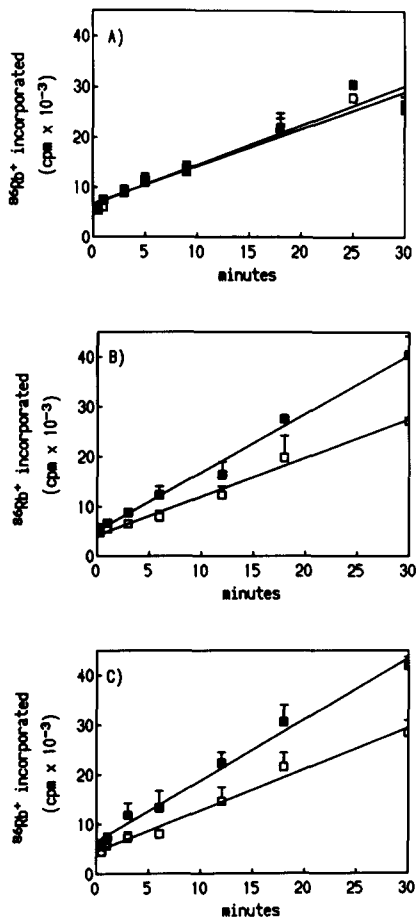

Fig. 4. $^{86}Rb^+$ uptake into yeast cells expressing different subunits of Na^+/K^+-ATPase. Yeast cells, grown over night at 30°C with 250 rpm agitation, were pelleted at 5000 × *g* for 10 min, were washed with water, and were pelleted again under the same conditions. The pelleted cells were resuspended in 2% glucose and stored on ice. 500 ml of the cells suspension were incubated for 30 min at 37°C with (□) or without (■) 0.1 mM ouabain in 10 mM Tris-HCl (pH 7.5), 5 mM PO_4(Tris) (pH 7.0), and 5 mM $MgCl_2$. Thereafter 2.5 mM $^{86}RbCl$ was added to each sample. The time dependent accumulation of $^{86}Rb^+$ into cells expressing either none (A) or the α- and β-subunits (B) or the α-, the β- and the γ-subunits simultaneously (C) was followed at 22°C. The reaction was interrupted by centrifugation for 3 min in an Eppendorf centrifuge. Non specifically-entrapped $^{86}RbCl$ was removed by resuspending the cells in cold water, followed by an additional centrifugation. Cells were dissolved in TS-1 tissue solubilizer prior counting for radioactivity. Data are shown as mean ± S.E. ($n = 3$).

4. Discussion

The results presented here indicate that the γ-subunit is not essential either for ouabain binding, or for the ATPase or phosphatase activities of Na^+/K^+-ATPase. The data also suggest that the participation of the γ-polypeptide in the formation of the ionophoric part of the sodium pump is very unlikely, since no effect of γ was observed on Rb^+ uptake by yeast cells expressing Na^+/K^+-ATPase or on Na^+ binding by the enzyme. These conclusions are in agreement with a previous investigation [25] in which the γ-subunit was removed from membranes by detergent extraction but ouabain-sensitive ATP hydrolysis was not affected. The absence in yeast cells of mRNA homologous to the γ-mRNA (Fig. 2) and the absence of protein homologous to the γ-protein (Fig. 3) also indicate that these results are not affected by an endogenous yeast γ-subunit that might interact with the αβ-subunit complex.

Although the data indicate that the γ-subunit is not required either for the enzymatic or the transport reactions of the pump, the results of these investiga-

tions do not allow us to exclude the possibility that the γ-polypeptide might be associated with some other functions of the sodium pump, such as the asymmetric distribution of the Na^+/K^+-ATPase that is observed in epithelial tissues, or in the association of the enzyme with the cytoskeleton. The γ-polypeptide may also participate in the physiological regulation of the Na^+/K^+-ATPase, possibly after phosphorylation by a protein kinase, as has been observed for phospholamban regulation of cardiac sarcoplasmic reticulum Ca^{2+}-ATPase [49]. The γ-subunit has been shown to have some amino acid sequence homology to phospholemman, the major target for cAMP-dependent protein kinase in the cardiac plasma membrane [50]. Investigations of a possible regulatory role for the γ-subunit in Na^+/K^+-ATPase must be done in a system in which Na^+/K^+-ATPase is normally found. Finally, the interaction of the γ-peptide with the αβ complex may not be preserved in the SDS-extracted membranes used for the ATPase and *p*-nitrophenylphosphatase assays reported here. It has been shown that detergent extraction of renal membranes containing Na^+/K^+-ATPase disrupts interactions between αβ and γ, but not between α and β [30]. Unlike the other measurements reported here, which can be made on intact yeast cells or on microsomal membranes from yeast cells, measurement of the ouabain-sensitive hydrolysis of ATP and *p*-nitrophenyl phosphate in yeast membranes expressing Na^+/K^+-ATPase requires extraction of the membranes with SDS. The γ-subunit was observed in microsomal membranes from yeast cells transformed with expression plasmids containing γ-subunit cDNA (Fig. 3), however, Western blots of SDS-extracted yeast membranes prepared from these cells were unable to detect this peptide or were inconclusive (data not shown). Thus, although the enzymatic and transport properties of Na^+/K^+-ATPase require only α- and β-subunits, a role for the γ-peptide in the modulation of enzymatic activity can not be excluded by the data reported here.

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References

[1] Schuurmans Stekhoven, F. and Bonting, S.L. (1982) Physiol. Rev. 61, 1–76.
[2] Skou, J.C. (1988) Methods Enzymol. 156, 1–25.
[3] Glynn, I.M. and Karlish, S.J.D. (1975) Annu. Rev. Physiol. 37, 13–54.
[4] Glynn, I.M. (1993) J. Physiol. 462, 1–30.
[5] Shull, G.E., Schwartz, A. and Lingrel, J.B. (1985) Nature 316, 691–695.
[6] McDonough, A.A., Geering, K. and Farley, R.A. (1990) FASEB J. 4, 1598–1605.
[7] Matsui, H. and Schwartz, A. (1968) Biochim. Biophys. Acta 151, 655–663.
[8] Forbush, B., III and Hoffman, J.F. (1979) Biochemistry 18, 2308–2315.
[9] Huang, W. and Askari, A. (1976) Arch. Biochem. Biophys. 175, 185–189.
[10] Allen, J.C., Entmann, M.L. and Schwartz, A. (1975) J. Pharmacol. Exp. Ther. 192, 105–112.
[11] Bodemann, H.H. (1981) Klin. Wochenschr. 59, 1333–1343.
[12] Geering, K., Meyer, D.I., Paccolat, M.-P., Kraehenbühl, J.-P. and Rossier, B.C. (1985) J. Biol. Chem. 260, 5154–5160.
[13] Noguchi, S., Mishina, M., Kawamura, M. and Numa, S. (1987) FEBS Lett. 225, 27–32.
[14] Horowitz, B., Eakle, K.A., Scheiner-Bobis, G., Randolph, G.R., Chen. C.Y., Hitzeman, R.A. and Farley, R.A. (1990) J. Biol. Chem. 265, 4189–4192.
[15] Eakle, K.A., Kim, K.S., Kabalin, M.A. and Farley, R.A. (1992) Proc. Natl. Acad. Sci. USA 89, 2834–2838.
[16] Jaisser, F., Canessa, C.M., Horisberger, J-D. and Rossier, B.C. (1992) J. Biol. Chem. 267, 16895–16903.
[17] Lutsenko, S. and Kaplan, J.H. (1993) Biochemistry 32, 6737–6743.
[18] Ruoho, A. and Kyte, J. (1974) Proc. Natl. Acad. Sci. USA 71, 2352–2356.
[19] Alexander, A. (1974) FEBS Lett. 45, 150–154.
[20] Forbush, B., III, Kaplan, J.H. and Hoffman, J.F. (1978) Biochemistry 17, 3667–3676.
[21] Collins, J.H., Forbush III, B., Lane, L.K., Ling, E., Schwartz, A. and Zot, A. (1982) Biochim. Biophys. Acta 686, 1–12.
[22] Rossi, B., Viulleumier, P., Gache, C., Balerna, M. and Lazdunski, M. (1980) J. Biol. Chem. 255, 9936–9941.
[23] Hall, C. and Ruoho, A. (1980) Proc. Natl. Acad. Sci. USA 77, 4529–4533.
[24] Rempeters, G. and Schoner, W. (1981) Eur. J. Biochem. 121, 131–137.
[25] Hardwicke, P.M.D. and Freytag, J.W. (1981) Biochem. Biophys. Res. Commun. 102, 250–257.
[26] Reeves, A.S., Collins, J.H. and Schwartz, A. (1980) Biochem. Biophys. Res. Commun. 95, 1591–1598.
[27] Collins, J.H., Kranias, E., Reeves, A.S., Bilezikjian, L.M. and Schwartz, A. (1981) Biochem. Biophys. Res. Commun. 99, 796–803.
[28] Racker, E. (1976) Trends Biochem. Sci. 1, 244.
[29] Collins, J.H. and Leszyk,J. (1987) Biochemistry 26, 8665–8668.
[30] Mercer, R.W., Biemesderfer, D., Bliss, D.P., Jr., Collins, J.H. and Forbush, B., III (1993) J. Cell Biol. 121, 579–586.
[31] Ito, H., Fukuda, Y., Murata, K. and Kimura, A. (1983) J. Bacteriol. 153, 163–168.
[32] Eakle, K.A., Bernstein, M. and Emr, S.D. (1988) Mol. Cell. Biol. 8, 4098–4109.
[33] Thomas, P.S. (1980) Proc. Natl. Acad. Sci. USA 77, 5201–5205.
[34] Laemmli, U.K. (1970) Nature 227, 680–685.
[35] Ammerer, G. (1983) Methods Enzymol. 101, 192–201.
[36] Jørgensen, P.L. (1974) Biochim. Biophys. Acta 356, 36–52.
[37] Robinson, J.D. (1969) Biochemistry 8, 3348–3355.

[38] Pitts, J.B.R. and Askari, A. (1971) Biochim. Biophys. Acta 227, 453–459.
[39] Esmann, M. (1988) Methods Enzymol. 156, 105–115.
[40] Erdmann, E. and Schoner, W. (1973) Biochim. Biophys. Acta 330, 302–315.
[41] Scheiner-Bobis, G., Fahlbusch, K. and Schoner, W. (1987) Eur. J. Biochem. 168, 123–131.
[42] Gaber, R.F., Styles, C. and Fink, G.R. (1988) Mol. Cell. Biol. 8, 2848–2859.
[43] Skou, J. (1960) Biochim. Biophys. Acta 42, 6–23.
[44] Vilsen, B., Andersen, J.P., Petersen, J. and Jørgensen, P.L. (1987) J. Biol. Chem. 262, 10511–10517.
[45] Rodriguez-Navarro, A. and Ortega, M.D. (1982) FEBS Lett. 138, 205–208.
[46] Glynn, I.M., Hara, Y. and Richards, D.E. (1984) J. Physiol. 351, 531–547.
[47] Inagaki, C., Lindenmayer, G.E. and Schwartz, A. (1974) J. Biol. Chem. 249, 5235–5140.
[48] Post, R.L., Sen, A.K. and Rosenthal, A.S. (1965) J. Biol. Chem. 240, 1437–1445.
[49] Sasaki, T., Inui, M., Kimura, Y., Kazuya, T. and Tada, M. (1992) J. Biol. Chem. 267, 1674–1679.
[50] Palmer, C.J., Scott, B.T. and Jones, L.R. (1991) J. Biol. Chem. 266, 11126–11130.