Role of the Transmembrane Domain of FXYD7 in Structural and Functional Interactions with Na,K-ATPase*

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Members of the FXYD family are tissue-specific regulators of the Na,K-ATPase. Here, we have investigated the contribution of amino acids in the transmembrane (TM) domain of FXYD7 to the interaction with Na,K-ATPase. Twenty amino acids of the TM domain were replaced individually by tryptophan, and combined mutations and alanine insertion mutants were constructed. Wild type and mutant FXYD7 were expressed in Xenopus oocytes with Na,K-ATPase. Mutational effects on the stable association with Na,K-ATPase and on the functional regulation of Na,K-ATPase were determined by co-immunoprecipitation and two-electrode voltage clamp techniques, respectively. Most residues important for the structural and functional interaction of FXYD7 are clustered in a face of the TM helix containing the two conserved glycine residues, but others are scattered over two-thirds of the FXYD7 TM helix. Ile-35, Ile-43, and Ile-44 are only involved in the stable association with Na,K-ATPase. Glu-26, Met-30, and Ile-44 are important for the functional effect and/or the efficient association of FXYD7 with Na,K-ATPase, consistent with the prediction that these amino acids contact TM domain 9 of the α subunit (Li, C., Grosdidier, A., Crambert, G., Horisberger, J.-D., Michielin, O., and Geering, K. (2004) J. Biol. Chem. 279, 38895–38902). Several amino acids that are not implicated in the efficient association of FXYD7 with Na,K-ATPase are specifically involved in the functional effect of FXYD7. Leu-32 and Phe-37 influence the apparent affinity for internal Na+ and extracellular K+ of Na,K-ATPase, probably by influencing the rebinding or release of Na+ to or from the extracellular medium (4).

In previous studies (5), we have shown that FXYD7 together with FXYD2 and FXYD4 are located in close vicinity of transmembrane (TM) 9 of the α subunit of Na,K-ATPase. In particular, Leu-964 and Phe-967 in TM9 contribute to the stable association, whereas Phe-956 and Glu-960 are involved in the transmission of the functional effect of FXYD proteins to Na,K-ATPase. Moreover, mutational analysis has revealed that the TM domain of FXYD7 plays a role in the structural and functional interaction of FXYD7 (6). Similar conclusions were drawn for FXYD2 and FXYD4 (7–9).

To better understand the functional importance of the TM domain of FXYD proteins and the individual contribution of each of the twenty amino acid residues, we have in the present study replaced the amino acids of the TM domain of FXYD7 by tryptophan individually or in combination and have investigated their roles in the structural and functional association with Na,K-ATPase.

MATERIALS AND METHODS

Site-directed Mutagenesis—Point mutations and alanine were introduced into the TM domain of mouse FXYD7 (4) by the PCR-based method as described by Nelson and Long (10). The insert of FXYD7 was subcloned into a pSD5 vector using EcoRI/NotI restriction sites. The nucleotide sequences of all constructs were confirmed by dideoxy functions of various tissues. In renal epithelial cells, Na,K-ATPase is exclusively expressed at the basolateral membrane and becomes the driving force for transepithelial Na+ reabsorption. In nervous tissue, Na,K-ATPase restores the Na+ and K+ gradients during action potentials and thus ensures neuronal excitability. In skeletal and heart muscle, the Na+ gradient coupled to Na+/Ca2+ exchanger activity controls the intracellular Ca2+ concentration, which influences muscle contractility.

The activity of Na,K-ATPase is subjected to short- and long-term regulation (1) mediated by the intracellular Na+ concentration, neurotransmitters, and hormones. Recently, members of the FXYD family (2) have been shown to be tissue-specific regulators of the Na,K-ATPase (3).

FXYD proteins are small membrane proteins that are characterized by an N-terminal FXYD motif, two highly conserved glycine residues in the TM domain, and a serine residue at the cytoplasmic end of the TM domain. FXYD7, an O-glycosylated protein, is only expressed in brain, in both neurons and glial cells (4). FXYD7 associates with αβ1 isoforms, but not with αβ2 isoforms, when expressed in Xenopus oocytes. Stable association of FXYD7 with Na,K-ATPase modifies its apparent affinity for external K+ by increasing by ~2-fold the Ki1/2 value for K+ over a large range of membrane potentials in the presence and absence of external Na+.

In addition, FXYD7 modifies the apparent affinity for extracellular Na+ of Na,K-ATPase, probably by influencing the rebinding or release of Na+ to or from the extracellular medium (4).

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sequencing. cDNAs for rat Na,K-ATPase α1 and β1 subunits were kindly provided by J. Lingrel.

**Protein Expression in Xenopus Oocytes**—cRNAs were obtained by in vitro transcription (11). Stage V-VI Xenopus oocytes were obtained as described (12). Oocytes were injected with cRNAs coding for rat Na,K-ATPase α1 and β1 subunit (10 and 1 ng/oocyte) either alone or together with cRNAs for wild type or mutant mouse FXYD7 (2 ng/oocyte). To ensure that FXYD7 is expressed in excess over Na,K-ATPase for association and function analysis, we determined in preliminary experiments that maximal association of FXYD7 with Na,K-ATPase is achieved after injection of 1 ng of wild type FXYD7 cRNA (data not shown). Oocytes were incubated in modified Barth’s solution in the presence of 0.8–1 mM cold methionine or to 24- and 48-h chase periods. Digitonin extracts were prepared after the pulse and chase periods, and immunoprecipitation was performed in non-denaturing conditions using an anti-FXYD7 antibody. Immunoprecipitates were separated on Tricine-polyacrylamide gels. gFXYD7, O-glycosylated form of FXYD7; fFXYD7, non-glycosylated form of FXYD7.

![Figure 1](image1.png)

**FIGURE 1. Expression and processing of FXYD7 mutants.** **A**, representation of the twenty amino acids of the transmembrane domain of FXYD7 that were mutated into alanine residues. **B**, examples of the expression pattern of FXYD mutants. Xenopus oocytes were injected with wild type or mutant FXYD7 cRNAs (2 ng), metabolically labeled for 6 h with [35S]methionine, and subjected to 24- and 48-h chase periods. Digitin extracts were prepared after the pulse and chase periods, and immunoprecipitation was performed in denaturing conditions using an anti-FXYD7 antibody. Immunoprecipitates were separated on Tricine-polyacrylamide gels. gFXYD7, O-glycosylated form of FXYD7; fFXYD7, non-glycosylated form of FXYD7.

![Figure 2](image2.png)

**FIGURE 2. Efficiency of association of FXYD7 mutants with Na,K-ATPase.** **A**, examples of the association efficiency of FXYD7 mutants. Xenopus oocytes were injected with wild type or mutant FXYD7 cRNAs (2 ng) together with rat Na,K-ATPase α1 (10 ng) and β1 (1 ng) cRNAs, metabolically labeled for 24 h with [35S]methionine, and subjected to a 48-h chase period. Digitonin extracts were prepared after the pulse and chase periods, and immunoprecipitation was performed in non-denaturing conditions using an anti-FXYD7 antibody (A) or an anti-α1 antibody (B). Immunoprecipitates were separated on SDS-polyacrylamide gels. **B**, quantifications of the association efficiency of FXYD7 mutants with Na,K-ATPase. Data from experiments as shown in panels A and B were quantitated. Represented are the amounts of α subunit co-immunoprecipitated with an anti-FXYD7 antibody (A) or an anti-α1 antibody (B). The amount of α subunit co-immunoprecipitated with wild type FXYD7 after a 48-h pulse period was arbitrarily set to 1. Black bars, association of Na,K-ATPase α subunit with FXYD7 after a 48-h pulse. White bars, association of Na,K-ATPase α subunit with FXYD7 after a 48-h chase. Shown are means ± S.E. from three different experiments. *, p < 0.05.

**RESULTS**

Expression and Processing of FXYD7 TM Mutants—To analyze the role of the TM domain of FXYD7 in the structural and functional interactions with Na,K-ATPase, we have 1) inserted an alanine residue in three different positions (Fig. 1A, Ala1, Ala2, Ala3) and 2) replaced individually twenty amino acids of the TM domain by tryptophan (Fig. 1A). Alanine insertion into an α helix is believed to dislocate the N-terminal part of the helix by 100° with respect to the C-terminal part and consequently impede the structural and/or functional continuity of the helix (16). Tryptophan scanning has been used successfully to determine functionally important amino acids in integral membrane proteins (16–18). Mutants were expressed in Xenopus oocytes, metabolically labeled, and immunoprecipitated with an FXYD7 antibody. The expression level of all mutants was comparable with that of wild type FXYD7 (3 out of 23 mutants are shown in Fig. 1B).
FXYD7 is O-glycosylated on three threonine residues (4). After a 24-h chase period, wild type FXYD7 and Q26W, T27W, G29W, M30W, T31W, L32W, A33W, T34W, M36W, V38W, G40W, and I44W mutants were completely processed from a core protein, seen after a 6-h pulse, to the glycosylated form (wild type FXYD7 and the I44W mutant are shown as examples in Fig. 1A). On the other hand, Ala1, Ala2, Ala3, V28W, I35W, F37W, L39W, I41W, I42W, I43W, and L45W exhibited a delayed processing as reflected by the presence of the core protein after a 24-h chase (for examples see Fig. 1B). However, all mutants were fully processed after a 48-h chase period, indicating that they are not severely misfolded and can exit the endoplasmic reticulum.

Efficiency of Association of FXYD7 Mutants with Na,K-ATPase—FXYD7 mutants were co-expressed with Na,K-ATPase α and β subunits in Xenopus oocytes, and the efficiency of association was studied by co-immunoprecipitations under non-denaturing conditions using an FXYD7 antibody. Examples of such co-immunoprecipitations are shown in Fig. 2A. The association efficiency of all FXYD7 mutants was quantitated as the amount of co-immunoprecipitated subunit with FXYD7 after a 24-h pulse period. Data shown are means ± S.E. of 2–8 different batches. *, p < 0.05;

The association efficiency of wild type FXYD7 with Na,K-ATPase associated by using an anti-FXYD7 (A) or an anti-α antibody (B). Immunoprecipitates were separated on SDS-polyacrylamide gels. C, quantification of the association efficiency of wild type or mutant FXYD7 with Na,K-ATPase was carried out as in Fig. 2. Black bars, association of Na,K-ATPase subunit with FXYD7 after a 24-h pulse period. White bars, association of Na,K-ATPase α subunit with FXYD7 after a 48-h chase period. Data shown are means ± S.E. from three different experiments. D–F, effects of FXYD7 mutants on the apparent K⁺ affinity of Na,K-ATPase. The K₁/₂ values were determined as in Fig. 3. Closed squares, Na,K-ATPase with wild type FXYD7. Open squares, Na,K-ATPase with FXYD7 mutants (panel D, F37A; panel E, V28W/I42W; panel F, V28W/I42W/L32W). Shown data are means ± S.E. of 8–11 oocytes from 4 different batches. *, p < 0.001; #, p < 0.05.
intracellular domain are essential for correct association with Na,K-ATPase. In agreement with a role of the transmembrane domain, single mutations of Gln-26, Gly-29, Met-30, Ala-33, Ile-35, Gly-40, Ile-43, and Ile-44 significantly decreased the association efficiency with Na,K-ATPase after a 48-h chase period (Fig. 2C).

Functional Effects of FXYD7 Mutants on Na,K-ATPase—FXYD7 increases the $K_{1/2}$ value for extracellular $K^+$ of Na,K-ATPase by $\sim-2$ fold over a large range of membrane potentials (4). We first determined the $K_{1/2}$ values for $K^+$ at $-50$ mV of Na,K-ATPase either expressed alone or together with wild type FXYD7 (Fig. 3A). None of the mutations completely abolished the regulatory effect of FXYD7. Ala2, Ala3, Q26W, G29W, and G40W mutants produced a significantly smaller change in the $K_{1/2}$ value for $K^+$ of Na,K-ATPase compared with that produced by wild type FXYD7, possibly reflecting the lower association efficiency. In contrast, the L32W, A33W, and I42W mutants had significantly higher $K_{1/2}$ values for $K^+$. On the other hand, Ala1, M30W, I35W, I43W, and I44W mutants produced $K_{1/2}$ values comparable with wild type FXYD7 despite their lower association efficiency (Fig. 2C). These results suggest that the degree of co-immunoprecipitation observed in the presence of digitonin does not necessarily reflect the actual proportion of complexes between Na,K-ATPase and the mutants in intact cells but rather the detergent sensitivity for dissociation of these complexes, which is higher than that of complexes including wild type FXYD7. Finally, despite their efficient association, the V28W mutant induced a decreased and the L32W and I42W mutants an increased $K_{1/2}$ value for $K^+$ of Na,K-ATPase.

Because FXYD7 mutations could have effects on the $K_{1/2}$ value for $K^+$ of Na,K-ATPase at membrane potentials other than $-50$ mV, we also evaluated the voltage dependence of the $K^+$ effect for each mutant. Fig. 3B shows the voltage dependence of the $K_{1/2}$ value for $K^+$ of Na,K-ATPase alone or together with wild type FXYD7. FXYD7 mutants that induced a decrease or an increase in the $K_{1/2}$ value for $K^+$ at $-50$ mV showed a voltage dependence of the $K_{1/2}$ value for $K^+$ similar to that produced by wild type FXYD7 (the G29W mutant is shown as an example in Fig. 3B). Similar results were obtained for FXYD7 mutants that did not change the $K_{1/2}$ value for $K^+$ at $-50$ mV (the L39W mutant is shown as an example in Fig. 3C). The only exception was the F37W mutant, which showed a slight modification of the voltage dependence of the $K_{1/2}$ value for $K^+$ of Na,K-ATPase at very negative membrane potentials compared with wild type FXYD7 (Fig. 3D).

Thus, Val-28, Leu-32, Ile-42, and Phe-37, which produce an increase or a decrease in the $K_{1/2}$ value for $K^+$ or a slight modification of its voltage dependence, are important for the functional effect of FXYD7 on the apparent $K^+$ affinity of Na,K-ATPase without being implicated in the association efficiency. To further confirm the functional significance of these residues, we replaced Phe-37 by an alanine residue that is less similar to phenylalanine than tryptophan and produced a double V28W/I42W and a triple V28W/I42W/L32W mutant. All mutants
associated efficiently with Na,K-ATPase, comparable with wild type FXYD7 (Fig. 4, A and C). As shown in Fig. 4D, the F37A mutant produced a different and much more pronounced effect on the voltage dependence of the $K^+$ affinity of Na,K-ATPase than the F37W mutant (Fig. 3D), confirming the role of Phe-37 in the $K^+$ effect of FXYD7. As shown in Fig. 3A, the V28W mutant decreased and the I42W mutant increased the $K_v$ value for $K^+$. Significantly, double mutations of Val-28 and Ile-42 abrogated the functional effect of FXYD7 (Fig. 4E). When a L32W mutation, which on its own produced a more pronounced effect on the $K_v$ for $K^+$ than wild type FXYD7 (Fig. 3A), was included into the V28W/I42W mutant, the functional effect on the apparent $K^+$ affinity was regained (Fig. 4F). Altogether, these results indicate that Val-28, Leu-32, Ile-42, and Phe-37 play a specific role in the transmission of the functional effect of FXYD7 on the apparent $K^+$ affinity of Na,K-ATPase.

FXYD7 not only has an effect on the apparent affinity for extracellular $K^+$ but also increases the apparent affinity for extracellular Na$^+$ of Na,K-ATPase (4). Extracellular Na$^+$ binding is reflected in a voltage-dependent inhibition of the $K^+$-activated pump current by extracellular Na$^+$ measured at 1 mM extracellular $K^+$ (20). As shown in Fig. 5A, FXYD7 increased the inhibition of the Na,K pump current by 100 mM extracellular Na$^+$ over a large range of membrane potentials and thus increased the apparent affinity for extracellular Na$^+$ of Na,K-ATPase. Most FXYD mutants produced an inhibition of Na,K pump currents by extracellular Na$^+$ similar to that produced by wild type FXYD7 (the I44W mutant is shown as an example in Fig. 5B). However, M30W (Fig. 5C), A33W (Fig. 5D), V28W (Fig. 5E), and I42D (Fig. 5F) mutants further increased the inhibition by extracellular Na$^+$. A double mutant, V28W/I42W (Fig. 5) abolished the effect of the I42W mutant observed at negative membrane potentials and decreased the effect of the V28W mutant observed at less negative and positive membrane potentials. These results show that Val-28 and Ile-42 are involved in the effect of FXYD7 on the apparent affinity for $K^+$ (Fig. 3) as well as on the apparent affinity for extracellular Na$^+$ of Na,K-ATPase and that they act in a synergistic way to produce their effect.

**DISCUSSION**

In this study, we have demonstrated the importance of the TM domain of FXYD7 in the interaction with Na,K-ATPase and have identified amino acids that are implicated in the efficient association and/or the functional effects of FXYD7 (Fig. 6). Our results show that Val-28, Leu-32, Phe-37, and Ile-42 in FXYD7 do not play a role in the efficient association with Na,K-ATPase but contribute to the functional effect of FXYD7 on the apparent affinity for extracellular Na$^+$ and/or $K^+$. Phe-37 and Leu-32 have an effect only on the apparent affinity for extracellular $K^+$, indicating that the intrinsic affinity of the $K^+$ binding site of Na,K-ATPase is modified. On the other hand, Val-28 and Ile-42 influence the apparent affinity for both extracellular $K^+$ and Na$^+$.

Mutations of other amino acids such as Gln-26, Ala-33, Gly-29, Met-30, and Gly-40 lead to a decrease in the association efficiency as well as a perturbation of the functional effect on Na,K-ATPase. In this case, it is difficult to decide whether the reduction in the functional effect observed after mutation of these residues is due to inefficient association of FXYD7 with Na,K-ATPase or to a direct effect of the mutated residue inside the Na,K-ATPase-FXYD7 complex. However, rather than a decreased function, mutation of Met-30 produces an increase in the inhibition of Na,K-ATPase by extracellular Na$^+$ and A33W an increase in the inhibition by extracellular Na$^+$ and an increase of the $K_v$ value for extracellular $K^+$. These results suggest a functional role of these amino acids independent of their role in the association efficiency.

Finally, mutation of Ile-35, Ile-43, and Ile-44 lead to a reduced association efficiency with Na,K-ATPase but do not perturb the functional effect of FXYD7. This result may indicate that co-immunoprecipitation experiments do not reflect the real amount of Na,K-ATPase-FXYD7 complexes in intact cells but rather give an indication of their detergent sensitivity. As indicated in Fig. 6, the strength of association of FXYD7 is determined by several amino acids in the TM domain of FXYD7. Any of the different contacts may weaken the strength of the association with the Na,K-ATPase $\alpha$ subunit and therefore affect efficiency of co-immunoprecipitation yet not prevent the association with the $\alpha$ subunit in native membranes.

A distinction between amino acids that determine the structural and functional interaction of FXYD proteins has previously also been observed in TM9 of the $\alpha$ subunit of Na,K-ATPase (5). Moreover, our data on FXYD7 are in line with results obtained with FXYD2 and FXYD4 that suggest that the functional interaction is different from that determining the detergent resistance of FXYD-Na,K-ATPase complexes (8). Interestingly, amino acids such as Ala-33, Ile-43, and Ile-44 that were found to be important for the stable interaction of mouse FXYD7 with Na,K-ATPase correspond to Ala-34, Leu-44, and Ile-45 in rat FXYD2 that were shown to be involved in the greater detergent resistance of FXYD2-Na,K-ATPase complexes compared with FXYD4-Na,K-ATPase complexes (8). Moreover, both in FXYD7 (this study) and in FXYD4 (8), mutations at positions Ile-42, Leu-39, and Met-36 in FXYD7 or the corresponding amino acids Leu-48, Ala-51, and Ala-54 in FXYD4 had no effect on the stability of interaction with Na,K-ATPase. These results strongly suggest that amino acids at similar positions determine the stability of interaction with Na,K-ATPase in all FXYD proteins.

The results of our study permit us to delineate two distinct regions of the TM helix with different importance for the structural and functional interaction of FXYD7 with Na,K-ATPase (Fig. 6). Region A does not contain any residue that is involved in the structural or functional association with Na,K-ATPase. On the other hand, region B, which contains all structural and functional determinants of FXYD7 and which covers two-thirds of the TM helix, is likely to face the potential binding pocket for FXYD proteins composed of TM2, TM4, TM6, and TM9 of the Na,K-ATPase $\alpha$ subunit (21). Although TM9 of the Na,K-ATPase $\alpha$ subunit was shown to be involved in the interaction with FXYD proteins (5), little is known about which part of domain B interacts with this TM domain. A docking model of Na,K-ATPase-FXYD7 complex predicts contacts of TM9 with Gln-26, Met-30, and Phe-37 of FXYD7 (5). These amino acids were shown in this study to be involved in the structural and/or functional interaction of FXYD7, which would be consistent with their interaction with TM9 of the $\alpha$ subunit. More studies are needed to position FXYD proteins in the binding pocket and to determine the TM domains of the $\alpha$ subunit and possibly the $\beta$ subunit of Na,K-ATPase that interact with FXYD proteins and contribute to the stable interaction and the functional effects. It is, however, clear that several TM domains of Na,K-ATPase must contribute to the interaction with FXYD proteins. Amino acids that are important for the stable interaction and the functional effects of FXYD proteins are present at least two faces of the TM helix. Interestingly, similar results were obtained for the inhibitory protein phospholamban of sarco (endo) plasmatic reticulum Ca$^{2+}$ ATPase (SERCA) that is supposed to be located also in a transmembrane groove made up of TM2, TM4, TM6, and TM9 (22).

It should be noted that no single mutation of amino acids in the TM domain completely abolishes the functional effects of FXYD7 and that amino acids involved in the functional effects are scattered over a large region of the FXYD7 TM helix. This result indicates that the functional
effects are produced by a complex interplay of FXYD7 interactions with different TM helices of Na,K-ATPase. Most likely, these interactions influence the conformational changes during the transport cycle and, in consequence, the cation affinities of Na,K-ATPase. Alternatively or in addition, a modification of the closely located third Na’ binding site (19) could explain some of the effects. For instance, mutation of Met-30 increases the inhibition of Na,K-ATPase by extracellular Na’/H+.

A docking model between Na,K-ATPase-FXYD7 complexes predicts contacts between Met-30 and TM9 of the Na,K-ATPase α subunit (5) that is part of the third Na’ binding site (19).

In conclusion, the present study has provided new insights into the structure-function relationship of FXYD proteins in general and FXYD7 in particular and has emphasized the important role of transmembrane interactions of FXYD proteins and Na,K-ATPase.

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