FXYD7, Mapping of Functional Sites Involved in Endoplasmic Reticulum Export, Association With and Regulation of Na,K-ATPase*

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The brain-specific FXYD7 is a member of the recently defined FXYD family that associates with the α1-β1 Na,K-ATPase isozyme and induces an about 2-fold decrease in its apparent K’ affinity. By using the Xenopus oocyte as an expression system, we have investigated the role of conserved and FXYD7-specific amino acids in the cellular routing of FXYD7 and in its association with and regulation of Na,K-ATPase. In contrast to FXYD2 and FXYD4, the studies on FXYD7 show that the conserved FXYD motif in the extracytoplasmic domain is not involved in the efficient association of FXYD7 with Na,K-ATPase. On the other hand, the conserved Gly10 and Gly29, located on the same face of the transmembrane helix, were found to be implicated both in the association with and the regulation of Na,K-ATPase. Mutational analysis of FXYD7-specific regions revealed the presence of an ER export signal at the end of the cytoplasmic tail. Deletion of a C-terminal valine residue in FXYD7 significantly delayed and decreased its O-glycosylation processing and retarded the rate of its cell surface expression. This result indicates that the C-terminal valine residue is involved in the rapid and selective ER export of FXYD7, which could explain the observed post-translational association of FXYD7 with Na,K-ATPase. In conclusion, our study on FXYD7 provides new information on structural determinants of general importance for FXYD protein action. Moreover, FXYD7 is identified as a new member of proteins with a regulated ER export, which suggests that, among FXYD proteins, FXYD7 has a particular regulatory function in brain.

The Na,K-ATPase uses the hydrolysis of ATP to exchange Na+ and K+ across the plasma membrane and to create and maintain the transmembrane Na+ and K+ gradients. The role of the Na,K-ATPase is essential for basic cellular homeostasis as well as for specialized tissue functions. In the kidney, for instance, the Na,K-ATPase is exclusively expressed in the basolateral membrane and thus becomes the driving force for net transepithelial Na+ reabsorption, which is necessary for the maintenance of the extracellular volume and blood pressure. In the nervous system, the presence of Na,K-ATPase permits a rapid restoration of the resting ionic conditions after action potentials and is thus of primary importance for a correct neuronal excitability.

At the molecular level, the Na,K-ATPase consists of an α- and a β-subunit. The catalytic α-subunit hydrolyzes ATP and transports the cations, and it also binds cardiac glycosides with high specificity. The β-subunit acts as a molecular chaperone, which plays a crucial role in the structural and functional maturation of the α-subunit (1). Several α and β isoforms (α4 and 3β) exist that may combine to form 12 possible α-β complexes with different transport and pharmacological properties (2, 3). Na,K-ATPase isoforms show a tissue-specific expression and, thus, may cope with different physiological demands.

The regulation of Na,K-ATPase activity is tight and complex and involves short and long term mechanisms mediated by hormones and neurotransmitters (for review see Ref. 4). Recently, a new regulatory mechanism has emerged, which involves association of the Na,K-ATPase with small type I membrane proteins of the newly defined FXYD protein family (5). This family contains 7 members that share a signature sequence consisting of the FXYD motif and 3 other conserved amino acid residues (for review see Ref. 6). We recently characterized FXYD7 (7), which is exclusively expressed in brain, both in neurons and glial cells. FXYD7 is subjected to O-glycosylation, which appears to be important for protein stability. Expressed in Xenopus oocytes, FXYD7 interacts with α1-β1, α2-β1, and α3-β1 isoforms, but not with α2-β2 Na,K-ATPase isoforms, whereas in the brain, FXYD7 can only be co-immunoprecipitated with an antibody against the α1-subunit and not with antibodies against the other α isoforms. FXYD7 modulates Na,K-ATPase transport properties in a different way than FXYD2 (the γ-subunit) (8, 9, 10), FXYD4 (CHIF) (8, 11), or FXYD1 (phospholemman) (12). After co-expression in Xenopus oocytes, FXYD7 has no effect on the apparent Na+ affinity of Na,K-ATPase but decreases the apparent K’ affinity over a wide range of membrane potentials, though it only affects α1-β1 and α2-β1 isoforms and not α3-β1 isoforms (7).

To better understand the regulatory effect on the Na,K-ATPase of FXYD7 proteins in general, and of FXYD7 in particular, we investigated, in the present study, the role of conserved and FXYD7-specific amino acids in FXYD7 properties, namely in its stability, its intracellular routing, its association with the Na,K-ATPase and in its functional effect on the apparent K’ affinity of Na,K-ATPase.

EXPERIMENTAL PROCEDURES

cDNAs—Mouse FXYD7 (7) served as a template for FXYD7 mutants (see Fig. 1). For truncated mutants (Nt-Δ15, Ct-Δ7, and ΔV80), a series of single PCR reactions was carried out with appropriate primers. PCR products were then digested and subcloned between EcoRI and NotI restriction sites into the pSd5 vector. Point mutations were introduced by using the PCR method described by Nelson and Long (13). Nt-FLAG

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Structure-Function Relationship in FXYD7

FXYD7 and Na,K-ATPase were constructed by PCR using a sense primer tailed with the appropriate sequence coding for the Na,K-ATPase (DYKDDDDK) placed upstream of the first ATG and the appropriate antisense primer. The nucleotide sequences of all constructs were confirmed by DNA sequencing. cDNAs for rat α1- and β1-subunits were kindly provided by J. Lingrel. All cDNAs were introduced into the pSD5 vector. CRNAs were prepared as in vitro translation (14).

Protein Expression in Xenopus Laevis Oocytes——Stage V–VI oocytes were obtained from Xenopus laevis as previously described (15). cRNAs coding for wild-type or mutant FXYD7 (2 ng/oocyte) were injected into oocytes in the presence or absence of cRNAs coding for the rat Na,K-ATPase α1 (10 ng/oocyte) and β1 (1 ng/oocyte) subunits as described in the figure legends. Under these conditions, wild-type, or mutant FXYD7 is expressed in excess of exogenous Na,K-ATPase and is not limiting for the association with Na,K-ATPase. To study protein expression and association, oocytes were incubated in modified Barth solution (MBS)1 containing 10 mM cold methionine. After the pulse and chase periods, oocyte extracts were prepared similar to the wild-type FXYD7 (Fig. 2). After expression in Xenopus oocytes, we tested the stability of these mutants, their processing, their association with the α1β1 Na,K-ATPase isozyme and their functional effect on the apparent K⁺ affinity of Na,K-ATPase.

RESULTS

Effects of Mutations in the N-terminal Extracellular Domain of FXYD7——The N-terminal extracellular domain of FXYD7 shows no homology to that of other FXYD proteins with the exception of the preserved conserved FXYD motif (FYFYDD) in FXYD7. To determine the role of conserved and FXYD7-specific regions in the N terminus of FXYD7, we produced an AAFA mutant lacking the FYFYDD motif, a Na-3T/A mutant lacking the 3 threonines, which are potential sites of O-glycosylation (7), and an N-terminally-truncated mutant (Nt–Δ15) (see Fig. 1). After expression in Xenopus oocytes, we tested the stability of these mutants, their processing, their association with the α1β1 Na,K-ATPase isozyme and their functional effect on the apparent K⁺ affinity of Na,K-ATPase.

As shown in Fig. 2, wild-type, metabolically labeled FXYD7 expressed in Xenopus oocytes without Na,K-ATPase was processed from a core protein of about 14 kDa, observed exclusively during a 6-h pulse period, to an O-glycosylated doublet of 18–19 kDa, quite stably expressed during a 24- and 48-h chase period (Fig. 2, A, lanes 1–3, and B). The AAFA mutant was processed similar to the wild-type FXYD7 (Fig. 2A, lanes 10–12). As previously described (7), the Na-3T/A mutant lacking the O-glycosylation sites Thr3, Thr4, and Thr9 was degraded during a 48-h chase period (Fig. 2, A, lanes 4–6, and C) indicating a role of O-glycosylation in the stability of FXYD7. Interestingly, truncation of 15 N-terminal amino acids of FXYD7 in a N-Δ15 mutant partially restored the stability of these mutants, their processing, their association with the α1β1 Na,K-ATPase isozyme and their functional effect on the apparent K⁺ affinity of Na,K-ATPase.

FXYD7 and Na,K-ATPase ΔV80 were constructed by PCR using a sense primer. The nucleotide sequences of all constructs were confirmed by DNA sequencing. cRNAs for rat α1- and β1-subunits were kindly provided by J. Lingrel. All cDNAs were introduced into the pSD5 vector. CRNAs were prepared as in vitro translation (14).

Protein Expression in Xenopus Laevis Oocytes——Stage V–VI oocytes were obtained from Xenopus laevis as previously described (15). cRNAs coding for wild-type or mutant FXYD7 (2 ng/oocyte) were injected into oocytes in the presence or absence of cRNAs coding for the rat Na,K-ATPase α1 (10 ng/oocyte) and β1 (1 ng/oocyte) subunits as described in the figure legends. Under these conditions, wild-type, or mutant FXYD7 is expressed in excess of exogenous Na,K-ATPase and is not limiting for the association with Na,K-ATPase. To study protein expression and association, oocytes were incubated in modified Barth solution (MBS)1 containing 10 mM cold methionine. After the pulse and chase periods, oocyte extracts were prepared similar to the wild-type FXYD7 (Fig. 2). After expression in Xenopus oocytes, we tested the stability of these mutants, their processing, their association with the α1β1 Na,K-ATPase isozyme and their functional effect on the apparent K⁺ affinity of Na,K-ATPase.

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The ability of wild-type and mutant FXYD7 to associate with the α1β1 Na,K-ATPase isozyme was tested after co-expression in Xenopus oocytes and by immunoprecipitation under non-denaturing conditions with a FXYD7 antibody. Our results

1 The abbreviations used are: MBS, modified Barth's solution; ER, endoplasmic reticulum; TGF, transforming growth factor; wt, wild type; Tricine, N-[2-hydroxy-1-bis(hydroxymethyl)ethyl]glycine.
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. Digitonin extracts were prepared after the pulse and the chase periods, and immunoprecipitation was performed in denaturing conditions using an anti-FXYD7 antibody. Immunoprecipitates were separated on Tricine-polyacrylamide gels. B, kinetics of O-glycosylation of FXYD7. The 18- and 19-kDa bands shown in A (lanes 1–3) were quantitated after the pulse and the chase periods. The amount of O-glycosylated FXYD7 after a 6-h pulse was arbitrarily set to 1. Shown are means ± S.E. of six experiments. C, degradation of Nt-3T/A and Nt-Δ15 mutants. The amounts of Nt-3T/A (triangles) and Nt Δ15 (circles) quantitated after a 6-h pulse were arbitrarily set to 1. Shown are means ± S.E. of five experiments.

indicate that association of wild-type FXYD7 with α1-β1 complexes is time-dependent and occurs post-translationally. Indeed, despite a similar expression level of the α-subunit after the pulse and the chase periods, as revealed by immunoprecipitation with an α antibody (Fig. 3B, lanes 1–3), the amount of α-subunit co-immunoprecipitated with a FXYD7 antibody (Fig. 3A, lanes 1–3, and C) increased progressively between the 6-h pulse and the 48-h chase. Association of the AFAA mutant with the α-subunit proceeded similar to that of the wild-type FXYD7 (Fig. 3A, lanes 10–12 and C) indicating that the FXYD motif does not play a role in the efficient interaction of FXYD7 with the Na,K-ATPase. As previously shown (7), the Nt-3T/A mutant (Fig. 3A, lanes 4–5 and C) associated with Na,K-ATPase at a level similar to wild-type FXYD7 and became stabilized, suggesting that lack of O-glycosylation does not prevent the post-translational association of FXYD7 with the Na,K-ATPase. In contrast, truncation of 15 N-terminal amino acids in the Nt-Δ15 mutant decreased significantly the amount of α-subunit co-immunoprecipitated with FXYD7 antibodies (Fig. 3, A, lanes 7–9 and C) indicating that structural integrity of the N terminus is required for efficient association with Na,K-ATPase.

As assessed by electrophysiological means, oocytes expressing Na,K-ATPase alone or together with wild-type FXYD7, with the Nt-3T/A mutant, the Nt-Δ15 mutant, or the AFAA mutant showed similar Na,K-pump currents (Table I) indicating that these N-terminal FXYD mutants do not influence the expression of functional Na,K-ATPase at the cell surface. As previously described (7), we observed an about 50% decrease in the apparent affinity for K+ of the Na,K-ATPase associated with wild-type FXYD7 (Fig 3D and Table I). A similar effect was produced by the Nt-3T/A and the AFAA mutant as well as by the less efficiently associated Nt-Δ15 mutant. This latter result can be explained by the assumption that the co-immunoprecipitation experiment performed in the presence of digitonin does not reflect the actual proportion of complexes between Na,K-ATPase and the Nt-Δ15 and AFAA 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. Altogether, the results suggest that the N-terminal, extracellular domain, including the conserved FXYD motif and the sugar moiety, is not implicated in the functional effect of FXYD7 on the Na,K-ATPase.

Fig. 2. Role of structural determinants in the N-terminal domain in the processing and stability of FXYD7. A, 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. Digitonin extracts were prepared after the pulse and the chase periods, and immunoprecipitation was performed in denaturing conditions using an anti-FXYD7 antibody. Immunoprecipitates were separated on Tricine-polyacrylamide gels. B, kinetics of O-glycosylation of FXYD7. The 18- and 19-kDa bands shown in A (lanes 1–3) were quantitated after the pulse and the chase periods. The amount of O-glycosylated FXYD7 after a 6-h pulse was arbitrarily set to 1. Shown are means ± S.E. of six experiments. C, degradation of Nt-3T/A and Nt-Δ15 mutants. The amounts of Nt-3T/A (triangles) and Nt Δ15 (circles) quantitated after a 6-h pulse were arbitrarily set to 1. Shown are means ± S.E. of five experiments.

Fig. 3. Role of structural determinants in the N-terminal domain in the association with Na,K-ATPase and in the functional effect of FXYD7. A and B, 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. Oocytes were metabolically labeled, and digitonin extracts were prepared as described in the legend to Fig. 2. Non-denaturing immunoprecipitations were performed using an anti-FXYD7 antibody (A) or an anti-α antibody (B). Immunoprecipitates were separated on SDS-polyacrylamide gels. C, quantification of α-subunit co-immunoprecipitated with wild-type FXYD7 (squares), the Nt-3T/A (diamonds), the Nt-Δ15 mutant (circles), or the AFAA mutant (triangles) with a FXYD7 antibody. The amount of α-subunit co-immunoprecipitated was corrected for the expression level of the α-subunit shown in B. The amount of α-subunit co-immunoprecipitated with wild-type FXYD7 after a 6-h pulse was arbitrarily set to 1. Shown are means ± S.E. of nine experiments for wild-type FXYD7, four experiments for the Nt-3T/A, three experiments for the Nt-Δ15 mutant, and five experiments for the AFAA mutant. D, three days after injection of rat α1 and β1 cRNAs in the presence or not of wild-type or mutant FXYD7, the external K+ activation constant (Ko1) of Na,K-ATPase was determined in the presence of 90 mM external Na+ at a holding potential of −50 mV as described under “Experimental Procedures.” Results are means of 8–10 oocytes from 2–3 batches of oocytes.
Effects of Mutations in the Transmembrane Domain of FXYD7—The transmembrane helix is the most conserved domain among FXYD proteins. With the exception of a conserved serine residue (Ser46 in FXYD7), which marks the putative end of the transmembrane domain, invariant residues such as the 2 conserved glycine residues (Gly29 and Gly40 in FXYD7) and nearly invariant amino acids in FXYD proteins are clustered on one side of the transmembrane helix (5).

Alanine replacement of the conserved glycine residue Gly29 or the conserved serine residue Ser46 did not change the stability or the processing of FXYD7 expressed in oocytes (Fig. 4A, compare lanes 1–3 to lanes 4–6 and lanes 16–18).

Mutation of Gly41 in FXYD2 (Gly40 in FXYD7) into an arginine (G40R), a positively charged residue. All of these mutants showed stability and processing similar to wild-type FXYD7 (Fig. 4A, compare lanes 16–18). On the other hand, alanine replacement of the conserved glycine (G29A or G40A) significantly decreased the association efficiency of FXYD7. Indeed, despite a similar expression level of the α-subunit (Fig. 4C, lanes 1–9), the amount of α-subunit co-immunoprecipitated with a FXYD7 antibody was considerably lower throughout the pulse/chase periods in oocytes expressing the G40A (Fig. 4, B, lanes 7–9 and D) or the G29A mutant (Fig. 4, B, lanes 4–6 and D) than in oocytes expressing wild type FXYD7 (Fig. 4, B, lanes 1–3 and D). Interestingly, replacement of Gly41 by tryptophan instead of alanine further decreased the association efficiency (Fig. 4B, lanes 13–15) and replacement by arginine completely abolished association with Na,K-ATPase (lanes 10–12).

Analysis of the functional effect of these mutants on the Na,K-ATPase transport properties showed that the efficiently associated mutant S46A produced an effect on the K+ activation of the Na,K-ATPase similar to wild-type FXYD7 (Fig. 5 and Table I). On the other hand, the glycine mutants that were impeded in their association efficiency produced no (G29A) or a partial effect (G40A and G40W) on the apparent K+ affinity (Fig. 5 and Table I). Interestingly, though the G40R mutant did not associate with the Na,K-ATPase (Fig. 4B, lanes 10–12) and...
did not impede expression of stable Na,K-ATPase (Fig. 4C, lanes 10–12), its presence led to premature death of some oocytes or to significantly reduced Na,K-pump currents in surviving oocytes. This excluded reliable measurements of the apparent \( K_p \) affinity. At present, we do not know the reasons for the deleterious effect on cell viability of the G40R mutant.

Altogether, these results point to the importance of the transmembrane domain of FXYD7, and in particular of the conserved glycine residues, both in the association with the Na,K-ATPase and in the modulation of its transport properties.

**Effects of Mutations in the C-terminal, Cytosolic Domain of FXYD7**—Like the extracellular domain, the cytosolic domain of FXYD7 is very distinct from that of other FXYD proteins. For our structure-function analysis, we concentrated on two distinctive sequence motifs namely a 56SRSES60 sequence, which contains 3 putative phosphorylation sites as predicted by the NetPhos 2.0 program (20), and a 79PGGGGV60 sequence at the C-terminal end of the protein (see Fig. 1).

FXYD7 in which Ser56, Ser58, and Ser60 were replaced by alanine (Ct-3S/A, abolishment of the phosphorylation sites) or by aspartic acid (Ct-3S/D, mimicry of phosphorylation) showed a stability and processing similar to wild-type FXYD7 (Fig. 6A, compare lanes 1–3 to lanes 7–12). Both mutants associated with Na,K-ATPase (Fig. 6B compare lanes 1–3 and lanes 7–12) and produced a functional effect similar to wild-type FXYD7 (Fig. 6D and Table I). Further experiments are necessary to investigate the possible functional role of phosphorylation in FXYD7.

The putative role of the 79PGGGGV60 sequence was assessed in a mutant lacking the seven last C-terminal amino acids (CtΔ7). Truncation of the PGGGGV sequence significantly delayed and decreased the processing of FXYD7 expressed in oocytes. Whereas part of the newly synthesized, wild-type FXYD7 population became glycosylated already during the 6-h pulse period (Fig. 6A, lane 1) and the total population after a 24-h chase period (lane 2), the CtΔ7 mutant was not processed during the pulse (lane 4), and only part of the newly synthesized CtΔ7 population was glycosylated after a 24-h chase period (compare lane 4 to lane 5). The remaining non-glycosylated CtΔ7 species were degraded during the chase period (lanes 4–6, 12-kDa band). Since O-glycosylation mainly occurs in an early Golgi compartment (for review see Ref. 21), this result indicates that the CtΔ7 mutant exit less rapidly from the ER than wild-type FXYD7. Association of Na,K-ATPase with the CtΔ7 mutant was somewhat less efficient than with wild-type FXYD7 (Fig. 6B, lanes 1–6). Interestingly, in oocytes co-expressing exogenous Na,K-ATPase, the proportion of O-glycosylated CtΔ7 increased after a 24- and a 48-h chase (compare Fig. 6, A and B, lanes 4–6) indicating that association of CtΔ7 with Na,K-ATPase abolishes the retention effect of the mutation.

In order to define more precisely the amino acids involved in the delayed processing of CtΔ7, we prepared a mutant in which the PGGGG motif was replaced by alanines but which still contained the terminal valine residue (Ct-P4G/A). As shown in Fig. 7A (lanes 16–18), this mutant, when expressed alone in oocytes, exhibited a time course of processing similar to wild-type FXYD7 (lanes 1–3) indicating that the terminal valine residue and not the PGGGG motif is involved in the normal processing of FXYD7. Indeed, deletion of the terminal valine (Val80) led to a similar delay in FXYD7 processing (Fig. 7A, lane 9) than that observed with the CtΔ7 mutant (Fig. 7A, lanes 4–6). Interestingly, replacement of Val80 by another hy-
48 h after cRNA injection, intact oocytes were subjected to a radioimmunolabeling assay using an 125I-labeled FLAG antibody as described under Experimental Procedures. Immunoprecipitates were separated on Tricine-polyacrylamide gels. Oocytes were injected with N-terminally epitope-flagged wild-type FXYD7 (black bars) or a ΔV80 mutant (white bars). 3, 6, 24, and 48 h after cRNA injection, intact oocytes were subjected to a radioimmunolabeling assay using an 125I-labeled FLAG antibody as described under "Experimental Procedures." Results are means ± S.E. from 10 oocytes from 5 batches of oocytes. In each experiment, the maximal 125I anti-FLAG binding (B_{max}) was determined by fitting of the binding curve, and the percentage of binding at each time point was calculated. B_{max}: wild-type FXYD7, 325 ± 17 cpm; ΔV80 mutant, 354 ± 44 cpm. **, p < 0.01.

**Fig. 7.** The C-terminal valine of FXYD7 acts as an ER exit signal. A, 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. Digitonin extracts were prepared and used for immunoprecipitation in denaturing conditions using an anti-FXYD7 antibody. Immunoprecipitates were separated on Tricine-polyacrylamide gels. B, oocytes were injected with N-terminally epitope-flagged wild-type FXYD7 (black bars) or a ΔV80 mutant (white bars). 3, 6, 24, and 48 h after cRNA injection, intact oocytes were subjected to a radioimmunolabeling assay using an 125I-labeled FLAG antibody as described under "Experimental Procedures." Results are means ± S.E. from 10 oocytes from 5 batches of oocytes. In each experiment, the maximal 125I anti-FLAG binding (B_{max}) was determined by fitting of the binding curve, and the percentage of binding at each time point was calculated. B_{max}: wild-type FXYD7, 325 ± 17 cpm; ΔV80 mutant, 354 ± 44 cpm. **, p < 0.01.

drophobic amino acid (V80A, lanes 10–12) but not by a charged amino acid (V80D, lanes 13–15), restored rapid processing of FXYD7. Finally, to substantiate that Val80 contributes to ER exit of FXYD7, we compared the kinetics of cell surface appearance of epitope-flagged wild-type FXYD7 and of the ΔV80 mutant by radioimmunooassays with anti-FLAG antibodies on intact oocytes. As shown in Fig. 7B, 3 and 6 h after cRNA injection, the cell surface expression of the ΔV80 mutant was significantly lower than that of wild-type FXYD7 though both proteins reached a maximal expression level after 24 h. Altogether these results indicate that the presence of a valine at the end of the intracellular domain is involved in the control of ER exit of FXYD7 and determines the rate of its cell surface expression.

**DISCUSSION**

The brain-specific FXYD7 belongs to the FXYD protein family and was shown to associate with and regulate Na,K-ATPase transport properties (7). FXYD7 is a small type I protein with three distinct parts, a N-terminal extracellular domain, a transmembrane domain and a C-terminal intracellular domain. In this study, we reveal structural determinants in each of these domains that are functionally relevant either for FXYD7 in particular or for FXYD proteins in general. FXYD7-specific parts of the extracellular domain are important for its stability, conserved amino acids in extracellular and the transmembrane domain are involved in the association, probably of all FXYD proteins, with the Na,K-ATPase, and, finally, the intracellular domain contains FXYD7-specific determinants involved in the intracellular routing of FXYD7.

**Determinants of the Stability and Intracellular Routing of Newly Synthesized FXYD7**—Our previous observation that mutations of threonines implicated in O-glycosylation leads to degradation of FXYD7 (7) indicates that O-linked sugar chains in the N terminus of FXYD7 contribute to its stabilization. Since O-glycosylation is a post-translational event that mainly occurs in different Golgi compartments (for review see Ref. 21), it is difficult to conceive how it can protect FXYD7 from the ER quality control system involved in the recognition and degradation of improperly processed or misfolded proteins (for review see Ref. 22). However, it may be postulated that newly synthesized FXYD7 recycles between the ER and an early Golgi compartment, and only escapes ER degradation if glycosylation occurs during its presence in the Golgi, which impedes retrograde transport to the ER and permits further routing to the plasma membrane. Significantly, association with Na,K-ATPase prevents the degradation of unglycosylated FXYD7 (Ref. 7 and this study). Since the association process most likely occurs in a post-ER compartment (see below), it may be inferred that association with Na,K-ATPase impedes retrograde transport of unglycosylated FXYD7 to the ER and thus prevents its degradation. In this study, we also show that truncation of 15 N-terminal amino acids partially protects FXYD7 from degradation. This observation may indicate that a degradation signal exists in the N-terminal part of FXYD7, which is attenuated in the truncated mutant or that this mutant has partially lost its ability to recycle to the ER.

In contrast to FXYD2, which is rapidly degraded in the ER if not associated with Na,K-ATPase (23), FXYD7 becomes stabilized by O-glycosylation in the Golgi and can be routed to the plasma membrane as an individual protein. In this study, we have identified a C-terminal valine residue which may be critically involved in the rapid exit from the ER e.g. from the site where newly synthesized and not yet O-glycosylated FXYD7 are degraded. Indeed, we observe that deletion of the most C-terminal valine residue leads to a delay in O-glycosylation and to the retention and degradation of an important part of the newly synthesized FXYD7 population in the ER. Mutation of the preceding Pro-Gly-Gly-Gly-Gly sequence into Pro-Ala-Ala does not abolish the functional effect of the C-terminal valine residue indicating that it works independently. It can, however, not entirely be excluded that the Pro-Ala-Ala sequence still has characteristics of a flexible spacer that may be necessary for proper functioning of the valine residue.

It has long been believed that soluble and membrane-bound proteins exit nonselectively from the ER and are packaged by default into transport vesicles (24) destined for the ER-Golgi intermediate compartment (25). Increasing experimental evidence supports an alternate mechanism (26), which predicts
that at least some proteins contain ER-export motifs that selectively concentrate these proteins into vesicles during the budding process. In certain proteins, diphenylalanine or diacidic motifs were shown to be required for selective ER export or efficient ER to Golgi transport. A similar role has been attributed to a C-terminal valine residue in proTGFα, MT1-MMP (27), and CD8 (28) with a more or less stringent effect on the ER exit process depending on the protein. By screening of databases, a single C-terminal valine residue was found in 9.8% of 488 human type I membrane proteins which is 1.7-fold higher than theoretically expected from the frequency of valine in proteins (29). Moreover, several polytopic membrane proteins such as plasma membrane Ca-ATPase, band 3 anion exchanger (30), various potassium channels etc have a putative valine signal. These results are consistent with the notion that regulation of ER export by C-terminal valine residues is a widely used mechanism.

At present, is now known how the C-terminal valine residue produces its functional effect. It has been suggested that, similar to other transport signals, C-terminal valine residues interact with COPII proteins which coat vesicles involved in ER export (29). On the other hand, C-terminal valine residues are often part of a motif, which determines interaction with PDZ binding domain containing proteins (31). Significantly, two hybrid screens using the C-terminal tail of proTGFα have indeed identified a specific interacting protein TACIP18, which is a PDZ protein (32). TACIP18 is located in initial compartments of the secretory pathway, in contrast to other members of the PDZ protein family, which interact with membrane proteins close or at the plasma membrane. TACIP18 interaction depends on the C-terminal valine residue in proTGFα but otherwise has a more relaxed specificity than other known PDZ proteins (32). Significantly, the PGGGV sequence present in FXYD7 does not belong to the classical PDZ binding motifs. Thus it remains to be established whether it represent a new class of such motifs.

More experimental work is needed to determine the mechanism of the ER export control of FXYD7 dependent on the C-terminal valine residue, and to identify its functional importance. Significantly, only FXYD7 but no other member of the FXYD family has an N-terminal valine residue suggesting that the role of an ER export control may be linked to the specific expression of FXYD7 in neurons and glial cells and/or to the particular requirements of the regulation of Na,K-ATPase expression and function in the brain.

Determinants in FXYD7 That Are Involved in the Interaction with and the Regulation of Na,K-ATPase Expression and Function—Up to now, the only functional role ascribed to FXYD7 is the isozyme-specific regulation of the cerebral Na,K-ATPase. As other Na,K-ATPase regulators of the FXYD family, FXYD7 mediates this function by direct and specific interactions with the α-β complex. The precise domain(s) or amino acids in FXYD proteins that are directly involved in the interaction with Na,K-ATPase are still unknown. Recent studies suggest that, both in FXYD2 and FXYD4, the transmembrane domain is mainly involved in the stable interaction with Na,K-ATPase (19, 33) though in FXYD2 the cytoplasmic C terminus may also play a role in this process (8). In the present study, we confirm recent observations (19) that the conserved Gly40, which is associated with a form of renal hypomagnesemia when mutated into an arginine residue in FXYD2 (18), is involved in Na,K-ATPase-FXYD protein interaction. Indeed, replacement of Gly40 in FXYD7 by Ala, Trp, or Arg progressively decreases its association with Na,K-ATPase. We also show that the second conserved Gly29 in the transmembrane domain, which is located on the same face of the TM helix (5), plays a critical role for efficient association. Finally, our mutational analysis of FXYD7 reveals that, in contrast to our previous observations made with FXYD2 and FXYD4 (8), the FXYD motif in the extracellular domain of FXYD7 does not affect the stability and/or the efficiency of the interaction with Na,K-ATPase.

As suggested by recent experimental evidence (33), the amino acids in FXYD proteins that are involved in the stable interaction with the Na,K-ATPase may not be the same as those involved in the functional effect, though they are also concentrated in the transmembrane domain. In our study, this could be reflected by the observation that some FXYD7 mutants affecting the N-terminal domain indeed decrease association efficiency in detergent but still produce the same functional effect on the K+ kinetics of Na,K-ATPase as wild type FXYD7. Nevertheless, a clear distinction between interaction sites involved in the stable association or in a specific functional effect is not obvious in all cases. Based on experiments with mimetic peptides, it has indeed been proposed that Gly41 (corresponding to Gly40 in FXYD7) is not primarily involved in Na,K-ATPase interaction but rather mediates the effect on the Na+ kinetics of FXYD2 (34). Knowing that FXYD7 has no effect on the apparent Na+ affinity of Na,K-ATPase, this result is difficult to reconcile with our results which show that a G40A mutant of FXYD7, decreases the association efficiency and completely abolishes the K+ effect. Obviously, interactions of Na,K-ATPase with single, conserved amino acids cannot explain the diverse functional effects of FXYD7 proteins.

In addition to structural information, our study also provides insight into dynamic aspects of the association process of FXYD7 with Na,K-ATPase. First, our results indicate that similar to phospholemman (12), efficient association of FXYD7 occurs post-translationally. At present it is not known in which compartment of the secretory pathway association takes place. Since we observe very inefficient interaction of Na,K-ATPase with newly synthesized non-glycosylated FXYD7, we can exclude the ER as an important cellular site of interaction. On the other hand, we observe that FXYD7, mutated in the glycosylation sites, becomes rapidly stabilized by the interaction with Na,K-ATPase. This is consistent with an association compartment close to the ER, for instance an early Golgi compartment. However, we cannot, however, exclude that association with Na,K-ATPase could occur even in later transport compartments or at the plasma membrane. What is the reason for the post-translational association of FXYD7 with Na,K-ATPase? In view of the presence of an ER-export signal in FXYD7, it is conceivable that Na,K-ATPase and FXYD7 are sorted into different transport vesicles in the ER and therefore exit the ER separately and with different rates.

In conclusion, we have further characterized one of the FXYD proteins, which act as tissue-specific regulators of Na,K-ATPase. Our results on FXYD7 provide information on structural determinants that are relevant for the mechanism of action of all FXYD proteins but also on FXYD7-specific structural and functional properties. Future experiments will be directed to show the physiological relevance of these FXYD7-specific characteristics in the functional context of cells in which FXYD7 is expressed.

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REFERENCES
1. Geering, K. (2001) J. Bioenerg. Biomembr. 33, 425–438
2. Blanco, G., and Mercer, R. W. (1998) Am. J. Physiol. 275, F633–F650
3. Crumbbert, G., Hasler, U., Beggah, A.-T., Yu, C., Modyanov, N. N., Hornisberger, J. D., Leleivre, L., and Geering, K. (2000) J. Biol. Chem. 275, 1976–1986
4. Therien, A. G., and Blustein, R. (2000) Am. J. Physiol. 279, C541–C566
5. Sweadner, K. J., and Rabl, E. (2000) Genomics 68, 41–56
6. Crumbbert, G., and Geering, K. (2003) Sci. STKE 166, RE1
7. Béguin, P., Crumbbert, G., Monnet-Tschudi, F., Udly, M., Hornisberger, J.-D.,
Garty, H., and Geering, K. (2002) EMBO J. 21, 3264–3273
8. Béguin, P., Crambert, G., Guennoun, S., Garty, H., Horisberger, J.-D., and Geering, K. (2001) EMBO J. 20, 3993–4002
9. Pu, H. X., Cluzeaud, F., Goldshleger, R., Karlish, S. J. D., Farman, N., and Blostein, R. (2001) J. Biol. Chem. 276, 20370–20378
10. Arystarkhova, E., Wetzel, R. K., Asinovski, N. K., and Sweadner, K. J. (1999) J. Biol. Chem. 274, 33183–33185
11. Garty, H., Lindzen, M., Scanzano, R., Aizman, R., Fuzesi, M., Goldshleger, R., Farman, N., Blostein, R., and Karlish, S. J. D. (2002) Am. J. Physiol. 283, F607–F615
12. Crambert, G., Fuzesi, M., Garty, H., Karlish, S., and Geering, K. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 11476–11481
13. Nelson, R. M., and Long, G. L. (1989) Anal. Biochem. 180, 147–151
14. Melton, D. A., Krieg, P. A., Rebagliati, M. R., Maniatis, T., Zinn, K., and Green, M. R. (1984) Nucleic Acids Res. 12, 7035–7056
15. Geering, K., Beggah, A., Good, P., Girardet, S., Roy, S., Schaer, D., and Jaunin, P. (1996) J. Cell Biol. 133, 1193–1204
16. Girardet, M., Geering, K., Frantes, J. M., Geser, D., Rossier, B. C., Kraehenbuhl, J.-P., and Bron, C. (1981) Biochem. J. 20, 6684–6691
17. Jaisser, F., Jaunin, P., Geering, K., Rossier, B. C., and Horisberger, J. D. (1994) J. Gen. Physiol. 103, 605–623
18. Mei, I. C., Koenderink, J. B., van Bokhoven, H., Assink, K. F., Grootenstege, W. T., de Pont, J. J., Bindels, R. J., Monnens, L. A., van den Heuvel, L. P., and Knors, N. V. (2000) Nat. Genetics 26, 265–266
19. Pu, H. X., Scanzano, R., and Blostein, R. (2002) J. Biol. Chem. 277, 20270–20276
20. Blom, N., Gammeltoft, S., and Brunak, S. (1999) J. Mol. Biol. 294, 1351–1362
21. Van den Steen, P., Rudd, P. M., Dwek, R. A., and Opdenakker, G. (1998) Crit. Rev. Biochem. Mol. Biol. 33, 151–208
22. Trombetta, E. S., and Parodi, A. J. (2003) Annu. Rev. Cell. Dev. Biol. 19, 649–676
23. Béguin, P., Wang, X. Y., Firsov, D., Puoti, A., Claeyss, D., Horisberger, J. D., and Geering, K. (1997) EMBO J. 16, 4256–4260
24. Rothman, J. E. (1994) Nature 372, 55–63
25. Hauri, H., Appenzeller, C., Kohn, F., and Nufer, O. (2000) FEBS Lett. 467, 32–37
26. Lodish, H. F. (1988) J. Biol. Chem. 263, 2107–2110
27. Urena, J., Merlos-Suarez, A., Baselga, J., and Arribas, J. (1999) J. Cell Sci. 112, 773–784
28. Nufer, O., Goldbrantsen, S., Degen, M., Kappeler, F., Paccaud, J. P., Tani, K., and Hauri, H. P. (2002) J. Cell Sci. 115, 619–628
29. Cordat, E., Li, J., and Reithmeier, R. A. (2003) Traffic 4, 642–651
30. Zouzoulas, A., Therien, A. G., Scanzano, R., Deber, C. M., and Blostein, R. (2003) J. Biol. Chem. 278, 18738–18743
31. Lindzen, M., Aizman, R., Lifshitz, Y., Lubarski, I., Karlish, S. J. D., and Garty, H. (2003) J. Biol. Chem. 278, 18738–18743
32. Fernandez-Larrea, J., Merlos-Suarez, A., Urena, J. M., Basela, J., and Arribas, J. (1999) Mol. Cell 3, 423–433
33. Lindzen, M., Aizman, R., Lifshitz, Y., Lubarski, I., Karlish, S. J. D., and Garty, H. (2003) J. Biol. Chem. 278, 18738–18743
34. Zougazas, A., Therien, A. G., Scanzano, R., Deber, C. M., and Blostein, R. (2003) J. Biol. Chem. 278, 40437–40441
