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Splice Variants of the Gamma Subunit (FXYD2) and Their Significance in Regulation of the Na,K-ATPase in Kidney

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The recent discovery of a family of single-span membrane proteins (the FXYD proteins) introduced a new direction to the rather complicated area of regulation of Na,K-ATPase. At least six members of the family have been shown to associate with the Na,K-ATPase in a cell- and tissue-specific manner, while four of them, namely the γ subunit (FXYD2), CHIF (FXYD4), phospholemman (FXYD1), and dysadherin (FXYD5) have been identified in kidney. All four exhibited different effects on the properties of the pump in heterologous expression systems. Taken along with their non-overlapping expression patterns in the nephron, this provides a potential structural basis for the segment-specific properties of the Na,K-ATPase that had been reported in a number of papers on kidney physiology.

This brief review summarizes our own contributions on structure/functional characterization of one of the family members, the γ subunit (FXYD2). The focus is on splice variants of γ, their structural similarity and yet distinct effects conferred to Na,K-ATPase.

KEY WORDS: FXYD family; endogenous inibitor; post-translational modification; stress response; growth control.

SPLICE VARIANTS AND STRUCTURAL FORMS OF γ

Analysis of human genomic sequence revealed that FXYD2 (the γ subunit), a 7.15 kDa protein, is encoded by a gene encompassing more than 9 kb and containing seven different exons (Sweadner *et al.*, 2000). Moreover, the first translated exon is alternatively spliced, giving rise to the splice variants γa and γb, which have different N-termini. Two different promoter regions were identified *in silico*, providing a potential basis for differential regulation of expression of the γ splice variants. A similar genomic organization can be observed in rat and mouse genomes.

Post-translational modification (PTM) of γ adds another layer of complexity to the FXYD2 structural forms. The first evidence for post-translational modification of γ was obtained from in vitro biosynthesis experiments. γb always migrated faster than γa after in vitro biosynthesis when translated in the absence of microsomes (Arystarkhova *et al.*, 2002a). When pancreatic microsomes were added to the reticulocyte lysates, the electrophoretic mobility of both γa and γb was decreased detectably, albeit to different extents. This implies that (a) both splice variants of the rat γ subunit were post-translationally modified, presumably at extracellular sites, and (b) despite extensive sequence identity, either different types of modification of newly translated proteins or the modification of different numbers of sites was employed. The data are consistent with the existence of γ in four different structural forms: γa, γa′, γb, and γb′, where the prime represents post-translational modification(s). Since mutations of Ser5/Ala and Thr2/Ala partially blocked the shift in mobility of γa synthesized in vitro, we proposed those sites to be involved in PTM (Arystarkhova *et al.*, 2002a). An additional potential site, Ser11, is found in both splice variants.

An independent piece of evidence for post-translational modification of γ was obtained from stable

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transfectants of γa and γb, which were generated in NRK-52E cells (normal rat kidney epithelial cell line) under the constitutive CMV promoter (Arystarkhova *et al*., 1999). Two groups of clones were identified in both cases: γa was seen either as a doublet (γa*, the term we use for a mixture of γa and γa′, i.e., partially modified) or as a single band with the electrophoretic mobility of the slower migrating species of the doublet (γa′, completely modified). γb clones had either faster (γb) or post-translationally modified, slower migrating species (γb′) (Arystarkhova *et al*., 2002a). Given that γa and γb are practically structurally identical, the data imply a differential post-translational modification apparently targeted to the spliced extracellular exon in γ. What other factors determine in each clone whether or not PTM takes place is unknown, but the phenotype of each clone is stable.

Interestingly, exposure of renal epithelial NRK-52E cells to different kinds of stress, such as hypertonicity, exogenous oxidants, heavy metal treatments, and heat shock, or exposure of other types of cells such as C6 glioma, PC12 pheochromocytoma, Caco2 colorectal adenocarcinoma cells to high NaCl caused an induction of the endogenous γa splice variant distinctively in a partially modified form (γa*). This implies that post-translational modification of γ (at least of γa) occurs in wild type cells that upregulate γa in response to physiological stimuli (Wetzel *et al*., 2004).

At the moment we do not know the nature of the post-translational modification(s) of γ; however, the prediction is that PTM is complex. There are no consensus sites for N-linked glycosylation in the extracellular domain of γ, but acylation or O-linked glycosylation of the molecule is likely. Interestingly, Ser5 is the only residue within the N-terminal spliced exon in γa that is conserved among the species (Fig. 1A). In contrast, the sequence of the N-terminal exon of γb is absolutely identical between species. Whether modification of Ser5 is indeed physiologically significant awaits further investigation.

SPLICE VARIANTS OF γ ARE DIFFERENTIALLY EXPRESSED ALONG THE NEPHRON

Although both splice variants are abundant in kidney, in renal tubules their expression patterns are different (Arystarkhova *et al*., 2002b). In the outer medulla, both γa and γb are abundantly expressed in the thick ascending limb (TAL) of the inner stripe (where thin descending and thick ascending segments of the loop of Henle are found). In contrast, γa staining is reduced greatly in the TAL of the outer stripe (where thick descending/proximal straight segments and thick ascending segments are found), but γb is still expressed there. The rest of the cortical γ stain is in segments that are negative for a marker of TAL, Tamm-Horsfall protein. Both splice forms are found in inner medullary collecting duct.

In cortex, the proportion of γb is markedly lower than in medulla, and γb is detected only in distal convoluted tubules (DCT) and connecting tubules (CNT) (Arystarkhova *et al*., 2002b). Conversely, we observed a low level of expression of γa in the proximal convoluted tubules (PCT) that correlates with the low abundance of the α1 subunit in these segments. No γb was detected in proximal tubule either in kidney sections or in Western blots of isolated PCT.

Such segment-specific distribution of γa and γb, which are practically identical in primary structure, may be indicative of distinct regulatory effects the splice variants confer to Na,K-ATPase. Unfortunately, at this moment no probes are available for detection of PTM(s) of γ splice forms in tissues. Since no post-translational modification was identified by MALDI analysis of the γ subunit from the outer medulla of Milan Hypertensive rats (Küster *et al*., 2000), the potential significance of PTM in renal physiology has received little attention even though cell-specific modification in γ-transfectants of mammalian cells has been easily observed. However, based on our own experience with cells in culture, where PTM occurs when γa is induced in response to variations in physiological conditions (Wetzel *et al*., 2004), the prediction is that expression of γ in modified form(s) occurs in vivo as well.

STRUCTURAL FORMS OF γ DIFFERENTIALLY INFLUENCE Na,K-ATPASE ACTIVITY

In general, the anatomical distribution of γ along the nephron correlates well with Na^+ affinities measured in microdissected permeabilized nephron segments. In rabbits, the lowest affinity for Na^+ was identified in PCT, somewhat lower in CTAL, and the highest in collecting duct (Barlet Bas *et al*., 1990). Thus the presence of γ is accompanied by lower affinity for Na^+, suggesting that expression of γ might selectively modify Na,K-ATPase properties in vivo.

To test the hypothesis that γ is a modulator of the kinetic properties of the pump we generated stable clones in NRK-52E cells, which have a proximal phenotype but which do not express γ under regular tissue culture conditions. We performed kinetic analysis on partially purified preparations of Na,K-ATPase from multiple individual clones. As mentioned above, stable clones expressing γa or γb with or without post-translational modification (PTM) were obtained during selection (Arystarkhova

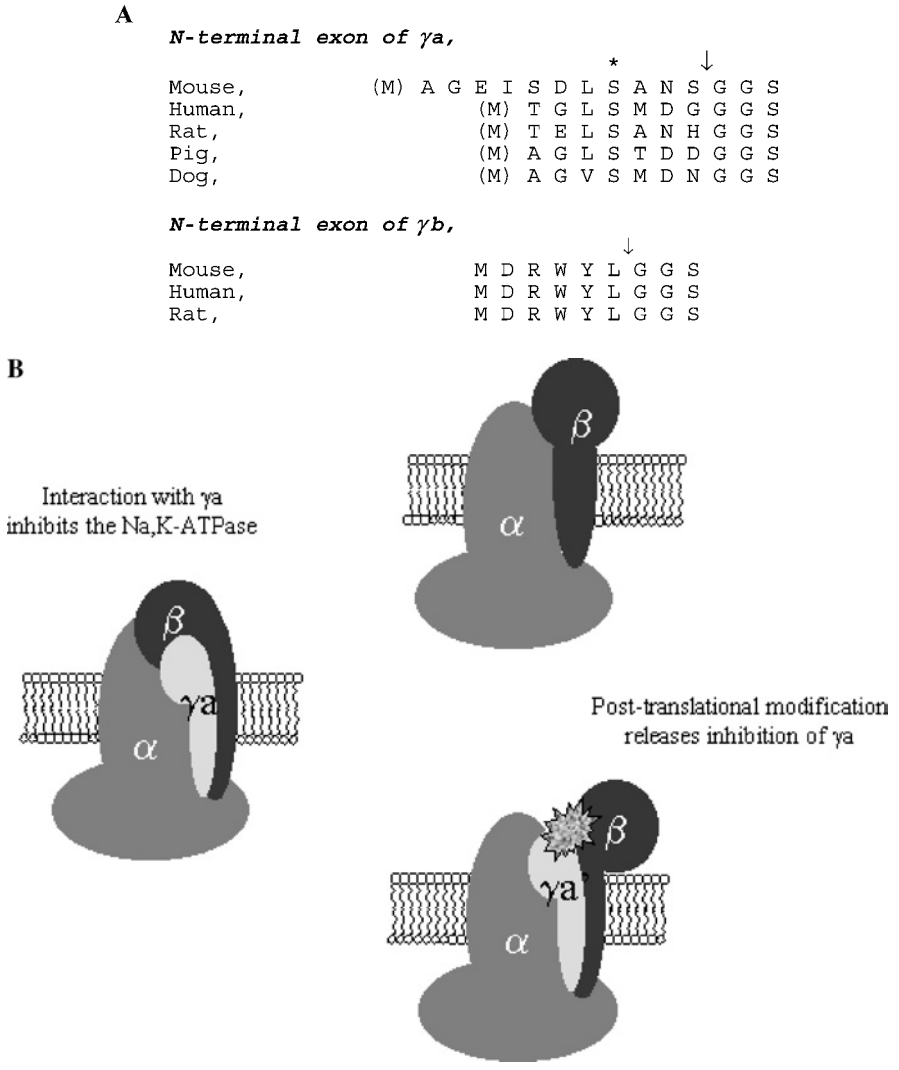

Fig. 1. (A) Sequence comparison of the N-terminal exons of γa and γb from different species. The conserved serine residue is marked with an asterisk. The arrows indicate the beginning of the second exon, which (like the rest of the molecule) is shared in γa and γb. (B) Schematic representation of $\alpha\beta\gamma$a complex and the potential role of post-translational modification (PTM) in regulation of pump activity.

et al., 1999, 2002a). A total of 15 different clones were analyzed, at least 3 clones for each of the 4 structural forms.

Remarkably, the four structural forms of the γ subunit appeared to present all of the possible permutations for reduction in ion affinity for the major physiological ligands: Na^+ alone, K^+ alone, and Na^+ and K^+. The apparent affinity for Na^+ was significantly reduced with γa^*, γb, or $\gamma b'$ expression implying that post-translational modification of γb did not influence this parameter of the pump. On the contrary, the effect on affinity for Na^+ was completely abolished in the clones expressing exclusively post-translationally modified $\gamma a'$ (Fig. 1B). The apparent affinity for K^+, on the other hand, was reduced in both γa^* and $\gamma a'$ clones but not in the γb-expressing cells. Post-translational modification of γb, however, appeared to be critical for a reduction in the affinity for K^+. The outcome that emerges is that depending on whether or which γ form is present, Na,K-ATPase affinities for its major physiological ligands, Na^+, K^+, both, or neither will be reduced (Table I).

We did not see any major changes in K_m for ATP between mock-transfected NRK-52E cells expressing any structural form of γ: in all cases affinity for ATP was higher compared to rat kidney membranes (Arystarkhova *et al*., 2002a).

Table I. Effects of Splice Variants of γ on Intrinsic Parameters of Na,K-ATPase and Cell Growth [Adapted and Modified from Arystarkhova *et al*. (2002a)]

	Na^+ affinity	K^+ affinity	ATP affinity	Cell growth
No gamma	(high)	(high)	(high)	(high)
γ-a*	↓	↓	No change	↓
γ-a′	No change	↓	↑	No change
γ-b	↓	No change	↑	↓
γ-b′	↓	↓	No change	↓

Generation of γ knockout mice and analysis of their Na,K-ATPase kinetics was used as an independent approach to assess the functional role of γ in renal tissue. The major effect was found on the apparent affinity for Na^+: there was a modest but statistically significant reduction in $K_{1/2}$ for cytoplasmic Na^+ in membrane preparations from FXYD2$^{-/-}$ vs. FXYD2$^{+/+}$ mice. No statistically significant change was detected for apparent affinity for K^+, whereas K_m for ATP was slightly reduced in the γ-knockout preparations (Jones *et al*., 2005).

Thus, two complementary approaches showed that reduction of apparent affinity for Na^+ is the major functional consequence of association of Na,K-ATPase with the γ subunit. On the other hand, the effects on apparent affinity for K^+ and for ATP are more likely to be cell-specific and may reflect more complex association of Na,K-ATPase with other cellular proteins.

SPLICE VARIANTS OF γ RESPOND DIFFERENTLY TO CELLULAR STRESS

As mentioned above, none of the splice variants were found in renal cell lines under normal cell culture conditions. However, expression of endogenous γ in a partially modified form (γa*) can be induced in NRK-52E by exposure to hyperosmotic medium (with NaCl or sucrose, but not with urea) (Wetzel *et al*., 2004).

The newly synthesized γ is co-localized with α at the plasma membrane. Functionally, induction of γa* results in a reduction of Na,K-ATPase activity, as predicted by transfectants, but the most affected parameter was the V_{max} with only small changes in the affinity for Na^+. (It should be stressed that variable levels of γ expression in stable transfectants made it hard to be quantitative about the V_{max}). Since hypertonicity is a complex signal affecting multiple signaling pathways, the inhibitory effect of γ on the pump activity was corroborated using the siRNA interference approach. Selective silencing of γ induction with the γ-specific siRNA probe completely prevented reduction of Na,K-ATPase activity in hypertonic medium, implying that the changes in activity are apparently caused by association of the $\alpha 1\beta 1$ complex with the *de novo* synthesized γa (Wetzel *et al*., 2004).

Whether modulation of Na,K-ATPase activity under hypertonicity conditions entails another mode of $\alpha\beta\gamma$ interaction as compared to γ-transfectants, is not yet clear. One of the possibilities is that association of $\alpha\beta$ with the newly synthesized γ may result in a conformational rearrangement of the complex with subsequent steric hindrance/exposure of potential sites of phosphorylation on α. Protein kinases and protein phosphatases are activated by hypertonicity. Functionally, reductions in both Na^+ affinity and V_{max} caused by expression of γ should have the effect of reducing pump activity since normally the concentration of Na^+ is rate-limiting.

Surprisingly, no induction of the γb splice variant was observed during acute hypertonic treatment of NRK-52E cells, whereas Capasso *et al*. (2001) demonstrated induction of both splice variants in mIMCD-3 cells, which are derived from mouse inner medulla collecting duct. This may be suggestive of cell-specific control of γ splice variant expression. Hypertonic conditions are the norm for medullary segments of the nephron, thus hypertonicity can represent a highly selective mode of regulation of γ expression. Nevertheless, in our hands, hypertonicity stimulated the induction of only the γa splice variant in rat cells other than renal origin—C6 glioma, PC12 pheochromocytoma, L6 myoblasts (Wetzel *et al*., 2004), as well as in human intestinal epithelial-like Caco-2 cells. This is especially interesting since γ as a protein is normally absent from the Na,K-ATPase in most other tissues. Similar responses to osmotic stress may underlie important physiological and pathophysiological consequences throughout the body.

Furthermore, our recent data suggest that expression of γa (but not γb) is increased in response to several other modes of genotoxic stress. Heat shock, treatment with heavy metals, and exogenous oxidants stimulated induction of γa* (Wetzel *et al*., 2004). Given that Na,K-ATPase consumes about 70% of the ATP supply in the cell, the inhibition of the ATPase activity via the γa subunit will reduce demand for ATP, and should be adaptive and beneficial for overcoming the apoptotic insult.

A novel extension of our basic hypothesis came from a microarray study of chemically-insulted hippocampal tissue showed γ mRNA to be up-regulated in brain (Kassed *et al*., 2004). Thus not only can γ expression regulate ion transport in the kidney, but it may be called up to reduce Na,K-ATPase activity in other tissues as a protective mechanism.

EXPRESSION OF γ IN VITRO CORRELATES WITH A DELAY IN CELL PROLIFERATION

In experiments with γ-transfected cells we noticed a correlation between reduction in Na^+ affinity and the rate of cell proliferation. γa^* transfectants had slower growth rate than mock-transfected cells or cells with fully modified $\gamma a'$ (Arystarkhova *et al*., 1999). Analysis of the γb or $\gamma b'$ transfectants revealed that their cell growth was similarly reduced compared to the mock-transfected cells (Arystarkhova *et al*., 2002a). Since all three groups of slow-growing clones possessed lower affinity for Na^+ than control or $\gamma a'$ clones, the slower growth apparently correlates with lower activity. The data suggest that culture conditions create a selective pressure against cells with high γ expression apparently because of the reduction of Na,K-ATPase affinity for Na^+. This may explain why most established renal cell lines lack constitutive γ expression.

Correlating with this, induction of endogenous γa in NRK-52E by hypertonicity was accompanied by a significant reduction in the rate of cell division (Wetzel *et al*., 2004). Transient arrest in cell growth (18–24 h as reported for mIMCD-3 cells) is a well-established phenotypic readout for hypertonic stress. In our case, the arrest was even longer, but could be abolished by selective knockdown of γ. To achieve this, cells were transfected with γ-specific or scrambled siRNA probes under isotonic conditions followed by exposure to hypertonic medium. Phenotypically, cell growth was retarded in the scrambled, but not the γ-siRNA probe-treated cells in hypertonic conditions. Thus, removal of a single protein, i.e., selective silencing of γ induction, resulted in escape from cell growth arrest even under continuing non-permissive hypertonic conditions.

The ultimate fate of the cells (proliferation, apoptosis and hypertrophy) is governed by several levels of control, including regulation by growth factors, signaling pathways, extracellular matrix and immediate response genes, as well as by cell cycle regulatory proteins within the nucleus. Our experimental data suggest that regulation of Na,K-ATPase through the FXYD proteins (γ) may represent yet another layer of control for cell proliferation and growth. There are two possible mechanisms. Changes in intracellular ion concentration associated with modulation of Na,K-ATPase via γ may affect expression of cell cycle regulators or proteins involved in DNA synthesis. Alternatively, association with the γ subunit may trigger assembly of Na,K-ATPase with protein partners in signaling modules similar to what has been observed with ouabain treatment. By either mechanism, expression of γ negatively correlates with the proliferative status of the cell and therefore may have adaptive value as part of a general cellular mechanism of regulation of cell growth.

ASSOCIATION WITH THE Na,K-ATPASE COMPLEX

Interaction of the splice variants of γ with the Na,K-ATPase was revealed by a co-immunoprecipitation approach. When rat kidney outer medulla membranes were solubilized with zwitterionic or non-ionic detergents, either CHAPS or $C_{12}E_8$, antibody against the α subunit brought down both splice variants of γ (Arystarkhova *et al*., 2002b). In the reverse experiment, specific antibodies against γa or γb co-precipitated not only the α subunit, but each other as well, supporting the existence of a multimeric architectural organization of the Na,K-ATPase complex in MTAL when both γ forms are co-expressed. Importantly, the activity of Na,K-ATPase was only slightly reduced upon solubilization with $C_{12}E_8$, thus supporting the specificity of the interaction between the splice variants of γ within the Na,K-ATPase complex. The most likely interpretation of co-immunoprecipitation of γ splice variants is association of Na,K-ATPase in MTAL into higher oligomeric complexes $[(\alpha\beta\gamma a)\text{-}(\alpha\beta\gamma b)]_n$, where n can be either 1 or (more likely) 2, i.e., dimers or tetramers of Na, K-ATPase.

Na,K-ATPase goes through a cycle of conformational changes during the turnover of the enzyme. Interestingly, pre-incubation of rat kidney outer medulla membranes with ligands controlling E2 or E2-P conformations, which are spatially more compact compared to E1, reduces the specific interaction between γ (either splice form) and the α subunit as detected by co-immunoprecipitation (Arystarkhova *et al*., 2002b). This was most obvious when (Na^+) conditions were compared directly with (Na^+, Mg^{2+}, and ATP) conditions, which induce E1 and E2-P conformations respectively. The data further support γ's functional integration into the Na,K-ATPase and suggest that the stability of association may depend on conformational rearrangements of the α (and/or β) subunits during enzyme turnover.

Thermodenaturation experiments provided other evidence for structural integration of γ into the Na,K-ATPase complex. Heating at 55°C caused radical re-organization of the C-terminal portion of the α subunit. The M8-M10 transmembrane spans of the catalytic subunit were expelled from the membrane along with γ, suggesting association of the latter with M8–M10 (Donnet *et al*., 2001). Interestingly, when γ is absent from Na,K-ATPase (in preparations from FXYD2$^{-/-}$ mice), heating even at

much lower temperature (41°C) caused a significant decrease of the activity of enzyme (Jones *et al*., 2005). Conversely, activity was sustained in Na,K-ATPase containing γ (in preparations from FXYD2$^{+/+}$ mice) suggesting a stabilizing role of γ against thermal inactivation.

FINAL REMARKS

In sum, this overview of our studies demonstrates that the product of a single gene, FXYD2 (the γ subunit), may serve as a multifunctional physiological adaptor required for the proper response of renal Na,K-ATPase to activation of specific signaling pathways, and it does so in a manner that is affected by expression of splice variants and post-translational modification. The expression of γ has an overriding effect on the rate of cell proliferation. The expression of γ also appears to be protective in the case of cell stress, possibly by reducing the consumption of ATP. Elucidation of a high-resolution structure of the Na,K-ATPase complex including different forms of γ will eventually unveil a detailed mechanism of action.

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