Structure-Function Relations of Interactions between Na,K-ATPase, the γ Subunit, and Corticosteroid Hormone-induced Factor*§

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Corticosteroid hormone-induced factor (CHIF) and the γ subunit of the Na,K-ATPase (γ) are two members of the FXYD family whose function has been elucidated recently. CHIF and γ interact with the Na⁺ pump and alter its kinetic properties, in different ways, which appear to serve their specific physiological roles. Although functional interactions with the Na,K-ATPase have been clearly demonstrated, it is not known which domains and which residues interact with the α and/or β subunits and affect the pump kinetics. The current study provides the first systematic analysis of structure-function relations of CHIF and γ. It is demonstrated that the stability of detergent-solubilized complexes of CHIF and γ with α and/or β subunits is determined by the trans-membrane segments, especially three residues that may be involved in hydrophobic interactions. The transmembrane segments also determine the opposite effects of CHIF and γ on the Na⁺ affinity of the pump, but the amino acids involved in this functional effect are different from those responsible for stable interactions with α.

CHIF is an aldosterone-induced gene, expressed only in kidney collecting duct and distal colon surface cells (10–12). CHIF is up-regulated by stimuli that enhance Na⁺ absorption and K⁺ secretion, i.e. Na⁺ deprivation and K⁺ loading (12–14). CHIF knockout mice exhibit an abnormality in water absorption that is secondary to a defect in electrolyte transport (15). γ too is a kidney-specific FXYD protein. However, the distribution of CHIF and γ along the nephron is quite different and is, in fact, mutually exclusive (8, 12). Expression of two γ splice variants (γa and γb) is moderate in proximal tubules and heavy in the medullary thick ascending limb; there is a difference in distribution of γa and γb in various distal cortical segments, with little or no expression of either γa or γb in the cortical connecting tubules and collecting duct and a low level of expression of γa in the inner medullary collecting duct (8, 16–18). CHIF on the other hand, is located only along the collecting duct and distal colon (8, 11, 12). Both CHIF and γ are immunoprecipitated by antibodies raised against the α subunit of the Na,K-ATPase, and the co-immunoprecipitation is efficient only in conditions that preserve native pump structure (8, 19). The effects of γ and CHIF on the pump kinetics are, however, quite different. γ raises the apparent affinity for ATP by shifting the E1-E2 conformational equilibrium toward E1, and reduces apparent affinity for cytoplasmic Na⁺ ions by increasing the affinity of cytoplasmic K⁺ ions as competitors for cytoplasmic Na⁺ ions (5, 17). On the other hand, CHIF increases the affinity of the pump for cell Na⁺ and has no effect on the affinity for ATP (7, 8).

Although the functional effects of CHIF and γ appear to be well established, it is not known which domains and which residues interact with the α and/or β subunits and affect the pump kinetics. The current study provides the first systematic analysis of structure-function relations of CHIF and γ. It is demonstrated that the stability in detergent of the complexes formed with the α and β subunits is determined by the transmembrane segments, especially the residues Gly⁴⁵, Met⁵⁵, and Ala⁶⁶. The trans-membrane segment also determines the opposite effects of CHIF and γ on the Na⁺ affinity, but the amino acids involved in this functional effect are different from those responsible for stable interactions with α.

EXPERIMENTAL PROCEDURES

cDNAs, Antibodies, and Transfected Cell Lines—cDNAs that contain the coding sequences of rat CHIF, γa and γb, subcloned into the mammalian expression vector pIRE2-hyg (Clontech), were described previously (8, 20). The various domains in CHIF were defined as follows: extracellular, Met⁷–Gln⁴⁸; transmembrane, Leu⁶⁰–Leu⁶⁷; and intracellular, Ser⁶⁷–Thr⁷⁵. These domains were replaced by the corresponding regions in rat γa or γb using standard recombinant DNA procedures and verified by sequencing. HeLa cells overexpressing the rat α1 subunit of Na,K-ATPase (HeLa-α1 cells, kindly provided by Dr. J. B. Lingrel, University of Cincinnati College of Medicine, This paper is available on line at http://www.jbc.org/
Cincinnati, OH) were transfected using Polyfect (Qiagen) according to the manufacturer’s instructions. Colonies expressing the FXYD constructs were selected in 400 μg/ml hygromycin B and tested by Western blotting. The following antibodies have been used: (a) a polyclonal antibody to the last 13 amino acids of rat CHIF (12); (b) a polyclonal antibody to the 10 C-terminal amino acids of γ (20); (c) a polyclonal antibody to the N-terminal residues MDRWYL of yb (20); (d) a monoclonal antibody directed against the N-terminal segment of the α subunit (6H, kindly provided by Dr. M. J. Caplan, Yale University School of Medicine, New Haven, CT) (5); and (e) a polyclonal antibody, and the yield decreased sharply at increased detergent concentration. The means ± S.E. of three to five experiments are depicted.

RESULTS

Previously, we demonstrated that CHIF, γ, and phospholemman are specifically co-immunoprecipitated with the α subunit of Na+,K-ATPase (6, 8). For both CHIF and γ co-immunoprecipitation was efficient only in conditions known to preserve the native pump conformations, namely when solubilization of membranes was done with C12E10 in the presence of Rb+ and ouabain. The particular material was removed by centrifugation, and the soluble fraction was immunoprecipitated with the anti-α antibody (6H), as described in Ref. 8. Total soluble proteins and immunoprophiles were blotted for the presence of FXYD and α as described previously (21, 22) using the following antibodies: anti- KETTY (1:3000), anti-CHIF (1:5000), and anti-γ (γ or N tail, 1:250). The blots were overlaid with horseradish peroxidase-coupled goat anti rabbit IgG (1:10,000) and analyzed by enhanced chemiluminescence. The abundance of FXYD message in stably transfected cell clones was determined by semi-quantitative PCR. RNA was extracted using TriReagent® (Molecular Research Center, Inc.), and cDNA was reverse transcribed from the poly(A)+ tail using SuperScript II RNase H (Invitrogen). It was amplified in LightCycler (Roche Applied Science) using specific primers corresponding to the FXYD and the pIRE- HYG sequences. The data were normalized by parallel amplification of GAPDH. 68Rb+ uptake was measured in monensin-permeabilized transfected HeLa-α1 cells as detailed in Refs. 8 and 23.

Fig. 1. Efficiency and detergent sensitivity of co-immunoprecipitation of FXYD/αβ complexes. HeLa-α1 cells stably transfected with either CHIF or yb were solubilized at the indicated concentrations of C12E10 in the presence of 10 mM RbCl and 5 mM ouabain. Nonsolubilized material was removed by centrifugation, and the proteins were immunoprecipitated from the detergent-solubilized fractions with anti-α antibody (6H). Part of the solubilized fraction and whole immunoprophiles were resolved electrophoretically and blotted with antibodies against CHIF or γ, as described under “Experimental Procedures.” A, a representative image depicting blotting of 5% of the total solubilized proteins (T) and all of the immunoprophile (IP), after solubilization at 0.35 and 1.5 mg/ml C12E10. yb runs as a doublet (γb and yb). B, the percentage of FXYD precipitated by the anti-α antibody was quantified by densitometric scans of the blots and plotted against the detergent concentration. The means ± S.E. of four of the six constructs, cell clones expressing the chimeric proteins at levels comparable with those of CHIF and γ in native tissue could be selected (Fig. 2B). As reported previously (5), yb was expressed in transfected cells as a doublet (yb and yb†), of which yb† could be a post-translationally modified protein. It is different from the doublet seen in kidney medulla, which reflects expression of γa and yb. A similar doublet (yb and yb†) was apparent in cells expressing γbγmC1α, whereas only a single band appeared in cells transfected with CαγmγC1α. Thus, yb† may carry a post-translational modification in the external domain of γ. Two chimeras that have the extracellular domain of γ and the trans-membrane segment of CHIF, γCmC1α and γCmγC1α, produced little or no protein. The same observation has been constructed and transfected in HeLa-α1 cells (Fig. 2A).
made irrespective of the γ splice variant used (γa or γb). Despite the lack of expression of these proteins, the chimeric cDNAs did transcribe mRNA at levels 30–200% of those of the nonchimeric constructs (not shown). Thus, the lack of expression of the protein may reflect inherent instability of these particular structures.

The four chimeras that were expressed were tested for co-immunoprecipitation with α and stability of the FXYD/α complex at increasing concentrations of C12E10 (Fig. 3). The key observation is that stability of the different chimera in C12E10 correlates with the origin of their transmembrane domain. Thus, the protein CeγmCi behaved like γ and could be effectively immunoprecipitated (>8%) at 1.5 mg/ml C12E10 (Fig. 3). On the other hand, exchanging the cytoplasmic domains or the N termini between the two FXYD proteins was without effect, i.e. CeγmCi and γmγnCi behaved as γ, whereas CmγmCi behaved as CHIF. Previous observations indicated that the phospholemman/α complex too is stable at relatively high concentrations of C10E12 (6). However, replacing the transmembrane domain of CHIF with that of phospholemman (P) did not substantially increase the immunoprecipitation efficiency at 2 mg/ml C12E10, i.e. the anti-α antibody immunoprecipitated 0.4 ± 0.2% (4) of the CHIF protein, 0.7 ± 0.4% (4) CmPmCi, and 7.0 ± 3.6% (3) phospholemman. Thus, there must be factors in addition to the transmembrane segments that are significant for stabilizing the interaction (see “Discussion”).

To further analyze the amino acids involved in the FXYD/α interactions, we have mutated different transmembrane residues and examined the consequences for stability of the CHIF/α complex. Initially, multiple mutations of transmembrane CHIF residues to the corresponding γ positions were tested (Fig. 4). The data indicated that mutagenesis can indeed increase immunoprecipitation efficiency at 2 mg/ml C12E10 and abolish the decrease in complex stability upon raising detergent concentration from 0.35 to 2 mg/ml C12E10.
also change the functional effects of this protein. Accordingly, effects on the pump kinetics of CHIF, γ, and the two CHIF triple mutants were compared (Fig. 6 and Table I). As expected, CHIF decreased and γ increased $K_{0.5}$ for Na$^+$ relative to the values measured in cell transfected with empty vector. The triple mutant A64L/M55I/A65L behaved essentially like CHIF and increased the apparent affinity to Na$^+$ even more than CHIF. Thus, these residues, which include two of the three amino acids that stabilize the CHIF/α complex, are not responsible for the different functional effects of CHIF and γ. Similarly, another triple mutation of residues that varies between CHIF and γ, Q38R/M42I/C49F, was without effect on function.

To further assess the functional role of different FXYD domains, we have tested the effects of the various chimeras on the affinity of the pump for cell Na$^+$. These experiments, summarized in Fig. 7, clearly demonstrated that although the triple mutations described above were without effect, the differences in Na$^+$ affinity conferred by CHIF or γ are governed by the transmembrane domain of these proteins. Thus, chimeras in which the C or N terminus of γ was replaced by the corresponding CHIF sequences (γ, Cγ, and Cγγ) behaved like γ and increased $K_{0.5}$. Similarly, a CHIF construct in which the C terminus was replaced by that of γ (CγCγ) behaved like CHIF. On the other hand, a CHIF construct with the trans-membrane domain derived from γ (CγCγ) did not express protein. Protein was expressed, however, if the chimera had, in addition, the triple mutation AMA → LIL. This mutated construct (γ, CγCγ) decreased $K_{0.5}$ like CHIF, although the mutation alone (A54L/M55I/A65L) was without effect. Taken together, the above data indicate that the transmembrane interactions also control the effects of CHIF and γ on the Na$^+$ affinity of the pump, but the interactions involved differ from those shown to control the FXYD/α complex stability. It should be noted that a source of error in these experiments is a variable level of FXYD expression in the transfected cells. Thus, if the FXYD/α stoichiometry is less than 1, the observed change in $K_{0.5}$ will be less than maximal. Accordingly, the only rigorous conclusion to be drawn from such experiments is whether a particular chimera increases or decreases Na$^+$ affinity. The observed magnitude of the effect could be an underestimation of the true one.

Fig. 4. Effects of point mutations on the immunoprecipitation efficiency of CHIF. A, alignment of the trans-membrane segments of rat CHIF, γ, and phospholemman. Amino acids numbers refer to CHIF. B, immunoprecipitation of CHIF and the two triple mutants. Stably transfected HeLa-a cells were solubilized in either 0.35 or 2.0 mg/ml C12E10, and the nonsolubilized material was removed by centrifugation. The soluble α/FXYD complexes were immunoprecipitated with an anti-α antibody and detected with an antibody against CHIF. C, immunoprecipitation of different mutated proteins at 2 mg/ml C12E10 is expressed as a percentage of the total expressed protein. The means ± S.E. of three or four experiments (numbers in parentheses) are depicted.

Fig. 5. Residues involved in the CHIF/α interactions. CHIF constructs carrying single and double mutations were expressed and assayed by co-immunoprecipitation with α. The means ± S.E. of six experiments are depicted. Significances of the difference from CHIF (+ and ***) and from the double mutant M55I/A65L (# and ##) were calculated by a two-tailed t test. * and #, p < 0.05; ** and ##, p < 0.01. Inset, wheel projection of the transmembrane domain of CHIF. G45, M55, and A56 are circled.
**DISCUSSION**

FXYD is a newly discovered family of single-span transmembrane proteins with a common extracellular motif (1). Four members of this group have been shown to interact with the a subunit of the Na,K-ATPase and alter the pump kinetics (3, 5–9). The working hypothesis is that FXYD proteins are tissue-specific regulators of the pump. Their function would be to adjust the pump kinetics in a specific tissue, cell type, or physiological state, without affecting it elsewhere.

CHIF and γ are two epithelial specific members of this family expressed primarily along the kidney nephron or in distal colon (CHIF) (8, 11, 12, 16, 17). The two proteins have opposite effects on the affinity of the pump for cytoplasmic Na⁺ (5, 8, 17) and interact differently with α (25). These differences have been used to study their structure-function relationships using chimeras formed between the two proteins, as well as point mutations. The major observation was that both the effect on the Na⁺ affinity and the stability of the complex in detergent are determined by the transmembrane segment, which is the most conserved region of the seven members of the FXYD protein family. Obviously, this does not exclude important structural and functional roles of the N and C termini. It has been reported that the FXYD sequence in the extracellular domain is itself essential for assembly of CHIF and γ with the pump (7). In addition, the anti-C-terminal antibody abrogates the effect of γ on ATP affinity but not that on sodium affinity, suggesting the existence of more than one interaction (5). It has been shown recently that both the C-terminal cytoplasmic and N-terminal extracellular sequences of the γ subunit affect the ATP affinity (26).

**TABLE I**

Effects of γ, CHIF, and CHIF with mutations in the trans-membrane segment on the apparent Na⁺ affinity

Ouabain-sensitive ³²P⁺ fluxes were measured for different Na⁺ activities as described under “Experimental Procedures.” The means ± S.E. of three to seven experiments (in parentheses) are shown.

| cDNA          | K⁺0.5 (mM) |
|---------------|------------|
| Empty vector  | 4.56 ± 0.30 (7) |
| CHIF          | 2.62 ± 0.50 (3) |
| γ             | 7.07 ± 1.02 (5) |
| γC³MγC⁻         | 1.41 ± 0.06 (3) |
| Cγγγγµγµ         | 1.54 ± 0.22 (5) |

One indication for stability of the FXYD/αβ complex in detergent is the efficiency of co-immunoprecipitation (Fig. 1). Because the soluble complexes preserve both the structural and functional integrity of the native complexes (27), we assume that this parameter is also indicative of protein/protein interactions that are relevant in the intact membrane. We found that the normal low stability of the CHIF/αβ complex can be increased by 4–10-fold to values characteristic of γ/αβ or phospholemman/αβ oligomers, by mutating one of three residues to the corresponding amino acids.
acids in \( \gamma \), i.e. G45A, M55I, or A56L. Helical wheel projection indicates that Gly\(^{45} \) and Ala\(^{46} \) face the same direction, whereas Met\(^{55} \) is oriented at an angle of \(-100^\circ \) (Fig. 5, inset). Thus, the above stabilizing mutations appear to be involved in interactions with two other helices in \( \beta \) and/or \( \alpha \). Interestingly, these residues appear to be well conserved among FXYD proteins species. All of the available CHIF sequences (mouse, rat, and human) have Gly at position 45, but all other FXYD have a well conserved Ala at this position (1). Similarly, other FXYD proteins have Ile, Leu, or Val in the positions corresponding to CHIF Met\(^{55} \) and Ala\(^{56} \).

The transmembrane domain also appears to govern at least one of the functional effects of CHIF and \( \gamma \). Exchanging these segments reversed the direction of the change in Na\(^+\) affinity, whereas substituting the N- or C-terminal sequences that lie outside the membrane was without effect on this parameter. Thus, intra-membrane interactions also determine at least one of the kinetic effects of these FXYD proteins. However, this functional interaction is different from that determining the stability of the complex because it is not affected by mutating \( ^{54}\text{AMA} \) into LIL. Three other mutations of nonconserved transmembrane residues (Q38R/M42L/C49F) were also ineffective in altering Na\(^+\) affinity. The effect on Na\(^+\) affinity must therefore be mediated by other residues or depends on a more complex interaction that requires a specific combination of the above residues. For example it is just conceivable that the \( ^{54}\text{AMA} \) to LIL mutation decrease \( K_{\text{ATP}} \) and secondarily \( K_{\text{Na}} \), thereby mask a \( \gamma \)-like effect on the \( K^+/\text{Na}^+ \) antagonism. To detect this kind of complex interaction, expression systems permitting more detailed functional analysis will be required. In conclusion, the present study provides the first systematic analysis of structure-function relations of FXYD proteins and highlights the important functional and structural role of the transmembrane segment.

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