Liddle's syndrome associated with a point mutation in the extracellular domain of the epithelial sodium channel γ subunit

Timo P. Hiltunen[a], Tuula Hannila-Handelberg[a], Noora Petäjäniemi[a], Ilkka Kantola[b], Ilkka Tikkanen[a], Jarmo Virtamo[c], Ivan Gautschi[d], Laurent Schild[d] and Kimmo Kontula[a]

Objective **To characterize novel type of mutations of the epithelial sodium channel (ENaC) β or γ subunits in patients with Liddle's syndrome, an autosomal dominant form of hypertension.**

Patients and methods **DNA samples from two probands with early-onset, treatment-resistant hypertension and suppressed plasma renin activity were initially screened for mutations in the C-terminal exons of the ENaC β or γ subunit genes, using amplification by polymerase chain reaction and direct DNA sequencing.**

Results **Two novel mutations causing Liddle's syndrome were identified. One mutation due to a single nucleotide insertion in the exon 13 of βENaC results in a frameshift at codon 601 and abrogates the PY motif similar to all the previously described ENaC mutations causing Liddle's syndrome. The other mutation, substituting serine for asparagine at codon 530 (Asn530Ser) of the extracellular loop of γENaC subunit, was found in a 25-year-old man with hypertension, hypokalemia, low plasma renin activity and low serum aldosterone levels. Hypertension and hypokalemia favorably responded to amiloride or triamterene administration both in the proband and his affected mother. Expression of the mutant Asn530Ser γENaC subunit in *Xenopus* oocytes demonstrated a two-fold increase in ENaC activity, compared with the wild-type, without a significant change in cell surface expression of ENaC. This suggests that the γENaC Asn530Ser mutation increases the channel open probability, and is consistent with an abnormally high sodium reabsorption in the distal nephron.**

Conclusions **This study describes the first mutation located in the extracellular domain of an ENaC subunit associated with an increased ENaC activity and Liddle's syndrome. *J Hypertens* 20:2383–2390 © 2002 Lippincott Williams & Wilkins.**

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[a]Department of Medicine, University of Helsinki, [b]Department of Medicine, University of Turku, [c]The National Public Health Institute, Helsinki, and [d]Institute of Pharmacology and Toxicology, University of Lausanne, CH-1005 Lausanne, Switzerland.

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Correspondence and requests for reprints to Kimmo Kontula, Professor of Medicine, Department of Medicine, University of Helsinki, 00290 Helsinki, Finland.
Tel/fax: 358 9 471 74013; e-mail: kimmo.kontula@hus.fi

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See editorial commentary page 2331

Introduction

Liddle's syndrome is an autosomal dominant form of hypertension with somewhat variable clinical expression. The clinical hallmarks of this syndrome were initially described in detail by Grant Liddle and coworkers in 1963 [1]: they involve an early onset of hypertension, diminished serum potassium levels, excessive urinary potassium excretion, suppressed plasma renin activity and blunted aldosterone secretion rates. Blood pressure (BP) can be normalized by treating the patients with the epithelial sodium channel blockers amiloride or triamterene, together with a low salt diet [2]. This suggested that hypertension was due to an abnormal sodium reabsorption in the distal nephron.

The amiloride-sensitive epithelial sodium channel (ENaC) is composed of three homologous subunits α, β and γENaC [3]. Genetic analysis of patients with Liddle's syndrome identified mutations in the ENaC β or γ subunits associated with the syndrome [4–6]. The mutations causing Liddle's syndrome remove or change the sequence of a conserved proline-rich motif (PPPxY sequence) called PY motif and present in the C-terminal ends of the β or γ subunit of ENaC [7,8]. This PY motif is important for appropriate regulation of the number of ENaC channels at the cell surface. The disruption of the PY motif in Liddle's syndrome leads to a retention of active ENaC at the cell surface causing increased sodium absorption in the distal

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nephron [9]. In addition to the ENaC gain of function mutations in Liddle's syndrome, ENaC loss of function mutations have been found in pseudohypoaldosteronism type I, a salt-loosing nephropathy [10]. In addition a number of amino acid polymorphisms have been identified in β and γ ENaC, but these genetic variants have not been unequivocally shown to cause increased channel activity nor high blood pressure [11,12].

Here, we describe two novel mutations associated with Liddle's syndrome in the Finnish population. One mutation deletes the PY motif in the C-terminus of βENaC and is consistent with all the ENaC mutations previously described in Liddle's syndrome. The second mutation is the first naturally occurring mutation in the extracellular domain of an ENaC subunit in a patient with Liddle's syndrome. This mutation causes ENaC gain of function, consistent with an increased sodium reabsorption in the distal nephron.

Methods

Probands with suspected Liddle's syndrome

Two patients clinically examined at the Hypertension Outward Patient Wards of the University Hospitals of Helsinki and Turku were suspected to have Liddle's syndrome because of early-onset, treatment-resistant hypertension, spontaneous hypokalemia and suppressed plasma renin activity (Table 1). They and their closest living relatives were invited to give blood samples for molecular studies of their ENaC subunit genes.

Patients with hypertension

The initial cohort was composed of all consecutive hypertensive patients ($n = 615$) admitted to the Hypertension Outpatient Ward, Helsinki University Central Hospital, between 1992–96. The causes of admittance included moderate-to-severe hypertension, suspicion of secondary forms of hypertension, or hypertension resistant to treatment with combination of at least two drugs. A letter with a request to donate a blood sample for genetic studies on hypertension was sent to those 598 individuals whose addresses became available. A total of 399 (183 males, 216 females; 67% of those contacted) individuals responded and visited the Hypertension Outpatient Ward of the Helsinki University Central Hospital, thus comprising our material of patients with moderate-to-severe hypertension.

Control individuals

To obtain a random sample from the background population, genomic DNA was extracted from 291 randomly selected healthy blood donors (148 males, 143 females) aged 40–50 years (mean, 45 years) visiting the Finnish Red Cross Blood Transfusion Service. Their residences represent the same capital area from where the hypertensive patients originated. In addition, another series of control population, designated as males with low–normal BP, were selected from individuals participating in the Alpha-Tocopherol, Beta Carotene (ATBC) cancer prevention trial [13]. A total of 29 133 smoking males, aged 50–69 years and living in the south-western part of Finland, relatively well matched with the origin of the hypertensive patients, were initially recruited for this cancer prevention study, comparing alpha-tocopherol, beta-carotene, and the two combined, against placebo, during a 5–8 year follow-up period. Patients with a history of previous myocardial infarction were excluded at the initiation of the ATBC study. During progression of the study, whole blood samples for DNA analysis were collected, and were available from 70% of the original study participants. We picked up the available blood samples from all ATBC participants fulfilling the following criteria: no known hypertensive disorder; no antihypertensive drugs ever in use; systolic blood pressure (SBP) and diastolic blood pressure (DBP) values $\leqslant 128$ and $\leqslant 84$ mmHg, respectively, at each blood pressure measurements repeated five times at 1-year intervals. The resulting material consisted of 175 men, whose mean ($\pm$ SD) SBP and DBP were 114.9 ± 5.4 and 73.7 ± 4.3 mmHg, respectively, at these measurements.

This study was approved by the Ethical Review Committee of the Helsinki University Central Hospital,

Table 1 **Clinical and biochemical characteristics of the two probands with mutant ENaC subunits**

Variable	Proband 1	Proband 2	Normal values
Mutation	βENaC Thr601→Frmshft	γENaC Asn530Ser	–
Sex/age (year)	Female/34	Male/28	–
Body mass index (kg/m^2)	18	20.5	20–25
Age at onset of hypertension	19	25	–
Blood pressure (mmHg)			
no treatment	190/120	180/120	–
with amiloride/triamterene	130/95	120/88	–
Serum potassium (mmol/l)	2.4	3.0	3.7–5.3
Serum sodium (mmol/l)	143	149	136–144
Plasma renin activity (μg/l per h)	0.6	0.1	2–5
Serum aldosterone (pmol/l)	ND	158	260–1000
24 h urinary excretion of aldosterone (nmol)	0.55	1.0	< 50

Plasma was used for potassium measurement (normal values, 3.3–4.8 mmol/l) in proband 1. ND, not determined.

and all patients and controls gave their informed consent.

DNA analysis

Genomic DNA was extracted from peripheral venous blood using standard techniques. The coding sequences of the C-terminal exons of β and γENaC of the two probands were amplified by polymerase chain reaction (PCR) using the following primers: 5′-CAAG AATGTGTGGCCTGAGC-3′ and 5′-ACAGTCTTGG CTGCTCAGTG-3′ (βENaC, fragment size 480 bp) and 5′-GAGGTTCCTCTTGATGGTGT-3′ and 5′-TGACTAGATCTGTCTTCTCA-3′ (γENaC, fragment size 448 bp). The volume of PCRs was 25 μl, and they contained 50 mmol/l KCl, 10 mmol/l Tris-HCl (pH 8.3), 1.5 mmol/l $MgCl_2$, 200 μmol/l of each of the dNTPs, 1 U of Taq polymerase (AmpliTaq Gold; Perkin Elmer, Foster City, California, USA), 0.6 (βENaC) or 0.7 (γENaC) μmol/l each of the primers, and 100 ng genomic DNA. The PCRs were carried out in a PTC-225 DNA Engine Tetrad thermal cycler (MJ Research Inc, Watertown, Massachusetts, USA) using a program of 95°C for 9 min in the first cycle, followed by 96°C for 1 min, 69 (βENaC) or 58 (γENaC) °C for 1 min, and 72°C for 1 min for 35 cycles, and final extension for 30 min at 72°C. Six microlitre aliquots of the PCR products were subjected to cycle sequencing from both strands in a volume of 13 μl containing 2 μl of Termination Ready Reaction Mix (AmpliTaq DNA polymerase FS, dNTPs and fluorescein donor dye-ddNTPs; Perkin Elmer) and 2 pmol of one of the PCR primers. The temperature program for sequencing involved 25 cycles with steps of 96°C for 15 s and 60°C for 4 min. The sequencing reactions were run through a vertical polyacrylamide gel according to manufacturer's instructions (ABI Prism 377; Perkin Elmer).

Screening for the two mutations among the 399 hypertensive patients, 291 healthy blood donors and 175 men from the ATBC Study was carried using PCR (see above) followed by restriction enzyme digestion and agarose/polyacrylamide gel electrophoresis. For the βENaC Thr601→Frmshft mutation, an aliquot (10 μl) of the PCR reaction was digested with 1 U of PflMI (New England Biolabs, Beverly, Massachusetts, USA) for 16 h at 37 °C. For the γENaC Asn530Ser mutation, 4 U of AluI (New England Biolabs) was used. The specific fragment sizes after digestions were 480 bp for the wild type and 306 and 175 bp for the βENaC Thr601→Frmshft mutation, and 72 bp for the wild type and 59 and 13 bp for the γENaC Asn530Ser mutation.

Functional expression of the γAsn530Ser ENaC

The γAsn530Ser mutation was introduced in the human γENaC cDNA as described previously using a mutagenic cassette flanked by unique SfiI and AspI restriction sites [14]. Healthy stage V and VI Xenopus oocytes were injected with mRNAs of α and β human ENaC wild type (wt) together with either γENaC wt or γENaC Asn530Ser mutant at a final concentration of 10 ng. Electrophysiological measurements were taken at 16–20 h after injection. ENaC activity was assessed by measurement of the amiloride-sensitive current (I_{Na} in μA) recorded at −100 mV with a two-electrode voltage clamp amplifier (TEV-200, DaganCorp., Minneapolis, Minnesota, USA) in a standard solution containing 110 mmol/l NaCl, 1.8 mmol/l $CaCl_2$, 10 mmol/l HEPES-NaOH, pH 7.35. The amiloride concentration used was 5 μmol/l in the bath solution. Six batches of oocytes were obtained from different *Xenopus* frogs in which five oocytes were tested for each αβγENaC wt or αβγENaC Asn530Ser mutant.

The FLAG reporter octapeptide was introduced into the extracellular domain of α and γ subunits, in order to be recognized by the extracellular anti-FLAG M_2 (M_2Ab) mouse monoclonal antibody (Kodak). M_2Ab was iodinated as described by Firsov *et al.* [9] to a specific activity of $5–10^{17}$ cpm/mol. For the binding assay, oocytes were transferred into a 2 ml Eppendorf tube containing MBS of the Na^+ activity used during the channel expression supplemented with 10% heat-inactivated calf serum, and incubated for 30 min on ice. The binding was started upon addition of 12 nmol/l ^{125}I-M_2Ab (final concentration) in a volume of 5–6 μl/oocyte. After 1 h of incubation on ice, the oocytes were washed eight times with 1 ml MBS supplemented with 5% heat-inactivated calf serum, and then transferred individually into tubes for γ counting containing 250 μl of the same solution. The samples were counted and the same oocytes were kept for subsequent measurement of the whole-cell current INa. Non-specific binding was determined from parallel assays on non-injected oocytes.

Results

Family with the βENaC Thr601→Frmshft mutation

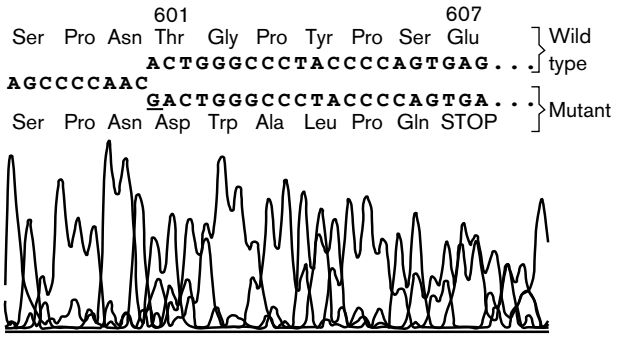
The proband (proband 1) was a female born in 1966 who was normotensive until the age of 19. In 1985, elevated blood pressure levels in the range of 190/115–120 mmHg were measured. Examinations at the university hospital revealed low serum potassium concentration on repeated occasions and suppressed plasma renin activity (Table 1). There was no evidence of renovascular hypertension or catecholamine overproduction. She was initially treated with the β-adrenergic antagonist metoprolol and hydralazine without response. One year later, triamterene 50 mg daily was added to the medication, with subsequent blood pressure values around 130/95 mmHg and an increase of serum potassium concentration to 3.8 mmol/l.

A heterozygous G insertion after the 256th nucleotide of exon 13 of the βENaC gene was detected in proband

1 (Fig. 1). The insertion occurs in the codon 601 coding for threonine localized in the intracellular domain of the βENaC subunit. This mutation causes a translational frameshift and is predicted to create a premature stop codon at position 607, thus deleting the PY motif of βENaC.

The proband's two children and her mother were carriers of the βENaC Thr601→Frmshft mutation, while her brother and father were not (Fig. 2). Hypertension was present in the mother. After treatment with amiloride 10 mg daily for 1 month, she became normotensive as judged by ambulatory blood pressure recording; the mean daytime blood pressure levels diminished from 174/90 to 141/82 mmHg and nighttime blood pressure levels from 152/74 to 130/73 mmHg. In addition, serum potassium concentration was normalized from 3.3 to 4.0 mmol/l.

Fig. 1

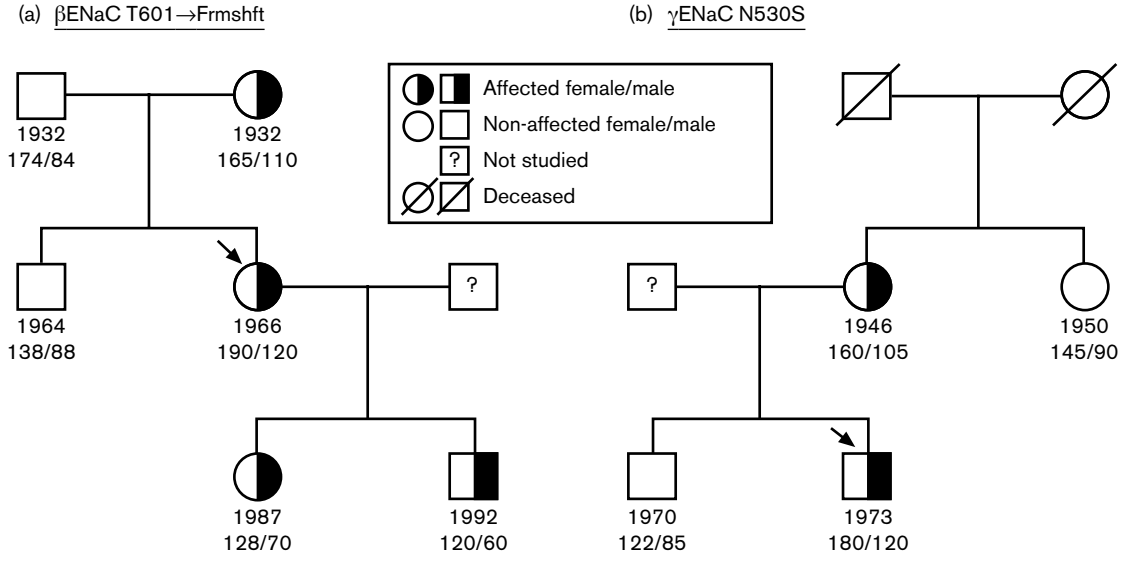


Sequence analysis of the βENaC Thr601→Frmshft mutation present in heterozygous form in proband 1. Insertion of G (underlined) after codon 600 is predicted to cause a translational frameshift and a premature stop codon at residue 607.

Family with the γENaC Asn530Ser mutation

The proband 2 born in 1973 was a healthy man until age of 25 years when he developed hypertension with blood pressure values in the range of 160–180/110–120 mmHg (Table 1). Subsequent examinations revealed low serum potassium concentrations (3.0 and 3.2 mmol/l) on several occasions. He was referred to the Hypertension Outpatient Clinic for suspected secondary hypertension. A captopril renogram was normal, whereas cardiac ultrasound examination revealed left ventricular hypertrophy with septal thickness of 12–15 mm. Serum aldosterone concentration was low, plasma renin activity was suppressed and hypokalemia was persistently present (Table 1). The patient was initially treated with amlodipine 5 to 10 mg daily, but with no response. Triamterene 50 mg daily was then combined, with a reduction of mean daytime blood pressure from 140/95 to 126/83 mmHg and an increase of serum potassium level to 3.7 mmol/l. Because of subsequent abdominal discomfort, amiloride 5 mg daily was substituted for triamterene, with maintenance of a good antihypertensive effect and further increase of serum potassium level to 4.7 mmol/l.

Fig. 2



The pedigrees of the two families with mutations of the ENaC subunits. (a) βENaC T601→Frmshft mutation, (b) γENaC N530S mutation. The affected/non-affected status (shown by symbols) is defined by the results of the DNA analysis. The year of birth and the blood pressure level (systolic/diastolic, mmHg) at the initiation of clinical studies are indicated for each individual, when available. The probands are shown by arrows.

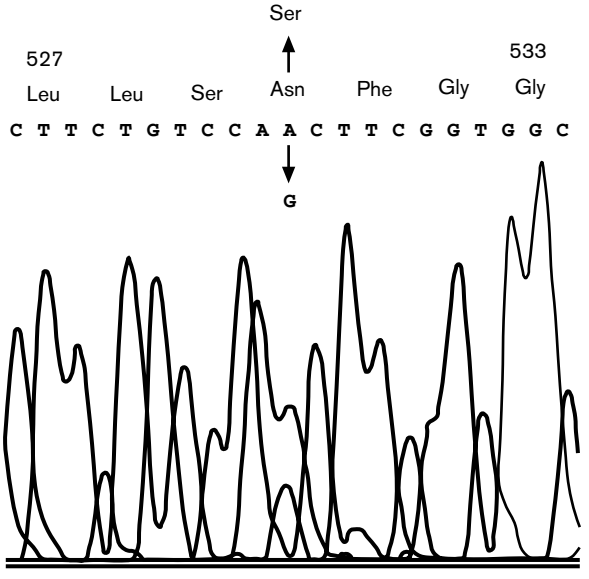
Upon screening of the DNA sample of the proband 2 for β or γENaC mutations, a heterozygous A to G missense mutation located 20 nucleotides after the start of exon 13 of the SCNN1G gene coding for the γENaC subunit was identified (Fig. 3). The mutation is predicted to substitute serine for asparagine at codon 530 (Asn530Ser). This residue is located in the extracellular domain of γENaC, 18 amino acid upstream of the putative second transmembrane domain.

All available family members were examined for the occurrence of the γENaC Asn530Ser mutation. The proband's mother with a history of hypertension from the age of 40 years carried the mutation, but his aunt (mother's sister) and normotensive brother were negative (Fig. 2). The proband's mother had low-normal serum potassium level (3.9 mmol/l) and low plasma renin activity (0.2 μg/l per h). During atenolol 50 mg daily, her blood pressure remained at 140/90 mmHg, but she became normotensive (110/80 mmHg) with a combination of atenolol 50 mg, hydrochlorothiazide 25 mg and amiloride 2.5 mg daily. The proband's father was not available for examination.

Screening for the occurrence of the βENaC Thr601→Frmshft and γENaC Asn530Ser mutations in Finnish subjects with and without hypertension

The βENaC Thr601→Frmshft mutation was not detected in any of the 399 patients with moderate to severe primary hypertension. Its occurrence was not screened for in the other groups. Except the proband 2 himself, there were no additional carriers of the γENaC Asn530Ser mutation among the 399 patients with moderate-to-severe hypertension. In contrast, one of the 291 healthy blood donors and one of the 175 control male subjects with low-normal blood pressure carried the mutation. Unfortunately, they were unavailable for more detailed clinical or laboratory examinations.

Fig. 3



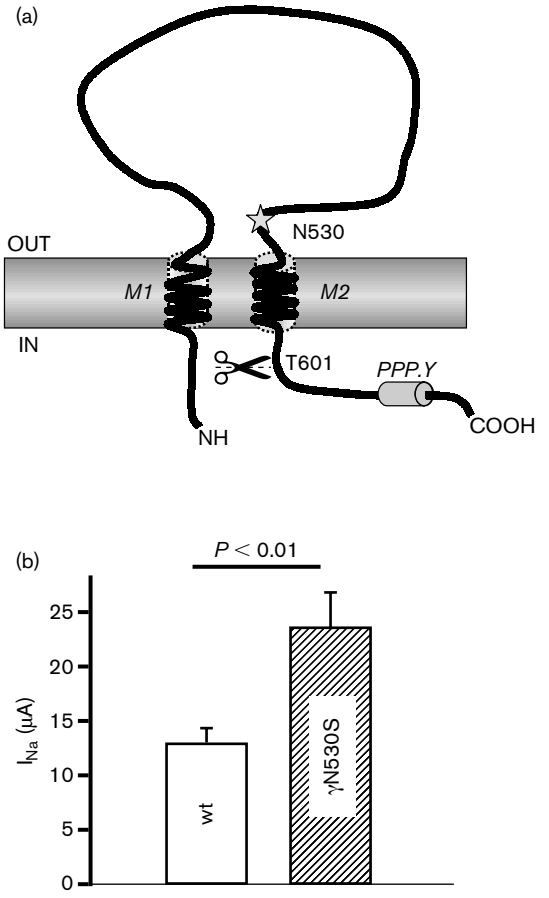
Sequence analysis of the γENaC Asn530Ser mutation present in heterozygous form in proband 2. The mutation substitutes serine for asparagine at codon 530.

Functional expression of the γENaC Asn530Ser mutation in Xenopus oocytes

The G nucleotide insertion at codon 601 of βENaC causes a frameshift mutation which abrogates the PY motif in the C-terminus of the βENaC subunit (Fig. 4a). So far all the non-sense, missense or frameshift mutations, which have been identified to cause Liddle's syndrome, mutate or delete the PY motif in the β or γENaC subunits. The functional consequences of these mutations in the PY motif have been extensively investigated and shown to increase the amiloride-sensitive current (I_{Na}) up to two- to four-fold over the wild-type ENaC [5,6,8,15]. We have therefore not repeated these experiments to show the increased I_{Na} of the βENaC Thr601→Frmshft mutation. In contrast to all the previously described Liddle's syndrome mutations that target the intracellular PY motif, the Asn530Ser missense mutation identified in the γENaC subunit of proband 2 is located in a conserved sequence of the extracellular loop that precedes the second transmembrane domain of the ENaC subunit. The amiloride-sensitive current measured in oocytes expressing the αβγAsn530Ser mutant ENaC was 23.9 ± 4.3 μA compared with 12.3 ± 1.8 μA (mean ± SE, $P = 0.008$) in oocytes expressing wt ENaC subunits (Fig. 4b). This highly significant 2.0 ± 0.2-fold (mean ± SE, $P = 0.0003$) higher amiloride-sensitive current expressed by the αβγAsn530Ser mutant ENaC indicates a higher channel activity at the cell surface. Other characteristics of ENaC function, such as ionic selectivity and sensitivity to amiloride, remained unchanged in the αβγAsn530Ser mutant (data not shown).

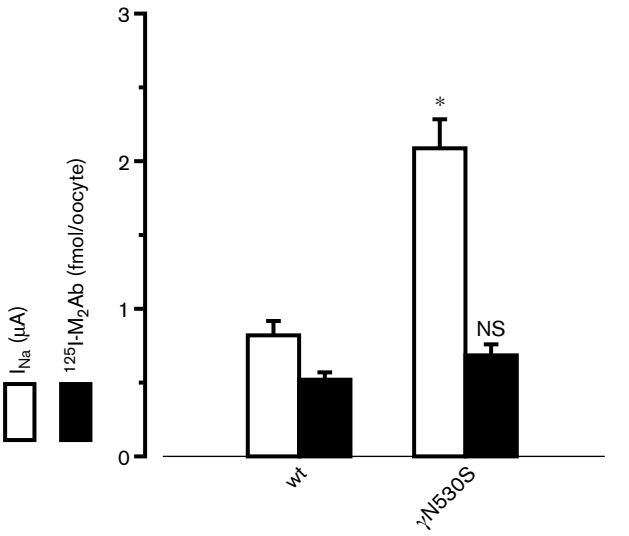
The higher amiloride-sensitive INa expressed in oocytes injected with the αβγAsn530Ser mutant ENaC can be due either to a higher density of ENaC molecules at the cell surface, or to a larger flux of Na^+ ions through the channel. To distinguish between these two possibilities, we compared the expression of wt ENaC and αβγAsn530Ser mutant ENaC at the cell surface using specific recognition of a FLAG epitope introduced in the extracellular domain of α and β ENaC by iodinated anti-FLAG antibodies (^{125}I-M_2Ab). In oocytes expressing ENaC, the specific binding of ^{125}I-M_2Ab reflects the number of channels at the cell surface, and INa measurements in the same oocytes the flux of Na^+ ions through the channel. The results obtained in these experiments show that in oocytes

Fig. 4



Location and functional expression of the γENaC Asn530Ser mutation. (a) Membrane topology of the γ or βENaC subunit with intracytoplasmic amino and carboxy termini, 2 transmembrane segments (M1 and M2) and a large extracellular loop. ☆ symbol locates the asparagine residue at codon 530 (N530) and the ✂ symbol the threonine residue at codon 601 (T601). (b) Amiloride-sensitive current (I_{Na}) expressed by ENaC wt and ENaC γAsn530Ser (γN530S) mutant (mean ± SE, $n = 40$ oocytes, obtained from five different frogs). Statistical significance was obtained from unpaired Student *t*-test.

Fig. 5



ENaC expression at the cell surface and channel activity of wt and αβγAsn530Ser (γN530S) mutant ENaC. Measurements of INa (open bars) and specific binding of ^{125}I-M$_2$Ab (filled bars) were performed in each expression of αβγ ENaC wt ($n = 35$) and αβγAsn530Ser ($n = 26$). Bars represent the mean ± SE. $^*P < 0.001$, NS, not significant.

expressing the αβγAsn530Ser mutant ENaC and a 2-fold higher INa, compared with oocytes expressing wt ENaC, surface expression as measured by specific binding of ^{125}I-M$_2$Ab was not significantly different (Fig. 5). These experiments indicate that a higher flux of Na^+ ions through the channel, instead of a higher number of ENaC expressed at the cell surface, accounts for the two-fold higher activity of αβγAsn530Ser mutant ENaC. Since the αβγAsn530Ser mutation did not change single channel conductance (data not shown), we conclude from these experiments that the αβγAsn530Ser mutation increases by two-fold the channel open probability.

Discussion

This paper describes two hitherto unreported mutations of the subunits of the human amiloride-sensitive epithelial sodium channel, one affecting the β and the other the γ subunit of ENaC. In both cases, the carrier status of the mutation was associated with clinical features compatible with Liddle's syndrome, including hypertension responsive to amiloride or triamterene diuretics, hypokalemia, suppressed plasma renin activity and very low urinary aldosterone excretion. There are no previous reports on the occurrence of molecularly established cases of Liddle's syndrome in the Finnish population, and the prevalence of this disease among the Finns is unknown.

At least nine different mutations of the βENaC subunit have been described in patients with Liddle's syndrome [4–6,16–20]. Non-sense or missense βENaC mutations underlying Liddle's syndrome described so far either remove or change the intracytoplasmic conserved PPPxY sequence in the βENaC C-terminus end. The βENaC Thr601→Frmshft mutation also deletes this PY motif similar to the other non-sense mutations reported previously, including the Arg597→Frmshft (deletion of C) and Thr594→Frmshft (insertion of C). These mutations invariably increase channel activity at the cell surface resulting in a higher rate of Na^+ transport in Xenopus oocytes where these ENaC mutants have been expressed [9]. Similar non-sense mutations deleting the C-terminus of γENaC have identical effects on channel function [5]. Therefore the functional consequences of the βENaC Thr601→

Frmshft mutation in increasing channel activity seems quite obvious and did not require functional analysis of this ENaC mutation.

Proline-rich motifs such as the PY motif are generally involved in protein–protein interactions. The PY motif of βENaC was shown to interact with a cytosolic protein called Nedd4 [21]. In the *Xenopus* oocyte expression system Nedd4 protein is an ubiquitin ligase which controls the number of active ENaC channels at the cell surface [22]; ENaC ubiquitination, a post-translational modification which adds small ubiquitin proteins on the N-terminus of ENaC, represents a signal for rapid endocytosis and degradation of ENaC in the cell. Disruption of this PY motif impairs ENaC retrieval from the plasma membrane and causes retention of active ENaC channel at the cell surface. This is the likely mechanism responsible for the increased Na^+ reabsorption in the distal nephron in patients with Liddle's syndrome [23]. The proband 1 with the βENaC Thr601→Frmshft mutation had early-onset hypertension, very low serum potassium level and documented suppression of the renin–aldosterone axis, and the patient responded favorably to triamterene treatment. Hypertension and hypokalemia in her mother with the same mutation were similarly amenable to treatment with amiloride. The βENaC Thr601→Frmshft mutation appears to be a rare cause of hypertension in Finns as no additional cases were detected upon screening of 399 individuals with moderate-to-severe hypertension.

Since the initial description of the γENaC Trp574Stop mutation in a patient with Liddle's syndrome, only one additional γENaC mutation (Trp576Stop) associated with this syndrome has been detected [5,20]. Other mutations have been identified in γENaC but none associated with hypertension [12]. The γAsn530Ser mutation described in the present study is the first mutation in the extracellular γENaC domain and associated with Liddle's syndrome. The scanty pedigree data do not permit an unequivocal conclusion on the cosegregation of the mutation and the clinical syndrome, but the relationship between a carrier status of the γAsn530Ser mutation and low renin, low aldosterone hypertension associated with hypokalemia and amiloride responsiveness is highly suggestive.

The amiloride-sensitive current expressed by the Asn530Ser mutant channel is approximately two-fold higher compared with the ENaC wild-type channel (Fig. 4), consistent with a channel gain of function. For comparison, the PY motif mutations causing Liddle's syndrome usually result in a more than two-fold increase in amiloride-sensitive current when expressed in Xenopus oocytes, suggesting that mutations of the PY motif are more efficient than the Asn530Ser mutation in increasing ENaC activity. A γENaC variant allele with Asn530Lys and Cys581Arg substitutions has been identified in one patient suffering from diabetic nephropathy and hypertension [24]. The γAsn530Lys mutation resulted in a two-fold increase in channel activity when expressed in *Xenopus* oocytes [25].

The molecular mechanism underlying the increase in channel activity caused by the γENaC Asn530Ser mutation differs from mutations in the PY motif of β and γ ENaC causing Liddle's syndrome. The increase in channel activity of αβγAsn530Ser was observed without any significant change in the surface expression compared with ENaC wt, nor a change in single channel conductance. Since the macroscopic current INa measured in oocytes expressing ENaC is the product of the number of channels expressed at the surface times the ENaC conductance and open probability, our results indicate that the αβγAsn530Ser increases the probability of the channel being open.

Recently, it has been reported that substitution of the Asn530 by a cysteine in γENaC and its covalent modification by sulfhydryl reagents rapidly stimulates channel activity [25]. This effect and its time course are highly suggestive of an activation/opening of ENaC at the cell surface by modification of the substituted cysteine at position 530 by sulfhydryl reagents. Residues at the corresponding positions in the α and β subunits that have been substituted with cysteine and subsequently modified by sulfhydryl reagents show similar effects on channel function. The region encompassing these residues in the extracellular loop is likely important for the control of channel openings and closures. Mutations of ENaC-related genes in *C. elegans* named degenerins are responsible for touch insensitivity and cause neuronal cell swelling and subsequent death. It was postulated that the degenerative phenotype of these mutations in *C. elegans* degenerins is due to the constitutive activation of a mechanosensitive cation channel involved in touch transduction [26]. Importantly, one degenerin mutation causing this degenerative phenotype is located in the extracellular domain at a position corresponding to Ser529, one residue upstream of the Asn530 in the γENaC subunit. These observations together with our results strongly suggest that the γAsn530Ser mutation in the extracellular vestibule of the channel leads to a hyperactive ENaC channel due to changes in the channel gating leading to an increase in the channel open probability. This mechanism underlying the gain of function of γAsn530Ser ENaC mutant would lead to abnormally high sodium reabsorption in the distal nephrons of our Liddle patient.

In addition to the proband 2 and his hypertensive mother, the γENaC Asn530Ser mutation was detected

in one middle-aged man, originally recruited to the ATBC Cancer Prevention Study and selected from its participants on the basis of repeatedly low-normal blood pressure levels, as well as in an apparently healthy blood donor whose blood pressure status was unknown. Unfortunately, our original study protocol was not designed to disclose health information of the control individuals, thus preventing more detailed examinations of these two mutation carriers or their relatives. The occurrence of the γENaC Asn530Ser mutation in a person with persistently normal blood pressure levels suggests that the γENaC Asn530Ser gain-of-function mutation may not be sufficient alone to cause hypertension despite an increase in distal sodium reabsorption. Indeed, other genes or environmental factors may protect individuals with an abnormally high ENaC activity from developing hypertension. These protective factors could include other genes controlling sodium reabsorption or dietary habits favoring low salt intake.

In conclusion, we report two mutations of the human epithelial sodium channel subunits associated with Liddle's syndrome in the Finns. One of these mutations (γAsn530Ser) is located in a conserved domain of the extracellular domain of γENaC preceding the second putative transmembrane segment and leads to a hyperactive channel likely due to an increased open probability. This mutation represents the first gain-of-function mutation associated with Liddle's syndrome that does not alter the PY motif in the C-terminus of β and γENaC and identifies another domain in the extracellular loop important for the control of ENaC activity. These data also indicate that a search for mutations of this molecular region of the γENaC subunit should be included in strategies aimed at screening for the occurrence of Liddle's syndrome in the population.

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