Interaction of FXYD10 (PLMS) with Na,K-ATPase from Shark Rectal Glands

CLOSE PROXIMITY OF Cys\(^{74}\) OF FXYD10 TO Cys\(^{254}\) IN THE A DOMAIN OF THE \(\alpha\)-SUBUNIT REVEALED BY INTERMOLECULAR THIOL CROSS-LINKING\(^{*}\)

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FXYD domain-containing proteins are tissue-specific regulators of the Na,K-ATPase that have been shown to have significant physiological implications. Information about the sites of interaction between some FXYD proteins and subunits of the Na,K-ATPase is beginning to emerge. We previously identified an FXYD protein in plasma membranes from shark rectal gland cells and demonstrated that this protein (FXYD10) modulates shark Na,K-ATPase activity. The present study was undertaken to identify the location of the C-terminal domain of FXYD10 on the \(\alpha\)-subunit of Na,K-ATPase, using covalent cross-linking combined with proteolytic cleavage. Treatment of Na,K-ATPase-enriched membranes with the homobifunctional thiol cross-linker 1,4-bismaleimido-2,3-dihydroxybutane resulted in cross-linking of FXYD10 to the \(\alpha\)-subunit. Cross-linking was not affected by preincubation with sodium or potassium but was significantly reduced after pre-incubation with the non-hydrolyzable ATP analog \(\beta\),\(\gamma\)-methyleneadenosine 5'-triphosphate (AMP-PCP). A peptic assay was developed, in which pepsin treatment of Na,K-ATPase at low pH resulted in extensive cleavage of the \(\alpha\)-subunit while FXYD10 was left intact. Proteolytic fragments of control and cross-linked preparations were isolated by immunoprecipitation and analyzed by gel electrophoresis. A proteolytic fragment containing FXYD10 cross-linked to a fragment from the \(\alpha\)-subunit could be localized on SDS gels. Sequencing of this fragment showed the presence of FXYD10 as well as a fragment within the A domain of the \(\alpha\)-subunit comprising 33 amino acids, including a single Cys residue, Cys\(^{254}\). Thus, regulation of Na,K-ATPase by FXYD10 occurs in part via cytoplasmic interaction of FXYD10 with the A domain of the shark \(\alpha\)-subunit.

The Na,K-ATPase is a member of the \(P_{\text{2}}\)-type ATPases superfamily, which also includes gastric H,K-ATPase and sarcoplasmic reticulum Ca\(^{2+}\)-ATPase (SERCA)\(^{1}\). The Na,K-ATPase is a heterodimeric membrane protein that pumps three sodium ions out of and two potassium ions into the cell, thereby establishing and maintaining the ion gradients for Na\(^+\) and K\(^+\) essential for cellular homeostasis. The enzyme consists of a catalytic \(\alpha\)-subunit that contains substrates and inhibitor binding sites and a glycosylated \(\beta\)-subunit that is important for proper expression and function of the overall Na,K-ATPase activity (2).

Recently, considerable attention has been directed at studying the role of pump-interacting proteins in the regulation of Na,K-ATPase activity. In kidney, the kinetic properties of Na,K-ATPase are modulated by interaction with the \(\gamma\)-subunit (Refs. 3–9 and see Ref. 10 for review). The \(\gamma\)-subunit (FXYD2) is a member of the FXYD family that includes seven mammalian members (11). To date, five of these members have been shown to regulate Na,K-ATPase in different tissues, mainly by modulating the apparent ion affinity of the pump (Refs. 12–18 and see Refs. 19–21 for reviews). The regulation of Na,K-ATPase by interaction with FXYD proteins is tissue-specific as evidenced from their heterogeneous expression patterns and tissue distribution. Of particular interest is the fact that interaction of the different FXYD family members with the \(\alpha\)-subunit at the cytoplasmic face does not lead to a unique functional effect on Na,K-ATPase (20), possibly owing (in part) to the significant dissimilarities found in the C-terminal domains of these proteins (11, 20).

We have previously identified and characterized an FXYD protein called FXYD10 (PLMS) that associates with and modifies the activity of shark Na,K-ATPase in vitro through interactions mediated by its transmembrane and cytoplasmic C-terminal domains (22). The functional effects of the C-terminal domain of FXYD10 on shark Na,K-ATPase were studied by using a preparation of Na,K-ATPase in which the C-terminal domain of FXYD10 is specifically cleaved by mild trypsin treatment, and it was demonstrated that FXYD10 is important in the phosphorylation and potassium deocclusion reactions, which are known to be controlled by \(\alpha\) domain movements (23). Consequently, we suggested that FXYD10 interacts with the \(\alpha\) domain of the shark \(\alpha\)-subunit (19, 23).

Little is known about the functional sites of interaction between FXYD proteins and Na,K-ATPase. Indeed, transmembrane domains of FXYD2 and FXYD4 were shown to be important in structural association with the \(\alpha\)-subunit of Na,K-ATPase (24). Mutational and expression studies have indicated

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank\(^{22}\)/EMBL Data Bank with accession number(s) AJ781093.

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\(^{\ddagger}\) The abbreviations used are: SERCA, sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase; AMP-PCP, \(\beta\),\(\gamma\)-methyleneadenosine 5'-triphosphate; DTT, dithiothreitol; PVDF, polyvinylidene fluoride; Tricine, N-tris(hydroxymethyl)methylglycine; TM, transmembrane; DMDB, 1,4-bismaleimido-2,3-dihydroxybutane; PKA, protein kinase A.

\(^{1}\) Ca\(^{2+}\)-ATPase: AMP-PCP, \(\beta\),\(\gamma\)-methyleneadenosine 5'-triphosphate; DTT, dithiothreitol; PVDF, polyvinylidene fluoride; Tricine, N-tris(hydroxymethyl)methylglycine; TM, transmembrane; DMDB, 1,4-bismaleimido-2,3-dihydroxybutane; PKA, protein kinase A.
the importance of transmembrane domain 9 (TM9) of the α-subunit in the stable association with some FXYD proteins as well as in functional interactions affecting ion affinities (25). Recently, Fuzesi et al. (26) have demonstrated interaction of the C terminus of FXYD2 with the S5 stalk segment of the pig kidney α-subunit in studies employing different cross-linking agents combined with protein fragmentation and mutational analyses.

The aim of the present study was to isolate and sequence cross-linked fragments from FXYD10 and the shark α-subunit to obtain information about the spatial interaction of FXYD10 and the α-subunit. We developed a proteolytic cleavage assay that allowed us to preferentially cleave the α-subunit, leaving FXYD10 intact, which is important in localizing the cross-linked fragments. This proteolytic assay was used to produce a minimum αFXYD10 cross-linked proteolytic fragment that could be subjected to sequence analysis. It was found that the C-terminal Cys\(^{14}\) of FXYD10 associates with the A domain of the α-subunit at Cys\(^{254}\) (numbering according to the shark α-subunit sequence, GenBank\(^{54}\) accession number AJ781093) upstream of the S3 stalk segment of the shark α-subunit.

**EXPERIMENTAL PROCEDURES**

**Materials**—1,4-Bismaleimidyld-2,3-dihydroxybutanate (DMDB) was from Pierce. The catalytic subunit of protein kinase A (PKA), AMP-PCP, Nε-cysteine, and pepsin were from Sigma-Aldrich. Polyvinylidene fluoride (PVDF) membranes, enhanced chemiluminescence reagents, protein A-Sepharose beads, and [\(^{32}\)P]ATP were from Amersham Biosciences. All other reagents were of the highest analytical grade available.

**Na,K-ATPase Preparation**—Purified Na,K-ATPase-containing membranes from the rectal gland of the shark *Squalus acanthias* were purified as described previously (27). Protein concentration was determined as described previously (28), using bovine serum albumin as a standard.

**PKA Phosphorylation**—PKA phosphorylation was performed in a typical assay mixture containing 50 mM HEPES, pH 7.5, 10 mM MgCl\(_2\), 1 mM EGTA, 100 nM Tris-ATP containing 0.3 μCi/μmol [\(^{32}\)P]ATP, 5–10 μg of protein, and 3 units of the catalytic subunit of PKA. The reaction was initiated by the addition of ATP, allowed to proceed for 20 min at 24 °C, and terminated by the addition of an equal volume of electrophoresis sample buffer (29).

**Gel Electrophoresis and Immunoblotting**—Protein fragments were separated using Tricine-based SDS-PAGE (3% stacking gel, 8% intermediate, and 15% resolving gel). For the detection of \(^{32}\)P-assisted kinase phosphorylation, gels were stained with Coomassie Blue and then analyzed by autoradiography. For the shark Na,K-ATPase, the three gels were transferred to PVDF membranes, washed three times with phosphate-buffered saline containing 5% Tween 20, and incubated overnight at room temperature with the primary antibody. Thereafter the membranes were washed with phosphate-buffered saline as above and incubated with goat anti-rabbit antibody for 2 h. After the final washings, the proteins were detected using enhanced chemiluminescence reagents. For the detection of the α-subunit fragments from shark rectal gland, the antibody NKA1002–1016 was used (a generous gift of Jesper V. Møller, Institute of Physiology and Biophysics, University of Aarhus). The FXYD10-specific antibody was developed as described previously (30). This antibody detects the N-terminal two-thirds of the protein as it binds to the N-terminal part after cleavage of the C terminus with mild trypsin treatment, and localization studies suggested that the antibody binds at the intracellular side of the membrane (23). Thus, it is suggested that the antibody binds at the motif K\(^{46}\)CRCK of FXYD10. This has also been indicated from the fact that the FXYD10 antibody binds to FXYD1 from rabbit skeletal muscle (data not shown), which has an identical sequence at this part of the protein and a completely different sequence at the C-terminal region (20).

**C-terminal end of FXYD10 and the α-subunit**—The cleavable cross-linker DMDB (spacer arm length, 10 Å) was used. Cross-linking was performed in a mixture containing 20 mM HEPES, pH 6.5, 2 mM EDTA, 20% glycerol, 12 μg of protein, and 0.10 mM DMDB (from a 20 mM stock dissolved in Me\(_2\)SO). The reaction was performed such that the volume of Me\(_2\)SO did not exceed 2% of the total volume. The reaction was started by adding the cross-linker for 25 min at 24 °C and terminated with the addition of the same volume of dithiothreitol (DTT) sample buffer containing 200 mM DTT, 4% SDS, 0.1% sodium azide, and 20% glycerol. The mixture was subjected to brief incubation at 37 °C before loading onto SDS gels. Cross-linked products in sample buffer were loaded onto SDS-Tricine gels as described above. In some other experiments, mercaptoethanol was used instead of DTT. Prior to the proteolytic assays (see below), cross-linking was terminated with 10 mM Cys (dissolved in 20 mM histidine, pH 7.0, containing 25% glycerol and freshly prepared each time) instead of DTT sample buffer. For measuring the effect of ligands on cross-linking, membranes were pre-incubated with increasing concentrations of sodium, potassium, or AMP-PCP (as indicated in Fig. 2) for 5 min at 24 °C before the addition of the cross-linker. Control experiments were run in parallel where the same volume of Me\(_2\)SO was added instead of the cross-linker. Additional negative control experiments were also performed in which cross-linking was carried out in the presence of 5–10 mM Cys added prior to addition of the cross-linker or using membranes pretreated with 2% SDS prior to cross-linking, where the native functional conformation of the enzyme is lost.

**Pepsin Digestion of Cross-linked Proteins**—Before proteolytic digestion, control and cross-linked membrane-bound shark Na,K-ATPase were washed with 25 mM imidazole, pH 7.2, and homogenized in a buffer containing 20 mM Tris, pH 5.0, 25% glycerol, and 1 mM EDTA at a protein concentration of 10 mg/ml. The proteolytic reaction (300 μl) was initiated by the addition of 2 μg of S-proteinase in Tris buffer, pH 7.2, in the presence of 20 mM ICy and 5 mM EDTA. Proteolysis was started with the addition of pepsin (pep/ protein ratio of 1:100), allowed to proceed for 1–2 h at 37 °C, and terminated with the addition of 2 μl of 5% NaOH, bringing the pH of the mixture to about 8.5, which irreversibly inhibits pepsin activity (31).

**Isolation of Cross-linked Proteolytic Fragments**—Based upon the fact that FXYD10 is not cleaved by pepsin under the conditions described above (see “Results”), proteolytic fragments produced after pepsin treatment were purified using pull-down experiments with protein A-Sepharose using an FXYD10-specific antibody. Post-proteolytic mixtures were mixed with solubilizing buffer containing (final concentrations) 50 mM HEPES, pH 7.0, 1 mM EDTA, 1 mM DTT, and 0.1% Nonidet 40. Solubilized protein fragments were mixed with prewashed complexes containing protein A-Sepharose beads equilibrated with the FXYD10 sample buffer containing 1 mM NaCl and two times with 20 mM EDTA. Dry beads were washed three times with solubilizing buffer, two times with solubilizing buffer containing 1 mM NaCl, and two times with 20 mM histidine, pH 7.2. Dry beads were either suspended in histidine buffer prior to PKA phosphorylation or treated with SDS mix prior to loading on SDS gels.

**Sequence Analysis**—Protein fragments were sequenced by Edman degradation at the protein structure Core Facility, University of Nebraska Medical Center. Mass spectroscopic analysis was performed at Alphalyse, Odense, Denmark.

**Homology Modeling of Shark α-subunit Sequence**—A homology model of the shark Na,K-ATPase α-subunit was built based on an alignment of the shark α-subunit sequence with that of the rabbit Na,K-ATPase α-subunit sequence and a homology model of the three-dimensional crystal structure of the rabbit SERCA 1a in the E1 conformation (Protein Data Bank code 1SU4, Ref. 32) using Swiss-Model in the “alignment mode” (33).

**RESULTS**

**Cross-linking of FXYD10 and the α-subunit**—The C-terminal end of FXYD10 contains three Cys residues (Cys\(^{43}\), Cys\(^{85}\), and Cys\(^{14}\)) (23), which could be possible targets for thiol cross-linkers. The presence of a Cys residue in the α-subunit in close proximity to one of the Cys residues of FXYD10 would allow covalent cross-linking of the two proteins. Therefore, the thiol cross-linking agent DMDB was used to test possible cross-linking between the α-subunit and FXYD10. DMDB is cleaved and inactivated in the periodate thus making it useful in positive control experiments following cross-linking and proteolytic cleavage. As seen in Fig. 1A, an immunoblot using an α-specific antibody showed that incubation of shark rectal gland membranes with DMDB resulted in the appearance of a band with higher molecular mass (about 100 kDa, lane 2) in addition to the native α-subunit normally running at 92 kDa in SDS gels (lane 1). A specific FXYD10 antibody detected only the upper 100-kDa band (Fig. 1B, lane 2), showing that the 100-kDa band represents an αFXYD10 complex. In the lower part of the gel the 15-kDa FXYD10 band is noted together with a 22-kDa band.
representing oligomeric FXYD10 (22), which is stabilized by the intermolecular cross-linking.

The efficiency of cross-linking was about 40%, as estimated from the measured area intensity of the scanned bands. Similar cross-linking efficiency has also been reported for cross-linking of FXYD2 to renal Na,K-ATPase after optimization of cross-linking (26). Cross-linking was found to decrease at increasing temperatures, being maximal at 10–24 °C and decreased at 37 °C (data not shown). DMDB in concentrations up to 500 μM was found not to influence the maximum hydrolytic activity of shark Na,K-ATPase (data not shown). Prolonged incubation of the membranes with the cross-linker (up to 2 h) did not further increase the efficiency of cross-linking.

Cross-linking was abolished in membranes pretreated with 2% SDS or 5–10 mM Cys (data not shown), indicating that cross-linking is occurring in functional enzyme preparations, where the native interaction between FXYD10 and the α-subunit is preserved. Further experiments in which membranes were solubilized with the non-ionic detergent octaethylene glycol mono-n-dodecyl ether (10 mM) (employed to exclude nonspecific intermolecular cross-links resulting from protein diffusion in the membrane) showed the same cross-linking pattern observed in Fig. 1 (data not shown). Finally, cross-linking at different enzyme dilutions also gave similar results (data not shown). The possibility of nonspecific cross-linking after solubilization of the membranes with SDS prior to gel electrophoresis was not likely because the cross-linking reactions were quenched with 5–10 mM DTT or Cys.

Effect of Na,K-ATPase Substrates on Cross-linking—The effect of Na,K-ATPase ligands on cross-linking was studied by pre-incubating the purified membranes with sodium, potassium, or the nonhydrolyzable ATP analog AMP-PCP prior to cross-linking. As seen in Fig. 2, pre-incubation of the Na,K-ATPase with increasing concentrations of either sodium (panel A) or potassium (panel B) did not affect the level of cross-linking of FXYD10 to the α-subunit. However, AMP-PCP significantly reduced cross-linking (Fig. 2C). Thus, incubation with 3 mM AMP-PCP reduced cross-linking by ~70%, in accordance with previous functional studies demonstrating that the regulatory effects of FXYD10 on the Na,K-ATPase are reduced at high ATP concentrations (23).

Characterization of the Cross-linked Cysteine(s) in FXYD10—The C terminus of FXYD10 can be specifically cleaved after incubation of shark rectal gland membranes with low concentrations of trypsin (23). In the present study the cleavage pattern of FXYD10 was studied by mass spectroscopy, which indicated that cleavage occurred C-terminal to Lys74. This cleavage will leave the N-terminal two-thirds of the protein (containing Cys25 and Cys45) associated with the membrane, whereas the soluble C-terminal fragment including Cys74 will be removed from the membranes by centrifugation. To test whether FXYD10 is cross-linked at Cys74 or at either Cys35/Cys45 we tested the ability of the C-terminally truncated FXYD10 to cross-link with the α-subunit. As seen in Fig. 3, cleaved FXYD10 does not cross-link with the α-subunit, strongly indicating that Cys74 in native FXYD10 is the site of cross-linking under the conditions used in this study.

Proteolytic Assays—Cross-linking of FXYD10 to the shark α-subunit indicates the existence of two closely associated domains in α and FXYD10, each containing a Cys residue. We initially anticipated that extensive trypsin digestion of cross-linked preparations would result in a fragment containing the C terminus of FXYD10 cross-linked to a fragment from the α-subunit. However, initial attempts to produce an α-FXYD10 cross-linked fragment using extensive trypsin digestion were unsuccessful because of extensive fragmentation of the C terminus of FXYD10, in contrast to the single-site cleavage previously observed after treatment of the membranes with a low trypsin concentration (23). As an alternative, we investigated the suitability of pepsin to cleave the α-FXYD10 complex in the hope that cleavage of FXYD10 at the Lys- and Arg-rich C-terminal phosphorylation motif could be avoided. Pepsin preferentially cleaves C-terminally to Phe, Leu, and Glu residues.

After incubation of the membranes with pepsin (see “Experimental Procedures”) extensive cleavage of the α-subunit occurred (Fig. 4A), and no intact α could be detected on the gel. Also the 19-kDa C-terminal fragment, which is resistant to trypsin cleavage in the presence of potassium, was almost completely degraded by the pepsin treatment. Surprisingly, immu-
The purified fragments resulted in three bands that reacted with the anti-FXYD10 antibody after SDS–PAGE, transferred to PVDF membranes, and immunoblotted with the FXYD10-specific antibody. In a recent study (25), the TM helical segment of three FXYD proteins, FXYD2, FXYD4, and FXYD7, has been proposed to be located in the M2, M9, M4, and M6 groove of the Na,K-ATPase because FXYD proteins like FXYD10 or FXYD2 are not cleaved by pepsin under the conditions used. Because the FXYD proteins all contain cytoplasmic Cys residues, thiol cross-linking studies on other FXYD proteins could be relevant using the approach described here.

**Discussion**

Cross-linking of FXYD10 to the α-Subunit—The crystal structure of SERCA 1a in various conformational states has revealed many important aspects about the structure and mechanism of P-type ATPases in general (34–39). The Na,K-ATPase α-subunit shares significant sequence similarity with SERCA 1a, especially in the C-terminal portion of the A domain, as well as in the N and P domains (40). Low-resolution structures have also suggested that both enzymes share significant structural homology, where the cytoplasmic domains are found very similarly arranged (41).

To obtain structural information about the location of the identified α-FXYD10 cross-link, multiple sequence alignment (ClustalW) of the shark Na,K-ATPase α-subunit and the α-subunit of SERCA1a was performed. This showed that Cys254 of the shark α-subunit is homologous to Ile214 of rabbit SERCA1a situated in the A domain. Homology modeling of shark Na,K-ATPase using the SERCA 1a in the E1 conformation as a template (Fig. 6) demonstrates that Cys254 (and Ile214) is positioned near the conserved TGES motif pointing toward the N domain in the E1 crystal structure (32). In a recent study (25), the TM helical segment of three FXYD proteins, FXYD2, FXYD4, and FXYD7, has been proposed to be located in the M2, M9, M4, and M6 groove of the Na,K-ATPase α-subunit, a position inferred from two-dimensional crystallographic data that indicated a center of mass outside of the 10 superimposed SERCA TM helices (42). More recent modeling studies have also used this position for docking of FXYD2 (26).
onto homology models of Na,K-ATPase. Because the TM region of FXYD proteins shares a high degree of homology, docking of FXYD10 to the same position of \( \text{Na}^{+}/\text{K}^{+}-\text{ATPase} \) would probably also be feasible. Actually, a similar position was assumed in a structural model for the association of the TM region of phospholamban with SERCA (43). If, as a first approach, this position is assumed for the transmembrane domain of FXYD10, its C-terminal Cys74 is well within the 10-Å cross-linking distance to Cys254 in the A domain, provided that the FXYD10 cytoplasmic tail, which appears to be largely unstructured (as evidenced from the Predict Protein Server, www.expasy.ch), is positioned as a rather straight extension from the TM domain toward Cys254 alongside S4 as indicated in the ribbon model in Fig. 6A. Such a position was also considered for FXYD2 (26) but was considered more unlikely than a model where the FXYD2 tail follows a groove to the right toward the L67 loop (stippled line in Fig. 6B), where Lys35 and Lys36 in the C-terminus of FXYD2 is cross-linked to either Lys347 or Lys352 in the S4 stalk segment. The latter model was favored for FXYD2, in part because of the insensitivity of this FXYD protein to digestion by trypsin. Contrary to FXYD2 the cytoplasmic tail of FXYD10 is very sensitive to proteolysis by trypsin (23) suggesting that it is more exposed than FXYD2.

When considering the proposed model, where the FXYD10 and FXYD2 transmembrane domains are located at the same position on the \( \text{Na}^{+}/\text{K}^{+}-\text{ATPase} \) α-subunit, a difficulty is that, relative to this position of FXYD10, Cys254 seems to be at the opposite face of the protein. Even though the cytoplasmic tail of FXYD10 is considerably longer than that of FXYD2 (34 versus 19 amino acids), a model where FXYD10 is located on the opposite side of the α-subunit in the M2, M4, M1, and M3 cleft would be more favorable. Furthermore, in such a model leucine-isoleucine zipper motifs in M1/M2 of the Na,K-ATPase could stabilize interactions with the FXYD10 TM domain, as suggested previously (19). It should be emphasized, however, that the molecular structures of FXYD proteins (as well as of the Na,K-ATPase α-subunit) are unknown at atomic resolution and that the cytoplasmic domain of the FXYD proteins appears to be largely unstructured, making interactive modeling uncertain. These proposed models will have to be confirmed by alternative meth-

Fig. 5. Identification of site of interaction of FXYD10 on the α-subunit. Control (labeled Ct) and DMDB cross-linked (labeled DMDB) proteins were digested with pepsin as described under “Experimental Procedures.” After isolation with pull-down experiments using the FXYD10 antibody, the purified cleavage products were either resolved with SDS-PAGE and immunoblotted with FXYD10 antibody (A) or phosphorylated with PKA in the presence of [32P]PATP followed by SDS-PAGE and autoradiography (B). After treatment with DMDB, a band running at 19 kDa appeared. This 19-kDa band is stained with the FXYD10 antibody (A, right lane) and phosphorylated with PKA (B, right lane). C, sequencing of the 19-kDa proteolytic fragment revealed the presence of FXYD10 and a fragment from the shark Na,K-ATPase α-subunit corresponding to the fragment Leu242/Glu243–Leu275 from the A domain (labeled FXYD10, see “Results”). Shown is part of the sequence of the shark α-subunit, deduced from cDNA sequence after cloning of the protein (GenBank™ accession number AJ781093). The α-subunit sequenced peptides are shown in red.

Table I

| Fragment         | Peptide number | Origin                                      | Sequence found                  | Yield  |
|------------------|----------------|---------------------------------------------|---------------------------------|--------|
| 19-kDa FXYD10α   | 1              | FXYD10 (Ref. 23)                            | MDPAGPDND                       | 24.6   |
|                  | 2              | Na,K-ATPase α-subunit A domain              | T244RNIAFFST                    | 26.8   |
|                  |                | (GenBank™ accession number AJ781093)        |                                 |        |
|                  | 3              |                                              | G261VVXYT                       | 27.2   |

When considering the proposed model, where the FXYD10 and FXYD2 transmembrane domains are located at the same position on the α-subunit, a difficulty is that, relative to this position of FXYD10, Cys254 seems to be at the opposite face of the protein. Even though the cytoplasmic tail of FXYD10 is considerably longer than that of FXYD2 (34 versus 19 amino acids), a model where FXYD10 is located on the opposite side of the α-subunit in the M2, M4, M1, and M3 cleft would be more favorable. Furthermore, in such a model leucine-isoleucine zipper motifs in M1/M2 of the Na,K-ATPase could stabilize interactions with the FXYD10 TM domain, as suggested previously (19). It should be emphasized, however, that the molecular structures of FXYD proteins (as well as of the Na,K-ATPase α-subunit) are unknown at atomic resolution and that the cytoplasmic domain of the FXYD proteins appears to be largely unstructured, making interactive modeling uncertain. These proposed models will have to be confirmed by alternative meth-

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**FIG. 5.** Identification of site of interaction of FXYD10 on the α-subunit. Control (labeled Ct) and DMDB cross-linked (labeled DMDB) proteins were digested with pepsin as described under “Experimental Procedures.” After isolation with pull-down experiments using the FXYD10 antibody, the purified cleavage products were either resolved with SDS-PAGE and immunoblotted with FXYD10 antibody (A) or phosphorylated with PKA in the presence of [32P]PATP followed by SDS-PAGE and autoradiography (B). After treatment with DMDB, a band running at 19 kDa appeared. This 19-kDa band is stained with the FXYD10 antibody (A, right lane) and phosphorylated with PKA (B, right lane). C, sequencing of the 19-kDa proteolytic fragment revealed the presence of FXYD10 and a fragment from the shark Na,K-ATPase α-subunit corresponding to the fragment Leu242/Glu243–Leu275 from the A domain (labeled FXYD10, see “Results”). Shown is part of the sequence of the shark α-subunit, deduced from cDNA sequence after cloning of the protein (GenBank™ accession number AJ781093). The α-subunit sequenced peptides are shown in red.

**Table I**

N-terminal sequences of the cross-linked trypsin-digested 19-kDa FXYD10-α fragment complex

| Fragment         | Peptide number | Origin                                      | Sequence found                  | Yield  |
|------------------|----------------|---------------------------------------------|---------------------------------|--------|
| 19-kDa FXYD10α   | 1              | FXYD10 (Ref. 23)                            | MDPAGPDND                       | 24.6   |
|                  | 2              | Na,K-ATPase α-subunit A domain              | T244RNIAFFST                    | 26.8   |
|                  |                | (GenBank™ accession number AJ781093)        |                                 |        |
|                  | 3              |                                              | G261VVXYT                       | 27.2   |
PLMS Association with A Domain of Na-pump α-Subunit

increased mobility of the A domain should accelerate the rate of the phosphorylation reaction, E1NaATP → E1 → E2 → E2P, and increase the ATP-supported deocclusion reaction, E2(K) → E1ATP (23). A similar effect is observed in studies of SERCA, in which cleavage of the A domain drastically decreases the rate of the E1P → E2P transition (44), indicating that the A domain is involved in this reaction. Moreover, the spontaneous mutation E233K in the rat α1-subunit of Na,K-ATPase (which resides in the A domain upstream of the S3 stalk segment) produced a functional enzyme species that had a faster rate of potassium deocclusion and higher ATP affinity, indicating that this loop is important for ATP interaction (45).

In summary, we developed a proteolytic assay using pepsin to extensively cleave the Na,K-ATPase α-subunit while leaving FXD1Y proteins intact. This assay enabled us to isolate a cleavage product containing FXD1Y0 cross-linked to the shark Na,K-ATPase α-subunit. Sequencing of the cross-linked cleavage products demonstrated interaction between the C-terminal domain of FXD1Y and a Cys254 positioned in the A domain of the α-subunit. Interaction of FXD1Y0 with the shark Na,K-ATPase A domain, restricting the A domain movement, explains the functional effects of FXD1Y regulation, including inhibition of turnover by decreasing the rate of phosphorylation.

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