Expression and Functional Role of the γ Subunit of the Na,K-ATPase in Mammalian Cells*

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The functional role of the γ subunit of the Na,K-ATPase was studied using rat γ cDNA-transfected HEK-293 cells and an antiserum (γC33) specific for γ. Although the sequence for γ was verified and shown to be larger (7237 Da) than first reported, it still comprises a single initiator methionine despite the expression of a γC33-reactive doublet on immunoblots. Kinetic analysis of the enzyme of transfected compared with control cells and of γC33-treated kidney pumps shows that γ regulates the apparent affinity for ATP. Thus, γ-transfected cells have a decreased $K_{ATP}$ as shown in measurements of (i) $K_{ATP}$ of Na,K-ATPase activity and (ii) $K^+$ inhibition of Na-ATPase at 1 μM ATP. Consistent with the behavior of γ-transfected cells, γC33 pretreatment increases $K_{ATP}$ of the kidney enzyme and $K^+$ inhibition (1 μM ATP) of both kidney and γ-transfected cells. These results are consistent with previous findings that an antiserum raised against the pig γ subunit stabilizes the $E_2(K)$ form of the enzyme (Therien, A. G., Goldshleger, R., Karlish, S. J., and Blostein, R. (1997) J. Biol. Chem. 272, 32628–32634). Overall, our data demonstrate that γ is a tissue (kidney)-specific regulator of the Na,K-ATPase that can increase the apparent affinity of the enzyme for ATP in a manner that is reversible by anti-γ antiserum.

The Na,K-ATPase is the sodium pump protein responsible for maintaining the electrochemical gradient present across the membranes of most animal cells (1). It consists of at least two subunits, α and β, each of which exists as one of several isoforms ($\alpha_1$, $\alpha_2$, $\alpha_3$, and $\alpha_4$ and $\beta_1$, $\beta_2$, and $\beta_3$; for review, see Ref. 2). The α subunit, also known as the catalytic subunit, contains the binding sites for the enzyme’s nucleotide and cation substrates, as well as the catalytic and regulatory (calcium-dependent and cAMP-dependent protein kinase C and A, respectively) phosphorylation sites. The role of the β subunit is less clear, but it is required for normal processing and expression of the enzyme and may have a role in regulating the interaction of cations with the α subunit (3). The different isoforms of the pump are expressed in a tissue- and develop-

ment-specific fashion and are believed to be distinct in both function and modes of regulation (2).

A small single-transmembrane protein called the γ subunit was originally believed to be a third subunit of the pump. It was discovered and shown to be larger (7237 Da) than first reported, but it still comprises a single initiator methionine despite the expression of a γC33-reactive doublet on immunoblots. Kinetic analysis of the enzyme of transfected compared with control cells and of γC33-treated kidney pumps shows that γ regulates the apparent affinity for ATP. Thus, γ-transfected cells have a decreased $K_{ATP}$ as shown in measurements of (i) $K_{ATP}$ of Na,K-ATPase activity and (ii) $K^+$ inhibition of Na-ATPase at 1 μM ATP. Consistent with the behavior of γ-transfected cells, γC33 pretreatment increases $K_{ATP}$ of the kidney enzyme and $K^+$ inhibition (1 μM ATP) of both kidney and γ-transfected cells. These results are consistent with previous findings that an antiserum raised against the pig γ subunit stabilizes the $E_2(K)$ form of the enzyme (Therien, A. G., Goldshleger, R., Karlish, S. J., and Blostein, R. (1997) J. Biol. Chem. 272, 32628–32634). Overall, our data demonstrate that γ is a tissue (kidney)-specific regulator of the Na,K-ATPase that can increase the apparent affinity of the enzyme for ATP in a manner that is reversible by anti-γ antiserum.

EXPERIMENTAL PROCEDURES

Antibodies—γC33 is a rabbit polyclonal antiserum raised against a peptide representing the C-terminal 10 amino acids of the γ subunit. In the experiments reported herein, γC33 was used, and a control nonimmune serum was obtained from the same rabbit prior to immunization. The peptide, KHRQVNEDEL, was synthesized at the Alberta Peptide Institute, University of Alberta, and used either as the free peptide for competition studies or linked to keyhole limpet hemocyanin and emulsified with Freund’s adjuvant before injection into rabbits. Antibody 6H is a mouse monoclonal antibody specific for the $\alpha_1$ isoform of the Na,K-ATPase, and was a generous gift from Dr. Michael Caplan, Yale University. Horseradish peroxidase-labeled secondary antibodies (donkey anti-rabbit) were purchased from BIO/CAN Scientific.

5′-RACE and pREP4-γ Synthesis—5′-Rapid amplification of cDNA ends (5′-RACE) 3 was carried out using CLONTECH Marathon-ready cDNA from rat kidney following the manufacturer’s instructions. Appropriate primers (see below) were synthesized, and the γ subunit gene sequence was amplified by polymerase chain reaction. The 5′-end primer contained a site for HindIII endonuclease (boldface), a Kozak sequence (underlined; see Ref. 13), and the first 24 bases of the γ subunit gene as determined by Mercer et al. (5) (GGGCGGACGTCGTCGACAGAGCTGCTGTACGACTACACAT). The 3′-end primer contained a BamHI endonuclease site (boldface) and bases complementary to the last 24 bases of the γ subunit gene as determined by Mercer et al. (5) (GGGGGATCCGTCACAGCTCTTTCATCGAATTC). The resulting DNA was then cleaved with these endonucleases and ligated into the corresponding sites of pREP4 vector (Invitrogen) to make pREP4-γ. Sequencing of the recombinant plasmid was carried out using a Pharmacia T7 sequencing kit. pREP4 and pREP4-γ DNA used for

The abbreviated version is: 5′-RACE, 5′-rapid amplification of cDNA ends.

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‡‡ The nucleotide sequence reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number AF129400.

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Expression of the γ Subunit in Mammalian Cells—Our earlier evidence of a modulatory role for the γ subunit on the conformational equilibrium of the Na,K-ATPase reaction was inferred from studies of the effects of an anti-γ antiserum on enzymatic activity. To evaluate directly the functional role of γ, it was essential to transfect cDNA encoding γ into mammalian cells devoid of γ. An additional goal of such experiments was to establish the basis for the existence of γ as a doublet in the rat (5) as it is in the Xenopus kidney (7). Accordingly, we first used 5′-RACE to ascertain that the previously reported cDNA of the rat γ subunit comprised the full-length sequence and, if not, whether the doublet in Western blots is secondary to the presence of an additional start codon in the mRNA for the γ subunit as is the case for Xenopus kidney (7). The resulting sequence, shown in Fig. 1, confirmed the presence of a single initiator methionine. However, the γ cDNA thus obtained encodes a protein of 66 rather than 58 residues, as originally reported (5), and corresponds to the sequence subsequently revised by Minor et al. (11). The calculated molecular mass is 7237 Da. The dichotomy may be the result of either a cloning artifact or, possibly, an isoform variant.3

Efforts to express γ in HeLa and HEK cells using a standard stable transfection system resulted in levels of expression that, compared with the kidney, were considered too low (γα < 0.1) given the relatively modest effects of anti-γ on the kidney enzyme. In an effort to increase the level of expression, we used the plasmid pREP4 that combines the advantages of "classical" transient and stable expression systems. In addition a hygromycin resistance gene, this plasmid contains an origin of replication that allows it to remain expressed episomally for several weeks in the nuclei of primate and canine cells. Thus, hygromycin can be used to select for cells that contain multiple copies of the gene (rather than just one). Accordingly, we subcloned the gene for γ (revised sequence shown in Fig. 1) in pREP4 and transfected HEK-293 cells with both recombinant and wild type plasmids. Membranes were made from the transfected HEK-pREP4-γ and control HEK-pREP4 cells, and the amount of γ subunit protein relative to a subunit protein was estimated by a comparison with kidney membranes using Western blot analyses of both the γ and α subunits.

The blots shown in Fig. 2 indicate that the γ doublet is present in both kidney and HEK-pREP4-γ membranes but not in control HEK-pREP4 membranes. The densities of the γ subunit doublet and α subunit band of HEK-pREP4-γ were compared with those of the kidney using several dilutions and varying times of exposure to film. We determined that pREP4-γ membranes contained 34 ± 12% (S.E.) of the amount of γ present in the kidney after normalizing for α, densities. Assuming that observations.

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the γα ratio of kidney is 1:1 (7, 15, 16), this indicates that the stoichiometry of the γα proteins in HEK-pREP4-γ ~ 1:3. That this ratio reflects γ associated with α was confirmed in Western blots of immunoprecipitates using the antibody 6H (not shown).

Functional Effects of γ—We showed earlier that binding of antibodies raised to the γ polypeptide doublet associated with the pig kidney Na,K-ATPase binds to the cytoplasmic tail of the γ subunit (12). This binding was associated with partial inhibition of the Na,K-ATPase activity. Moreover, inhibition varied as a function of conditions that affect the rate-limiting step(s) during steady-state hydrolysis, for example varying pH. Thus, the inhibition (~30%) observed under conditions of optimal concentrations of substrates and at pH 7.4 decreased as the pH level increased and increased as pH decreased. We concluded that the antiserum caused a shift in the $E_I \leftrightarrow E_2(K)$ equilibrium toward $E_2(K)$.

To maximize the inhibitory effect of the antiserum, particularly for tests of the effect of γ in the transfected cells in which the γα ratio is lower than in the kidney medulla, we tested the prediction that inhibition would be greater at suboptimal ATP concentrations, under which conditions the $E_2(K) \rightarrow E_I$ sequence becomes even more rate-limiting (17). For these experiments, a 10-residue peptide representing the C-terminus of the γ subunit was synthesized and used for the production of γC33 antiserum, allowing confirmation of the specificity of the antiserum toward γ subunit. The critical implication of this result is that antiserum caused a 1.8-fold increase in $K_{ATP}$ (for values, see inset in Fig. 4). $V_{max}$ for γC33-treated enzyme was 78% that for nonimmune serum-treated enzyme. The critical implication of this result is that anti-γ reverses an increase in affinity effected by the γ subunit. This hypothesis was tested in HEK-pREP4-γ cells and HEK-pREP4 cells.

We first compared the effect of γC33 on HEK-pREP4-γ, HEK pREP4 cells, and kidney enzymes, all assayed at 10 μM ATP. The experiment (not shown) indicated that γC33 caused 33 ± 2

![Fig. 2. Western blot analysis of rat kidney, HEK-pREP4-γ, and HEK-pREP4 membranes. Immunoblotting was carried out as described under “Experimental Procedures.” Lane 1, 2.0 μg of rat kidney membranes; lane 2, 30 μg of HEK-pREP4-γ membranes; lane 3, 30 μg of HEK-pREP4 membranes.](image-url)
and 82 ± 15% inhibition of HEK-pREP4-γ and kidney enzymes, respectively, and had no effect on the activity of HEK-pREP4 cells. This inhibition is consistent with the aforementioned relative amounts of γ in kidney versus HEK-pREP4-γ cells. Experiments were then carried out to determine whether the γ subunit has any effect on $K_{\text{ATP}}^*$. The plots shown in Fig. 4 indicate that the HEK-pREP4-γ enzyme has a significantly higher affinity for ATP compared with control HEK-pREP4 enzyme (for $K_{\text{ATP}}^*$ values, see inset). The γ-mediated-1.3-fold decrease in $K_{\text{ATP}}^*$ in these cells, although modest, is in fact similar to the effect of γ in the kidney membranes, taking into account the lower α:γ ratio in the transfected cells (approximately one-third that of kidney membranes). This being the case, we used a more sensitive assay of ATP affinity to magnify the effect of γ and to determine whether anti-γ antisera can reverse its effects. This assay takes advantage of the fact that $K^+$ inhibits Na-ATPase activity at a very low (1 μM) ATP concentration under which condition the (low affinity) ATP-activated $K^+$ deocclusion reaction becomes rate-limiting. Accordingly, this inhibition decreases as the affinity for ATP at its low affinity binding site increases (18). As shown in Fig. 5A, $K^+$ is less effective at inhibiting the Na-ATPase activity of pumps of γ-transfected membranes than of control membranes. Experiments were then carried out to test and compare $K^+$ inhibition, and the effect of anti-γ thereupon, of the enzyme of the kidney medulla, HEK-pREP4-γ, and HEK-pREP4. Fig. 5B shows the percentage inhibition at 0.2 mM KCl of these pumps in the presence of nonimmune versus immune serum. Whereas preincubation of kidney and pREP4-γ pumps with γC33 effected 2.1- and 1.5-fold increases in $K^+$ inhibition, respectively, no γC33-mediated change was detected for HEK-pREP4 pumps.

### DISCUSSION

The successful transfection of the γ subunit into mammalian cells with sodium pumps devoid of this subunit has enabled the direct analysis of the functional role of this Na,K-ATPase-associated protein. Although the γ subunit does not appear to be necessary for normal Na,K-ATPase activity (7, 15, 19), its role as a modulator of function is consistent with its appearance in a tissue (kidney)-specific manner.

Recently, Béguin et al. (7) have shown that the rat γ subunit lowers the affinity of the pump for $K^+$ in cRNA-injected Xenopus oocytes, at least in the absence of Na$^+$. A γ-mediated decrease in $K_{\text{ATP}}^*$ could explain this increase in $K_{\text{ATP}}^{0.5}$ for $K^+$ because, as a first approximation, ATP and $K^+$ affinities are inversely related (20). However, that result may be confounded by the use of cRNA synthesized using the original sequence for rat γ (5). In a recent report, the human γ subunit was shown to induce cation channel activity in Xenopus oocytes (11), consistent with several reports of other channel-inducing membrane expressions.
peptides (8, 9, 21). These proteins have homology with the γ subunit but are generally larger, and some contain possible protein kinases C and A phosphorylation sites at their C-terminal ends that are not present in the γ subunit (5, 8–10). Although we have no information regarding such a role in our transfected cells, it should be pointed out that the sequence of the putative human γ subunit reported in the aforementioned study contains 30 extra amino acids at its N terminus (11) that are absent in rat γ (cf. Fig. 1). Whether the rat γ subunit also has a channel function and/or this extended N terminus confers a particular functional role in forming channels in Xenopus oocytes remains to be determined.

The N-terminal sequence of the rat γ subunit reported here and by Minor et al. (11) is different from the one originally reported (5). That it is the correct sequence is substantiated by the following observations. First, the γ subunit doublet present in membranes of transfected cells corresponds in size to that of kidney membranes (Fig. 2). Second, the presence of a lysine residue at position 13 (Fig. 1) where a glutamate was originally reported (5) is in accordance with the finding that the upper band of the rat γ subunit is cleaved by trypsin (treatment of intact right-side-out microsomes (12)). Third, preliminary results using matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectroscopy indicate that the pig kidney γ subunit has a length of between 64 and 67 residues, consistent with a length of 66 residues reported here and in Ref. 11.

The presence of two distinct bands of γ has been the subject of some controversy. Whereas Mercer et al. first showed that a single RNA species could yield two protein products evidenced on Western blots using an artificial translation system (5), Béguin et al. (7) showed that in X. laevis the two bands were secondary to the presence of two distinct start codons (7). Our results with 5′-RACE analysis preclude the presence of distinct ATG codons for the rat protein, indicating instead that post-translational modifications are involved, because transfection of HEK-293 cells with a gene containing single start and stop codons yielded two bands of similar mobilities to those of the kidney γ subunit. In addition, preliminary mass spectroscopy results are consistent with the notion that the difference between the two bands is the result of post-translational modifications. The ratio of the densities of the upper to the lower band between γ subunits of kidney and transfected HEK membranes (see Fig. 2) suggest tissue-specific variations in post-translational modifications. Whether each band has some distinct role remains to be determined.

Overall, our results suggest an interaction between the Na,K-ATPase and the C-terminal tail of the γ subunit that regulates ATP affinity and that is reversible upon binding of antibodies to γ. The finding that γ increases the apparent affinity for ATP in γ-transfected cells is completely concurrent with the effect of anti-γ on the αβγ pump of the kidney tubule. Moreover, under conditions in which K+ sensitivity of Na,K-ATPase at low ATP concentration is used as a sensitive marker of differences in ATP affinity, the reversal of the γ effect by anti-γ is similar with the enzyme of γ-transfected cells and the kidney medulla. These similarities underscore our earlier interpretation of the effect of γ from analysis of the effects of the anti-γ antisera. Whether the increased apparent affinity for ATP is, in fact, a true increase in affinity or a reflection of an alteration in conformational equilibrium toward $E_1^γ$ form(s) is unclear and requires further analysis, as does the question of whether the difference in ATP affinity can be evidenced in a change in apparent affinity for extracellular K+. Whatever the case, it is the change in ATP affinity that is likely to be of major physiological relevance.

The increase in apparent affinity for ATP effected by γ is approximately 2-fold, as evidenced in either the effect of anti-γ on the kidney enzyme or of γ transfected into HEK cells, extrapolating the ratio of $γ_0$ in HEK-pREP-γ to that of the kidney. Such a change in apparent affinity may be of critical physiological importance. Although other physiological functions may be served by the γ subunit (as suggested recently by Jones et al. (22)), an almost 2-fold shift in ATP affinity is a potentially important regulatory mechanism. The γ subunit may serve to preserve the pumping activity in cells or conditions in which the ATP level falls suddenly. Relevant to this notion is the observation that the renal outer medulla is highly prone to anoxia because it works on the brink of anoxia even in normal circumstances (23, 24). That the γ subunit effect is reversible upon addition of anti-γ antibodies further underscores its physiological relevance. It may be hypothesized that, like the anti-γ antibodies, some cytosolic factor binds to the γ subunit and disrupts its interactions with the enzyme. Mutational analysis of the C-terminal 10 amino acids that comprise the epitope reactive with anti-γ may provide information on specific residues involved in α-γ interactions.

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