BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **252,** 208–213 (1998)
ARTICLE NO. RC989625

Gene Structure of the Human Amiloride-Sensitive Epithelial Sodium Channel Beta Subunit

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Received September 28, 1998

ENaC functions in the transport of sodium ions across epithelial cells and consequently regulates blood volume and pressure. ENaC complex includes at least three different subunits, alpha, beta, and gamma, which are developmentally regulated and differentially controlled by aldosterone. In this study, we determined the exon–intron organization of the beta ENaC subunit by sequencing genomic DNA from three subjects from three different ethnic groups. The results showed that the coding region of the human βENaC gene (SCNN1B) extends from exon 2 to exon 13. No polymorphism was observed in the examined subjects, indicating strict conservation of the coding region sequence. The introns of beta subunit gene are located at exactly the same positions as in the alpha and gamma subunits, although these proteins share only 26–32% sequence identity. These results thus elucidate the gene structure of the beta subunit and indicate that exon–intron architecture of the three genes encoding the three subunits of ENaC have remained highly conserved despite the divergence of their sequences.

The amiloride-sensitive epithelial sodium channel (ENaC) is expressed in specific tissues and functions in the transport of sodium across epithelial cells (1–3). ENaC is located on the apical membrane facing the lumen, and allows movement of sodium from the lumen into the epithelial cell. The sodium reabsorbed by ENaC is then extruded from the epithelial cell back into the blood stream by the Na/K ATPase that is located at the opposite basolateral membrane of the cell. The reabsorption of sodium by ENaC is accompanied by an osmotic uptake of water to maintain a constant extracellular Na^+ concentration. This changes blood volume and consequently affects blood pressure. Thus, ENaC plays an important role in electrolyte homeostasis and the control of blood volume and blood pressure (1–3).

ENaC is expressed at the highest levels in epithelial cells lining the distal colon, the distal part of the renal tubule, respiratory airways, and in the duct of several exocrine glands, such as salivary and sweat glands. Cloning and expression of cDNAs revealed that ENaC complex is made up of at least three different subunits α, β and γ, which are developmentally regulated, selectively expressed and differentially controlled by steroid hormone aldosterone, and other regulatory elements (1–4). Neither β nor γ subunit gives rise to Na^+ currents when expressed individually, while expression of the α-subunit alone induces a very small amiloride-sensitive Na^+ current (5). By contrast, coexpression of all three ENaC subunits results in large currents with the functional protein (5). A recent study suggests that functional ENaC channel is composed of four subunits, two alpha subunits separated by beta and gamma subunits (6). ENaC activity may be regulated by interacting with other proteins like CFTR (cystic fibrosis transmembrane conductance regulator), α-spectrin, Nedd4, and CAP, a channel activating protease (7–10).

In humans, mutations in ENaC subunits have been shown to cause multi-system form of pseudohypoaldosteronism (PHA) type I, which is a salt-wasting disease (11–15), and Liddle's syndrome which is characterized by hereditary hypertension (16–18). These two diseases respectively result from either the loss of function of ENaC or excess activity of ENaC (12–18). Genetic analysis in 11 families with multi-system PHA indicated that the disease was linked to the chromo-

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The nucleotide sequence data reported in this paper will appear in DDBJ, EMBL, and GenBank Nucleotide Sequence Databases under Accession Numbers AJ005383–AJ005393.

Abbreviations used: ENaC, amiloride-sensitive epithelial sodium channel; PHA, pseudohypoaldosteronism; TM, trans-membrane.

0006-291X/98 $25.00

TABLE 1

Primer Sets and PCR Conditions Used to Amplify Segments of the Human βENaC Gene

Primer set	Exon	Forward	Reverse	[$MgCl_2$] (mM)	Temp. (°C)
B2	2	ATGCCTCTCTGCAGGTGCCAC	AGCTGTGCACTCCGGGGCCAC	4.0–6.0	60
B3	3	TTCCCCCTAACCAGCCCTCTC	CATTGCTTGATATGTGCCCAG	4.0–6.0	60
B4	4	TGGCCTCCACAGTGTAGCCTC	CCGACTGTCCGTAGGTGCCAG	4.0–6.0	60
B5	5	CCTGCCCTGCAGCTGATGCTG	GGTTAAAGCCTCATGGCTCTG	4.0–6.0	60
B6	6	CGCAGCCCTCACCCCACCCTC	GCCCTTGGGCTCCGGCCATAC	4.0–6.0	60
B7	7	AAGCAACCCCTCTAAACACAG	AGGCGTGCACCACCTTCCCAC	4.0–6.0	60
B8	8	CCTGTGTTCTCTCATTATGAAC	GATCCCCCGTGCCCCCGCTC	4.5	56
B9	9	AACCTCTTGGCCGCCTTTCTG	TGTGCCCGCCCACCCGCACTC	4.0–6.0	60
B10	10	GCAGGGACCACAACAGGCCTG	GTGGTTGCAAAAGTTGCCATC	1.5	56
B11	11	GATGGCAACTTTTGCAACCAC	CCAGCCCCGCCCAGGCTCAG	4.0–6.0	60
B12	12	GGCCCATCTCGCTGCCTCCTG	AGGGCTGGGGTATTGGGAGAC	4.0–6.0	60
B13	13	CAAGAATGTGTGGCCTGAG	GTCCAGCGTCTGCAGACGCAG	1.5	56

Note. All sequence are listed in the 5′-3′ direction. Temp. refers to the annealing temperature.

some 12p13.1 region containing the gene for αENaC and chromosome 16p12.2-13.11 region containing the gene for β and γ ENaC (13). The proximity of β and γ suggests that they derive from a common ancestor by a gene duplication event, a view which is supported by the observation that in the colon the β and γ subunits are controlled by aldosterone whereas αENaC is constitutively expressed (19, 20).

The gene sequences of α and γ ENaC subunits have been determined (21, 22). Yet, the genomic structure of the β subunit has not been reported. In this study, we determined the exon-intron organization of the βENaC gene in three human subjects, one normal and two patients with multi-system PHA. Our results reveal that the sequence of βENaC shows no polymorphism in the examined subjects from different ethnic groups, and that exon-intron organization of the three genes encoding the three subunits of ENaC have remained nearly identical despite the divergence of the sequences.

MATERIALS AND METHODS

Subjects. In this study we sequenced genomic DNA from three individuals, one normal subject of Sephardic Jewish origin (IH), and two patients with multi-system form of PHA, patient 013 of Syrian origin, and patient 023 of English origin. Both PHA patients were the offsprings of consanguineous marriages, consistent with the autosomal recessive transmission of the disease. Patient 013 had raised urinary sodium in the presence of hyponatremia, hyperkalemia, increased plasma renin activities, elevated serum aldosterone levels, but normal renal and adrenal functions (13). In this patient, sodium was raised in sweat, saliva and stool indicating multi-system involvement. Patient 023 was born after a pregnancy complicated by polyhydraminos and died on day 4 with hyponatremia and hyperkalemia. The infant required large Na supplementation but did not respond to mineralocorticoids. Genetic linkage analysis using microsatellite markers (13) indicated that PHA was linked to chromosome 16 in both patients.

DNA isolation. Genomic DNA was extracted from white blood cells as described by Miller et al. (23). Proteins were digested by proteinase K, and "salted out" using NaCl. DNA was then precipitated and resuspended in 10 mM Tris, 1 mM EDTA, pH 8.0.

PCR. The coding regions of βENaC were amplified by PCR using genomic DNA as a template. The primers used were either in introns or in the exon-intron junctions (12) (Table 1). PCR reactions were conducted in 30 μl containing 50 mM KCl, 1.5–6.0 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.8), 200 μM dNTPs, 0.08% Nonidet P-40, 0.5 U Taq polymerase (recombinant), 0.5 μM of each primer and 100 ng of human genomic DNA. After an initial denaturation step at 94°C for 4 min, PCR was conducted for 30 cycles with denaturation at 94°C for 45s, annealing at 56–60°C for 45s and extension at 72°C for 45s.

To determine some intron sequences, long range PCR was performed in a similar 30 μl reaction containing double the concentration of dNTPs and primers as well as three fold higher concentration of Taq polymerase. The initial denaturation temperature used was 92°C for 4 min and PCR was conducted for 25 cycles with denaturation at 92°C for 30s, annealing at 58–60°C for 50s and extension at 72°C for 90s, followed by 2 cycles with denaturation at 92°C for 30s, annealing at 58–60°C for 1 min and extension at 72°C for 90s. In the last cycle, the products were kept at the extension temperature for 7–10 min. In these studies we used the primer set B9f-B10r to determine the complete sequence of intron 9, and the primer set B10f-B11r to sequence intron 10.

PCR products were visualized on 10% polyacrylamide gels, purified by high pure PCR-product purification kit (Boehringer Mannheim), and used directly for sequence analysis.

DNA sequencing. DNA sequences were determined by the dideoxy chain termination method (24) using the Thermosequenase radiolabelled Terminator Cycle Sequencing kit and α[^{33}P]ddNTPs (1500Ci/mmole or 55.5 TBq/mmole; Amersham), following the standard protocol supplied by the manufacturer. In brief, 0.045 μCi of each of four Redivue α[^{33}P] dideoxy nucleotide (G, A, T, C) terminators were used to radiolabel four different DNA sequencing reactions by the Thermosequenase DNA polymerase. Initial denaturation and subsequent cycling conditions were performed as described in PCR with only one specific primer. The reaction products were then denatured by stop solution containing 95% formamide and heating at 70°C for 2–10 min. The products were then electrophoresed on DNA sequencing gels (6% acrylamide, 7M urea and 1×TBE) at 50W constant power for 4–8 hr.

Autoradiography was carried out using Kodak BioMax MR films. In all cases, DNA sequences were confirmed by sequencing both strands of the DNA.

TABLE 2

Exon–Intron Organization of the Human βENaC Gene

		Exon				Intron		
No.	Accession No.	Start	End	Size (bp)	3′-End	5′-End	3′-End	5′-End of exon
1	AC002300	1	119	119	CCCGAGCAG	gtatga...	atgcctctctgcag	GTGCCACT
2	AJ005383	120	438	319	CCCTTCAA	gtaggt...	ctctccccatccag	GTATTCCAAA
			104		ProPheLy			sTyrSerLys
3	AJ005384	439	712	274	ATGAGACTA	gtaagt...	ggtggcctccacag	TGTAGCCTC
		104	195		MetArgLeu			CysSerLeu
4	AJ005385	713	903	191	AACTACCG	gtgaga...	gctgtttcttttag	GAACTTCACG
		196	259		AsnTyrAr			gAsnPheThr
5	AJ005386	904	1007	104	GAATTCG	gtgagt...	caccctccccacag	GCCTGAAGTTG
		259	294		GluPheG			lyLeuLysLeu
6	AJ005387	1008	1171	164	GTACTCGTG	gtatgg...	ccctctaaacacag	GACAAGCTT
		294	348		VAlLeuVal			AspLysLeu
7	AJ005388	1172	1279	108	TCCATCCAG	gtggga...	cctaccctccccag	GCCTGTCTT
		349	384		SerIleGln			AlaCysLeu
8	AJ005389	1280	1397	118	GACTGGG	gtgagc...	tgtctcctgcgcag	CCCATTGCTAC
		385	424		AspTrpA			laHisCysTyr
9	AJ005390	1398	1473	76	TCCTGCAA	gtgagt...	ccgttctctttcag	TGACACCCAG
		424	449		SerCysAs			nAspThrGln
10	AJ005390	1474	1531	58	GCCTCCGAG	gtgaga...	ttcttgggttccag	GACTGGATT
		449	468		AlaSerGlu			AspTrpIle
11	AJ005391	1532	1593	62	CTGAGCAG	gtgagc...	gctgcctcctgcag	GAAGGGAATT
		469	489		LeuSerAr			gLysGlyIle
12	AJ005392	1594	1669	76	GCCAATAAC	gtgagt...	cctgttccccacag	ATCGTCTGG
		489	514		AlaAsnAsn			IleValTrp
13	AJ005393	1670						
		515						
Consensus					AG	gtaagt...	yyyyyyyyyyncag	G

Note. For each exon, the first line of "Start" and "End" refer to the numbering of the mRNA sequence (27), and the second line, the numbering of the amino acid sequence.

RESULTS AND DISCUSSION

Exon–intron organization of the βENaC gene. In this study, we determined the sequences of the coding region and exon-intron junctions of the human βENaC gene (SCNN1B) by directly sequencing genomic DNA from three subjects. The sequence of the 5′-untranslated region has been recently reported and includes only one exon and one intron (25). Our sequencing results indicate that the coding region of the human βENaC gene extends from exon 2 to exon 13. Hence, in the translated region of the gene there are 11 introns (Table 2, Figure 1). Thus, the intron and exon numbers used here represent the entire coding region of the βENaC gene.

All the splice junctions determined in this study conform strictly to the GT/AG rule of intron splice junction sequences (26). Determination of these splice sites is important, as some cases of PHA emanate from splice-site mutations (14).

Determination of the intron positions of the βENaC gene allowed us to compare the exon-intron organization of the three subunits of the ENaC (Figure 1). The β and γ subunits are located on chromosome 16p12.2-13.11, whereas the α subunit is located on chromosome 12p13.1 (13, 27). Sequence identity between β and γ subunit protein sequences is 32%, while identity between α and β or γ subunit is 26–28%. These similarities indicate that β and γ subunits are, as expected by their proximity, evolutionarily more closely related.

Although 26 to 32% identity between the coding sequences of the ENaC subunits indicate significant divergence of these sequences, the introns of all three proteins appear at exactly the same positions when these sequences are aligned based on their sequence homology (Figure 1). The amino acids at some intron locations (introns 6, 8, 9, 12) are different yet, all three sequences can be aligned in the regions spanning these introns without ambiguity. Thus, the relative positions of introns were maintained in the face extensive mutations in the exon-intron junctions. In some multigene families such as the chloride channel genes, the exon-intron organization is not conserved (28).

The α, β, and γ ENaC subunit sequences contain two approximately 20 residue long very highly hydrophobic segments. These two domains (highlighted in Figure 1) probably function as transmembrane (TM) domains that span the membrane. The TM domains are located on exon 2 and exon 13, which are the first and the last exons of the coding region of the gene. Thus, the seg-

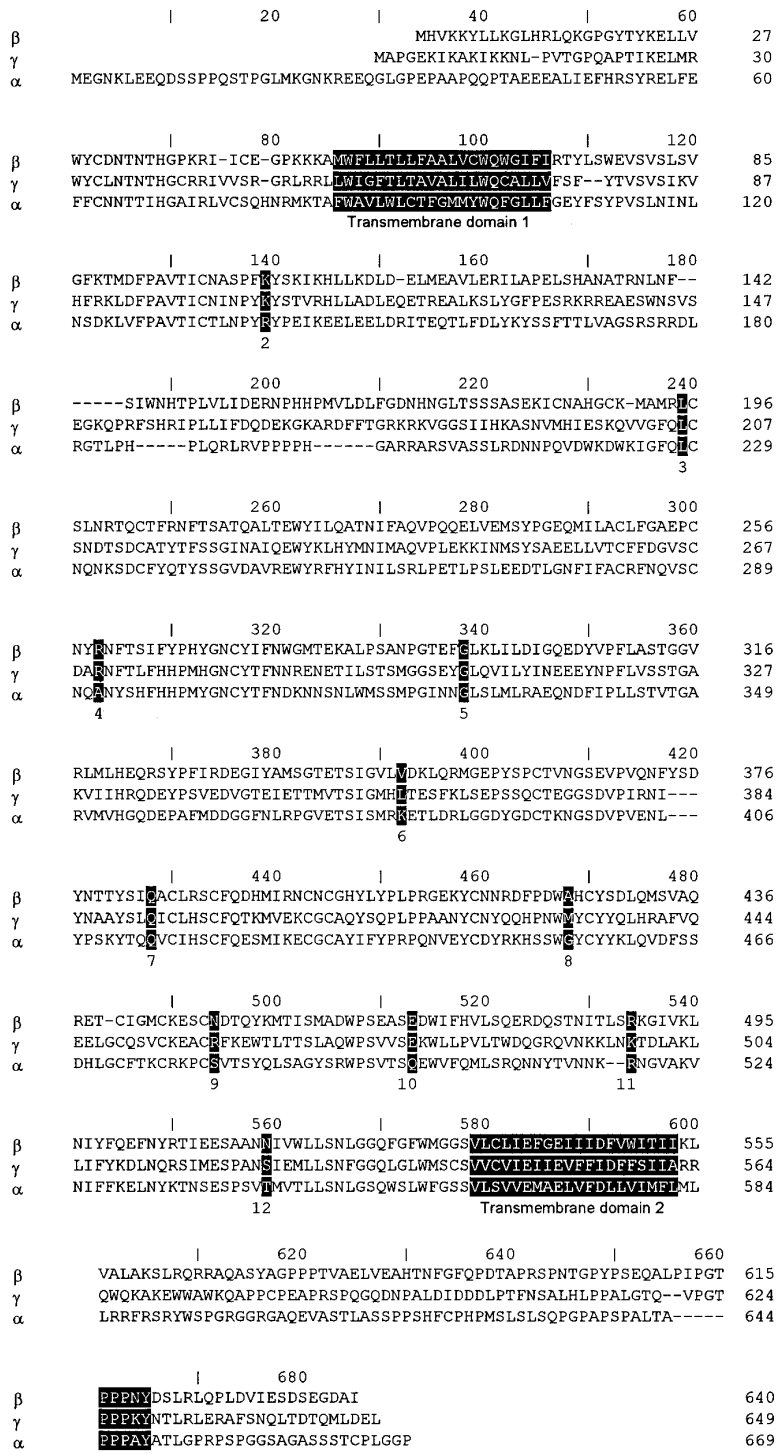

FIG. 1. Positions of introns in the genes encoding for the α, β, and γ subunits of the epithelial sodium channel. The three sequences were aligned using the pileup program (38). The single residues shaded mark the positions of introns which are located either in or at the end of that codon. The numbers below the shading mark the number of the intron. There are altogether 13 exons and 12 introns. Exon 1 and intron 1 are not shown as both are located in the 5′-untranslated region (UTR) (see Table 2).

ments encoded by exons 2 and 13 are mostly located in the cytoplasm, whereas a large segment of about 470 residues, bounded by TM-1 and TM-2, project to the outside of the cell.

Among all exons, the amino and carboxy segments of the ENaC subunits encoded by exons 2 and 13 respectively are the least conserved, both in terms of their sequences and length of the coded segments (Figure 1).

These segments probably interact with other molecules in the cytoplasmic side of the cell. The divergence of these two regions may be related to the evolutionary development of subunit specific interactions. In the face of this divergence, a Pro-Pro-Pro-X-Tyr (PPPXY) motif appears in all subunits (Figure 1). The elucidation of the mutations causing Liddle's syndrome revealed that this motif plays a major role in the function of the ENaC (29). A cytosolic protein named Nedd4 is presumed to bind to this motif mediating ubiquitin-dependent down-regulation of Na^+ channel activity (30–32).

Polymorphism of the ENaC genes. The role of ENaC in blood pressure regulation and the finding of hereditary hypertension causing mutations in ENaC subunits of Liddle's Syndrome patients led to the hypothesis that polymorphisms in ENaC subunit sequences may be responsible for other forms of blood pressure disorders (33, 34). Recent reports indicate that a mutant form of the βENaC with a T594M substitution that was observed in 6% of African-Americans but not in Caucasian subjects may be associated with hypertension (34, 35).

Our sequencing of the gene from three individuals of distinct ethnic origin revealed no differences in the encoded protein sequences. This lack of polymorphism, though in a limited population, point out that the ENaC subunit gene sequences are tightly conserved, and that mutations in these genes probably reduce evolutionary survival. Our sequencing showed two differences from the previously determined mRNA sequence: In all subjects we observed a C instead of a G at 1068, and a C instead of a T at 1174. The former changes the residue from a Gly in mRNA to an Ala. We do not know whether these differences represent a polymorphism or sequencing errors. In patients 013 and 023 linkage analysis showed that PHA type I is linked to chromosome 16. Thus, the mutations in these patients may be in the gamma subunit rather than the beta subunit sequenced in this study.

The sequencing of the carboxy-terminal portion of the βENaC gene in an unselected cohort of Japanese patients with essential hypertension did not reveal any mutations in this region (36). Similarly, the C-terminal regions sequences of α, β, and γ subunits of ENaC in hypertensive rat strains were determined to examine the possibility that hypertension may be associated with a mutation in this region (37). The results of the study however, did not show any differences in sequence, leaving open the possibility of mutations at other sites on these proteins or other factors involved in blood-pressure regulation (37).

The T594M mutation noted above raise the possibility that other low-frequency polymorphic hypertension associated mutations may be found by more extensive sequencing of different populations. The sequence information provided in this study should facilitate further studies on this subject.

ACKNOWLEDGMENTS

This research was supported by the UK–Israel Science and Technology Research Fund under the auspices of the Ministry of Science (Israel), Medical Research Council (UK) and Action Research. We are grateful to Dr. Deepak Saxena for assistance in DNA sequence analysis.

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