Cloning and functional studies of splice variants of the \( \alpha \)-subunit of the amiloride-sensitive \( \mathrm{Na}^+ \) channel

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Cloning and functional studies of splice variants of the \( \alpha \)-subunit of the amiloride-sensitive \( \mathrm{Na}^+ \) channel (hENaC) have been recently cloned (17–19, 38), and it is known that mutations of hENaC subunits are associated with Liddle’s syndrome (11, 12, 28, 33) and pseudohypoaldosteronism type 1 (5, 31). ENaC is a key component in regulating the rate of transepithelial \( \mathrm{Na}^+ \) transport across epithelia (21, 24, 25). ENaC is composed of at least three homologous subunits, \( \alpha \), \( \beta \), and \( \gamma \), and the \( \alpha \)-subunit (\( \alpha \)ENaC) is critical to the formation of an ion conductive membrane pore, whereas the \( \beta \)- and \( \gamma \)-subunits (\( \gamma \)ENaC) greatly potentiate the level of expressed \( \mathrm{Na}^+ \) currents in heterologous expression systems (4, 18). In recent experiments in mice, gene knockout of \( \alpha \)ENaC was lethal within 40 h of birth due to failure of pulmonary fluid clearance, clearly demonstrating the critical role of \( \alpha \)ENaC in forming a functional \( \mathrm{Na}^+ \) channel complex in vivo (13).

Electrophysiological studies of different epithelial systems have shown a wide variability of ion selectivity, inhibitory profiles by amiloride analogs, and/or single-channel conductances (1, 21, 24). The reasons for this functional variability are poorly understood at present. In the present study, we have characterized h\( \alpha \)ENaC by molecular cloning and functional expression studies in Xenopus oocytes. The results demonstrate three alternatively spliced variants of h\( \alpha \)ENaC with altered function and suggest that alternate splicing of the h\( \alpha \)ENaC may be involved in the functional regulation of ENaC activity.

MATERIALS AND METHODS

Isolation of full-length h\( \alpha \)ENaC subunit. The full-length coding region of h\( \alpha \)ENaC was obtained from the human lung cell line H441, using RT-PCR. The H441 cells were obtained from the American Type Culture Collection (ATCC HTB-174) and were previously shown to contain h\( \alpha \)ENaC (19). RT-PCR was performed as previously reported (22, 23); mRNAs were extracted from cultured H441 cells using the Micro mRNA purification kit (Pharmacia Biotech). cDNA was synthesized by oligo(dT)-priming methods using avian myeloblastosis virus RT, and DNA was amplified for 35–40 cycles in a programmable thermal controller (PTC-100, MJ Research) with Taq DNA polymerase (Boehringer Mannheim).

We designed PCR primers to amplify the entire coding region of h\( \alpha \)ENaC. The forward primer corresponded to nucleotide positions 82–97 (bp; numbered according to GenBank no. L29007), modified at the 5’ end to create a BamH I restriction site, and the reverse primer corresponded to bp 2096–2110 modified at the 5’ end to create an Xho I restriction site. PCR products were separated by electrophoresis on 1% agarose gel, purified using GeneClean II (Bio 101), and cloned into the pCR II vector (Invitrogen). The plasmid inserts were analyzed by restriction enzyme analysis and/or nucleotide sequence determination. Nucleotide sequences were determined by using an ABI 373 automated DNA sequencer at the University of Alabama at Birmingham Center for AIDS Research DNA Sequencing Core. Both strands of the inserts (prepared via Qiagen column) were sequenced. For in vitro transcription and oocyte expression studies, the h\( \alpha \)ENaC inserts were subcloned into pCDNA3 after digestion with BamH I and Xho I.

Tissue distribution of h\( \alpha \)ENaC. To determine tissue distribution of h\( \alpha \)ENaC splice variants, PCR was performed on human cDNA libraries constructed from eight different human tissues (QUICK-Screen human cDNA library panel; Clontech), which were done using two different vectors, \( \lambda \)gt10 and \( \lambda \)TriplEx, with the exception of brain tissue (which was done only in \( \lambda \)TriplEx vector). PCR primers were designed so that the coamplified PCR products derived from either control or splice variants could be readily distinguishable on 2% agarose gel. To examine tissue distribution of prematurely truncated h\( \alpha \)ENaC (h\( \alpha \)ENaCx), the forward primer corresponded to bp 473–491 and the reverse primer corresponded to bp 1010–1033. Tissue distribution of the 19-amino acid-deleted h\( \alpha \)ENaC (h\( \alpha \)ENaC-19) was examined using the forward primer corresponding to bp 968–989 and the reverse primer corresponding to bp 1171–1192. Tissue distribution of the 22-amino acid-inserted h\( \alpha \)ENaC (h\( \alpha \)ENaC+22) was examined using the forward primer corresponding to bp 1302–1325 and the reverse primer corresponding to bp 1459–1483.

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In vitro transcription and translation. The hαENaCs cloned into pcDNA3 were linearized with XhoI for the preparation of cRNA. The full-length coding cDNAs of hENaC β- and γ-subunits, kindly provided by Dr. M. J. Welsh (University of Iowa, Iowa City, IA), were subcloned into pcDNA3 and linearized with XhoI. Linearized plasmid DNA was purified by phenol-chloroform extraction. In vitro transcription was carried out using the mMESSAGE mMACHINE T7 kit (Ambion) according to the manufacturer’s instructions. The integrity of in vitro transcribed cRNA was analyzed by electrophoresing through 1.2% agarose-2.2 M formaldehyde denaturing gel. In addition, the cRNA was translated in vitro into biotin-labeled proteins in the presence of reticulocyte lysates and biotin-lysine-tRNA (Boehringer Mannheim). Biotin-labeled hαENaCs were separated on 10% SDS-polyacrylamide gel and transferred to a polyvinylidene difluoride membrane. The transferred hαENaCs were detected with a streptavidin-alkaline phosphatase conjugate. The color reaction was initiated by adding substrate solution containing 420 µg/ml nitro blue tetrazolium and 188 µg/ml 5-bromo-4-chloro-3-indolyl phosphate in 100 mM Tris-150 mM NaCl-50 mM MgCl2, and stopped by several changes of distilled water.

Expression of hαENaC in Xenopus oocytes. Adult female Xenopus laevis were obtained from Xenopus I (Ann Arbor, MI). Oocytes were isolated and defolliculated in the presence of 2 mg/ml collagenase type A (Boehringer Mannheim). Stage V-VI oocytes were selected by visual inspection and maintained at 18°C in ND-96 Ringer solution (in mM: 96 NaCl, 2.4 KCl, 2 CaCl2, 1.8 MgCl2, and 5 HEPES, pH 7.4) supplemented with 5% horse serum (Life Technologies). Oocytes were injected with 50 nl of the transcribed cRNAs (5 ng of cRNA for each α-, β-, and γ-subunit) using a microinjector (Drummond Nanoject, Drummond Scientific). Electrophysiological recordings were performed 2–3 days postinjection in ND-96 Ringer solution using a two-electrode voltage-clamp system. The current-voltage relationship was obtained by stepping for 500 ms from a holding potential of −40 mV to −120 to +20 mV, in 20-mV increments. To determine Na+ selectivity of the expressed ENaC activity, the external Na+ was replaced with either Li+ or K+.

Exon-intron boundary of hαENaC. Genomic DNA was sequenced to determine exon-intron boundaries of the extracellular domain of hαENaC. Human genomic DNAs were obtained from human B lymphoblast cell lines, using a QIAamp tissue kit (Qiagen). PCR primers corresponding to either coding or intron regions of hαENaC were synthesized and used to amplify relevant genomic DNAs. Sequence information regarding intron PCR primers was obtained from the report by Chang et al. (5), who previously showed that hαENaC was encoded by at least 13 separate exons. Amplified genomic DNA was cloned into pCR II and analyzed as described above.

RESULTS

Cloning of alternative splice variants of hαENaC. To determine whether hαENaC has alternatively spliced isoforms and, if so, whether the splice variants display functional differences, we examined the coding sequences of hαENaC mRNAs. Using PCR primers that were designed to amplify full-length coding sequences, we obtained and characterized three novel splice variants of hαENaC from the human lung epithelial cell line, H441 (Fig. 1). Wild-type hαENaC was readily
identified. The first splice variant, referred to as h\textsubscript{ENaC}x, contained a premature stop codon in the extracellular domain, whereas the second (h\textsubscript{ENaC}−19) and the third (h\textsubscript{ENaC}+22) splice variants contained a deletion of 19 amino acids and an addition of 22 amino acids in the extracellular domain, respectively. Nucleotide sequences of these h\textsubscript{ENaC}s were confirmed by restriction mapping (Fig. 1B) and complete nucleotide sequencing analyses.

Sequencing results showed that h\textsubscript{ENaC}x was the shortest isoform (1793-bp PCR product) due to deletions at two extracellular regions: bp 767–957 (191-bp deletion) and 1061–1117 (57-bp deletion). The h\textsubscript{ENaC}−19 (1984 bp) had a 57-bp deletion at nucleotides 1061–1117; h\textsubscript{ENaC}+22 was the longest splice isoform (2107 bp), with a 66-bp insertion at nucleotide position 1441. The wild-type h\textsubscript{ENaC}, 2041 bp, was nearly identical to the previously published h\textsubscript{ENaC} sequence (19, 38).

Minor nucleotide changes were detected in the cloned h\textsubscript{ENaC}s compared with the previously published h\textsubscript{ENaC} sequence (19). There was one nucleotide change from A to G at bp 587 in h\textsubscript{ENaC}x, which would change the corresponding amino acid from threonine to alanine. There were two nucleotide changes in h\textsubscript{ENaC}−19: 1) C to A at bp 1309 without an amino acid change and 2) A to G at bp 2069 with an amino acid change from threonine to alanine. The wild-type h\textsubscript{ENaC} had six nucleotide changes: 1) G to A at bp 164 with an amino acid change from glutamic acid to lysine, 2) T to C at bp 375 with an amino acid change from methionine to threonine, 3) T to G at bp 393 with an amino acid change from leucine to arginine, 4) G to A at bp 1222 without an amino acid change, 5) C to T at bp 1575 with an amino acid change from serine to phenylalanine, and 6) A to G at bp 2069 with an amino acid change from threonine to alanine. There were two nucleotide changes in h\textsubscript{ENaC}+22: 1) A to G at bp 1897 without an amino acid change and 2) A to G at bp 2069 with an amino acid change from threonine to alanine.

These nucleotide changes could represent a cloning artifact that occurred during sequencing or DNA amplification or accurate nucleotide changes occurring in cells during alternative RNA splicing or RNA editing.

Tissue expression of h\textsubscript{ENaC}. To confirm that the nucleotide deletion and insertion observed in the splice variants were not due to a cloning artifact, we designed PCR primers that would amplify the area containing either the deletion or insertion sites from the h\textsubscript{ENaC} cDNAs. Three different PCR primer sets were used for coamplification of 1) h\textsubscript{ENaC}x and h\textsubscript{ENaC}−19, 2) h\textsubscript{ENaC}−19 and h\textsubscript{ENaC}x, and 3) h\textsubscript{ENaC}+22 and h\textsubscript{ENaC}x from H441 cells, respectively. The results show that the mRNA levels for h\textsubscript{ENaC}x, h\textsubscript{ENaC}−19, and h\textsubscript{ENaC}+22 were very low in abundance compared with the wild-type h\textsubscript{ENaC} in H441 cells (Fig. 2).

Coamplification of h\textsubscript{ENaC} splice variants with wild-type h\textsubscript{ENaC} from 15 different human cDNA libraries showed that the wild-type h\textsubscript{ENaC} was the most abundantly expressed h\textsubscript{ENaC} mRNA species in all tissues in which its expression was detected. The highest level of h\textsubscript{ENaC} expression was observed in kidney and lung. Moderate levels of h\textsubscript{ENaC} expression were detected in liver and pancreas, and weak expression was detected in heart and placenta. Negligible levels of expression were detected in brain and skeletal muscle. This pattern of h\textsubscript{ENaC} expression is similar to the previous reports based on Northern blot analysis (19, 38). The general expression level of h\textsubscript{ENaC}x, h\textsubscript{ENaC}−19, and h\textsubscript{ENaC}+22 was negligible in most cDNA libraries except in the heart and lung cDNA libraries (Fig. 2B).

h\textsubscript{ENaC}−19 expression as translated proteins and in Xenopus oocytes. The h\textsubscript{ENaC}−19 and wild-type h\textsubscript{ENaC} were transcribed in vitro with T7 RNA polymerase to generate cRNAs. These cRNAs were either...

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**Fig. 2.** Tissue distribution of h\textsubscript{ENaC} subunits. h\textsubscript{ENaC}x (A), h\textsubscript{ENaC}−19 (B), and h\textsubscript{ENaC}+22 (C) were coamplified with wild-type h\textsubscript{ENaC} from 15 independently prepared human cDNA libraries derived from brain (B), heart (H), kidney (K), liver (LV), lung (LG), pancreas (PA), placenta (PL), and skeletal muscle (SM). H441 cells were used as a control to coamplify splice variants of h\textsubscript{ENaC} subunit mRNAs. Results show that control h\textsubscript{ENaC}x is the most abundantly expressed h\textsubscript{ENaC} subunit mRNA in H441 cells, compared with splice variants. Same results were obtained in tissue distribution studies, suggesting that h\textsubscript{ENaC}x, h\textsubscript{ENaC}−19, and h\textsubscript{ENaC}+22 are all minor transcripts in normal conditions. Highest level of h\textsubscript{ENaC} expression was detected in kidney and lung, followed by liver, pancreas, heart, and placenta. Negligible levels of h\textsubscript{ENaC} expression were detected in brain and skeletal muscle. Interestingly, a high level of h\textsubscript{ENaC}−19 expression (asterisk) was observed in heart cDNA library. A weak expression of h\textsubscript{ENaC}−19 (asterisk) was also observed in lung cDNA library.
injected into Xenopus oocytes for functional expression or translated with the reticulocyte lysate system to confirm their ability to make a protein. For example, in vitro translation of h\(_{\alpha}ENaC\)-19 and wild-type h\(_{\alpha}ENaC\) (as shown in Fig. 3) produced proteins of 74 ± 2 and 77 ± 2 kDa, respectively, consistent with the expected size of 19-amino acid-deleted or wild-type h\(_{\alpha}ENaC\)s (19).

The h\(_{\alpha}ENaC\) cRNAs were expressed in Xenopus oocytes in combination with human \(\beta\) - and \(\gamma\) -subunits of ENaC, which are known to potentiate the level of amiloride-sensitive currents in oocytes in combination with human \(\alpha\) ENaC (as shown in Fig. 3) produced proteins of 74\(\times\)19 kDa and ENaC and ENaC \(\beta\) - and \(\gamma\) -subunit cRNAs (Fig. 4). Amiloride-sensitive currents at \(-100\) mV holding potential averaged \(-1,479 ± 193\) nA with 96 mM NaCl and \(-2,240 ± 245\) nA with 96 mM LiCl in the bath, respectively (\(n = 11\) oocytes in each series). Replacement of the bath solution with 96 mM KCl did not generate any amiloride-sensitive currents, consistent with previous reports by other laboratories (4, 18).

![Fig. 3: A: in vitro-transcribed cRNA of h\(_{\alpha}ENaC\)-19 (lane 1) and wild-type (WT) h\(_{\alpha}ENaC\) (lane 2) analyzed on 1.2% agarose-2.2 M formaldehyde denaturing gel. B: in vitro translated proteins (arrows) of h\(_{\alpha}ENaC\)-19 (lane 1) and wild-type h\(_{\alpha}ENaC\) (lane 2) analyzed on 10% SDS-polyacrylamide gel. Lane 3 represents a control (CT) in vitro translation experiment (without cRNA addition), showing that \(-31\) kDa represents a nonspecific band. Note that cRNAs as well as translated proteins corresponding to h\(_{\alpha}ENaC\)-19 are smaller than those of control h\(_{\alpha}ENaC\), consistent with sequence information.](image-url)

In contrast to wild-type h\(_{\alpha}ENaC\), substantially reduced levels of amiloride-sensitive currents were observed in oocytes with h\(_{\alpha}ENaC\)-19 and equal amounts of ENaC \(\beta\) - and \(\gamma\) -subunit cRNAs (\(n = 18\) oocytes); amiloride-sensitive currents at \(-100\) mV averaged \(-119 ± 30\) nA with 96 mM NaCl in the bath. We also tried to express h\(_{\alpha}ENaC\)-19 cRNAs with 25 ng cRNA, an amount that was fivefold greater than the normal amount of \(\alpha\)-subunit (5 ng cRNA) that was injected into each oocyte. Despite the increased amount of injected cRNA, there was not any increase in the amiloride-sensitive currents (\(n = 4\) oocytes). Furthermore, we coexpressed h\(_{\alpha}ENaC\)-19 cRNA with carboxy terminus-truncated \(\beta\)-subunits (K100 construct, Oh and Warnock, unpublished data) together with wild-type hENaC \(\gamma\)-subunit, but still substantially reduced levels of amiloride-sensitive currents (\(-179 ± 38\) nA; \(n = 11\) oocytes) were observed. The carboxy terminus-truncated ENaC \(\beta\)-subunit is a gain-of-function mutation that has been shown to increase expressed amiloride-sensitive ENaC activity in oocytes by at least threefold (26). The possible dominant negative effect of the h\(_{\alpha}ENaC\)-19 on wild-type ENaC expression was examined by coexpressing a 1:1 mixture of wild-type h\(_{\alpha}ENaC\) and h\(_{\alpha}ENaC\)-19 together with \(\beta\)- and \(\gamma\)-subunits. The results showed that there was no dominant negative effect of the h\(_{\alpha}ENaC\)-19 on wild-type ENaC expression; amiloride-sensitive currents at \(-100\) mV averaged \(-972 ± 206\) nA with 96 mM NaCl in the bath (\(n = 20\) oocytes).

We were surprised to find very little amiloride-sensitive current in oocytes expressing the h\(_{\alpha}ENaC\)-19 splice variant. Therefore, to eliminate any undefined mutations and/or nucleotide sequencing errors, we exchanged a 501-bp fragment of the extracellular region of the wild-type h\(_{\alpha}ENaC\) with the homologous h\(_{\alpha}ENaC\)-19 region (444 bp; encompassing the 57-bp deletion site) after digestion of both plasmids with EcoRI and SacII. After confirmation of the successful exchange of sequences between the wild-type h\(_{\alpha}ENaC\) and h\(_{\alpha}ENaC\)-19 by restriction mapping and sequencing analyses, cRNA was synthesized in vitro from the linearized new h\(_{\alpha}ENaC\)-19 construct (19-amino acid-deleted construct based on the wild-type h\(_{\alpha}ENaC\) sequences) and the new wild-type h\(_{\alpha}ENaC\) (control h\(_{\alpha}ENaC\) construct based on the h\(_{\alpha}ENaC\)-19 sequences). The cRNAs were then expressed in oocytes together with hENaC \(\beta\) - and \(\gamma\)-subunit cRNAs. Large
amiloride-sensitive currents were observed in oocytes expressing the new wild-type h\(\alpha\)ENaC; amiloride-sensitive currents at \(-100\, \text{mV}\) holding potential averaged \(-2,900 \pm 677\, \text{nA}\) with 96 mM NaCl in the bath (\(n = 8\) oocytes). In contrast, an extremely low level of amiloride-sensitive currents was observed in oocytes expressing the new h\(\alpha\)ENaC\(_{219}\) (\(n = 26\) oocytes). Therefore, it is likely that the loss of function in the h\(\alpha\)ENaC\(_{219}\) is due to the specific deletion of 19 amino acids from the extracellular domain of h\(\alpha\)ENaC and not from undetected nucleotide errors and/or minor nucleotide differences between the wild-type h\(\alpha\)ENaC and h\(\alpha\)ENaC\(_{219}\). In this respect, it is interesting to note that artificial modification of the extracellular domain involving different regions from the deleted 19 amino acids in h\(\alpha\)ENaC, such as deletion of a putative amiloride-binding site (3, 14) or insertion of a FLAG epitope (6), can produce Na\(^+\) currents in oocytes, further strengthening the hypothesis that the 19 amino acids deleted in the h\(\alpha\)ENaC\(_{219}\) splice variant have a critical role in the assembly and/or functional expression of the ENaC complex.

To confirm further the importance of the 19 amino acids deleted in the h\(\alpha\)ENaC\(_{219}\), we have constructed a rat homologue (r\(\alpha\)ENaC\(_{219}\)) to h\(\alpha\)ENaC\(_{219}\) by substituting the corresponding 19 amino acids with 2 novel amino acids using PCR-directed in vitro mutagenesis. Coexpression of wild-type rat \(\alpha\)-, \(\beta\)-, and \(\gamma\)-subunits produced large amiloride-sensitive currents at \(-100\, \text{mV}\) holding potential, averaging \(-3,063 \pm 672\, \text{nA}\) with 96 mM NaCl in the bath (\(n = 5\) oocytes). In contrast, greatly reduced amiloride-sensitive currents were observed in oocytes expressing r\(\alpha\)ENaC\(_{219}\) in combination with wild-type rat \(\beta\)- and \(\gamma\)-subunits; amiloride-sensitive currents at \(-100\, \text{mV}\) averaged \(-403 \pm 144\, \text{nA}\) with 96 mM NaCl in the bath (\(n = 8\) oocytes; Fig. 5).
hαENaCx and hαENaC+22 expression in Xenopus oocytes. The cRNAs of hαENaCx and hαENaC+22 were synthesized in vitro, and their functional competence was tested in the Xenopus oocyte expression system. Amiloride-sensitive currents were undetectable in oocytes expressing hαENaCx (n = 12 oocytes), which is consistent with previous observations (16). Interestingly, often undetectable or very small (~20 nA) and unstable amiloride-sensitive currents were observed in oocytes expressing hαENaC+22 (n = 17 oocytes), which was another unexpected finding. Therefore, similar to what was done with the hαENaC−19 splice variant, we exchanged the extracellular region of the wild-type hαENaC with the homologous hαENaC+22 region to eliminate any undefined mutations and/or nucleotide sequencing errors in hαENaC+22. In oocytes expressing this newly constructed hαENaC+22 together with wild-type human β- and γ-subunits, amiloride-sensitive currents were negligible (n = 16 oocytes), suggesting that the cysteine-rich domain of hαENaC where 22 amino acids are inserted is also a critical region for normal function of the ENaC complex.

Determination of exon-intron splicing junctions in hαENaC. Alternative splicing of the primary RNA transcript is one of the key mechanisms for generating structural and functional diversity of many membrane proteins (29). At present, the genomic structure of the hαENaC has not been published, although a single gene has been localized to chromosome 12 by several laboratories (19, 20, 37). Functional isoforms corresponding to splice variants of hαENaC could be derived from alternative RNA splicing mechanisms. To test this hypothesis, exon-intron boundaries of the extracellular domain of hαENaC (emphasizing the area encompassing the 57-bp deletion site) were determined using genomic DNA-PCR and subsequent nucleotide sequence analysis. The results demonstrated an exon-intron splice junction at the 5′ end of the 57-bp deletion site (Fig. 6A). Two more exon-intron splice junctions were identified downstream from the 57-bp deletion site. Detailed examination of the 57-bp deletion region demonstrated conserved nucleotide sequences for the 5′ and 3′ splice sites and a pyrimidine-rich tract near the 3′ splice site, which are the consensus sequences recognized by spliceosomes (29). Therefore, as a mechanism for generating the hαENaC−19 splice variant, it can be speculated that these 57 bp can be spliced out under certain circumstances using an internal splicing site of an exon, which is one well-known RNA splicing pattern (29).

Fig. 5. Expression of rat αENaC−19 in oocytes. Amiloride-sensitive Na+ currents recorded at a holding potential of −100 mV after expression of wild-type rat αENaC (Rat-WT) or rat αENaC−19 (Rat−19) together with wild-type rat β- and γ-subunits. Note substantially reduced amiloride-sensitive currents with rat αENaC−19 expression, consistent with finding in hαENaC−19 expression.

Fig. 6. A: exon-intron boundaries in extracellular region of hαENaC. In present study, 3 exon-intron splicing sites were identified (arrows). The 19 amino acids deleted in hαENaC−19 are boxed; there is an exon-intron splicing site at 5′ end. Nucleotide sequences of this deleted region (57 bp) contain conserved sequences for 5′ and 3′ splice sites as well as a pyrimidine-rich tract near 3′ splice site, suggesting that these 57 bp can be recognized by spliceosomes and spliced out in certain conditions. B: sequence alignment of hαENaC+22 with splice variants identified from rat taste tissue (rαENaCa and rαENaCb) (16) and chicken cochlear cDNA library (ccαENaC) (15). Results show that these splice variants use same splicing site in extracellular domain (arrows).
DISCUSSION

The present study demonstrates the presence of three novel splice variants of the extracellular domain of h\(_{\alpha ENaC}\) subunits, which appear to be derived from alternative RNA splicing mechanism. Furthermore, functional expression studies of these splice variants demonstrate that they generate highly reduced or often undetectable amiloride-sensitive Na\(^+\) currents in Xenopus oocytes, indicating the importance of the extracellular domain in the expression of functional ENaC complexes in the membrane. Similar observations have been made in an ENaC-related invertebrate gene, degenerin, which has been found to contain an extracellular regulatory domain; this regulatory domain is not found in any of the mammalian ENaC proteins (7). Caenorhabditis elegans, which express a mutant degenerin, MEC-4, with a nine-amino acid deletion at this regulatory domain, undergo neurodegeneration, suggesting that this region negatively regulates degenerin function (8). Therefore, it seems that the ENaC gene superfamly contains an important regulatory domain in the extracellular region, which can cause either gain of function or loss of function. The present study also emphasizes the importance of carefully examining the full-length coding regions of ENaC mRNAs, because rather subtle changes that may not be detected by commonly used mRNA detection methods (i.e., Northern blot and in situ hybridization analyses) may produce dramatically altered function in a cell. In this respect, there have been several reports demonstrating the presence of mRNAs in certain cells or tissues without any corresponding proteins and/or functional activity of the mRNAs (9, 10, 32). It is possible that relatively minor changes, such as the alternatively spliced 57-bp deletion or 66-bp insertion described herein, could account for the loss of functional activity in these other examples.

The presence of splice variants of the \(\alpha\)ENaC subunit has recently been described in rat taste tissues (16) and in a chick cochlear cDNA library (15). In rat taste tissue, two \(\alpha\)-subunit splice variants were found with nucleotide deletions that introduced a premature stop codon at the extracellular domain and resulted in prematurely truncated proteins. Interestingly, these two splice variants share the same splicing site with the h\(_{\alpha ENaC+C+22}\) variant (Fig. 6B). One of these splice variants from rat taste tissue was functionally characterized to show that the truncated protein that lacks the second transmembrane domain failed to generate amiloride-sensitive Na\(^+\) currents when expressed in Xenopus oocytes. Killick and Richardson (15) have found a splice variant of the \(\alpha\)-subunit of chick ENaC with the addition of two exons of 163 and 276 bp into the extracellular domain. The second exon of this splice variant is introduced at the same splicing site as the h\(_{\alpha ENaC+C+22}\) variant (Fig. 6B), suggesting that this splicing site is commonly used in various species to produce splice variants of the \(\alpha\)ENaC subunit. Taken together, these findings indicate that in addition to transcriptional regulation of ENaC subunit genes, alternative RNA splicing might represent an additional mechanism for the regulation of ENaC activity.

Linkage analysis has demonstrated an association of ENaC gene mutations with pseudohypoaldosteronism type 1, an inherited human disorder characterized by salt wasting, hyperkalemic acidosis, and unresponsiveness to mineralocorticoids, consistent with loss-of-function mutations of the ENaC complex (5, 31). Chang et al. (5) found two different premature truncations of the h\(_{\alpha ENaC}\) occurring either before the first transmembrane domain or before the second transmembrane domain. Expression of these mutations in Xenopus oocytes generated no amiloride-sensitive currents (5), consistent with the pathophysiology of pseudohypoaldosteronism type 1. The h\(_{\alpha ENaC}\) splice variant identified in this study represents another isoform of prematurely truncated h\(_{\alpha ENaC}\), and the lack of generation of amiloride-sensitive currents in h\(_{\alpha ENaC}\)-expressing oocytes is consistent with the observation by Chang et al. (5).

Although the mechanisms for physical assembly of ENaC complexes and targeting and maintenance of ENaCs in the plasma membrane have not yet been defined, the loss of ENaC activity in oocytes expressing h\(_{\alpha ENaC–19}\) or h\(_{\alpha ENaC +22}\) could be explained by any of the following mechanisms. First, the h\(_{\alpha ENaC}\) splice variant-containing ENaCs may not function properly in the plasma membrane due to the critical involvement of the missing 19 amino acids or the disrupted cysteine-rich domain in h\(_{\alpha ENaC+C+22}\). Second, the assembly of h\(_{\alpha ENaC}\) with \(\beta\)- and \(\gamma\)-subunits may not be proper, so that the final assembled h\(_{\alpha ENaC}\) splice variant-containing ENaC complexes cannot be inserted into the membrane and/or are unstable in the cytoplasm. The possibility of improper assembly of ENaC complexes and/or abnormal insertion of h\(_{\alpha ENaC}\) splice variant-containing ENaC complexes is supported by the observations that there was no increased ENaC activity even after injection of 25 ng of h\(_{\alpha ENaC–19}\) cRNA into the oocyte (a fivefold increase compared with the usual amount) and that coexpression with carboxy terminus-truncated \(\beta\)-subunit and wild-type \(\gamma\)-subunit did not result in any increased ENaC activity. It has been shown that this truncation mutation of the \(\beta\)-subunit described in the original pedigree with Liddle's syndrome can cause abnormally regulated, highly activated ENaC complexes when expressed in oocytes (26, 30, 40). A recent approach described by Firsov et al. (6), who expressed FLAG epitope tagged ENaC in oocytes and quantitated the expression level of ENaC at the cell surface using \(^{125}\)I-labeled anti-FLAG monodonal antibody, may be useful in the future to distinguish between assembly and impaired function of ENaC expressed at the cell surface.

ENaC expression is not restricted to Na\(^+\)-absorptive epithelial cells. In this study we have detected weak-to-moderate levels of h\(_{\alpha ENaC}\) mRNA expression in liver, pancreas, heart, and placenta, where ENaC activity has not been reported (Fig. 2). Interestingly, the expression of ENaC mRNAs in nonepithelial tissues has been consistently reported by several laboratories (15, 18,
The patch-clamp technique has also been used to demonstrate the presence of ENaC-like channel activities in vascular smooth muscle cells (34), thyroid cells (35), human B lymphocytes (2), and brain endothelial cells (36). If ENaC mRNAs are processed to make functional ENaC proteins in these cells, then their physiological roles may be very different from the classical role of ENaCs in absorptive epithelia.

Identification of alternatively spliced variants of the αENaC subunit in human (this study) and in other species (15, 16) indicates a possible heterogeneity of multimeric ENaC structure and, possibly, varied functional roles of ENaCs in different tissues. Under certain circumstances, it is possible that certain splice variants of the αENaC subunit can be preferentially produced via alternative RNA splicing to serve as a regulatory component for ENaC activity. In this respect, the three functional ENaC splice variants described herein, which show a loss of channel function in oocytes, may play a role as a negatively acting component for ENaC activity. In this respect, the three functional ENaC splice variants described herein, which show a loss of channel function in oocytes, may play a role as a negatively acting component for ENaC activity. In this respect, the three functional ENaC splice variants described herein, which show a loss of channel function in oocytes, may play a role as a negatively acting component for ENaC activity. In this respect, the three functional ENaC splice variants described herein, which show a loss of channel function in oocytes, may play a role as a negatively acting component for ENaC activity. In this respect, the three functional ENaC splice variants described herein, which show a loss of channel function in oocytes, may play a role as a negatively acting component for ENaC activity.

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