Modulation of Na,K-ATPase by the γ Subunit

STUDIES WITH TRANSFECTED CELLS AND TRANSMEMBRANE MIMETIC PEPTIDES

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The enzymatic activity of the Na,K-ATPase, or sodium pump, is modulated by members of the so-called FXYD family of transmembrane proteins. The best characterized member, FXYD2, also referred to as the γ subunit, has been shown to decrease the apparent Na⁺ affinity and increase the apparent ATP affinity of the pump. The effect on ATP affinity had been ascribed to the cytoplasmic C-terminal end of the protein, whereas recent observations suggest that the transmembrane (TM) segment of γ mediates the Na⁺ affinity effect. Here we use a novel approach involving synthetic transmembrane mimetic peptides to demonstrate unequivocally that the TM domain of γ effects the shift in apparent Na⁺ affinity. Specifically, we show that incubation of these peptides with membranes containing αβ pumps modulates Na⁺ affinity in a manner similar to transfected full-length γ subunit. Using mutated γ peptides and transfected proteins, we also show that a specific glycine residue, Gly-41, which is associated with a form of familial renal hypomagnesemia when mutated to Arg, is important for this kinetic effect, whereas Gly-35, located on an alternate face of the transmembrane helix, is not. The peptide approach allows for the analysis of mutants that fail to be expressed in a transfected system.

The Na,K-ATPase or sodium pump is an integral membrane protein found in the cells of virtually all higher eukaryotes and is one of the most important systems involved in cellular energy transduction (1, 2). It catalyzes the electrogenic exchange of three intracellular Na⁺ for two extracellular K⁺ ions energized by the hydrolysis of one molecule of ATP. The transporter plays a major role in ion homeostasis, and, in epithelia, the sodium gradient created by the pump also plays an important role in secondary active transport mechanisms that are necessary for Na⁺-dependent reabsorption of a variety of solutes including sugars and amino acids.

There is an increasing body of evidence that members of a family of membrane proteins, the so-called FXYD family (3), associate with and modulate the kinetic behavior of the sodium pump (for a recent overview, see Ref 4). Members of this family of proteins are small, single transmembrane (TM) proteins characterized by an N-terminal PFXYD motif that remains invariant in all mammals. There are at least seven known family members, of which several appear to modulate the kinetic behavior of the pump in a tissue-specific manner (5–8). To date, the γ “subunit” of the renal Na,K-ATPase is the best characterized member (reviewed in Refs. 9 and 10). Gamma, or FXYD2, exists as two main splice variants, γα and γβ, with distinct as well as overlapping localization along the nephron (11). Mass spectrometry of γα and γβ indicate that they differ only in the N terminus; in rat γα, TELSANH is replaced by Ac-MDRWYL in γβ (12). Previous studies using membrane fragments isolated from γ-transfected rat α1-HeLa cells have shown that γ serves at least two distinct functions in regulating the pump, and the effects are similar for both variants (11, 13). These distinct effects of γ include the following: (i) an increase in the apparent affinity for ATP; and (ii) an increase in Kᵢ/Naᵢ competition at cytoplasmic Na⁺ activation sites, as seen by an increase in Kᵢ/Naᵢ at high [K⁺]. Previous studies have suggested that the effect on ATP affinity is mediated by the cytoplasmic C-terminal domain of the protein (5, 14), whereas effects on Kᵢ/Naᵢ antagonism may be associated with the TM domain (15).

In this study, we have used a novel strategy to assess directly the functional effects of the TM region of FXYD2 whereby peptides corresponding to the TM domain of γ are added directly to membranes derived from cells devoid of the protein. In addition, we describe a mutagenesis approach to structure/function analysis of γ by using both transfected cells and peptides. A residue of particular interest is Gly-41, because it is replaced by Arg (G41R) in a familial form of renal magnesium wasting (16) and is invariant throughout the FXYD family. The other residue examined is Gly-35 because of its location at the alternate face of the membrane relative to Gly-41 (17). Effects of peptides with either of these Gly residues replaced by Arg or Leu are compared with those of the full-length transfected γ proteins (wild-type or mutants of the γβ variant) expressed in cultured rat α1-transfected HeLa cells. This approach allows for assessment of the role of the TM region alone and provides an opportunity to distinguish the effects of mutations on biogenesis and routing to the plasma membrane from the direct effects on association and kinetic modulation of αβ pumps.

EXPERIMENTAL PROCEDURES

Mutagenesis, Transfection, Tissue Culture, and Membrane Preparations—Point mutations were introduced into γβ cDNA subeloned into

1 The abbreviations used are: TM, transmembrane; PFO, perfluoro-octanoate; Scr, scrambled (peptide); WT, wild-type.
the pIRES expression vector and then transfected into HeLa cells stably expressing the rat α1 subunit of the Na,K-ATPase (α1-HeLa cells) kindly provided by Dr. J. B. Lingrel as described previously (14). Unless indicated otherwise, cellular membranes from the stably transfected cells were prepared as described by Jewell and Lingrel (18). Polyacrylamide Gel Electrophoresis and Western Blotting—SDS-PAGE and Western blotting were carried out as described previously (11), and following transfer to polyvinylidene difluoride membranes, the lower half was analyzed with a polyclonal γ antibody (γC32 raised against the C-terminal KHRQVNEDEL peptide and essentially the same as γC33 described previously in Ref. 19), and the upper half was analyzed with anti-α1 subunit A277 (Sigma).

Co-immunoprecipitation—The method used is a modification of Gage et al. (20). Briefly, 250 μg of membranes, prepared from ybWT- and yb(mutant)-transfected cells as described previously (18), were resuspended and incubated at room temperature for 30 min in immunoprecipitation buffer containing 50 mM imidazole, pH 7.5, 1 mM EDTA, 10 mM RbCl, and 5 mM ouabain in a final volume of 250 μl. Polyoxyethylene 10 lauryl ether (C12E10) (250 μl of a 2 mg/ml solution in water) was then added (final concentration, 1 mg/ml), and the suspension was incubated for 30 min at 4 °C with end-to-end rotation. Following centrifugation at 4 °C for 30 min at 16,000 × g, the supernatant was added to 50 μl of bovine serum albumin (1 mg/ml)-treated protein A-Sepharose beads and incubated for 30 min at 4 °C, and the beads were then centrifuged to remove non-specifically bound proteins. The supernatant was removed, and a 20-μl aliquot was taken for SDS-PAGE. To another 450-μl aliquot, 90 μl of Rbc1 (118 μl final concentration) and 30 μl of γC32 anti-serum (stored at −20 °C in 50% glycerol) were added, and the suspension was further incubated for 4 h at 4 °C with end-to-end rotation. The suspension was then mixed with protein A-Sepharose beads (100 μl) and incubated overnight at 4 °C. Beads were washed six times with immunoprecipitation buffer without ouabain, and the immunoprecipitated proteins retained by the beads were eluted with 100 μl of SDS-PAGE sample buffer containing 5% β-mercaptoethanol at 37 °C for 30 min. A 20-μl aliquot of the eluate and the 10-μl sample removed before addition of antisera were resolved by SDS-PAGE followed by Western blotting.

Cell Surface Biotinylation—Transfected α1-HeLa cells were grown to ~80% confluence in 6-well plates. The surface biotinylation is a modification of Stephan et al. (21). Briefly, the cell surface biotinylation reaction was carried out on ice for one 20-min period using sulfosuccinimidyl-2-(biotinamido)ethyl-1,3-dithiopropionate (sulfo-NHS-SLS-biotin) (Pierce). After biotinylation, each well was rinsed briefly twice with a phosphate-buffered saline (PBS), 0.1 mM CaCl2, and 1 mM MgCl2 (CM) solution containing 100 mM glycine and then treated with the same solution for 30 min to ensure complete quenching of unreacted sulfo-NHS-SLS-biotin. The cells were then lysed for 45 min with 500 μl of L1 buffer (1% Triton X-100, 1% SDS in 150 mM NaCl, 5 mM EDTA, and 50 mM Tris-HCl, pH 7.5, containing 10 μg/ml each leupeptin and pepstatin and 1 mM phenylmethylsulfonyl fluoride). Each well was then scraped, and the sample was collected and centrifuged at 18,000 × g for 10 min. One 20-μl aliquot of supernatant (total fraction, T) was taken for protein determination (Lowry method) and another (5 μl), for Western blotting. The biotinylated surface-exposed proteins were isolated by incubating a 150-μg (~85 μl) fraction in L2 buffer (L1 buffer without SDS; final volume, 500 μl) with 100 μl of streptavidin-agarose beads (Pierce) overnight at 4 °C with gentle rotation. The samples were then centrifuged to separate beads and supernatant (unbound fraction, U), and an aliquot of U (15 μl = 2.5 μl of T) was taken for Western blotting. The beads were washed three times with L2 buffer, twice with high salt L2 buffer (L2 buffer with 500 mM NaCl and 0.1% Triton X-100), and then with L3 buffer with 50 mM Tris-HCl, pH 7.5. The biotinylated proteins were eluted from the beads (Fraction B) by incubation in 100 μl of SDS-PAGE sample buffer containing 5% β-mercaptoethanol at 85 °C for 10 min and an aliquot (30 μl = 25 μl of T) was taken for Western blotting.

Peptide Synthesis—Synthetic peptides corresponding to residues 23–45 of the TM segment of wild-type yb, mutant TM peptides with residues 23–45 of C32 anti-serum (stored at −20 °C in 50% glycerol) and each of the above-mentioned glyG41R and glyG35L mutants is the failure to detect post-translational modification of γ. Thus, in Western blots of multiple clones of rat α1-HeLa cells transfected with WT yb and each of the above-mentioned mutants (experiments not shown), the yb chain appeared as a doublet (yb and yb′; cf. Ref. 11) in WT yb and the conservative mutants ybG41L and ybG35L but not in the ybG41R and ybG35R mutants. We then looked at the functional properties of the various yb-transfected cells. Table I summarizes measurements of apparent Na⁺/affinity of Na,K-ATPase measured at high K⁺ concentration (100 mM) of membranes isolated from the mock-transfected (control) as well as the WT and mutant yb-transfected cells. The results indicate that ybG35L behaves like WT yb in that it decreases the apparent affinity for Na⁺; KNa⁺ is increased 2.5-fold by ybG35L and nearly 2-fold by yb. In contrast, ybG41L, like ybG41R, does not increase KNa⁺. Unfortunately, ybG35R mutants were expressed at levels too low for meaningful kinetic analysis.

Effects of Transmembrane Mimetic Peptides—Studies of the effects of peptides comprising the TM sequence of γ (γ-TM),
Western blotting using anti-
immunoprecipitate (IP procedures. A, membranes were solubilized and immunoprecipitated with γC32 as described under “Experimental Procedures.” Aliquots of the total solubilized protein (PreIP) and the immunoprecipitate (IP) were resolved by SDS-PAGE and analyzed by Western blotting using anti-γC32. As described, the aliquots of PreIP and IP represent 5 and 45 μg, respectively, of the original membrane protein. B, cell surface biotinylation was carried out as described under “Experimental Procedures.” For the unbound (U) and bound (B) fractions, the amounts analyzed by Western blot detection were 0.5 and 5 times the amounts of the total fraction (T) analyzed, respectively. For the biotinylation analysis in panel B, the amount of material analyzed with respect to the original cell number was ~1/100th that used in the co-immunoprecipitation analysis shown in panel A; for chemiluminescent detection, the time of exposure of the polyvinyl difluoride membrane to x-ray film was 60–120 times greater in panel B compared with panel A. n.d., expression of γbG35R was very low as shown by the PreIP (A) and the T fraction (B).

**TABLE I**

| Membranes | $K_{Na}$ at 100 mM $K^+$ |
|-----------|------------------------|
| Control (mock) | 4.8 ± 0.2 |
| γb         | 8.3 ± 0.4 |
| γbG41R     | 5.3 ± 0.2 |
| γbG41L     | 4.5 ± 0.02 |
| γbG35R     | n.d.* |
| γbG35Lα    | 10.7 ± 0.8 |

* p < 0.01 for γb and γbG35L compared to control membranes. n.d., not determined due to low expression.

G41R (γG41R-TM), G41L (γG41L-TM), G35R (γG35R-TM), and G35L (γG35L-TM) γ as well as a “scrambled” peptide (“γScr-TM”) were carried out with synthetic peptides containing “lysine tags” at both the N and the C termini. These tags overcome the inherent insolubility of these α-helical TM peptides, thus aiding in their synthesis and purification, but retain their native TM oligomeric states (17, 22). Fig. 3 shows the Na⁺ activation profile measured at high (100 mM) K⁺ concentration without and with the various TM peptides. Compared with γ-Scr-TM, the addition of either γ-Scr or γG35L-TM peptide to γ-devoid membranes isolated from rat α1-HeLa cells increases $K_{Na}$. The increase effected by G35L is even greater than seen with the WT peptide, similar to the greater effect of γbG35L compared with γb in γ-expressing cells (described above). In contrast, γG41R-TM, γG41L-TM, and γG35R-TM did not increase $K_{Na}$ above that observed with the control scrambled peptide (γScr-TM). A nonspecific effect of peptide addition is evident in a small but consistent increase in $K_{Na}$ seen irrespective of whether γScr-TM or any of the three mutants, γG35R-TM, γG41L-TM and γG41R-TM, is added. The other notable kinetic effect of the full-length γ chain, the decrease in $K_{ATP}$, was not seen with γ-Scr-TM (experiments not shown), consistent with earlier evidence (14) that this effect is mediated primarily by the cytoplasmic C terminus.

We showed previously that $K_{Na}$ is a linear function of $K^+$ concentration, approximating a simple competitive model of cytoplasmic K⁺/Na⁺ competition, i.e. $K_{Na} = K_Na(1 + [K^+] / K_c)$, and, that the main effect of either γa or γb was not due to an increase in $K_{Na}$ but rather to a decrease in $K_c$. To further assess the specificity of the mimery of the TM peptides, we determined $K_{Na}$ as a function of $K^+$ concentration and, thus, values of $K_{Na}^*$ (K*) when ($K^+ = 0$) and $K_{Na}^*$ (the apparent K⁺ affinity for competition with cytoplasmic Na⁺). The results shown in the inset to Fig. 3 support the conclusion that γ γ-Scr-TM and γG35L-TM peptides, like their full-length counterparts (see table in Ref. 11), decrease $K_{Na}$ with little effect on $K_{Na}$.

As shown in Fig. 4, in control experiments carried out with the γ-Scr peptide and/or the γG35L-TM peptide, an increase in $K_{Na}$ seen with mock-transfected cell membranes is seen also with γbG41R-transfected but not γb-transfected membranes. Thus, the kinetic effects of the mimetic peptides are highly specific in that they are seen only with pumps not already associated (mock transfected) or minimally associated (γb-G41R) with full-length γ chains. As expected, γG41L-TM and γG41R-TM peptides had no effect on the $K_{Na}$ values of pumps in any of the transfected systems.

**DISCUSSION**

The experiments described herein unequivocally show that the effect of γ (FXYD2) on the $K_{Na}$ values of Na,K-ATPase is mediated solely by the TM domain of the protein, as suggested recently (15). Thus, peptides comprising only this region of γ simulate functional effects obtained in assays performed on membranes isolated from wild-type and mutant γb-transfected α1-HeLa cells. The mimetic effects of the peptides underscore...
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Fig. 4. Effect of TM peptides on membranes isolated from γ b and ybG41R transfected rat α1-HeLa cells. $K_{\text{Na}}$ values were determined as in Fig. 3. Activity with TM peptides added to either α1-HeLa (control), yb-α1-HeLa, or γbG41R-α1-HeLa membranes are presented as the fold change in $K_{\text{Na}}$ compared with membranes with no peptide added, normalized to 1.0. Error bars are S.E. of at least three experiments. In the absence of added peptide, values of $K_{\text{Na}}$ were 4.8, 8.3, and 5.3 mM for control, γ b, and ybG41R membranes, respectively (see Table I). Data for control membranes are taken from Fig. 3.

The results of the present study provide insight into the role of two Gly residues, Gly-35 and Gly-41, which reside on opposite faces of the helix (see inset of Fig. 3 with Table I).

The feasibility of using TM peptides to evaluate kinetic effects of TM helices, including instances in which adequate expression is problematic, as is the case of the yb-G35R mutant. TM mimetic peptides offer the unique advantage of examining the function of the TM domain without the complexity of long-range effects of extramembranous regions. In fact, concern over this complexity was mentioned by Lindzen et al. (15) who raised the possibility that a change in $K_{\text{ATP}}$ effected by the cytoplasmic domain may mask an apparent effect of γ TM on $K_{\text{Na}}$/Na$^+$ antagonism, which may account for the larger decrease in $K_{\text{K}}$ by γ-TM and γG35L-TM compared with the analogous transfectants (compare inset of Fig. 3 with Table I).

The observation that both the yb-G35R (γ b) and ybG41R (11) mutants show either minimal expression or processing and trafficking led us to consider more conservative mutations in our attempts to understand the role of these residues in modulating pump function. We therefore studied G35L and G41L mutants in experiments with both transfected cells and synthesized peptides. These mutants circumvent the energetic cost associated with inserting a positively charged residue within the hydrophobic core of the membrane and are therefore more relevant in experiments with both transfected cells and synthesized peptides. These mutants are not only less important in understanding the role of the native glycine residues. This consideration may not only explain the low processing/trafficking of Arg mutants but also the lack of effect of the γG35R-TM peptide on sodium pump kinetics (Fig. 3).

The studies with both transfected cells and peptides provide evidence that disruption of a Gly residue (Gly-41) on one face of the γ helix but not on the other (Gly-35) abrogates the increased $K_{\text{Na}}$/Na$^+$ antagonism effect by γ as seen by a notable increase in $K_{\text{Na}}$ at a high K$^+$ concentration. Thus, the abolition of the kinetic effect is seen not only with the disrupting mutation G41R replacement associated with autosomal dominant renal magnesium wasting (16) but also with the conservative mutation, G41L. Previous results from our laboratory (14) suggested that Gly at position 41 is essential for normal trafficking to the cell surface and association with the pump. However, even when the cellular processing and trafficking problems are circumvented, as is the case with the G41L mutant and the peptide experiments presented herein, mutating this residue fails to increase $K_{\text{Na}}$ above levels seen with a control scrambled peptide. Importantly, yb-G41L appears to be expressed at the surface (Fig. 2B) and to associate, at least to an extent, with the α subunit (Fig. 2A), confirming a role for Gly-41 in mediating one of the kinetic effects of γ and not primarily in αβγ association. Such a dissociation between residues important for the functional effects of γ and its interaction with the sodium pump is consistent with the findings of Lindzen et al. (15).

In contrast to Gly-41, Gly-35 on the opposite face of the helix does not appear to be functionally important, because the G35L mutation does not abrogate kinetic function. Our results also suggest that Gly-35 is not involved in association of γ with the G35L complex, because yb-G35L was able to co-immunoprecipitate with the α subunit (Fig. 2A). The latter finding is consistent with recent experiments showing that the residues important for this association are on a different side of the helix (see Fig. 3 in Ref. 15).

As described previously (17), γ-TM and γG35R-TM peptides form oligomers in the weak detergent PFO, but mutants of Gly-41 (γG41R-TM and γG41L-TM) do not. Using PFO-PAGE analysis (see Ref. 17), we have confirmed that the γG35L-TM peptide can form oligomers in PFO (not shown), revealing a potential correlation between the effects of γ peptides on $K_{\text{Na}}$ and their ability to form oligomers in PFO. The physiological relevance of this observation remains to be investigated.

Mimetic TM peptides reconstituted in detergent micelles have been used previously in structural studies of membrane proteins (22, 25, 26) and have been shown to inhibit membrane protein function (27–29). However, to our knowledge, the results reported here constitute an unique example of a TM peptide that can display specific kinetic effects attributable to the TM domain of a membrane protein. The remarkable mimetic properties of the TM peptides and the expressed γ mutants have particular significance. The failure of the G41R and G41L mutants to affect $K_{\text{Na}}$, whether added as TM peptides to cell membranes or expressed as full-length proteins in transfected cells, underscores the conclusion that Gly-41, which is associated with renal magnesium wasting when mutated to Arg, is important for the effect of the γ subunit on $K_{\text{Na}}$/Na$^+$ antagonism and possibly for oligomerization of the protein, whereas Gly-35 is not.

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