Liddle Disease Caused by a Missense Mutation of β Subunit of the Epithelial Sodium Channel Gene

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Abstract

Mutations in β or γ subunit of the epithelial sodium channel (ENaC) have been found to cause a hereditary form of human hypertension, Liddle syndrome. Most of the mutations reported are either nonsense mutations or frame shift mutations which would truncate the cytoplasmic carboxyl terminus of the β or γ subunits of the channel, suggesting that these domains are important for the normal regulation of this channel. We sequenced ENaC in a family with Liddle syndrome and found a missense mutation in β subunit which predicts substitution of Tyr by His at codon 618, 2 bp downstream from a missense mutation (P616L) that has been reported recently. Presence of this mutation correlates with the clinical manifestations (hypertension, hypokalemia, suppressed aldosterone secretion) in this kindred. Functional expression studies in the *Xenopus* oocytes revealed constitutive activation of the Y618H mutant indistinguishable from that observed for the deletion mutant (R564stop) identified in the original pedigree of Liddle. Our data suggest that the region between Pro616 and Tyr618 is critically important for regulation of ENaC activity. (*J. Clin. Invest.* 1996. 97:1780–1784.) Key words: hypertension • hereditary disease • pseudoaldosteronism • kidney • amiloride

Introduction

The aldosterone-regulated and amiloride-sensitive epithelial Na^+ channel (ENaC)[1] is composed of at least three protein subunits (encoded by three different genes) that share ~ 35% sequence homology with each other (1, 2). Each subunit (α, β, γ) has a large extracellular loop and two membrane spanning domains with short cytoplasmic amino and carboxyl termini. Functional expression studies in the *Xenopus* oocyte system have shown that the α subunit alone can induce Na^+ channel activity whereas βrENaC and γrENaC are unable by themselves to induce an amiloride-sensitive Na^+ current; if, however, the β and γ subunits are coexpressed with the α subunit, then amiloride-sensitive Na^+ current is greatly enhanced (2).

Liddle syndrome is an hereditary form of human hypertension with autosomal dominant inheritance. Its clinical features are characterized by severe hypertension, hypokalemia, suppressed secretion of aldosterone, and a clear-cut response to inhibitors of sodium transport in the distal nephron, but not to mineralocorticoid antagonists (3, 4). These characteristics had led to a hypothesis that the hypertension in this disorder is caused by excessive reabsorption of Na^+ in the distal nephron and that mutations in Na^+ channel subunits may be an underlying mechanism if these mutations cause constitutive activation of ENaC activity. Clinical observations (4), indicating that renal transplantation completely corrected abnormalities observed in this disorder, further supported the speculation that this disorder is caused by an intrinsic renal defect in the regulation of Na^+ channel activity. This hypothesis could be directly examined once the subunits of the ENaC were cloned. In 1994 Shimkets et al. (5) reported mutations in the β subunits of ENaC (βENaC) in five kindreds of Liddle syndrome including Liddle's original one. Subsequently, a mutation in the γ subunit was also reported (6). All of these mutations were either stop codons or frame shifts which result in the truncation of carboxyl termini of the β or γ subunit of ENaC, indicating that some part of the COOH-terminal cytoplasmic domain of both β and γ subunits is important for normal regulation of ENaC. Recently, a missense mutation within the distal proline-rich region (P616L) of the carboxyl terminus of the β subunit has been reported to cause Liddle syndrome (7).

We report herein a family with Liddle syndrome whose abnormality is caused by another missense mutation in the carboxyl terminus of the β subunit of ENaC. The functional significance of this abnormality was confirmed in the *Xenopus* oocyte expression system. The analysis of missense mutations should provide new insights into the regulatory mechanisms of ENaC, and are more informative in this regard than truncating nonsense mutations.

Methods

DNA sequencing. Genomic DNA was prepared from peripheral leukocytes of the family members by standard methods. PCR primers for amplification of βENaC were designed according to Shimkets et

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1. *Abbreviations used in this paper:* ENaC, epithelial sodium channel; RELP, restriction enzyme length polymorphism; Y618H, Tyr to His mutation at codon 618.

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al. (5). The final exon of human βENaC was amplified by PCR using outer primers. 100 ng of the genomic DNA was made up to 50 μl of a PCR mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 400 nM of each PCR primer (outer primer), 200 μM each dNTP, 0.01% gelatin, and 0.5 U of *Taq* polymerase (AmpliTaq; Cetus Corp., Emeryville, CA). PCR consisted of 35 cycles of denaturing at 94°C for 1 min, annealing at 55°C for 1 min, and polymerization at 72°C for 1 min. 1 μl of the first PCR products was transferred to the second PCR reaction with nested 5′- and 3′- primers. The composition of the PCR mixture was the same as the first PCR mixture except for the primers. M13-forward and M13-reverse primer sequences were attached to the 5′terminus of the 5′- and 3′- nested primers, respectively, to facilitate direct sequencing by an automated DNA sequencer (model 373S; Applied Biosystems Japan, Chiba, Japan). The PCR products were sequenced with the PRISM dye termination kit (Applied Biosystems Japan), according to the manufacturer's instructions, on both strands. The sequencing primer was M13-forward primers for the sense strand and M13-reverse primer for the antisense strand.

Nucleotide sequences of the PCR and sequencing primers were as follows: M13-forward: 5′-TGTAAAACGACGGCCAGT-3′; M13-reverse: 5′-CAGGAAACAGCTATGACC-3′; 5′ outer primer: 5′-ATCGTCTGGCTGCTCTCGAATCTG-3′; 3′ outer primer: 5′-TTAGATGGCATCACCCTCACTGTC-3′; 5′ nested primer: 5′-TGTAAAACGACGGCCAGTTGGCCAGTTTGGCTTCTGGATG-3′ (M13-forward sequence is underlined); and 3′ nested primer: 5′-CAGGAAACAGCTATGACCCGTCCAGCGGCTGCAGACGCAG-3′ (M13-reverse sequence is underlined).

Restriction enzyme length polymorphism (RELP) analysis of the final exon of human βENaC gene. Because the direct sequencing of the final exon of human βENaC gene revealed that the variant allele from the patients contained the unique mutation in codon 618 creating the NlaIII restriction enzyme site, the RELP analysis was performed on the family members. 5 μl of the first PCR products was digested with 10 U of NlaIII and was electrophoresed on 2% agarose gel in 1× TBE. The PCR product of 381 bp was cleaved into two fragments of 319 and 62 bp if it contained the mutation at codon 618.

Functional expression study in Xenopus oocytes. The Tyr to His mutation at codon 618 of the β subunit of rat ENaC (rENaC) was generated by site-directed mutagenesis using a three-step PCR protocol. The first step used a 5′ mutagenic primer, a 3′ primer carrying an unrelated anchoring sequence, and the βrENaC cDNA as template to amplify a cDNA fragment with the desired mutation. In the second step the PCR product was elongated. The third step amplified the mutated cDNA using a 3′ primer which primes the synthesis of DNA containing the unrelated sequence. The final PCR product carrying the desired mutation was digested and ligated into the βrENaC cDNA wild-type at the Sac1 and Spe1 cleavage sites. The mutation was verified by sequencing.

cRNA of α, β, and γ subunits was synthesized in vitro, and 5–8 ng cRNA encoding the three α, β, γ subunits was injected into stage V and VI oocytes. 20–24 h after injection, amiloride-sensitive Na^+ current expressed by the injected oocytes was measured using the two electrode voltage-clamp technique. The amiloride-sensitive current was measured at a membrane potential of −100 mV in the presence or absence of 5 μM amiloride in the bathing medium. The bathing medium contained (mM): NaCl 115, KCl 5, $CaCl_2$ 1.8, Hepes-NaOH 10 (pH 7.2). A mean amiloride-sensitive current was determined in five to eight oocytes originating from the same animal, and injected either with the βrENaC wild-type, the β subunit deletion mutant at position R564 (βR564stop), or the βY618H mutant, together with αrENaC and γrENaC. The experiment was repeated eight times with oocytes from different animals. Statistical significance of the results was found using Student's *t* test.

Results

Clinical features of the family. This family affected with Liddle syndrome has been described in part in the Japanese literature in 1976 (8). In that report, clinical characteristics of two young brothers 17 and 21 yr old, who both had severe hypertension, were carefully examined. Each subject had low serum potassium level, completely suppressed plasma renin activity, and low urinary excretion of aldosterone. Both subjects did not respond to a therapy with spironolactone, but responded to triamterene with normalization of hypertension, hypokalemia, and renin and aldosterone levels. Based on these findings, these two patients were diagnosed as having Liddle syndrome and were followed for several years before being lost to follow-up. Recently, we relocated these two patients and found that one has chronic renal failure caused by nephrosclerosis and receives hemodialysis, and the other regularly takes antihypertensive medications. We analyzed genomic DNA of these patients and found a mutation in βENaC (see below).

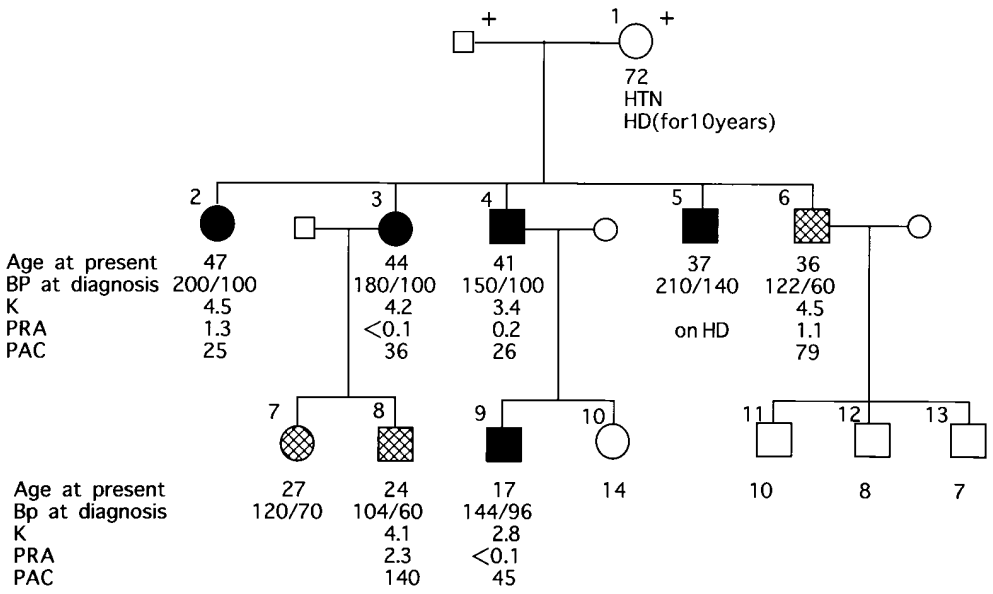

Figure 1. Pedigree of a family with Liddle disease. Individuals with the Y618H mutation are shown as filled symbols; individuals lacking the mutation are shown as hatched symbols; unexamined individuals are shown as open symbols. Below the symbols, in descending order, are shown the ages of subjects (in years as of 1995), blood pressure at diagnosis (in mmHg), the serum potassium level (meq/liter), the plasma renin activity (ng/ml/h, normal range, 0.3–2.9), and the plasma aldosterone concentration (pg/ml, normal range, 36–240). Case 1 died at age 72, had hypertension (*HTN*), and had been treated by hemodialysis (*HD*).

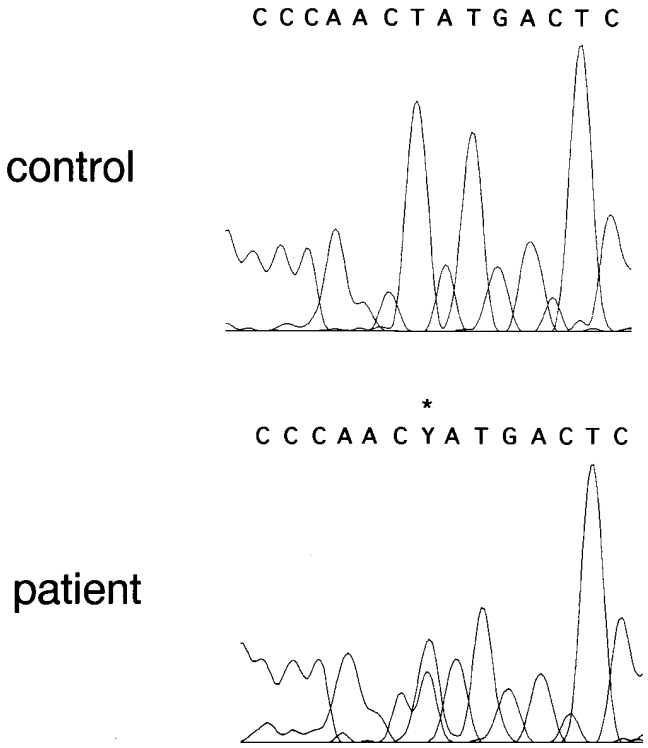

Figure 2. Identification of the mutation by direct sequencing of the PCR-amplified β subunit DNA. As indicated by asterisk, both T and C signals were observed in an affected subject, while only T signals were observed in a normal subject.

We then expanded our study to the other members of this family. Fig. 1 shows this kindred; cases 4 and 5 are the index cases. The ages of family members at present (1995), blood pressure at diagnosis, and most recent blood chemistry data (the serum potassium level, plasma renin activity, and plasma aldosterone concentration) are shown in Fig. 1. The patients took their regular antihypertensive medications (Ca^{2+} antagonists) when we collected blood samples. The mother of the index cases was known to have hypertension and had been treated with regular hemodialysis starting at 62 yr old for chronic renal failure probably due to nephrosclerosis. She died in 1992 at 72 yr old for unknown reasons. No further clinical data are available, but her clinical course was strongly compatible with Liddle syndrome. In addition to the mother, three additional family members were clinically diagnosed as having Liddle syndrome (see Fig. 1). Cases 2 and 3 have a history of hypertension and are receiving regular antihypertensive medication. Although they did not show hypokalemia (4.5 and 4.2 meq/liter), they had a low plasma aldosterone concentration. This is consistent with a recent observation in Liddle's original pedigree that hypokalemia is not a universal finding among affected members (4).

DNA sequencing and RELP analysis. To detect a mutation in βENaC, a 381-bp segment encoding the cytoplasmic carboxyl terminus of βENaC (codon 513– 638) was amplified and directly sequenced. As shown in Fig. 2, two signals for T and C were observed in an affected member instead of single T observed in a normal subject, indicating the presence of a mutation T→C in one allele with the other allele being intact. This point mutation predicts a missense mutation of Tyr 618 to His (Y618H). The presence of this mutation could be easily detected by RELP analysis because this mutation creates a new recognition site for NlaIII. PCR products of the family members were digested by NlaIII and electrophoresed (Fig. 3). In the DNA from affected members, a smaller DNA band at 319 bp which represents a truncation by digestion was observed as well as the 381-bp normal allele. On the other hand, this band was not observed for the unaffected members. The result of this genetic analysis is also shown in Fig. 1. Genetic identification (marked by filled circles or squares in Fig. 1) nicely matched with the clinical diagnosis. This result strongly suggests that the Y618H mutation causes Liddle syndrome in this family. The mode of inheritance is clearly autosomal dominant; the male to male transmission between cases 4 and 9 is evidence against X-linked inheritance. We similarly amplified and sequenced carboxyl termini of α (codon 571–669, references 9 and 10) and γ (codon 556–650, reference 6) ENaC and could not find any other mutations in these family members (not shown).

Functional expression in Xenopus oocytes. Previous expression studies of the rENaC have shown that deletions in the carboxyl terminus of the β or γ subunit (6, 11) as well as a single point mutation in the β subunit (7) cause Liddle syndrome and induce an increase in the amiloride-sensitive current expressed by the mutant channel in the oocyte system, indicative of constitutive activation of the ENaC. To assess the functional consequences of the Y618H mutation, we expressed the mutant channel in *Xenopus* oocytes and compared the resulting amiloride-sensitive current with that obtained for the rENaC

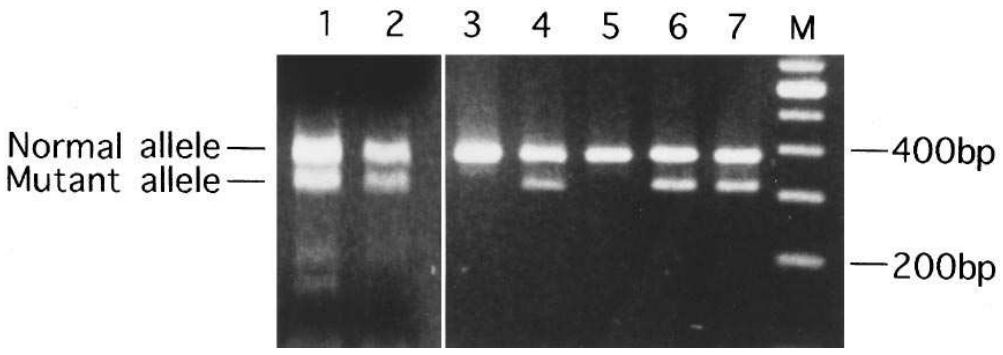

Figure 3. RELP analysis of the PCR-amplified β subunit DNA from the family members. The PCR product of 381 bp was cleaved into two fragments of 319 and 62 bp if it contained the mutation at codon 618. *Lanes 1*, *2*, *4*, *6*, and *7* shows DNA from affected members (cases 4, 9, 5, 2, and 3, respectively), and *lanes 3* and *5* are DNA from an unrelated normal subject and an unaffected member of the kindred (case 6), respectively. *M*, size marker for DNA.

wild-type and the β deletion mutant (R564stop) found in the original kindred described by Liddle. The experiments in Fig. 4 show that the β subunit R564 deletion mutant compared with rENaC wild-type displays a 3.0±0.4-fold increase (P = 0.002) in the amiloride-sensitive current, consistent with the results obtained in previous experiments (11). The Y618H mutant exhibited a 2.6±0.4-fold (P = 0.006) greater amiloride-sensitive current than the rENaC wild-type, a value indistinguishable from that obtained for the deletion mutant (R564 stop). These results indicate that the Y618H mutation like the R564 deletion mutation results in an increased channel activity and a stimulation of Na^+ transport. These experiments show at the molecular level that the Y618H mutation can account for the constitutive channel activation and is the proximate cause of the low-aldosterone hypertension in this kindred.

Discussion

Our previous report described two brothers affected by Liddle syndrome (8), but further studies on other members of the family had not been performed. The present study, performed 19 yr later, includes clinical and genetic examinations which clearly show that other siblings of the index brothers are also affected by the disease, which is transmitted to the next generation. Although not confirmed, the inheritance may come from the mother of the index brothers (case 1). The father of the index brothers did not have hypertension and died 3 yr ago at 74 yr old from a malignant tumor of urinary bladder. It is noteworthy that the clinical and genetic diagnoses matched nicely in the present analysis of the extended kindred. PCR amplification followed by RELP enabled us to obtain a rapid and reliable diagnosis of this family, although this specific RELP result is relevant only to the Y618H mutation. This ease of diagnosis was in sharp contrast to the difficulty we had 19 yr ago with these patients, providing an example of the powerful contribution of molecular biology to clinical medicine.

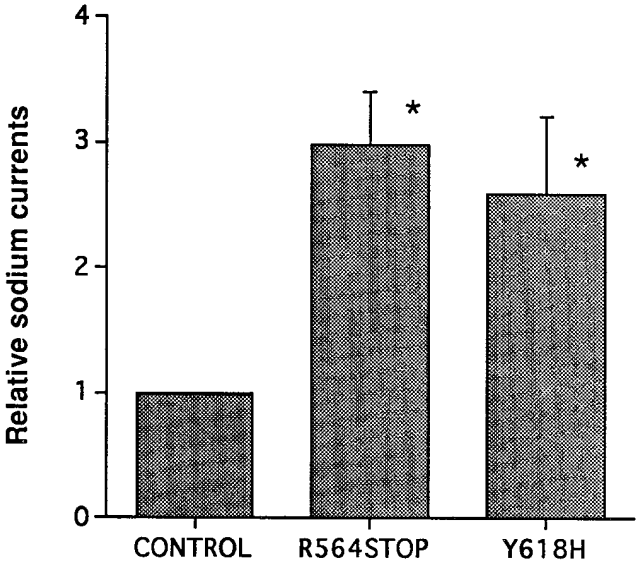

Figure 4. Relative increase in amiloride-sensitive current expressed by βR564stop mutant and βY618H mutant in the oocytes. Amiloride-sensitive current measured at −100 mV was normalized for the values obtained with αβγ ENaC wild-type, and bars show mean±SEM of eight experiments. Absolute amiloride-sensitive current values were 5.07±1.53 μA for αβγ, 13.8±4.7 μA for R564stop mutant, and 13.8±5.06 μA for Y618H mutant. P values are 0.002 and 0.006 for R564stop mutant and Y618H mutant, respectively.

The previously reported mutations which cause Liddle syndrome and constitutive activation of ENaC delete ~ 90% of the amino acid sequence constituting the carboxyl terminus of β or γ subunits (5, 6). Among the possible target sequences in the β and γ carboxyl termini involved in this phenotype, a proline-rich domain and a putative protein kinase C phosphorylation site are conserved among the two subunits. Very recently, Hansson et al. (7) reported another kindred affected by Liddle syndrome, and analysis of the genes encoding ENaC in affected patients revealed a missense mutation substituting Pro by Leu at codon 616 of the β subunit. This mutation (P616L) was shown to increase the amiloride-sensitive Na^+ current when expressed in *Xenopus* oocytes, suggesting that a point mutation or a deletion of the distal proline-rich region in the carboxyl terminus of the β subunit is responsible for the constitutive activation of channel activity observed in Liddle disease.

In this study we show that another point mutation substituting the adjacent Tyr to His at codon 618 also causes Liddle disease and enhances channel activity when the mutant is expressed in *Xenopus* oocytes. The Y618H mutation was functionally equivalent to the deletion mutation at R564 identified in the original Liddle kindred (Fig. 4). Analysis of missense mutations instead of truncation or frame shift mutations may help to better define the functional domains for the regulation of channel function. The P616L and Y618H mutants reveal a target sequence responsible for Liddle disease that encompasses a proline-rich region and a tyrosine that are highly conserved among the β and γ subunit isoforms. These missense mutations seem to exclude the putative protein kinase C phosphorylation site (S620-R622) in the carboxyl terminus of the β subunit as a target sequence for mutations causing Liddle syndrome. The proline-rich domains present in the carboxyl termini of ENaC subunits share a SH3 binding motif, suggesting that these regions might be important for interaction with SH3 domain–containing proteins (12, 13). SH3 domains are found in many proteins involved in tyrosine kinase signaling and proteins such as cytoskeletal components (14, 15). Recently, a similar proline-rich domain of the α subunit carboxyl terminus has been shown to bind SH3 domains of spectrin in vitro (16), suggesting that protein–protein interactions may be involved in the regulation of channel activity (11). It is tempting to speculate that phosphorylation of Tyr 618 by tyrosine kinase may modulate the binding of the proline-rich domain with its binding partner, thus regulating the channel activity of ENaC. Modulation of epithelial Na^+ transport by many signaling systems, including tyrosine kinase, has been demonstrated (17, 18). Further studies are needed to directly address this possibility.

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References

1. Canessa, C.M., J. Horisberger, and B.C. Rossier. 1993. Epithelial sodium channel related to proteins involved in neurodegeneration. *Nature (Lond.).* 361:467–470.

2. Canessa, C.M., L. Schild, G. Buell, B. Thorens, I. Gautschi, J. Horisberger, and B.C. Rossier. 1994. Amiloride-sensitive epithelial Na channel is made of three homologous subunits. *Nature (Lond.).* 367:463–467.

3. Liddle, G.W., T. Bledsoe, and W.S. Coppage. 1963. A familial renal disorder simulating primary aldosteronism but with negligible aldosterone secretion. *Trans. Am. Assoc. Physiol.* 76:199–213.

4. Botero-Velez, M., J.J. Curtis, and D.G. Warnock. 1994. Liddle's syndrome revisited: a disorder of sodium reabsorption in the distal tubule. *N. Engl. J. Med.* 330:178–181.

5. Shimkets, R.A., D.G. Warnock, C.M. Bositis, C. Nelson-Williams, J.H. Hansson, M. Schambelan, J.R. Gill, S. Ulick, R.V. Milora, J.W. Findling, et al. 1994. Liddle's syndrome: heritable human hypertension caused by mutations in the β subunit of the epithelial sodium channel. *Cell.* 79:407–414.

6. Hansson, J.H., C. Nelson-Williams, H. Suzuki, L. Schild, R. Shimkets, Y. Lu, C. Canessa, T. Iwasaki, B. Rossier, and R.P. Lifton. 1995. Hypertension caused by a truncated epithelial sodium channel γ subunit: genetic heterogeneity of Liddle syndrome. *Nat. Genet.* 11:76–82.

7. Hansson, J.H., L. Schild, Y. Lu, T. Wilson, R. Shimkets, C. Nelson-Williams, B.C. Rossier, and R.P. Lifton. 1995. A de novo missense mutation of the β subunit of the epithelial sodium channel causes hypertension and Liddle's syndrome, identifying a proline-rich segment critical for regulation of channel activity. *Proc. Natl. Acad. Sci. USA.* 92:11495–11499.

8. Matsui, N., M. Matsuda, T. Shiigai, S. Koshikawa, and Y. Yoshikawa. 1976. Two brothers affected by Liddle's syndrome. *Saishinigaku.* 31:1005–1009. (Published in Japanese)

9. Voilley, N., E. Lingueglia, G. Champigny, M.-G. Mattei, R. Waldmann, M. Lazdunski, and P. Barbry. 1994. The lung amiloride-sensitive Na channel: biophysical properties, pharmacology, ontogenesis, and molecular cloning. *Proc. Natl. Acad. Sci. USA.* 91:247–251.

10. McDonald, F.J., P.M. Snyder, P.B. McCray, and M.J. Welsh. 1994. Cloning, expression, and tissue distribution of a human amiloride-sensitive Na channel. *Am. J. Physiol. (Lung Cell. Mol. Physiol.).* 266:L728–L734.

11. Schild, L., C.M. Canessa, R.A. Shimhetes, I. Gautschi, R.P. Lifton, and B. Rossier. 1995. A mutation in the epithelial sodium channel causing Liddle disease increases channel activity in the Xenopus laevis oocyte expression system. *Proc. Natl. Acad. Sci. USA.* 92:5699–5703.

12. Ren, R., B.J. Mayer, P. Cicchetti, and D. Baltimore. 1993. Identification of a ten-amino acid proline-rich SH3 binding site. *Science (Wash. DC).* 259: 1157–1161.

13. Pawson, T. 1995. Protein modules and signaling networks. *Nature (Lond.).* 343:573–580.

14. Drubin, D.G., J. Mulholland, Z.M. Zhu, and D. Botstein. 1990. Homology of a yeast actin-binding protein to signal transduction proteins and myosin-I. *Nature (Lond.).* 343:288–290.

15. Cohen, G.B., R. Ren, and D. Baltimore. 1995. Modular binding domains in signal transduction proteins. *Cell.* 80:237–248.

16. Rotin, D., D. Bar-Sagi, H. O'Brodovich, J. Merilainen, V.P. Lehto, C.M. Canessa, B.C. Rossier, and G.P. Downey. 1994. An SH3 binding region in the epithelial Na channel (αrENaC) mediates its localization at the apical membrane. *EMBO (Eur. Mol. Biol. Organ.) J.* 13:4440–4450.

17. Matsumoto, P.S., A. Ohara, P. Duchatella, and D.C. Eaton. 1993. Tyrosine kinase regulates epithelial sodium transport in A6 cells. *Am. J. Physiol.* 264:C246–C250.

18. Rossier, C.B., C.M. Canessa, L. Schild, and J.D. Horisberger. 1994. Epithelial sodium channels. *Curr. Opin. Nephrol. Hypertens.* 3:487–496.