Structural and functional interaction sites between Na,K-ATPase and FXYD proteins

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Running title: Interaction sites between Na,K-ATPase and FXYD proteins
Several members of the FXYD protein family are tissue-specific regulators of Na,K-ATPase which produce distinct effects on its apparent K\(^+-\) and Na\(^+-\) affinity. Little is known about the interaction sites between the Na,K-ATPase \(\alpha\) subunit and FXYD proteins that mediate the efficient association and/or the functional effects of FXYD proteins. In this study, we have analyzed the role of the transmembrane segment TM9 of the Na,K-ATPase \(\alpha\) subunit in the structural and functional interaction with FXYD2, FXYD4 and FXYD7. Mutational analysis combined with expression in Xenopus oocytes reveals that F\(^{956}\), E\(^{960}\), L\(^{964}\) and F\(^{967}\) in TM9 of the Na,K-ATPase \(\alpha\) subunit represent one face interacting with the 3 FXYD proteins. L\(^{964}\) and F\(^{967}\) contribute to the efficient association of FXYD proteins with the Na,K-ATPase \(\alpha\) subunit whereas F\(^{956}\) and E\(^{960}\) are essential for the transmission of the functional effect of FXYD proteins on the apparent K\(^+-\) affinity of Na,K-ATPase. The relative contribution of F\(^{956}\) and E\(^{960}\) to the K\(^+-\)-effect differs for different FXYD proteins probably reflecting the intrinsic differences of FXYD proteins on the apparent K\(^+-\)-affinity of Na,K-ATPase. In contrast to the effect on the apparent K\(^+-\)-affinity, E\(^{956}\) and F\(^{960}\) are not involved in the effect of FXYD2 and FXYD4 on the apparent Na\(^+-\)-affinity of Na,K-ATPase. The mutational analysis is in good agreement with a docking model of the Na,K-ATPase/FXYD7 complex which also predicts the importance of F\(^{956}\), E\(^{960}\), L\(^{964}\) and F\(^{967}\) in subunit interaction. In conclusion, by using mutational analysis and modeling, we show that TM9 of the Na,K-ATPase \(\alpha\) subunit exposes one face of the helix that interacts with FXYD proteins and contributes to the stable interaction with FXYD proteins, and mediates the effect of FXYD proteins on the apparent K\(^+-\)-affinity of Na,K-ATPase.
Na,K-ATPase transports 3 Na⁺ against 2 K⁺ across the plasma membrane of animal cells by using the energy of the hydrolysis of ATP. Its main task is the maintenance of the transmembrane Na⁺ and K⁺ gradients that permit to maintain the cell volume and the membrane potential. Moreover, Na⁺ gradients provide the energy for many secondary transport systems of vital importance. In addition to these basic functions, Na,K-ATPase is involved in many specialized tissue functions such as transepithelial Na⁺ transport, and muscle and neuronal excitability.

Na,K-ATPase is an oligomeric protein. The α subunit hydrolyzes ATP, becomes phosphorylated during the catalytic cycle and transports the cations (1). The β subunit is a molecular chaperone which is necessary for the correct membrane insertion of the α subunit and also modulates the transport properties of the α subunit (2). Four α and 3 β isoforms have been identified which potentially can form 12 different isozymes with different transport and pharmacological properties (3, 4).

In agreement with its important physiological role, Na,K-ATPase is finely regulated. Established regulatory mechanisms include changes in the intracellular Na⁺ concentration that produce short-term regulation of the Na,K-ATPase transport rate, phosphorylation of the α subunit by PKA and PKC that influences the distribution of Na,K-ATPase between the plasma membrane and intracellular stores, and long term regulation that increases the number of Na,K-ATPase units (5). Recently, a novel regulatory mechanism has been identified which involves tissue- and isozyme-specific interactions between Na,K-ATPase and small membrane proteins of the FXYD protein family (6).

The FXYD protein family contains 7 members that are characterized by one transmembrane domain and a signature sequence that contains the FXYD motif and 3 other conserved amino acids (7). FXYD2 or the γ subunit of Na,K-ATPase (8) was the first FXYD protein that was identified as a specific modulator of renal Na,K-ATPase (9, 10, 11, 12, 13).
It is now well established that also FXYD1 (phospholemman) (14), a phospholemman-like protein from shark (15), FXYD4 (CHIF) (11, 16) and FXYD7 (17) also play a tissue-specific role in Na,K-ATPase regulation. Significantly, each of these auxiliary subunits produce a distinct functional effect on Na,K-ATPase which is adapted to the physiological needs of the tissues in which they are expressed.

The functional effects of FXYD proteins on Na,K-ATPase have extensively been studied but the molecular basis of these effects is unknown and very little is known on the interaction sites in the Na,K-ATPase and the FXYD proteins that mediate the efficient association between these two proteins and transmit the functional effects of FXYD proteins on Na,K-ATPase. Experiments based on thermal denaturation suggest that association of FXYD2 occurs with transmembrane (TM) domains 8-10 (18). Moreover, a recent model (19) deduced from an electron cristallographic analysis at 9.5 A resolution of renal Na,K-ATPase, and by taking as a basis the high resolution structure of the Ca-ATPase (20), predicts that FXYD2 is located in a pocket made up of TM9, TM6, TM4 and TM2 of the Na,K-ATPase \(\alpha\) subunit. In this study, we investigated the role of TM9 of the Na,K-ATPase \(\alpha\) subunit in the structural and functional interaction with FXYD proteins. For this purpose, we produced a model of the Na,K-ATPase \(\alpha\) subunit to determine amino acids in the TM9 helix which point to M2 and which could potentially interact with FXYD proteins. These amino acids were substituted individually or in combination by alanines and the effects of these mutations on the efficiency of association with different FXYD proteins and on their functional effects were tested after co-expression in \textit{Xenopus} oocytes.

Our results indicate that TM9 of the Na,K-ATPase \(\alpha\) subunit is involved in the interaction with FXYD proteins. Different domains of interaction could be identified that are either involved in the stable association with FXYD proteins or in the functional effect produced by FXYD proteins on the apparent \(K^+\)-affinity of Na,K-ATPase. The interaction of
FXYD proteins with TM9 of the Na,K-ATPase α subunit is supported by modeling of the 
Na,K-ATPase α subunit/FXYD7 complex.
Materials and Methods

Side-directed mutagenesis and chimeras

The rat Na,K-ATPase α1 subunit was modified by silent mutagenesis to introduce one restriction site (AccI) at nucleotide position 2893 and then point mutations were introduced by the PCR-based method as described by Nelson and Long (21). The insert of the TM9 was subcloned into a pSD5 vector using AccI/DraIII restriction sites. The nucleotide sequences of all constructs were confirmed by dideoxy sequencing. cDNAs for rat α1 and β1 subunits were kindly provided by J. Lingrel. The construction of the chimera α1-AL1 containing Met1 to Arg941 of the human Na,K-ATPase α1 subunit and Asn960 to Tyr1039 of the human, non-gastric H,K-ATPase α subunit (AL1) has been described previously (22).

Protein expression in Xenopus oocytes and metabolic labelling

cRNAs were obtained by in vitro transcription (23). Stage V-VI Xenopus oocytes were obtained as described (24). Oocytes were injected with cRNAs coding for wild type or mutant rat Na,K-ATPase α1 subunits (10 ng/oocyte) or for AL1 (10 ng/oocyte) (25) or α1-AL1 (10 ng/oocyte) together with cRNAs for the rat Na,K-ATPase β1 subunit (1 ng/oocyte) or rabbit, gastric H,K-ATPase β subunit (1 ng/oocyte) (kindly provided by G. Sachs) with or without cRNAs coding for human FXYD2a (11), rat FXYD4 (11) or mouse FXYD7 (2 ng) (17). Oocytes were incubated in modified Barth’s solution (MBS) in the presence of 0.8 - 1 mCi/ml 35S-methionine (Easy Tag Express [35S] Protein Labeling Kit, PerkinElmer) at 19°C and subjected to a 6 h pulse and to 24 h, 48 h and 72 h chase periods in MBS containing 10 mM cold methionine. Microsomes were prepared after each chase period as described previously (24).
Immunoprecipitation and quantification of association between FXYD proteins and Na,K-ATPase

FXYD2 and FXYD4 were co-immunoprecipitated with wild type or mutant Na,K-ATPase α1 subunits under non-denaturing conditions as described (24) by using an α1 subunit antibody (26). Since co-immunoprecipitation of FXYD7 by an α subunit antibody is not very efficient, association of FXYD7 with Na,K-ATPase was tested by using an FXYD7 antibody (17) under non-denaturing conditions. Association of FXYD2 with AL1 and the α1-AL1 chimera was tested by using a AL1 antibody (24) under non-denaturing conditions. Immunoprecipitated proteins were loaded on SDS-polyacrylamide gels (5-13%) and revealed by fluorography. Proteins were quantified by laser densitometer (KB Ultrascan 2202) and calculations were performed as described in the legends to figures. Statistical analysis was performed by unpaired Student’s t-tests.

Electrophysiological measurements

The functional effect of mutations in the Na,K-ATPase α1 subunit expressed with or without FXYD proteins was assessed by studying the apparent affinity for external K+ or internal Na+ of Na,K-ATPase. Electrophysiological measurements were performed 3 days after injection of Xenopus oocytes with rat, wild type α1 and β1 or mutant α1 and β1 cRNAs alone or together with FXYD cRNAs, by using the two-electrode voltage clamp technique. Measurements of the apparent external K+ affinity were carried out as described previously (11) in the presence of 1 μM ouabain which inhibits the endogenous oocyte Na,K-pump, but not the expressed ouabain-resistant rat Na,K-ATPase. The maximal Na,K-pump current and the apparent K+ affinity (K_1/2K+), measured in the presence of 100 mM external Na+, were obtained by fitting the Hill equation to the data using a Hill coefficient of 1.6 (27). Measurements of the apparent Na+-affinity of Na,K-ATPase were performed as described
previously (28) in oocytes co-expressing rat, wild type or mutant Na,K-ATPase α1 and β1 subunits, and the rat epithelial Na⁺ channel α, β and γ subunits in the presence or absence of FXYD proteins. The Hill equation was fitted to the experimental data by using a Hill coefficient of 3 (28). Curves with a correlation coefficient lower than 0.90 were rejected. Only oocytes with a Imax of more than 100 nA and with an initial internal Na⁺-concentration of less than 10 mM were used for the determination of K₁/₂Na⁺ values. Statistical analysis was performed by unpaired Student’s t-test.

**Protein Modeling**

Based on our previous homology model of the Bufo Na-K-ATPase (29) in the E1 conformation, a model of the FXYD7/Na-K-ATPase complex was build using an evolutionary algorithm. The details of the calculations will be presented separately (Grosdidier et al., in preparation). In brief, starting from an arbitrary conformation of the FXYD7 helix in the vicinity of TM2 and TM9 of the Bufo Na,K-ATPase α subunit, the FXYD7 coordinates were refined using two operators translating or rotating the FXYD7 helix around a random axis. Two other operators were designed to rationalize the search, performing rotations around, or translations along the axis of the modeled fragment of FXYD7. The fifth and last operator was a semi-stochastic interpolator combining two high-scoring complexes to generate a new position and orientation of the FXYD7 fragment. After each operator was applied, a short energy minimization of the FXYD7 helix as well as TM9 and TM2 residues was performed using the CHARMM program (30). The minimization consisted of 30 steps of Steepest Descents followed by 50 steps of Adopted Basis Newton-Raphson.

The fitness of a complex was defined as its total enthalpic energy calculated by CHARMM using the CHARMM22 (31) parameters with a soft Van Der Waals potential. An
implicit lipid layer was added by means of a dielectric switch in the Generalized Born-Simple
Switch solvation model (32, 33) with its default parameter set. No mutation data were used in
the conformational space search, nor in the ranking of the complexes.

The best scoring complexes generated during the evolutionary search were clustered,
based on heavy atom RMSD values and a contact list was generated for each cluster.
Results

The TM9-TM10 region of the Na,K-ATPase α subunit is involved in interaction with FXYD2

We have previously shown (11) that non-gastric H,K-ATPase does not associate with FXYD2 or FXYD4. To test whether the TM8-TM10 region of the Na,K-ATPase α subunit is involved in the interaction with FXYD2 as suggested by biochemical evidence (18), we produced a chimera containing the sequence of the Na,K-ATPase α1 subunit up to TM8 and that of the human, non-gastric H,K-ATPase α subunit (AL1) (25) including TM9 and TM10 (see Fig.1). This chimera (α1-AL1) was expressed in Xenopus oocytes together with the H,K-ATPase β subunit and FXYD2 and the efficiency of FXYD2 association with this chimera was compared to that with wild type Na,K-ATPase α1 subunit and with AL1. As previously shown (11), in metabolically labeled oocytes expressing the Na,K-ATPase α1 and β1 subunit and FXYD2, the β subunit and FXYD2 could be co-immunoprecipitated with an antibody against the Na,K-ATPase α subunit over prolonged chase periods (Fig.2A, lanes 4-6). On the other hand, no association of FXYD2 is observed in oocytes co-expressing the wild type AL1 (lanes 7-9) despite a similar expression of FXYD2 (Fig.2B, lanes 4-9). The association efficiency of FXYD2 with the chimeric α1-AL1 was reduced by about 70% (Fig.2A, lanes 10-12, Fig.2C) after 48 h and 72 h chase periods indicating that the TM9-TM10 region of the Na,K-ATPase α subunit is involved in the stable association with FXYD2.

The role of TM9 of the Na,K-ATPase α subunit in the stable interaction with FXYD proteins

Since a model deduced from a crystallographic analysis of renal Na,K-ATPase predicts that TM9 of the α subunit is in close proximity to FXYD2 (19), we tested the role of TM9 in the structural and functional interaction with FXYD proteins. We produced a model of the
TM9 helix (29) and identified I$_{953}$, F$_{956}$, E$_{960}$, L$_{964}$ and F$_{967}$ that could be directed to FXYD proteins and potentially could form an interaction face. We substituted these amino acids of the rat α1 isoform, located on the same side of the TM9 helix, either individually or in combination by alanine residues (see Fig.1), expressed these α mutants together with rat β1 subunits and FXYD2 in *Xenopus* oocytes and tested their association efficiency with FXYD2 by performing non-denaturing immunoprecipitations with a Na,K-ATPase α subunit antibody (Fig.3A). All α mutants were synthesized similar to the wild type α subunit, associated with the β subunit and became stabilized over prolonged chase periods. Determinations of the ratios between associated FXYD2 and the α subunit show that the I/A mutant (Fig.3B a) and the FE/AA mutant (Fig.3B d) did not affect the stable association of FXYD2. On the other hand, the LF/AA (Fig.3B b) and the ILF/AA (Fig.3B c) significantly reduced the association efficiency after a 48 h or a 72 h chase period indicating a less stable association of FXYD2 with these α mutants. The 5A (Fig.3B e) mutant, in which all 5 amino acids were replaced by alanines, reduced the association efficiency of FXYD2 up to 50% after a 72 h chase period. Similar to FXYD2, FXYD4 (Fig.4A and B) associated efficiently with the FE/AA mutant but not with the ILF/AAA mutant. FXYD7 (Fig.4C and E) association was slightly less efficient with the FE/AA mutant after a 72 h chase period than with the wild type α subunit and decreased by 44% and 54% with the ILF/AAA mutant after a 48 h and a 72 h chase period, respectively. Altogether, these results indicate that TM9 of the Na,K-ATPase α subunit and in particular L$_{964}$ and/or F$_{967}$ contribute to the stable interaction with FXYD proteins.

*The role of TM9 of the Na,K-ATPase α subunit in the functional effect of FXYD proteins*

As shown in Fig.5, the I/A, the IL/AA and the ILF/AAA α mutants had an effect *per se* on the voltage dependence of the $K_{1/2}$ value for $K^+$ (Fig.5A). The FE/AA and the E/A mutants slightly increased the $K_{1/2} K^+$ over the whole potential range tested compared to the
wild type α1 subunit, and the F/A mutant showed a pronounced effect at very negative membrane potentials (Fig. 5B). Interestingly, E\textsuperscript{960} in the FE/AA mutant has been suggested to be part of the cation ‘occlusion’ gate of Na,K-ATPase (34) though substitution by alanine did not reveal any importance of this amino acid in the cation dependence of Na,K-ATPase activity (35).

FXYD2 (11), FXYD4 (11) and FXYD7 (17) have distinct effects on the voltage-dependence of the apparent K\textsuperscript{+}-affinity of Na,K-ATPase (see Fig. 7A a, Fig. 7B a and Fig. 7C a). We investigated whether TM9 of the Na,K-ATPase α subunit is implicated in the functional effect of FXYD proteins on the K\textsubscript{1/2}\textsuperscript{K+} value of the Na,K-ATPase.

FXYD2, co-expressed with the I/A (Fig. 6 b), the IL/AA (Fig. 6 c) or the ILF/AAA (Fig. 6 d) mutant, retained a functional effect on the voltage-dependence of the K\textsubscript{1/2}\textsuperscript{K+} of Na,K-ATPase (compare Fig. 6 b, c, d to Fig. 6 a). On the other hand, when FXYD2 was co-expressed with the FE/AA α mutant, its functional effect on the voltage-dependence of the K\textsubscript{1/2}\textsuperscript{K+} of Na,K-ATPase was completely lost (compare Fig. 7A b to Fig. 7A a). F\textsuperscript{956} and E\textsuperscript{960} in TM9 of the Na,K-ATPase α subunit are also important for the K\textsuperscript{+}-effect of FXYD4 and FXYD7. The FE/AA mutant significantly decreased the functional effect of FXYD4 (compare Fig. 7B b to Fig. 7B a) and completely abolished that of FXYD7 (compare Fig. 7C b to Fig. 7C a). We also investigated the relative contribution of F\textsuperscript{956} and E\textsuperscript{960} to the K\textsuperscript{+}-effect of the 3 FXYD proteins. Significantly, the E/A mutant contributed significantly to the abolishment of the functional effect of FXYD2 (Fig. 7A d) and FXYD4 (Fig. 7B d) and the F/A mutant to a lesser extent (Fig. 7A c and Fig. 7B c). On the other hand, the E/A mutant permitted a full functional effect of FXYD7 (Fig. 7C d) whereas the F/A mutant reduced or modified the functional effect of FXYD7 on the voltage-dependence of the K\textsuperscript{+}-affinity of Na,K-ATPase (Fig. 7C c). Thus, F\textsuperscript{956} and/or E\textsuperscript{960} which do not contribute to the stable
interaction of FXYD proteins with the Na,K-ATPase α subunit, are involved in the functional effect of FXYD proteins on the apparent K⁺-affinity of the Na,K-ATPase.

Some FXYD proteins such as FXYD2 and FXYD4 have not only an effect on the apparent affinity of Na,K-ATPase for extracellular K⁺ but also on the apparent affinity for intracellular Na⁺ (11). As shown in Fig.8, FXYD2 decreases whereas FXYD4 increases the apparent Na⁺-affinity of the Na,K-ATPase. We investigated whether F956 and E960 in the Na,K-ATPase α subunit are involved not only in the K⁺-effect but also in the Na⁺-effect of FXYD2 and FXYD4. When FXYD2 or FXYD4 was co-expressed with the FE/AA α mutant, the K₁/₂ Na⁺ of the Na,K-ATPase decreased to a similar extent than when FXYD2 or FXYD4 was co-expressed with the wild type Na,K-ATPase α subunit (Fig.8) indicating that F956 and E960 do not contribute to the functional effect of FXYD proteins on the apparent Na⁺-affinity of Na,K-ATPase.

Docking of FXYD7 on homology model of the Na⁺,K⁺-ATPase α subunit

The docking of FXYD7 on the previously described homology model of the Na⁺,K⁺-ATPase α subunit (29) was performed by a genetic algorithm. No mutation data were taken into account during the conformational space search. A total of 540 complexes were generated, from which the 10% complexes with the lowest energy were extracted and clustered based on heavy atom RMSD values (Fig.9). Two clusters were identified; the cluster A (83% of the conformers) had a mean energy of -22676 kcal/mol and the cluster B (17% of the conformers) a mean energy of -22613 kcal/mol. Both clusters share the same anchoring of FXYD7 in the cleft near the extracellular space, with contacts involving residues Q²⁶ (cluster A) and T²⁷ (cluster B) of FXYD7, with F⁹⁶⁷ from TM9 (Fig.10). FXYD7 interacts with residues I⁹⁵³, F⁹⁵⁶, E⁹⁶⁰, L⁹⁶⁴ and F⁹⁶⁷ and L⁹⁶⁸ from TM9 and Y¹⁴⁹, I¹⁴³, R¹⁵⁶ and I¹⁵⁷ from TM2. Two residues of FXYD7 (M³⁰ and F³⁷) are overrepresented in the contact list. M³⁰ is
close from both L\textsuperscript{964} and L\textsuperscript{968} (TM9) and I\textsuperscript{142} (TM2). Two alternative rotamers for F\textsuperscript{37} were isolated in each cluster, with favorable interactions with either F\textsuperscript{956} (TM9) or with Y\textsuperscript{149} (TM2). Next to F\textsuperscript{37}, V\textsuperscript{38} also interacts with Y\textsuperscript{149} (TM2). Two hydrophobic residues of FXYD7, I\textsuperscript{44} and L\textsuperscript{45}, fill the widest part of the TM9-TM2 groove, stabilized by several contacts with I\textsuperscript{953}, and I\textsuperscript{157} (structures from cluster B) or R\textsuperscript{156} (aliphatic part of the sidechain, structures from cluster A).
Discussion

By site-directed mutagenesis and protein modeling, we provide evidence for an interaction of FXYD proteins with TM9 of the Na,K-ATPase which contributes to the efficient association and the functional effect of FXYD proteins on the apparent K⁺-affinity of Na,K-ATPase.

Based on crystallographic analysis of renal Na,K-ATPase, previous studies by Hebert et al. (19) predict that FXYD2 or the γ subunit is located in a pocket made up of TM9, TM6, TM4 and TM2 of the Na,K-ATPase α subunit. Based on this hypothesis and using the structure of the SERCA pump (20), we produced a model of TM9 (29) and determined amino acids that point to M2 and could potentially interact with FXYD proteins. Though, we cannot entirely exclude that substitution of these amino acids by alanine could have some indirect effects on the global conformation of the Na,K-ATPase α subunit, our results rather suggest that the predicted side of the TM9 helix of the Na,K-ATPase α subunit indeed represents at least one of the interaction faces with FXYD proteins. All amino acids tested are well conserved in different Na,K-ATPase α isoforms which is compatible with the observation that after expression in Xenopus oocytes, FXYD proteins associate with all α isoforms (9, 17).

Significantly, in the interaction face of TM9 we can distinguish regions with different functional roles. The L⁹⁶⁴/F⁹⁶⁷ region contributes to the stable interaction between the Na,K-ATPase α subunit and FXYD proteins but not to the functional effect of FXYD proteins on the apparent K⁺-affinity of Na,K-ATPase. The lack of a partial loss of the functional effect of FXYD proteins despite a partial loss of the association efficiency in the LF/AA mutant can be explained if one assumes that the co-immunoprecipitation experiments do not reflect the real amount of Na,K-ATPase/FXYD protein complexes in the cell but rather an increased detergent sensitivity. Since the LF/AA mutant only partially abolishes the interaction with
FXYD proteins, it is likely that not only TM9 but also other TM helices of the TM9, TM6, TM4 and TM2 binding pocket in the Na,K-ATPase α subunit contribute to the structural interaction of FXYD proteins. This hypothesis is also supported by the fact that in non-gastric H,K-ATPase and gastric H,K-ATPase α subunits, amino acids at the positions of L964 and F967 in Na,K-ATPase are not clearly distinct to explain that FXYD proteins do not associate with these P-type ATPases.

In contrast to the L964/F967 region, the F956/E960 region does not contribute to the stable interaction between Na,K-ATPase and FXYD proteins but it transmits the K+ -effect of FXYD proteins to Na,K-ATPase. Substitution of F956/E960 by alanines completely abolishes the effect of FXYD2 and FXYD7 on the apparent K+ -affinity of Na,K-ATPase and strongly reduces that of CHIF. Moreover, it is interesting to note that the contribution of F956 and E960 to the K+ -effect of different FXYD proteins is different. Though the precise interpretations of the effect of single mutations are complex, it is clear that the E/A mutant contributes significantly to the loss of the functional effect of FXYD2 but it permits the full functional effect of FXYD7. The differential roles of amino acids in the TM9 helix of the Na,K-ATPase α subunit in the K+ -effect of FXYD proteins is likely to reflect the intrinsic differences in the modulation of the apparent K+ -affinity of Na,K-ATPase by different FXYD proteins. FXYD proteins significantly differ in their N- and C-terminal region but show a significant sequence homology in the TM region. Nevertheless, specific sequence differences exist in the TM domain of FXYD proteins which could allow interactions with TM9 of the Na,K-ATPase α subunit resulting in different functional effects.

In contrast to the K+ -effect, the Na+ -effect of certain FXYD proteins such as FXYD2 and FXYD4 is not mediated by F956 and E960 in the TM9 of the Na,K-ATPase α subunit. A recent study using chimeric proteins between FXYD2 and FXYD4 has revealed that the transmembrane segments of these FXYD proteins determine the opposite effect of FXYD2
and FXYD4 on the apparent Na\(^+\)-affinity of the Na,K-ATPase (36). Together with our observations, these results suggest that not only the structural but also the functional interaction with FXYD proteins is determined by multiple interaction sites.

The model of FXYD7 docked with the Na,K-ATPase \(\alpha\) subunit in the E1 conformation is in excellent agreement with the experimental data. All residues in TM9, I\(^{953}\), F\(^{956}\), E\(^{960}\), L\(^{964}\) and F\(^{967}\), identified by the mutagenesis analysis are involved in contacts between FXYD7 and the Na,K-ATPase \(\alpha\) subunit in the model. In addition, the model predicts I\(^{142}\), Y\(^{149}\), R\(^{156}\), I\(^{157}\) in TM2, and Q\(^{26}\), M\(^{30}\), V\(^{38}\), F\(^{37}\), I\(^{41}\), I\(^{44}\) and L\(^{45}\) in FXYD7 as interaction sites. Interestingly, the stabilizing interactions involving L\(^{964}\) and F\(^{967}\) in TM9 are similar in both clusters. G\(^{29}\) and G\(^{40}\) in FXYD7, the substitution of which have previously been shown to significantly affect the association efficiency with Na,K-ATPase (37) were not predicted to form favorable contacts by the docking. It remains to be shown whether substitution of these glycine residues could perturb correct folding of FXYD proteins, thus limiting complex formation. Alternatively, it remains to be investigated whether there might be a correlation between the potential implication of G\(^{40}\) in oligomer formation (38) and the association efficiency of G\(^{40}\) mutants in FXYD7 as well as in FXYD2 (39). Finally, the residues suggested by the docking study only, such as , Y\(^{149}\), I\(^{157}\), R\(^{156}\), I\(^{142}\), L\(^{968}\) in the Na,K-ATPase \(\alpha\) subunit, and the predicted amino acids in FXYD7, are good candidates for further mutagenesis analysis.

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References


**Figure Legends.**

Fig.1: *α*-mutants and chimera. (A) Linear models of the Na,K-ATPase α1 subunit, the non-gastric H,K-ATPase α subunit (AL1) and the α1-AL1 chimera in which the transmembrane (TM) domain 9 and 10 and C-terminus of the human Na, K-ATPase α1 subunit were replaced by those of the human non-gastric H, K-ATPase. Indicated is Met1 and Arg941 of the Na,K-ATPase α1 subunit, and Asn960 and Tyr1039 of AL1 which are comprised in the α1-AL1 chimera. Black and hatched rectangles indicate the TM domains of the Na,K-ATPase α1 subunit and non-gastric H,K-ATPase α subunit, respectively. (B) Amino acid sequences of TM9 of rat, wild type (wt) and mutant Na,K-ATPase α1 subunit.

Fig.2: *FXYD2 associates less efficiently with α1-AL1 than with the wild type Na,K-ATPase α1 subunit.* (A) Xenopus oocytes were injected with rat Na, K-ATPase α1 subunit, human non-gastric H,K-ATPase α subunit (AL1) or α1-AL1 chimera cRNAs (10ng) together with rat β1 cRNA (1ng) or rabbit gastric H,K-ATPase β subunit cRNA (1 ng) in the presence (lanes 4-12) or absence (lanes 1-3) of human FXYD2 cRNA (2ng). Oocytes were metabolically labeled for 6 h with 35S-methionine and subjected to 24, 48 and 72 h chase periods. Microsomes were prepared and immunoprecipitations were performed in non-denaturing conditions using a Na,K-ATPase α1 subunit antibody (lanes 1-6, 10-12) or an AL1 antibody (lanes 7-9). Immunoprecipitates were migrated on SDS-polyacrylamide gels. (B) Aliquots of samples described in (A) were loaded on SDS-polyacrylamide gels without immunoprecipitation. Indicated are the positions of the α subunits, the core-glycosylated (c β) and the fully glycosylated (f β) subunits and of FXYD2. (C) Quantification of data shown in (A). Shown are the ratios between FXYD2 and the Na,K-ATPase α1 subunit (black bars) or the α1AL1 chimera (white bars) after 48 h and 72 h chase periods. One out of 2 experiments with similar results is shown.
Fig. 3: **TM9 of the Na,K-ATPase α1 subunit contributes to the stable association with FXYD2.**

(A) *Xenopus* oocytes were injected with cRNAs for wild type or mutant rat Na, K-ATPase α1 subunits (10 ng), and rat β1 subunit (1 ng) together with human FXYD2 (2 ng), metabolically labeled for 6 h with ^35^S-methionine and subjected to 24, 48 and 72 h chase periods. Microsomes were prepared and immunoprecipitations were performed under non-denaturing conditions using a Na,K-ATPase α1 subunit antibody. Immunoprecipitates were migrated on SDS-polyacrylamide gels. Indicated are the positions of the α subunit, the core-glycosylated (cβ) and the fully glycosylated (fβ) β subunits and of FXYD2. (B) Quantification of data shown in (A). Shown are the ratios between FXYD2 and the α subunit. The ratio between FXYD2 and wild type α1 subunit after 24 hours of chase was arbitrarily set to 1. FXYD2/wild type α1 (closed squares); FXYD2/mutant α1 (open squares). I/A mutant (B a); LF/AA mutant (B b); ILF/AAA mutant (B c); FE/AA mutant (B d); 5A mutant (B e). Data are means ± SE of 4-8 experiments, * = p < 0.05.

Fig. 4: **TM9 of the Na,K-ATPase α1 subunit contributes to the stable association with FXYD4 and FXYD7.** (A, C and D) *Xenopus* oocytes were injected with cRNAs for wild type or mutant rat Na, K-ATPase α1 subunits (10 ng) and rat β1 subunit (1 ng), together with FXYD4 (2 ng) (A) or FXYD7 (2 ng) (C, D), metabolically labeled for 6 h with ^35^S-methionine and subjected to 24, 48 and 72 h chase periods. Microsomes were prepared and immunoprecipitations were performed under non-denaturing conditions using a Na,K-ATPase α1 subunit antibody (A, D) or a FXYD7 antibody (C). Immunoprecipitates were migrated on SDS-polyacrylamide gels. Indicated are the positions of the α subunit, the core-glycosylated (cβ) and the fully glycosylated (fβ) β subunits and of FXYD4 and FXYD7. (B) Quantification of data shown in (A). Shown are the ratios between FXYD4 and the α subunit.
The ratio between FXYD4 and wild type α1 subunit after 24 hours of chase was arbitrarily set to 1. FXYD4/wild type α1 (closed squares); FXYD4/mutant α1 (open squares). FE/AA mutant (B a); ILF/AAA mutant (B b). (E) Quantification of data shown in (C). Shown is the amount of α subunit co-immunoprecipitated by a FXYD7 antibody corrected for the total amount of α subunit expressed as revealed by immunoprecipitations with an α subunit antibody (D). The amount of α subunit co-immunoprecipitated by a FXYD7 antibody after a 24 h chase was arbitrary set to 1. Wild type α1 (closed squares); mutant α1 (open squares). FE/AA mutant (E a); ILF/AAA mutant (E b). Data are means ± SE of 3-4 experiments, * = p < 0.05.

Fig.5: Effects of alanine substitutions in TM9 of the α1 subunit on functional properties of Na,K-ATPase. (A and B) Oocytes were injected with cRNAs for rat, wild type or mutant Na, K-ATPase α1 subunits (10 ng) and for rat β1 subunit (1 ng). Three days after cRNA injection, K1/2 K+ values of the Na,K-ATPase as a function of the membrane potential were determined as described in Material and Methods. Inset: Maximal Na,K-pump currents (I max). For clarity, no symbols for significance are indicated. In (A): P < 0.01, wild type (closed squares) vs I/A (open squares), IL/AA (open triangles) and ILF/AAA (open circles) mutants between −130 mV and −50 mV, vs I/A and ILF/AAA mutants between 10 mV and 30 mV. In (B): P < 0.01, wild type (closed squares) vs FE/AA (open squares) at all membrane potentials except at −130 mV and 30 mV; p < 0.01, wild type vs F/A (open triangles) at all membrane potentials; p < 0.05, wild type vs E/A (open circles) at all membrane potentials except at −130 mV.

Fig.6: I953, L964 and F967 in TM9 of the Na,K-ATPase α1 subunit are not involved in the functional effect of FXYD2 on the apparent K+ -affinity of Na,K-ATPase. Oocytes were
injected with cRNAs for rat, wild type or mutant Na,K-ATPase α1 subunits (10 ng) and for rat β1 subunit (1 ng) in the presence of cRNA for FXYD (2 ng). Three days after cRNA injection, K_{1/2} K^{+} values of the Na,K-ATPase as a function of the membrane potential were determined. Wild type α1 subunit (a), I/A (b), IL/AA (c) or ILF/AAA (d) mutants expressed without FXYD2 (closed squares); wild type or mutant α1 subunits expressed with FXYD2 (open squares). Data are means ± SE of of 11-40 oocytes from 3-6 different batches. *= p < 0.05.

Fig.7: F^{956} and/or E^{960} in TM9 of the Na,K-ATPase α1 subunit are involved in the functional effect of FXYD2, FXYD4 and FXYD7 on the apparent K^{+}-affinity of Na,K-ATPase. Oocytes were injected with cRNAs for wild type or mutant rat Na, K-ATPase α1 subunit (10 ng) and the rat β1 subunit (1 ng) together with cRNAs for FXYD2 (A), FXYD4 (B) or FXYD7 (C). Three days after cRNA injection, K_{1/2} K^{+} values of the Na,K-ATPase as a function of the membrane potential were determined. Wild type α1 subunit (A a, B a, C a), FE/AA (A b, B b, C b), F/A (A c, B c, C c) or E/A (A d, B d, C d) mutants expressed without FXYD protein (closed squares); wild type or mutant α1 subunits expressed with FXYD proteins (open squares), with FXYD2 (A), with FXYD4 (B) or with FXYD7 (C). Imax values were 323.5 ± 22.4 nA, 455.6 ± 23 nA, 252.3 ± 17.5 nA, and 262.7 ± 27 nA in oocytes injected with α/β, α/β/FXYD2, α/β/FXYD4 and α/β/FXYD7 cRNAs, respectively. Data are means ± SE of 9-40 oocytes from 2-6 different batches. *= p < 0.05.

Fig.8: F^{956} and/or E^{960} in TM9 of the Na,K-ATPase α1 subunit are not involved in the functional effect of FXYD2 and FXYD4 on the apparent Na^{+}-affinity of Na,K-ATPase. Oocytes were injected with cRNAs for wild type or mutant rat Na, K-ATPase α1 subunits (10 ng) and the rat β1 subunit (1 ng) alone or together with FXYD2 or FXYD4 (2 ng). After 2
days of incubation, oocytes were injected with rat epithelial Na\(^+\) channel (ENaC) cRNAs (0.5 ng \(\alpha\), \(\beta\), and \(\gamma\) subunit cRNAs). The measurement of the apparent affinity for internal Na\(^+\) of Na,K-ATPase was performed one day after injection of ENaC cRNA. Intracellular Na\(^+\)-activated Na, K-pump currents were recorded with 100 mM extracellular Na\(^+\) at \(-50\) mV. * = \(p < 0.05\); ** = \(p < 0.001\). Data are means \(\pm\) SE of 9-27 oocytes from 2-7 different batches.

Fig.9: Docking results. Left: Overview of the Na,K-ATPase \(\alpha\) subunit, showing TM2 and TM9, and two average structures from cluster A (green) and B (red). The location of the membrane is shown in transparent. Right: Detailed view of the interaction between TM9 (left) and TM2 (right) and two average structures of FXYD7, from cluster A (green) and B (red). Heavy atoms of residues involved in the interaction are shown in blue for the Na,K-ATPase \(\alpha\) subunit, in green or in red for cluster A and B.

Fig.10: Contact list derived from the docking calculations. See text for details.
**FIG. 1**

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Met1

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FIG. 1
Fig. 2

Figure 2

A

B

C

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Bar chart of FXYD2/α (arbitrary units)

Chase period (h)

48 72

FXYD2/α (arbitrary units)

0.0 2.0 4.0

Fig. 2
Fig. 3
Fig. 4
Fig. 5

A

K1/2 K+ (mM)

I max (nA)

-150 -100 -50 0 50

Membrane potential (mV)

B

K1/2 K+ (mM)

I max (nA)

-150 -100 -50 0 50

Membrane potential (mV)
Fig. 6
Fig. 7

A

WT

FE/AA

F/A

E/A

B

C

K1/2 K+ (mM)

Membrane potential (mV)

FXYD2

FXYD4

FXYD7

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Fig. 8

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K 1/2 Na⁺ (mM)
Extracellular space

Intracellular space

TM2 TM9

membrane

Na,K-ATPase - α subunit

F967
L964
E960
F956
I953

Q26
L968
M30
T27
E960
I41
F37
V38
I44
L968

I142
Y149
R156
I157
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residues
Structural and functional interaction sites between Na,K-ATPase and FXYD proteins
Ciming Li, Aurelien Grosdidier, Gilles Crambert, Jean-Daniel Horisberger, Olivier Michielin and Kaethi Geering

J. Biol. Chem. published online July 2, 2004

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