The γ Subunit Modulates Na\(^+\) and K\(^+\) Affinity of the Renal Na,K-ATPase*

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The Na\(^+\),K\(^+\)-ATPase catalyzes the active transport of ions. It has two necessary subunits, α and β, but in kidney it is also associated with a 7.4-kDa protein, the γ subunit. Stable transfection was used to determine the effect of γ on Na,K-ATPase properties. When isolated from either kidney or transfected cells, αβγ had lower affinities for both Na\(^+\) and K\(^+\) than αβ. A post-translational modification of γ selectively eliminated the effect on Na\(^+\) affinity, suggesting three configurations (αβ, αβγ, and αβγ\(^\#\)) conferring different stable properties to Na,K-ATPase. In the nephron, segment-specific differences in Na\(^+\) affinity have been reported that cannot be explained by the known α and β subunit isoforms of Na,K-ATPase. Immunofluorescence was used to detect γ in rat renal cortex. Cortical ascending limb and some cortical collecting tubules lacked γ, correlating with higher Na\(^+\) affinities in those segments reported in the literature. Selective expression in different segments of the nephron is consistent with a modulatory role for the γ subunit in renal physiology.

The renal control of Na\(^+\) and K\(^+\) balance is complex and entails ensembles of apical and basolateral transporters that play specialized roles in different segments of the nephron. One of the most physiologically important transporters is the Na,K-ATPase, or sodium pump, which is crucial for the absorptive, secretory, and concentrating capacity of the kidney. Small changes in Na,K-ATPase ion affinities can have important physiological effects both directly on transepithelial ion and fluid transport and indirectly through the ion gradients used by other transporters (1, 2). In permeabilized microdissected nephron segments, Na,K-ATPase has increased affinity for Na\(^+\) in thick ascending limb compared with proximal convoluted tubule and has still higher affinity in cortical collecting tubule (3–6). Although isoforms of α and β can have different ion affinities (7), isoforms unique to the nephron segments with higher Na\(^+\) affinities have not been detected (8–12), leaving the mechanism unexplained.

A small single-span membrane protein, member of a protein family that has ion channel activity in oocytes, copurifies with renal Na,K-ATPase α and β subunits and appears as a doublet on gels (13, 14). Known as the γ subunit, it is not present in all tissues, and its role in the Na,K-ATPase has long been an enigma (15–17). As an ancillary subunit it is likely to play a modulatory role. It has been reported to increase ATP affinity when expressed in mammalian cells (18, 19) and to affect the voltage sensitivity of K\(^+\) activation when expressed in oocytes (20). Here evidence is presented for its role in the control of the most basic property of Na,K-ATPase, the affinity for Na\(^+\) and K\(^+\). Most significantly, analysis of independent clones points to a functional role for post-translational modification of the γ subunit.

**EXPERIMENTAL PROCEDURES**

**Enzyme Preparations**—Na,K-ATPase was purified from rat, pig, and dog kidney by SDS extraction (21), which produces membrane bound, active enzyme. Crude membranes from renal cell lines and transfectants were treated with SDS at 0.56 mg/mg protein and sedimented on 7–30% sucrose gradients. Specific activities of the Na,K-ATPase from rat kidney and NRK-52E were 1200–1500 and 120–150 μmol of P/mg/hr, respectively.

**Gels**—Electrophoresis was in SDS-Tricine\(^*\) gels (22). Proteins were transferred to nitrocellulose, incubated with antibodies, and detection was with chemiluminescence (23). Antibody to γ—A rabbit antibody (RCT-G1) was raised against the peptide corresponding to the last 14 amino acids (CGG-SKKHRQVNEDEL) of rat γ, conjugated to keyhole limpet hemocyanin (KLH).

**ATP Hydrolysis**—Na,K-ATPase activity was measured in media containing 3 mM TriS-ATP, 3 mM MgCl\(_2\), 30 mM histidine, pH 7.3, and various concentrations of K\(^+\) (0–20 mM) with [Na\(^+\)] fixed at 140 mM. Reactions were performed for 30 min at 37 °C with and without 3 mM ATP, and ouabain-sensitive P\(_i\) release was measured colorimetrically.

**Transfections**—cDNA for the γ subunit was obtained by RT-PCR from total rat kidney RNA (CLONTECH, Palo Alto, CA). The following primers were based on a nucleotide sequence for rat γ in the GenBank\(^\#\) dbEST database (AA801241) plus EcoRI and BamHI restriction sites for unidirectional cloning: forward primer, 5'-GGATTCGGT-GGCTGGGGAAATGAC-3'; reverse primer, 5'-CGCGGATCCCACTGTCCTCTTCCATGGG-3'. Gel-purified DNA was ligated into pRRES vector (CLONTECH). Several clones containing the full-length cDNA of γ were verified by PCR and nucleotide sequencing. The N-terminal protein sequence is predicted to be MTLSANHGG, corresponding to the corrected sequence reported by Minor et al. (14) and different from that deposited in GenBank\(^\#\) (X70062). Transfection was with cationic liposomes (Clonfectin, CLONTECH). Antibiotic (G418) was added after 48 h, and resistant colonies formed in 15–20 days.

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\# The abbreviations used are: Tricine, N-[2-hydroxy-1,1,1-tris(hydroxymethyl)ethyl]glycine; RT-PCR, reverse transcriptase polymerase chain reaction.


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### RESULTS

Cryostat sections were made from rat kidney fixed with periodate-lysine-paraformaldehyde. Sections were double-labeled with mouse anti-α1 (McK1, 1:4) and rabbit anti-γ (RCT-G1, 1:500), stained with Cy3-conjugated anti-mouse and fluorescein isothiocyanate-conjugated anti-rabbit secondary antibodies, and examined with a Bio-Rad MRC 1024 Laser Sharp confocal microscope.

**Properties of γ Transfectants**—While Na,K-ATPase from kidney contains γ, this is not true of mammalian kidney cell lines (18). NRK-52E, an epithelial line of rat kidney cells, expressed the same Na,K-ATPase α1 and β1 isoforms found in rat kidney, but γ could not be detected with specific antibodies (Fig. 1) or by RT-PCR (not shown). The Na⁺ and K⁺ affinities of partially purified Na,K-ATPase with and without γ were compared (Table I, top). The apparent affinities for both ions were substantially higher (1.5–2-fold) in enzyme from NRK-52E (α1β1γ1) than from renal medulla (α1β1). Two other renal cell lines, LLC-PK1 (pig) and MDCK (dog), also lacked γ (not shown), and again cell-derived enzyme without γ had higher affinities for Na⁺ than renal enzyme with it (Table I, top).

To determine whether γ was responsible for the kinetic difference, stable transfectants of NRK-52E were generated. Expression of γ was detected on Western blots (Fig. 1). By densitometric analysis, the level was 15–20% of the level in the kidney enzyme, normalized to the amount of α1. Two groups of clones were distinguished by the mobility of γ; those expressing a doublet (as in kidney preparations) and those expressing only the band with slower mobility. The distinction between clones proved to be critical for the kinetic properties of the Na,K-ATPase. The clones that expressed a γ doublet showed reduced affinity for Na⁺ as well as K⁺, while the clones with only one

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**Table I**

| γ                  | K<sub>0.5</sub>Na⁺ | Hill coefficient | K<sub>0.5</sub>K⁺ | Hill coefficient | n  |
|--------------------|--------------------|------------------|--------------------|------------------|----|
| Kidney vs. cell lines |                    |                  |                    |                  |    |
| Rat kidney         | +                  | 8.39 ± 0.49      | 2.3 ± 0.2          | 0.85 ± 0.09      | 4  |
| NRK-52E            | −                  | 5.41 ± 0.12      | 1.9 ± 0.1          | 0.44 ± 0.03      | 4  |
| Pig kidney         | +                  | 9.54 ± 0.56      | 2.1 ± 0.2          | 2.0 ± 0.02       | 4  |
| LLC-PK1            | +                  | 4.75 ± 0.25      | 2.5 ± 0.3          | 2.4 ± 0.2        | 3  |
| Dog kidney         | +                  | 8.23 ± 0.39      | 2.4 ± 0.2          | 2.4 ± 0.2        | 3  |
| MDCK               | −                  | 5.32 ± 0.38      | 1.8 ± 0.2          | 1.8 ± 0.03       | 4  |
| NRK-52E transfectants |                |                  |                    |                  |    |
| Mock-transfected   | −                  | 5.27 ± 0.17      | 1.9 ± 0.1          | 0.43 ± 0.027     | 3  |
| Doublet            | +                  | 7.79 ± 0.29      | 2.1 ± 0.1          | 0.69 ± 0.036     | 6  |
| Upper band         | +                  | 4.75 ± 0.24      | 2.5 ± 0.1          | 0.71 ± 0.04      | 4  |

Data were analyzed by nonlinear regression using the Sigma Plot Scientific Graph System (Jandel Scientific, San Rafael, CA). Na⁺ and K⁺ affinity; Hill coefficient

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**Fig. 3.** The γ doublet results from a post-translational modification near the N terminus. a, right-side-out sealed renal medullary vesicles (lane 1, control) were trypsinized at a trypsin:protein ratio of 1:3 for 60 min at 37 °C (lane 2) (24). The γ doublet was reduced to a single band of lower M<sub>r</sub> that still stained with the antiserum against the C terminus. b, purified enzyme from rat renal medulla was treated with 1 M Tris, pH 11 (lane 1, control), or 1 M hydroxylamine, pH 11 (lane 2), at 37 °C overnight (23). The upper γ band was reduced in size to that of the lower band by hydroxylamine, and no smaller species were generated.

**Fig. 4.** Renal cortex stained with α1- and γ-specific antibodies shows nephron segments with α1 but no γ. a, α1 stain, which was brightest in distal convoluted tubule and cortical thick ascending limb. Proximal tubule was stained, but more lightly. b, γ stain, which was localized with α1 in distal convoluted tubule but not cortical thick ascending limb, where the γ stain was at the level of background. c, two-color image, scale bar 50 μm. DCT, distal convoluted tubule; CTAL, cortical thick ascending limb; PCT, proximal convoluted tubule.
band showed reduced affinity for K$^+$ only (Fig. 2). This relationship held true in three independent clones with doublets and three clones with single bands. The ion affinities for all of the tested clones are compared in Table I, bottom.

Post-translational Modification of $\gamma$—In the literature, doublets of mammalian $\gamma$ appeared during in vitro synthesis in the presence of pancreatic microsomes (17), but not in their absence (20), which suggests that there might be post-translational processing in the endoplasmic reticulum. The $\gamma$ subunit does not have a signal sequence, but trypsin treatment of pig kidney right-side-out sealed microsomal vesicles reduced the doublet to a single band, consistent with a cleavage site near the extracellular N terminus (18). We digested right-side-out rat renal medulla vesicles with trypsin (24). Both bands of the $\gamma$ doublet were reduced to one smaller band, from 7.6 and 6.8 kDa to 5.6 kDa (Fig. 3a), predicting cleavage at Lys-13, analogous to the cleavage of pig $\gamma$ in extensively digested purified enzyme (25). The disappearance of the doublet implies that the modification lies extracellularly in the first 13 amino acids (MTELSANHGGSAK).

Hydroxylamine treatment was used to determine whether the modification was because of acylation of the protein (23, 26). At basic pH, hydroxylamine shifted the slower-migrating band but left the faster-migrating band unchanged (Fig. 3b). At neutral pH neither band was affected (not shown), and there is no Asn-Gly in the sequence for hydroxylamine to cleave (27). This suggests a hydroxyester linkage, possibly fatty acid acylation, to Ser or Thr. There are three Ser and Thr residues in the N-terminal segment that are conserved in $\gamma$ from rat, human, and mouse. Modification of Lys-13 is unlikely because trypsin still cleaved, but modification of the N terminus was not ruled out. The Na,K-ATPase $\alpha_1$ subunit in kidney is also modified by a group that is labile to basic hydroxylamine, but this modification is intracellular (23).

Localization of $\gamma$ in the Kidney—We examined the distribution of $\gamma$ in rat kidney by double-label confocal microscopy, using the $\alpha_1$ subunit of the Na,K-ATPase for comparison because Na,K-ATPase is known to be expressed at higher levels in some nephron segments than in others (Fig. 4). The anti-$\gamma$ antibodies were raised against the C terminus and should detect $\gamma$ forms with or without the posttranslational modification. Distal convoluted tubule (DCT) stained brightly for both $\alpha_1$ and $\gamma$, and proximal convoluted tubule (PCT) stained weakly for both. Other segments, however, including cortical collecting tubules (CCT) (not shown), expressed $\alpha_1$ without any $\gamma$ detected above the background of secondary antibody stain. Thus the expression of $\gamma$ is segment-specific in the rat nephron.

DISCUSSION

The most notable consequence of $\gamma$ expression was that the affinities of the Na,K-ATPase for its substrates, Na$^+$ and K$^+$, could be modified independently. In clones expressing the doublet, as well as enzyme purified from renal medulla (which has the doublet), affinities for both ions were reduced in concert. This is not easy to explain by a shift between Na,K-ATPase E1-E2 conformations as suggested by Blosein and co-workers (19) but could be understood as a consequence of physical association of $\gamma$ with portions of $\alpha$ comprising the ion binding sites. The stoichiometry of $\gamma$ relative to $\alpha$ was lower than in renal medullary Na,K-ATPase, however, which raises the question of whether $\gamma$ acts exclusively as a subunit. $\gamma$ has been shown to form an ion channel in the absence of $\alpha$ (14). It has not been ruled out that the observed effects on ion affinities result from an indirect modulatory pathway activated by its presence.

NRK-52E is morphologically heterogeneous, lending credibility to the idea that subclones may have different phenotypes with respect to $\gamma$ modification. It is intriguing that the clones with fully modified $\gamma$ have alteration of K$^+$ affinity alone, while the half-modified clones show alteration of both ion affinities. The modification appears to nullify a major functional consequence of association with $\gamma$.

The anatomical distribution of $\gamma$ is consistent with Na$^+$ affinities measured in different nephron segments: the presence of $\gamma$ is accompanied by lower affinity for Na$^+$. This suggests that expression of $\gamma$ selectively modifies Na,K-ATPase properties in vitro. Whether the post-translational modification demonstrated here has a specific cellular distribution among $\gamma$-positive nephron segments remains to be determined.

Pathways that alter ion affinities of kidney Na,K-ATPase consequent to hormone treatment or electrolyte dietary restriction have been reported (4, 5, 28, 29). Their molecular mechanisms are still obscure, however, and could entail the expression and/or modification of $\gamma$. Regardless of whether $\gamma$ acts as a subunit or a channel, the evidence here indicates that it is an important contributor to the control of renal transport physiology.

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