SEMINARS IN
NEPHROLOGY

FXYD Proteins: Tissue-Specific Regulators of the Na,K-ATPase

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Work in several laboratories has led to the identification of a family of short single-span transmembrane proteins named after the invariant extracellular motif: FXYD. Four members of this group have been shown to interact with the Na,K-adenosine triphosphatase (ATPase) and alter the pump kinetics. Thus, it is assumed that FXYD proteins are tissue-specific regulatory subunits, which adjust the kinetic properties of the pump to the specific needs of the relevant tissue, cell type, or physiologic state, without affecting it elsewhere. A number of studies have provided evidence for additional and possibly unrelated functions of the FXYD proteins. This review summarizes current knowledge on the structure, function, and cellular distribution of FXYD proteins with special emphasis on their role in kidney electrolyte homeostasis.

KEYWORDS FXYD, Na,K-ATPase, CHIF, gamma-sybunit, Phospholemman

The Na,K-adenosine triphosphatase (ATPase) actively pumps Na^+ out of cells and K^+ into cells and maintains the characteristic transmembrane electrochemical gradients of Na^+ and K^+. This function is important particularly in kidney and other epithelia where Na,K-ATPase, in the basolateral membrane of the cells, mediates the vectorial transepithelial transport of Na^+ and K^+, as well as a variety of essential secondary transport processes. As could be expected for a protein with such a central physiologic role, Na,K-ATPase is regulated closely at several levels. Known mechanisms include long-term transcriptional regulation of α and β subunits by hormones (eg, mineralocorticoids, thyroid hormones) or during development, as well as short-term kinetic regulation involving changes in catalytic activity or trafficking to and from the cell membrane (eg, insulin, dopamine).[1] The Na,K-pump consists of the catalytic α subunit and β subunit required for correct folding, stabilization, and expression of the α/β complex at the cell membrane. There are several isoforms of both subunits, α (α1-4) and β (β1-3), expressed in a tissue- and developmental-dependent fashion, and having distinct kinetic properties.[2] The classic plant-derived inhibitor of the Na,K-pump, ouabain, increases the force of contraction of heart muscle. Ouabain also is synthesized endogenously in adrenal cortex and hypothalamus.[3] Endogenous ouabain is thought to be involved in the generation of essential hypertension.[4] Ouabain also has an important signaling function, mediating gene expression and cell growth via mitogen-activated protein kinase pathways.[5]

Recently, a new and unique mode of regulation of the Na,K-ATPase has been discovered. It involves interactions between the α/β complex and a group of short single-span transmembrane proteins termed the *FXYD* proteins.[6-8] Earlier studies suggested different and apparently unrelated functions for the 7 members of the FXYD family. Recently, however, it has become apparent that at least 4 members of this group interact specifically with the Na,K-ATPase, and alter the pump kinetics. Such effects have been shown for FXYD1 (phospholemman [PLM]),[9] FXYD2 (the γ subunit of Na,K-ATPase [γ]),[10,11] FXYD4 (corticosteroid hormone induced factor [CHIF]),[12,13] and FXYD7.[14] Thus, the working hypothesis is that FXYD proteins function as tissue-specific modulators of Na,K-ATPase, which adjust or fine-tune its kinetic behavior to the specific needs of the given tissue, cell type, or physiologic state, without affecting it elsewhere.[7,8] Although γ, CHIF, and FXYD7 appear to behave as modulatory subunits, PLM can be phosphorylated[15-17] and therefore it may be classified more as a classic regulatory subunit that alters the pump properties in response to external signals. This review summarizes existing data on the structural and

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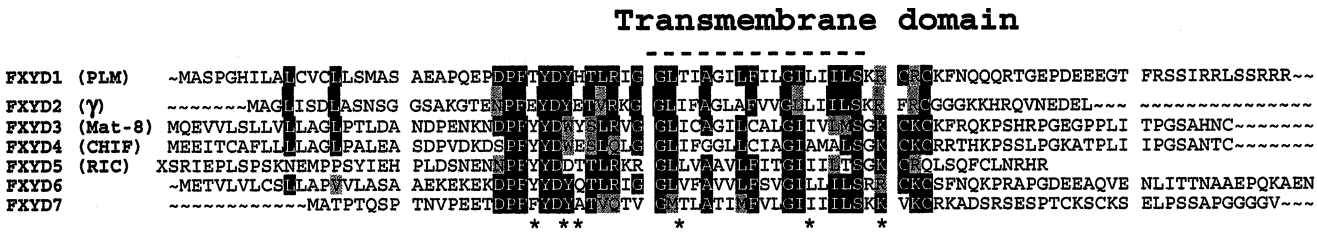

Figure 1 Sequence alignment of the FXYD family. The sequences correspond to mouse proteins. In RIC (FXYD5) there are an additional 105 N-terminal residues marked as X. The 6 invariant amino acids are marked by asterisks.

functional interactions between the Na,K-ATPase and FXYD proteins and discusses their putative physiological role(s) with special emphasis on regulation of the Na,K-ATPase by γ and CHIF in kidney.

Primary Structure of the FXYD Proteins

FXYD proteins are short polypeptides (>100 amino acids) with a single transmembrane domain with or without a signal peptide (Fig. 1). The only exception is Related to Ion Channel (RIC), which has an atypically long N-terminal sequence. Analysis of membrane topology performed for γ, CHIF, and FXYD7 established that they are type I single-span membrane proteins, with an extracellular N-terminus and cytoplasmic C-terminus.[10,13,14,18,19] Alignment of FXYD sequences for several species revealed 6 invariant amino acids (Fig. 1, asterisks). These are as follows: the FXYD motif in the extracellular domain, 2 glycine residues within the membrane, and a serine residue at the membrane-cytoplasm interface. Two additional characteristic features are the following[1]: marked homology among family members is observed in a stretch of 35 amino acids around the transmembrane domain but not outside this region; and helical wheel projection of the transmembrane segment shows that the conserved residues are located preferentially on one face of the helix,[6] suggesting that this face is important for the functional and/or structural interaction.[2] The genomic organization of the 7 FXYD members is unusual in that each transcript is made up of at least 6 exons and that the above 35 amino acid conserved region is formed by 3 separate exons.[20] This might serve to enable high structural diversity, although structural diversity is not well documented experimentally. Multiple transcripts have been reported for γ and PLM only.[21,22] At the protein level, however, only γ exists as 2 splice variants, γa and γb.[23] Two additional species designated γa′ and γb′ can be resolved electrophoretically in different cells transfected with a single plasmid (γa or γb, respectively).[11,24]

Tissue Distribution of FXYD Proteins

As a summary of FXYD protein distribution: γ is expressed in kidney, CHIF in kidney and colon, PLM in heart and skeletal muscle, and FXYD7 in brain. Other FXYD proteins have not been detected at the protein level. The detailed expression patterns of γ (γa and γb), CHIF, and PLM in kidney provides insights into to their possible physiologic roles. Immunohistochemistry of intact rat kidney shows that γ is expressed at high levels in thick ascending limb of the loop of Henle (TALH) and distal convoluted tubule (DCT), at lower levels in PCT,[11,25] and at low but detectable levels in inner medullary collecting duct (IMCD) cells.[26] CHIF is expressed in cortical collecting duct (CCD)[19] and also in IMCD.[26] In general, γ and CHIF are not expressed in the same cells[27] (for an exception, see later). The use of splice variant–specific antibodies of γ have revealed overlapping but also distinct expression patterns of γa and γb in different nephron segments.[11,28] In PCT both splice variants are detected at low to moderate levels but γa predominates over γb. In medullary thick ascending limb of the loop of Henle (mTALH) both γa and γb are expressed at high levels, in cortical thick ascending limb of loop of Henle (cTALH) only γb, and in macula densa γa but not γb was observed.[11] In one study only γb was detected in the DCT.[28] In the other study γa was assigned to CCD cells,[11] but the nephron segment could have been DCT because the marker (AQP2) also is located in DCT.

Although initially γ was not detected in IMCD of intact rat kidney,[11,29] more sensitive measurements using laser scanning confocal immunofluorescence showed the presence of γ in IMCD.[26] In the initial quarter of IMCD, γa was found in intercalated cells but not in principal cells, which express CHIF. This exclusive expression of CHIF but not γ in principal cells is similar to that in CCD. However, in the middle segment of IMCD all principal cells expressed both γa and CHIF. γb was not detected in IMCD. By immunoelectron microscopy both γ and CHIF were located at the basolateral surface of the relevant cells.

PLM has been found in selected structures of both brain and kidney. In brain it was detected in cerebellum and enriched in choroids plexus, colocalized with Na,K-ATPase.[30] In kidney PLM has been found in extraglomerular mesangial cells and afferent arterioles, but not in nephrons, suggesting a role in renal blood flow and tubuloglomerular feedback.[25] This contrasts with expression of γa in the adjacent macula densa epithelial cells.[11]

Association of FXYD Proteins With the Na,K-ATPase

Specific association of PLM, γ, CHIF, and FXYD7 with the α/β complex has been shown by co-immunoprecipitation

Table 1 Effects of FXYD Proteins on the Pump Kinetics

FXYD Protein	Experimental System	Kinetic Parameter	Fold Change	Reference
PLM	*Xenopus* oocytes	$K_{1/2}$ Na^+	1.8	9
		$K_{1/2}$ K^+	≤ 1.7*	
		V_{max}	No change	
	Based on inhibitory effect of an antibody	$K_{1/2}$ Na^+	No change	30
		V_{max}	1.4	
PLMS	Proteolytic digestion	V_{max}	0.7	17
Gamma	*Xenopus* oocytes	$K_{1/2}$ K^+	≤ 1.5–2[1]	10
	Based on inhibitory effect of an antibody	K'_{ATP}	0.5	32
	Transfected mammalian cells	K'_{ATP}	0.5	11
		K_{12} Na^+	1.7	
	Reconstitution with γ peptide	$K_{1/2}$ Na^+	2–3	34
CHIF	*Xenopus* oocytes	$K_{1/2}$ Na^+	0.64	13
		$K_{1/2}$ K^+	1.8[1]	
	Transfected mammalian cells	$K_{1/2}$ Na^+	0.3	12
		$K_{1/2}$ K^+	No change	
FXYD7	*Xenopus* oocytes	$K_{1/2}$ Na^+	No change	14
		$K_{1/2}$ K^+	1.3–1.9	

*In this case variations of $K_{1/2}$ with membrane potential and the presence of external Na^+ were noted. The effects of PLM and FXYD7 also depend on the α isoform used.

experiments in native membranes and expression systems.[9,10,12-14,31] Efficient co-immunoprecipitation of γ and CHIF was apparent only if detergent solubilization was performed under conditions known to preserve native protein structure, suggesting that the co-immunoprecipitation is specific.[12] In *Xenopus* oocytes expressed γ or CHIF are unable to reach the plasma membrane without expressed α/β subunits, providing further evidence of specific associations.[10,13]

The stoichiometry of α/β/FXYD associations is an important question. In purified renal Na,K-ATPase, containing both γa and γb, the ratio γ:α:β is close to 1:1:1.[23,31] This is suggestive of an equal mixture of 1:1:1 α/β/γa and α/β/γb complexes, but also could be consistent with equal amounts of an α/β/γa/γb mixed complex and α/β complexes without γ. However, co-immunoprecipitation experiments now have excluded α/β/γa/γb complexes.[12] In conditions that preserve native protein structure, anti-γa precipitates γa but not γb from renal Na,K-ATPase, whereas an antibody that does not discriminate between the 2 splice variants (anti–γC-terminus) precipitates both γa and γb (and also α and β). This indicates the presence of both α/β/γa and α/β/γb but not α/β/γa/γb complexes. A report that inferred the possibility of α/β/γa/γb used detergent conditions that inactivated the enzyme, and might have caused aggregations of macromolecular complexes such as α/β/γa-α/β/γb.[28] In renal papilla membranes anti-γC immunoprecipitates γ but not CHIF and anti-CHIF precipitates CHIF (and α plus β) but not γ.[12] Because γ and CHIF largely are expressed in different nephron segments the experiment does not rigorously exclude mixed α/β/γ/CHIF complexes. However, co-immunoprecipitation experiments of γ and CHIF co-expressed to the same level in the same Hela cell definitively exclude α/β/ γ/CHIF complexes (Lindzen et al, unpublished data).

Functional Effects of FXYD Proteins on the Pump Kinetics

Functional effects of FXYD proteins have been shown for PLM, γ, CHIF, and FXYD7 and are summarized in Table 1. Kinetic effects of γ were detected either by co-expression with the α/β subunits in *Xenopus* oocytes and mammalian cells or by neutralization interactions with a specific anti-γ antibody.[10,11,18,32,33] In oocytes, γ reduces affinity to cell Na^+ and evokes a small increase in the extracellular K^+ affinity, which varies with voltage.[10] In cultured mammalian cells, γ increases apparent affinity for ATP by shifting the E1-E2 conformational equilibrium toward E1, and reduces apparent affinity for cytoplasmic Na^+ by making cytoplasmic K^+ a better competitor,[11,32,33] and slightly reduces extracellular K^+ affinity.[24] The reduced cytoplasmic Na^+ affinity is also detected in isolated membrane incubated with a mimetic peptide corresponding to the transmembrane domain of γ.[34] In HeLa and HEK293 cells no significant differences in functional effects are found between γb and γa or between γb and γb′ (HeLa cells) and γa and γa′ (HEK293 cells).[11] Similarly in *Xenopus* oocytes functional differences between γa and γb are not detectable.[13] Differences between γa and γa′ on Na^+ affinity and between γb and γb′ forms on K^+ affinity have been reported after expression in NRK-52E cells.[24,33] Whether these differences are physiologically significant is uncertain because no γa′ and very little γb′ are detected in intact kidney membranes.[23] Also, in transfected cells γa′ and γb′ are expressed in a cell-specific fashion.[11] Although γ was discovered by labeling with a photoactive ouabain derivative,[35] expression of γ does not affect the apparent affinity for ouabain inhibition, at least in *Xenopus* oocytes.[13]

The functional effects of CHIF in *Xenopus* oocytes[13] and mammalian cells[12] are different and, in fact, opposite to those of γ. In HeLa cells, the expression of CHIF increases the affinity for cell Na^+ by 2- to 3-fold, but has no effect on the affinity for external K^+ or ATP.[12] A similar decrease in $K_{1/2}$ for cell Na^+ was observed in *Xenopus* oocytes.[13] In this system, however, an increased $K_{1/2}$ to external K^+ was measured as well. This effect was seen only at high voltages and the presence of external Na^+.[13] The opposite functional effects of CHIF and γ on the affinity of the pump to cell Na^+ are consistent with their different patterns of expression along the nephron, as well as the induction of CHIF by mineralocorticoids (see later).

When expressed in *Xenopus* oocytes, PLM interacts with both the α1β1 and α2β1 isoforms and decreases the internal Na^+ affinity of the pump by approximately 2-fold, and external K^+ affinity by a small amount.[9] Although PLM, similar to γ, appears to decrease apparent Na^+ affinity, it is not clear that the mechanism or mode of interaction with the α subunit are the same. In the heart, this effect could allow an effective response of the pump to increases of cell Na^+ associated with cardiac activity. Effects of PLM also have been described in bovine choroid plexus membranes.[30] In this system, an anti-PLM antibody reduces maximal rate without affecting the Na^+ affinity. An FXYD protein that specifically interacts with the Na,K-ATPase also has been identified in shark rectal glands.[17,36] This protein was termed *PLMS* because of its PLM-like phosphorylation sites (see later). However, its nearest mammalian homolog is mammary tumor marker 8 (Mat-8) and, hence, its physiologic roles could be quite different from those of PLM. Proteolysis of the C-terminus of PLMS, which presumably impairs its interaction with the α/β complex, increases Vmax, which is opposite to the effect reported for PLM.[17,30]

FXYD7 is the fourth family member whose functional interaction with the Na,K-ATPase has been shown. In *Xenopus* oocytes, FXYD7 decreases the apparent K^+ affinity of the pump when expressed with α1β1 or α2β1, but not with α3β1.[37] In brain this protein was found to interact with α1β1 isoforms only. Thus, FXYD7 appears to exhibit an isoform-specific interaction with the pump. The remaining 3 FXYD proteins (Mat-8, RIC, and FXYD6) have not been tested yet for functional interactions with the Na,K-ATPase.

In summary, different effects of 4 FXYD proteins have been shown in expression systems as well as isolated membranes and purified pump complexes. Although the in vitro kinetic effects of FXYD proteins are rather small, they may be highly significant in the physiological context. One also should bear in mind that the application of an antibody or proteolytic digestion may not eliminate fully the functional FXYD/pump interaction. Also, expression systems may lack additional components or posttranslational modifications essential for the full response. Overall, although the earlier-described experiments show effects of FXYD proteins on pump kinetics, the mechanistic details are far from being understood fully.

Physiological Role of FXYD Proteins

The distinct functional effects of γ or CHIF on the Na,K-pump presumably serve the different physiological needs of the cells in which γ and CHIF are expressed. For example, the medullary TALH segments contain high levels of Na,K-ATPase (α/β/γ complexes) and are characterized by a very high rate of Na^+ pumping and transepithelial Na^+ reabsorption, which generate the renal salt gradients. An increased ATP affinity may allow maintenance of Na,K-pump rates in response to rapid decreases in ATP levels accompanying anoxic episodes, caused by the high energy requirements and low blood flow in this region of the kidney. A decreased cytoplasmic Na^+ affinity could allow the pump to respond sensitively to increases of cytoplasmic Na^+ at higher set-point levels of cytosolic Na^+. By contrast, CHIF could be expected to serve the crucial role of the CCD in K^+ homeostasis and its regulation by mineralocorticoids. A CHIF-induced increased Na^+ affinity should adapt the pumping rate to a lower cell Na^+ resulting from the low luminal Na^+, and respond effectively to increases in cytosolic Na^+ associated with mineralocorticoid-induced Na^+ permeability at the luminal surface, as well as maintaining the driving force for Na^+ entry and the transepithelial potential, which drive Na^+ reabsorption from the luminal fluid. The regulatory interaction of CHIF presumably underlies a higher affinity of the Na,K-pump for cytosolic Na^+ observed in cortical collecting ducts compared with other nephron segments.[38] It is not clear whether effects of γ on extracellular K^+ affinity are physiologically significant because extracellular K^+ (4.5 mmol/L) is close to saturating the pump.

One approach to better understand the physiologic role of FXYD proteins is the phenotypic analysis of knockout mice models and genetic disorders associated with mutations in these proteins. To date, only 2 such examples have been provided: CHIF knockout mice[39,40] and mutation of human γ causing dominant hypomagnesemia/hypocalcuria.[41] CHIF knockout mice are viable, fertile, and under normal conditions cannot be distinguished from wild-type litter mates.[39] After K^+ loading or Na^+ deprivation they manifest mild kidney phenotypes such as increased glomerular filtration rate and urine volume but no difference in the fractional excretion of Na^+ and K^+. Short-circuit current measurements in distal colon show a 2-fold decrease in the amiloride-blockable Na^+ absorption and the forskolin-induced Cl^- secretion.[40] These observations are consistent with a specific decrease in Na^+,K^+-pumping rate in the collecting duct and distal colon under electrolyte stress (K^+-rich or Na^+-depleted diets). It is assumed that this primary deficit is well compensated by the more proximal nephron segments, whereas the secondary volume imbalance is compensated only partly by regulation of the glomerular filtration rate.

A single nucleotide mutation in human γ has been linked to familial-dominant renal hypomagnesemia.[41] The mutation causes replacement of a conserved transmembrane glycine by an arginine. As a result, γ fails to be incorporated into the

plasma membrane and accumulates in the Golgi. The distal convoluted tubule is known to play an important role in determining the final urinary excretion of Mg^{2+} even though it reabsorbs only about 10% of the filtered Mg^{2+}.[42] The loss of Mg^{2+} could be an indirect effect of absence of the γ. For example, a reduced ATP affinity and Na,K-ATPase activity could lead to increased cell Na^+ and secondary reduction of Mg^{2+} entry into the distal convoluted tubule.[43] Again, the underlying assumption is that the primary effect of such impairment on Na^+ and K^+ transport is compensated fully by nephron segments that do not express γ, and only secondary effects that are specific to the γ-enriched segment are manifested.

Adaptation of cultured mouse IMCD3 cells to hypertonic salt conditions (from 300 mOsm to 600 and 900 mOsm NaCl) is accompanied by amplified synthesis of organic osmolytes and many proteins, including Na,K-ATPase α and β subunits.[44] Both γa and γb subunits are also expressed on adaptation to hypertonicity, whereas the IMCD3 cells cultured in isotonic media express neither γa nor γb.[45] The process involves the C-terminal Jun kinase and phosphatidylinositol 3-kinase signaling pathways. By contrast, the adaptive expression of the α subunit does not involve the C-terminal Jun kinase and phosphatidylinositol 3-kinase kinase signaling pathways. Expression of the γ subunit depends on Cl^- and not Na^+,[46] whereas adaptation of α and β subunit expression is dependent on Na^+. The findings are consistent with an essential role of γ in the adaptive response in IMCD3 cells to hypertonicity. In the face of increased passive Na^+ influx, presumably the cells adapt by increasing their Na pumping capacity (α/β units) and, in addition, γ to fine-tune the pump kinetics (reduced Na^+ and increasing ATP affinity). However, the striking fact that different signaling pathways are used to control expression of α/β and γ, and the fact that inhibition of the C-terminal Jun kinase pathway is associated with loss of cell viability, raises the question whether the role of the γ subunit is solely to alter pump kinetics. Detection of γa in intact rat kidney IMCD[26] suggest that observations on cultured mouse IMCD are relevant to physiologic adaptations to hypertonicity.

The γ subunit is expressed in mouse embryos and appears to be required for the cavitation process to the blastocyst stage.[47] Interestingly, γ is expressed at both basolateral and luminal surface of the trophoectoderm epithelial cell layer, which drives fluid transport, although the α/β subunits are concentrated at the basolateral surface. This supports the notion of a role that is additional to the modulation of the pump.

Structure/Function Relations of FXYD Proteins

Recent studies on the FXYD-α/β interactions indicate the existence of multiple sites of interaction. An initial indication came from the fact that the anti–γC-terminus abrogates the effect of γ on the apparent ATP affinity in renal Na,K-ATPase or Hela cells transfected with γ, but not that on the K:Na antagonism.[11,32] Also, in *Xenopus* oocytes, the extracytoplasmic FXYD motif is required for stable γ and CHIF interaction with α/β, whereas cytoplasmic, positively charged residues are necessary for efficient association of γ and for CHIF's functional effects.[13] Expression in Hela cells of γ with either 4 or 10 C-terminal residues or 7 N-terminal residues deleted removes the effect of γ on ATP affinity, but does not affect the K:Na antagonism.[43] Replacement of the deleted 7 N-terminal residues by 7 alanines restored the effect on ATP affinity. The conclusion is that different regions mediate the different functional effects and effects can be long range.

In a systematic recent study of roles of the different segments, a series of γ-CHIF chimeric molecules was prepared in which extracellular, transmembrane, and cytoplasmic sequences were interchanged.[48] The chimera were expressed in Hela cells together with rat $\alpha1\beta1$ and the stability of the α/β/FXYD chimera was determined in a co-immunoprecipitation assay taking advantage of the fact that the α/β/CHIF complex is more sensitive to excess detergent than $\alpha/\beta/\gamma$. Stability of the α/β/FXYD chimera was found to depend on the origin of the transmembrane segment. Chimerae with transmembrane segments derived from CHIF were less stable (similar to CHIF itself), whereas chimerae with transmembrane segments derived from γ behaved like γ. By exchanging residues ^{55}MA in the transmembrane segment of CHIF with the corresponding IL residues in γ, the stability reverted to that of $\alpha/\beta/\gamma$. A similar effect was obtained by mutating ^{45}G in the transmembrane segment of CHIF to the corresponding γ residue (A).[48] Helical wheel projection shows that the 3 CHIF residues identified to be important for the stability of the CHIF-α/β complex face 2 different planes of the membrane (ie, they are likely to interact with at least 2 different helixes on α and/or β).

The opposite functional effect of γ and CHIF on apparent Na^+ affinity also is determined by the origin of the transmembrane segments. Namely, a CHIF construct whose transmembrane segment was replaced by that of γ decreased the Na^+ affinity similar to the effect seen for γ and vice versa.[48] On the other hand, exchanging the extracellular or C-terminal domains was without effect. However, different residues determine the functional effect and stability. Mutating ^{45}G into A and ^{55}MA into IL did not alter the effect of CHIF on the pump kinetics. The functional role of the transmembrane segments has been confirmed in a study showing that peptides corresponding to the transmembrane segment of γ reduce apparent Na^+ affinity of the α/β complex in Hela cell membranes, as found previously for full-length transfected γ.[34] Peptides with G41R or G41L substitutions did not mediate the effect, showing that ^{41}G is an important residue, perhaps being involved in the α/β-γ associations, whereas ^{35}G on the opposite face of the helix was not important.

Although not directly relevant to α/β-FXYD interactions, there is evidence that FXYD proteins can undergo self-oligomerization. Oligomers of both native γ[36] and peptides corresponding to the transmembrane segment[49] are detected in perfluoroctanoate (PFO) gels. Oligomerization is not observed for peptides with the G41R mutation but is retained in G35L mutants, suggesting that it depends on a specific inter-

action. The γ sequence LAFVVGLLILLS is similar to the sequence LAXXVGXXIGXXI known to mediate homo-oligomerization.[50] The functional role of oligomerization, if any, is unknown.

Where do FXYD proteins interact with the α/β complex? Direct structural information on Na,K-ATPase–FXYD interactions is as meager as structural information on the Na,K-ATPase itself. Cryoelectron microscopy of renal Na,K-ATPase at 9 to 10 Å resolution identified electron densities assumed to correspond to transmembrane helices of α, β, and γ subunits.[51] Based on analogy of 10 α subunit helices to Ca-ATPase helices, additional electron densities were assigned to transmembrane helices of β and γ subunits. The γ subunit helix was proposed to lie in a groove bounded by M2, M4, M6, and M9 of the α subunit. This is analogous to the proposed location of phospholamban in the Ca-ATPase.[52]

A denaturation study suggested that γ might interact in the M8-M10 region.[53] Recently, a role for M9 of the α subunit has been inferred from effects of mutants in M9 on stability of α/β-γ, CHIF, or FXYD 7 complexes, and their functional consequences, studied in *Xenopus* oocytes.[54] L964 and F967 were important for stability of the complexes, whereas F956 and E960 were required for mediation of effects of the FXYD protein on K^+ affinity. Thus, stabilizing and functional interactions were separable as found also in mutational studies in CHIF.[48] Interestingly, the F956 and E960 mutations did not alter effects of γ and CHIF on Na^+ affinity, implying that still other interactions mediate these effects. Modeling of the FXYD helix is consistent with docking in the M9, M2, M4, and M6 groove.

Direct evidence for structural interactions has been obtained by covalent cross-linking. Specific cross-links between γ and α subunits of renal Na,K-ATPase have been located to residues in the cytoplasmic tail of γ and a short cytoplasmic sequence following M4 of the α subunit (S4) ([55] and Fusezi et al, unpublished data). The interaction at the cytoplasmic surface appears to mediate the conformational effect of γ, which raises ATP affinity, and also is consistent with the location of the transmembrane segment in the M9, M6, M4, and M2 groove. Specific β-γ cross-links also have been observed in extracellular sequences of β and γ. In colonic membranes, α-CHIF and β-CHIF cross-links also can be detected. Thus, a similar disposition of γ and CHIF with respect to the α/β complex is likely.

By combining data from different approaches, structural modeling of FXYD proteins with homology models of the α subunit should be possible. This will be facilitated by the determination of the structure of FXYD proteins by nuclear magnetic resonance (NMR) methods, which appears to be within sight.[56] Eventually, it must be hoped, the molecular structure of Na,K-ATPase with FXYD proteins will become available.

Do FXYD Proteins Have Additional Functions?

Most FXYD proteins were identified because of properties that seemingly are unrelated to the Na,K-ATPase. Thus, PLM was purified as the major phosphoprotein in myocardium,[57] which mediates or regulates Cl^- and osmolytes transport.[58,59] FXYD3 (Mat-8) has been cloned as an oncogene-induced mammary protein with PLM-like Cl^- channel activity.[60,61] CHIF and γ too were found to manifest ionic channel activity in expression systems.[62-64] γ evokes a large nonspecific conductance that allows permeation even of inulin, whereas the CHIF-induced conductance is K^+ specific and may reflect activation of KCNQ1 channels.[62,65] FXYD5 (Related to Ion Channels, RIC, or dysadherin) has been cloned as E2a-Pbx1–induced messenger RNA[66] and was shown to down-regulate E-cadherin and promote metastasis.[67] These observations increase the possibility that FXYD proteins perform other cellular functions in addition to modulation of the pump kinetics and, in particular, that they modulate other ion transporters.

It also has been shown that overexpressing PLM in cultured rat myocytes altered the contraction and Ca^{2+} transient amplitudes in a manner observed after myocardial infarction.[68-70] Opposite effects were evoked when the PLM expression was suppressed by using antisense sequences. Evidence was provided that these effects are likely to reflect direct regulation of the Na^+/Ca^{2+} exchanger by PLM rather than changes in cell Na^+ caused by modulating the Na,K-ATPase.

The evidence that γa and γb are expressed in TALH cells as separate α/β/γa and α/β/γb complexes, and yet γa and γb do not show remarkable differences in their functional effects,[11,13] imply the possibility of separate roles in the cellular setting. Recently it has been reported that γa is detected without γb in preparations of rat renal caveolar membrane.[71] Because caveoale are the site of many signaling pathways, and a ouabain-dependent signaling pathway,[5] leading to activation of mitogen-activated protein kinase and cellular hypertrophy, and γ has recently been located in caveoale membranes,[72] the possibility exists that γa is involved in a signaling role. Recall that γ subunit was identified with a ouabain affinity label.[35] As an even more striking example, γ and CHIF recently have been detected within the same cells of native IMCD.[26] Because γ and CHIF have opposite effects on the Na^+ affinity but are not found in mixed complexes α/β/γ/CHIF,[73] this finding clearly raises the issue of whether there are additional roles for γ or CHIF. Alternatively, this may suggest a regulatory mechanism by which some post-translational modification will determine interaction of α with either γ or CHIF.

In summary, accumulating evidence suggest functional effects of FXYD proteins other than modulation of the Na,K-pump kinetics. Some of them might be nonphysiological responses caused by overexpression. However, it is not implausible that some FXYD proteins could have dual functions and interact with more than one target protein to produce synergistic physiologic effects. For example, PLM could affect both the cardiac Na,K-pump and Na^+/Ca^{2+} exchanger in a way that, depending on its state of phosphorylation, promotes either Ca^{2+} accumulation and cardiac contractility or Ca^{2+} extrusion and cardiac relaxation. CHIF might function to activate both the Na,K-ATPase and basolateral K^+

channels. Clearly, further investigation is required to establish or exclude these effects of FXYD proteins.

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