Impaired routing of wild type FXYD2 after oligomerisation with FXYD2-G41R might explain the dominant nature of renal hypomagnesemia

Edinio R. Cairo [a], Thomas Friedrich [b], Herman G.P. Swarts [a], Nine V. Knoers [c], René J.M. Bindels [d], Leo A. Monnens [e], Peter H.G.M. Willems [a], Jan Joep H.H.M. De Pont [a], Jan B. Koenderink [f,*]

[a] Department of Biochemistry, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
[b] Max-Planck-Institute of Biophysics, Department of Biophysical Chemistry, Marie-Curie-Street 15, D-60439 Frankfurt am Main, Germany
[c] Department of Human Genetics, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
[d] Department of Physiology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
[e] Department of Pediatrics, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands
[f] Department of Pharmacology and Toxicology, Nijmegen Centre for Molecular Life Sciences, Radboud University Nijmegen Medical Centre, P.O. Box 9101, 6500 HB Nijmegen, The Netherlands

Abstract

Autosomal dominant renal hypomagnesemia, associated with hypocalciurea, has been linked to a G to A mutation at nucleotide position 121 in the *FXYD2* gene, resulting in the substitution of Gly with Arg at residue 41 of the protein. FXYD2, also called the Na,K-ATPase γ-subunit, binds to Na,K-ATPase and influences its cation affinities. In this paper, we provide evidence for the molecular mechanism underlying the dominant character of the disorder. Co-immunoprecipitation experiments using tagged FXYD2 proteins demonstrated that wild type FXYD2 proteins oligomerise. Moreover, FXYD2-G41R also shows oligomerisation with itself and with the wild type protein. In the case of FXYD2-G41R, however, formation of homo-oligomers was prevented by addition of DTT or introduction of the C52A mutation. Finally, we demonstrated that artificial glycosylation of the wild type FXYD2 is reduced when co-expressed with FXYD2-G41R. These data indicate that binding of FXYD2-G41R to wild type FXYD2 subunit might abrogate the routing of wild type FXYD2 to the plasma membrane thus causing the dominant nature of this mutation.

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1. Introduction

Na,K-ATPase is an integral membrane protein, responsible for the transport of Na^+ and K^+ ions across the cell membrane. Na,K-ATPase consists of a catalytic α-subunit and a chaperone β-subunit. In some tissues, like the kidney, however, a third non-obligatory subunit is present. In 1978 Forbush et al. [1] identified this small polypeptide that could be labeled with an ouabain derivate and that directly associated with Na,K-ATPase. This third subunit, also called the Na,K-ATPase γ-subunit, is one of seven mammalian members of the FXYD family [2]. FXYD proteins are small type I membrane proteins that all contain the FXYD motif and show a tissue-specific expression pattern.

FXYD2 is expressed in the nephron. Although its exact localization has been the subject of various studies, the results seem to be somewhat at variance [3–5]. Recent research, however, indicates different distribution patterns for the two splice variants of FXYD2 (FXYD2a and FXYD2b), and differences in distribution between the outer and inner medulla of the rat kidney [6]. Studies have shown that FXYD2 can change the Na,

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K-ATPase affinity for K^+ in a membrane potential dependent manner [3,4] and that it reduces the apparent affinity for Na^+ [7,4]. In addition, it has been shown that FXYD2 regulates the apparent affinity for ATP [5].

The two splice variants of FXYD2 differ in their seven N-terminal residues but otherwise have identical chains [8,9]. It has been shown that at any given time Na,K-ATPase associates exclusively with either FXYD2a or FXYD2b [10]. If the FXYD2 subunit is not associated with Na,K-ATPase it will not remain stable and is not efficiently transported to the plasma membrane [3]. Studies indicate that the transmembrane domain of FXYD2 binds to the groove formed by M2, M6, and M9 of the Na,K-ATPase α-subunit [11], whereas the location of the cytoplasmatic part is still questionable [12]. In addition, it appears that the extracellular loop between M7 and M8 is the focal region for γ−α−β interactions [13]. Mahmmoud et al. [14] could isolate FXYD2 oligomers when the detergent sodium dodecylsulphate (SDS) was replaced by perfluoro-octanoic acid (PFO, a detergent that tolerates weak interactions) during polyacrylamide gel electrophoresis. Furthermore, synthetic peptides comprising the FXYD2 transmembrane region self-associate in PFO [15].

Previously we reported a heterozygous $121G \rightarrow A$ mutation in the *FXYD2* gene, leading to a G41R substitution in FXYD2, as the cause of dominant renal hypomagnesemia associated with hypocalciuria [16]. It has been shown that the G41R mutant fails to interact with Na,K-ATPase and does not travel to the plasma membrane when expressed in *Xenopus laevis* oocytes or HeLa cells [17,18]. Exactly how a mutation in FXYD2 induces hypomagnesemia as observed in patients is, however, still not known. It is also unclear how this heterozygous mutation can cause a dominant phenotype as we previously reported that the disorder is not caused by haploinsufficiency [16]. The aim of the present study was to provide a mechanism for the dominant nature of autosomal dominant renal hypomagnesemia. We show that this dominance might be explained by impaired routing of wild type FXYD2 due to oligomerisation of wild type and mutated FXYD2 proteins.

2. Materials and methods

2.1. Construction of expression constructs

pTLN constructs coding for the N-terminally FLAG-tagged (DYKDDDDK) and N-terminally VSV-tagged (YTDIEMNRLGK) human FXYD2a were used for the *X. laevis* oocyte expression system [17]. The rat Na,K-ATPase α₁- and β₁-subunits, were subcloned into the pTLN vector, suitable for the *X. laevis* oocyte expression system. Site-directed mutagenesis was used to generate the mutants described in this paper. All introduced mutations were verified by sequencing.

2.2. Expression in X. laevis oocytes

X. laevis were sacrificed and parts of the ovaries were removed. Oocytes were isolated by incubation for 2 h in modified Ringer's solution (90 mM NaCl, 2 mM KCl, 2 mM CaCl₂, 5 mM MOPS, pH 7.4, 30 units/l penicillin and 30 mg/l streptomycin) containing 2 mg/ml collagenase A. Prophase-arrested oocytes of stages V and VI were selected for cRNA injection. cRNA synthesis was carried out with the SP6-mMessage mMachine kit (Ambion, Austin, TX); HpaI-linearized pTLN vectors were used as templates. For the expression of the α-, β-,

and γ-subunits, oocytes were injected with 15 ng, 2 ng, and 2 ng, respectively, of the corresponding cRNAs. After injection, the oocytes were incubated for 3 days at 18 °C in modified Ringer's solution [19].

2.3. Two-electrode voltage clamp

Two-electrode voltage clamp experiments were essentially performed as described by Koenderink et al. [19]. Before measurements, oocytes were kept for 30 min in LS buffer (110 mM NaCl, 2.5 mM Na-citrate, 10 mM MOPS, Tris, pH 7.4) and 30 min in PS buffer (100 mM NaCl, 1 mM CaCl₂, 20 mM tetra-ethylammonium chloride (TEACl), 5 mM BaCl₂, 5 mM NiCl₂, 5 mM MOPS/Tris (pH 7.4)) to elevate intracellular Na^+ concentration. Measurements in the absence of K^+ were performed in Na^+ test solution (100 mM NaCl, 20 mM TEACl, 5 mM BaCl₂, 5 mM NiCl₂, 5 mM MOPS/Tris (pH 7.4) and 10 µM ouabain to inhibit endogenous Na,K-ATPase). For K^+ titration experiments and stationary pump current recordings, 0.3–10 mM K^+ were replaced for equimolar amounts of Na^+. Heterologously expressed Na,K-ATPase was inhibited by 10 mM ouabain during control measurements. Experiments were carried out at room temperature (22–24 °C). To determine the apparent $K_{0.5}$ values (apparent K^+ affinity ($K_{1/2}K^+$) for stimulation of Na^+/K^+ pump current by extracellular $[K^+]$), the amplitudes of stationary currents measured for a series of membrane potentials (−160 mV to +60 mV) were fitted by a Hill equation with set Hill coefficient of 1.6.

2.4. Preparation of total membranes

Oocytes were disrupted by passing them ca. 20 times through a standard 200 µl Gilson pipette in homogenization buffer (10 µl/oocyte; 250 mM sucrose, 0.5 mM EDTA, 5 mM Tris/HCl pH 7.4, and "Complete Protease Inhibitor" (according to the instructions of the manufacturer, Roche Diagnostics GmbH, Mannheim, Germany)). The yolk granules were removed by centrifugation (1000 ×g, 4 °C, 3 min) and the supernatant was collected in a new tube. This step was repeated twice. Subsequently, the membranes (plasma membranes and intracellular membranes) were pelleted by centrifugation (16,000 ×g, 4 °C, 30 min). Finally, the pellet was resuspended in homogenization buffer (4 µl/oocyte) and the samples were stored at −20 °C.

2.5. Immunoprecipitation

Immunoprecipitation was essentially performed as described by Garty et al. [10]. 40 µl of total membranes (ca. 10 oocytes) was added to 60 µl buffer I (33.3 mM Tris–HCl (pH 7.4), 1.67 mM EDTA, 33.3 mM NaCl, 0.167 mg/ml oligomycin to force Na,K-ATPase in a conformation favorable for FXYD2 binding, and 1 tablet 'Complete Protease Inhibitor' (Roche)/10 ml) and incubated 30 min at 4 °C. 100 µl buffer II (20 mM Tris–HCl (pH 7.4), 1 mM EDTA, 20 mM NaCl, 0.1 mg/ml oligomycin, 2 mg/ml C₁₂E₁₀, and 1 tablet of 'Complete Protease Inhibitor' (Roche)/10 ml) was added and incubated for 20 min at 4 °C under repeated vortexing (5 min intervals). After adding 800 µl of buffer III (20 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, 0.1 mg/ml oligomycin, and 1 mg/ml C₁₂E₁₀) solutions were centrifuged (16,000 ×g at 4 °C, 30 min). The supernatant was added to 25 µl ANTI-FLAG M2-Agarose affinity gel beads (Sigma-Aldrich) and rotated overnight at 4 °C. Beads were washed 5 times in cold buffer IV (20 mM Tris–HCl, 1 mM EDTA, 100 mM NaCl, and 0.2 mg/ml C₁₂E₁₀), solubilized in 30 µl Laemmli buffer [20] and analyzed on 15% SDS-PAGE gels.

2.6. Western blotting

Total membrane fractions of *X. laevis* oocytes were solubilized in Laemmli buffer and analyzed on SDS-PAGE gels containing 15% acrylamide according to Laemmli [20]. For immunoblotting, the separated proteins were transferred to Immobilon-P membranes (Millipore, Co., Bedford, MA). The FXYD2-subunits were detected with the anti-FLAG antibody M2 (Sigma-Aldrich) or the anti-VSV-G antibody P4D5 [21], whereas the α-subunit of Na,K-ATPase was detected with antibody C356-M09 [19]. Primary antibodies were detected using an anti-mouse or anti-rabbit secondary antibody, labeled with horseradish peroxidase (DAKO A/S, Denmark).

2.7. Glycosylation

Deglycosylation was performed on membranes isolated from oocytes (co-) expressing wild type and G41R FXYD2 in which a G3N mutation was introduced. 9 µl of membrane sample was denatured by adding 1 µl denaturation buffer (5% SDS and 10% mercaptoethanol) after which the mixture was incubated at 50 °C for 10 min. Subsequently 1.5 µl 500 mM sodium phosphate pH 7.5, 1.5 µl 10% NP-40, 1 µl water and 1 µl N-Glycosidase F was added (for negative control glycosidase was replaced with water), incubated at 37 °C for 1 h and analyzed on SDS-PAGE.

3. Results

To determine the effect of a FLAG-tagged human FXYD2a subunit on the apparent K^+ affinity of Na,K-ATPase we investigated the voltage dependence of the stationary currents ($I–V$ curves) of Na,K-ATPase with and without FLAG-tagged FXYD2 at different extracellular K^+ concentrations. The current in the absence of K^+ was subtracted from those in the presence of 0.15, 0.5, 1.5, 5, or 15 mM K^+. Upon the addition of 10 mM ouabain the currents ceased and baseline was restored, indicating that the currents were driven by heterologously expressed Na,K-ATPase. The apparent $K_{0.5}$ value for stimulation of Na,K-ATPase current by extracellular K^+ was determined from the voltage dependence of stationary currents at different $[K^+]$ (see "Materials and methods"). The (voltage-dependent) $K_{0.5}$ of the wild type enzyme at 100 mM Na^+ had a minimum of 0.4 mM at −20 mV and increased to 0.6 mM at 60 mV and 2.4 mM at −160 mV, respectively (Fig. 1). In the presence of FXYD2 the enzyme had its maximal apparent K^+ affinity at −40 mV ($K_{0.5}=0.5$ mM). This value decreased when the plasma membrane was depolarized ($K_{0.5}=1.0$ mM at 60 mV) or hyperpolarized ($K_{0.5}=1.7$ mM at −180 mV; see Fig. 1). FXYD2 increased the apparent K^+ affinity of Na,K-ATPase at high negative membrane potentials and decreased the apparent

Fig. 2. Interaction between Na,K-ATPase α, β, and γ. Membrane isolations, immunoprecipitation, and immunoblotting were carried out as described under Materials and methods. (A) Total membrane isolations of injected oocytes expressing Na,K-ATPase subunits as indicated. (B) Co-immunoprecipitation targeted against the FLAG-tag of the FXYD2 subunit revealing strongly diminished interaction between the α-subunit and the γ-subunit containing the G41R mutation compared to the wild type FXYD2 ($n=3$).

K^+ affinity at less negative and positive membrane potentials, similarly as previously reported [3,7]. When FXYD2 was replaced with FXYD2-G41R, however, the oocytes showed high leak conductance and no reliable electrophysiological results could be obtained (data not shown). The results from the oocytes injected with wild type FXYD2 confirm that our expression system is functional, and that the presence of the FLAG-tag most likely has no adverse effect on the human FXYD2 protein.

Previous studies have demonstrated that FXYD2 binds to Na,K-ATPase [3,7,10,22–24]. To further validate our experimental setup we solubilized the total membrane fraction of *Xenopus* oocytes with $C_{12}E_{10}$ according to Garty et al. [10] and immunoprecipitated the FLAG-tagged FXYD2 subunit with an anti-FLAG antibody. The samples were subjected to SDS-PAGE and immunoblotting after which the presence of both FXYD2 and the Na,K-ATPase α-subunit was visualized with specific antibodies. The Na,K-ATPase α-subunit was co-expressed with the wild type FXYD2 and the FXYD2-G41R (Fig. 2A). Co-immunoprecipitation of the Na,K-ATPase α-subunit is evident when it is co-expressed with the wild type FXYD2 protein (Fig. 2B, lane 2). When the α-subunit is co-expressed with the G41R mutant, however, the amount of detected association is strongly diminished (Fig. 2B, lane 3) indicating that the G41R mutation destabilizes association between FXYD2 and Na,K-ATPase.

The lack of binding of the FXYD2-G41R mutant to Na,K-ATPase, however, cannot explain the dominant nature of autosomal dominant renal hypomagnesemia. We previously presented evidence that deletion of a single FXYD2 allele does not give rise to hypomagnesemia [16]. This finding together with previous observations that FXYD2 can form oligomers [14,15] led us to hypothesize that the mutant FXYD2 subunit could have an effect on the routing of the wild type FXYD2 subunit. To investigate the interaction between FXYD2 subunits, we carried out co-immunoprecipitation experiments using differentially tagged FXYD2 proteins. Both wild type and FXYD2-G41R proteins were expressed in *X. laevis* oocytes (Fig. 3A). Co-immunoprecipitation experiments showed that VSV-tagged FXYD2 proteins are co-immunoprecipitated with
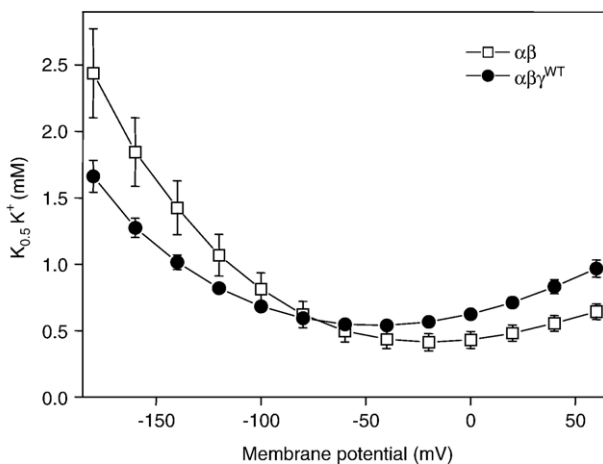

Fig. 1. Voltage dependence of the $K_{0.5}$ value of K^+-dependent current stimulation in injected *Xenopus laevis* oocytes expressing Na,K-ATPase α-, β-, and γ-subunits as indicated, at 100 mM Na^+. The $K_{0.5}$ values were determined by fitting the voltage dependence of extracellular K^+-activated Na,K-ATPase current with a Hill equation for the Na,K-ATPase, with and without FXYD2 (Average of 5–6 oocytes (2 batches)±SEM). The presence of FXYD2 clearly shifts the range of the apparent $K_{0.5}$ values.

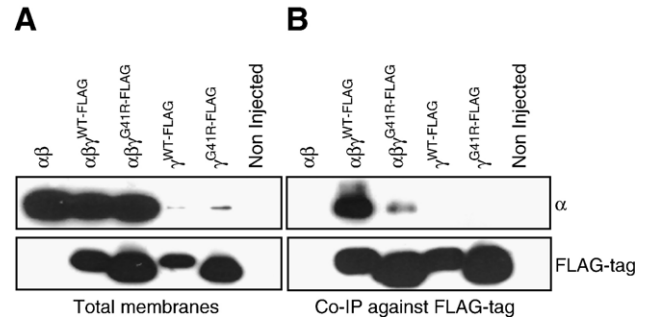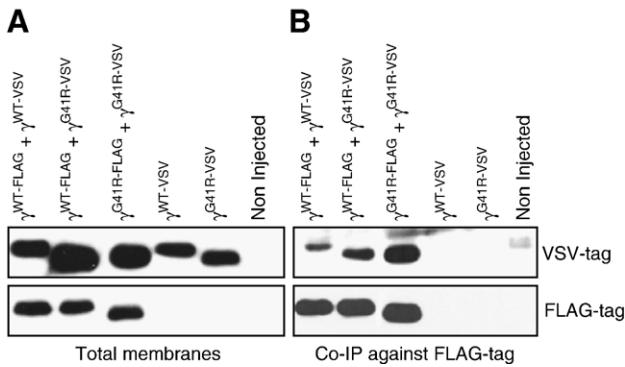Fig. 3. Oligomerisation of FXYD2. Membrane isolations, immunoprecipitation, and immunoblotting were carried out as described under Materials and methods. (A) Total membrane isolations of injected oocytes showing expression of all proteins. (B) Immunoprecipitation targeted against the FLAG-tag shows oligomerisation of the FLAG-tagged and VSV-tagged FXYD2 proteins ($n=3$).

FLAG-tagged FXYD2 proteins in all combinations shown (Fig. 3B), indicating that they form oligomers consisting of at least two subunits, irrespective of the absence or presence of the G41R mutation.

To investigate whether the conserved cysteine at position 52 had any effect on oligomerisation the reducing agent dithiothreitol (DTT) was excluded during the total membrane sample preparation for SDS-PAGE. The immunoblot not only showed the monomeric FXYD2 signal around 15 kDa, but also bands with decreased mobility, of which a band around 25–30 kDa in particular suggests the presence of FXYD2 oligomers (Fig. 4). The presence of *N*-ethylmaleimide (NEM), a well-known cysteine alkylator, during the membrane isolation steps did not change these results (data not shown), indicating that the disulphide bridges are not an artifact formed during the membrane isolation steps. These data suggest that in our experiments the formation of a disulphide bridge is involved in FXYD2 oligomerisation.

To study the role of this disulphide bridge, co-immunoprecipitation experiments were performed in the presence or absence of 1 mM DTT during the entire immunoprecipitation

Co-IP against FLAG-tag

Fig. 5. Oligomerisation of FXYD2 under reducing and non-reducing conditions. Expression, membrane isolations, immunoprecipitation, and immunoblotting were carried out as described under Materials and methods. FLAG-tagged FXYD2 was immunoprecipitated with and without DTT. The immunoprecipitate was blotted and the presence of FLAG-tagged and VSV-tagged FXYD2 proteins was detected with specific antibodies. Addition of DTT to the immunoprecipitation reaction resulted in the abolishment of oligomerisation between FXYD2-G41R mutants and showed an apparent decrease in oligomerisation between the wild type/G41R mutants. The wild type FXYD2 situation seemed unaffected ($n=3$).

reaction. Without DTT the results were the same as in the previous immunoprecipitation experiments with all subunits showing association regardless of the G41R mutation. Under the reducing conditions wild type subunits still show association and wild type/mutant FXYD2 proteins also still co-immunoprecipitated (Fig. 5, lanes 5–7), but FXYD2-G41R proteins no longer show co-immunoprecipitation (Fig. 5, lane 8).

Since there is only a single cysteine in FXYD2 that can account for the previous results, we mutated Cys[52] into an Ala and repeated the co-immunoprecipitation experiments. The expression level of this mutated FXYD2 protein was similar to that of the wild type (Fig. 6, lanes 1–4). The interaction between this FXYD2-C52A and the wild type FXYD2 is sufficient for co-immunoprecipitation (Fig. 6, lane 5). As soon as the G41R
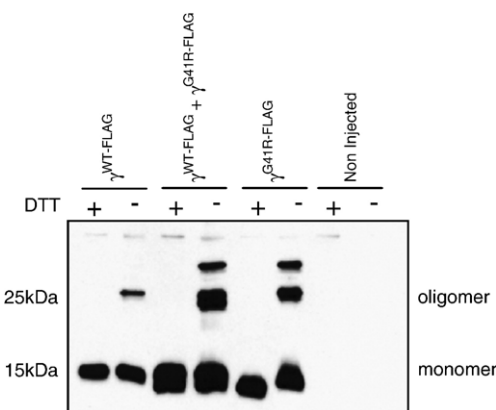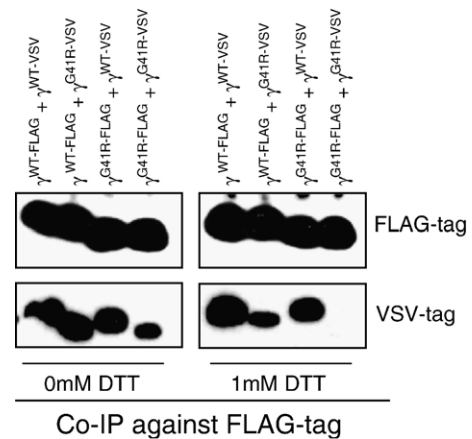

Fig. 4. Native SDS-PAGE electrophoresis of the FXYD2 subunit. Total membrane isolations and immunoblotting were performed as described in Materials and methods with and without DTT as indicated. Under non-reducing conditions FLAG-tag signals can be seen at heights indicating the presence of FXYD2 oligomers ($n=2$).
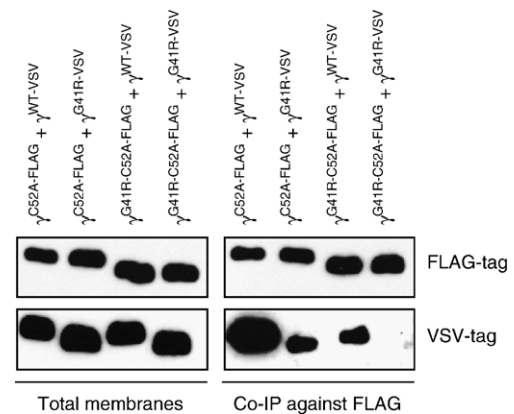

Fig. 6. Oligomerisation of FXYD2-C52A mutants. Membrane isolations, immunoprecipitation, and immunoblotting were carried out as described under Materials and methods. Mutation of Cys[52] into Ala shows the same effect as adding DTT to the reaction as seen in Fig. 5, indicating the role of Cys[52] in forming FXYD2 oligomers ($n=3$).
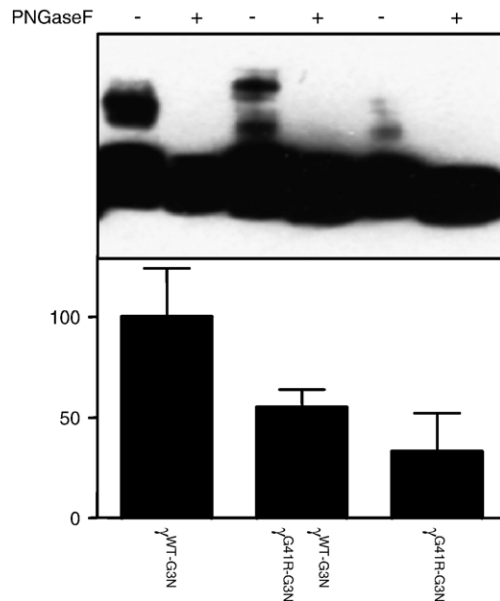
Fig. 7. Glycosylation of FXYD2-G3N and FXYD-G41R/G3N. An artificial glycosylation site was introduced in wild type FXYD2 and FXYD-G41R. Membranes of *Xenopus laevis* oocytes injected with equal amounts of cRNA of FXYD2-G3N, FXYD-G41R/G3N and their combination were treated with PNGase F and immunoblotted as described under Materials and methods. The pixel density of the three experiments was averaged (±SEM) and plotted below a typical immunoblot.

mutation was also present in one of the FXYD2 subunits co-immunoprecipitation diminished (Fig. 6, lanes 6 and 7). When the G41R mutation was present in both FXYD2 subunits in addition to the C52A mutation no interaction was apparent (Fig. 6, lane 8), similar to the co-immunoprecipitation experiments using DTT.

We previously demonstrated with immuno-cytochemical methods that the G41R-mutated FXYD2 is misrouted in the present system [17]. Due to the balance between over-expression and the detection limit we could not show with immuno-cyto-chemical methods that wild type FXYD2 is absent in the plasma membrane when G41R-mutated FXYD2 is co-expressed. To demonstrate that FXYD2-G41R influences FXYD2-WT routing we introduced a glycosylation site (G3N) in both FXYD2 proteins. These mutants were (co-)expressed in *X. laevis* oocytes and the isolated membranes were treated with PNGase F and analyzed on SDS-PAGE (Fig. 7). The FXYD2-G3N showed decreased mobility on SDS-PAGE, which was absent after treatment with PNGase F, indicating glycosylation of the protein. The FXYD2-G41R/G3N protein showed less glycosyl-ation. When both the wild type FXYD2 and the G41R mutant are co-expressed, the amount of glycosylation is reduced. In addition, the pattern of glycosylation of the wild type and mutant FXYD2 seems to differ. This might be due to core glycosylation of the mutant versus full glycosylation of the wild type enzyme.

4. Discussion

In this study we show that oligomerisation of FXYD2 might explain the dominant character of renal hypomagnesemia. Our studies demonstrate that the G41R-mutated human FXYD2 subunit is not able to interact with Na,K-ATPase. We also provide evidence for the formation of FXYD2 oligomers that in the first instance are unaffected by the G41R mutation. However, in the presence of DTT, or when Cys^{52} in FXYD2 was mutated, the interaction between two FXYD2-G41R molecules was absent, whereas the interaction between wild type FXYD2 and FXYD2-G41R was still present, but weakened. In addition, artificial glycosylation of FXYD2-WT is decreased when co-expressed with FXYD2-G41R, indicating that routing of wild type FXYD2 could be hampered due to the co-expression of FXYD2-G41R. Binding of FXYD2-G41R to the wild type FXYD2 subunit therefore may abrogate the regulation of Na,K-ATPase and thereby underlie the dominant nature of this mutation.

We demonstrated that the human FLAG-tagged FXYD2 protein influences the apparent K$^+$ affinity in a membrane potential dependent manner. The effect is in agreement with published results for wild type FXYD2 [3]. FXYD2 was shown to increase the apparent K$^+$ affinity of Na,K-ATPase at high negative membrane potentials and decrease the apparent K$^+$ affinity at less negative membrane potentials, suggesting a shift in the E_1–E_2 equilibrium towards the E_1 conformation [7]. Though we cannot exclude the possibility of other FXYD subunits being endogenously expressed, it is not very likely that they influenced the results obtained, because we compared oocytes that heterologously express FXYD2 with those that do not express FXYD2. Introduction of the G41R mutation in FXYD2, that was identified in patients with hypomagnesemia [16], resulted in oocytes with a high leak conductance. We were not able to determine the apparent affinity for K$^+$ in these oocytes from different current experiments. Previously, Crambert et al. showed that the introduction of the homologous G40R mutation into FXYD7 also induced increased mortality in injected oocytes [25]. This indicates that the over-expression of these mutants disrupts normal physiological processes in the *X. laevis* system and thus making electrophysiological experiments more difficult.

Since the FXYD family members possess their highest level of homology within their transmembrane domains and Gly^{41} is conserved among all known FXYD proteins, this amino acid is likely to be structurally and/or functionally significant. We demonstrated that FXYD2 also binds to the Na,K-ATPase α-subunit and that binding of FXYD2-G41R to the α-subunit is virtually abolished, which is in agreement with the findings of Pu et al. [18].

This is the first report that demonstrates oligomerisation of recombinant FXYD2 proteins by co-immunoprecipitation. It was shown previously that pig renal FXYD2 and synthetic peptides that comprise the FXYD2 transmembrane region oli-gomerise when the detergent sodium dodecylsulphate (SDS) was replaced by perfluoro-octanoic acid (PFO) in polyacrylamide gel electrophoresis [14,15]. Co-immunoprecipitation experiments, however, provided no evidence for complexes containing more than one FXYD protein for each αβ-complex [10,12]. In this regard it is interesting to compare the function of FXYD2 with the regulatory role of phospholamban (PLN) on the SERCA

pump. PLN only associates with SERCA if it is in a monomeric state and the pentameric PLN represents a reservoir that dissociates to provide regulatory monomeric PLN subunits [26]. In addition, the oligomeric state of FXYD2 may explain the finding that this small protein has been reported to exhibit cation channel activity [27] by forming channels in the plasma membrane.

We observed co-immunoprecipitation between FXYD2-G41R subunits and also between FXYD2-G41R and wild type subunits. In contrast to our results, Therein and Deber, did not find oligomerisation of G41R mutants in their study using synthetic peptides comprising the TM domain of FXYD2 [15]. This discrepancy could be explained by the absence of intracellular and extracellular FXYD2 domains in their study. Indeed, our study showed that the presence of a Cys residue at position 52 in the cytosolic domain is important for the stable interaction of G41R-FXYD2. This Cys is highly conserved among the FXYD family members (only in FXYD5 it is a Glu) and might play a stabilizing role in oligomerisation. Our results indicate that the introduction of the G41R mutation in the transmembrane domain weakens the interaction between the subunits, yet in non-reducing conditions the disulphide bridge is still capable of yielding normal association between subunits and thus normal co-immunoprecipitation. Only when both sub

units contain the G41R mutation in a reducing environment, we do not observe co-immunoprecipitation. Although the existence of a disulphide bridge in the cytosol is debatable, our studies indicate that without the formation of a disulphide bridge, FXYD2-G41R subunits are still capable of forming oligomers with wild type subunits. Moreover, in the absence of disulphide bridges the pool of monomeric G41R mutants is not diminished by the formation of FXYD2-G41R oligomers. Therefore, more monomeric FXYD-G41R subunits are available to associate with wild type subunits.

The dominant mechanism of the disorder was investigated in two individuals with an 11q23.3-ter deletion that includes the *FXYD2* gene. Both had normal serum Mg^{2+} level, showing that rather than haploinsufficiency, the presence of FXYD2-G41R causes hypomagnesemia, consistent with a dominant-negative mechanism [16]. The formation of FXYD2 oligomers could explain the dominant nature of the disease. Whereas the wild type FXYD2 protein is present at the plasma membrane the G41R mutant is not [17,18]. With the introduction of an artificial glycosylation site in both FXYD2 subunits (G3N), we showed that only the wild type subunit is fully glycosylated, indicating that it is completely processed. The G41R mutant is hardly glycosylated and the pattern of glycosylation is altered. This altered pattern might be due to core glycosylation that is characteristic for ER retention. These observations might imply that the G41R mutant is retarded before full glycosylation occurs. When both wild type and G41R proteins are expressed the glycosylation is also reduced and the pattern altered. It is likely to assume that the mutated FXYD2 protein binds to the wild type and prevents the routing of the wild type FXYD2 to the plasma membrane.

In this study we show that FXYD2 wild type and G41R proteins are present in an oligomeric status. The amino acids that form the oligomerisation interface and the exact cellular location of hetero-oligomers of wild type and FXYD2-G41R remain to be established in future experiments. The oligomerisation experiments together with the glycosylation experiments support our hypothesis that binding of FXYD2-G41R to wild type FXYD2 subunit might abrogate the routing of wild type FXYD2 to the plasma membrane thus causing the dominant nature of this mutation.

Acknowledgement

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