FXYD Proteins Stabilize Na,K-ATPase

AMPLIFICATION OF SPECIFIC PHOSPHATIDYLSERINE-PROTEIN INTERACTIONS

Received for publication, September 12, 2010, and in revised form, January 9, 2011 Published, JBC Papers in Press, January 12, 2011, DOI 10.1074/jbc.M110.184234

Neeraj Kumar Mishra1, Yoav Peleg1, Erica Cirri2,6, Talya Belogus1, Yael Lifshitz4, Dennis R. Voelker1, Hans-Juergen Apell6, Haim Garty1, and Steven J. D. Karlish3

From the 1Department of Biological Chemistry and 2Israel Structural Proteomics Centre, Weizmann Institute of Science, Rehovoth 76100, Israel, the 3Department of Biology, University of Konstanz, Konstanz 78464, Germany, and the 4Department of Medicine, National Jewish Medical Research Center, Denver, Colorado 80206

FXYD proteins are a family of seven small regulatory proteins, expressed in a tissue-specific manner, that associate with Na,K-ATPase as subsidiary subunits and modulate kinetic properties. This study describes an additional property of FXYD proteins as stabilizers of Na,K-ATPase. FXYD1 (phospholemman), FXYD2 (γ subunit), and FXYD4 (CHIF) have been expressed in Escherichia coli and purified. These FXYD proteins associate spontaneously in vitro with detergent-soluble purified recombinant human Na,K-ATPase (α1β1) to form α1β1FXYD complexes. Compared with the control (α1β1), all three FXYD proteins strongly protect Na,K-ATPase activity against inactivation by heating or excess detergent (C12E8), with effectiveness FXYD1 > FXYD2 > FXYD4. Heating also inactivates E1 ↔ E2 conformational changes and cation occlusion, and FXYD1 protects strongly. Incubation of α1β1 or α1β1FXYD complexes with guanidinium chloride (up to 6 M) causes protein unfolding, detected by changes in protein fluorescence, but FXYD proteins do not protect. Thus, general protein denaturation is not the cause of thermally mediated or detergent-mediated inactivation. By contrast, the experiments show that displacement of specifically bound phosphatidylserine is the primary cause of thermally mediated or detergent-mediated inactivation, and FXYD proteins stabilize phosphatidylserine-Na,K-ATPase interactions. Phosphatidylserine probably binds near trans-membrane segments M9 of the α subunit and the FXYD protein, which are in proximity. FXYD1, FXYD2, and FXYD4 co-expressed in HeLa cells with rat α1 protect strongly against thermal inactivation. Stabilization of Na,K-ATPase by three FXYD proteins in a mammalian cell membrane, as well the purified recombinant Na,K-ATPase, suggests that stabilization is a general property of FXYD proteins, consistent with a significant biological function.

The Na,K-pump or Na,K-ATPase consists of a catalytic α subunit, with 10 trans-membrane segments, and a glycosylated β subunit, with one trans-membrane segment, associated in a 1:1 αβ complex that forms the minimal functional unit (1, 2). The Na,K-ATPase usually also contains an auxiliary subunit of the FXYD protein family (3–5). The α subunit contains the functional sites for ATP, transported cations, and cardiac glycoside inhibitors. The β subunit is required for maturation and stability of the α subunit (6). There are four α and three β isoforms, expressed and regulated in a tissue- and development-specific fashion. The different combinations of αβ isoforms exhibit somewhat different functional properties adjusted to the physiological requirements of the tissues (7).

FXYD proteins are a family of small regulatory proteins (3–5). FXYD proteins have a single trans-membrane segment, an extracellular N terminus and cytoplasmic C terminus, and are named after the conserved FXYD sequence in the extracellular domain. In mammals, there are seven homologous members (FXYD1–7) that are expressed in a tissue-specific fashion. They are often referred to by their common names as follows: FXYD1 (phospholemman (8)); FXYD2 (γ subunit of Na,K-ATPase (9)); FXYD3 (mammary tumor marker 8 (Mat-8) (10)); FXYD4 (CHIF (11)); FXYD5 (protein “related to ion channel” (12)); FXYD6 (phosphohippolin (13)); and FXYD7 (no common name (14)). FXYD proteins are not essential for Na,K-ATPase function but modulate the kinetic properties of Na,K-ATPase, adapting the rates and cation affinities of active Na+ and K+ transport to the physiological requirements of different cells. Effects of FXYD proteins on parameters such as Km, K0.5Na+, K0.5K+, Km,ATP, and Vmax are significant, but the magnitude is usually modest, usually 2-fold or less (reviewed extensively in Refs. 4, 5, 15, 16). Nevertheless, these effects are thought to have important consequences for homeostasis of cation balance, see for example Ref. 17. FXYD2–7 are modulators of Na,K-ATPase activity, the effects of which depend on the relative expression and association of αβ and the FXYD protein in the αβFXYD complex. FXYD1 is more strictly a regulator, in the classical sense, because it is phosphorylated by protein kinases (such as PKA and PKC) in response to external hormone signals, and its effects are altered accordingly (8, 18, 19).

The abbreviations used are: CHIF, corticosteroid hormone-induced factor; C12E8, octaethylene glycerol monododecyl ether; DDM, n-dodecyl β-o-maltopyranoside; SOPE, 1-stearyl-2-oleoyl-sn-glycero-3-phosphoethanolamine; SOPS, 1-stearyl-2-oleoyl-sn-glycero-3-phospho-L-serine; PSTD, phosphatidylserine decarboxylase; PS, phosphatidylserine; TEV, tobacco etch virus; Tricine, N(2-hydroxyethyl)glycine.

1 Postdoctoral fellow of the Feinberg Graduate School, Weizmann Institute of Science.
2 Member of the Graduate School of Chemical Biology of the University of Konstanz.
3 To whom correspondence should be addressed. Tel.: 972-8-934-2278; Fax: 972-8-934-4118; E-mail: Steven.Karlish@weizmann.ac.il.

© 2011 by The American Society for Biochemistry and Molecular Biology, Inc. Printed in the U.S.A.
FXYD Proteins Stabilize Na/K-ATPase

Recently, the structures of pig kidney Na,K-ATPase at 3.5 Å (20) and shark rectal gland Na,K-ATPase at 2.4 Å resolution (21) have been determined. In the renal Na,K-ATPase structure, the α subunit, trans-membrane segment of the β subunit, and trans-membrane segment of FXYD2 were resolved, and the latter is in proximity to M9 of the α subunit. The shark Na,K-ATPase structure (with FXYD10) confirmed these features, and extracellular segments of the β subunit and of FXYD10 protein containing the conserved FXYD sequence were also resolved. The aromatic residues in FXYDY were shown to interact with both α and β subunits. The cytoplasmic domain was not resolved. Mutation data also show that residues in M9 of α are involved in both the structural and functional interaction with FXYD2, FXYD4, and FXYD7 (22). There is also good evidence that trans-membrane segments of both FXYD2 and FXYD4 are responsible for both structural interactions with αβ and modulation of $K^{+}$/Na$^{+}$ (23), but different residues are involved in the two types of effects, and details of functional interaction are not known.

In view of the modest effects of FXYD proteins on the kinetic parameters (2-fold or less), there is an obvious question whether kinetic regulation of Na,K-ATPase activity is their sole function. One relevant observation is that Na,K-ATPase (porcine αβ1 or human αβ1 or αβ2), expressed in the yeast Pichia pastoris (24) and purified in a detergent-soluble functional state (25, 26), is strongly stabilized against thermal inactivation by FXYD1, also expressed in P. pastoris (27). As we have described extensively, it is necessary to add exogenous phosphatidylserine (PS) together with the detergent (C$_{12}$E$_{8}$) to the purified recombinant to maintain functional stability (25–27). Several observations indicate that, in the absence of added phospholipids, the Na,K-ATPase is inactivated by the C$_{12}$E$_{8}$ or DDM that displace endogenous lipids. The endogenous lipids are replaced by exogenous PS, which interacts specifically with the αβ complex, in the absence of FXYD proteins (25, 26). These observations include structural specificity of the phospholipid headgroup and fatty acyl chains, SOPS being the optimal phospholipid, additional specific stabilization of cholesterol interacting with the SOPS, different efficacies of C$_{12}$E$_{8}$ or DDM to inactivate, and the necessity to increase the SOPS concentration at increasing detergent concentrations to maintain activity, suggesting competition between the phospholipid and detergent (26). In the case of the unstable detergent-soluble αβ1 isofrom complex, it was possible to show directly a much lower “affinity” of added SOPS for protection against thermal inactivation compared with the more stable αβ1 (27).

An important observation, which forms the basis of this study, was that FXYD1 associates spontaneously with either purified porcine or human αβ1 and αβ2 isofrom complexes to form αβFXYD1 complexes, which were protected further against thermal inactivation (27, 28). A key finding in relation to the mechanism was that, after reconstitution with FXYD1, a functional Na,K-ATPase complex (αβ1FXDY1) was obtained even without addition of exogenous SOPS (27). As discussed in Ref. 27, this was interpreted to mean that FXYD1 stabilizes interactions of endogenous phospholipids on the protein, but the nature of the phospholipid was unknown.

Stabilization of the purified recombinant Na,K-ATPase by FXYD1 raises a number of issues, which are the focus of this study. First, how general is this effect, i.e. do all FXYD proteins stabilize Na,K-ATPase? Observations that renal Na,K-ATPase from FXYD2 knock-out mice are more thermolabile than the wild-type (29) and that expression levels of α1 and α2 subunits are reduced in mouse cardiac membranes depleted of FXYD1 (30) are compatible with the direct stabilizing effects of FXYD2 and FXYD1, but they could have alternative explanations. Second, what is the mechanism of the thermo-stabilizing effect of FXYD proteins? To address both of these questions, we have expressed FXYD1, FXYD2, and FXYD4 in Escherichia coli, purified the proteins, and looked directly at the stability properties of purified α1β1 versus α1β1FXDY complexes and established the mechanism. Finally, if stabilization by FXYD proteins has biological significance, it should also be detectable in intact mammalian cells. This point has been addressed by looking at the thermal stability of Na,K-ATPase expressed in HeLa cells, without or with co-expressed FXYD1, FXYD2, and FXYD4, as described previously (31, 32).

Experimental Procedures

Materials

DDM (catalog no. D310) and C$_{12}$E$_{8}$ (25% w/w, catalog no. O330) were purchased from Anatrace. Synthetic SOPS (sodium salt) was obtained from Avanti Polar Lipids and stored as a chloroform solution. BD Talon metal affinity resin (catalog no. 635503) was obtained from Clontech. TEV protease was obtained from Invitrogen or was prepared in the Israel Structural Proteomics Centre. All other materials were of analytical grade.

Expression of FXYD Proteins in E. coli and Purification

DNA Manipulations—Cloning of the different FXYD genes was performed in the expression vector pET28-TevH (33), harboring an N-terminal His$_{6}$ tag followed by TEV protease cleavage site. Cloning of rat FXYD4 (rat CHIF), human FXYD1 (human PLM), and rat FXYD2a (rat γ-a) was performed by insertion of the genes into the KpnI and NotI sites of the expression vector. Integration of human FXYD4 (human CHIF) and human FXYD2b (human γ-b), to the pET28-TevH vector was performed immediately following the TEV protease cleavage sites, using the restriction-free cloning procedure (34). In the latter case, no additional Thr was present downstream to the TEV protease cleavage site in contrast to cloning using restriction enzymes (see Table 1). Primers used for cloning are listed in supplemental Table S1. Prior to protein expression, the integrity of the different genes was confirmed by DNA sequencing.

FXYD Protein Expression—Protein expression of the cloned genes was performed using C41 (FXYD1 and FXYD2) or BL21(DE3) (FXYD4) E. coli cells (Novagen). Cells were grown overnight at 37 °C. The next day, cultures were diluted 1:100 in fresh LB medium containing kanamycin (30 μg/ml) and grown for an additional 2–2.5 h until $A_{600}$ nm was 0.6 – 0.8. Pro-

---

5 Previously, it was suggested that the trans-membrane helix lies in a crevice between M2, M6, and M9 (see Ref. 4), but the structures now show this to be incorrect.
tein expression was induced by the addition of isopropyl d-thiogalactopyranoside at a final concentration of 0.6 mM for about 16 h at 16 °C (FXYD1 and FXYD2) or 37 °C (FXYD4).

Membrane Preparation from E. coli Cells—After overnight induction, the cells were harvested by centrifugation and resuspended in a lysis buffer containing 50 mM Tricine/Tris, pH 7.4, 1 mM EDTA, 10 μg/ml DNase I, and 0.1 mM phenylmethylsulfonyl fluoride. The cells were disrupted using a high pressure emulsifier (EmulsiFlex C5, AVESTIN), followed by centrifugation at 10,000 × g for 15 min at 4 °C. The supernatant was centrifuged at 200,000 × g for 2 h at 4 °C. The pellet was resuspended in 50 mM MOPS/Tris buffer + 1 mM EDTA and 2 M urea, pH 7.8, followed by incubation on ice for 1 h with stirring. The resuspended membranes were then diluted 4-fold with 50 mM MOPS/Tris + 1 mM EDTA solution and centrifuged again at 200,000 × g for 2 h. The pellet was resuspended in 20 mM Tricine/Tris, pH 7.4, 20% glycerol, 200 mM NaCl, and protease inhibitors.

Purification of Recombinant FXYD Proteins and TEV Protease Treatment—Bacterial membranes suspended to a final concentration of 4 mg/ml in the medium containing 200 mM NaCl, 50 mM Tricine/Tris, pH 7.4, 10 mM imidazole, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), 20% (v/v) glycerol were homogenized (glass-Teflon) with a solution containing 8 mM/ml n-dodecyl β-maltoside (i.e. final DDM, 2:1 mg/mg protein). The insoluble material was removed by ultracentrifugation at 150,000 × g for 30 min. The supernatant was incubated overnight at 4 °C with BD Talon beads (Co2+–chelate) at a ratio of 1 ml of beads per supernatant from 100 mg of total membrane protein. The beads were washed twice with 10 bead volumes of “wash buffer” containing 100 mM NaCl or KCl or no added salt, 50 mM Tricine/Tris, pH 7.4, 20% (v/v) glycerol, 10 mM imidazole, 0.1 mg/ml C12E8, 0.1 mg/ml SOPS (unless stated otherwise), 0.01 mg/ml cholesterol. The FXYD proteins were eluted by mixing the beads at 4 °C with 1 bead volume of a solution containing 250 mM imidazole, 100 mM NaCl or KCl, or no salt, 50 mM Tricine/Tris, pH 7.4, 0.1 mg/ml C12E8, 0.1 mg/ml SOPS (or the indicated concentration), 0.01 mg/ml cholesterol, and 20% glycerol (“elution buffer”), with rotation for 1 h. Protein concentrations were determined by the BCA method using BSA as a protein concentration standard. Purified FXYD proteins were dialyzed against 50 mM Tricine/Tris buffer, pH 7.4, 40% glycerol, 100 mM NaCl or KCl or no added salt overnight at 4 °C with continuous stirring, using a Gelfast dialysis filter (cut-off 6–8 kDa). The dialysis exchanges the soluble small molecules in the solution, but detergent–lipid micelles (~60–80 kDa) are retained together with the protein/detergent/lipid mixed micelles. TEV protease was added to the dialyzed proteins in the ratio of 1:10 (w/w), followed by incubation either at 4 °C for overnight or 4 h at room temperature.

Purification of Human αβ1 and Reconstitution of αβ1FXYD Complexes

The human αβ1 complex was purified from P. pastoris membranes as described earlier (25, 26). The protein was normally eluted at 0.3–0.4 mg/ml in the elution buffer. In some cases, the SOPS concentration was varied from 0 to 0.1 mg/ml (see Figs. 10 and 11). For reconstitution of αβ1β and FXYD proteins on the beads, BD Talon beads pre-bound with αβ1 complex were centrifuged, and all but 1 bead volume of the supernatant was removed. The beads were incubated for at least 4 h with TEV protease-treated FXYD proteins at about 10:1 molar ratio with respect to αβ1 at 4 °C with rotation, followed by two washes with 10 bead volumes of wash buffer and elution with 1 bead volume of elution buffer, as described above. For reconstitution of the purified αβ1FXYD complex in solution (Figs. 4–11), the soluble FXYD proteins were added to the soluble αβ1 complexes at a molar ratio of about 10:1 or at varying ratios in titration experiments (e.g. Fig. 5), followed by incubation for at least 4 h on ice.

Thermal Inactivation, Detergent Inactivation, Na,K-ATPase Activity

The αβ1 or αβ1FXYD complexes were diluted to 0.2 mg/ml with elution buffer, incubated at room temperature for 15 min, and then heated at 45 °C for the indicated times, lightly centrifuged to return any evaporated and condensed water to the sample, and removed to ice. Alternatively, C12E8 was added at the indicated concentrations and incubated at 25 °C for 15 min followed by 5 min at 37 °C and then removed to ice for 15 min. At 15-s intervals, aliquots of protein (0.1 μg) were diluted into the standard reaction medium (125 mM) (130 mM NaCl, 20 mM KCl, 3 mM MgCl2, 25 mM histidine, pH 7.4, 1 mM EGTA, 1 mM ATP) and assayed for Na,K-ATPase activity at 37 °C. Na,K-ATPase activity of HeLa cell membranes was measured at 0.5 mM ATP. P, release was measured with a malachite green dye to detect the phosphomolybdate (P, Color Lock, Innova Biosciences). P, release was measured over 2, 4, and 6 min, and the slope was calculated from the slope of the time course by linear regression analysis, the error of determination of each slope being within 5–7% (r ≥ 0.99). The percent of control was calculated from the slopes before and after heating or in the absence or presence of detergent. Figures containing points without error bars represent the average of two experiments. Figures containing points with error bars represent the average of three experiments ± S.E. The specific Na,K-ATPase activity of the different preparations was in the range 8–17 μmol per min per mg of protein.

Treatment with Phosphatidylserine Decarboxylase (PSD)—Phosphatidylserine decarboxylase was prepared from E. coli following the general procedures described (35). Briefly, bacterial spheroplast membranes were subjected to 5% Triton X-100 solubilization, pH 5.1, and 70% acetone precipitation, protein resolubilization at pH 7.4 with Tris acetate and 1% Triton X-100, ion exchange chromatography with DE52, gel filtration chromatography with Sephadex G-150, and further ion exchange chromatography using Waters Accell QMA medium. The final homogeneous enzyme preparation had an activity of 51 mmol/min/mg of protein or 400 ng of PS/min/μl under optimal conditions at 37 °C. PSD was incubated with the αβ or αβFXYD1 complexes at 1:10 (v/v) ratio for different times up to 100 min at 37 °C. Na,K-ATPase activity was then measured.

Fluorescence Measurements—Tryptophan and fluorescein fluorescence were measured using a Varian spectrophotometer. Membranes expressing human αβ1 were labeled with 1 μM FITC at pH 9, and the fluorescein-labeled αβ1 complex...
**FXYD Proteins Stabilize Na/K-ATPase**

was purified as described (36). The fluorescein-labeled α1β1FXYD1 complex was produced by adding FXYD1 at a molar ratio of ~10:1 overnight at 4 °C. The protein was incubated at 20 °C for 30 min and was then either heated at 45 °C for the times indicated in Fig. 6 or not heated (control). Fluorescein fluorescence was measured with excitation at 495 nm and emission at 520 nm with both slits adjusted to 5 nm. The labeled protein was added to 2 ml of the following solution: 150 mM choline chloride, 10 mM Hepes (Tris), pH 7.5, and fluorescence changes were recorded upon addition of RbCl (20 mM) or NaCl (50 mM).

Tryptophan fluorescence was measured with an excitation 285 nm, and emission was measured between 300 and 400 nm.

**Steady-state Fluorescence Measurements with the Electrochromic Styril Dye RH421**—The experiments were carried out in a PerkinElmer Life Sciences 50B fluorescence spectrophotometer as described recently (37). The experiments were performed in buffer containing 25 mM imidazole, 1 mM EDTA, 5 mM MgCl2, pH 7.2. Subsequently, 200 nM RH421 and 9–10 μg/ml of Na,K-ATPase reconstituted with or without FXYD1 were added to the thermostatted cuvette (20 ± 0.5 °C) and equilibrated until a stable fluorescence signal, $F_0$, was obtained, which corresponds to an $E_1$ state with 1.2–1.5 $H^+$ bound. To stabilize the ion pump in the defined states, the following substrate additions were made: 50 mM NaCl (Na$\times$E1), 100 μM ATP (E$\beta$-P), and 20 mM KCl (turnover condition, in which mostly the $E_1$ Na$\times$ state is populated). To allow a comparison between different titration experiments, normalized fluorescence changes with respect to $F_0$, $\Delta F/F_0 = (F - F_0)/F_0$ were calculated.

**Preparation of HeLa Cells Membrane and ATPase Activity**—Sodium iodide-treated membranes from HeLa cells expressing rat α1 (and native human β1) together with co-expressed rat FXYD1, FXYD2, or FXYD4 were prepared as described previously (see Ref. 38 and references therein). Prior to the final suspension, the membranes were washed in a medium containing 10 mM Tricine/Tris, pH 7.2, and 20% glycerol to remove NaI and were resuspended in this medium. The expression of FXYD proteins was tested by Western blotting. The total Na$^+$,K$^+$-ATPase activity in intact HeLa cells membrane was measured by subtraction of ouabain-inhibited ATPase activity obtained in the presence of 10 mM ouabain from the measured activity without ouabain. Membranes were incubated overnight at 4 °C with 0.01 mg/ml of C$_{12}$E$_8$ (“unmasking”) in a medium containing 10 mM Tricine/Tris buffer, pH 7.2, 3 mM MgCl$_2$, 1 mM EGTA/Tris, without or with 10 mM ouabain. The membranes were then heated at 45 °C or not heated, and Na$^+$,K$^+$-ATPase activity was measured with and without 10 mM ouabain in the reaction mixture.

**RESULTS**

Fig. 1 presents experiments demonstrating purification of the human FXYD proteins expressed in the *E. coli* membranes, using metal chelate affinity chromatography (BD Talon), and digestion with TEV protease to remove the His tag and TEV protease site. Similar results were obtained for rat FXYD proteins (see Table 1 for all sequences). The results of many experiments showed that the FXYD proteins were purified to about 50%, as judged by the Coomassie stain of the gels. FXYD proteins stain poorly with Coomassie dye, by comparison other proteins, such as the standards in this gel. Thus, the estimate of 50% purity may be an underestimate. The purified but uncleaved FXYD1, FXYD2, and FXYD4 all ran with an apparent mass of 13–14 kDa and with apparent mass of ~11–12 kDa after cleavage with TEV. The TEV protease was added after elution of FXYD proteins from the beads and dialysis to remove imidazole, which inhibits TEV. The His tag-TEV protease site fragment and the His-tagged TEV protease themselves could also be removed by adding BD Talon beads, leaving only the soluble FXYD protein in solution (data not shown). Western blots using anti-FXYD1- and FXYD2-specific antibodies confirmed these findings.6

As described previously, FXYD1 expressed in *P. pastoris* associates spontaneously with either the human α1-porcine β1 or human α2-porcine β1 complexes of Na,K-ATPase bound to BD Talon beads to form the αβFXYD1 complex, which is strongly protected against thermal inactivation (27, 28). In those experiments, the FXYD1 was not purified. The experiments in Fig. 2 examined the ability of all three purified human proteins, FXYD1, FXYD2, and FXYD4, to spontaneously reconstitute with the human α1β1 and protect against thermal inactivation. The purified TEV-treated detergent-soluble FXYD proteins were incubated with BD Talon beads bound with the human α1β1 complex (at a molar ratio of FXYD:α1β1 ~10:1), for 12 h at 4 °C, and the beads were then washed and α1β1FXYD complexes eluted in the normal way. Because other data suggested that the eluted α1β1 complex was less stable in KCl compared with NaCl,7 the experiment also tested whether

---

6 In some early experiments, the uncleaved His$_6$-TEV-FXYD1 protein appeared to run on SDS-PAGE with a mass of 26 kDa, but with a mass of 12 kDa after removal of the His tag by TEV protease cleavage. Although there is no direct evidence, the observation suggested that a heavy metal was present and cross-linked the uncleaved His$_6$-TEV-FXYD1 protein via the His tag. In any event, the stabilizing effect of FXYD1 was quite unaffected whether or not it was cleaved with TEV protease (see for example Fig. 3).

7 H. Haviv and S. J. D. Karlish, manuscript in preparation.
the complexes could be formed spontaneously in both NaCl and KCl and protect against thermal inactivation. Gels of the purified αβ1 and reconstituted αβ1FXYD1 complexes have been presented previously (27, 28), and so the following experiments focus only on the stabilizing effects of the FXYD proteins. In Fig. 2 the Na,K-ATPase activity of the human αβ1, αβ1FXYD1, αβ1FXYD2, and αβ1FXYD4 complexes was measured after incubation at 45 °C for the indicated times. In the NaCl-containing medium (Fig. 2A), the purified human FXYD1 protected human αβ1 strongly against thermal inactivation at 45 °C, similar to the effect of unpurified FXYD1 expressed in P. pastoris. This observation, of course, provides evidence that the αβ1FXYD1 complex was formed on the BD Talon beads and was eluted from the beads into solution. The average specific Na,K-ATPase activities of αβ1 and αβ1FXYD1 complexes in four separate preparations were 9.42 ± 1.05 and 13.5 ± 1.32 μmol/min/mg protein, respectively. The difference of 30% is due to partial inactivation of the control during the preparation, as shown previously (28). By comparison with FXYD1, both FXYD2 and FXYD4 protected against thermal inactivation at 45 °C to a significant but lesser degree with the order FXYD1 > FXYD2 > FXYD4 (Fig. 2A). Another experiment demonstrated that rat FXYD2a and FXYD4 stabilized the human αβ1 complex very similarly to the human FXYD2b and FXYD4, as in Fig. 2A (data not shown). Fig. 2B shows the effects of the human FXYD proteins in a KCl-containing medium. A quite different pattern was observed to that in Fig. 2A. First, the control αβ1 complex was much more easily thermally inactivated than in the NaCl-containing medium (seen also in Figs. 4 and 9). Second, although FXYD1 protected the enzyme very strongly also in the KCl-containing medium, FXYD2 and FXYD4 were ineffective. In view of the significant protective effects of FXYD2 and FXYD4 in the NaCl-containing medium, this lack of effect in the KCl-containing medium was surprising. In this protocol, the excess of the unbound FXYD protein is removed by washing prior to elution of the αβ1FXYD complex, and it is conceivable that the more weakly bound FXYD proteins dissociate from the αβ1 complex during the washing. Because of this factor, the different effects of FXYD1, FXYD2, and FXYD4 in either NaCl- or KCl-containing media could be explained by different degrees of dissociation of the αβ1FXYD complexes, during the washing.

To distinguish whether there are different maximal effects of bound FXYD proteins or different degrees of dissociation, we have examined protective effects of FXYD proteins added directly to the eluted detergent-soluble αβ1 complexes. These are conditions in which the αβ1 complexes and FXYD proteins are expected to interact spontaneously in solution and reach binding equilibrium (Figs. 3–5) (9–12). In the experiment of Fig. 3, TEV protease-treated or -untreated FXYD1 and FXYD2 were incubated with the αβ1 complex (at a molar ratio of about 10:1) for 4 h on ice and were then heated or not heated at 45 °C for 60 min, prior to measurement of Na,K-ATPase activity. The control sample was largely inactivated by the heating. FXYD1, either TEV protease-treated or -untreated, protected almost completely, whereas FXYD2, either TEV protease-treated or -untreated, protected partially. In many subsequent experiments, TEV protease treatment was discontinued because the His₆-TEV sequence does not affect the ability to protect against thermal inactivation. Note that the enzymes used for Fig. 3 were eluted without added NaCl and are particularly sensitive to thermal inactivation (compare the control with the 60-min time point in Fig. 2). Nevertheless, FXYD1 protected largely against the thermal inactivation. Fig. 4 shows time courses of thermal inactivation in either NaCl- or KCl-containing media, after reconstitution of αβ1FXYD1, αβ1FXYD2b, and αβ1FXYD4 complexes by the solution

### Table 1: Amino acids sequence of the different FXYD proteins

| Gene    | Tag sequence* (His + Tev) | Protein sequence |
|---------|---------------------------|-----------------|
| Rat FXYD2a | MGSSHHHHHHHSAGENLYTQG   | EANGPVDKGSFYYDWSLQLQGMIFGGGLCLAGIAMALSGKCKCRHNTPLPSLPEKVTLTPTGAST* |
| Rat FXYD2b | MGSSHHHHHHHSAGENLYTQG   | TELSANHGGSAKGTENFPFYYDTEYTRKGGLGLAFGVYVLILLSKSRRFSCGSGKHKQVYNEDEK* |
| Human FXYD4 | MGSSHHHHHHSSAGENLYTQG   | ERVTALLLLLLATLAEANDFPAKNDPFYDWKQNLSGLICLGLILAIAGIAAVL5GKCKKCSSQK |
| Human FXYD2b | MGSSHHHHHHSSAGENLYTQG   | HSYVPEKAIPLTPTGASATC* |
| Human FXYD1 | MGSSHHHHHHSSAGENLYTQG   | DRWYLGISIFXDVDFFYYDYTEVRNGLIFAGLAFYVILILLSRBRCGSGKHEQNNDEEP* |
|           |                           | KAESFKEHDFTTVDYQSLQ6GLVIAGILFILGLILSVRRRCCKENQQQR7GEPEDEEGTFRSSIRLLSTRR* |

* TEV protease recognition site is underlined.
equilibration method of Fig. 3. In the NaCl-containing medium (Fig. 4A), the inactivation patterns of α1β1 and its FXYD complexes are quite similar to that with the eluted complexes observed in Fig. 2A, namely a very strong protection by FXYD1 and significant but weaker protection by FXYD2 and FXYD4. In another experiment, the rat FXYD2a and FXYD4 showed slightly better, but still partial, protection compared with the human FXYD2b and FXYD4 proteins (data not shown). The striking finding with the KCl-containing medium (Fig. 4B) is that, by contrast with the lack of protection seen in Fig. 2B, both FXYD2 and FXYD4 protected quite strongly in these conditions, even though less, again, than FXYD1. Re-plots of the data to compare the stability in the two ionic media show clearly that, whereas the control is much more thermally labile in the KCl- than the NaCl-containing medium, there is little or no difference between the KCl- than the NaCl-containing medium in the Na,K-ATPase activity was measured, and the percent of control was calculated. The data points represent the average ± S.E. of three separate experiments.

FIGURE 3. Stabilization of α1β1 complexes by reconstitution with FXYD proteins in solution. The α1β1 complexes were eluted without added salt and incubated with TEV-treated or untreated FXYD1 and FXYD2 for 4 h on ice at a molar ratio of FXYD:α1β1 = 10:1. The samples were then heated for 60 min at 45 °C or not heated, and Na,K-ATPase activity was measured. The values represent the average of two separate experiments.

FIGURE 4. Time course of thermal inactivation of α1β1 and α1β1FXYD complexes. Control α1β1 complexes were prepared in the presence of either 100 mM NaCl (A) or 100 mM KCl (B), and α1β1FXYD1, α1β1FXYD2, and α1β1FXYD4 complexes were then produced by reconstitution in solution as in Fig. 3. Na,K-ATPase activity was measured after incubation at 45 °C for the indicated times. The data points represent the average ± S.E. of three separate experiments.

FIGURE 5. Titration curves for protection against thermal inactivation FXYD1, FXYD2, and FXYD4. Control α1β1 complexes were prepared in the presence of either 100 mM NaCl or 100 mM KCl as in Fig. 4. FXYD1 (A), FXYD2 (B), and FXYD4 (C) were added at the indicated concentrations, incubated on ice for 4 h, followed by heating for 60 min at 45 °C or not heated (control), and then Na,K-ATPase activity was measured, and the percent of control was calculated. The data points represent the average ± S.E. of three separate experiments.
FXYD Proteins Stabilize Na/K-ATPase

FXYD2 \approx FXYD4. Furthermore, the lack of protection by FXYD2 and FXYD4 in the KCl-containing medium in the conditions of Fig. 2B is attributable to dissociation of these FXYD proteins from the α1β1FXYD complex prior to elution of the protein.

Alternative ways of demonstrating protection by FXYD1 against thermal inactivation, with mechanistic insights into the functional changes, are presented in Figs. 6 and 7. The experiment in Fig. 6 utilized fluorescein-labeled recombinant α1β1 complex for study of E1 ↔ E2 conformational changes (as described recently (36) and under “Experimental Procedures”). The enzymes used in Fig. 6 (and also Fig. 7) were eluted without added NaCl. The fluorescence changes of the control observed upon addition of Rb⁺ and then Na⁺ ions to the labeled protein, suspended initially in a Na⁺- and Rb⁺-free medium, reflect the conformational change E1 → E2(2Rb) and then the reverse change E2(2Rb) → E1Na3Na. The amplitude of the fluorescence change \( \Delta F \) of the total fluorescence in the control is proportional to the number of pumps undergoing the conformational transition. Evidently, the signal is rapidly lost upon heating the labeled enzyme at 45 °C for 5 and 15 min (0 min, \( \Delta F \) 15.2%, 5 min, 4.5%, 15 min, 0% respectively). Upon incubation with FXYD1, the fluorescence change of the reconstituted α1β1FXYD1 complex increased somewhat to \( \Delta F \) 19% compared with 15.2% for the control. The striking finding is that heating at 45 °C up to 60 min reduced the signals only to a minor extent (0 min, \( \Delta F \) 19%; 10 min, 17.5%; 30 min, 17.8%; and 60 min, 14.5%, respectively) thus demonstrating a very strong protective effect of the FXYD1.

The effects of thermal inactivation on electrogenic partial reaction of the pump cycle were studied by fluorescence experiments with the electrochromic styril dye RH421, as described (37). According to the underlying physical principle of electrochromy (“Stark effect”), the fluorescence is linearly related to electric field strength (or charge density, here the number of charges in the membrane). Thus, the proportion of active pumps (i.e. with a full complement of bound cations) is detected by linearly proportional changes of the fluorescence level (39).

In Fig. 7, the time course of the fluorescence upon addition of NaCl (50 mM), ATP (100 μM), and KCl (20 mM) is shown before and after a 1-h incubation at 45 °C for Na,K-ATPase reconstituted without (Fig. 7A) and with a 10-fold excess of FXYD1 (Fig. 7B). Both Na⁺ binding and the ATP-induced partial reaction, \( E1Na3Na + ATP \rightarrow E1P(Na3Na) + ADP \rightarrow E2PNa3Na \rightarrow E2P + 3Na⁺ \), are reduced by the reduction of the active ion pumps. Again it is shown that FXYD1 protects effectively against thermal inactivation. In the absence of FXYD1, Na⁺ binding capacity was reduced to \(~69\%\) of the initial value and enzyme phosphorylation to \(~17\%\). In the presence of FXYD1, the respective numbers were \(~95\%) and \(~62\%). Corresponding experiments were performed also after thermal incubations of 10 and 30 min. The normalized fluorescence levels are plotted against incubation time after addition of NaCl (Fig. 7C) and ATP (Fig. 7D). FXYD1 stabilizes almost quantitatively access to the binding sites in the E1 conformation and Na⁺ binding. The ATP-induced partial reaction of which the last reaction step, Na⁺ release in the P-E2 conformation, as monitored by RH421, is more sensitive to thermal inactivation than Na⁺ binding. Again, however, FXYD1 has a clear protective effect. At short incubation times, a small but significant enhancement of Na⁺ binding and ATP-induced activity was observed. A similar phenomenon was observed previously in measurements of Na,K-ATPase activity and was attributed to activation of a fraction of dormant pumps by equilibration with the lipid (26, 36).

Figs. 8–12 concern the mechanism of inactivation and protection by FXYD proteins. In principle, thermal inactivation might be the result of heat-induced unfolding of the α1β1 protein complex, and FXYD proteins might protect against unfolding. One way to study protein unfolding is with denaturants such as urea or guanidinium chloride, as described recently for native Na,K-ATPase (40). The experiment in Fig. 8 examined the effect of increasing concentrations of guanidinium chloride (0–6 M) on unfolding of the protein as detected by a shift in the intrinsic protein fluorescence spectrum, from an emission maximum of 330–360 nm (Fig. 8A). In any condition, the difference of the fluorescence emission maximum at 330 and 360 nm divided by the maximum at 330 nm \( (F_{330} - F_{360})/F_{330} \) (100) provides a measure of the percent of native folded structure. As can be seen in Fig. 8B, this value fell progressively as the guanidinium chloride concentration was raised from 0 to 6 M, and none of the FXYD proteins protected against this unfolding. The lack of protection by the FXYD proteins against a denaturant makes it unlikely that thermal inactivation is the result of a general unfolding of the protein (see under “Discussion”).

As mentioned in the Introduction, α1β1FXYD1 complexes prepared as in Ref. 27 are active even without added exogenous SOPs, by contrast with the control α1β1 complex, which is largely inactive without added lipid. In Ref. 27, we argued that the FXYD1 stabilizes an endogenous yeast phospholipid (e.g. PS) on the protein, although it was not possible to know which yeast lipid is involved. The idea that FXYD proteins stabilize interactions of phosphatidylserine with the protein leads to the
hypothesis that thermal inactivation of the detergent-soluble protein is an accelerated form of detergent-mediated inactivation caused by displacement of specifically bound phospholipids (see “Discussion”). This has been tested in four types of experiments. First, we have looked extensively at inactivation of $\alpha_1\beta_1$ and $\alpha_1\beta_1\text{FXYD}$ complexes by the detergent C$_{12}$E$_8$. Fig. 9 compared inactivation of $\alpha_1\beta_1$ and the three $\alpha_1\beta_1\text{FXYD}$ complexes by increasing concentrations of C$_{12}$E$_8$, above those already in the elution medium, in both NaCl- and KCl-containing media. A re-plot of the data in Fig. 9 emphasizes the effects of NaCl- and KCl-containing media (supplemental Fig. S2). The first point of interest is that the $\alpha_1\beta_1$ complex itself is much more sensitive to C$_{12}$E$_8$ in the KCl-containing compared with the NaCl-containing medium, mirroring exactly the response to thermal inactivation (seen in Fig. 4). Second, FXYD1 protects the enzyme very strongly in both NaCl- and KCl-containing media, and FXYD2 and FXYD4 also protect significantly, so that the final order of effects is FXYD1 > FXYD2 > FXYD4.
the presence of the FXYD proteins, the difference between NaCl- and KCl-containing media largely disappeared, similarly, again, to the pattern for thermal inactivation. A small difference from the thermal inactivation experiments is that FXYD2 protects against C12E8 better than FXYD4, and there is a biphasic effect of detergent in the case of FXYD4. In essence, however, the characteristics of detergent inactivation observed in Fig. 9 are similar to the thermal inactivation in Fig. 4, suggesting that they represent essentially the same phenomenon, i.e. thermal inactivation is indeed an accentuated detergent-induced inactivation. A second and more direct test of the notion that FXYD proteins stabilize specific interactions of PS with the α1β1 complex, we have looked at activity of the protein eluted at different SOPS concentrations and then reconstituted or not with FXYD1, FXYD2, and FXYD4 (Fig. 10). To compare activities of the different preparations at increasing SOPS concentrations, the activities (ν) have been normalized by calculating the ratio of activity at a particular SOPS concentration to that at 0.1 mg/ml SOPS (ν0.1) and expressed as ν/ν0.1. The experiment in Fig. 10A shows that FXYD1 strongly increases and FXYD2 and FXYD4 moderately increase the "apparent affinity" for SOPS, because the curves for dependence of Na,K-ATPase activity on SOPS are shifted to the left compared with the control. In the presence of FXYD1, in particular, 0.02 mg/ml SOPS sufficed to preserve full Na,K-ATPase activity. In addition, as seen in Fig. 10B, after heating for 15 min at 37 °C, the control curve was shifted significantly to the right, although the curves with the FXYD proteins were shifted only slightly, and the difference between the control and curves with FXYD proteins was accentuated.

Fig. 11 presents a third test of the mechanism of detergent inactivation and protection by FXYD1. If, as inferred previously (26), SOPS interacts specifically with the protein and is displaced by the detergent, one could expect that the SOPS and detergent should compete. If the FXYD protein stabilizes the SOPS on the protein, competition between the phospholipid and detergent should be minimal in the same conditions. The predicted features are observed in Fig. 11. The concentration of C12E8 required to inactivate the α1β1 complex was prepared with 0.02, 0.03, or 0.1 mg/ml SOPS and reconstituted with FXYD1 as in Fig. 10. C12E8 was added at the indicated concentrations, and the complexes were incubated at room temperature (25 °C) for 20 min prior to determination of Na,K-ATPase activity. The data points represent one of two similar experiments.

FIGURE 9. Detergent-induced inactivation. Protection by FXYD1, FXYD2, and FXYD4. α1β1 or α1β1FXYD complexes were prepared with 100 mM NaCl (A) or 100 mM KCl (B) as in Fig. 4. Excess C12E8 was added at the indicated concentration, and incubated as described under "Experimental Procedures," and Na,K-ATPase activity was measured. The data points represent the average of two separate experiments.

FIGURE 10. FXYD1, FXYD2, and FXYD4 increase apparent affinity for SOPS. α1β1 or α1β1FXYD complexes were prepared as in Fig. 4 with varying concentrations of SOPS (0–0.1 mg/ml). All preparations were diluted to a protein concentration of 0.1 mg/ml, and Na,K-ATPase activity was measured. A, activity measured immediately after elution. B, activity measured after incubation at 37 °C for 15 min. The y axis labeled Na,K-ATPase-ν/ν0.1 refers to the ratio of Na,K-ATPase activity relative to that at 0.1 mg/ml of SOPS (ν/ν0.1).

FIGURE 11. Competitive displacement of bound SOPS by C12E8 and stabilization of bound SOPS by FXYD1. The α1β1 complex was prepared with 0.02, 0.03, or 0.1 mg/ml SOPS and reconstituted with FXYD1 as in Fig. 10. C12E8 was added at the indicated concentrations, and the complexes were incubated at room temperature (25 °C) for 20 min prior to determination of Na,K-ATPase activity. The data points represent one of two similar experiments.
FXYD Proteins Stabilize Na/K-ATPase

FIGURE 12. Enzymatic decarboxylation of SOPS inactivates the Na,K-ATPase and FXYD1 protects. The experiment represents the Na,K-ATPase activity after incubation with PSD as a percentage of the control activity not incubated with PSD for both the αβ and αβ:FXYD1 complexes. The data are the average of two independent experiments.

A fourth and conclusive test of the mechanism of inactivation, and protection by FXYD1, involved the use of PSD, a bacterial enzyme that specifically converts PS to phosphatidylethanolamine (Fig. 12). As shown in a previous paper (26), phosphatidylethanolamine is unable to maintain Na,K-ATPase activity of the eluted enzyme. Therefore, it could be expected that conversion of the SOPS to SOPE would be accompanied by inactivation of the enzyme. By contrast, if the mechanism of protection by FXYD1 indeed involves stabilization of binding of specifically bound SOPS, the bound SOPS could be inaccessible to the PSD, and therefore activity could be protected by the bound FXYD1. The result in Fig. 12 represents the average of two experiments and shows conclusively that this prediction is correct.

A final observation addressed the question whether FXYD proteins stabilize the Na,K-ATPase when expressed in mammalian cell membranes. For this purpose, we have utilized HeLa cells expressing rat α1 (and native human β1) together with co-expressed rat FXYD1, FXYD2, or FXYD4 (Fig. 13). These cells have been used extensively to study both structural interactions and functional effects of the three FXYD proteins (23, 27, 41). A point that should be taken into account is the recent observation that HeLa cells express FXYD5 (42). Thus, effects of other expressed FXYD proteins are over and above those of FXYD5 itself (see also under “Discussion”). Cells were grown and membranes were prepared as described previously. The inset of Fig. 13 shows Western blots using rat-specific anti-FXYD1, FXYD2, or FXYD4 antibodies to confirm that the cells expressed the three different FXYD proteins, respectively. The membranes were incubated overnight at 0 °C, with low concentrations of C₁₂E₈ (demasking), with and without ouabain (10 mM), and were then heated at 45 °C for up to 16 h, or not heated. The ouabain-inhibited fraction of the total ATPase activity (∼40%) was then measured. Fig. 13 presents one of two similar experiments. The Na,K-ATPase activity of the control membranes (αβ) was thermally inactivated quite slowly at 45 °C (∼50% in 4 h), and all three FXYD proteins protected strongly against the thermal inactivation before eventually losing activity at 16 h. An interesting feature of the experiment is that the FXYD1 appeared to protect less well than FXYD2 and FXYD4 in the initial 4 h, but a larger fraction of initial activity was preserved at 16 h compared with FXYD2 and FXYD4, which were essentially completely inactivated. As pointed out previously (28), in cells with co-expressed αβ and FXYD subunits, it is not possible to ensure 1:1 stoichiometric ratios of FXYD:αβ, by contrast with in vitro reconstitution of FXYD with αβ, which does allow experimental control of the stoichiometry (for example, as in Fig. 5). An economical explanation of the result in Fig. 13 could assume that the stoichiometry of FXYD1:αβ is less than 1 and is lower than the molar ratio of FXYD2:αβ or FXYD4:αβ subunits. On this assumption, the initial rate of thermal inactivation should include that of both the αβFXYD1 complex and the unbound αβ subunits, and it could be faster than the rate of inactivation of αβFXYD2 and αβFXYD4 complexes. Over several hours, the αβ subunits could be completely inactivated, and the remaining αβFXYD1 inactivated slower than the αβFXYD2 and αβFXYD4 complexes. Thus, a higher fraction of initial activity could remain for αβFXYD1 at the longest time (16 h). In any event, whether or not this is the correct explanation, it is clear that FXYD1, FXYD2, and FXYD4 all protect the Na,K-ATPase against thermal inactivation in the intact mammalian membrane (at least compared with the possible effects of FXYD5 (42)).

DISCUSSION

The central finding of this study is that three human FXYD proteins, FXYD1, FXYD2, and FXYD4, protect human Na,K-ATPase α1β1 against thermal and detergent inactivation of the reconstituted detergent-soluble purified α1β1FXYD1 complexes and amplify specific SOPS-protein interactions. In addition, co-expression of all three rat FXYD proteins with rat α1 in intact HeLa cell membranes protects against thermal inactivation. The extent of stabilization varies in the order FXYD1 > FXYD2 ≫ FXYD4 but can be very large, certainly by comparison with the modest ∼2-fold effects on functional parameters of Na,K-ATPase activity (Kₒ Na, Kₒ K, Kₐ ATP, and Vₘₐₓ). Protection against thermal inactivation by the purified FXYD1, expressed in E. coli, confirms our previous observations of thermal stabilization of human α1β1 and α2β1 by unpurified...
FXYD1 expressed in \textit{P. pastoris} (27). Protection by purified human FXYD2b and also rat FXYD2a is consistent with the observation that the renal Na,K-ATPase of mice with FXYD2 knocked out is more thermally labile than that of wild-type mice, which express FXYD2 heavily in the kidney (29). In light of all these findings, stabilization of Na,K-ATPase may be a general feature of all FXYD proteins. Two questions arise. First, what is the detailed mechanism of stabilization by FXYD proteins? Second, what is the biological significance of stabilization of Na,K-ATPase by FXYD proteins?

\textbf{Mechanism of Stabilization by FXYD Proteins}—The similar features of thermally and detergent-induced inactivation observed in Figs. 4 and 9, including parallel degrees of protection by the three FXYD proteins, and the effects in Na\textsuperscript{+} - and K\textsuperscript{+}-containing media, show that these reflect essentially the same phenomenon. Taken together with the experiment of Fig. 10 showing directly that all three FXYD proteins raise the apparent affinity of SOPS, and Figs. 11 and 12 showing specific stabilization of SOPS on the protein by FXYD1, the clear-cut conclusion is that the thermally mediated or detergent-mediated inactivation of Na,K-ATPase activity involves displacement of specifically bound SOPS, and FXYD1 prevents or strongly reduces displacement of the bound SOPS. This mechanism is, of course, consistent with specific stabilizing effects of phosphatidylserine described previously in Refs. 25–27, and an inference in the previous paper that FXYD1 stabilizes a lipid-protein interaction, albeit via an unknown yeast phospholipid (27).

Concerning regions of the FXYD proteins involved in this stabilizing effect, protection against both thermal and detergent inactivation, the similar effects of Na\textsuperscript{+} and K\textsuperscript{+} ions (in Figs. 4, 5, and 9) and the FXYD protein-induced increase in the SOPS “affinity” (Fig. 10) indicate that the trans-membrane segments of the FXYD proteins play an important role. Conversely, the differences in stabilizing effects between FXYD1, FXYD2, and FXYD4 (Figs. 4, 5, and 9), which are large in some conditions (e.g. Fig. 2B, in which lack of effects of FXYD2 and FXYD4 are attributed to their dissociation), implies that interactions of the conserved FXYD sequence itself are not predominant.

The experiments with the denaturant guanidinium chloride (Fig. 8) are also revealing. In general, one could expect that that water-soluble chemical denaturants, like urea or guanidinium chloride, would preferentially unfold domains of membrane proteins exposed to the water, by comparison with the less accessible trans-membrane segments, that are also stabilized by hydrophobic interactions of side chains with the lipid and internal H-bonds in the \textalpha-helical peptide backbone (43). Indeed, a very recent study has shown directly that urea unfolds the extramembrane domains of Na,K-ATPase, and in particular, the cytoplasmic domain is more accessible and sensitive to perturbations by urea than the trans-membrane sector, and lipid-protein interactions are also insensitive to the denaturant (40). Thus, the lack of protection by FXYD proteins against unfolding by guanidinium chloride argues strongly against the involvement of the extramembrane domains of both the \alpha, \beta, and the FXYD proteins in the stabilizing interactions and, by exclusion, in favor of intramembrane interactions. The result quoted above that human FXYD2b and rat FXYD2a (with different N-terminal sequences see Table 1) protect similarly against thermal inactivation also precludes a major role for the extracellular segments preceding the FXYD motif.

Previous work supports the conclusion that interactions of the trans-membrane segments of the FXYD proteins are prominent in the FXYD-\alpha\beta complex. For example, we have shown that co-immunoprecipitation of FXYD4 with \alpha\beta is more sensitive to the detergent C\textsubscript{12}E\textsubscript{10} when compared with FXYD2, and the differences are attributable to residues in the trans-membrane segment (equivalent to 44IL45 versus 55AV56 and Ala-34 versus Gly-45 for human FXYD2 versus FXYD4, respectively) (23). Conversely, in experiments with \textit{Xenopus} oocytes, although the FXYD motif was shown to be required for correct assembly of FXYD2 and FXYD4 with \alpha\beta (44), this was not the case for FXYD7, but rather the two conserved glycine residues within the trans-membrane segment were important (Gly-41 and Gly-30 in human FXYD2). These observations argue against an overriding role of the FXYDY motif in the FXYD-\alpha\beta interaction (45). Similarly the human G41R mutation of FXYD2, associated with hereditary renal Mg\textsuperscript{2+}-wasting, disrupts assembly of FXYD2 with the \alpha subunit and trafficking of FXYD2 to the cell membrane (46, 47). The residues identified in these studies are all on one face of the trans-membrane segment of the FXYD protein (Gly-30, Ala-34, Gly-41, 44IL45 in human FXYD2) and interact with M9 of \alpha (see Fig 14). Although the aromatic residues in FXYDY bridge both \alpha and \beta subunits, these interactions of the FXYD subunit appear to be less predominant than the trans-membrane interactions. They could, rather, orient the trans-membrane segment of the FXYD protein for correct interaction with M9 of the \alpha subunit.

Fig. 14 presents two views of the interaction of the FXYD protein (FXYD10) with \alpha and \beta subunits of the shark rectal gland Na,K-ATPase in the 2.4-Å resolution structure determined recently (21). The trans-membrane segment of the FXYD protein interacts with only one trans-membrane segment of the \alpha subunit (M9) confirming the finding in Ref. 20,
and the aromatic residues of the FXYDY sequence interact with both α and β subunits (Fig. 14, left). According to the evidence presented in this study, the trans-membrane interactions of the FXYD protein are the major determinants of the stabilizing effect, whereas the interactions of the FXYD sequence itself are less important. Thus, we propose (Fig. 14, right) that SOPS is bound specifically near the trans-membrane segment of the FXYD protein and trans-membrane segments of the α subunit, and the FXYD protein amplifies the SOPS-α interactions, making it less easy for the detergent to displace the bound SOPS. As shown in Fig 14 (right) there are two possible interactive positions for SOPS, on either side of the FXYD protein, in the crevices between M8, M9, and M10 or M2, M6, and M9 of the α subunit. Of course, this model is subject to further proof. In forthcoming papers, we shall provide independent evidence for binding of lipid molecules between both M8, M9, and M10 and M2, M6, and M9. The concept in Fig. 14 (right) is also consistent with a finding that anionic lipids affect kinetic effects of FXYD10 on shark rectal gland enzyme (48).

Another interesting feature concerns the large difference in stability of the protein in NaCl- or KCl-containing media in the absence of the FXYD protein (Na+ > K+), but little or no difference in the presence of FXYD proteins (Na ≈ K) (Figs. 4 and 9 and supplemental Figs. S1 and S2). A possible explanation can be proposed on the basis of the likely position of a Na-specific third cation site between M6, M8, and M9 (see Ref. 49), adjacent to the trans-membrane segment of the FXYD protein (Fig. 14, right). In the absence of both the FXYD protein and the bound Na ion, M9 may be relatively mobile and the bound SOPS easily displaced by the detergent. A Na ion bound in the Na-selective site may stabilize M9 and the bound SOPS more effectively than bound K ions, reducing the ability of the detergent to remove the SOPS. By contrast, in the presence of the FXYD protein, M9 and the SOPS may be immobilized, when either Na or K ions are bound, so that the SOPS is less easily displaced, in either condition.

Inactivation of Na,K-ATPase activity by heating or excess detergent may be attributed to irreversible changes in disposition of trans-membrane segments that affect the ability to sustain $E_1 \leftrightarrow E_2$ conformational changes or occlude cations. Previously, for example, it was shown that heating of renal Na,K-ATPase induces an irreversible change in topological organization of the M8-M10 trans-membrane segments (50, 51). The experiments with both fluorescein-labeled protein and RH421, especially those in the absence of FXYD1, add some insights into the mechanism of inactivation. First, Na+ binding, detected by the RH421 signal, is inactivated by about 30% after incubation at 45 °C for 1 h (Fig. 7C). Second, the ATP-dependent change in the RH421 signal, indicative of the subsequent steps $E_1N_a3 + ATP \rightarrow E_1P(Na3) + ADP \rightarrow E_2PNa3 \rightarrow E_2P + 3Na^+$, is more sensitive and is inactivated over 80% after incubation at 45 °C for 1 h (Fig. 7D). The RH421 experiments do not distinguish whether phosphorylation and Na+ occlusion, $E_1P(Na3) + ATP \rightarrow E_1P(Na3) + ADP$, or the conformation transition, $E_1P(Na3) \rightarrow E_2PNa3$, itself are preferentially inactivated, because the dye monitors the subsequent Na+ release. The results with the fluorescein-labeled enzyme (Fig. 6) demonstrate, however, that the transition $E_1 \rightarrow E_2$ when Rb+ is bound is strongly inactivated by heating. Thus, this observation suggests that the transition $E_1P(Na3) \rightarrow E_2PNa3$ with Na+ bound may be the most sensitive to thermal inactivation in the Na+-translocating pathway. In any event, as observed in Fig. 7, FXYD1 protects against inactivation of both cation occlusion and the $E_1-E_2$ conformational changes.

A final point concerns the experiment with the HeLa cell membranes (Fig. 13). In these conditions, of course, no detergent is present. The Na,K-ATPase activity is much less thermally labile than in the detergent-soluble purified recombinant protein. Nevertheless, all three FXYD proteins protect strongly against thermal inactivation. With the knowledge of the mechanism of the protective effects of FXYD proteins in the purified recombinant protein, one could assume that heating increases the chance of displacement of the PS from its specific binding site(s) with the accompanying irreversible inactivation, and the FXYD protein protects against that event. Because FXYD5 is expressed natively in HeLa cells (42), the observed stabilizing effects of FXYD1, FXYD2, and FXYD4 are over and above any such effects of FXYD5 itself (or any other natively expressed FXYD protein). In any case, protection by all three expressed FXYD proteins against thermal inactivation in a native mammalian cell membrane is consistent with the notion that this phenomenon has biological significance.

**Biological Significance of Stabilization by FXYD Proteins**—Cellular proteins are subject to a variety of stress conditions that may affect their folding and compromise their biological function. A key cellular feature is to identify misfolded proteins and either re-fold them with the aid of chaperones or direct them to degradation in the lysosome or proteosome. In the case of membrane proteins, this is accomplished by the endocytotic pathway. The plasma membrane is in a dynamic equilibrium with the endosomal compartment. In the endosome, membrane proteins undergo a “quality check” and are either recycled back to the cell surface or are targeted to the lysosome (52). In addition to the physiological temperature of 37 °C that can be considered normal “stress,” oxidative stress (either ischemia or hypoxia) is a common type, leading to the formation of free radicals and damage to cell proteins. Interestingly, FXYD proteins are particularly abundant in organs that are sensitive to oxidative stress, *i.e.* heart (FXYD1), brain (FXYD6 and FXYD7), and kidney (FXYD2, FXYD4, and FXYD5) (3). As discussed below, FXYD2 plays an important role in the response of kidney cells to osmotic stress.

In principle, stabilization of the Na,K-ATPase at physiological temperature (37 °C) by FXYD proteins could, over time, increase the pump density by reducing degradation or endocytosis, thereby increasing Na,K-pumping capacity. Conversely, lack of association of FXYD proteins with αβ subunits may destabilize and lead to increased degradation or endocytosis of the protein and lower levels of Na,K-ATPase activity. A number of examples illustrate this hypothesis, although, it must be admitted, direct evidence is lacking. One relevant observation is that, by contrast with the specific expression pattern of FXYD2 in native kidney tissue (53), FXYD2 is not normally expressed in

---

8 E. Kapri-Pardes, A. Katz, and S. J. D. Karlish, manuscript in preparation.
cultured renal cells (54, 55), although other FXYD proteins such as FXYD1 can be expressed in renal cells (42). However, in conditions that are more stressful than normal culture media, it is possible to induce expression of FXYD2 in kidney cells (IMCD3, NRK-52E), for example by adapting them to higher than normal hypertonicity (56–58) and also to some extent by heat shock, oxidative stress, and heavy metals (58). These observations have led to the suggestion that FXYD2 adapts the pump function and cell survival to different stress conditions. Adaptation of IMCD3 cells to hypertonicity has been studied in detail. In particular, induction of FXYD2 in the IMCD3 cells adapted to hypertonic media is essential, because knockdown of the FXYD2 by siRNA is lethal to the IMCD3 cells in the hypertonic media (59). Interestingly, both α and β subunits and FXYD2 are up-regulated, but the signaling pathways used for the subunits are different (α, β transcription are regulated by Na\(^{+}\)\_cyt and FXYD2 by Cl\(^{-}\)\_cyt, respectively) (56, 60). It might appear paradoxical that adaptation to hypertonicity is associated with increased levels of αβ subunits, which should increase the total Na/K-pumping capacity of the cells, and also FXYD2, which reduces the apparent affinity for Na\(^{+}\)\_cyt and could reduce the Na/K-pumping capacity. However, if the stabilization effect of FXYD2 leads to compensatory increased pump density this could off-set the effect of a reduced Na\(^{+}\)\_cyt affinity. Another example concerns the G41R mutant of human FXYD2 associated with autosomal dominant renal hypomagnesia (46, 47). As reviewed in Ref. 61, the distal convoluted tubule is the site of active renal Mg\(^{2+}\) absorption and regulation of Mg\(^{2+}\) balance. Although it is known that the G41R mutant FXYD2 does not associate with the αβ units, the mechanism of Mg\(^{2+}\) loss is not known (46, 47). In relation to the hypothesis raised here, it seems possible that, over time, the lack of association with FXYD2 G41R and loss of stabilization of αβ subunits reduces the density of pumps in the distal convoluted tubule, thereby compromising the ability of the cell to maintain the driving force for active Mg\(^{2+}\) transport (trans-epithelial voltage, Na\(^{+}\) gradients). A recent paper reports that induction of FXYD2 in human proximal tubule cells by hypertonic media is associated with increased Na,K-ATPase activity and α subunit expression, but the increase in Na,K-ATPase activity and α subunit density is blunted in the cells from patients with the G41R mutation, thus associating the two phenomena (62).

Another example has been described in connection with mice deficient in FXYD1. In these mice, which also showed increased cardiac mass and ejection fraction, Na,K-ATPase activity was found to be significantly depressed, due both to reduced α subunit expression and apparently a lower turnover rate (30). Interestingly, although the level of α1 was decreased by about 20%, α2 was much more decreased, by about 60%. As we have reported, α2 seems to be intrinsically much less stable than α1, and it also associates less well with FXYD1 (27, 63). Thus, the deficiency of FXYD1 may be more destabilizing for α2 than for α1.

Although these examples are suggestive, they do not prove that expression levels of αβ subunits and FXYD proteins are necessarily correlated in response to stress. Indeed, it must be born in mind that stress can lead to exchange of expression of one FXYD protein for another in association with the Na,K-ATPase, namely FXYD2a for FXYD1 in kidney cells exposed to hyperosmotic media (42). This finding could imply that FXYD proteins are obligatory rather than subsidiary subunits of Na-K-ATPase, and responses of Na-K-ATPase to cellular stress involve interchanging different FXYD proteins rather than expressing one particular member of the family. Of course, not all stress need have the same consequence for the cell. Obviously, the hypothesis that expression levels of FXYD proteins and the α subunit are correlated could be tested directly by looking at the lifetime and steady-state levels of the α subunit in cells at the physiological temperature, and different conditions, when associated or not associated with FXYD proteins.

Acknowledgments—We thank Drs. Haim Haviv and Adriana Katz for helpful comments on the manuscript.

REFERENCES

1. Jorgensen, P. L., Hakansson, K. O., and Karlish, S. J. (2003) *Annu. Rev. Physiol.* 65, 817–849
2. Kaplan, J. H. (2002) *Annu. Rev. Biochem.* 71, 511–535
3. Sweadner, K. J., and Rael, E. (2000) *Genomics* 68, 41–56
4. Garty, H., and Karlish, S. J. (2006) *Annu. Rev. Physiol.* 68, 431–459
5. Geering, K. (2006) *Am. J. Physiol. Renal Physiol.* 290, F241–F250
6. Geering, K. (2008) *Curr. Opin. Nephrol. Hypertens* 17, 526–532
7. Blanco, G., and Mercer, R. W. (1998) *Am. J. Physiol.* 275, F633–F650
8. Palmer, C. J., Scott, B. T., and Jones, L. R. (1991) *J. Biol. Chem.* 266, 11126–11130
9. Mercer, R. W., Biemesderfer, D., Bliss, D. P., Jr., Collins, J. H., and Forbush, B., 3rd. (1993) *J. Cell Biol.* 121, 579–586
10. Morrison, B. W., Moorman, J. R., Kowdley, G. C., Kobayashi, Y. M., Jones, L. R., and Leder, P. (1995) *J. Biol. Chem.* 270, 2176–2182
11. Attali, B., Latter, H., Rachamim, N., and Garty, H. (1995) *Proc. Natl. Acad. Sci. U.S.A.* 92, 6092–6096
12. Ino, Y., Gotoh, T., Sakamoto, M., Tsukagoshi, K., and Hirohashi, S. (2002) *Proc. Natl. Acad. Sci. U.S.A.* 99, 365–370
13. Yamaguchi, F., Yamaguchi, K., Tai, Y., Sugimoto, K., and Tokuda, M. (2001) *Brain Res. Mol. Brain Res.* 86, 189–192
14. Béguin, P., Crambert, G., Monnet-Tschudi, F., Uldry, M., Horisberger, J. D., Garty, H., and Geering, K. (2002) *EMBO J.* 21, 3264–3273
15. Blostein, R., Pu, H. X., Scanzano, R., and Zouzoulas, A. (2003) *Ann. N.Y. Acad. Sci.* 986, 420–427
16. Sweadner, K. J., Arystarkhova, E., Donnet, C., and Wetzel, R. (2003) *Ann. N.Y. Acad. Sci.* 986, 382–387
17. Zouzoulas, A., Dunham, P. B., and Blostein, R. (2005) *J. Membr. Biol.* 204, 49–56
18. Shibata, S., Roy, S., Schaefer, D., Horisberger, J. D., and Geering, K. (2008) *J. Biol. Chem.* 283, 476–486
19. Bossuyt, J., Despa, S., Han, F., Hou, Z., Robia, S. L., Lingrel, J. B., and Bers, D. M. (2009) *J. Biol. Chem.* 284, 26749–26757
20. Morth, J. P., Pedersen, B. P., Toustrup-Jensen, M. S., Sørensen, T. L., Petersen, J., Andersen, J. P., Vilsen, B., and Nissen, P. (2007) *Nature* 450, 1043–1049
21. Shinoda, T., Ogawa, H., Cornelius, F., and Toyoshima, C. (2009) *Nature* 459, 446–450
22. Li, C., Grosdidier, A., Crambert, G., Horisberger, J. D., Michielin, O., and Geering, K. (2004) *J. Biol. Chem.* 279, 38895–38902
23. Lindzen, M., Aizman, R., Lifshitz, Y., Lubsarski, I., Karlish, S. J., and Garty, H. (2003) *J. Biol. Chem.* 278, 18738–18743
24. Strugatsky, D., Gottschalk, K. E., Goldshleger, R., Bibi, E., and Karlish, S. J. (2003) *J. Biol. Chem.* 278, 46064–46073
25. Cohen, E., Goldshleger, R., Shainskaya, A., Tal, D. M., Ebel, C., le Maire, M., and Karlish, S. J. (2005) *J. Biol. Chem.* 280, 16610–16618
26. Haviv, H., Cohen, E., Lifshitz, Y., Tal, D. M., Goldshleger, R., and Karlish, S. J. (2007) *Biochemistry* 46, 12855–12867

MARCH 18, 2011•VOLUME 286•NUMBER 11 JOURNAL OF BIOLOGICAL CHEMISTRY 9711
FX2 Proteins Stabilize Na/K-ATPase

27. Lifshitz, Y., Petrovich, E., Haviv, H., Goldshleger, R., Tal, D. M., Garty, H., and Karlish, S. J. (2007) Biochemistry 46, 14937–14950
28. Lifshitz, Y., Lindzen, M., Garty, H., and Karlish, S. J. (2006) J. Biol. Chem. 281, 15790–15799
29. Jones, D. H., Li, T. Y., Arystarkhova, E., Barr, K. J., Wetzel, R. K., Peng, J., Markham, K., Sweadner, K. J., Fong, G. H., and Kidder, G. M. (2005) J. Biol. Chem. 280, 19003–19011
30. Jia, L. G., Donnet, C., Bogaev, R. C., Blatt, R. J., McKinney, C. E., Day, K. H., Berr, S. S., Jones, L. R., Moorman, J. R., Sweadner, K. J., and Tucker, A. L. (2005) Am. J. Physiol. Heart Circ. Physiol. 288, H1982–H1988
31. Garty, H., Lindzen, M., Scanzano, R., Aizman, R., Fuzesi, M., Goldshleger, R., Farman, N., Blostein, R., and Karlish, S. J. (2002) Am. J. Physiol. Renal Physiol. 283, F607–F615
32. Lindzen, M., Gottschalk, K. E., Fuzesi, M., Garty, H., and Karlish, S. J. (2006) J. Biol. Chem. 281, 5947–5955
33. Peleg, Y., and Unger, T. (2008) Methods Mol. Biol. 426, 197–208
34. van den Ent, F., and Lowe, J. (2006) J. Biochem. Biophys. Methods 67, 67–74
35. Dowhan, W., Wickner, W. T., and Kennedy, E. P. (1974) J. Biol. Chem. 249, 3079–3084
36. Belogus, T., Haviv, H., and Karlish, S. J. (2009) J. Biol. Chem. 284, 31038–31051
37. Habeck, M., Cirri, E., Katz, A., Karlish, S. J., and Apell, H. J. (2009) Biochemistry 48, 9147–9155
38. Price, E. M., and Lingrel, J. B. (1988) Biochemistry 27, 8400–8408
39. Pedersen, M., Roudna, M., Beutner, S., Birmes, M., Reifers, B., Martin, H. D., and Apell, H. J. (2002) J. Membr. Biol. 185, 221–236
40. Babavali, M., Esmann, M., Fedosova, N. U., and Marsh, D. (2009) Biochemistry 48, 9022–9030
41. Pu, H. X., Cluzeaud, F., Goldshleger, R., Karlish, S. J., Farman, N., and Blostein, R. (2001) J. Biol. Chem. 276, 20370–20378
42. Arystarkhova, E., Donnet, C., Munoz-Matta, A., Specht, S. C., and Sweadner, K. J. (2007) Am. J. Physiol. Cell Physiol. 292, C1179–C1191
43. White, S. H., and Wimley, W. C. (1999) Annu. Rev. Biophys. Biomol. Struct. 28, 319–365
44. Beguin, P., Crambert, G., Guennoun, S., Garty, H., Horisberger, J. D., and Geering, K. (2001) EMBO J. 20, 3993–4002
45. Crambert, G., Li, C., Swee, L. K., and Geering, K. (2004) J. Biol. Chem. 279, 30888–30895
46. Meij, I. C., Koenderink, J. B., van Bokhoven, H., Assink, K. F., Groenestege, W. T., de Pont, J. J., Bindels, R. J., Munnens, L. A., van den Heuvel, L. P., and Knoers, N. V. (2000) Nat. Genet. 26, 265–266
47. Pu, H. X., Scanzano, R., and Blostein, R. (2002) J. Biol. Chem. 277, 20270–20276
48. Cornelius, F., and Mahmoud, Y. A. (2007) Biochemistry 46, 2371–2379
49. Li, C., Capendeguy, O., Geering, K., and Horisberger, J. D. (2005) Proc. Natl. Acad. Sci. U.S.A. 102, 12706–12711
50. Goldshleger, R., Tal, D. M., and Karlish, S. J. (1995) Biochemistry 34, 8668–8679
51. Arystarkhova, E., Gibbons, D. L., and Sweadner, K. J. (1995) J. Biol. Chem. 270, 8785–8796
52. Krebs, M. P., Noorwez, S. M., Malhotra, R., and Kaushal, S. (2004) Trends Biochem. Sci. 29, 648–655
53. Pihakaski-Maunsbach, K., Vorum, H., Honore, B., Tokonabe, S., Frokiaer, J., Garty, H., Karlish, S. J., and Maunsbach, A. B. (2006) Am. J. Physiol. Renal Physiol. 291, F1033–F1044
54. Arystarkhova, E., Wetzel, R. K., Asinovski, N. K., and Sweadner, K. J. (1999) J. Biol. Chem. 274, 33183–33185
55. Therien, A. G., Goldshleger, R., Karlish, S. J., and Blostein, R. (1997) J. Biol. Chem. 272, 32628–32634
56. Capasso, J. M., Rivard, C., and Berl, T. (2001) Proc. Natl. Acad. Sci. U.S.A. 98, 13414–13419
57. Capasso, J. M., Rivard, C. J., Enomoto, L. M., and Berl, T. (2003) Ann. N.Y. Acad. Sci. 986, 410–415
58. Wetzel, R. K., Pascoa, J. L., and Arystarkhova, E. (2004) J. Biol. Chem. 279, 41750–41757
59. Capasso, J. M., Rivard, C. J., and Berl, T. (2006) Am. J. Physiol. Renal Physiol. 291, F1142–F1147
60. Capasso, J. M., Rivard, C. J., Enomoto, L. M., and Berl, T. (2003) Proc. Natl. Acad. Sci. U.S.A. 100, 6428–6433
61. Dai, L. J., Ritchie, G., Kerstan, D., Kang, H. S., Cole, D. E., and Quamme, G. A. (2001) Physiol. Rev. 81, 51–84
62. Cairo, B. E., Swarts, H. G., Wilmer, M. J., Willems, P. H., Levchenko, E. N., De Pont, J. J., and Koenderink, J. B. (2009) J. Membr. Biol. 231, 117–124
63. Crambert, G., Fuzesi, M., Garty, H., Karlish, S., and Geering, K. (2002) Proc. Natl. Acad. Sci. U.S.A. 99, 11476–11481