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Truncated beta epithelial sodium channel (ENaC) subunits responsible for multi-system pseudohypoaldosteronism support partial activity of ENaC

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ABSTRACT

Aldosterone regulated epithelial sodium channels (ENaC) are constructed of three homologous subunits. Mutations in the α-, β- and γ-ENaC subunit genes (SCNN1A, SCNN1B and SCNN1G) are associated with multi-system pseudohypoaldosteronism (PHA), and mutations in the PY motif of carboxy-terminal region of β and γ subunits are associated with Liddle syndrome of hereditary hypertension. In this study we identified two frameshift mutations in the SCNN1B alleles of a female infant diagnosed with multi-system PHA inherited from her parents. This is the first case of PHA in an Ashkenazi family in Israel. The p.Glu217fs (c.648dupA in exon 4) and p.Tyr306fs (c.915delC in exon 6) mutations produce shortened β-ENaC subunits with 253 and 317 residues respectively instead of the 640 residues present in β-ENaC subunit. Expression of cRNAs carrying these mutations in *Xenopus* oocytes showed that the mutations drastically reduce but do not eliminate ENaC activity. The findings reveal that truncated β-ENaC subunits are capable of partially supporting intracellular transport of the other two subunits to the membrane and the final assembly of a weakly active channel together with normal α- and γ-ENaC subunits. Moreover, these results enhance our understanding of the long-term consequences of these types of mutations in PHA patients.

1. Introduction

Steroid hormone aldosterone that is synthesized and secreted from the zona glomerulosa of adrenal cortex [1] is one of the major regulators of electrolyte homeostasis and blood volume [2]. Secretion of aldosterone is regulated mainly by the renin-angiotensin system [3]. The major site of action of aldosterone in electrolyte regulation is epithelial cells in the distal tubule of kidney where aldosterone induces expression of epithelial sodium channel (ENaC) subunits increasing ENaC activity at the apical cell surface [4,5]. Sodium ions that enter the epithelial cells via ENaC are pumped out into the interstitial space via Na/K ATPase located on the opposite basolateral membrane. Enhanced sodium transfer from the lumen of kidney tubule into epithelial cells increases interstitial fluid osmolarity. Water then freely flows in the same direction to maintain similar osmolarity of compartments. Consequently, aldosterone actions result in increases in blood volume and blood pressure [2,6].

Epithelial sodium channels are constructed of three homologous subunits named as α-, β- and γ-ENaC that are embedded in the membrane with two trans-membrane segments [6–8]. The amino and carboxy-terminal domains of these subunits are located in the cytoplasm, while the bulk of their structure is exposed outside of the cell, forming part of the funnel that directs ions from the lumen into the pore of ENaC, and from there into the epithelial cell.

Mutations in the genes that encode for ENaC subunits are associated with two independent hereditary diseases: multi-system pseudohypoaldosteronism (PHA) [9–17], and Liddle syndrome of hereditary hypertension [18,19]. Multi-system PHA is observed as an autosomal recessive disease, whereas Liddle syndrome shows autosomal dominant inheritance.

Multi-system PHA is manifested as a syndrome of aldosterone unresponsiveness that leads to severe salt-wasting in early infancy [20]. Most of the mutations responsible for PHA have been identified in the gene encoding the α subunit of ENaC [15]. There are only a few reports of PHA causing mutations in the genes encoding the β and γ subunits. Mutations responsible for the Liddle syndrome

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have been observed so far solely at the carboxy-terminal of the β and γ subunits. This region includes a PY (Pro-Pro-Pro-x-Tyr) motif that binds Nedd4-2, an ubiquitin ligase. Ubiquitylation of this motif eventually leads to removal of the protein from the membrane by endocytosis. Truncation of this motif by a nonsense mutation in the carboxy-terminal region, or a missense mutation in the PY motif, inhibits ubiquitylation of the subunit. As a consequence, channels accumulate at the membrane leading to enhanced activity of ENaC. Thus, while mutations associated with multi-system PHA reduce ENaC activity, mutations associated with the Liddle syndrome enhance ENaC activity [21].

In our examination of a multi-system PHA patient we identified two independent frame-shift mutations in the gene encoding the β-ENaC leading to drastic truncation of the carboxy-terminal region of β-ENaC. Previous studies indicated that the cell surface expression of functional ENaC is dependent on the presence of all three ENaC subunits. We undertook the present study to examine the question whether a severely truncated β-ENaC subunit missing more than 50% of its primary structure can partially support cell surface expression and activity of ENaC. For this purpose we generated the corresponding mutant forms of human β-ENaC by site-directed mutagenesis of normal human cDNAs and examined the activities of the mutant forms expressed in *Xenopus* oocytes that were microinjected with corresponding cRNAs.

2. Subjects and methods

2.1. Subjects

The female infant with multi-system PHA was born full term (gestational age: 40 weeks) weighing 3.385 kg as the second sibling of non-consanguineous Ashkenazi Jewish parents and presented with extreme lethargy at 8 days of age with a 1-day history of poor feeding. Laboratory evaluation on admission showed very high serum K^+ levels and hyponatremia. Trans-tubular potassium gradient was 2.4, indicating very low renal potassium excretion. Sweat Cl^- concentration was also high, consistent with salt losing from multiple target organs of aldosterone. Her weight at 1 year was 7.035 kg, and height 71 cm. Laboratory findings are presented in Table 1.

To examine possible presence of identified mutations we randomly selected a control population 25 healthy Ashkenazi Jewish residents of Israel.

2.2. DNA isolation and sequencing

Genomic DNA was extracted from lymphocytes of the subjects and was analyzed by sequencing using an ABI 310 Genetic Analyzer as previously described [22]. ABI Sequencer traces were analyzed using BioEdit and CodonCode Aligner software. Gene mutations are described using the Human Genome Variation Society recommendations for the description of DNA sequence variants [23].

Table 1
PHA patient laboratory data on admission at 8 days of age.

	PHA patient	Normal for neonates
Serum Na^+	132 mmol/l	136–146
Serum K^+	9.2 mmol/l	3.5–5
Serum aldosterone	>2770 pmol/l	<3050
Serum plasma renin activity	>15 ng angiotensin/ml/h	<10
Sweat Cl	137 mmol/l	<40
Urine trans-tubular K^+ gradient	2.4	>4

2.3. Expression of ENaC in Xenopus oocytes

We cloned the three cDNAs encoding for the α, β and γ subunits of human ENaC in plasmid pGEM-HJ for expression in *Xenopus* oocytes [17]. We generated mutant forms of β-ENaC using a recently described site-directed mutagenesis method [22]. Both the wild type and the mutated forms were completely sequenced using an ABI 310 Genetic Analyzer to verify the sequences.

The cDNAs were transcribed *in vitro* using T7-RNA polymerase to generate complementary RNAs (cRNAs). The cRNAs (3 ng for each subunit) were microinjected into immature stage V–VI *Xenopus* oocytes that were dissociated with 0.3 mg/ml type 1A collagenase [24]. The oocytes were incubated at 18 °C in ND-96 medium (in mM: 96 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, and 5 HEPES, pH 7.4) containing 2.5 mM sodium pyruvate, 50 μg/ml gentamicin and 10 μM amiloride. ENaC dependent amiloride-sensitive whole-cell inward Na^+ current was measured 2–3 days after cRNA injection using the two-electrode voltage-clamp method while oocytes were clamped at −80 mV and continuously perfused with ND-96 +10 μM amiloride and ND-96 alternately at 22 °C. Data were collected and analyzed using pClamp software (Axon Instruments).

2.4. Statistical analysis

The activity of each mutant cRNA was tested in independent experiments with at least 10 oocytes in each run. Measurements were carried out in a double blind protocol, without knowledge of the injected cRNA. Significance of the difference between means was analyzed by Student's *t*-test.

3. Results

3.1. PHA associated mutations in the β-ENaC gene

Patient's laboratory data on admission including hyperreninemia and hyperaldosteronism (Table 1) were highly suggestive of multi-system PHA. Subsequently she required large quantities of NaCl (60 mmol/kg/day), sodium bicarbonate and Kayexalate therapy to maintain the serum sodium and potassium within the normal range. Apart from electrolyte disturbances she also suffered from failure to thrive, chronic nasal discharge, seborrhea like skin eruptions, and recurrent respiratory infections. Thus, the general clinical picture was clearly consistent with multi-system PHA.

Sequencing of the patient's genomic DNA revealed two heterozygous mutations, an insertion in exon 4 (c.648dupA) and a deletion in exon 6 (c.915delC) of the β-ENaC gene (Figs. 1 and 2). These mutations shift the reading-frame of the coding sequence for human β-ENaC, starting at Glu217 and Tyr306, respectively (the mutation was on codon for Asp305, but the first residue that was affected by the frame-shift was Tyr306). According to the Human Genome Variation Society nomenclature, at protein sequence level these mutations should be marked as p.Glu217fs and p.Tyr306fs.

These results established that the patient was clearly a compound heterozygote that would have truncated β-ENaC subunits. The mutant sequence of the first allele has an open-reading-frame (ORF) of 253 residues, and the second allele has an ORF of 317 residues, while the normal allele codes for β-ENaC that 640 residues.

To identify the origin of the mutations we sequenced the ENaC genes of both parents. Their genomic sequences showed that they are both heterozygote carriers, each parent carrying one of the alleles observed in the patient (Figs. 1 and 2).

Sequencing of the β-ENaC gene exons 4 and 6 from a control sample of 25 Jewish Ashkenazi individuals (representing 50 alleles) did not reveal any mutation in the sequenced segments. Thus,

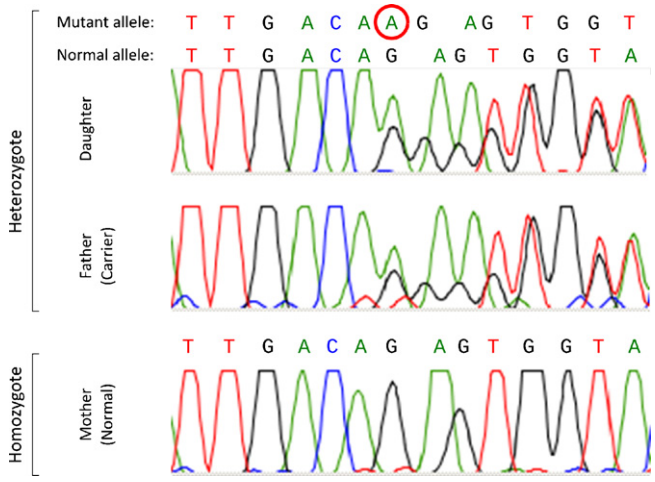

Fig. 1. Sequence of the β-ENaC gene segment with a heterozygous mutation in exon 4. The insertion mutation (c.648dupA) causes a reading-frame shift in the codon for Glu217. The nucleotide A marked with a red circle was observed only in the mutant allele. While the mother of the patient is a normal homozygote, the father of the patient is a heterozygote carrying the same mutant allele that was observed in the patient. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

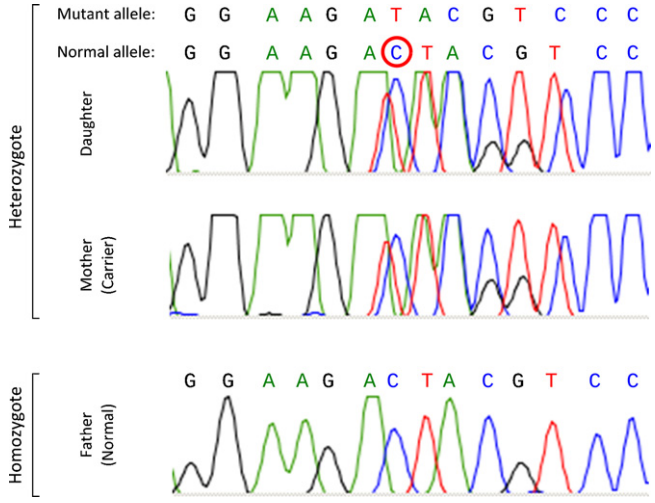

Fig. 2. Sequence of the β-ENaC gene segment with a heterozygous mutation in exon 6. The deletion mutation (c.915delC) causes a reading-frame shift in the codon for Tyr306. The nucleotide C marked with a red circle was deleted in the mutant allele. While the father of the patient is a normal homozygote, the mother is a heterozygote carrying the same mutant allele that was observed in the patient. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

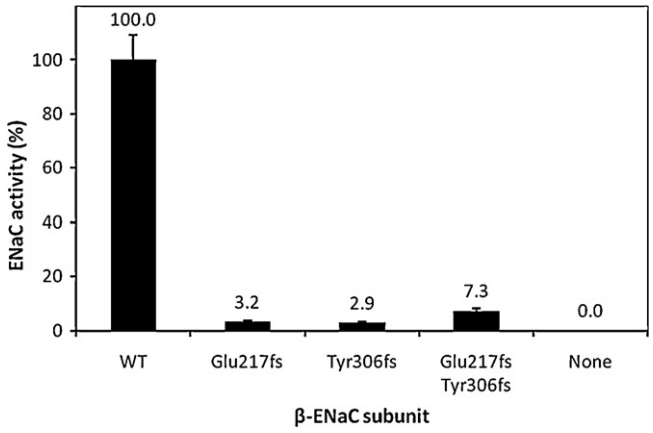

Fig. 4. Relative levels of Na^+ conductance assayed in *Xenopus* oocytes microinjected with normal α-, β- and γ-ENaC cRNAs (WT), or with normal α and γ and mutated β-ENaC cRNAs carrying a single mutation (p.Glu217fs or p.Tyr306fs). For the experiments in the first three columns the oocytes were injected with 3 ng of each cRNA as noted in Section 2. For the experiment in the fourth column (Glu217fs + Tyr306fs) oocytes were injected with 3 ng of each of α and γ cRNAs and 1.5 ng of each of two β-ENaC cRNAs with p.Glu217fs and p.Tyr306fs mutation, respectively. For the experiment in column 5 oocytes were injected with α and γ cRNAs without β cRNA (None). The columns represent the mean ± S.E.M. of measurements in 33, 21, 21, 11 and 10 oocytes for each of the columns, respectively. The mean for WT was standardized to 100%.

the mutations do not appear to be frequent in the Ashkenazi population.

3.2. Na^+ conductance in oocytes expressing mutated β-ENaC

We reconstituted human ENaC in *Xenopus* oocytes using normal and mutated cloned cDNAs. Examination of the Na^+ conductance in individual oocytes indicates clearly that the mutant cRNAs support partial activity of ENaC. In oocytes expressing ENaC, channel activity is inhibited by inclusion of amiloride in the medium that blocks ion conductance directly by binding to the pore of the channel. For measurement of the sodium current, superfusion medium is changed to medium without amiloride. The removal of the inhibitor amiloride exposes the channel activity resulting in an increase in inward current (Fig. 3). In oocytes expressing either of the two mutant β subunits, a significant current was observed. Yet, in oocytes expressing α and γ cRNAs (αγ) without β cRNA no change in current could be detected (Fig. 3).

In independent experiments with oocytes injected with β-ENaC cRNA carrying the p.Glu217fs mutation the average Na^+ conductance activity observed was ~3% of normal ENaC (Fig. 4). Similarly, in oocytes injected with β-ENaC cRNA carrying the p.Tyr306fs

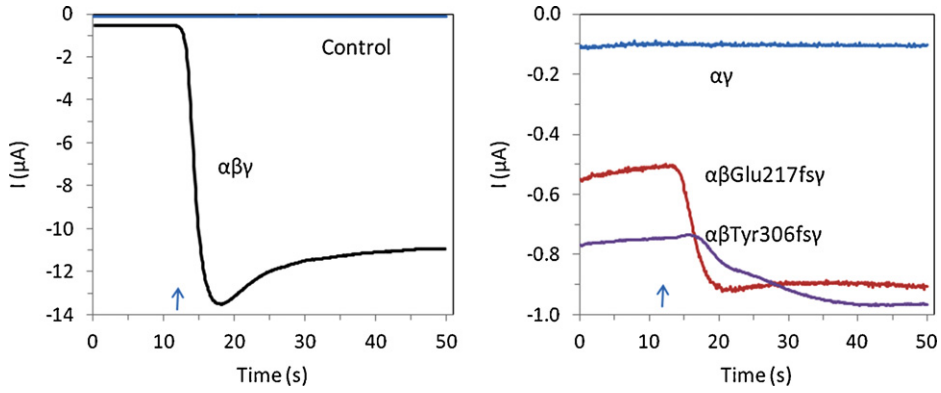

Fig. 3. Representative recordings of sodium current (I_{NA}) in oocytes expressing different combinations of ENaC subunits. Superfusion medium was changed to medium without amiloride at 12 s (marked by arrow) in the traces shown.

mutation the average Na^+ conductance activity was also ~3% of normal ENaC (Fig. 4). Surprisingly, in oocytes injected with both mutant cRNAs (1.5 ng of each for a total of 3 ng) we observed significantly higher activity (Fig. 4). This doubling of the activity could not be ascribed to doubling the amount of the injected cRNA, as for this experiment we injected half the amount of cRNA (1.5 ng versus 3 ng) for each mutant. As noted above, oocytes injected with α and γ cRNAs without β cRNA showed no detectable channel activity.

4. Discussion

In this study we identified two heterozygous frame-shift mutations in the paternal and maternal alleles coding for β-ENaC subunit in a patient with multi-system PHA. This is the first case of PHA in an Ashkenazi family in Israel. Similar mutations were not detected in a sample of 50 alleles from Ashkenazi subjects. Thus the mutations, similar to other PHA causing mutations, do not appear to be frequent in this population. We know of only one previous report on mutations in the β-ENaC subunit of an Ashkenazi Jewish patient from the USA [11]. Since the residues affected by the mutations were not specified in this report [11], we could not determine their relationship to the mutations identified in our patient.

4.1. Partial activity of ENaC reconstituted with truncated subunits

The p.Glu217fs and p.Tyr306fs mutations produce shortened β-ENaC subunits with 253 and 317 residues respectively instead of the 640 residues present in the normal β-ENaC [7]. Expression of cRNAs coding for these mutant subunits in *Xenopus* oocytes revealed that these mutations lead to drastic reductions in ENaC activity, but nonetheless are capable of supporting partial activity of ENaC (Figs. 3 and 4).

Both molecular studies on multi-system PHA patients [15], and *in vitro* studies [25–27] indicate that all three subunits are required for full ENaC activity. A series of studies suggest that the subunits do not reach the membrane independently, but rather, they form an oligomeric complex in the endoplasmic reticulum and only then are transported *en bloc* to the apical membrane [27]. The partial activity that we observed with normal α- and γ-ENaC and truncated β-ENaC indicates that the short N-terminal segment of β-ENaC is capable of supporting oligomeric assembly of the three subunits and the subsequent transport of the complex to the cell surface. Previous studies on expression of α-ENaC with Arg492Stop and Arg508Stop mutations showed that a truncated α-ENaC is also capable of supporting ENaC activity [13,28]. Currently we do not what are the specific points of interactions between subunits prior to their transport. The question of what are the specific domains of the subunits necessary for *en bloc* transport to the membrane may be examined by probes that can be used to track intracellular trafficking of the re-engineered subunits.

Another surprising finding of the present study was that co-expression of both β-ENaC subunits with p.Glu217fs and p.Tyr306fs mutations led to a doubling of the ENaC activity as compared to activity observed with only one mutant β-ENaC subunit (Fig. 4). This suggests that there may be a synergistic effect of the different mutants on the oligomerization and/or subsequent membrane assembly and function of the ENaC complex. In epithelial cells *in vivo* probably both alleles are expressed together. Thus, in the cells of the PHA patient partial ENaC activity with both mutants expressed together may be higher than that we observed with a single mutated β-ENaC subunit.

In some previous studies partial sodium conductance was observed after expression of only two human ENaC subunits, i.e. αγ, and αβ [28]. In our studies consistently we failed to detect amiloride-sensitive sodium currents in oocytes expressing only two subunits (Figs. 3 and 4). The current we see with all three subunits αβγ are similar to those observed by others. Thus, the possibility of a lower activity in our system cannot be invoked to explain this discrepancy. It is possible that oocytes isolated from *Xenopus* grown at different locations may have functional differences. Bonny et al. [28] have noted that the low currents they observed when only two subunits were expressed could not be due to expression of endogenous subunits as they could not detect endogenous expression of ENaC subunits in *Xenopus* oocytes. Our observation of lack of any measurable current in the presence of only two subunits confirm that there is no endogenous amiloride-sensitive channel in *Xenopus* oocytes.

4.2. Clinical implications of mutant ENaC activity

Previous studies of Liddle syndrome showed that truncation of the carboxy-terminal domain of β-ENaC leads to enhancement of ENaC activity [21,29]. Thus, the β-ENaC mutations described here would be expected to have two independent opposing effects within epithelial cells: (1) inhibition of ubiquitination of ENaC and consequent lower rate of endocytotic removal of the channel; (2) inhibition of ENaC activity as a result of lower rate of membrane transport and lower activity in the membrane.

In Liddle syndrome, inactivation of the PY motif in β-ENaC by a missense mutation leads to enhancement of ENaC presence in the membrane without a negative effect on ENaC activity. Similarly, truncation of a relatively small carboxy-terminal region as a result of a nonsense mutation also seems to have the major effect of enhancing "survival" of ENaC, rather than damaging channel function.

In the case of the mutations described here, the PHA phenotype of the patient clearly indicates that the damage to ENaC function is great and that, if there is any enhanced "survival" of the channel, it is not sufficient to compensate for the reduced channel activity. Thus, multi-system PHA symptoms emerge as the predominant effect of the β-ENaC mutations found in our patient. It remains to be seen if there are intermediate cases of β-ENaC mutations that lead to a balancing between the two opposing effects noted above.

Recently we reported that in the long-term (up to 20 years) there is an age-dependent normalization in the urinary Na/K ratios of multi-system PHA patients accompanied by an exaggerated renin-aldosterone system response probably contributing to age-dependent amelioration [17]. Based on the partial activity of ENaC reconstituted with a truncated subunit we suggest that even the small residual activity ENaC may be important in contributing to improved sodium balance with age. In a case with missense mutation that displays much higher channel activity (~40% of wild type) the salt losing is less severe and improvement with age occurs earlier [17]. As discussed before [17], we do not know the physiological mechanisms responsible for the age-dependent normalization, but the residual activity of the mutated channel that we observed may be a key factor for understanding this long-term process. This issue may be clarified if we find a case of multi-system PHA without any residual activity of channel, i.e. 100% loss of ENaC activity.

The present findings should also be useful for prenatal diagnosis and early treatment of multi-system PHA. If undetected during the first week of life, multi-system PHA can lead to neonatal death. But, if detected, the patients may lead near normal lives on a life-long diet of high salt. The identification of these mutations facilitates future screening of PHA patients with a similar ethnic background.

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