Structural and functional interactions between FXYD5 and the Na\(^+\)-K\(^+\)-ATPase

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Lubarski I, Karlish SJ, Garty H. Structural and functional interactions between FXYD5 and the Na\(^+\)-K\(^+\)-ATPase. Am J Physiol Renal Physiol 293: F1818–F1826, 2007. First published September 19, 2007; doi:10.1152/ajprenal.00367.2007.—FXYD5 is a member of a family of tissue-specific regulators of the Na\(^+\)-K\(^+\)-ATPase expressed in kidney tubules. Previously, we have shown that FXYD5 interacts with the \(\alpha\)-subunits of the Na\(^+\)-K\(^+\)-ATPase and increases its \(V_{\text{max}}\) (Lubarski I, Pihakas-Kaunasb K, Karlish SJ, Maunsbach AB, Garty H. J Biol Chem 280: 37717–37724, 2005). The current study further characterizes structural interaction and structure-function relationships of FXYD5. FXYD5/FXYD4 chimeras expressed in Xenopus laevis oocytes have been used to demonstrate that both the high-affinity association with the pump and the increase in \(V_{\text{max}}\) are mediated by the transmembrane domain of FXYD5. Several amino acids that participate in the high-affinity interaction between FXYD5 and the \(\alpha\)-subunit of the Na\(^+\)-K\(^+\)-ATPase have been identified. The data suggest that different FXYD proteins interact similarly with the Na\(^+\)-K\(^+\)-ATPase and their transmembrane domains play a key role in both the structural interactions and functional effects. Other experiments have identified at least one splice variant of FXYD5 with 10 additional amino acids at the COOH terminus, suggesting the possibility of other functional effects not mediated by the transmembrane domain. FXYD5 could be specifically bound to wheat germ agglutinin beads, indicating that it is glycosylated. However, unlike previous findings in metastatic cells, such glycosylation does not evoke a large increase in the size of the protein expressed in native epithelia and X. laevis oocytes.

RIC; FXYD; glycosylation; sodium pump

FXYD IS A FAMILY OF single-span transmembrane proteins named after an invariant extracellular motif (18, 38). The seven family members specifically interact with the Na\(^+\)-K\(^+\)-ATPase and modulate its kinetic properties. The tissue distribution and kinetic effects of each FXYD protein are different. Therefore, it is generally assumed that FXYD proteins are tissue-specific auxiliary subunits or regulators of the Na\(^+\)-K\(^+\)-ATPase, which adjust their kinetic properties to specific needs of the cells in which they are expressed, without affecting them elsewhere (14, 18, 19). In particular, the kidney collecting duct was shown to express three different FXYD proteins. These are FXYD2 (the \(\gamma\)-subunit of Na\(^+\)-K\(^+\)-ATPase), FXYD4 (CHIF), and FXYD5 (RIC). They exhibit specificity to intercalated vs. principle cells (28, 30, 33), respond to stimuli such as osmotic stress and mineralocorticoid levels (2, 5, 12, 13), and have different effects on pump kinetics (3, 4, 6, 7, 17, 26, 28, 31, 39). Their differential expression and functions should provide a flexible mechanism to enable the Na\(^+\) pump in the kidney to respond efficiently to a variety of extracellular stimuli and different physiological conditions (18).

Accumulating data suggest additional roles for some FXYD proteins, and it is possible that they also regulate other transporters (18). In particular, data have been accumulated that FXYD1 (phospholemman) also modulates activity of the cardiac Na\(^+\)/Ca\(^2+\) exchanger (1, 37, 42, 43). FXYD proteins are type I membrane proteins with an intracellular COOH terminus, a single transmembrane domain, and an extracellular NH\(_2\) terminal that may have a signal peptide. Usually, the extracellular NH\(_2\) terminal is shorter than 40 amino acids. The only exception is FXYD5, which has an atypically long extracellular domain of more than 140 amino acids.

Previously, we have studied the biochemical and functional properties of FXYD5 (28). Using specific antibodies, the cellular and tissue distribution of FXYD5 as well as its interaction with the Na\(^+\)-K\(^+\)-ATPase were elucidated. FXYD5 was found to be a ~24-kDa protein that is particularly expressed in the basolateral membrane of epithelial cells in the kidney, intestine, and lung. It is specifically immunoprecipitated by antibodies to the \(\alpha\)-subunit of the Na\(^+\)-K\(^+\)-ATPase and vice versa. Coexpressing FXYD5 with the Na\(^+\)-K\(^+\)-ATPase in Xenopus laevis oocytes elicits more than a twofold increase in the \(V_{\text{max}}\) of the pump, without affecting the \(K_{0.5}\) for external K\(^+\) (28). Other studies, however, have identified FXYD5 as a 50- to 55-kDa highly glycosylated protein termed dysadherin, expressed in metastatic but not normal cells (20, 35, 40). Dysadherin was associated with downregulation of E-cadherin, increased cell motility, decreased aggregation, and metastasis (20, 34, 35).

The current study further characterizes properties of FXYD5 in normal tissue and expression systems and demonstrates that, as in other FXYD proteins, its transmembrane domain plays a key role in the structural and functional interactions with the Na\(^+\)-K\(^+\)-ATPase. Additional evidence indicates that in normal tissue this protein is not extensively glycosylated, although some glycosylation is apparent. Finally, at least one FXYD5 splice variant with 10 additional COOH-terminal residues has been identified.

EXPERIMENTAL PROCEDURES

cDNA clones. cDNAs of mouse FXYD5, rat \(\alpha\)-1-, and pig \(B\)-subunits of the Na\(^+\)-K\(^+\)-ATPase were described before (28). For coprecipitation experiments, the \(B\)-subunit was tagged in its NH\(_2\) terminus with 10 histidines. FXYD5 mutants in which residues A150, I160, and L161 were replaced by the corresponding FXYD4 residues (G, M, and A, respectively) and FXYD5/FXYD4 chimeras were expressed in kidney tubules.
prepared by standard recombinant DNA techniques using overlapping oligonucleotides. In these chimeras, the extracellular, transmembrane, and cytoplasmic segments of mouse FXYD5 were defined as M1-K144, R145-S163, and G164-R178, respectively. The extracellular domain of FXYD5 (M1-R145) was also subcloned into a PET28 vector upstream and in frame with a 6XHis tag and expressed in *Escherichia coli*. For expression in *X. laevis* oocytes, cDNAs were subcloned between 5'- and 3'-sequences of *X. laevis* β-globin in pGEM- or pBluescript-derived vectors (25). cRNAs were synthesized from linearized plasmids using T7 RNA polymerase. All constructs were verified by sequencing.

**Antibodies.** Polyclonal antibodies against COOH-terminal peptides of FXYD4 and FXYD5 were described before (17, 28). In addition, a monoclonal antibody was prepared against a GST-FXYD5 fusion protein expressed in *E. coli*. The antibody was found to react with an NH2-terminus epitope on FXYD5. An antibody to the NH2-terminus of the α1-subunit of Na+−K+−ATPase (6H) was kindly provided by Dr. M. J. Caplan (Yale University School of Medicine). An antibody to the β-subunit was described elsewhere (36). Monoclonal anti-hemagglutinin A (HA) antibody was purchased from Santa Cruz Biotechnology.

**Tissue and cell preparations.** Mice (ICR) were euthanized using CO2 gas, and various organs were excised and rinsed in ice-cold HEPES buffer composed of 250 mM sucrose, 25 mM histidine, 1 mM EDTA, pH 7.2, and a cocktail of protease inhibitors (1 mM PMSF, 10 μg/ml aprotinin, 10 μg/ml leupeptin, 2 μg/ml pepstatin A). They were cut into small pieces and homogenized using a Polytron PT 2100 (4 × 6-s pulses at setting 31, Kinematica Switzerland). Tissue homogenates were sedimented for 15 min at 4000 g at 4°C to remove cell debris. The supernatants were further centrifuged for 90 min at 20,000 g at 4°C. The supernatants (cytosol) were saved, and the pellets (membranes) were suspended in HEPES buffer + protease inhibitors. Protein content was determined by the method of Lowry.

HeLa cells cultured under standard conditions were transiently transfected with FXYD5 using polyfect (Qiagen) and assayed for protein expression after reaching confluency. Na+−K+−ATPase was purified from pig kidney medulla as described elsewhere (21). A cell suspension highly enriched in dendritic cells was obtained from spleens of C57BL/6 mice using monoclonal antibodies to CD11c according to the manufacturer’s protocol.

**RT-PCR.** RNA was isolated from mouse kidney medulla and cortex or dendritic cells using TRI Reagent (Molecular Research Center). Reverse transcription was performed with a Super-Script II Reverse Transcriptase kit for RT-PCR (Invitrogen) according to the manufacturer’s instructions using 1.0 μg of total RNA. Two sense primers denoted I and III were used (Table 1). The first corresponds to a coding sequence of mouse FXYD5 upstream the FXYD motif, and the second to a 5'-FXYD5 sequence. Antisense primers (II and IV in Table 1) correspond to the 3'-sequences of different transcripts predicted by expressed sequence tag (EST) entries. PCR products were ligated into a pGEM-T Easy vector (Promega) and sequenced from the vector ends.

**Heterologous expression in *X. laevis* oocytes.** Batches of stage V-VI oocytes were injected with aliquots of 50 nl containing 10 ng rat α1 cRNA, 7 ng pig 10XHis-tagged β1 cRNA, and 3 ng cRNA transcribed from different FXYD5 constructs. The oocytes were incubated for 3 days at 20°C and used for assaying surface expression of the pump, coprecipitation of pump/FXYD complexes, and Na+−K+−ATPase activity. For coprecipitation assays, oocytes were homogenized in a glass teflon homogenizer in a buffer containing 10 mM HEPES, pH 7.9, 83 mM NaCl, 1 mM MgCl2, and protease inhibitor cocktail (1 mM PMSF, 20 μg/ml leupeptin, and 20 μg/ml pepstatin A). Homogenates were first centrifuged twice at 1,000 g for 10 min at 4°C for yolk removal and then at 10,000 g for 20 min to yield a microsomal pellet that contained ~90% of the heterologously expressed proteins. Membranes were stored at -80°C in 10 mM MOPS-Tris (pH 7.2), 1 mM EDTA, 25% glycerol, and protease inhibitor cocktail. Protocols of all experiments involving animals were approved by the Weizmann Institute Institutional Animal Care and Use Committee.

**Coprecipitation of FXYD5 proteins and the Na+−K+−ATPase.** Co-precipitation assays were done in oocytes expressing various FXYD constructs together with α1- and 10XHis-tagged β1. Unless otherwise indicated, membranes were first solubilized in a buffer containing 5 mM Tris, pH 7.6, 10 mM RbCl, and 1 mg/ml C12E10. The detergent-solubilized membranes were centrifuged for 30 min at 50,000 g, the supernatant was collected, and RbCl and imidazole were added to final concentrations of 100 and 20 mM, respectively. For coprecipitation, the detergent-solubilized membranes were incubated overnight under swirling at 4°C with Ni2+−NTA beads (10 μl beads/1 mg protein). Beads were sedimented and washed three times in solubilization buffer containing 1 mg/ml C12E10, 20 mM imidazole, 5 mM Tris, pH 7.6, and 100 mM RbCl. Bound proteins were eluted with 250 mM imidazole and dissolved in SDS sample buffer. The eluted proteins and an aliquot of the total detergent-solubilized membranes were resolved on either 10 or 7.5% acrylamide Tris/tricine/SDS gels. Proteins were transferred to polyvinylidene difluoride membranes in CAPS buffer plus 10% methanol at 13 V for 90 min. The blots were blocked in 5% milk for 1 h at room temperature and cut into several pieces according to predicted sizes of the bands of interest. These were incubated overnight at 4°C with one of the following antibodies: anti-FXYD5 (either polyclonal or monoclonal,1:500), anti-FXYD4 (1:500), anti-α (1:1,000), anti-β (1:4,000), or anti-HA (1:1,000). Bound antibodies were visualized by ECL following binding with horseradish peroxidase-coupled goat anti-rabbit or goat anti-mouse IgG (1 h, room temperature, 1:5,000). Each observation was confirmed in at least three independent experiments.

**Biotinylation of surface-expressed proteins.** Groups of 15–20 oocytes were incubated under gentle rotation for 1 h at 4°C with 1 mM freshly made sulfo-NHS-SS-biotin (Pierce). Incubation was done in ND-94 medium composed of: 94 mM NaCl, 2 mM KCl, 1.8 mM CaCl2, 1 mM MgCl2, and 5 mM HEPES, pH 7.4. They were washed four times with cold ND94 and homogenized in a buffer composed of 20 mM Tris, 5 mM MgCl2, 5 mM Na2HPO4, 1 mM EDTA, 80 mM sucrose, and protease inhibitors (1 mM PMSF, 20 μg/ml leupeptin, and 20 μg/ml pepstatin A), pH 7.4. Homogenization was done on ice by passing oocytes six times through a 21-gauge needle, and another six times through a 27-gauge needle. Homogenates were centrifuged at 200 g for 5 min at 4°C to pellet the yolk, and the supernatants were then centrifuged for 20 min at 14,000 g at 4°C to separate membranes and cytosol. The membrane fractions were dissolved in TBS-Triton buffer, composed of 150 mM NaCl, 10 mM Tris, pH 7.5, and 1% Triton X-100, incubated on ice for 30 min, and centrifuged for 20 min at 14,000 g at 4°C. The Triton-solubilized membrane proteins obtained from 15–20 oocytes were incubated under gentle rotation for 1 h at 4°C with 50 μl streptavidin-agarose beads (Pierce). The beads were precipitated by brief centrifugation, washed three times with TBS-Triton buffer, suspended in SDS sample buffer, and heated for 5 min at 90°C to release the biotinylated proteins. These proteins were resolved electrophoretically and analyzed on Western blot as above.

**Lectin binding assay.** Purified pig kidney Na+−K+−ATPase and FXYD5-expressing oocytes were homogenized in 1% Triton X-100, 150 mM NaCl, and 50 mM HEPES (pH 7.4) with or without 200 mM

Table 1. Primers used to amplify FXYD5 isoforms

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Description</th>
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<tr>
<td>I</td>
<td>AGGATCGGAGCTCTGACGCG</td>
<td>Sense FXYD5 coding region</td>
</tr>
<tr>
<td>II</td>
<td>AGACAGATTGAGCTTAAAGCG</td>
<td>Antisense 3' of FXYD5</td>
</tr>
<tr>
<td>III</td>
<td>CTGCGAGGAGCTTGGACCGG</td>
<td>Sense 5' of FXYD5</td>
</tr>
<tr>
<td>IV</td>
<td>CAACAGAGGAGAGCAGCTG</td>
<td>Antisense Upstream exon 9</td>
</tr>
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The insoluble fraction was removed by centrifugation, and the soluble proteins were incubated with agaroase wheat germ agglutinin beads (Vector Laboratories) overnight at 4°C with rotation. Beads were washed three times with the incubation buffer and another three times with water. Immobilized proteins were then eluted with 100 mM acetic acid and analyzed by probing with anti-FXYD5 antibody.

Effects of FXYD proteins on the pump activity were measured as the initial rate of Na⁺K⁺-ATPase-mediated 86Rb⁺ fluxes in oocytes. Measurements were done in the presence of either 10 µM ouabain, which fully blocks the endogenous X. laevis pump but not the expressed rat Na⁺K⁺-ATPase, or 2 mM ouabain, which blocks both orthologs. Oocytes were first loaded with Na⁺ for 2 h by incubation in a K⁺-free medium composed of: 80 mM Na-gluconate, 0.82 mM MgCl₂, 0.41 mM CaCl₂, 10 mM NMDG-HEPES, pH 7.4, 5 mM BaCl₂, and 10 mM tetraethylammonium chloride. Na⁺-loaded oocytes were divided into two groups of seven to eight oocytes and incubated for an additional 15 min at 25°C in a solution containing 90 mM NaCl, 1 mM CaCl₂, 1 mM MgCl₂, 5 mM BaCl₂, 10 mM HEPES, pH 7.4, and either 10 µM or 2 mM ouabain. 86Rb⁺ uptake was then initiated by the addition of 5 mM KCl+5 µCi/ml 86RbCl. The uptake was stopped 12 min later by a 15-fold dilution and five washings in an ice-cold incubation solution containing 5 mM nonradioactive RbCl. Oocytes were then counted individually for 86Rb⁺ uptake. Statistical significance was determined by Student’s t-test.

RESULTS

FXYD5 splice variants. FXYD5 sequences deposited in public databases indicate the possible existence of at least two FXYD5 isoforms which differ in their COOH tails. The previously reported FXYD5 sequence is characterized by a very short cytoplasmic COOH-terminal sequence of only 15 amino acids (top sequence in Fig. 1A). At least 15 mouse EST entries from different cDNA libraries predict a 92-nucleotide deletion (codon and produce a longer protein with 10 additional COOH-terminal residues (middle sequence of Fig. 1A). Such an isoform, however, is not observed in publicly available human EST entries. RT-PCR has been used to confirm expression of this new variant. Primers I and II in Table 1 should amplify 396- and 307-bp products corresponding to the shorter and longer COOH-terminal proteins, respectively. Amplifying mouse kidney RNA resulted in a 396-bp species only (left arrow in Fig. 1B). Since some of the EST entries predicting the long form came from a dendritic cell library, we attempted to amplify this form also from dendritic cell RNA. In this case, a minor, smaller fragment was observed in addition to the predominant 396-bp species (right arrow in Fig. 1B). It was sequenced and found to correspond to the new variant with 10 extra COOH-terminal amino acids. The fact that this species was amplified from dendritic but not kidney RNA does not necessarily indicate differential expression of the two forms. It may be due to the large heterogeneity of the kidney and a much lower fraction of FXYD5-expressing cells, resulting in a lower abundance of this species in the total RNA preparation. Since none of the EST entries predicting the new species contains the whole coding region, it is in principle possible that this isoform differs also in its NH₂ terminus. To test for this possibility, RT-PCR of dendritic cells was repeated using primer III, corresponding to a sequence that is upstream of the AUG start codon of FXYD5. The product sequence showed no NH₂-terminal differences and confirmed that the additional COOH-terminal residues are the only difference between the two isoforms.

An FXYD5 protein with an entirely different COOH terminus is reported in Swissprot (P97808-1, bottom sequence in Fig. 1A). This sequence is predicted by a single mouse EST entry (BC031112). We indeed could amplify this species from dendritic cell RNA using primers I and IV (asterisk in Fig. 1B). These primers amplified a very low abundance 413-bp product with a sequence identical to that of BC031112 and P97808-1. Aligning BC031112 with the genomic sequence of FXYD5 indicates that this species is likely to represent an incompletely spliced mRNA, in which the intron between exons 8 and 9 is still present, and not necessarily a different protein.

Monoclonal anti-FXYD5 antibody. Previous studies by Hirohashi and coworkers (20, 34, 35, 40) have suggested that FXYD5 is a heavily O-glycosylated protein of 50–55 kDa that expresses specifically in tumor cells. On the other hand, we have observed that FXYD5 is abundantly expressed in several native tissues as a 24-kDa polypeptide, in agreement with its calculated molecular weight (28). Arystarkhova et al. (5) have recently reported that an anti-FXYD5 antibody labels in HEK293 and HeLa cells both 17- to 20-kDa and 28- to 35-kDa polypeptides, while in LLC-PK1 cells a 50-kDa protein was detected. They suggested that these correspond to core and glycosylated forms of FXYD5. Thus the glycosylation state and size of FXYD5 in native tissue may require additional corroboration by a second antibody. Therefore, a monoclonal antibody against recombinant FXYD5 has been raised. To confirm its specificity and identify the protein region interacting with this antibody, we have constructed various FXYD5/FXYD4 chimeras, expressed them in X. laevis oocytes, and probed them with different antibodies. All FXYD constructs were successfully expressed as evidenced by their labeling by polyclonal antibodies directed to the COOH-terminal sequences of either FXYD4 or FXYD5 (Fig. 2A). The size of the expressed proteins was either 24 or 7 kDa, depending on the origin of the ecto domain (i.e., FXYD4 or FXYD5). Blotting the same preparations with the new monoclonal anti-FXYD5
antibody demonstrates that this antibody is directed to an extracellular NH2-terminal sequence (Fig. 2B). It recognizes all FXYD5/FXYD4 chimeras, of which the NH2-terminal sequences originate from FXYD5 (i.e., RRC, RCC, and RCR), but none of the other chimeras. The fact that the monoclonal antibody recognizes an extracellular epitope was further confirmed by Western blotting of lysates from *Escherichia coli* transformed with the extracellular domain of FXYD5. In this case, the antibody recognized an 20-kDa band in isopropyl-β-D-thiogalactoside-stimulated but not in nonstimulated cells (Fig. 2C).

The new antibody interacts more weakly with FXYD5 than the previously described polyclonal anti-COOH tail antibody (cf. Fig. 2A vs. 2B). However, since it recognizes an extracellular epitope it should cross-react with all three isoforms depicted in Fig. 1A. Another observation in these experiments was that the coexpression of FXYD5 with FXYD4 chimeras tends to reduce glycosylation of the β-subunit of the Na+–K+–ATPase. This is apparent from the increased electrophoretic mobility compared with the fully glycosylated form of β and is particularly notable when FXYD constructs with extracellular and transmembrane segments originating from FXYD5 are coexpressed with FXYD5 and some FXYD4 chimeras (Fig. 3).

Tissue distribution and glycosylation of FXYD5. Next, we tested expression of FXYD5 in different tissues using the new antibody. The antibody labeled an ~24-kDa polypeptide with electrophoretic mobility and tissue distribution similar to those reported by us before with the polyclonal antibody (28) (Fig. 4A). In some preparations, a faint high-molecular-weight species was observed as well. This could be either a dimeric form of FXYD5, a glycosylated species, or a nonrelated protein. The prominent 28- to 35-kDa band reported by Arystarkhova et al. (5) in HEK293 and HeLa cells was not visualized in native tissue or transfected cells (Fig. 4). The data confirm that FXYD5 is expressed in native membranes as a 24-kDa protein. It should also be noted that
since the antibody is directed to an extracellular epitope, it will cross-react with all the above-described splice variants.

While the above experiments show no major 50- to 55-kDa FXYD5 species in normal tissue nor in the X. laevis expression system, some O-glycosylation of this protein is likely due to the high abundance of S, T, and P residues and its weak homology to mucins. Glycosylation may also account for the difference between the apparent molecular weight of the protein (24 kDa) and the calculated value of 17 kDa (assuming cleavage of the signal peptide). FXYD5 lacks N-glycosylation sites, and its electrophoretic mobility is not affected by treatment with the peptide N-glycosidase. (28). Another way to test for the presence of sugar moieties is to assay for lectin binding. In this case, detergent-solubilized membrane proteins are incubated with lectins covalently attached to agarose beads in the presence and absence of competing sugars, and the beads are assayed for immobilized protein. Such an assay requires dissociation between FXYD5 and the Na\(^+\)-K\(^+\)-ATPase to avoid FXYD5 binding through the heavily glycosylated \(\beta\)-subunit of the pump. We therefore first tested for stability of the \(\alpha\beta\)/FXYD5 complex in various detergents. It was found that while FXYD5 effectively co precipitates with \(\alpha\beta\) when membranes are solubilized in \(C_{12}E_{10}\) (see Ref. 28 and data below), no such coprecipitation is apparent in Triton X-100 (Fig. 5A). Accordingly, lectin binding experiments were done in Triton X-100-solubilized membrane. Incubating Triton X-100-solubilized membranes from oocytes expressing \(\alpha\) and FXYD5 with agarose wheat germ agglutinin beads resulted in binding of FXYD5 to the beads (Fig. 5B). Such binding could be prevented by the presence of 200 mM \(N\)-acetylglucosamine (GlcNac), suggesting that it is mediated by a specific interaction of the protein sugar moieties with the beads. To test for glycosylation in native tissue, we have used purified pig kidney Na\(^+\)-K\(^+\)-ATPase prepared as described elsewhere (21). This preparation contains a considerable amount of FXYD5, although it is more enriched with \(\alpha\) (Fig. 5C). In this case, too, FXYD5 could be immobilized on lectin beads in the absence but not in the presence of GlcNac (Fig. 5D). Thus FXYD5 is indeed glycosylated, but this presumably O-glycosylation does not evoke a large increase in size.

**FXYD5-Na\(^+\)-K\(^+\)-ATPase interactions.** The above FXYD5/FXYD4 chimeras have also been used to identify FXYD5 domains participating in the interaction with the \(\alpha\beta\)-subunits of the Na\(^+\)-K\(^+\)-ATPase. It has been shown before that the detergent-solubilized \(\alpha\beta\)/FXYD5 complex is more stable than the corresponding \(\alpha\beta\)/FXYD4 oligomer, and efficient \(\alpha\)/FXYD5 coimmunoprecipitation is observed under more stringent conditions than those preserving interactions between \(\alpha\beta\) and FXYD4 (28). Thus comparing coprecipitation efficiencies of different FXYD4/FXYD5 chimeras with \(\alpha\beta\) should identify the FXYD5 domain responsible for the extra stability of the complex in detergent. Accordingly, FXYD4 and FXYD4/FXYD5 chimeras were expressed in X. laevis oocytes together with \(\alpha\) and 10XHis-tagged \(\beta\). Microsomes were extracted and dissolved in \(C_{12}E_{10}\), and pump complexes were isolated using Ni-NTA beads. These were assayed for the relative amounts of \(\alpha\), \(\beta\), and FXYD on Western blots. The data summarized in Fig. 6A demonstrate that the high coprecipitation efficiency of FXYD5 is determined by its transmembrane domain. Thus chimeras with transmembrane domains originating from FXYD5 (RRC and CRC) coprecipitate with \(\alpha\beta\) at high efficiency, irrespective of the origin of the NH\(_2\)-terminus.\(^1\) A similar result was obtained before by comparing coimmunoprecipitation efficiencies of \(\alpha\beta\)/FXYD4 and \(\alpha\beta\)/FXYD2 (26).

In this case, it was established that the extra stability of \(\alpha\beta\)/FXYD2 is due to three transmembrane residues. In FXYD4, these residues are G41, M55, and A56. Mutating them to the corresponding FXYD2 residues (A, I, and L) resulted in

\(^1\) This experiment was limited to chimeras whose COOH-terminal sequence originates from FXYD4 to assay all preparations with the same antibody.
an αβ/FXYD4 complex with stability comparable to that of αβ/FXYD2. In FXYD5, the equivalent positions are A150, I160, and L161, i.e., the same amino acids found in FXYD2. Accordingly, we have mutated them to the FXYD4 residues (A150G, I160M, and L161A) and assayed for effects on complex stability. As seen in Fig. 6B, a FXYD5 construct carrying these mutations alone or in combination could still be precipitated by αβ, but the coprecipitation efficiency was

Fig. 5. Binding of FXYD5 to wheat germ agglutinin. A: X. laevis oocytes were injected with cRNA mixtures coding for α, 10XHis-tagged β-Na⁺-K⁺-ATPase, and FXYD5. Microsomes were extracted 3 days later, dissolved in a buffer containing 1% Triton-X-100, and proteins associated with the 10Xhis tag were precipitated using Ni-NTA beads. Five percent of the total Triton X-100-soluble fraction (tot.) and the whole volume of pulled down proteins (pd) were resolved electrophoretically and transferred to a blotting membrane. The membrane was cut into high-, medium-, and low-MW segments that were blotted with antibodies to α, β, and FXYD5. B: Triton-solubilized oocyte membranes were incubated with agaroase wheat germ agglutinin beads with and without 200 mM GlcNac as described in EXPERIMENTAL PROCEDURES. Ten percent of the total detergent-solubilized fraction (total) and the whole volume of proteins eluted from the beads (bound) were resolved electrophoretically, transferred to a blotting membrane, and blotted with anti-FXYD5 antibody. C: pig kidney microsomes (Mic.) and purified Na⁺-K⁺-ATPase (Pur.) were resolved electrophoretically, transferred to a blotting membrane, and the membrane was cut to high- and low-MW regions and blotted with antibodies to α and FXYD5, respectively. D: purified Na⁺-K⁺-ATPase was dissolved in 1% Triton X-100 and incubated with agaroase wheat germ agglutinin beads with and without 200 mM N-acetylglucosamine. Lectin-bound proteins were resolved electrophoretically, transferred to a blotting membrane, and blotted with anti-COOH-terminal FXYD5 antibody.

Fig. 6. Coprecipitation of αβ with FXYD constructs. X. laevis oocytes were injected with cRNA mixtures coding for α, 10XHis-tagged β-Na⁺-K⁺-ATPase, and either FXYD4 or FXYD4/FXYD5 chimeras (A) or FXYD5 and various mutants (B). One group of oocytes was injected with FXYD5 but no αβ (Con). Microsomes were extracted 3 days later and solubilized in 3 mg/ml C₁₂E₁₀. Pump complexes were precipitated using Ni-NTA beads. The pulled down proteins (pd) and 5% of the total C₁₂E₁₀-solubilized proteins (tot.) were resolved electrophoretically, transferred to a blotting membrane, cut to low-, medium-, and high-MW segments, and assayed by blotting with antibodies to α, β, and either FXYD4 (A) or FXYD5 (B).
considerably lower than that of the wild-type. Thus the structural interactions between FXYD5 and the Na\(^+\)-K\(^+\)-ATPase appear to involve the same membrane domains and residues identified in FXYD2.

Structure-function relationships of FXYD5. FXYD4/FXYD5 chimeras have also been used to identify the domain(s) involved in the functional effects of FXYD5. Previously, we have shown that expressing FXYD5 in *X. laevis* oocytes together with αβ increases the \(V_{\text{max}}\) of the pump, an effect not seen for FXYD4 (28). This is further demonstrated in Fig. 7A, which depicts the initial rates of \(^{86}\text{Rb}^+\) uptake measured in the presence of 10 μM or 2 mM ouabain in oocytes expressing Na\(^+\)-K\(^+\)-ATPase and different FXYD proteins. Since under the experimental conditions intracellular Na\(^+\) and extracellular K\(^+\) are much higher than the pump \(K_{1/2}\) values for these ions, changes in the initial rate of ouabain-sensitive \(^{86}\text{Rb}^+\) uptake reflect changes in \(V_{\text{max}}\). Figure 7A also demonstrates that a chimera in which the transmembrane domain originates from FXYD5 and the extracellular and intracellular domains come from FXYD4 has a higher ouabain-sensitive \(^{86}\text{Rb}^+\) uptake, like that of FXYD5 itself. This is further demonstrated in Fig. 7B, which compares pump activities in oocytes injected with a chimera in which the extracellular, transmembrane, and intracellular domains of FXYD4 were replaced by the corresponding FXYD5 domain. Again, the transmembrane domain of FXYD5 evokes the increase in pump \(V_{\text{max}}\), while the cytoplasmic or extracellular domains have no effect. Interpretation of these data depends, however, on the assumption that the different FXYD constructs have no effect on the pump surface expression so that a change in the pump-mediated \(^{86}\text{Rb}^+\) uptake does not stem from a change in the number of surface-expressed pump units. To confirm this assumption, we have determined surface expression of αβ by biotinylating the surface of intact oocytes and quantifying the amount of extracted α and β proteins that are immobilized on streptavidin beads. As demonstrated at the bottom of Fig. 7B, the surface expression of Na\(^+\)-K\(^+\)-ATPase was not affected by the coexpression of various FXYD proteins and the amount of α and β bound to streptavidin were similar or even lower in oocytes expressing CRC. Thus FXYD5 does indeed increase \(V_{\text{max}}\), and the effect is mediated by its transmembrane domain.

DISCUSSION

The current study provides further characterization of FXYD5 in native tissue and expression systems. Using a monoclonal antibody to an extracellular epitope, we confirmed that in normal tissue FXYD5 exists as a ~24-kDa polypeptide that is particularly expressed in the spleen, lung, kidney, and heart. These findings are different from previous reports indicating that in metastatic cells FXYD5 is a heavily O-glycosylated 50- to 55-kDa polypeptide (20, 34, 35, 40). In principle, it is possible that the monoclonal antibody used in these studies detects a 50- to 55-kDa cancer-related glycoprotein that is different from FXYD5. However, some of the data reported were obtained using small interfering RNA, making a strong link between the FXYD5-related sequence and the 50- to 55-kDa protein (35). A possible explanation for these differences is that FXYD5 has different degrees of O-glycosylation in normal and cancer cells. It is well known that transformation to malignancy is associated with a change in O-glycosylation.

![Fig. 7. Effects of FXYD5 and FXYD5/FXYD4 chimeras on Na\(^+\)-K\(^+\)-ATPase activity. Na\(^+\)/K\(^+\)-ATPase activity was measured as ouabain-sensitive \(^{86}\text{Rb}^+\) uptake in Na\(^+\)-loaded X. laevis oocytes, as described in EXPERIMENTAL PROCEDURES. A: \(^{86}\text{Rb}^+\) uptake was measured in groups of oocytes expressing the α- and β-subunits of the pump with and without different FXYD constructs. Measurements were done in the presence of 2 mM ouabain (open bars) or 10 μM (shaded bars) ouabain. Means ± SE of values (by Student’s t-test) obtained in 7–8 oocytes are depicted. The flux mediated by the pump is shown in filled bars. B: ouabain-sensitive fluxes in oocytes injected with αβ with and without different FXYD constructs are shown. The figure depicts means ± SE of the pump-mediated fluxes from 3–5 experiments (numbers shown in brackets) using 7–8 oocytes in each experiment for each condition. Data are expressed as percentage of the pump activity in oocytes injected with αβ alone. In FXYD5 and CRC, the flux measured was significantly higher than the control (*P < 0.01). Bottom: representative Western blot comparing the amount of surface biotinylated α and β in oocytes injected with different FXYD constructs.](http://ajprenal.physiology.org/Downloadedfrom://ajprenal.org)
of adhesion molecules and mucins, in particular (e.g., Refs. 9–11 and 27). Also, the excessive glycosylation of mucins is known to block binding to adhesion proteins and hence, down-regulates E-cadherin, as observed (20). The possibility that transformation causes FXYD5 glycosylation is also consistent with the observation of high-molecular-weight bands in cultured (transformed) cells (5). Some glycosylation of the 24-kDa species is apparent since this protein specifically associates with wheat germ agglutinin beads. Previously, we have shown that treating FXYD5-containing membranes with peptide N-glycosidase has no effect on the electrophoretic mobility of this protein (28). In addition, the ecto domain of FXYD5 lacks consensus N-glycosylation sites. Taken together with the fact that 50 of the 122 extracellular residues of FXYD5 are serines, threonines, and prolines, it is likely that some of these residues are O-glycosylated. Such glycosylation may account at least for part of the difference between the apparent molecular weight of 24 kDa and the calculated value of 17 kDa (assuming cleavage of the signal peptide).

FXYD5/FXYD4 chimeras expressed in X. laevis oocytes have been used to study structure-function relationships and structural interactions between FXYD5 and the αβ-subunits of Na^+-K^+-ATPase. Differences in stabilities of αβ/FXYD5 and αβ/FXYD4 complexes in detergent have been used to identify domains and residues involved in the above interactions. Transmembrane interactions were found to play a key role in the association of FXYD5 with αβ and determine stability of the αβ/FXYD5 complex in detergent. It was further demonstrated that three transmembrane residues are particularly important for the physical interaction of FXYD5 with the pump. Taken together with previous data on other FXYD proteins (23, 24, 26), it appears that different FXYD proteins interact similarly with the Na^+-K^+-ATPase and their different functional effects are likely to stem from a small number of key residues.

It was unexpected but of interest that coexpression with FXYD5 decreases to some extent glycosylation of the β-subunit. The somewhat lower broadband seen under these conditions is similar to that observed following sialidase treatment (data not shown). Such an effect may indicate higher endoplasmic reticulum retention of the β-subunit when co-expressed with FXYD5. However, experiments determining the amount of surface biotinylated β seem to argue against such a possibility. Another possibility is a direct FXYD5-β interaction, which hinders glycosylation to some extent. FXYD5 might interfere with addition of sialic acid residues at all sites or interfere with glycosylation at one or other of the three sites. A direct FXYD5-β interaction would fit well with our previous finding that both FXYD2 and FXYD4 can be specifically cross-linked to the extracellular domain of β (16). Interference with glycosylation of the β-subunit might also explain neatly the original findings of Hirohashi and coworkers (20, 35). Epithelial cell-cell adhesion and cell polarity are known to require E-cadherin (29), and the β-subunit of the Na^+-K^+-ATPase is also known to be essential for E-cadherin-mediated cell-cell adhesion and cell polarity, and also suppression of invasiveness of cancer cells (32). It has been shown recently that normal glycosylation of the β-subunit is essential for production of the stable association of the pump with the adherens junction and plays an important role in cell-cell contact formation (41). Thus interference of FXYD5 with glycosylation of the β-subunit might destabilize its interactions with E-cadherin and the adherens junction and thus account for the lowered expression of E-cadherin, decreased cell-cell contact, increased motility, and metastasis. A reduction in β glycosylation has also been observed for FXYD3, and in this case the effect was linked to the signal peptide of the protein (15).

As before (28), we found that coexpression of FXYD5 with the Na^+-K^+-ATPase increases the V_max of the pump by about twofold. Other potential kinetic effects of this FXYD protein, e.g., on the affinity for cell Na^+ or ATP, have not been tested as yet. In principle, the increase in V_max may stem from a higher cell surface expression of αβ. This possibility was, however, excluded by quantifying the amount of surface biotinylated pump units in intact oocytes. It is therefore apparent that FXYD5 increases the turnover rate of the pump. Such an effect can be the result of an increase in the rate of one of the rate-limiting conformational changes, i.e., E1P ↔ E2P or E2(K)ATP ↔ E1ATP. Such effects are usually accompanied by changes in the apparent affinity to ligands. However, FXYD5 was found to have no effect on K_1/2 to external K^+, and effects on affinities to Na^+ and ATP cannot be readily measured in the current system. It was further demonstrated that the increase in V_max is mediated by the transmembrane domain of FXYD5. This is similar to the influence of the transmembrane domains of FXYD2 and 4 on the apparent affinity to cell Na^+ (23, 26, 44). On the other hand, the effect on the pump’s affinity to ATP involves the COOH-terminal domain of FXYD2 (39).

Finally, we have identified at least one FXYD5 splice variant with 10 extra residues in the COOH tail of the protein. Functional effects of these residues have not been tested, because the increase in V_max is evoked by the transmembrane helix. However, it is possible that the new variant affects affinity to ATP. FXYD5 splice variants have been reported also for FXYD2 and FXYD3 (8, 22). In both cases, differential expression of the two isoforms was observed, and in the case of FXYD3 different functional effects were noted as well.

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GRANTS

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REFERENCES


