[Frontiers in Bioscience 10, 2604-2610, September 1, 2005]

THE γ SUBUNIT OF NA/K-ATPASE: AN EXCEPTIONAL, SMALL TRANSMEMBRANE PROTEIN

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1. ABSTRACT

The γ subunit has been characterized as a fine-tuning modulator of the Na/K-ATPase expressed in kidney tissues. This small single transmembrane domain protein interacts with the α subunit of Na/K-ATPase to increase affinity for ATP and decrease affinity for Na allowing medullary cells to continue pump activity under reduced cellular ATP levels. The γ subunit is undetectable in kidney cell cultures grown under isotonic conditions and expression is induced with acute or chronic exposure to hypertonicity. The γ subunit demonstrates remarkable regulatory complexity including induction by chloride ions rather than sodium, the differential expression of at least 2 isoforms, involvement of separate MAP kinase signaling pathways for transcription (JNK) and translation (PI3K) as well as cell type regulation of expression. Mutation in the transmembrane domain of the γ subunit has been implicated in cases of primary hypomagnesemia. Alternative roles have been established for the γ subunit in embryonic development and quite possibly additional functions in cell signaling as yet unrecognized may be possible.

2. INTRODUCTION

The first report of the γ subunit of the Na/K-ATPase was in 1972 by Rivas and coworkers who found the presence of a small hydrophobic protein from membrane preparations that bound ouabain and was thought to be a fragment of the original Na/K-ATPase (1). Later, Forbush III and coworkers termed this small transmembrane protein an acidic "proteolipid" component of the Na/K-ATPase (2). They characterized this small acidic proteolipid that co-purified with the renal Na/K-ATPase from pig kidney outer medulla. It was determined to have an apparent molecular weight of 12,000 daltons and covalently bound ouabain and the photoaffinity ouabain derivate NAB-ouabain. Since that time, research has focused on the structural relationship of the γ subunit with the α and β subunits of Na/K-ATPase and its physiological role in ion transport.

The Na/K-ATPase, itself, is an oligomeric P-type pump that was first reported by Jens C. Skou (1997 Nobel prize winner in Chemistry) from crab nerve preparations almost 50 years ago (3). Early work identified the protein

Table 1. Summary of FXYD family members and modulation of Na/K-ATPase

FXYD protein	Name	Source:GenBank #	Tissue Location	Protein Amino Acids	Ouabain Binding	Change in Na/K-ATPase affinity			References
						Na	K	ATP	
FXYD1	Phospholemman (PLM)	***r***: U72246 ***h***: U72245 ***c***: M63934 ***s***: AJ556170	Brain, heart, skeletal muscle, rectal glands (shark)	72-94	-	↓↓	↓	nd	14,53,54
FXYD2	γ subunit includes splice variants (isoforms) γa and γb	***m***: X70060 ***m***-γa: AY626243 ***m***-γb: AY626244 ***r***: X70062 ***b***: X70059 ***Sh***: X70061 ***h***: AF241236	kidney	65-66	+	↓	-	↑	15,49,55
FXYD3	Mammary Tumor Marker-8 (MAT-8)	***h:***X93036 ***p***:AB015759	mammary tumors	85-88	nd	nd	nd	nd	16,56
FXYD4	Corticosteroid Hormone-Induced Factor (CHIF)	***r***: L41254 ***h:***BC054876	Kidney, colon	87	-	↑↑	↓↓	n.d.	17,36,55
FXYD5	Related to Ion Channel (RIC)	***m***: U72680 ***h:***BC009642	Spleen, lung skeletal muscle, testis	178	nd	nd	nd	nd	18,55
FXYD6	Phosphohippolin (Php)	***m***:BC042579 ***h***: AY358976 ***r:***BC072528	brain kidney	93-95	nd	nd	nd	nd	55,57
FXYD7		***h***: BC018619	brain	80	nd	-	↓↓	nd	55,56

r: rat (*Rattus norvegicus*); m: mouse (*Mus musculus*); h: human (*homo sapiens*); c: canine; sh: sheep; p: pig; b: bovine; s: shark (*Squalus acanthias*); n.d.: no data.

for its ATP hydrolyzing activity that was stimulated by increased concentrations of sodium and potassium. Later the main function of Na/K-ATPase was recognized as a pump that transports intracellular Na ions out and K ions into the cell against the electrochemical gradient at the expense of ATP hydrolysis. For each mole of ATP hydrolyzed, the Na/K-ATPase moves 2 K ions into the cell and three Na ions out of the cell. Sodium reabsorption is the major function of the Na/K-ATPase in the kidney. In humans, more than 600 grams of Na is reabsorbed per day, utilizing over 2 kilograms of ATP in the process (4). One result of this ion transport is the generation of a transmembrane potential. This is important for nerve transmission and muscle contraction. This membrane potential may also be considered a form of cellular energy that may be used for cotransport of amino acids and sugars into the cell against a concentration gradient.

The Na/K-ATPase is composed of an α-subunit of ~ 113 kD and a heavily glycosylated β subunit with an apparent size of ~ 55 kD. Both the α and β subunits have been demonstrated to have multiple isoforms (5,6). The α-subunit contains a binding site for ATP as well as for the cardiac glycoside inhibitor, ouabain. The β-subunit acts as a specific molecular chaperone and guides proper folding of the α subunit (7-9). While the γ subunit is not required for activity of the Na/K-ATPase as with the β subunit, its role has been thought of as fine-tuning the activity of Na/K-ATPase (5).

2. THE γ SUBUNIT OF NA/K-ATPASE AND THE FXYD PROTEIN FAMILY

From the first report of the γ subunit as co-purifying with Na/K-ATPase from renal tissues, a large amount of data indicating this strong association has accumulated. Crystallization of the Na/K-ATPase with and without the γ subunit has been a difficult task as association with membrane lipids hampers analysis. However, density maps obtained by electron crystallography at 9.5 Angstroms suggest that the γ subunit may be positioned close to a cavity formed by M2, M6 and M9 transmembrane domains of the α-subunit (10). Thermal denaturation experiments on purified Na/K-ATPase preparations showed that γ subunit is lost from the membrane together with COOH-terminal membrane spans of the α-subunit, suggesting that the γ subunit may be associated with transmembrane domains M8 through M10 of the α-subunit (11). A more recent report using mutational analysis and modeling shows that the γ subunit interacts with one face of the M9 helix of the α subunit (12). Additional technical advances may soon allow definitive crystallography results to be obtained to verify the exact association of the γ subunit with Na/K-ATPase (S. Karlish personal communication).

In addition to the γ subunit, a number of other small single transmembrane proteins have been identified and their relatedness was described by Sweadner and Rael (13). These single domain, small transmembrane proteins were termed the FXYD family based on sequence similarity. This motif Phenylalanine, any amino acid, Tyrosine and Asparagine are always present at the beginning of the transmembrane domain from the N terminal. In mammals, seven different members of this family have been identified, including: phospholemman (PLM or FXYD1) (14), the γ subunit (FXYD2 or "proteolipid component of the Na/K-ATPase) (2,15), mammary tumor marker 8 (MAT-8, or FXYD3) (16), corticosteroid hormone-induced factor (CHIF, or FXYD4) (17), related to ion channel (RIC, or FXYD5) (18), Phosphohipoplin (FXYD6) (19), and FXYD7 (20). All of these proteins share a signature sequence of six conserved amino acids comprising the FXYD motif in the NH_2-terminus, and two glycines and one serine residue in the transmembrane domain. The NH_2- and COOH-termini are variable among FXYD proteins. A summary is presented in Table 1 and many excellent reviews on the FXYD family have recently been published (21,22).

Elucidation of the codifying sequence for many members of the FXYD family have been completed and data entered into GenBank. This information has allowed cloning experiments to be performed to assess the impact of the γ subunit on the transport activity of Na/K-ATPase after expression of the proteins in *Xenopus* oocytes (23,24), Sf-9 cells (24), HeLa cells (25-28) and HEK cells (29). Data indicates the FXYD protein modifies the activity of Na/K-ATPase via changes in affinity for Na, K, or ATP (see Table 1 for comparisons).

4. RENAL EXPRESSION OF THE γ SUBUNIT

In at least one tissue, the kidney medulla, cells must function under near anoxic conditions in which ATP levels are decreased (30,31). Under such conditions, continued cation pumping is requisite where water and solute reabsorption and secretion are required. The γ subunit interacts with Na/K-ATPase to alter its affinity for ATP allowing continued activity under ATP-depleted conditions. Such continued pumping is crucial to proper kidney function. The γ subunit thus acts as fine tuning for the Na/K-ATPase.

Expression of the γ subunit in renal tissues has been established in the plasma membrane and co-localizes with the Na/K-ATPase (32). Furthermore localization appears to be restricted to the basolateral membrane of cells. Studies employing kidney cell cultures acutely challenged to osmotic stress or adapted to hypertonic conditions also demonstrates shuttling of the γ subunit to the plasma membrane and co-localization with the Na/K-ATPase (33). Minor and coworkers found that viral transfected cells can express γ subunit that is delivered to the plasma membrane independently of other subunits of the Na/K-ATPase (24).

Three members of the FXYD family have been found in kidney tissues (15,34-36). Phospholemman, the γ subunit and CHIF have been shown to be expressed in distinctively different areas of the kidney. Interestingly, only one FXYD family member is expressed in each cell type or location. Localization studies have been performed by several researchers. Immunohistochemistry examination of kidney tissues has demonstrated that the γ subunit is localized in proximal convoluted tubule (PCT), the medullary part of the thick ascending limb of Henle's loop (mTAL), the cortical part of the TAL (cTAL) and in the cortical collecting duct (CCD). While initially Arystarkhova and coworkers reported the γ subunit was not present in the inner medulla (32), subsequent studies by Maunsbach and laboratory studies with mIMCD3 cells indicate that γ subunit is present in the inner medullary collecting duct (37).

5. γ SUBUNIT ISOFORMS

The α and β subunits of Na/K-ATPase are known to have multiple isoforms. Four α and three β isoforms potentially permit the formation of 12 different isozymes (38,39). The current understanding is that different isoforms allow for variation in the activity of the Na/K-ATPase depending on the cell type and physiological circumstances (38). Two isoforms have also been described for the γ subunit (40,41). The sequence analysis of the γ subunit gene has identified at least two different isoforms differing only in their most NH_2-terminal amino acids. In kidney tissues and mIMCD3 cells exposed to hypertonic conditions, the expression of γ_a and γ_b is not equal with the smaller γ_b isoform in greater abundance. The function of the different isoforms has recently been studied (27) and may provide an additional level of fine tuning of the Na/K-ATPase. Differential expression of the γ subunit isoforms was determined based on kidney tissue cell type (42). While both isoforms are present in some tissues as described above, γ_a is found alone in the macula densa and principal cells of the cortical collecting duct whereas γ_b is present alone in cortical TAL.

6. γ SUBUNIT IS EXPRESSED IN KIDNEY CELL CULTURE UNDER OSMOTIC STRESS

Early reports identified the lack of expression of FXYD proteins in cultured kidney cells including MDCK, LLC-PK, and NRK-52E (43). Capasso and coworkers determined that mIMCD3 acutely exposed to hypertonicity by addition of NaCl expressed the γ subunit in addition to an increased expression of Na/K-ATPase (44). Furthermore, when mIMCD3 cells were stably adapted to increased hypertonicity by a slow step-wise increase in medium osmolarity with added NaCl, these cells expressed even greater levels of γ subunit protein.

7. ION MEDIATORS OF γ SUBUNIT EXPRESSION

Acute modulation of the Na/K-ATPase occurs in response to changes in the intracellular concentration of Na and, under resting cellular conditions, intracellular concentration of Na is limiting for the Na/K-ATPase transport activity. Moreover, certain peptide hormones and neurotransmitters that stimulate protein kinase A (PKA) or protein kinase C (PKC) phosphorylates the α subunit, which affects the transport properties or the distribution of the Na/K-ATPase between the plasma membrane and intracellular stores, or both (4,5). Finally, long-term regulation of Na/K-ATPase is mediated by hormonal control by aldosterone and thyroid hormones which alter α and β subunit genes transcription and ultimately produce an increased number of Na/K-ATPase units at the cell surface.

Initial studies with cultured kidney cells (IMCD3) revealed that the addition of NaCl to the medium resulted in increased expression of both subunits of Na/K-ATPase as well as the γ subunit. On closer evaluation it was determined that it was the chloride ion and not sodium that induced stimulation of the γ subunit (45). This fact is puzzling as the gamma subunit has not been implicated in the transport of chloride ions.

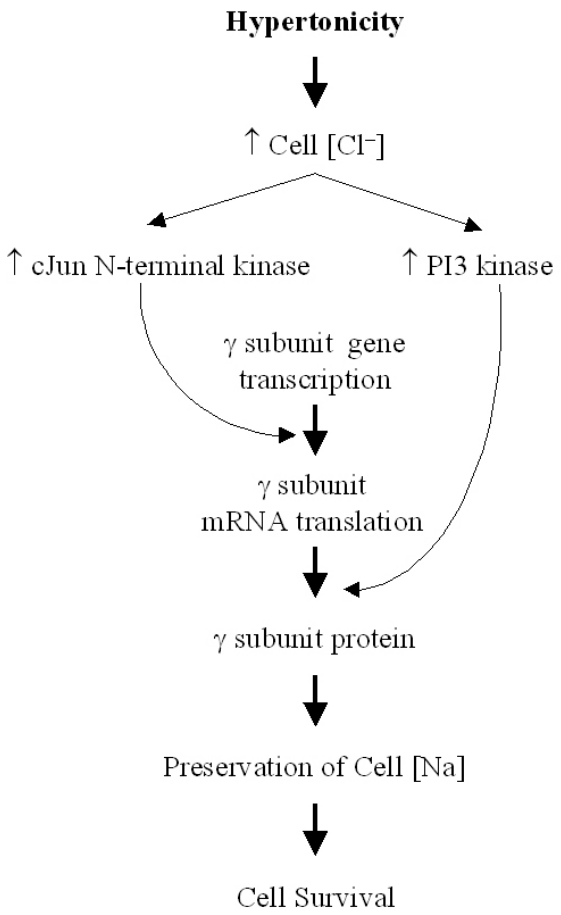

Figure 1. Proposed signaling pathways involved in the regulation of the γ subunit protein induced under hypertonic conditions (47).

8. COMPLEX REGULATION OF THE γ SUBUNIT

The upregulation of the γ subunit under hypertonic conditions is dependent on both the c-Jun N-terminal kinase (JNK) and PI3 kinase signaling pathways (46). This regulation is entirely different from the regulation of the α and β subunits of Na/K-ATPase. Recent preliminary studies have further identified the complex nature of this regulation (Figure 1) as inhibition of JNK with specific inhibitors abolishes both the γ subunit mRNA and protein expression while PI3 kinase inhibitors do not interfere with mRNA levels but totally abolishes protein expression (47). These data indicate that separate signaling pathways differentially regulate the expression of the γ subunit under osmotic stress. The regulation of the γ subunit has been shown to be independent of the P38 and ERK signaling pathways. It is noteworthy that these pathways have been implicated in the osmoresponse and expression of transcriptional elements including TonEBP.

Analysis of new sequence information for γ_a and γ_b isoforms in mouse including both 5' and 3' UTRs (GenBank AY626243 and AY626244, respectively) indicate a polyprimidine motif in the 5' end of the message. This motif is characteristic of P70 regulated proteins (48). The mechanism responsible for differential expression of γ_a versus γ_b has not as yet been elucidated.

9. EXPRESSION OF THE γ SUBUNIT BY CELL TYPE

As described in section 4, FXYD proteins are differentially expressed in discrete cells within the kidney. Recently we tested this level of regulation by transfecting both mIMCD3 and the less osmoresponsive M1 cell line with a complete γ transcript including both 5' and 3' UTRs. Both cell types produced the γ message while only the mIMCD3 cells translated the message to protein (47). These results are different from that reported by Minor and coworkers and is probably due to the lack of 5' and 3' UTRs on the message that are responsible for this regulation. This cell type based regulation of the γ protein represents an exceptionally complex level of control over expression and the mechanism for this regulation is still unknown.

10. γ SUBUNIT AND HUMAN DISEASE

Mutation of a conserved glycine residue in the transmembrane domain of FXYD2 is associated with cases of human primary hypomagnesemia (49,50). Studies investigating the effect of the G41R mutant of FXYD2 revealed that the mutation abolishes its interaction with Na/K-ATPase, which results in the failure of FXYD2 protein to traffic to the cell surface and to modulate Na/K-ATPase (25). Because the expression of the Na/K-ATPase at the cell surface is not affected in cells expressing the G41R mutant, the hypomagnesemia appears to be an indirect consequence of the loss of Na/K-ATPase modulation by the γ subunit. Nothing is known, however, about the cellular mechanism that link the abrogation of the γ subunit modulation of Na/K-ATPase to the loss of magnesium, increased Ca absorption, and hypocalciuria observed in these patients.

11. ALTERNATIVE FUNCTION OF THE γ SUBUNIT

Substantial information has been accumulated that indicates the major role for the γ subunit is the modulation of Na/K-ATPase. However, in considering the differences in signaling and regulation for the γ subunit as compared to the α and β subunits of Na/K-ATPase, alternative functions may be a possibility.

The γ subunit has been implicated in embryonic development. During embryonic development, the γ subunit is not only present in the basolateral membrane of blastocyst cells, which express Na/K-ATPase α1 and β1 isozymes, but also in the apical membrane, which is devoid of Na/K-ATPase isozymes (18,51,52). Disruption of γ subunit expression with antisense mRNA delays blastocoele formation. Jones and coworkers concluded that γ subunit has a role in transepithelial Na reabsorption, and hence in blastocoele formation, that is independent of its association with the Na/K-ATPase (51).

Finally, considering the extensive level of regulation (transcriptional, translational, and cell type) together with the presence of multiple isoforms, the complexity of the γ subunit is substantial in relation to the relatively minor effect ("fine-tuning") of the Na/K-ATPase activity. Therefore, alternative functions such as cell signaling and as yet undiscovered roles may in fact exist.

12. CONCLUSIONS

From its early identification, the γ subunit was found to be associated with the Na/K-ATPase. A large number of studies have since all but assured its primary role as modulation of this ion transporter. As is true for most membrane proteins, exact crystallization analysis has been stymied. Undaunted, researchers have identified the co-localization and effect of the γ subunit on Na/K-ATPase activity and determined it to be a fine modulator. However, this protein is surprising in its induction by chloride rather than sodium or potassium. In addition, the signaling pathways involved are quite different from that of Na/K-ATPase. Regulation at both the transcriptional and translational level, as well as by cell type, provides for complex regulation of this protein. In cell culture studies employing mIMCD3 cells, the γ subunit appears to be crucial to survival and adaptation to hypertonic stress. The identification of γ subunit's participation in cellular development suggests that this protein may in fact serve alternative functions.

13. ACKNOWLEDGEMENTS

This work was supported by National Institutes of Health Grants DK-19928 and DK-66544.

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Key Words: γ subunit, Na/K-ATPase, transmembrane, regulation, Review

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