Structural Interactions between FXYD Proteins and Na\(^{+}\),K\(^{+}\)-ATPase

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Interactions of rat FXYD4 (corticosteroid hormone-induced factor (CHIF)), FXYD2 (\(\gamma\)), or FXYD1 (phospholemman (PLM)) proteins with rat \(\alpha\)1 subunits of Na\(^{+}\),K\(^{+}\)-ATPase have been analyzed by co-immunoprecipitation and covalent cross-linking. In detergent-solubilized membranes from HeLa cells expressing both \(\gamma\) and CHIF or CHIF and hemagglutinin A-tagged CHIF, mixed complexes of CHIF and \(\gamma\) or CHIF and hemagglutinin A-tagged CHIF with \(\alpha/\beta\) subunits are undetectable. This implies that the \(\alpha/\beta\) FXYD protomer is the major species in detergent solution. A lipid-soluble cysteine-cysteine bifunctional reagent, dibromobimane, cross-links CHIF to \(\alpha\) in colonic membranes but not \(\gamma\) or PLM to \(\alpha\) in kidney or heart membranes, respectively. Sequence comparisons of the FXYD proteins suggested that Cys-49 in the trans-membrane segment of CHIF could be involved. In detergent-solubilized HeLa cell membranes, dibromobimane cross-links wild-type CHIF to \(\alpha\) but not the C49F mutant, and also the corresponding F36C mutant but not wild-type \(\gamma\)b, and F48C but not wild-type PLM. C140S, C338A, C804A, and C966S mutants of the \(\alpha\) subunit have been expressed. Only the C140S mutant prevents cross-linking with CHIF. The data demonstrated the proximity of trans-membrane segments of CHIF, \(\gamma\), and PLM to M2 of \(\alpha\). Molecular modeling is consistent with location of the trans-membrane segment of all FXYD proteins between M2, M6, and M9 and the proximity of Cys-49 of CHIF or Phe-36 of \(\gamma\) with Cys-140 of M2. Cross-linking also demonstrated CHIF-\(\alpha\) and CHIF-\(\beta\) proximities in extra-membrane regions, similar to the evidence for \(\gamma\)-\(\alpha\) and \(\gamma\)-\(\beta\) cross-links.

The Na\(^{+}\),K\(^{+}\)-ATPase actively transports Na\(^{+}\) and K\(^{+}\) ions across cell membranes and is responsible for maintaining the normal trans-membrane gradients of Na\(^{+}\) and K\(^{+}\) ions. As could be expected for a protein with such a central physiological role, Na\(^{+}\),K\(^{+}\)-ATPase is closely regulated at several levels. A unique mode of regulation of the Na\(^{+}\),K\(^{+}\)-ATPase has been described recently, involving interactions between the \(\alpha/\beta\) complex and the FXYD proteins (1–4). The FXYD family consists of seven short single-span trans-membrane proteins (extracellular N terminus), named after the conserved FXYD motif in the extracellular segment (5). The FXYD proteins show a tissue-specific expression pattern. For example, \(\gamma\) is expressed in kidney, CHIF\(^{+}\) in kidney and colon, PLM in heart and skeletal muscle, and FXYD7 in brain. In kidney \(\gamma\) is expressed as two splice variants, \(\gamma\)a and \(\gamma\)b (5, 6). Six members of the family, FXYD1 (PLM), FXYD2 (\(\gamma\)), FXYD3 (Mat-8), FXYD4 (CHIF), FXYD5 (related to ion channel), and FXYD7, are now known to interact specifically with the Na\(^{+}\),K\(^{+}\)-ATPase and to alter the pump kinetics in characteristic and different ways (2–4). Thus, the working hypothesis is that FXYD proteins function as tissue-specific modulators of Na\(^{+}\),K\(^{+}\)-ATPase, which adjust its kinetic behavior to the special needs of the given tissue, cell type, or physiological state (3, 4, 7, 8).

Functional interactions between the \(\gamma\) subunit and the Na\(^{+}\),K\(^{+}\)-ATPase have now been studied extensively. The \(\gamma\) subunit has been found to raise the apparent affinity for ATP, shifting the E1-E2 conformational equilibrium toward E2 (9–11), and reduces the apparent affinity for cytoplasmatic Na\(^{+}\) by making cytoplasmatic K\(^{+}\) a better competitor and slightly reduces extracellular K\(^{+}\) affinity (11–13). In Xenopus oocytes and whole HeLa cells with a membrane potential of ~50 mV or more hyperpolarized, \(\gamma\) somewhat raises extracellular K\(^{+}\) affinity (13, 14). Exposure of cultured renal cells to hyperosmotic solutions has been reported to be associated with a significantly reduced V\(_{\text{max}}\) value of Na\(^{+}\),K\(^{+}\)-ATPase in parallel with induction of expression of \(\gamma\) and the effect is blocked by silencing \(\gamma\) expression with small interfering RNA (15). By contrast, however, transfection of \(\gamma\) into Xenopus oocytes or HeLa cells is not associated with significant effects on V\(_{\text{max}}\) values (13, 14). In renal microsomes isolated from \(\gamma\) knock-out mice, the major effect appears to be that on the cytoplasmic Na\(^{+}\) affinity (16). CHIF raises the apparent affinity for cell Na\(^{+}\) by ~2–3-fold, the reverse effect to that of \(\gamma\) (13, 17). The opposite functional effects of CHIF and \(\gamma\) on the apparent Na\(^{+}\) affinity are consistent with their different patterns of expression along the nephron and physiological roles (reviewed in Refs. 2–4).

The structural interactions of FXYD proteins with \(\alpha/\beta\) subunits are not well understood, but information is accumulating (4). Structure-function studies have supplied good evidence for multiple sites of interaction involving both trans-membrane segments and the extra-membrane domains. Based on a series of \(\gamma\)-CHIF chimeric molecules in which extracellular, trans-membrane, and cytoplasmic sequences were interchanged, the stability of the FXYD-\(\alpha/\beta\) complex in detergent and the effects on the apparent Na\(^{+}\) affinity were found to be determined by the origin of the trans-membrane segment (18). The latter conclusion was confirmed in a study showing that peptides corresponding to the

\(^{4}\) The abbreviations used are: CHIF, corticosteroid hormone-induced factor; PLM, phospholemman; HA, hemagglutinin A; DOPC, 1,2-oleoyl-sn-glycero-3-phosphocholine or dioleoyl phosphatidylcholine; DOPG, 1,2-oleoyl-sn-glycero-3-(phospho-cytidino- or dioloyl phosphatidylserine; C$_{12}$E$_{6}$, polyoxyethylene(10)dodecylether; DB, dibromobimane; Ni-NTA, nickel-nitrilotriacetic acid; NHS-ASA, N-hydroxy-succinimidyl-4-azidosalicylic acid; DSTD, disuccinimidyl tartrate; EDC, 1-ethyl-3,3-dimethylpropylcarbodiimide; IRES, internal ribosome entry site; HPLC, high pressure liquid chromatography; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; Tricine, N-[2-hydroxy-1-bis[2-hydroxyethyl]amino]glycine; SERCA, sarcoplasmic reticulum Ca\(^{2+}\)-ATPase.\n
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trans-membrane segment of γ reduce apparent Na⁺ affinity of the α/β complex in HeLa cell membranes (19). In summary, trans-membrane segments mediate the effects of γ and CHIF on cation affinities, whereas the extra-membrane segments mediate the effect of γ on apparent ATP affinity.

On the basis of cryoelectron microscopy of renal Na⁺,K⁺-ATPase, the γ subunit helix was proposed to lie in a groove bound by M2, M6, and M9 of the α subunit (20). A denaturation study also suggested that γ might interact in the M8 to M10 region (21). A role for M9 of the α subunit has been inferred from the effects of mutants in M9 on the stability of α/β-γ, α/β-CHIF, or α/β-FXYD7 complexes, and the functional consequences of the mutations have been studied in Xenopus oocytes (22). Modeling of the FXYD helix was consistent with docking in the groove between M2, M6, and M9 in the α subunit. In a recent study we have used covalent cross-linking to obtain direct evidence for proximities between the γ subunit and α and β subunits of pig kidney Na⁺,K⁺-ATPase and in HeLa cells expressing rat α and γ subunits (23).

The inferred position of the α-γ cross-links was at Lys-54 and Lys-55 in the cytoplasmic domains of γ and Lys-347 of α (pig γ and α1 numbering), which lies in the cytoplasmic stalk 54 emerging from M4. This site of cross-linking in the cytoplasmic stalk overlaps the groove between M2, M6, and M9 and supports the proposed location of the trans-membrane segment. Based on the cross-linking and other data on α-γ proximities, we modeled interactions of the trans-membrane α-helix and an unstructured cytoplasmic segment SKRLRCGGKKHR of γ with a homology model of the pig α1 subunit. According to the model, the trans-membrane segment fits in a groove between M2, M6, and M9, and the cytoplasmic segment interacts with loops L6/7, L8/9, and stalk S5.

Because the model in Ref. 23 is based on limited information, its major value is that it provides a framework for further experimentation designed to test its predictions and to modify it as necessary. One objective of this study has been to obtain more direct evidence for the location of the trans-membrane segment of the FXYD by using primarily a hydrophobic cysteine-cysteine cross-linker, dibromobimane, with a range of 3.2–6.6 Å (24–26). The experiments utilize HeLa cells expressing rat α1 subunit and γ, CHIF, PLM, or the appropriate mutants. A related objective of this study has been to define the stoichiometry of α/β/FXYD interactions by co-immunoprecipitation assays. Previously, we showed that α/β subunits of purified renal Na⁺,K⁺-ATPase or renal microsomal membranes could be co-immunoprecipitated with either γa or γb but not both γa and γb and similarly with either CHIF or γb but not with both CHIF and γ (17, 27). A possible problem with the experiment with CHIF and γ is that CHIF and γ are largely expressed in different nephron segments, and thus membranes must have been derived from different cell types. Thus, the current series of experiments have been analyzed by co-immunoprecipitation assays whether different FXYD proteins, expressed in the same HeLa cells, interact simultaneously with α/β subunits. Together, the experiments provide information on the likely stoichiometric ratios of α/β/FXYD and the location of the trans-membrane segment of FXYD proteins. Part of these data have been published previously in an abstract form (28).

**MATERIALS AND METHODS**

**DNA Manipulations**—The coding regions of rat CHIF, γ, γb, and PLM were subcloned into the BamHI/BstXI site of the mammalian expression vector pIREs-hyg (Clontech). This vector has an internal ribosome entry site (IRES) that enables translation of the cloned cDNA and the hygromycin-resistant gene from a single RNA species. Thus, a better correlation is achieved between hygromycin resistance and high level expression of the desired protein. For transfection of more than one FXYD protein, the second round of transfection utilized a pIREs vector that confers puromycin resistance (pIREs-P, kindly provided by Dr. S. Hobbs, Royal Cancer Hospital). Hemagglutinin A (HA) and His₉₀ tags were introduced in the C termini (between the last amino acid and the stop codon) of CHIF and rat α1, respectively. The tags were added by PCR using appropriate oligonucleotides, and the modified constructs were subcloned into pIREs-P (CHIF) and pCDNA-3 (α1) vectors. A pig β subunit tagged in its N terminus with His₉₀ (29) was excised from the pGAP2α vector (Invitrogen) and subcloned into pIREs-P. Point mutations were introduced in FXYD proteins and the α1 and β1 subunits of Na⁺,K⁺-ATPase by PCR using overlapping primers. All constructs were verified by sequencing.

**Transfection and Selection of HeLa Cells**—HeLa cells overexpressing the rat α1 subunit of Na⁺,K⁺-ATPase (HeLa-α1, kindly provided by Dr. J. B Lingrel, University of Cincinnati College of Medicine) were transfected with FXYD constructs by Polyfect (Qiagen) according to the manufacturer’s instructions. Colonies stably expressing various FXYD constructs were selected in 400 μg/ml hygromycin B, and membranes were tested for protein expression by Western blotting. HeLa-α1 cells expressing different wild-type and mutated FXYD proteins (CHIF, γb, and PLM) were transfected with the His₉₀-β construct in pIREs-P, and colonies expressing His₉₀-β were selected in 2 μg/ml puromycin. For co-expression of two different FXYD proteins, single colonies expressing rat α1 and CHIF were transfected with constructs containing γb or HA-CHIF cloned into pIREs-P, and transfectants were selected in 2 μg/ml puromycin. HeLa cells expressing six histidine-tagged rat α1 and its mutants were selected at 1 μM ouabain. This concentration of ouabain fully blocks the endogenous (human) but not the transfected (rat) pump.

**Immunoprecipitation Assays**—HeLa cell membranes were suspended in a buffer containing 25 mM imidazole and 1 mM EDTA, pH 7.5, and either 20 mM TrisHCl, 20 mM NaCl plus 0.1 mg/ml oligomycin (incubated for 25 min at room temperature), or 10 mM RbCl plus 5 mM ouabain (incubated for 20 min at room temperature). Membranes were solubilized at 0 °C by adding C₁₁E₁₀ to a final concentration of 1 mg/ml (unless otherwise indicated) and a final protein concentration of ~0.5 mg/ml. The detergent-solubilized membranes were centrifuged for 30 min at 100,000 × g, and the supernatant was collected, and TrisHCl, NaCl, or RbCl was added to a final concentration of 100 mM. An ~40-μg aliquot of protein was removed (total protein samples), and the rest was subjected to immunoprecipitation. Total protein samples were delipidated by adding 4 volumes of methanol/ether (2:1) and incubation at −20 °C overnight. The suspensions were spun down, and the pellets were dried and dissolved in SDS-PAGE sample buffer. For immunoprecipitation (17), the detergent-solubilized proteins (~300 μg) were incubated for 4 h at 4 °C with an appropriate antibody (anti-α, anti-γC, anti-CHIF, anti-HA, at 1:50 dilutions). Protein A-Sepharose was added (50-μl volumes of washed and preincubated beads), and the suspensions were further incubated with swirling overnight at 4 °C. The beads were sedimented and washed three times in the original imidazole/EDTA buffer plus TrisHCl, NaCl, or RbCl plus 0.2 mg/ml C₁₁E₁₀. They were suspended in SDS-PAGE sample buffer and resolved on 10% Tricine gels together with 40-μg aliquots of the total delipidated membrane proteins. Proteins were transferred to polyvinylidene difluoride membrane filters in CAPS buffer plus 10% methanol (12 V, 1.4 h). The immunoblots were carried out using polyclonal antibodies to the C termini of α (anti-KETYY, 1:3,000), CHIF (1:1,000), HA (1:10,000), or γ (anti-γC 1:1,000).

**Enzyme and Membrane Preparations**—Purified renal Na⁺,K⁺-ATPase (30), rat colonocyte membranes (31), and rat cardiac membranes (32) were prepared as described previously. Plasma membranes were prepared from transfected HeLa-α1 cells as described in Ref. 17.
Dibromobimane-induced Cross-linking—Colonocyte membranes were solubilized with 0.25 mg/ml C12E10 in a medium containing 10 mM HEPES, pH 8.0, 20 mM NaCl, and 0.1 mg/ml oligomycin. Renal Na+/K+-ATPase and cardiac membranes were solubilized in similar solutions, but containing 1 or 1.5 mg/ml C12E10, respectively. Unsolubilized protein was removed by centrifugation. The soluble protein was treated with 1–2 mM dibromobimane, added in 3 aliquots each time for 20 min. In some cases the protein was diluted a further 10-fold with the solubilization buffer before addition of the dibromobimane. The reaction was stopped by addition of 50 mM dithiothreitol. Protein was precipitated by addition of 2% SDS and 4 volumes of methanol/ether (2:1 v/v).

HeLa cells expressing the rat α1 subunit were stably transfected with His6-tagged β subunit and wild-type or mutant FXYD proteins (CHIF, γ, or PLM). Alternatively, HeLa cells expressing rat CHIF were transfected with His6-tagged rat wild-type or mutant α1 subunits. Membranes were solubilized in 2 mg/ml C12E10 in the presence of 25 mM imidazole, 10 mM RbCl, and 5 mM ouabain. Na+/K+-ATPase complexes were bound to Ni-NTA beads by incubation for 1 h at 4 °C, and the beads were washed twice with 10 volumes of the solubilization buffer containing also 0.3 mg/ml of DOPC/DOPS (2:1 w/w) and then 30 mM imidazole to remove contaminant proteins. The washed beads were then incubated for 2 h at 4 °C and another hour at room temperature with 0.02 mM DB or diluent. The beads were solubilized in SDS-PAGE sample buffer, and proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride paper, and the bands were visualized in immunoblots using the appropriate antibodies (anti-CHIF, anti-γC, or anti-PLM, respectively).

Cross-linking Induced by NHS-ASA, DST, and EDC—C12E10-solubilized or intact colonocyte membranes were treated with NHS-ASA, DST, and EDC as described for renal Na+/K+-ATPase (23). In the case of NHS-ASA the intact colonocyte membranes were first reacted in the dark at pH 9.5, and then the membranes were solubilized with 0.25 mg/ml C12E10 in a medium containing 10 mM HEPES, pH 8.0, 20 mM NaCl, and 0.1 mg/ml of oligomycin, prior to illumination with UV light.

Modeling the α-FXYD Interaction—The trans-membrane domains of γ and CHIF were positioned manually between the helices M2, M6, and M9 of a homology model of the rat α1 subunit of Na+/K+-ATPase. The homology model was obtained as described previously (23, 33). For both γ and CHIF, three different starting conformations with identical backbone but varying side chain conformations were generated. From each starting conformation, 100,000 structures were generated by randomly rotating the helix 0–360° around the long axis, translating it −3 to +5 Å toward or away from the center of the bundle, respectively, translating the helix ±10 Å along its long axis, and tilting the CHIF helix relative to the fixed angles of the TM helices of the α1 subunit by ±20°. The coordinate transformations were done using the program package CNS parameter set (34, 35). Each such generated conformation was subjected to 50 steps of Powell minimization, and the interaction energy of the FXYD helix with the α1 subunit was calculated. A dielectric constant of 20 was used for the electrostatic interactions. The rotation was sampled in 1° bins and the tilt in 0.1° bins, and the transmembrane and translational shifts were sampled in 0.1 Å bins. The average energy of each bin was evaluated independently by using Boltzmann averaging of all conformations within this bin. A similar procedure has been shown to reliably model four helix bundles (36, 37).

RESULTS

Co-immunoprecipitation of FXYD Proteins with α/β Complexes—Figs. 1 and 2 document the results of immunoprecipitation experiments, which indicate the possible stoichiometric ratios of rat α/β subunits with or without co-expressed rat CHIF, HA-tagged CHIF, or γ in detergent-soluble complexes isolated from HeLa cells. To obtain cultures expressing two FXYD proteins, single colonies of transfected cells expressing CHIF were grown and then transfected with yb or HA-CHIF in pIRES-P, and colonies were selected in hygromycin plus puromycin and grown. This procedure ensures that all the cells of the transfected culture express both CHIF and either yb or HA-CHIF and that both FXYD proteins are exposed to α/β subunits and to each other in the cellular context. Co-immunoprecipitation experiments were done with HeLa cell membranes dissolved in C12E10 in the presence of Rb+ plus ouabain, conditions that we have shown previously to optimize the yields of co-immunoprecipitated α/β and FXYD proteins (17). For the experiment of Fig. 1, the detergent-solubilized membranes from HeLa cells expressing CHIF and γ together, or CHIF or γ alone, were immunoprecipitated with anti-γC or anti-CHIF or with monoclonal antibody, which recognizes an epitope near the N terminus of the α subunit (6H). The immunoprecipitated proteins were resolved by SDS-PAGE, and Western blots were probed with either anti-CHIF or anti-γC. γ is expressed as two bands as observed previously in HeLa cells (referred to as yb and yb′ (11)). The result is quite clear-cut. Although antibody 6H immunoprecipitates both CHIF and γ, and both anti-γ and anti-CHIF immunoprecipitate α, anti-CHIF immunoprecipitates CHIF but not γ, and anti-γ immunoprecipitates γ but not CHIF. The experiments
do not detect significant amounts of mixed complexes containing both CHIF and γ. Judging by the amounts of total (T) and immunoprecipitated protein applied to the gels, and the intensity of the chemiluminescent signals, the efficiency of co-immunoprecipitation of CHIF or γ with α is about 1–2% of the total CHIF and about 5% of total γ, similar to efficiencies observed previously (17, 18). Immunoprecipitation of CHIF by anti-CHIF and especially by γ by anti-γ is significantly more efficient than co-immunoprecipitation by α. This is seen in all three cell types and could indicate either or both that there is an excess of CHIF over α/β subunits or that the α/β/γ and α/β/CHIF complexes are partially dissociated in the conditions of the immunoprecipitation assays. Fig. 2 presents a similar experiment with membranes expressing CHIF and HA-CHIF, using anti-CHIF, which recognizes both CHIF and HA-CHIF (which are distinguished by their different electrophoretic mobilities) and anti-HA, which recognizes only HA-CHIF. Anti-CHIF immunoprecipitates both CHIF and HA-CHIF, whereas anti-HA immunoprecipitates only HA-CHIF. Anti-α antibody 6H1 immunoprecipitates both CHIF and HA-CHIF. Again, mixed complexes containing both CHIF and HA-CHIF were not detected. As discussed under the “Discussion,” both co-immunoprecipitation experiments suggest that the protomeric complexes α/β/γ, α/β/CHIF, or α/β/HA-CHIF, respectively, are the major species present in the detergent solution.

**Dibromobimane-induced Cross-linking of CHIF to the α Subunit in Native Colonicocyte Membranes**—Rat colonic membranes express high levels of CHIF mRNA and protein especially after feeding the rats high K+ or low Na+ diets (31, 38, 39). Membranes obtained from such colonicocytes are suitable for demonstrating α-CHIF cross-links. For this purpose we have screened a number of cysteine-reactive bifunctional reagents. The most informative reagent turned out to be dibromobimane (DB), which is a rigid molecule, soluble in organic solvents with a cross-linking range 3–6.6 Å (25, 26). The experiment in Fig. 3 compared the results of treatment of detergent-solubilized rat colonicocyte membranes, pig renal Na+,K+-ATPase enriched in γ, and rat cardiac membranes, which express PLM. It is striking that DB (at 1–2 mM) produced an α-CHIF cross-link and also a CHIF-CHIF cross-link with colonicocyte membranes, but essentially no cross-linked species of either γ in renal Na+,K+-ATPase or PLM in cardiac membranes. The α-CHIF cross-linked band was also detected with anti-KETYY (not shown). Incubation of the C12E10-solubilized colonicocytes with DB in the presence of SDS eliminated all cross-links. In addition, the CHIF-CHIF cross-linked species could be largely eliminated by a 10-fold dilution, whereas the α-CHIF cross-link was unaffected. The latter control experiments indicate that we are dealing with a specific intra-molecular cross-link between proximal cysteine residues of CHIF and the α subunit. Additional minor cross-linked bands were also seen in the colonicocytes and pig kidney Na,K-ATPase (Fig. 3, asterisks) but were not investigated further. Because DB induced cross-linking of CHIF but not of γ or PLM in native membranes, one could hypothesize that the cross-link involves a cysteine unique to CHIF. The position of cysteine residues of rat CHIF, γ, and PLM seen in the sequence alignment of Fig. 4 provides a clue to the basis of the selectivity of DB. All three FXYD proteins have a conserved cysteine in the cytoplasmic segment (C63, CHIF numbering), and both CHIF and PLM have an additional cytoplasmic cysteine (Cys-61), but only CHIF has a cysteine in the trans-membrane segment (Cys-49), whereas both γ and PLM have a phenylalanine residue at this position. Thus, one could hypothesize that Cys-49 is the cysteine involved in the α-CHIF cross-link.

**Dibromobimane-induced Cross-linking of FXYD Proteins with the α Subunit in HeLa Cells**—The hypothesis just proposed was tested by looking at DB-induced α-FXYD cross-links in HeLa cells (Figs. 5 and 6). The pig β1 subunit with a His10 tag engineered at the N terminus was transfected into HeLa cells expressing rat α1 cells, together with wild-type or mutant CHIF, γ, and PLM. The His10 tag was introduced at the N terminus of the β subunit, rather than elsewhere in the molecule, because of our recent work showing that when a tag is incorporated at this position the recombinant enzyme is fully functional (29). Membranes were then isolated and dissolved with C12E10 in conditions leading to optimal interactions of the FXYD proteins and α/β subunits (17). The detergent-soluble α/His-β complex was attached to Ni2+–NTA beads, which were washed with a medium containing 30 mM imidazole to remove contaminant proteins. The recent work shows that the porcine α-His10β complex is purified by at least 100-fold when eluted from the beads with 250 mM imidazole (29). Fig. 5 shows two important features, which validate the use of the beads in the present context. First, after incubating the soluble membrane preparations containing α, β, and CHIF (Fig. 5, soluble) with the beads, and washing with 30 mM imidazole to remove contaminant proteins, α, β, and CHIF are eluted together with 250 mM imidazole (Fig. 5, beads). Second, after washing the beads with 30 mM imidazole, further washing with 0.1% SDS removes α and CHIF, but not β, which remains bound via the His10 tag and is released only with 250 mM imidazole. The experiment shows that rat α1 and CHIF are noncovalently associated with the porcine His10β subunit, which was attached to the beads as a rat α1/porcine His10-β/...
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ChIF complex, and SDS dissociates α and CHIF from the bound αβ/β CHIF complex. For cross-linking of FXYD proteins associated with this α subunit, beads with bound αβ/β FXYD complexes were then incubated with DB, and finally, α, FXYD, and αFXYD cross-linked complexes were eluted with the SDS-PAGE sample buffer. The experiment in Fig. 6A shows cross-linking of CHIF with α and a control for specificity showing that prior denaturation of the bound αβ/CHIF complex with SDS prevents cross-linking. We have shown recently that added phospholipids are required to maintain recombinant Na+,K+-ATPase functional during purification by Ni-NTA chromatography, presumably by maintaining intact the structural organization of the transmembrane segments (29). Fig. 6B shows that the efficiency of the DB-induced cross-link is much improved when phospholipids (DOPC and DOPS) are included in the wash solution. We have reported that the α-CHIF interaction is easily disrupted even by the nonionic detergent C12E10 (18). Thus, the finding in Fig. 6B suggests that the phospholipids maintain intact native interactions between trans-membrane segments of CHIF and α and β subunits and that the cross-link occurs on a functional form of the αβ/CHIF complex. Other experiments showed that the α-CHIF cross-link was optimal at pH 7.5 and at a concentration of 0.02 mM DB. The fact that a much lower concentration of DB was required for cross-linking α and CHIF on the beads compared with native colonicyn membranes (1–2 mM) is not surprising, because the excess lipid and other proteins of native membranes could solubilize or react with the DB, respectively, reducing its effective concentration in the vicinity of the αβ/CHIF complex. This result provides further supporting evidence for the selectivity of the DB-induced α-CHIF cross-linking on the beads. The cross-linked band in Fig. 6 was detected with anti-CHIF. In principle, the cross-link should also be detectable with an anti-α antibody and run just above the α subunit, but in practice the resolution was not good enough, and the α subunit in the cross-linked sample ran as a broader band compared with the α subunit itself (not shown). The experiment of Fig. 7 examines the involvement of the cytosolic residue within the trans-membrane segment in the DB-induced cross-link. Wild-type CHIF or a C49F mutant, wild-type γ or a F36C mutant, and wild-type PLM or a F48C mutant were expressed together with rat α1 and His10β1 subunits, and the DB cross-linking was carried out in the optimal conditions described in Fig. 6. To detect the cross-linked bands, blots were probed with anti-CHIF, anti-γC, or anti-PLM antibodies, respectively. The result is clear-cut. Wild-type CHIF but not γ or PLM are cross-linked to α, as found also in native membranes. The C49F mutation prevents the α-CHIF cross-linking, whereas either the F36C mutation in γ or the F48C mutation in PLM produce cross-linked α-γ or α-PLM bands, respectively. Thus, the experiment demonstrates proximity of either the cysteine or phenylalanine residue in the transmembrane segment of CHIF, γ, or PLM, respectively, to a cysteine residue in the α subunit.

The model in Ref. 23 with the trans-membrane segment of γ docked into the groove between M2, M6, and M9 predicts that Phe-36 in γ,

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**FIGURE 5.** Expression of α1-His10β1/FXYD complexes in HeLa cells. Purification on Ni2+-NTA beads. Membrane (300 μg) prepared from HeLa cells expressing rat α1 and CHIF and pig His10β1 were suspended in a medium containing 10 mM Tris, pH 7.4, 20 mM Tris, pH 7.4, 100 mM RbCl, and 5 mM ouabain. Protein was eluted either with 250 mM imidazole or by washing the beads with 0.1% SDS for 1 min or 1 h. Aliquots of soluble protein (Soluble) or proteins eluted with 250 mM imidazole (Beads) or SDS were applied to gels. Immunoblots were developed with anti-KETTY (α), anti-β, or anti-CHIF antibodies.

**FIGURE 6.** DB-induced cross-linking of CHIF to α on Ni2+-NTA beads. A, HeLa cells stably expressing α, His10β1, and CHIF were solubilized in 2 mg/ml C12E10 in the presence of Rb+ and ouabain. Na+,K+-ATPase complexes were purified on Ni-NTA beads and incubated for 2 h at 4 °C and another hour at room temperature with 0.02 mM DB or diluted (control). SDS (0.1%) was added to one sample prior to the incubation (SDS + DB). The beads were solubilized in SDS sample buffer, resolved on SDS-polyacrylamide gel, and blotted with purified anti-CHIF antibody. Only the high molecular weight range containing α-CHIF cross-links is depicted. All samples contained equal amounts of CHIF (not shown). B, Na+,K+-ATPase complexes were purified in the presence or absence of 0.05 mg/ml phosphatidylcholine and phosphatidylserine (lipid).

**FIGURE 7.** Involvement of Cys-49 of CHIF in the CHIF–α cross-link. HeLa cells overexpressing β-His10β1 were transfected with wild-type and mutated CHIF, γ, and PLM constructs. Membranes were solubilized in 2 mg/ml C12E10 in the presence of Rb+ and ouabain. Na+,K+-ATPase complexes were bound to Ni-NTA beads by incubation for 1 h at 4 °C, and the beads were washed twice with 10 volumes of the solubilization buffer containing also 0.3 mg/ml of DOPC/DOPS (2:1 w/v) and 30 mM imidazole to remove contaminant proteins. The washed beads were then incubated for 2 h at 4 °C and another hour at room temperature with 0.02 mM DB or diluent. The beads were solubilized in SDS sample buffer, resolved on SDS-polyacrylamide gel, and blotted with purified anti-FXYD antibody. Only the high molecular weight range containing α-FXYD cross-links is depicted. All samples contained equal amounts of the corresponding FXYD.
equivalent to Cys-49 of CHIF, is oriented toward M2. Therefore, we have mutated the candidate cysteine in M2, C140S, and as a control we have made also C338A (M4), C804A (M6), and C966S (M9) (Fig. 8). The choice of residues to replace cysteines was made because it has been shown previously that these specific mutations do not inactivate the Na\(^+\),K\(^+\)-ATPase (40). The His\(_{10}\)-tagged wild-type and mutant rat \(\alpha\) subunits were expressed with CHIF in the HeLa cells to allow purification of the expressed \(\alpha\) subunits and associated FXYD proteins on Ni-NTA beads, and so allow discrimination between transfected rat \(\alpha\) and the endogenous human \(\alpha\) subunit. Selection of transfected cells was done in 1 \(\mu\)mol ouabain, a concentration that should kill nontransfected HeLa cells but not cells expressing active rat Na\(^+\),K\(^+\)-ATPase (40) because of insensitivity of the rat enzyme for ouabain. Thus, both the wild-type and mutant rat pumps expressed in the transfecants must be active in order to keep the cells alive. After cross-linking of \(\alpha/\beta\) complexes attached to the beads with the associated CHIF, the cross-linked product was detected with anti-CHIF. In the case of the wild type with associated CHIF, the DB-induced cross-link was easily detected. Fig. 8 shows that the C140S mutation in M2 fully blocks the DB-induced \(\alpha\)-CHIF cross-link, whereas the C338A, C804A, and C966S mutations did not block the cross-link. Overall, the cross-linking experiments show clearly that Cys-49 in the trans-membrane segment of CHIF or the analogous residues of \(\gamma\), Phe-36, and Phe-48, are oriented toward Cys-140 in M2 of the \(\alpha\) subunit.

**CHIF-\(\alpha\) and CHIF-\(\beta\) Cross-links in Intact Colonocyte Membranes Induced by NHS-ASA, DST, and EDC**—With the aim of establishing whether the overall disposition of both CHIF and \(\gamma\) with respect to \(\alpha\) and \(\beta\) subunit is similar, Fig. 9 shows that three reagents NHS-ASA, DST, and EDC, which we have described recently for detection of extramembrane \(\alpha/\gamma\) and \(\beta/\gamma\) cross-links in purified renal Na\(^+\),K\(^+\)-ATPase (23), can also be used to cross-link CHIF to \(\alpha\) and \(\beta\) subunits in rat colonocytes membranes. In general the results are similar to those in Ref. 23 except that NHS-ASA cross-links CHIF to both \(\alpha\) and \(\beta\) subunits, whereas NHS-ASA cross-linked primarily \(\gamma\) to \(\alpha\) subunits in renal Na\(^+\),K\(^+\)-ATPase. DST cross-linked CHIF to both \(\alpha\) and \(\beta\) subunits, and EDC cross-linked CHIF to the \(\beta\) subunit, similar to effects of DST and EDC in renal Na\(^+\),K\(^+\)-ATPase (23). The control experiments in which membranes solubilized with 2% SDS prior to addition of the cross-linker showed no cross-linked products provide evidence for specificity of the cross-linking. A difference between DST-induced cross-linking of the \(\gamma/\alpha\) and CHIF-\(\alpha\) is that the \(\gamma/\alpha\) cross-link is amplified in a Na\(^+\)-containing medium compared with a Rb\(^+\)-containing medium, indicating a preference for the E\(_{1}\)Na over the E\(_{2}\)(Rb) conformation, whereas that is not the case for CHIF-\(\alpha\), as seen in Fig. 9. Thus these observations indicate that, with some difference in detail, both \(\gamma\) and CHIF come into proximity with both \(\alpha\) and \(\beta\) subunits.

**DISCUSSION**

**Oligomeric State of \(\alpha/\beta\) Subunits and FXYD Proteins**—The results of the co-immunoprecipitation experiments (Figs. 1 and 2) are consistent with the existence of \(\alpha/\beta\)-FXYD complexes, such as \(\alpha/\beta/\gamma\) and \(\alpha/\beta/\text{CHIF}\), but provide no evidence for complexes containing more than one FXYD protein, such as \(\alpha/\beta/\gamma/\text{CHIF}\) or \(\alpha/\beta/\text{CHIF}-\text{HA-CHIF}\), dimeric complexes, such as \(\alpha/\beta/\gamma/\alpha/\beta/\text{CHIF}\) or \(\alpha/\beta/\text{CHIF}-\alpha/\beta/\text{HA-CHIF}\), or higher order mixed complexes. Similarly, our previous experiments with native renal Na\(^+\),K\(^+\)-ATPase did not detect mixed complexes such as \(\alpha/\beta/\gamma/\alpha/\beta/\gamma\) or \(\alpha/\beta/\gamma/\alpha/\beta/\gamma/\beta\) (17). These inferences are based on co-immunoprecipitation of each FXYD protein with \(\alpha/\beta\) but lack of co-immunoprecipitation with other FXYD proteins. Because the term “lack of co-immunoprecipitation” depends on the detection limits of the Western blots, the more accurate statement would be that, in these conditions, mixed complexes are not major components and are present, if at all, below the detection limits. It is also important to emphasize that the experiments were done with C\(_{12}\)E\(_{10}\)-sulfonated membranes in the presence of Rb\(^+\) and ouabain, conditions that are optimal for immunoprecipitation of both \(\gamma\) and CHIF and preservation of \(\gamma/\alpha/\beta\) or CHIF-\(\alpha/\beta\) interactions (17). A possible objection to the main conclusion of the co-immunoprecipitation experiments, would arise if the stoichiometric ratios of FXYD: \(\alpha/\beta\) were far below 1. If, say the molar ratio of both \(\gamma/\alpha\) and CHIF-\(\alpha\) in the same cells was 1:100, the molar fraction of mixed complexes such as \(\alpha/\beta/\gamma/\alpha/\beta\) could be 1:10,000, and the amount might fall below the detection limit. In this condition the possibility of such a mixed complex could not be excluded. A similar argument applies to the statistical possibility of dimeric complexes such as \(\alpha/\beta/\gamma/\alpha/\beta\) or mixed complexes such as \(\alpha/\beta/\gamma/\alpha/\beta/\gamma\) and \(\alpha/\beta/\gamma/\alpha/\beta/\gamma/\beta\). However, there is prior evidence that for native pig kidney Na\(^+\),K\(^+\)-ATPase the \(\alpha/\beta\) : \(\gamma/\alpha/\beta\) ratio is close to 1:1:1, and \(\gamma/\alpha\) and \(\gamma/\beta\) are present in roughly equal proportions. (6, 41). In the current HeLa cell experiment with CHIF and HA-CHIF, the two FXYD proteins are expressed at roughly equal levels and give detectable functional effects.
showing that the molar ratio of CHIF:α/β cannot be far below 1. Furthermore, as mentioned under “Results,” the higher efficiency of immunoprecipitation of γ or CHIF by anti-γ or anti CHIF, compared with co-immunoprecipitation by anti-α, is consistent, if anything, with an excess of γ or CHIF compared with α in the HeLa cells. Thus, this objection does not seem to be applicable.

Another possible caveat concerning the possibility of dimeric species, such as ι/β/γ-α/β/γb, is that because the HeLa cells express both rat and human α1, it is conceivable that heterodimers between rat and human α1 subunits are precluded by nonconservation of amino acids involved in oligomerization, but not necessarily homodimers between identical α subunits. Again, this argument cannot apply to the previous experiments with pig kidney Na⁺,K⁺-ATPase with only the one species of α1, which still shows no detectable mixed complexes of α/β with γa and γb.

As discussed in the Introduction, the lack of mixed complexes of γ and CHIF in renal papilla membranes could have been due to the fact that γ and CHIF are expressed in different nephron segments and are not exposed to each other and α/β subunits in the cellular context. The current experiments avoid this difficulty with either γ and CHIF or CHIF and HA-CHIF because both FXYD proteins are expressed in the same cells and are exposed to each other and to the α/β complexes. Note again that this type of objection could not apply to the result with γa and γb from native pig kidney Na⁺,K⁺-ATPase, because both γa and γb are strongly expressed in cells of the thick ascending limb of Henle in red outer medulla from which the membranes are prepared.

The oligomeric state of the Na⁺,K⁺-ATPase has been investigated for many years but is still a controversial subject. On the one hand, there is evidence supporting the α/β protomer as the predominant structural and minimal functional unit of Na⁺,K⁺-ATPase activity (42–46), and on the other hand, a variety of evidence supports the existence of dimers or higher order oligomers of the α/β heterodimer as well as experiments interpreted to mean that the oligomeric structure is necessary for Na⁺,K⁺-ATPase activity (reviewed in Refs. 47 and 48). In effect, the FXYD proteins serve as tags of α/β complexes to obtain evidence on possible α/β oligomeric interactions. Our results are in agreement with previous evidence that the α/β protomer is the principal structural form and minimal functional unit of the Na⁺,K⁺-ATPase in the C₁₂E₈ solution, based on sedimentation equilibrium and velocity experiments (42, 44). The same conclusion concerning purified recombinant Na⁺,K⁺-ATPase in n-dodecyl-β-maltoside has emerged recently, based on sedimentation velocity and size-exclusion HPLC measurements (29). Similarly, fluorescence energy transfer experiments using fluorescent oxubain derivatives, and also chemical modification, suggest that the α/β protomer is the major species of the membrane-bound form of duck salt gland Na⁺,K⁺-ATPase (45). Because this preparation lacks γ or any low molecular weight subunits (49), the predominant α/β protomer structure is not attributable to the presence or absence of FXYD proteins. Of the evidence supporting the dimer or higher oligomer hypothesis, the closest in experimental approach to the present work is the demonstration of interactions of different α isoforms or tagged α subunits expressed in insect cells (50–52). After co-expression in insect cells of α1 and α2 isoforms, or recently of α subunits tagged with His₉, or the FLAG epitope, co-immunoprecipitation of α1 and α2 isoforms or His₉ and FLAG-tagged α subunits has been observed. This indicates that the protein can exist as a dimer or higher order oligomer in the detergent solution. The observations in Refs. 50 and 52, suggesting the existence of α/β dimers or oligomers, are different from those in this study. However, it is known that dimeric and higher order oligomeric as well as α/β protomeric forms of renal Na⁺,K⁺-ATPase dissolved in C₁₂E₈ can be detected in varying ratios, as detected by size-exclusion HPLC, when the protein is stabilized in different conformational states (53) or in the presence of different detergent and lipid concentrations (54). Thus, it is conceivable that the protein from different sources is stabilized in different oligomeric states, because of different detergent and lipid interactions, which play a crucial role in maintaining the soluble protein in a native structural and functional state (29, 43). In other words, the reported observations and our observations may not be incompatible, particularly because the quantitative fraction of α subunits undergoing the interactions detected by co-immunoprecipitation assays is not known and may not be high. Overall, the present experiments support the evidence for α/β protomers as the major structural and functional unit of Na⁺,K⁺-ATPase in detergent solution but do not exclude the possibility that dimers or higher order oligomers can form in some conditions, or exist in the membrane-bound state.

The conclusions on the oligomeric state of the α/β subunits and FXYD proteins are, of course, relevant to the DB-induced cross-linking. Lack of mixed complexes of α/β subunits with more than one FXYD protein make it most unlikely that there are separate domains of interaction of the α subunit with the trans-membrane segment of the different FXYD proteins.
Cross-linking of FXYD and α/β Subunits—The cross-linking experiments with DB show directly that the trans-membrane segments of γ, CHIF, and PLM are in proximity to M2 of the α subunit. Previously, the location of the trans-membrane segment in the groove between M2, M6, and M9 was inferred on the basis of electron microscopy of renal Na$_\text{v}$,K$^+$-ATPase (20), functional effects of mutations in M9 (22), and cross-linking of the cytoplasmic domain of the γ subunit to S4 of the α subunit (23). Specifically, the current experiments show that Cys-49 of CHIF is cross-linked to Cys-140 in M2 of the α subunit, because either the C49F or the C140S mutation specifically interferes with the cross-link. Similarly, the F36C mutation of γ and F48C mutation of PLM lead to DB-induced cross-links, indicating that the equivalent residues Phe-36 of γ or Phe-48 of PLM are also in proximity with Cys-140. The general conclusion could be that the trans-membrane segments of all FXYD proteins interact with the α subunit in the groove between M2, M6, and M9.

Models of a Subunit and Trans-membrane Segments of CHIF and γ—Fig. 10, A and B, present models of trans-membrane segments of rat CHIF and γ, respectively, interacting with trans-membrane segments M2, M6, and M9 of a homology model of the rat α1 subunit, based on the Ca$^{2+}$-ATPase crystal structure in the E, ATP bound conformation. These models are similar to the model in Ref. 23 but focus on the trans-membrane segments, and it is seen that both the longitudinal position and rotation of the trans-membrane segment of both γ and CHIF are predicted to be quite similar. In particular, the rotational orientation of both proteins with respect to the M2 segment of the α subunit fits very well with the DB-induced cross-linking of CHIF, γ (F36C), and PLM (F48C). The orientation of both Cys-140 in M2 and Cys-49 (CHIF) or Phe-36 (γ) is toward each other and the lipid membrane, and at a distance of 6.5 and 6.1 Å, respectively. The latter point is crucial because the cysteine residues must be accessible to the cross-linking reagent in the medium and at a distance compatible with DB cross-linking (3–6 Å) (26). By contrast, if either or both Cys-140 (α) and Cys-49 (CHIF) were oriented toward the inside of the groove cross-linking efficiency would be low, even if the cysteines were in proximity. Another point of interest is that Met-55 and Ala-56 of CHIF and Leu-42 and Ile-43 of γ point toward M9/M6 and M2, respectively. This is consistent with our previous finding that both residues are important for the α-CHIF and α-γ interaction, and are in proximity with more than one trans-membrane helix of α (18). Thus, both the cross-linking and the previous mutation data support this rotational orientation of the trans-membrane segment of CHIF, γ, PLM, and hence, presumably, of all the other FXYD proteins. Another point to note is that the longitudinal position of the trans-membrane segments of CHIF and γ also appears to be similar. Thus, the functional difference between CHIF and γ, to raise or lower the apparent affinity for cytoplasmic Na$^+$ ions, respectively, (13, 17), could be the product of differences of detailed interactions of the side chains of CHIF and γ in trans-membrane segments. Experiments are underway to identify residues responsible for the specific functional effects of trans-membrane segments of γ and CHIF.

It is of interest that phospholamban, the single trans-membrane protein regulator of cardiac SERCA-type Ca$^{2+}$-ATPases, is also known to dock into the groove between M2, M6, and M9 (55). Another regulator, sarcolipin, is able to form a ternary complex with SERCA and phospholamban (55, 56). Sarcolipin and phospholamban appear to bind to the same regulatory site in SERCA. However, in a ternary complex, phospholamban occupies the regulatory site, and sarcolipin binds to the exposed side of phospholamban and to SERCA. This finding of a ternary complex of phospholamban and sarcolipin with SERCA is, of course, different from our findings, which have not been demonstrated complexes with more than one FXYD protein.

**Interactions of Extra-membrane Regions of FXYD Proteins**—A significant point concerns the interactions of cytoplasmic and extracellular domains of the different FXYD proteins. The chemical specificity of NHS-ASA (lysine-nucleophile), DST (lysine-lysine), and EDC (carboxyl-lysyl) is such that the cross-links are predicted to be in hydrophilic regions of the protein. In the case of γ-α and γ-β cross-links, this was shown to be the case and can also be assumed for CHIF-α and CHIF-β cross-links. The overall indication from Fig. 9 and the results in Ref. 23 is that extra-membrane regions of both CHIF and γ interact with both α and β subunits. γ is known to raise the apparent affinity for ATP, by stabilizing the E1 conformation, via an interaction near the cytoplasmic C terminus (9, 57). CHIF does not have such an effect (17), and there is also a detailed difference in the DST-induced cross-link in that the γ-α but not CHIF-α cross-link is amplified in a Na$^+$-containing medium. The model in Ref. 23 predicts that the C-terminal region of γ interacts with the cytoplasmic stalk region S5 and loop L6–7. It is not known where the cytoplasmic domain of CHIF interacts. A recent finding that the C terminus of the FXYD protein PLMS of shark rectal gland Na$_{v}$,K$^+$-ATPase is cross-linked to the A domain of the α subunit (58) is different from the prediction of the model in Ref. 23. The dissimilar sequences of the extra-membrane segments and dissimilar functional and structural interactions of different FXYD proteins, attributable to their cytoplasmic domains, raise the question whether all FXYD proteins interact with the same cytoplasmic domain of α but differ in detailed interactions of specific side chains. Alternatively the cytoplasmic domains of different FXYD proteins could interact with different cytoplasmic domains of α. We are currently carrying out experiments to distinguish between these alternatives.

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