HNF-1B specifically regulates the transcription of the γa-subunit of the Na⁺/K⁺-ATPase

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A R T I C L E   I N F O

A B S T R A C T

Hepatocyte nuclear factor-1B (HNF-1B) is a transcription factor involved in embryonic development and tissue-specific gene expression in several organs, including the kidney. Recently heterozygous mutations in the HNF1B gene have been identified in patients with hypomagnesemia due to renal Mg^{2+} wasting. Interestingly, ChIP–chip data revealed HNF-1B binding sites in the FXYD2 gene, encoding the γ-subunit of the Na⁺/K⁺-ATPase. The γ-subunit has been described as one of the molecular players in the renal Mg^{2+} reabsorption in the distal convoluted tubule (DCT). Of note, the FXYD2 gene can be alternatively transcribed into two main variants, namely γa and γb.

In the present study, we demonstrated via two different reporter gene assays that HNF-1B specifically acts as an activator of the γa-subunit, whereas the γb-subunit expression was not affected. Moreover, the HNF-1B mutations H69fsdelAC, H324S325fsdelCA, Y352finsA and K156E, previously identified in patients with hypomagnesemia, prevented transcription activation of γa-subunit via a dominant negative effect on wild type HNF1-B. By immunohistochemistry, it was shown that the γa- and γb-subunits colocalize at the basolateral membrane of the DCT segment of mouse kidney. On the basis of these data, we suggest that abnormalities involving the HNF-1B gene may impair the relative abundance of γa and γb, thus affecting the transcellular Mg^{2+} reabsorption in the DCT.

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1. Introduction

HNF-1B is a transcription factor that is critically involved in the early vertebrate development and embryonic survival. Although it was first identified in the liver [1], it is also highly expressed in the pancreas, kidney, lung, ovary, testis and gastrointestinal tract [2]. In kidney, it is expressed exclusively in the tubular epithelial cells along all nephron segments [3]. In the past decades, candidate gene strategies have led to the identification of several targets that are regulated by HNF-1B, such as the cystic disease genes PKHD1, PKD2, UMOD, and SOCS-3 [4–6].

HNF-1B belongs to a family of homeodomain-containing transcription factors. It consists of a POU-specific (Pit-1, OCT1/2, UNC-86; POU_S) and an atypical POU homeodomain (POU_H) that mediate DNA binding to the consensus sequence 5′-RGTTAATNAT-TAAC-3′. HNF-1B forms homo or heterodimers with the structurally related HNF-1A in a stable complex that includes the dimerization cofactor DCoH. In humans, heterozygous mutations of HNF1B result in several congenital kidney and urinary tract abnormalities, as well as a variety of extrarenal phenotypes [7,8]. Since deletion of the entire HNF1B gene is frequently found in human patients, it seems likely that a gene dosage effect is involved. However, some mutated factors behave as dominant negative proteins that may possibly inactivate the wild type protein [9]. Recently, novel mutations in the HNF1B gene, both *de novo* and inherited, have been described [10,11].

Hypomagnesemia, although not deeply investigated, is often reported in patients carrying HNF1B defects [11,12]. Interestingly, Adalat et al. described five cases of HNF1B whole-gene deletions, two splice site mutations and one frame-shift mutation being associated with renal malformations and hypomagnesemia (<0.65 mmol/L) due to a specific renal defect in the transcellular Mg^{2+} transport in the distal convoluted tubules (DCT). The DCT plays an important role in fine-tuning the plasma Mg^{2+} levels. Here, Mg^{2+} is actively reabsorbed from the pro-urine into the cell via the transient receptor potential channel melastatin subtype 6 (TRPM6) [13] and subsequently extruded to the blood via an unknown mechanism. In the recent years, several new proteins have

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been linked to active renal Mg^{2+} handling by directly affecting TRPM6 or by altering the driving force for Mg^{2+} influx via the channel [14].

Of note, bioinformatics prediction tools in combination with functional genomic approaches confirmed the presence of HNF-1B binding sites in the FXYD2 gene, encoding the regulatory γ-subunit of the Na^+/K^+-ATPase [15]. Interestingly, the missense mutation Gly41Arg in the same protein was previously identified as the underlying defect of isolated dominant hypomagnesemia associated with hypocalciuria (IDH; OMIM 154020) [16]. The γ-subunit (FXYD2) is a type I transmembrane protein, mostly expressed in the kidney [17]. Its gene can be alternatively transcribed into two main variants, namely γa and γb, which differ only at their extracellular N-termini [18]. The two γ-subunit isoforms modulate the Na^+/K^+-ATPase affinities for its major physiological ligands [19]. In particular, this pump is responsible for maintaining the normal transmembrane gradients of Na^+ and K^+, which in the kidney drive the trans-epithelial salt reabsorption.

In the present study, we investigated the role of wild type HNF-1B and HNF-1B H69fsdelAC, K156E, H324S325fsdelCA, Y352finsA on the alternative transcription of the two human γ-subunit variants. Via two different approaches based on reporter genes, we were able to show that wild type HNF1B specifically induces the γa-subunit transcription whereas all HNF1B mutants partially prevented it, probably due to a dominant negative effect on the wild type transcription factor.

2. Material and methods

2.1. Parvalbumin–EGFP mice

The transgenic parvalbumin–EGFP mice were obtained from the University of Heidelberg, Germany [20]. Parvalbumin is a Ca^{2+}-binding protein predominantly distributed in the early distal convoluted tubule (DCT) of the kidney [21]. Briefly, the enhanced green fluorescent protein (EGFP) was expressed under the control of the parvalbumin gene promoter by bacterial artificial chromosome (BAC) transgene, resulting in the specific EGFP-labeling of the parvalbumin-expressing DCTs.

2.2. DNA constructs

The human FXYD2 region from -1300 bp upstream the FXYD2b exon, till exon 2 was obtained by amplification of genomic DNA, using a high fidelity DNA polymerase (Phusion, Finnzymes), and the PCR product was cloned into a pGEM-T Easy vector (Promega). Subsequently, an EGFP SV40 polyA terminator product was amplified from a previous construct and cloned downstream of the FXYD2 genomic sequence.

HNF-1B full-length cDNA was amplified by PCR from HNF-1B pCMV-SPORT6 (clone IRATp970A0421D, ImaGenes), subcloned into the bicistronic expression vector pCINeo IRES GFP and HA-tagged at the N-terminus. HNF-1B H69fsdelAC, H324S325fsdelCA, Y352finsA
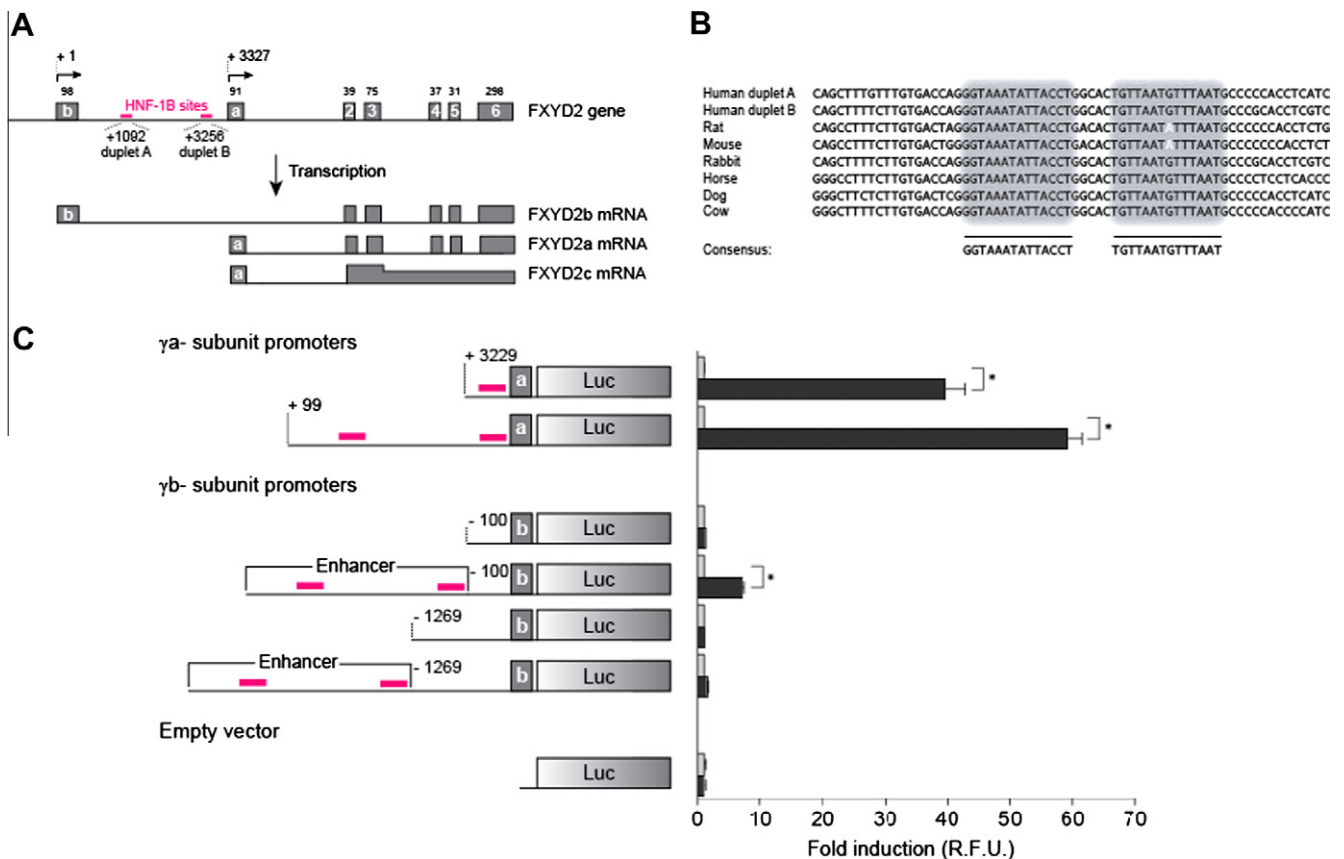
Fig. 1. HNF-1B promotes the γa-subunit transcription initiation. (A) Organization of the human FXYD2 gene and its alternative transcripts. Each exon is indicated with its length. Numbering of the nucleotides starts from the transcription initiation site of FXYD2b. Alternative transcription can be initiated at exon-b (+1) or exon-a (+3327). HNF-1B sites are arranged as duplets at positions +1092 and +3256. ⟶ Transcription initiation site. (B) Alignment of the promoter region -1000 from the start site of exon-a of different species revealed highly conserved HNF-1B binding sites arrange as a duplet. For the human FXYD2 gene, both duplet A (+1092) and duplet B (+3327) are shown. (C) Luciferase assay. HEK293 cells were transiently transfected with luciferase constructs carrying the γa- and γb-subunit regulatory elements. The promoter activity was tested with (black bars) or without (grey bars) HNF-1B stimulation. R.F.U.: Renilla Firefly Units. Error bars indicate SEM ($n = 3$). *$P < 0.05$, compared with non-stimulated condition.

and K156E were obtained by site-directed mutagenesis according to the manufacturer's guidelines (Stratagene, La Jolla, USA). For co-transfection experiments with FXYD2-EGFP pGEM T-Easy, the IRES GFP was removed from the bicistronic HNF-1B constructs.

Firefly luciferase constructs were obtained by amplification of the promoter regions of interest using FXYD2-EGFP pGEM T-Easy as a template and subcloned into pGL3-Basic vector (Promega).

All constructs were verified by sequence analysis.

2.3. Cell culture and transfection

Human Embryonic Kidney cells (HEK293) were grown in Dulbecco's modified Eagle's medium (Bio Whittaker-Europe, Verviers, Belgium) containing 10% (v/v) fetal calf serum, 2 mM L-glutamine and 10 µg/ml ciproxin at 37 °C in a humidity-controlled incubator with 5% (v/v) CO_2. The cells were transiently transfected with the respective constructs using polyethylenimine (Polysciences Inc.) cationic polymer and analyze for protein or mRNA expression at 48 h post-transfection.

2.4. Immunohistochemistry

Immunohistochemistry was performed as previously described [22]. In short, staining of serial sections was performed on 7-µm

cryosections of periodate-lysine-paraformaldehyde-fixed kidney samples from parvalbumin–EGFP mice. The mouse kidney cryosections were incubated overnight at 4 °C with the following primary antibodies: rat anti-γb (1:2000) raised against the N-terminus peptide MDRWYLGG of the mouse γb isoform conjugated to tetanus toxoid as a carrier [17], and rat anti-γa (1:50) raised against the N-terminus peptide MAGEISDLSANS of the mouse γa isoform (kind gift from Prof. Dr. Gerald Kidder, The University of Western Ontario). Sections were then incubated with Alexa Fluor 594 conjugated secondary antibodies. Photographs of the entire cortex were taken with the microscope Zeiss Axio Imager 1 microscope (Oberkochen, Germany) equipped with the fluorescence lamp HXP120 Kubler Codix.

2.5. Immunocytochemistry

HEK293 cells, which lack endogenous HNF-1B [23], were transiently transfected with wild type and mutant HA-HNF1-B pCINeo IRES GFP. 48 h after transfection the cells were seeded on glass and fixed with 4% w/v paraformaldehyde (PFA) for 30 min at room temperature, followed by permeabilization for 15 min with 0.2% v/v Triton X-100 in PBS supplemented with 0.1% w/v BSA. After incubation overnight with a 1:100 dilution of a mouse anti-HA antibody (6E2, Cell Signalling), cells were washed three times with
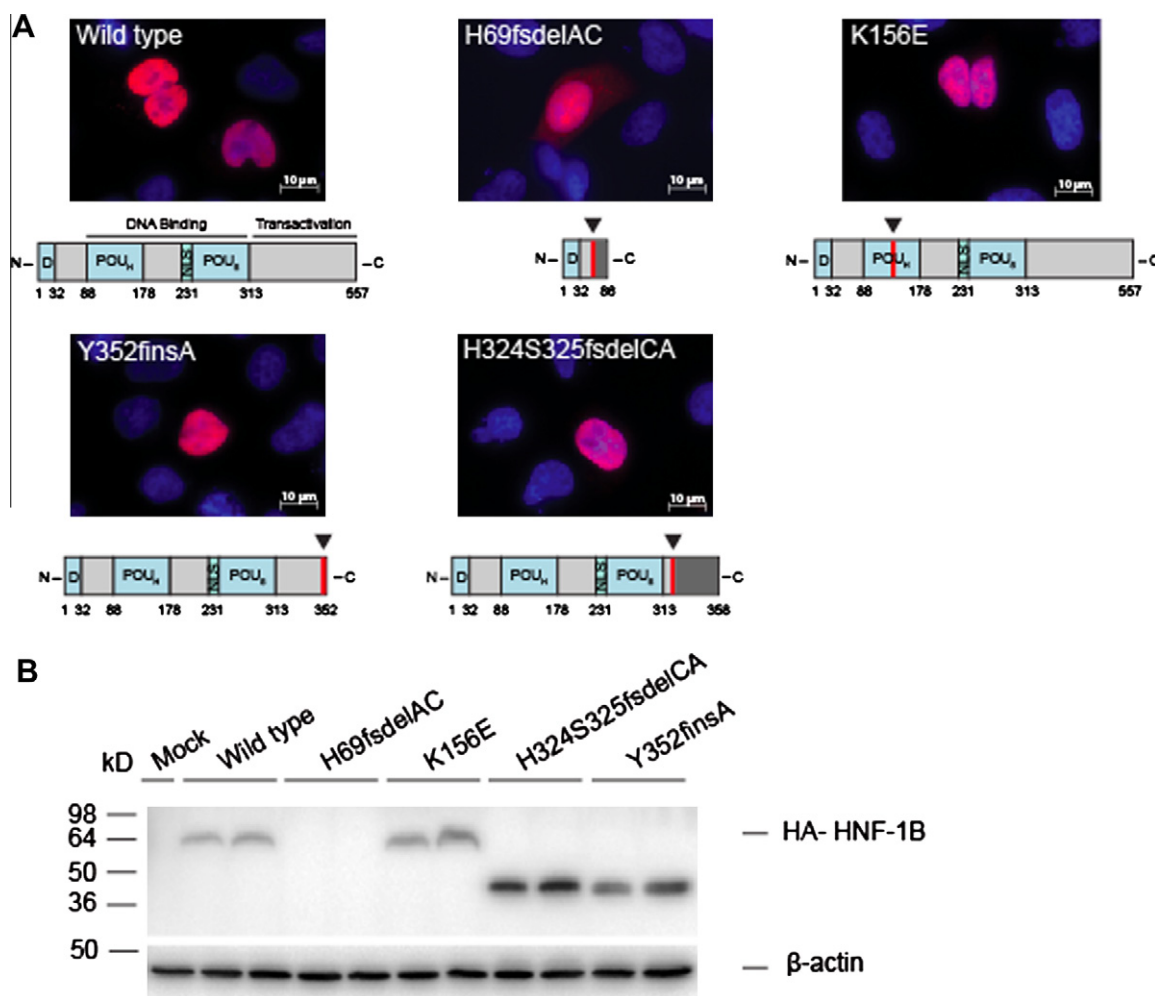
Fig. 2. Characterization of the human HNF-1B mutants. (A) Intracellular localization of wild type HNF-1B and HNF-1B mutants in transiently transfected HEK293 cells. Red: HA tag; blue: DAPI (nuclear marker); D: dimerization; POU$_H$: POU homeodomain; POU$_S$: POU-specific. Scale bars: 10 µm. (B) HA-HNF-1B wild type (65.5 kDa) and its mutants H69fsdelAC (9.47 kDa), K156E (65.5 kDa), H324S325fsdelCA (41.5 kDa) and Y352finsA (39.4 kDa) were transiently transfected in HEK293 cells and checked for expression via immunoblotting. Mock plasmid was transfected in HEK293 cells as negative control. β-actin was included to check for equal loading. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

PBS-CM (1 mM MgCl$_2$, 0.1 mM CaCl$_2$) and incubated with a secondary Alexa Fluor 594 coupled goat anti-mouse antibody (1:100) for 45 min at room temperature. Cells were washed three times with PBS-CM prior mounting with DAPI–Vectashield (Vector Laboratories, Burlingame, CA, USA).

2.6. Immunoblotting

Mouse kidney protein lysate was prepared as previously described [24]. HEK293 cells were lysed in 1x Laemmli sample buffer containing 100 mM dithiothreitol and protein inhibitors, subsequently denatured for 30 min at 37 °C, and then subjected to SDS–PAGE. Immunoblots were incubated with the following antibodies: mouse anti-HA (1:500), rabbit anti-γb (1:5000), rabbit anti-γa (1:500) or rabbit anti-γ (1:500) raised against the C-terminus of the γ-subunit (kind gift from Prof. Dr. Steven J.D. Karlish, Rehovot, Israel) [25]. Subsequently, blots were incubated with sheep horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Sigma, MO, USA) and then visualized using the enhanced chemiluminescence system (ECL, Pierce).

2.7. Luciferase reporter assay

In a 12-well plate, 700 ng of the promoter firefly luciferase plasmids and 100 ng of the HNF-1B pCINeo plasmid were transfected into HEK293 cells. For standardization of the transfection efficiency, 20 ng of *Renilla* luciferase plasmid CMV-pRL was used as a reference. Firefly and *Renilla* luciferase activities were measured with the Dual-Luciferase Reporter Assay (Promega).

2.8. Quantitative Real-time polymerase chain reaction analysis

HEK293 cells were transiently cotransfected in a 6-well with 1.75 μg DNA of FXYD2-EGFP and 30 ng of either wild type or mutant HNF1-B. The potential dominant negative effect was investigated by co-transfection of 15 ng of wild type HNF1-B and 15 ng of mutant HNF1-B. The total amount of DNA was kept equal using the pCINeo empty vector. Total RNA isolation and reverse transcription were performed, as described previously [26]. The cDNA was used to determine mRNA expression levels by CFX96 Real-Time PCR detection system (Bio-Rad) both of the target genes of interest and of the housekeeping gene hypoxanthine–guanine phosphoribosyl transferase (HPRT) as an endogenous control.

2.9. Data analysis

All results presented are based on a minimum of three different experiments. Values are expressed as means ± SEM. Statistical significance ($P < 0.05$) was determined using one-tailed Students t-test or one-way ANOVA with Bonferroni's procedure.
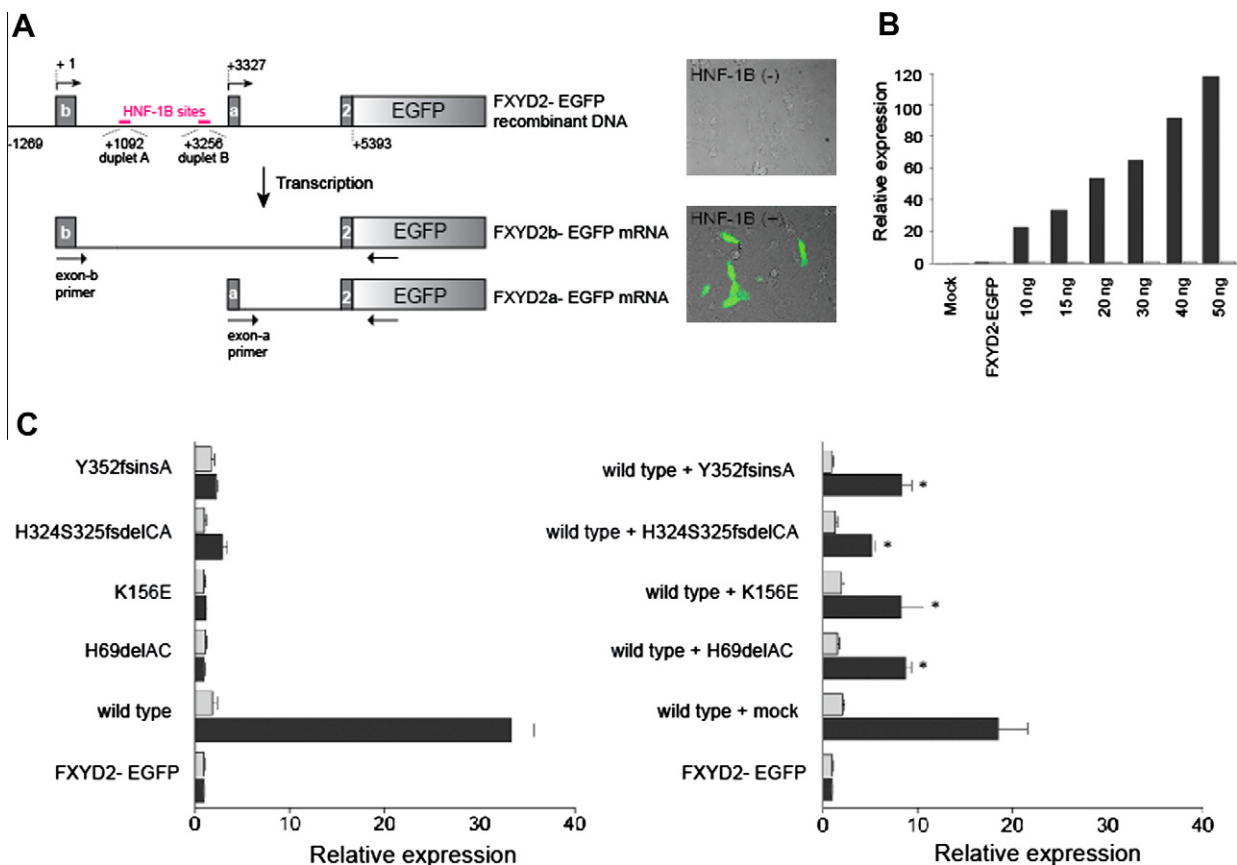
Fig. 3. Study of the alternative transcription of the human FXYD2 gene by wild type HNF-1B and its mutants. (A) Scheme of the human FXYD2-EGFP construct. FXYD2a- and FXYD2b-EGFP transcripts were detected by use of an isoform-specific forward primer, spanning the exon–exon junction, and an EGFP reverse primer. γ-EGFP fusion proteins can be detected in HEK293 cells 48 h after co-transfection of FXYD2-EGFP recombinant DNA with HNF-1B. Scale bars: 20 μm. (B) FXYD2a-EGFP (black bars) and FXYD2b-EGFP (grey bars) mRNA levels after co-transfection with increasing DNA quantities of wild type HNF-1B. (C) (Left panel) Wild type HNF-1B stimulates FXYD2a-EGFP (black bars) but not FXYD2b-EGFP (grey bars) mRNA synthesis. (Right panel) FXYD2a-EGFP mRNA expression is significantly reduced by a dominant negative effect of HNF-1B H69fsdelAC, K156E, H324S325fsdelCA and Y352finsA on the transactivation activity of wild type HNF-1B. Error bars indicate SEM ($n = 3$). *$P < 0.05$, compared to FXYD2a-EGFP mRNA stimulation by wild type HNF-1B.

3. Results and discussion

3.1. HNF-1B enhances FXYD2a but not FXYD2b transcription

FXYD2 gene (Gene ID: 486) maps on chromosome 11q23 and consists of seven exons spanning 9.2 kb of genomic DNA [15]. Three transcripts are associated to this gene, with FXYD2a (NM001680) and FXYD2b (NM021603) being the main ones. Promoter elements were identified around the alternative start sites for exon-b and exon-a, that encode different N-termini of the γ-subunit protein (Fig. 1A). The highly conserved HNF-1B binding sites localize within the first intron of the human FXYD2 gene (Fig. 1B), at positions +1092 and +3256 from the transcription initiation site of FXYD2b and act as enhancer elements [12]. Nevertheless, it is unknown whether HNF-1B sites may act as upstream regulatory elements for γa and/or downstream enhancer elements for γb. In order to elucidate the HNF-1B transactivation activity on the alternative transcription of FXYD2b, the first intron of the human FXYD2 gene was cloned upstream to the proximal (−100) and distal (−1269) promoter of the FXYD2b isoform and linked to a luciferase reporter gene. HEK293 cells were transfected with the promoter-reported plasmid and luciferase activity was measured after HNF1-1B stimulation. The enhancer moderately affected γb promoter activity at a proximal but not at a distal position (Fig. 1C). Noteworthy, γa promoter activity was substantially enhanced by HNF-1B (Fig. 1C). We conclude that HNF-1B specifically acts as an activator of the γa-subunit.

3.2. Characterization of HNF-1B mutants

In this study, three frameshift mutations (H69fsdelAC, Y352finsA, H324S325fsdelCA), of which one associates with hypomagnese-mia, and one missense mutation (K156E) were tested for its transactivation activity on the alternative transcription of the FXYD2 gene. Noteworthy, HNF-1B K156E, Y352finsA and H324S325fsdelCA have an intact nuclear localization sequence and, therefore, localized to the nucleus whereas HNF-1B H69fsde-lAC was mainly retained in the cytosol (Fig. 2A) [27]. Importantly, all mutants were checked for equal expression as shown in Fig. 2B. HNF-1B H69fsdelAC was not detectable at the expected molecular weight of 10 kD, probably due to the faster protein turnover or the different exposure of the HA epitope compared to the other mutants.

3.3. HNF-1B mutants have a dominant negative effect on wild type HNF-1B transcription of FXYD2a

The human genomic region spanning from −1269 to +5393 downstream the FXYD2b exon was cloned in front and in frame with the EGFP reporter gene (Fig. 3A, left panel). Upon stimulation with exogenous HNF-1B in HEK293 cells, alternative transcription takes place eventually translating two EGFP fusion transcripts into two proteins that differ in their N-terminus. The effectiveness of the induction was monitored by fluorescence microscopy (Fig. 3A, right panel). Dose–response assays with increasing amounts of wild type HNF-1B indicated 30 ng as the amount of DNA that generates the half maximal induction of the FXYD2a-EGFP transcription (Fig. 3B).

To determine the effect of the HNF-1B mutants on the transcription of FXYD2a and FXYD2b, the FXYD2-EGFP plasmid was transiently transfected into HEK293 cells and alternative transcripts levels were determined by use of isoform-specific primers after HNF-1B induction. As expected, FXYD2a-EGFP mRNA was stimulated by wild type HNF1B whereas the mutant transcription factors
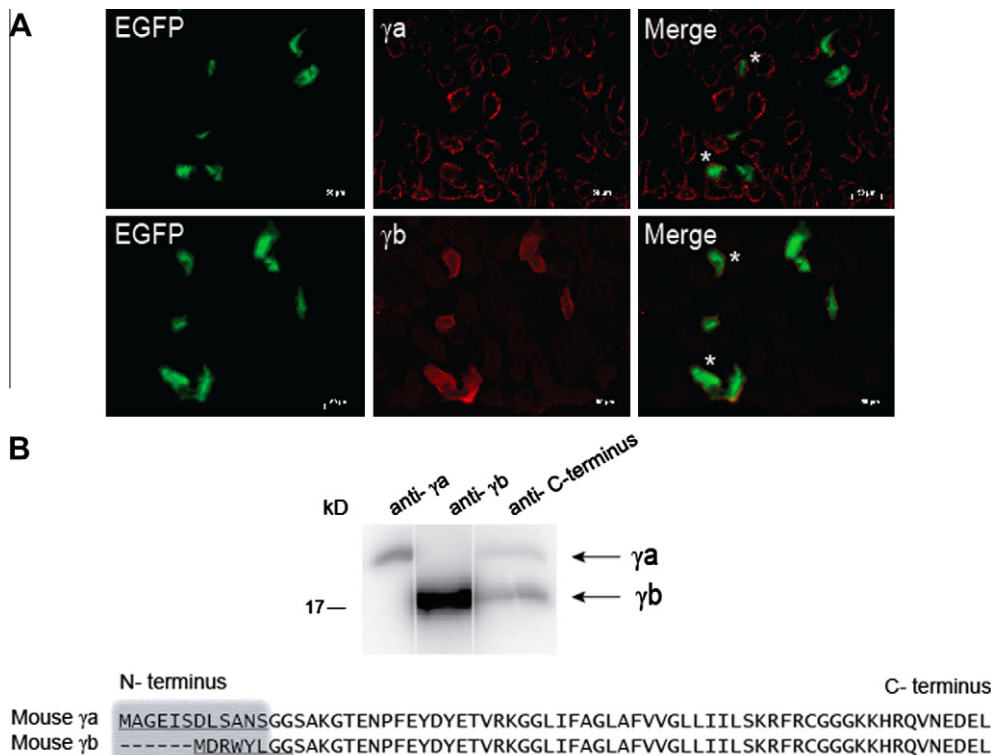
Fig. 4. Immunohistochemical detection of γa- and γb-subunits in the DCT of the kidney. (A) Staining for γa- and γb-subunits (red; upper and lower panel, respectively) of serial kidney sections from parvalbumin–EGFP mice. In green, the EGFP-positive DCTs. The asterisks in the merged panels indicate representative overlapping distal tubules on serial sections intensively stained for γa and γb. Scale bars: 50 μm. (B) (Left panel) A protein lysate from mouse kidney was resolved in a wide lane of a 16% SDS–PAGE gel. The blot was cut into three pieces and stained with anti-γb, anti-γa or anti-γ C-terminus antibodies. The faster-migrating band was identified as the γb-subunit. The epitopes recognized by the isoform-specific antibodies are underlined in the alignment of the mouse γa- and γb-subunits. (For interpretation of the references in color in this figure legend, the reader is referred to the web version of this article.)

lost their transactivation properties. FXYD2b-EGFP mRNA was in all cases not affected (Fig. 3C, left panel).

Considering the autosomal dominant inheritance of HNF1B mutations, a potential dominant effect was tested by co-transfection of equal amounts of plasmid DNA encoding wild type HNF-1B, HNF-1B mutants or mock in HEK293 cells. The induction of FXYD2a transcription in HEK293 cells coexpressing wild type HNF-1B and each of the HNF-1B mutants was significantly reduced in comparison with cells coexpressing wild type HNF-1B and mock plasmid (Fig. 3C, right panel). To date, HNF1B K156E has not been associated with hypomagnesemia, but plasma Mg^{2+} concentrations in HNF1-B mut[+] patients remain to be extensively investigated.

3.4. γa- and γb-subunits colocalize in the DCT of the kidney

In order to investigate γa and γb expressions in the DCT, parvalbumin–EGFP mouse kidney sections were stained with antibodies raised against the specific N-terminus of each γ isoform. Both γ-subunits are expressed at the basolateral membrane (Fig. 4A), where the Na^+/K^+-ATPase is also localized along the nephron [28]. The staining of γb was clearly restricted to the EGFP-positive DCTs, whereas γa-associated immunofluorescence showed staining of several tubular segments in the kidney cortex, in agreement with previous localization studies [18]. Noteworthy, using serial kidney sections, we demonstrated that EGFP-positive tubules show overlap with the immunopositive staining of both γ variants (Fig. 4A).

The specificity of the γa and γb antibodies was further investigated via immunoblot on a mouse kidney lysate. Due to posttranslational modifications, γ-subunit migrates on SDS–PAGE gels as a doublet with γb migrating faster than γa [29]. As expected, staining for the C-terminus of the protein showed two bands (Fig. 4B). Nevertheless, in our experimental conditions both variants were detected at a much higher molecular weight compared to the predicted size (7.18 kDa γa, 7.34 kDa γb) [29]. Probably other still unknown factors that influence gel migration are involved, as anti-γa and anti-γb antibodies clearly recognized the upper and the lower band of the doublet, respectively (Fig. 4B).

In conclusion, our data indicate that mutations in the HNF1-B gene affect the expression of γa-subunit. We speculate that reduced amount of γa at the basolateral membrane of the DCT may influence Mg^{2+} reabsorption via three putative mechanisms: (i) changes in the equilibrium between γa and γb expression can impair the Na^+/K^+-ATPase activity and cause an imbalance in salt reabsorption. Recently, it has been shown that dysfunction of the K^+ channel Kir4.1 in the basolateral membrane of the DCT inhibits the Na^+/K^+-ATPase via loss of recycling, renders the membrane potential less negative, and thereby reduces Mg^{2+} reabsorption through TRPM6 [30,31]; (ii) γ-subunit variants can oligomerize and behave as an inward-rectifying cation channel that can mediate extrusion of Mg^{2+} to the blood [32]. Nevertheless, this hypothesis seems unlikely due to the unfavorable electrochemical gradient across the basolateral membrane; (iii) γa and γb may interact with a still unknown Mg^{2+} extrusion mechanism, involving a primary or secondary active transporter. In the future, functional studies may validate one of these hypotheses.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2010.11.108.

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