Mutations in subunits of the epithelial sodium channel cause salt wasting with hyperkalaemic acidosis, pseudohypoaldosteronism type 1

Sue S. Chang[1,2], Stefan Grunder[3], Aaron Hanukoglu[4], Ariel Rösler[5], P.M. Mathew[6], Israel Hanukoglu[7], Laurent Schild[3], Yin Lu[1], Richard A. Shimkets[1], Carol Nelson-Williams[1], Bernard C. Rossier[3] & Richard P. Lifton[1,2]

Autosomal recessive pseudohypoaldosteronism type I is a rare life-threatening disease characterized by severe neonatal salt wasting, hyperkalaemia, metabolic acidosis, and unresponsiveness to mineralocorticoid hormones. Investigation of affected offspring of consanguineous union reveals mutations in either the α or β subunits of the amiloride-sensitive epithelial sodium channel in five kindreds. These mutations are homozygous in affected subjects, co-segregate with the disease, and introduce frameshift, premature termination or missense mutations that result in loss of channel activity. These findings demonstrate the molecular basis and explain the pathophysiology of this disease.

[1]*Howard Hughes Medical Institute, Department of Genetics, Boyer Center for Molecular Medicine,* [2]*Section of Nephrology, Department of Medicine, Yale University School of Medicine, New Haven, Connecticut 06510, USA* [3]*Institut de Pharmacologie et de Toxicologie, Universite de Lausanne, Rue du Bugnon 27, CH-1005 Lausanne, Switzerland* [4]*Department of Pediatrics, E. Wolfson Hospital, Holon, and Tel-Aviv University, Sackler School of Medicine, Tel Aviv, Israel* [5]*Department of Endocrinology and Metabolism, The Hebrew University of Jerusalem, Hadassah Medical Center, Jerusalem, Israel* [6]*Department of Pediatrics, Dhahran Health Center, Dhahran, Saudi Arabia* [7]*Research Institute, College of Judea and Samaria, Ariel, and Institute of Endocrinology, Sourasky Medical Center, Tel-Aviv, Israel*

Correspondence should be addressed to R.P.L.

Pseudohypoaldosteronism type I (PHA1) is a rare salt wasting disease characterized by an often fulminant presentation in the neonatal period with dehydration, hyponatraemia, hyperkalaemia, metabolic acidosis, failure to thrive and weight loss despite normal renal glomerular filtration and adrenal function[1–4]. PHA1 is suspected when these infants fail to respond to mineralocorticoids, and the diagnosis is supported by the finding of an elevated plasma aldosterone concentration and plasma renin activity[2–4]. Treatment includes sodium chloride supplementation and treatment with an ion-binding resin or dialysis to reduce life-threatening hyperkalemia[1–6]. Death in the neonatal period is common if the diagnosis is not made.

PHA1 kindreds showing both autosomal recessive and dominant transmission have been described[7]. Cases in recessive kindreds typically show mineralocorticoid resistance in the kidney, sweat and salivary glands, and colonic mucosa[4,7–9]; where measured, parents of these cases have had normal aldosterone and renin levels[4,7]. In contrast, kindreds supporting dominant transmission have also been reported, and in some of these have been shown to have disease limited to the kidney[4,7,10,11]. Clinical signs and metabolic abnormalities of some patients improve in the first several years of life, allowing discontinuation of therapy[1,2,4,5,7]; it has been suggested that these patients are most often those with dominant transmission[7].

The pathogenesis of this syndrome has not been elucidated. The triad of renal salt wasting, hyperkalemia and failure to respond to mineralocorticoids is most compatible with a renal defect in the distal nephron[1,12]. While mineralocorticoid receptor levels in affected patients have been found to be low[13–15], molecular studies have revealed no evidence for a primary defect in the mineralocorticoid receptor[16,17].

Electrogenic transepithelial sodium transport is the rate limiting step in sodium reabsorption in the distal nephron, the distal colon, salivary and sweat glands, and lung epithelia[18]. In the kidney, this electrogenic

Table 1 Characteristics of index cases of PHA1 kindreds

Kindred	Location	Ethnicity	Age	Na^+	K^+	PAC	Mutation
PHA K10	Saudi Arabia	Saudi	7 d	124	7.7	1.87	*αENaC* I68fr
PHA K13	Saudi Arabia	Saudi	1 d	126	11.2	6.28	*αENaC* I68fr
PHA K14	Saudi Arabia	Saudi	8 d	128	10.9	15.16	*αENaC* I68fr
PHA K3	Israel	Iranian Jew	9 d	125	10.0	14.27	*αENaC* R508stop
PHA K8	Israel	Arabic	19 d	133	8.2	1.00	*βENaC* G37S
PHA K12	Saudi Arabia	Pakistani	235 d	107	6.9	3.24	none
PHA K7	Saudi Arabia	Sudanese	5 d	112	11.0	8.64	none

Age, age at clinical presentation (days); Na^+, serum sodium concentration (mM), normal 138–142; K^+ serum potassium concentration (mM), normal 3.5–5.0; PAC, plasma aldosterone concentration (g/l), normal 1–95. fr, frameshift. Kindreds PHA K10, K13 and K14 are all Saudi natives ascertained in Dhahran but not known to be related to one another.

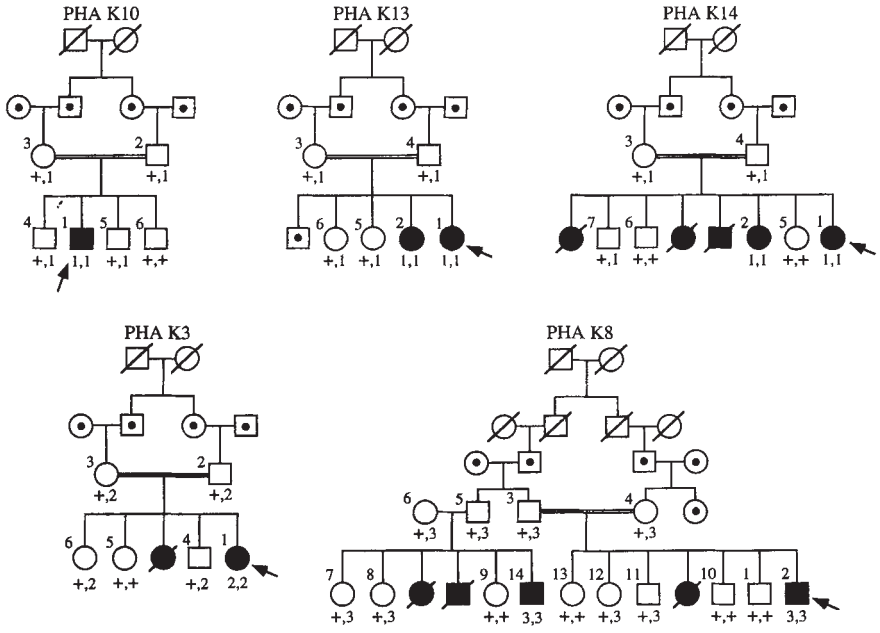

Fig. 1 The familiy relationships of 5 PHA1 kindreds in which mutations have been identified. All affected subjects are the product of consanguineous union. Subjects classified as affected are indicated by filled symbols; unaffected subjects are indicated by unfilled symbols; deceased subjects are indicated by a diagonal line; index cases are indicated by an arrow; living subjects who were not sampled are indicated by dots. Within each kindred, each sampled individual is identified by a unique number, which is shown above and to the left of their respective symbol. Below each symbol, the SSCP genotype at either *αENaC* (PHA K10, K13, K14 and K3) or *βENaC* (PHA K8) is shown. The symbol + denotes the normal SSCP variant, and the numbers 1, 2, and 3 indicate the *αENaC* codon 68 frameshift, *αENaC* codon 508 stop, and *βENaC* G37S mutations, respectively.

sodium transport is positively regulated by aldosterone[19] and is mediated by the amiloride-sensitive epithelial sodium channel (ENaC). This channel is composed of at least three subunits of similar structure[20,21], each with intracellular amino and carboxy termini, two transmembrane spanning domains, and a large extracellular loop. In humans, *αENaC* is present on human chromosome 12, while β and γ are tightly linked on chromosome 16 (ref. 22).

Mutations resulting in constitutive activation of ENaC activity have been shown to cause an autosomal dominant form of hypertension, Liddle's syndrome[22–25], which is characterized by volume expansion, hypokalemia and alkalosis; this finding raises the possibility that mutations causing loss of function of ENaC activity could cause the converse phenotype of volume depletion, hyperkalemia and acidosis characteristic of patients with PHA1.

Mutations in *αENaC* in PHA1

The α subunit of ENaC is required for ENaC activity[21],

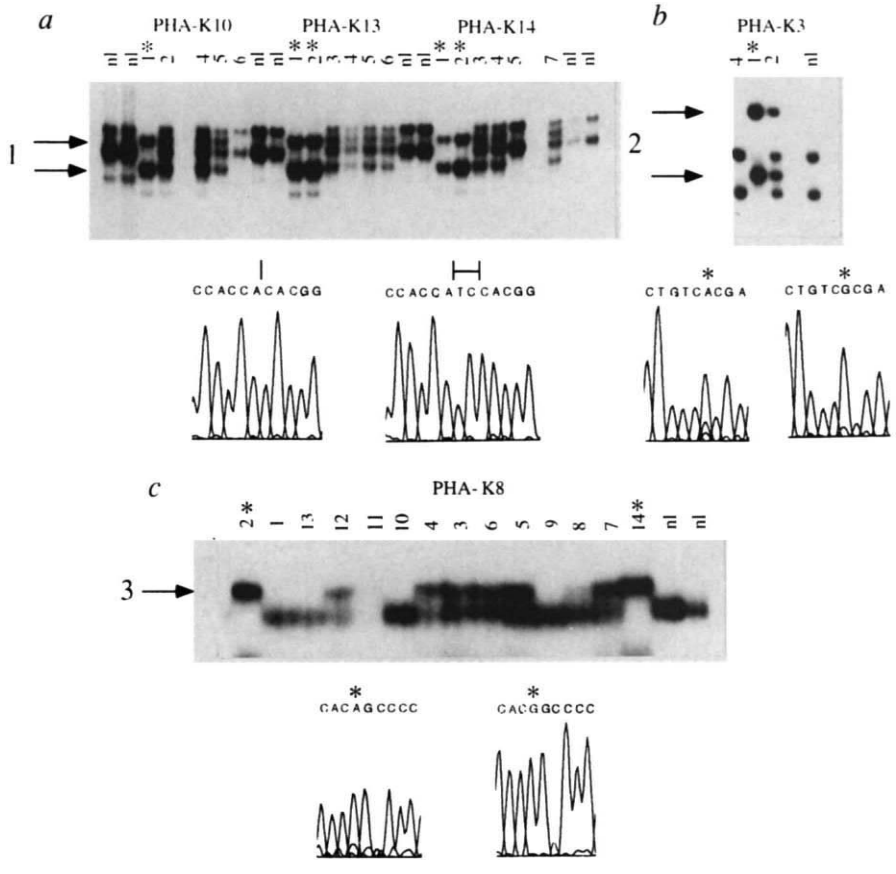

Fig. 2 Mutations in *ENaC* subunits in PHA1 patients. In each panel, variants identified by SSCP in PHA1 kindreds are shown. Individuals are numbered as in Fig. 1, and representative autoradiograms are shown. SSCP genotypes, as well as marker genotypes, were confirmed from at least two independent amplifications for each individual. Affected individuals are indicated by an asterisk, and the novel SSCP variants that are homozygous in affected subjects are indicated by arrows; these variants are numbered as in Fig. 1. At the bottom of each panel, the DNA sequence of the mutant allele (left) and corresponding wild-type allele (right) is shown; deleted bases are indicated by a bracket in (*a*), and single base substitutions are indicated by asterisks in (*b*) and (*c*). *a*, Affected subjects in PHA kindreds K10, K13 and K14 are homozygous for the same 2 base pair deletion introducing a frameshift at codon 68 of *αENaC*, and this variant co-segregates with the disease. The DNA sequence extending from the last two bases of codon 66 to the first two bases of codon 70 of the wild type sequence are shown in the sense orientation. The last two bases of codon I68 are absent in the mutant. *b*, Homozygous variant in *αENaC* introducing a premature termination codon at codon 508 of PHA K3. DNA sequence encoding amino acids 507–509 are shown in the antisense orientation; in the sense orientation, CGA encoding R508 is mutated to TGA, encoding stop508. *c*, Homozygous variant in *βENaC* encoding G37S in affected subjects of PHA K8. DNA sequence of codons 36–38 are shown in the sense orientation. The sequence GGC encoding G37 is mutated to AGC encoding S37.

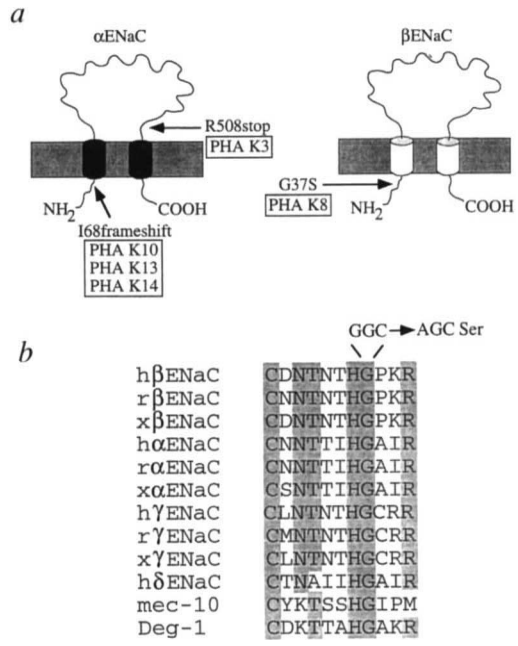

Fig. 3 *a*. The effects of mutations in α and *βENaC* in PHA1 kindreds. ENaC subunits are drawn as spanning the plasma membrane twice[40], and amino and carboxyl termini are indicated. Arrows indicate the position of identified mutations in each subunit. The kindreds in which each mutation is found are indicated. Mutation in *αENaC* introduces a frameshift mutation at codon 68 proximal to the first transmembrane domain; this mutation is found in 3 kindreds. A mutation in PHA K3 introduces a premature termination codon into the extracellular domain of αENaC, and a mutation in *βENaC* in PHA K8 introduces a missense mutation, changing glycine at residue 37 to serine. *b*, G37S mutation in *βENaC* occurs in a conserved ENaC segment. Amino acid sequences preceding the first transmembrane domain of different members of the ENaC family are shown. The prefix h, r, and x denote genes from human, rat and *Xenopus laevis*, respectively[20,21,29–32]; mec-10 and Deg-1 are from *C. elegans* [33,34]. Those residues that are identical in α, β and γ subunits from all species are shaded. The completely conserved glycine at position 37 of hβENAC is mutated to serine in PHA kindred 8.

and consequently loss of function mutations in this gene could result in a syndrome similar to PHA1. Affected subjects arising from consanguineous union are expected to be homozygous for the same mutant allele at the trait locus. In contrast, random loci will be homozygous for an ancestral allele with a likelihood of 1 in 16 in the offspring of 1st cousins and 1 in 64 in the offspring of second cousins, providing a powerful test of linkage[26]. Accordingly, we used knowledge of the intron–exon structure of *αENaC* and single-strand conformational polymorphism (SSCP) to screen for molecular variants in exons and intron–exon boundaries of this gene in PHA1 patients. Affected subjects in four of the seven kindreds (Table 1, Fig. 1, see Methods) showed novel *αENaC* variants that in each case were homozygous in all affected subjects in each kindred (Fig. 2*a*,*b*); no other missense variants or variants altering consensus splice sites were identified. In each case the parents were heterozygous for these variants and none of the unaffected siblings inherited two copies of the variant, demonstrating co-segregation of these variants with PHA1 in these kindreds (Figs 1, 2).

That these variants are homozygous by descent from a great-grandparent is supported by the finding that these variants are rare (absent in 160 alleles from unrelated subjects who do not have PHA1) and that two highly polymorphic loci tightly linked to *αENaC*, *D12S314* and *D12S93* (ref. 27), are each homozygous in affected subjects of these kindreds (data not shown).

Affected subjects in three of the Saudi kindreds showed indistinguishable homozygous variants in exon 2 of *αENaC* (Table 1, Fig. 2*a*). DNA sequence analysis of the variant in these kindreds revealed a 2 base pair deletion at codon I68, introducing a frameshift mutation (Fig. 2*a*). This mutation disrupts the encoded protein prior to the first transmembrane domain (Fig. 3*a*); the encoded protein bears no similarity to the normal protein from amino acid 68–144, where a termination codon ends translation.

The DNA sequence of the homozygous *αENaC* variant in kindred PHA K3 reveals a single base substitution changing codon R508 from CGA to TGA and introducing a premature termination codon (Table 1, Figs 2*b*, 3*a*). This codon is in the extracellular domain, and thus results in a protein containing a normal first transmembrane domain, part of the extracellular domain and missing the second transmembrane domain as well as the intracytoplasmic C terminus.

Both of these mutations result in αENaC subunits that lead to loss of ENaC channel activity since an intact second transmembrane domain is required for normal channel activity (ref. 28; L.S. and B.C.R., unpublished observations). These mutations can thus explain the pathogenesis of PHA1 in these families.

Mutation in *βENaC* in PHA1

Identification of mutations in *αENaC* in four PHA1 kindreds leaves open the question of whether other kindreds also harbour mutations at this locus, or alternatively whether there might be mutations at other loci that account for the disease in these remaining kindreds.

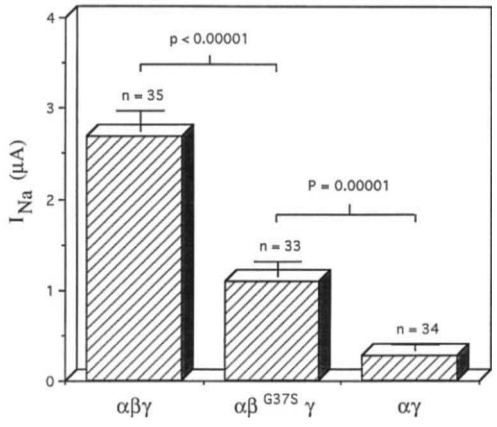

Fig. 4 Effect of βENaC G37S on amiloride-sensitive Na+ channel activity in *Xenopus* oocytes. cRNAs encoding normal or mutant ENaC subunits were co-injected into *Xenopus* oocytes, and the resulting amiloride-sensitive sodium current was measured. βENaC containing the G37S mutation was co-expressed with α and γ subunits (represented as αβ37Sγ). Oocytes injected either with normal α, β and γ or only α and γ subunits (αβγ and αγ, respectively) served as controls. The mean of the absolute values of the amiloride-sensitive sodium current obtained from 33 to 35 oocytes from 5 different batches of oocytes is shown. Error bars represent the SEM. The p values for t-tests comparing activity of mutant and wild-type channels are indicated.

Table 2 Primers used to amplify coding regions of *ENaC* subunits

Primer	Exon	Forward	Reverse
A-1	1	ACCCTTGCTCTCTCCAATCCAC	GAACTCGATCAGGGCCTCCTC
A-2	2	CTGCAACAACACCACCATCCAC	GGGGCAGAGGGACTAACCGAC
A-3	3	AGCTCCTTCACCACTCTCGTG	GGACCCTCAGGCGCTGCAAG
A-4	3	AGCTCCTTCACCACTCTCGTG	GTCAGGAAAGGAGCGGAGCCCATG
A-5	4	CCTCTGACTCTAGTCTCTGTGTC	GGAGCCAGGCAGGACTGACTC
A-6	5	GACCCTACTCTCTCTTTTCCTG	CGCCATGGAGCAAGCAGGGAG
A-7	6	GCCAACTCTGCTCTCTCTGCAC	CCTTCCAGGCCTCCCAGTCAG
A-8	7	CACGGAATCAGGTTGGGCCTC	CACGGAATCAGGTTGGGCCTC
A-9	8	CCTCTCCACCCTCCTCCCTTC	GGGGCTCCCTGGAGTCTCAC
A-10	9	ACAGGCATCTCTCTGTACCCAC	TGGCTCGGTAACCTGTATTCTAC
A-11	10	AACACTGAGCACCTTTCTCCATC	ACCCATCCCTTCCCCACACTC
A-12	11	GACCTTGATGACACCCCCATTC	CAGGGACCAGGGCAGGACTG
A-13	12	TCTTCCCACCCTCTGTCCCAC	CAGGCTCCATCCAGGCACGAC
A-14	13	AGAACCCTCTGTCCCATCGTC	CTGGAGACCAGTATCGGCTTC
A-15	13	GTCTGTGGTGGAGATGGCTGAG	GCCTGGGTGGGACAAGGACAG
A-16	13	GGTAGCCTCCACCCTGGCATC	GCCTTGGTGTGAGAAACCTCTC
B-1	1	ATGCCTCTCTGCAGGTGCCAC	AGCTGTGCACTCCGGGGCCAC
B-2	2	TTCCCCCTAACCAGCCCTCTC	CATTGCTTGATATGTGCCCAG
B-3	3	TGGCCTCCACAGTGTAGCCTC	CATCTCTACTAGCTCCTGCTG
B-4	3	TGGCCTCCACAGTGTAGCCTC	CCGACTGTCCGTAGGTGCCAG
B-5	4	CCTGCCCTGCAGCTGATGCTG	GGTTAAAGCCTCATGGCTCTG
B-6	5	CGCAGCCCTCACCCCACCCTC	GCCCTTGGGCTCCGGCCATAC
B-7	6	AAGCAACCCCTCTAAACACAG	AGGCGTGCACCACCTTCCCAC
B-8	7	CCTGTGTTCTCTCATTATGAAC	GATCCCCCGTGCCCCCGCTC
B-9	8	AACCTCTTGGCCGCCTTTCTG	TGTGCCCGCCCACCCGCACTC
B-10	9	GCAGGGACCACAACAGGCCTG	GTGGTTGCAAAAGTTGCCATC
B-11	10	GATGGCAACTTTTGCAACCAC	CCAGCCCCGCCCAGGCTCAG
B-12	11	GGCCCATCTCGCTGCCTCCTG	AGGGCTGGGGTATTGGGAGAC
B-13	12	CAAGAATGTGTGGCCTGAG	AAAGTTGGTGTGGGCCTCCAC
B-14	12	CACCAACTTTGGCTTCCAGCC	GGCTGCTCAGTGAGTTTCAG
B-15	12	CTGGTGGCCTTGGCCAAGAG	GTCCAGCGTCTGCAGACGCAG
G-1	1	GTCCCATCCTCGCCATG	CTGCAACATCAACCCCTACAA
G-2	2	CCCTCTCCCTGACTTTTCCTC	AATGAGAAGGTGAAATCTTACC
G-3	3	CGCATCTCCTCTTATTCACAG	AGAGCAGCATTCTCTCCTGAC
G-4	4	GACCCATTTTCTTCCTCCATAG	CCTTGGCACAGGTTTCCTTAC
G-5	5	CAGGTGGTCTTATCCTCCCAG	CTCCAAGCCTATGGAAATGAG
G-6	6	GAGGACAGGGCTGAGTGTGTG	CAGGGCTGGGTGCCCCTGCCA
G-7	7	TCCTGGGTCTCCTCTTTCAGA	CTGGAGCTGGGTCTCACTCAC
G-8	8	GCCCTCTCCCTTGTCCCTCAG	GTTCCCCACTCTGCCCACCG
G-9	9	CGCTTTCTCTCTCCGTTGTAG	GAACAGGGTAGAGGTAACTTAC
G-10	10	TTCACCTGTTGGAATTTTGCAG	GAAGGAAGCCACTCTACTCAC
G-11	11	TTGATGGTGTGGCTTGGCCTG	TACGGGGAGCTTCTGGACATG
G-12	11	GCAGAAAGCCAAGGAGTGGTG	GATCTGTCTTCTCAACCCTGC

Primers are all presented 5'–3'. A,B and G refer to primers for *αENaC*, *βENaC* and *γENaC*, respectively. Primers A13R, A14F, B13R, B14F and R, B15F, G1F and R, G11R and G12F are in coding regions; the remainder are in introns or untranslated regions.

We tested for mutations in the β and γ subunits of ENaC by systematic screening of the exons of these genes. This screening in all PHA1 kindreds revealed a single variant altering the encoded protein in PHA K8 (Fig. 2*c*). This kindred is particularly informative because two unaffected brothers had affected offspring, one of these via union with a second cousin, the other via a spouse of uncertain relationship (Fig. 1). The affected third cousins are homozygous for the same variant, while none of their unaffected siblings or relatives are homozygous for this variant; moreover, this variant is absent in 160 alleles of unrelated healthy subjects. In addition, genotypes of marker loci tightly linked to *βENAC, D16S412, D16S417* and *D16S420* (ref. 23) are all homozygous in these affected subjects but not their unaffected relatives, strongly supporting the identity by descent of the observed mutation.

DNA sequence analysis reveals that this *βENaC* variant substitutes serine for glycine at amino acid 37 of βENaC (Figs 2*c*, 3). While the cytoplasmic amino termini of α, β and γENaC generally show little amino acid sequence identity with one another, it is noteworthy that G37 is in a segment that shows homology among all members of the extended ENaC family ranging from humans to *C. elegans* (Fig. 3*b*)[20,21,29–34]. The functional significance of the G37S variant was assessed by expression of this βENaC variant in conjunction with normal α and γ subunits in *Xenopus* oocytes as described[23–25]. We compared the amiloride-sensitive Na^+ current measured by 2-electrode voltage clamp in oocytes expressing the wild-type ENaC, ENaC containing the mutant β subunit, and channels containing only α and γ subunits (Fig. 4). In order to compare levels of ENaC proteins in oocytes expressing wild-type and mutant channels, subunits were immunoprecipitated from oocyte membranes using specific antibodies to each subunit[35]. The results demonstrated indistinguishable levels of each subunit in oocytes expressing wild type and mutant ENaC (data not shown). Comparison of Na+ currents in oocytes expressing wild-type or mutant ENaCs demonstrate a highly significant reduction in ENaC activity in oocytes expressing the mutant β subunit (40% of wild-type activity, $P < 0.00001$). Oocytes expressing the mutant β subunit, however, still have significantly higher activity than channels expressing no β subunit ($P = 0.00001$), suggesting that this mutation does not result in complete loss of function.

The strong evidence of co-segregation of *βENaC* G37S with PHA in this kindred and the loss of function demonstrated on expression indicates the functional significance of this mutation, revealing genetic heterogeneity of PHA1.

Discussion

The finding of independent mutations in ENaC subunits which co-segregate with PHA1, are homozygous by descent in affected offspring of consanguineous union, and result in diminished ENaC activity constitute proof that mutations in subunits of the epithelial sodium channel cause autosomal recessive PHA1.

We have thus far identified functional mutations in five of seven consanguineous kindreds studied; these mutations occur in either the α or β subunits of ENaC, demonstrating genetic heterogeneity of the trait. In the two kindreds in which mutations so far have not been identified, one case is homozygous for all markers tightly linked to the *β-γENaC* locus — raising the possibility of an undetected mutation; the other case is not homozygous for any loci linked to *β-γENaC*, and is homozygous for only one of two loci tested linked to *αENaC*. This latter subject presented at eight months of age (Table 1), later than typical PHA1 subjects, raising the possibility that this patient might have a somewhat different clinical syndrome. These findings leave open the question of whether additional loci will prove to contribute to the pathogenesis of recessive forms of PHA1.

In contrast to the recessive kindreds described here, some PHA1 kindreds have been reported to show autosomal dominant transmission[7,10,11]. Since ENaC is a multimeric channel, it is possible that some *ENaC* mutations could have dominant negative function, with one defective gene product sufficient to disrupt normal assembly of a large fraction of channels. Further investigation of such kindreds will be required to evaluate this possibility.

Knowledge that PHA1 can result from loss of function mutations in ENaC provides the basis for a

detailed understanding the pathogenesis of this disease. Affected neonates have a primary defect in renal sodium reabsorption mediated via this channel. The consequence is salt wasting, leading to intravascular volume depletion; this results in a marked increase in secretion of renin and consequently aldosterone, in an effort to restore plasma volume. However, because ENaC is defective, renal sodium reabsorption cannot be appropriately increased, resulting in persistent intravascular volume depletion. In addition, sodium reabsorption via ENaC is indirectly coupled to K^+ secretion and H^+ secretion in the distal nephron. As a result, the loss of ENaC function impairs the ability to secrete K^+ and H^+, contributing to hyperkalemia and metabolic acidosis; these features are further worsened by poor perfusion of tissues due to hypovolaemia. In addition to this renal defect, parallel defects altering ENaC function in the colon and sweat glands may further augment salt wasting.

One puzzling clinical feature of PHA1 has been the observation that some affected children 'grow out' of the disease, and eventually they can stop supplemental dietary salt. It has been proposed that such patients usually if not always have autosomal dominant disease[7]. Consistent with this distinction, the subjects reported here all show recessive transmission and all remain dependent on supplemental dietary salt. It will consequently be of interest to determine whether kindreds showing clear-cut dominant transmission or cases with sporadic disease who improve with age harbour mutations in ENaC subunits.

ENaC plays a major role in the removal of salt and water from the alveolar space in the lung[36]. This finding has been emphasized by an *αENaC* knock-out mouse that shows neonatal lethality due to respiratory failure, apparently from an inability to clear fluid from the alveolar space[37]. It consequently is of interest that some PHA1 patients have concurrent respiratory problems[8]; interestingly, patient PHA K3-1, who has a truncated αENaC, has a history of recurrent respiratory infections. Nonetheless, these patients do not have a clinical picture of acute respiratory distress syndrome (ARDS), raising the question of whether the *αENaC* mutations result in complete knock-outs of ENaC activity or whether the portion of αENaC expressed in these patients is sufficient to provide some residual ENaC function *in vivo*, for example by permitting assembly or targeting of other subunits to the apical membrane. Further investigation of these channels and the pulmonary manifestations in these patients will consequently be of interest.

Identification of the molecular basis of this disease provides the means for prenatal genetic testing, which may prove to be of clinical benefit in preventing early death from this disease in kindreds known to be segregating this trait. Affected subjects in all 3 native Saudi PHA1 kindreds are homozygous for the identical variant, almost certainly by descent from a remote common ancestor. Since these kindreds are not known to be related to one another, this finding suggests that this mutation will prove to be a predominant cause of PHA1 in that country.

These findings bring the number of genes in which mutation causes primary renal salt wasting in humans to three: the two genes identified herein, and mutations in the thiazide-sensitive sodium-chloride cotransporter that cause Gitelman's syndrome[38], a disorder characterized by primary renal tubular salt wasting in association with hypokalemic metabolic alkalosis. These findings underscore the primary role of the kidney in regulating intravascular volume and controlling the ionic composition of the vascular space.

Finally, it is noteworthy that mutations in ENaC subunits cause two diseases: loss of function mutations — causing salt wasting and PHA1; and gain of function mutations — causing hypertension and Liddle syndrome. That extreme variation in ENaC activity either augments or reduces sodium reabsorption and blood pressure in humans motivates the further examination of these genes and their regulators in the pathogenesis of human blood pressure variation.

Note added in proof: Strautnieks *et al.* (*Hum. Mol. Genet.* 5, 293–299 (1996)) have recently found linkage of autosomal recessive PHA1 to 16p12.2–13.11 and 12p13.1-ter, regions containing the *α* and *β-γENaC* loci.

Methods

PHA kindreds. Seven PHA1 kindreds containing 10 living affected subjects were ascertained in Saudi Arabia and Israel (Table 1). Two of these kindreds, PHA K10, and PHA K3 have been previously reported[6,7]. All affected subjects were the product of consanguineous union, supporting autosomal recessive transmission (Fig. 1). Most subjects were diagnosed in the neonatal period, and all had clinical features of severe dehydration, hypotension, hyponatraemia, hyperkalemia, and metabolic acidosis despite normal glomerular filtration rate. Plasma renin activity and aldosterone concentrations were markedly elevated. No subjects had signs of abnormal virilization. Multi-organ involvement was documented in PHA K3 (ref. 7). Several index cases had siblings who died with a similar syndrome in the first days of life (Fig. 1). Clinical management consisted of dietary sodium supplementation and use of an ion binding resin or dialysis to reduce potassium levels. The constellation of clinical features permitted definitive diagnosis of PHA1 in all affected subjects.

Genotyping and SSCP. SSCP of all coding exons of *α*, *β*, and *γENaC* was performed using specific primers to amplify exons or exon fragments of exons 150–250 bp from genomic DNA by PCR as described[22]. Forty-three sets of primers were used (Table 2), based on the cloning[29,30] and characterization of the intron–exon organization of each genomic locus (Lu *et al.*, manuscript in preparation). Primers are in introns with the exception of large coding exons in which overlapping primer sets in exons are employed. PCR was performed using specific primers and genomic DNA as template, and products were fractionated on non-denaturing gels as described[38]. Novel SSCP conformers were identified by autoradiography, purified, and subjected to direct DNA sequence analysis as described[38]. In all cases, DNA sequences were confirmed by sequencing both DNA strands. Genotypes of markers closely linked to *α* or *β-γENaC* were determined by polymerase chain reaction using specific primers and genomic DNA as template by standard methods. Markers tightly linked to the *β-γENaC* locus were genotyped as described[22]. Marker loci linked to *αENaC* were identified by use of an RFLP detected by hybridizing rat *αENaC* cDNA to *Taq*I-digested human genomic DNA. Genotyping of 166 individuals in CEPH kindreds revealed linkage of *αENaC* to loci *D12S314* and *D12S93* (lod score of 8.3 for linkage to *D12S314* at a recombination fraction of 4%), with a peak multipoint lod score localizing the gene 2 cM telomeric to *D12S314*. Genomic DNA of subjects from PHA kindreds was prepared from venous blood by standard methods[39].

Construction of rat $\beta ENaC_{37S}$. Serine was substituted for glycine at residue 37 of rat $\beta ENaC$ cDNA by site-directed mutagenesis using a mutagenic primer and PCR. PCR was performed using rat $\beta ENaC$ cDNA[21] as a template, a sense mutagenic primer (5'–CCAACACACACAGCCCCAAAC–3') extending from nucleotide 170 to 190 (codons 33–39) of the $\beta ENaC$ cDNA sequence and altering nucleotide 181 from G to A, and a reverse or antisense primer (5'–CTTGACCTTGGAGTACTGGAAG–3'), extending from nucleotide 378 to 400. After PCR, this product was purified and used as a primer in conjunction with the vector Sp6 primer to direct PCR using the rat $\beta ENaC$ cDNA as a template. The resulting product contained the desired mutation, and was cleaved at a unique *Eco*RI cleavage site in vector sequence and a unique *Sca*I site in codon 146. This fragment was purified and substituted for the corresponding wild-type sequence in the $\beta ENaC$ cDNA. The structure and sequence of the resulting mutant construct was confirmed by DNA sequencing.

Expression studies of normal and mutant ENaC. Complementary RNAs (cRNA) of each α, β and γ subunit were synthesized *in vitro*. Equal saturating concentrations of each subunit cRNA (3 ng total cRNA of each subunit / oocyte) were injected into stage V to VI oocytes as described[25]; cRNAs injected together were normal α, β and γ subunits; normal α and γ subunits plus mutant β subunits; normal α and γ subunits alone with no β subunits. Oocytes from the same frog were injected on the same day with wild-type or mutant constructs. Twenty-four hours after injection, whole-oocyte currents were measured using two-electrode voltage clamp technique in a medium containing: 120 mM NaCl, 2.5 mM KCl, 1.8 mM $CaCl_2$, 10 mM HEPES-NaOH pH 7.2. The expressed ENaC channel activity was assessed by measurement of the amiloride-sensitive Na current, defined as the difference between the Na current recorded at a membrane potential of -100 mV in the absence and presence in the medium of 5 μM amiloride. The results were analysed by T-test.

Comparable expression-levels of the wild-type β subunit and one containing the G37S mutation in *Xenopus* oocytes were ensured by immunoprecipitation. Oocytes injected with cRNAs encoding either α, β and γ, or α and γ or α, βG37S and γ subunits were labeled for 14 h with (^{35}S)methionine, and microsomal membranes were prepared. The three subunits were immunoprecipitated under denaturing conditions with specific antisera[35] and immunoprecipitates were separated on a 8% SDS-polyacrylamide gel.

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