Hum Genet (2003) 112 : 91–97
DOI 10.1007/s00439-002-0855-7

ORIGINAL INVESTIGATION

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Functional polymorphisms in the mineralocorticoid receptor and amirolide-sensitive sodium channel genes in a patient with sporadic pseudohypoaldosteronism

Received: 21 June 2002 / Accepted: 17 September 2002 / Published online: 25 October 2002

Abstract Pseudohypoaldosteronism (PHA) is characterized by urinary salt-wasting in infancy resulting from a congenital resistance to aldosterone involving the genes for the mineralocorticoid receptor (MR) and the amiloride-sensitive sodium channel (ENaC). We identified, in a Japanese patient with sporadic PHA, three homozygous substitutions in the MR gene: $G^{215}\rightarrow C^{215}$, $A^{754}\rightarrow G^{754}$ ($Ile^{180}\rightarrow Val^{180}$), $C^{938}\rightarrow T^{938}$ ($Ala^{241}\rightarrow Val^{241}$), which had previously been reported to occur in healthy populations. Luciferase activities induced by MR with either $G^{215}\rightarrow C^{215}$, $Ile^{180}\rightarrow Val^{180}$, or $Ala^{241}\rightarrow Val^{241}$ substitution were significantly lower than those for wild-type MR with aldosterone at concentrations ranging from 10^{-11} to 10^{-9} M, 10^{-8} M, or 10^{-11} to 10^{-6} M, respectively. A homozygous A→G substitution of the donor splice site of αENaC intron 4 was found in the patient. The corresponding cDNA exhibited a normal structure, suggesting that this substitution does not alter the splice. The results suggest that each of three MR polymorphisms identified in our patient is functionally and structurally heterogeneous. We hypothesize that two or more "functional" polymorphisms, any of which exhibits only slight effects on MR or ENaC function and is alone incapable causing PHA, may in the right allelic combination induce the negative salt-conservation characteristic of PHA.

Introduction

Pseudohypoaldosteronism (PHA), a congenital condition that presents in infancy with urinary salt-wasting and failure to thrive, has been reported in over 70 patients (Cheek and Perry 1958; Speiser et al. 1986), approximately one fifth of whom have the familial form. All patients possess renal tubular unresponsiveness to aldosterone, and some also exhibit multiple mineralocorticoid target tissue involvement, including the sweat and salivary glands and the colonic epithelium. In kindreds with PHA, both an autosomal dominant and a recessive form of genetic transmission have been reported (reviewed by Speiser et al. 1986; Kuhnle 1997).

The mechanism by which aldosterone regulates sodium transport in target tissues involves both the mineralocorticoid receptor (MR) and amiloride-sensitive sodium channel (ENaC). ENaC is expressed in the apical membrane with MR being expressed in the cytosol or nucleus in the epithelial cells of the distal convoluted tubule in the kidney and other tissues involved in salt conservation, including the sweat and salivary glands and the colonic epithelium.

Mutations in ENaC or MR have been reported in autosomal recessive kindreds with PHA and a sporadic Japanese patient (Chang et al. 1996; Strautnieks et al. 1996; Adachi et al. 2001), or autosomal dominant kindreds with PHA (Geller et al. 1998; Tajima et al. 2000), respectively. One MR allele is knocked out in affected members in the kindred described by Geller et al. (1998) suggesting that full expression of both MR alleles is required for normal salt conservation. However, patients with PHA, especially with sporadic PHA, do not always exhibit abnormalities in the genes for ENaC or MR (Arai et al. 1994, 1995, 1999; Corvol and Funder 1994; Komesaroff et al. 1994; Zennaro et al. 1994). Polymorphisms in the genes for MR or ENaC are found not only in patients with PHA, but are also normal features of the population at large. The effects of these polymorphisms on MR and ENaC functions have been little studied, and the pathophysiology of this syndrome thus remains to be elucidated.

To begin to clarify the pathophysiology of this disorder, we examined the gene sequences for MR and the α, β, and γ subunits of ENaC in a Japanese patient with sporadic PHA and her parents. We then carried out in vitro

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functional studies on the MR gene variants containing the identified substitutions.

Subject and methods

Subject

The patient was 3-year-old female born, by spontaneous vaginal delivery, at 35 weeks and 5 days with a body weight of 2004 g. She presented at 8 days of age with abdominal distention, severe hyperkalemia (serum K^+: 7.8–7.9 mmol/l; normal range at this age: 3.0–7.0 mmol/l) and hyponatremia (serum Na^+: 128–134 mmol/l; normal range at this age: 134–146 mmol/l) caused by urinary sodium loss (6.8 mmol/day). Her blood pressure was 68/38 mmHg. Blood gas measurements revealed metabolic acidosis with respiratory compensation, whereas her renal and adrenal functions were normal. The plasma aldosterone concentration of the patient at 28 days of age (1623.6 ng/dl; normal range at this age: 38.2±21.0 ng/dl) was extremely high, and plasma renin activity (10.8 ng/ml per hour; normal range at this age: 5.7±3.0 ng/ml per hour) was elevated.

A diagnosis of pseudohypoaldosteronism was made following an extensive evaluation at the Department of Pediatrics, Yamanashi Medical School. Her urinary sodium excretion was 13.5 mmol/day when sodium intake was 2 g/day, whereas it was 18.2 mmol/day when sodium intake was restricted to 0.43 g/day at the age of 9 months. Fludrocortisone (Florinef) was administered at 0.5 mg/day with sodium intake being restricted to 0.43 g/day. Her urinary sodium excretion before and after administration of fludrocortisone with sodium intake restriction were 18.2 mmol/day and 18.1 mmo l/day, respectively. Her urinary sodium excretion did not alter according to the amount of sodium intake or administration of fludrocortisone, suggesting that she had aldosterone resistance. The sodium concentrations in the saliva and sweat of the patient were within the normal range (10 mmol/l and 34 mmol/l, respectively), which suggested that aldosterone resistance was present only in the kidney. She presented poor weight gain when aged 10–20 days, before starting sodium replacement. She was the only patient with PHA, and there was no consanguinity in her family. Both parents were healthy and exhibited no abnormalities in electrolyte levels, plasma renin activity, or plasma aldosterone concentrations.

Following the diagnosis, the patient was maintained, until 2 years of age, on high doses of salt. After cessation of salt-replacement therapy, the patient maintained a preference for salty food, suggesting that the patient still took a large amount of salt.

Sequencing of genomic DNA

After obtaining informed parental consent for DNA analysis, genomic DNA was isolated by standard techniques from white blood cells collected from both the patient and her parents. Polymerase chain reaction (PCR) of genomic DNA fragments was performed in a final volume of 25 µl containing 100 ng genomic DNA, 20 pmol sense and antisense oligonucleotide primers, 200 µM each dNTP, 10 mM TRIS-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.625 U *Taq* Polymerase (Takara Shuzo, Shiga, Japan). Following an initial denaturation step of 5 min at 95°C, 30 cycles of amplification were carried out in a Gen Amp PCR System 9600 (PE Biosystems, Foster City, Calif.). Each cycle consisted of 30 s denaturation at 94°C, 30 s annealing at 52°C, and 45 s extension at 72°C. The extension time for the last cycle was 4 min. Primer sets for the amplification of α, β, and γENaC subunit genes were as described by Chang et al. (1996). The MR gene primers were designed to amplify each exon except exon 2 (Table 1). The genomic DNA fragments of α, β, and γENaC and MR genes were sequenced directly by Big Dye terminator cycle sequencing with an ABI 377 automated DNA sequencer (PE Biosystems).

Sequencing of αENaC cDNA

Isogen (Nippon Gene, Toyama, Japan) was used to extract total RNA from peripheral lymphocytes by the acid-guanidinium thiocyanate-phenol-chloroform method (Chomczynski and Sacchi 1987). Contaminating genomic DNA was removed by mixing 15 U RNase-free DNase I (Roche Diagnostic, Mannheim, Germany) with 2 µg total RNA in 15 mM TRIS-HCl (pH 8.3), 75 mM KCl, 2 mM each dNTP, 30 U RNase inhibitor. The solution was incubated 30 min at 37°C followed by 5 min at 75°C to inactivate the DNase. For first-strand cDNA synthesis, 7.5 U reverse transcriptase from murine leukemia virus (Roche molecular systems, Branchburg, N.J.), 3.75 µM random hexamer, 3.75 µM oligo d(T) primer and 7.5 mM $MgCl_2$ were added to the solution of total RNA, treated with DNase, and incubated 1 h at 42°C. PCR was performed as described above in a final volume of 25 µl containing 1 µl of the products of the first-strand cDNA under the same condition as described above. The sense primer was 5'TAGCGTGGCCTCCGGCTTGC3' at cDNA positions 694–713 located in exon 3; the antisense primer was 5'GTAGTCACAGTACTCCACGT3' at position 1404–1423 located in exon 8.

Construction of plasmids pRShMR-C^{215}, pRShMR-Val^{180}, and pRShMR-Val^{241}

Expression vectors of human MR (hMR) including the substitution of $G^{215}\rightarrow C^{215}$ (pRShMR-C^{215}), $Ile^{180}\rightarrow Val^{180}$ (pRShMR-Val^{180}), or $Ala^{241}\rightarrow Val^{241}$ (pRShMR-Val^{241}) were constructed. pRShMR-C^{215} was constructed by replacing an *Eag*I/*Sac*I fragment including the hMR nucleotide 203–728 of plasmid pRShMR (Arizza et al. 1987) with the $G^{215}\rightarrow C^{215}$ nucleotide substitution. With pRShMR as a template, PCR was carried out as described above with a sense primer that included both the C^{215} substitution and an *Eag*I restriction enzyme site (5'CCCGGCCGAGGCAGCGATGGAGACCA3' at cDNA positions 201–226) and an antisense primer that included a *Sac*I restriction enzyme site (5'CCGAGCTCCCAG-

Table 1 Primers used to amplify the exons of MR

Exon	5' Primer	3' Primer
1b	5'-AGGGACGCCGCTGTCAACGC-3'	5'-GAGGCCCGGCCGCCGCCGCT-3'
1a	5'-CTCTTCGGGCCAATAGGAAG-3'	5'-TATCTGGACATGTGACCCGC-3'
2	5'-GCTAGAAATCGTTGCTCATA-3'	5'-GCAAATGCAATTTTAGAATG-3'[a]
	5'-TCTGACTCTGGGAGCTCCGT-3'[a]	5'-GCCATTGTTAAAAGCCCTAT-3'[a]
	5'-GGAAGTAGAGAGTGCCATCT-3'[a]	5'-ACTGGCCAGCTTAATATTGT-3'
3	5'-TGAATTCTTGTATCGAACCA-3'	5'-GCAGCATGTTAAATGTGCCT-3'
4	5'-GAGATTTCAAGGCAGGAAAG-3'	5'-CTAGTGTACTCTATTTTAAC-3'
5	5'-GTTCCTATTGACATAACAAG-3'	5'-ACCAGTGTGAGTCAGCATAT-3'
6	5'-GAATTGATTTGAGTCTGTCA-3'	5'-TGGAATGTACTTAAATGCTA-3'
7	5'-TCTGCTCCATGTTGAAGCCA-3'	5'-CAGTCTGGGTTTGATAATGA-3'
8	5'-GAGATTTAGTATCTAACCTC-3'	5'-TACCTGCCAAGATGCTAAAG-3'
9	5'-TCTAAATGTTGTCATTTGAG-3'	5'-GATCCAGGGTCGGAAAGGAG-3'

[a]Primers targeting exon sequences; remaining primers targeting intron sequences

AGTCAGACAT3' at cDNA positions 709–728). The PCR product and wild-type pRShMR were digested with restriction endonucleases *Eag*I and *Sac*I (New England Biolabs, Beverly, Mass.) in a separate reaction. After purification with standard techniques, the digested fragments were ligated with T_4 ligase (Takara Shuzo).

pRShMR-Val^{180} and pRShMR-Val^{241} were constructed by replacing a *Sac*I/*Bam*H1 fragment of hMR corresponding to nucleotides 723–1267 with sequences containing one of the nucleotide substitutions ($A^{754}\rightarrow G^{754}$ or $C^{938}\rightarrow T^{938}$). To create the *Sac*I/*Bam*H1 fragment with the desired substitutions, a *Sac*I/*Bam*H1 fragment of hMR was subcloned into multicloning sites between T7 and SP6 promoters of plasmid pGEM-3Z (Promega, Madison, Wis.). Two PCRs were first carried out with pGEM-3Z and the *Sac*I/*Bam*H1 fragment as a template. One set of PCR was performed with the T7 promoter primer and an antisense primer containing either the $A^{754}\rightarrow G^{754}$ or $C^{938}\rightarrow T^{938}$ substitution (5'CTTTTAACAACGGCGCGCATG3' at cDNA positions 744–764 or 5'TCGATTTTCAACATTAGGGGA3' at cDNA positions 928–948, respectively). Another reaction was performed with a sense primer containing either the $A^{754}\rightarrow G^{754}$ or $C^{938}\rightarrow T^{938}$ substitution (5'CATGCGCGCCGTTGTTAAAAG3' at cDNA positions 744–764 or 5'TCCCCTAATGTTGAAAATCGA3' at cDNA positions 928–948, respectively) and the SP6 promoter primer. A second PCR was carried out with these PCR fragments as templates for the T7 and SP6 promoter primers. These PCR fragments were, in turn, digested with restriction endonucleases SacI and *Bam*H1 (Roche Diagnostics). To cut the *Bam*H1/*Ava*I and the *Ava*I/SacI fragments, pRShMR was digested with restriction endonucleases *Bam*H1 and *Ava*I or with *Ava*I and *Sac*I (Roche Diagnostics). After purification, the three digested fragments were ligated with T_4 ligase (Takara Shuzo).

The entire cDNA insert, including the ligation sites of the recombinant plasmids, was sequenced and confirmed to possess substitutions of either $G^{215}\rightarrow C^{215}$, $A^{754}\rightarrow G^{754}$, or $C^{938}\rightarrow T^{938}$ with no other base changes. The recombinant plasmids were designated as pRShMR-C^{215}, pRShMR-Val^{180}, and pRShMR-Val^{241}, respectively.

In vitro function of mutated hMRs (pRShMR-C^{215}, pRShMR-Val^{180}, pRShMR-Val^{241})

Cos7 cells were grown in Dulbecco's modified Eagle's medium (GIBCO Invitrogen Corporation, Grand Island, N.Y.) supplemented with 10% fetal bovine serum at 37°C in an atmosphere of 5% CO_2. Twenty-four hours before transfection, cells were removed from their culture flasks by trypsinization, resuspended in supplemented medium, and plated in 24-well plates at a concentration of 10^5 cells/well. Wild-type hMR and mutated hMRs (0.1 μg) were respectively co-transfected in the presence of lipofectin reagent (Life Technologies, Gaithersburg, Md.) with 0.5 μg reporter plasmid (pL-TRluc) having a mouse mammary tumor virus luciferase promoter in which glucocorticoid response elements were coupled to the luciferase gene (Bresnick et al. 1990). Another luciferase reporter plasmid, pRL-TK (0.1 μg; Promega), was employed as an internal standard for transfection efficiency. Cells were incubated in either normal charcoal-stripped medium or charcoal-stripped medium with aldosterone (Sigma-Aldrich, St. Louis, Mo.) at concentrations ranging from 10^{-11} to 10^{-6} M. Forty-eight hours after transfection, cells were washed twice with phosphate-buffered saline and incubated for 15 min at room temperature with a lysis buffer (Promega). The luciferase activity of cell lysates was measured by the dual-luciferase assay method (Promega). Experiments were carried out in 9–11 plates for each aldosterone concentration. Luciferase activity data were expressed as mean ± SE of the mean and analyzed by the Mann Whitney U test; $P<0.05$ was considered to be statistically significant.

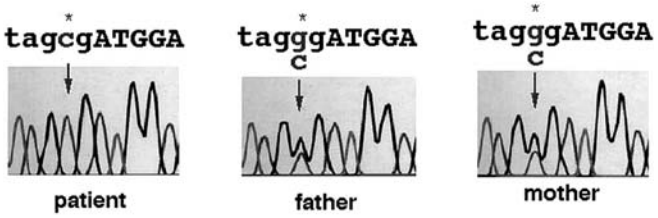

Fig. 1 Mutation in the Kozak sequence of the mineralocorticoid receptor (MR). *Asterisk*/*arrow* Homozygous G→C substitution in the MR Kozak sequence of the patient (*left panel*); the father (*middle panel*) and mother (*right panel*) are heterozygous

Results

Sequencing of MR and ENaC genomic DNA and αENaC cDNA

We identified a homozygous substitution ($G^{215}\rightarrow C^{215}$) at position –2 preceding the start codon in exon 2, which was located in the so-called Kozak sequence surrounding the translation initiation site (Fig. 1, Table 2). In the MR gene of the patient, a homozygous conservative amino acid substitution $A^{754}\rightarrow G^{754}$ (Ile$^{180}\rightarrow$Val^{180}) and a homozygous nonconservative amino acid substitution $C^{938}\rightarrow T^{938}$ (Ala$^{241}\rightarrow$Val^{241}) were also found in exon 2 (Table 2). Both parents were heterozygous for the $G^{215}\rightarrow C^{215}$ and Ile$^{180}\rightarrow$Val^{180} substitutions and homozygous for the Ala$^{241}\rightarrow$Val^{241} substitution.

The patient was homozygous for an A→G substitution at the second position of the donor splice site of intron 4 of the αENaC gene, a substitution for which her parents were both heterozygous (Fig. 2, Table 2). To determine whether this nucleotide substitution altered the splice site and consequent skipping of exon 4, we amplified the cDNA fragment of αENaC from exon 3 to exon 8 for both the patient and her parents. The structure of the αENaC cDNA was normal in all cases (Fig. 2). A homozygous nonconservative amino acid substitution $C^{1006}\rightarrow G^{1006}$ (Pro$^{336}\rightarrow$Ala^{336}) in βENaC and three homozygous amino acid

Table 2 Results of sequencing of the MR and the ENaC genes of the patient with PHA and her parents (*MR* mineralocorticoid receptor, *aENaC* α subunit of the amiloride-sensitive sodium channel, *PHA* pseudohypoaldosteronism)

	Amino acid substitution	Nucleotide position	Exon	Normal nucleotide	Nucleotide change in the patient	Father	Mother
MR							
1	Met^{1}–2	215	2	g	c/c	g/c	g/c
2	Ile$^{180}\rightarrow$Val^{180}	754	2	A	G/G	A/G	A/G
3	Ala$^{241}\rightarrow$Val^{241}	938	2	C	T/T	T/T	T/T
αENaC							
1	–	957+2	4	a	g/g	a/g	a/g

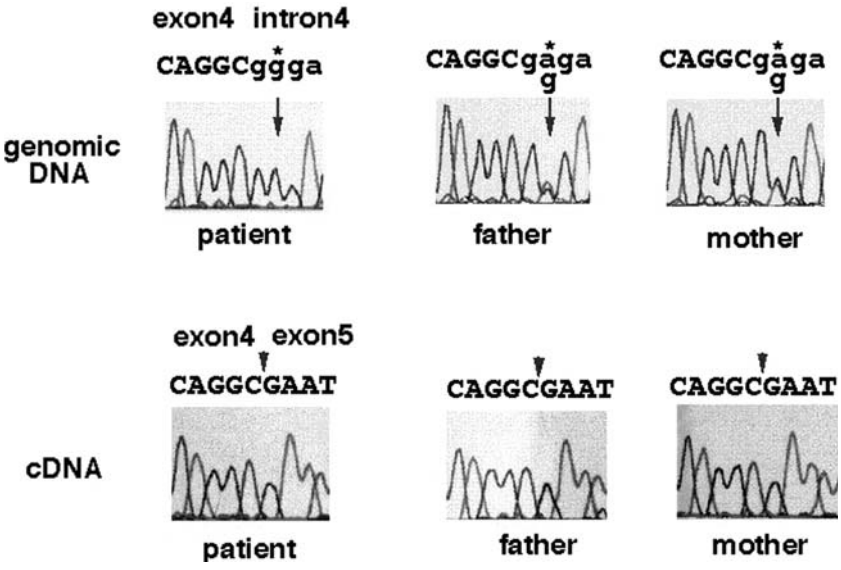

Fig. 2 DNA sequences of the substitution at the donor splice site of the α subunit of amiloride-sensitive sodium channel (αENaC) intron 4 for the patient and her parents. *Upper panel* Genomic sequences of the exon-intron boundary region of exon 4. *Asterisk*/*arrow* Homozygous A→G substitution at the donor splice site of αENaC intron 4 (patient, *left panel*); the father (*middle panel*) and mother (*right panel*) are heterozygous. *Lower panel* cDNA sequences at the boundary (*arrow*) of exon 4 and exon 5 indicating an identical splice structure for the patient and parents

substitutions, viz., $T^{554}\rightarrow A^{554}$ ($Trp^{178}\rightarrow Arg^{178}$), $C^{1526}\rightarrow G^{1526}$ ($Pro^{501}\rightarrow Ala^{501}$), and $T^{1862}\rightarrow G^{1862}$ ($Ser^{614}\rightarrow Ala^{614}$), in γENaC were also identified in the patient. We previously reported that these four amino acid substitutions occurred in all 25 subjects of a control population (Arai et al. 1999).

Functional study of mutated MRs

Luciferase activity induced by pRShMR-C^{215} was significantly lower than that induced by wild-type pRShMR when cells were incubated with aldosterone at concentrations ranging from of 10^{-11} to 10^{-9} M (P<0.05 or 0.01), but the difference disappeared with aldosterone at concentrations ranging from 10^{-8} to 10^{-6} M (Fig. 3A). Luciferase activity induced by pRShMR-Val^{180} was significantly higher than that induced by wild-type pRShMR when cells were incubated with aldosterone at a concentration of 10^{-10} M (P<0.01), whereas the opposite relationship was observed at a concentration of 10^{-8} M (P<0.05; Fig. 3B). Luciferase activity induced by pRShMR-Val^{241} was lower than that induced by wild-type pRShMR when cells were incubated with aldosterone at concentrations ranging from 10^{-11} to 10^{-6} M (P<0.05 or 0.01; Fig. 3C).

Discussion

A homozygous substitution ($G^{215}\rightarrow C^{215}$), which occurs at an allele frequency of 39% in human populations (Ludwig et al. 1998), has been identified at position –2 preceding the start codon of the MR gene in our patient with PHA. The lower luciferase activities observed for pRShMR-C^{215} compared with wild-type MR, with aldosterone at concentrations ranging from 10^{-11} to 10^{-9} M (corresponding to physiological concentrations), may be attributable to a lower translation efficiency of the $G^{215}\rightarrow C^{215}$ variant, resulting in the synthesis of fewer MR molecules. Because the structure of the open-reading frame of this variant does not differ from that of the wild-type MR gene, its DNA-binding or ligand-binding function may also be inferred to be the same. The substitution of a C nucleotide for T at position –5 preceding the mRNA start codon in the Kozak sequence of glycoprotein Ibα results in more efficient translation (Afshar-Kharghan et al. 1999). On the other hand, a previous report of sequences surrounding Kozak sequences indicates that, for the most efficient translation, the nucleotide at position –3 and +4 preceding the start codon should be either A or G (Kozak 1984). Indeed, the deletion of two nucleotides at position –2 and –3 preceding the start codon of the α^{+}-thalassemic gene has been documented as decreasing the efficiency of α-chain mRNA translation, resulting in the defective output of globin α-chains in patients with α^{+}-thalassemia (Morle et al. 1985). Recently, a single base polymorphism (C→T at position –4 preceding the start codon) within the Kozak sequence of the mRNA encoding coagulation factor XII has been demonstrated to influence profoundly the plasma concentrations of this protein in human (Kanaji et al. 1998). The substitution of the less common T allele at this position produces a new ATG codon upstream of the regular ATG codon; this brings about a marked decrease in plasma factor XII levels (Kanaji et al. 1998). These reports suggest that single nucleotide changes in the Kozak sequence significantly alter translation efficiency. The lower luciferase activity induced by pRShMR-C^{215} observed in the present study is congruent with these earlier reports and suggests that the $G^{215}\rightarrow C^{215}$ substitution in the MR Kozak sequence may reduce the translation efficiency of the MR.

The luciferase activity of pRShMR-Val^{241} was found to be lower than that of wild-type MR in response to every concentration of aldosterone, and the dose-response curve in luciferase activity for pRShMR-Val^{180} was shifted to the left compared with wild-type MR. These two amino acid substitutions are located in the A/B domain of MR

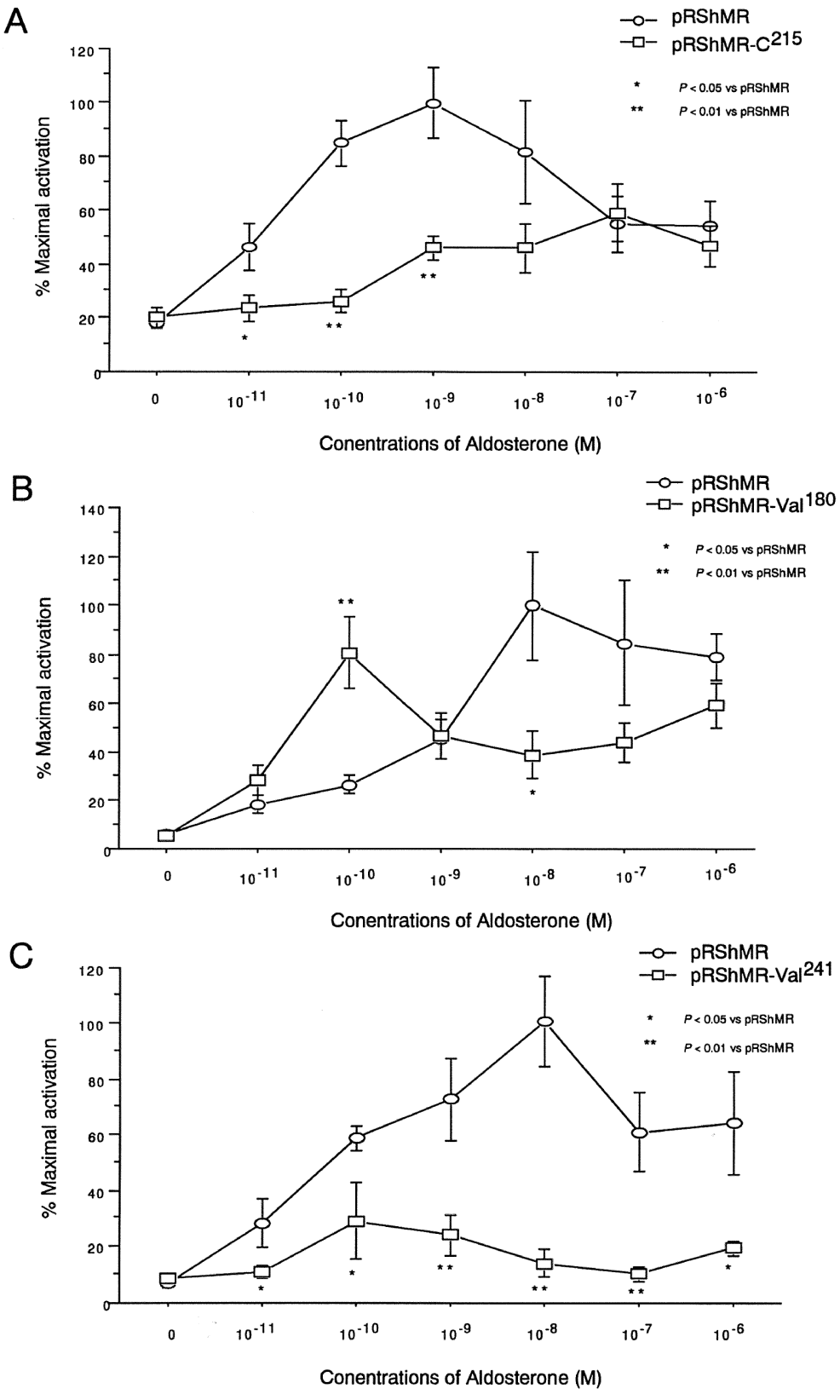

Fig. 3A–C Luciferase activity induced in vitro by plasmids containing wild-type (pRShMR) and mutant mineralocorticoid receptors. **A** pRShMR-C^{215}. **B** pRShMR-Val^{180}. **C** pRShMR-Val^{241}. Values are means (*error bars* SE). *Asterisks* Significance from wild-type under the Mann-Whitney U test: $P<0.05$ (*), $P<0.01$ (**)

and are encoded by exon 2. A recent study of deletion mutants of the rat A/B region has shown that they exhibit reduced transactivation activity, suggesting that the MR A/B domain has an important role in the transactivation function (Fuse et al. 2000). However, the hMR N-terminal deletion mutant, which lacks amino acids 59–247, exhibits a transactivation activity two-fold higher than that of wild-type MR, indicating that the amino acids between

59 and 247 play a role in inhibiting transactivation activity in human MR (Govindan and Warriar 1998). Consequently, the amino acid substitutions of $Ile^{180} \rightarrow Val^{180}$ or $Ala^{241} \rightarrow Val^{241}$ may affect MR transactivation function, although these amino acid substitutions by themselves may not result in a disorder.

We have also identified, in our patient, a homozygous A→G mutation at the second position of the donor splice site of intron 4 in αENaC. However, examination of the structure of the corresponding cDNA has revealed no exon skipping, suggesting that this substitution in the splice site neither alters the cDNA structure nor causes any disorder by itself. However, since a substitution in a splice site has been previously reported to affect splicing efficiency (Li and Pritchard 2000), we cannot rule out the possibility that this intronic substitution may affect the splicing efficiency of αENaC.

We have previously reported that both carbenoxolone and fludrocortisone normalize serum electrolytes in a patient with PHA (Arai et al. 1994). The physiological concentrations of circulating aldosterone in healthy subjects are 10^{-9} to 10^{-10} M, whereas those of patients with PHA are approximately 10^{-8} M. Plasma aldosterone concentrations of our patient, whose electrolytes were in the normal range at the age of 2 years, were actually 100-fold higher than normal. Given the preference of our PHA patient for salty food, it is likely that the presence of high concentrations of ligands for MR, combined with a highly dietary salt intake, compensates for her defective salt-conservation system including MR function.

We have previously shown that the heterozygosity or homozygosity frequency of the Val^{241} or Val^{180} substitution in a population of healthy subjects was 48%, 38%, 22%, and 1.5%, respectively, and that the frequency of simultaneous substitutions of Val^{241} and Val^{180} was 22% in healthy subjects (Arai et al. 1994). Moreover, the frequency of the co-occurrence of the Ala^{663} in αENaC and these two MR substitutions was only 7% in this population of healthy subjects (Arai et al. 1999). Although there is no report of the frequency of the co-occurrence of these three MR substitutions (C^{215}, Val^{241}, and Val^{180}), the co-occurrence frequency of MR and ENaC substitutions decreases with the increase in polymorphisms in normal population. In contrast, three of four (75%) patients with PHA showing multiple tissue resistance to aldosterone had two MR substitutions (Val^{241} and Val^{180}) and one ENaC substitution (Arai et al. 1999). There may be other gene abnormalities still undiscovered in the salt-conservation system. A single nucleotide polymorphism in the interleukin-1β (IL-1β) gene in combination with smoking or a polymorphism of the IL-1 receptor gene has been reported to be associated with early-onset periodontitis (Parkhill et al. 2000). This finding suggests that a single nucleotide substitution involving a slight reduction in the function of the protein may induce the disease if it co-occurs with certain environmental factors or another single nucleotide polymorphism. The results of the present study suggest that the three MR polymorphisms identified in the patient with sporadic PHA are not "true" polymorphisms in the sense that the alleles do not exhibit any significant functional differentiation; the polymorphisms described here appear to alter MR function. We therefore hypothesize that PHA can be brought about by the combined effects of two or more "functional" polymorphisms, each of which alone exhibits little influence on MR or ENaC function and physiological characteristics, but which together induce negative salt conservation that is characteristic of PHA.

Acknowledgements We thank Dr. R. M. Evans and Dr. G. L. Hager for kindly providing us with plasmids pRShMR and pL-TRluc, respectively.

References

Adachi M, Tachibana K, Asakura Y, Abe S, Nakae J, Tajima T, Fujieda K (2001) Compound heterozygous mutation in the γ subunit gene of ENaC (1627delG and 1570–1 G→A) in one sporadic Japanese patient with a systemic form of pseudohypoaldosteronism type 1. J Clin Endocrinol Metab 86:9–12

Afshar-Kharghan V, Li CQ, Khoshnevis-Asi M, Lopez JA (1999) Kozak sequence polymorphism of the glycoprotein (GP) Ibα gene is a major determinant of the plasma membrane levels of the platelet GP Ib-IX-V complex. Blood 94:186–191

Arai K, Tsigos C, Suzuki Y, Irony I, Karl M, Listwak S, Chrousos GP (1994) Physiological and molecular aspects of mineralocorticoid receptor action on pseudohypoaldosteronism: a responsiveness test and therapy. J Clin Endocrinol Metab 79: 1019–1023

Arai K, Tsigos C, Suzuki Y, Listwak S, Zachman K, Zangeneh F, Rapaport R, Chanoine J-P, Chrousos GP (1995) No apparent mineralocorticoid receptor defect in a series of sporadic cases of pseudohypoaldosteronism. J Clin Endocrinol Metab 80:814–817

Arai K, Zachman K, Shibasaki T, Chrousos GP (1999) Polymorphisms of amiloride-sensitive sodium channel subunits in five sporadic cases of pseudohypoaldosteronism: do they have pathologic potential? J Clin Endocrinol Metab 84:2434–2437

Arriza JL, Weinberger C, Cerelli G, Glaser TM, Handelin BL, Housman DE, Evans RM (1987) Cloning of human mineralocorticoid receptor complementary DNA: structural and functional kinship with the glucocorticoid receptor. Science 237: 268–275

Bresnick EH, John S, Berard DS, LeFebvre P, Hager GL (1990) Glucocorticoid receptor-dependent disruption of a specific nucleosome on the mouse mammary tumor virus promoter is prevented by sodium butyrate. Proc Natl Acad Sci USA 87: 3977–3981

Chang SS, Grunder S, Hanukogulu A, Rösler A, Mathew PW, Hanukogulu I, Schild L, Lu Y, Shimkets RA, Nelson-Williams C, Rossier BC, Lifton RP (1996) Mutations in subunits of the epithelial sodium channel cause salt wasting with hyperkalemic acidosis, pseudohypoaldosteronism type 1. Nat Genet 12:248–253

Cheek DB, Perry J (1958) A salt wasting syndrome in infancy. Arch Dis Child 33:252–256

Chomczynski P, Sacchi N (1987) Single-step method of RNA isolation by acid guanidium thiocyanate-phenol-chloroform extraction. Anal Biochem 162:156–159

Corvol P, Funder J (1994) Editorial: the enigma of pseudohypoaldosteronism. J Clin Endocrinol Metab 79:25–26

Fuse H, Kitagawa H, Kato S (2000) Characterization of transactivation property and coactivator mediation of rat mineralocorticoid receptor activation function-1 (AF-1). Mol Endocrinol 14: 889–899

Geller DS, Rodriguez-Soriano J, Vallo Boado A, Schifter S, Bayer M, Chang SS, Lifton RP (1998) Mutations in the mineralocorticoid receptor gene cause autosomal dominant pseudohypoaldosteronism type I. Nat Genet 19:279–281

Govindan MV, Warriar N (1998) Reconstitution of the N-terminal transcription activation function of human mineralocorticoid receptor in a defective human glucocorticoid receptor. J Biol Chem 273:24439–24447

Kanaji T, Okamura T, Osaki K, Muroikawa M, Shimoda K, Hamasaki N, Niho Y (1998) A common genetic polymorphism (46 C to T substitution) in the 5'-untranslated region of the coagulation factor XII gene is associated with low translation efficiency and decrease in plasma factor XII level. Blood 91: 2010–2014

Komesaroff PA, Verity K, Fuller PJ (1994) Pseudohypoaldosteronism: molecular characterization of the mineralocorticoid receptor. J Clin Endocrinol Metab 79:27–31

Kozak M (1984) Point mutations close to the TUG initiator codon affect the efficiency of translation of rat preproinsulin in vivo. Nature 308:241–246

Kuhnle U (1997) Pseudohypoaldosteronism: mutation found, problem solved? Mol Cell Endocrinol 133:77–80

Li M, Pritchard H (2000) Characterization of the effect of mutations in the putative branchpoint sequence of intron 4 on the splicing within the human lecithin:cholesterol acyltransferase gene. J Biol Chem 275:18079–18084

Ludwig M, Bolkenius U, Wickert L, Bidlingmaier F (1998) Common polymorphisms in genes encoding the human mineralocorticoid receptor and the human amiloride-sensitive sodium channel. J Steroid Biochem Mol Biol 64:227–230

Morle F, Lopez B, Henni T, Godet J (1985) α-Thalassaemia associated with the deletion of two nucleotides at position –2 and –3 preceding the AUG codon. EMBO J 4:1245–1250

Parkhill JM, Hennig BJW, Chapple ILC, Heasman PA, Taylor JJ (2000) Association of interleukin-1 gene polymorphisms with early-onset periodontitis. J Clin Periodontol 27:682–689

Speiser PW, Stoner E, New MI (1986) Pseudohypoaldosteronism: a review and report of two cases. In: Chrousos GP, Loriaux DI, Lipsett MB (eds) Mechanisms and clinical aspects of steroid hormone resistance. Plenum, New York, pp 173–195

Strautnieks SS, Thompson RJ, Gardiner RK, Chung E (1996) A novel splice-site mutation in the γ-subunit of the epithelial sodium channel gene in three pseudohypoaldosteronism type 1 families. Nat Genet 13:248–250

Tajima T, Kitagawa H, Yokoya S, Tachibana K, Adachi M, Nakae J, Suwa S, Katoh S, Fujieda K (2000) A novel missense mutation of mineralocorticoid receptor gene in one Japanese family with renal form of pseudohypoaldosteronism type 1. J Clin Endocrinol Metab 85:4690–4694

Zennaro MC, Borensztein P, Jeunemaitre X, Armanini D, Soubrier F (1994) No alteration in the primary structure of the mineralocorticoid receptor in a family with pseudohypoaldosteronism. J Clin Endocrinol Metab 79:32–38