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Short Sequence Paper

Cloning of an organ of Corti anion exchanger 2 isoform with a truncated C-terminal domain ☆

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Abstract

We have isolated a cDNA clone from a guinea pig organ of Corti library encoding a new isoform of the Anion Exchanger 2 (AE2) protein. This cDNA clone shows an 83 bp deletion in the region that encodes the membrane domain of AE2. Analysis of the overlapping regions of genomic and cDNA clones indicates that the missing portion does not correspond exactly to a constitutive exon. The alternate splicing process that generates this transcript involves internal donor and acceptor sites which introduces a shift in the open reading frame. The resulting polypeptide has a conserved cytoplasmic N-terminal domain but the membrane C-terminal domain has only two of the fourteen membrane spanning regions. An affinity-purified antipeptide antibody to the novel C-terminus detects an 89 kDa polypeptide which agrees with the molecular mass predicted from the cDNA.

Keywords: Anion exchanger; Alternative splicing; AE2; (Organ of Corti)

Anion exchangers (AE) are a family of closely related, integral membrane proteins of ubiquitous occurrence which facilitate the exchange of anions across the plasma membrane and provide a structural linkage between the bilayer and the membrane cytoskeleton. They are the product of three distinct genes, each of which transcribes multiple forms of mRNA [1]. The products of these genes are designated AE1, AE2, and AE3 and are expressed in a tissue-specific manner [1]. All three genes are known to generate alternative spliced transcripts [2–4]. Hydrophobicity plots and physicochemical methods indicate that all of the AEs have two domains. The C-terminal domain contains the membrane spanning region of the molecule which is thought to traverse the lipid bilayer up to fourteen times [1]. This domain is essential for catalyzing the anion exchange across the plasma membrane. The N-terminal domain protrudes into the cytoplasm where it binds several cytosolic and cytoskeletal proteins [1]. The region of highest sequence conservation among the polypeptide products of the three AE genes occurs in the membrane domain. In contrast, considerable differences are commonly found in the cytoplasmic domain. The cytoplasmic domain of the AE1 proteins is approx. 320–400 amino acids long, but it is approx. 700 amino acids in the AE2 and AE3 proteins.

The functions associated with AE proteins are particularly relevant to the mammalian hearing organ, the organ of Corti. Plasma membrane linkages to underlying membrane skeleton are critically important for the auditory function [5–7]. Previous results have shown that members of the AE protein family are present in the organ of Corti [8]. In order to determine the molecular nature of the AE isoforms present in the sensory epithelium, we have screened an organ of Corti cDNA library and cloned an alternative spliced transcript that encodes a novel isoform of AE2, named AE2α.

A fragment of mouse kidney AE1 cDNA [9] was amplified by PCR (5′ AGG TGT TGG CTG CTG TCA TC 3′, upper primer and 5′ GAG GTT TGG GCT TCA TCA CA 3′, lower primer). A fragment of ~450 bp from nucleotides 1394 to 1851 encoding a region within the conserved intramembranous domain, was obtained. A guinea pig organ of Corti cDNA library (kindly provided

☆ The sequence data reported in this study have been deposited in the GenBank data base (accession Nos. U20523 and U20524).

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by J. Fex and E. Wilcox [10]) was plated at a density of 10^5 colonies/150 mm plate. Replica filters were prepared from 10 plates using standard procedures and hybridized at reduced stringency (overnight at 42° C in the presence of 6 × SSPE, 10 × Denhardt's solution, 1% SDS, 100 μg/ml denatured salmon sperm DNA and 50 ng of the ^{32}P-labeled mouse band 3 cDNA fragment, at a specific activity of $> 5 \cdot 10^8$ cpm/μg). Medium stringency washes were performed twice for 15 min each at 55° C in the presence of 2 × SSPE, 0.1% SDS and then for 30 min in 1 × SSPE, 0.1% SDS at the same temperature. The filters were examined by autoradiography, and six positive clones were isolated under these conditions. Plasmid DNA was purified using the Magic™ plasmid purification kit (Promega) and analyzed by restriction endonucleases digestion. Clones were sequenced by using the dideoxy chain-termination method. Reactions were performed initially with fluorescent dye-labeled M13-21 and Reverse primers, and then with sequence-specific primers using the fluorescent dye-labeled dideoxy terminators (Applied Biosystems). The sequencing protocol for dye-primers was established at 95° C-30 s, 55° C-30 s, 70° C-60 s, for 15 cycles and other 15 cycles at 95° C-30 s, 70° C-60 s. Dye-terminators were 96° C-30 s, 50° C-15 s, 60° C-4 min for 25 cycles. Samples were run on a 373A automated DNA sequencer (Applied Biosystems). Sequences were analyzed using the DNASTAR software and data base searches were run against the EMBL-GenBank Release 75 and the Swiss-PIR-Translated Genbank Release 24. Only two clones showed a significant DNA similarity with anion exchanger sequences. They scored a similarity index above 95% with rat, mouse and human AE2 mRNA. These cDNA clones were ~ 2.7 kb and ~ 3.6 kb as determined by the release of the inserts from the vector using the restriction enzymes *Not*I and *Sal*I. Identical 3′ end sequences indicated that both clones belong to the same class.

The complete nucleotide sequence of the longer insert (~ 3.6 kb) was determined (Fig. 1). This insert corresponds to a near full length AE2 cDNA, missing about 200 bp of coding sequence from the 5′ end. Its sequence is highly homologous to the conventional AE2 cDNA. However, a segment of 83 bp is deleted at position 1929. Due to this missing segment, a shift in the open reading frame is introduced. While the conventional AE2 cDNA encodes about 520 residues after nucleotide 1929, the new reading frame has a stop codon at position 2190, only 260 bp downstream the deletion point. Analysis of the nucleotide sequence of the 2.7 kb clone showed identical deletion and

Fig. 1. AE2α sequence. Nucleotide and predicted amino acid sequences of the AE2α cDNA. Asterisk represents stop codon.

shift in the open reading frame. The sequences were confirmed in the complementary strands of both clones.

We analyzed the genomic structure to determine whether the missing portion corresponds to a complete exon. A region including the alternative splicing junction of the novel isoform AE2α was cloned from guinea pig genomic DNA by PCR. Two primers (5′ GCC GCT ACC CCC ACT ACC 3′ and 5′ CAA AAG CAC CAG CCA GAA 3′) designed from nucleotides 1833–2070 of the AE2α sequence, were selected. PCR reactions contained 100 pmoles of primers in a 100 μl volume. Reactions proceeded at 95° C-1 min, 55° C-2 min and 72° C-2 min, for 35 cycles. A PCR product of approx. 500 bp was obtained using genomic guinea pig DNA as template. The PCR product was gel purified and subcloned into pCR™ II (Invitrogen), using the TA cloning system. The constructs were introduced by electroporation into *Escherichia coli* DH10B (BRL) and the recombinant clones were sequenced.

Sequence analysis of this clone shows the presence of two introns of 76 and 92 bp, respectively (Fig. 2). The sequences surrounding the exon/intron boundaries follow the consensus GT-AG rule. By splicing these introns, an open reading frame for AE2 is generated. Although there is not available information regarding the exon-intron structure of the AE2 gene, the exon-intron boundaries of this guinea pig genomic fragment seems to correlate with the structure of the mouse AE1 gene [9], so that the 76 bp intron would correspond to the mouse AE1 intron 11, and the 92 bp to intron 12. Therefore, we refer to these introns as n11 and n12, respectively. The exons are numbered according to the intron located at its 3′ end. When this genomic clone is aligned to the both insert cDNA sequences, the homologies show that not only intron n11 has been removed but also the flanking sequences from the adjacent exons n11 and n12 are removed as well. This is accomplished by using alternative donor and acceptor sites. The donor site, located 24 bp upstream of the exon n11-intron n11 splicing junction, is atypically CAG/CGAT. In contrast, the acceptor site, placed 59 bp downstream the intron n11-exon n12 boundaries, fits the consensus with the sequence TCCAG/GGTG. Therefore, analysis of the overlapping regions on the genomic and cDNA clone indicates that the deleted region includes a complete intron as well as the two flanking regions from the adjacent exons.

In AE2α the splicing process uses internal donor and acceptor sites. The conventional AE2 cDNA is generated by removing the 76 bp intron n11 which donor and acceptor sites are G/GTAAA and CCCCTAG/G, respectively, being both compatible with the GT-AG rule. However, the processing of AE2α utilizes different sites located within exon sequences at 24 bp upstream and 59 bp downstream the sides of this intron. The 5′ splice site used in this case is unusual. Its sequence is CAG/CGATCA and does not follow the GT-AG rule. The acceptor site follows the rule, being CTCCAG/G. This alternate mode

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GCCGCTACCCCCACTACCTGAGTGACTTCCGCGATGCGCTTGACCC
GCAGTGCCTGGCTGCTGTCATCTTCATCTACTTTGCGGCTCTGTCT
CCAGCGATCACCTTTGGAGGGCTGTTGGgtaaaggagtgctctggggaggagc
ggggtgtacagagtacagggcccagctcctgaacctgtccctgacccctagGA
GAGAAGACGCATGACCTGATAGGTGTGTCGGAGCTCATCATGTCCAAAGCCCT
CCAGGGTGTCATTTTCTGCCTGCTGGGGGCTCAGCCACTGCTGGTGA
TTGGCTTCTCGGGGCCGCTGCTGGTCTTCGAGGAAGCCTTCTACTC
Ggtagggctctccttgccctgcaccagtgctgttctctccctgtcctcctctt
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CTGGCTGGTGCTTTTG
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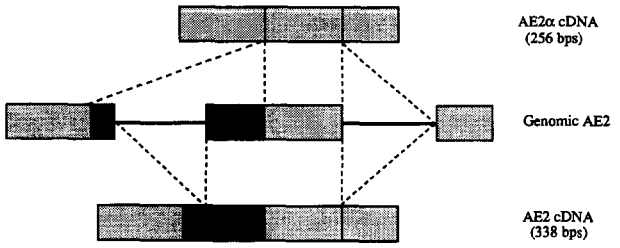

Fig. 2. Genomic DNA sequence of the alternatively spliced region of the AE2 gene. (A) Genomic guinea pig DNA was amplified with specific PCR primers. Exons are shown in boldface, introns in lower case, and exon spliced portions in upper case. (B) Schematic comparison of the AE2 genomic structure with AE2 and AE2α cDNAs. Single lines represents introns and boxes exons. Darker areas in boxes correspond to deleted portions of the exons. Dotted lines illustrate spliced sequences.

of splicing has been described in other genes, such as in the dopa decarboxylase gene [11]. and the genes for prohormones of the gastrin-releasing peptides [12]. Studies have shown that the presence of the AG dinucleotide in the splice acceptor sequence is indispensable to accomplish the processing [13]. However, less strict requirements exist for the donor site. Evidence shows that a donor site having one dinucleotide different from the canonical GT (GC, for instance) can still be spliced [14]. Moreover, some non-conforming donor splice sites, such as GC and CT have been found [15].

A comparison of the rabbit AE2 and the guinea pig AE2α sequences shows 88% similarity for the first 600 amino acids of the N-terminal (cytoplasmic) domain. The C-terminal region shows no significant similarity with AE2. AE2α only contains two hydropathic regions in comparison with the fourteen present in the conventional AE as shown in the Kyte-Doolittle plot (Fig. 3). The analysis of the Karplus flexibility profile was used to propose the limits of each domain. The cytoplasmic domain of this novel isoform is likely to keep the structural role played by AEs linking the cytoskeleton to the plasma membrane. In contrast, the absence of binding sites for DIDS as well as other anion transport inhibitors in AE2α suggest that the anion exchanger activity could be completely or partially abolished. According to the hydropathy plot and the flexibility profiles (Fig. 3), AE2α would only have two probable transmembrane domains instead of the

14 transmembrane helices typical in AEs. The analysis of the AE2α transmembrane region shows no significant similarities with any protein sequence in the data banks. Evidence for the existence of another C-truncated isoform has been recently published [16]. 14-AE3p, an isoform of AE3 present in rat brain lacking the entire transmembrane domain, is generated through an alternative splicing process similar to that suggested for AE2α.

Tissue expression of AE2α was checked through RT PCR on guinea pig cDNA samples from several organs. Total RNA was isolated from the organ of Corti as well as several other guinea pig tissues [17]. Poly(A)$^+$ mRNA was purified using magnetic beads coupled to oligo(dT)$_{25}$ (Dynabeads®, DYNAL). cDNA was synthesized using oligo(dT) and SuperScript™ RT (BRL). The set of primers utilized was the same as the one used for the amplification of the genomic clone. PCR reactions were allowed for 38 cycles at 95° C-1 min, 52° C-2 min, and 72° C-2 min. Amplification products were fractionated by agarose gel electrophoresis and transferred to a nitrocellulose membrane. DNA was fixed to the membrane by UV cross-linking using a Stratagene Stratalinker® 1800. To verify the nature of the amplified PCR products, the blot was tested with a 21-mer internal oligonucleotide probe from position 1981 (5′ CTC GGG GCC GCT GCT GGT CTT 3′). Hybridization proceeded overnight at 55° C, in the presence of 6× SSPE, 10× Denhardt's solution, 1% SDS, 100 μg/ml denatured salmon sperm DNA, and 5 pmoles of the 5′ end ^{32}P-labeled oligonucleotide. The oligonucleotide specific activity was $> 10^8$ cpm/μg. Washes were done twice at room temperature for 15 min each, in 6× SSPE, 0.1% SDS with a final wash of 20 min at 55° C, in the same buffer, followed by autoradiography. Two bands of 338 bp and 256 bp were obtained. These bands had the expected sizes for the portion flanked by the primers on both, the standard AE2 and the spliced message. They hybridized when probed with an internal oligoprobe (Fig. 4). The 256 bp fragment was subcloned and sequenced showing 100% similarity with AE2α. The internal oligoprobe also labeled three other bands of 370 bp, 210 bp and 150 bp. These bands could represent other uncharacterized spliced products.

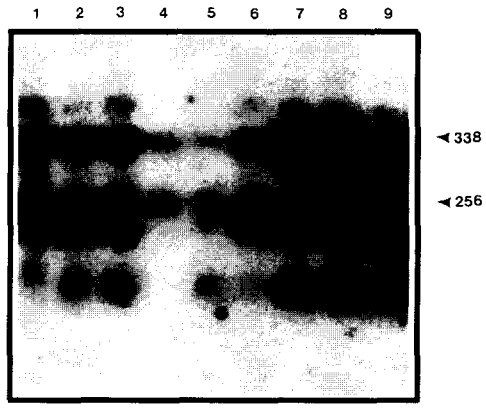

Fig. 4. Tissue distribution of AE2α mRNA. Poly(A)$^+$ from several tissues was analyzed by RT-PCR. Primers described in text were used to amplify the region that flanks the deletion. The amplified products were fractionated by agarose gel electrophoresis and blotted. The membrane was probed with a 21-mer internal oligonucleotide. Two expected bands of 338 bp and 256 bp were obtained in all the tissues tested. Their sizes correspond to the conventional AE2 mRNA and to the AE2α spliced message, respectively. The 256 band was sequenced, showing an exact identity with AE2α. Three other bands of 370, 210 and 150 bp are also labeled with the internal probe. They could represent other still unknown splice messages. Tissue lanes are guinea pig mRNA from: 1, brain; 2, pancreas; 3, tongue; 4, retina; 5, organ of Corti; 6, rib cage (bone, muscle, and connective tissues); 7, liver; 8, stomach; and 9, kidney.

An anti-AE2α specific antiserum was raised against a peptide localized within the novel C-terminal domain. The peptide NH_2-LHPGDLRLPHLP-COOH was synthesized by Bio-Synthesis (Lewisville, TX). It corresponds to amino acids 708 to 719 in the novel AE2α (Fig. 2). A polyclonal antibody against this peptide was raised in rabbits by TSI Labs (Kensington, MD). The antibody was affinity-purified using Immunopure Ag/Ab immobilization kit (Pierce, Rockford, IL). Whole extracts from cochlea and tongue proteins were resolved in 10% SDS-PAGE gels and transferred to nitrocellulose using a MilliBlot SDE Transfer

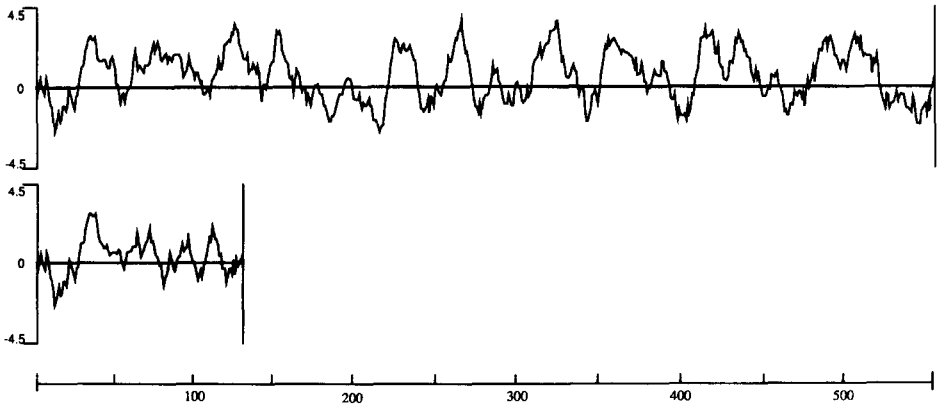

Fig. 3. Hydropathy profiles of AE2 (top) and AE2α (bottom). Hydropathy profiles of rabbit AE2 and AE2α were determined according to the procedure of Kyte and Doolittle using a window of 9 amino acids. Amino acid numbers are plotted on the abscissa. Hydropathy scores are plotted on the ordinate.

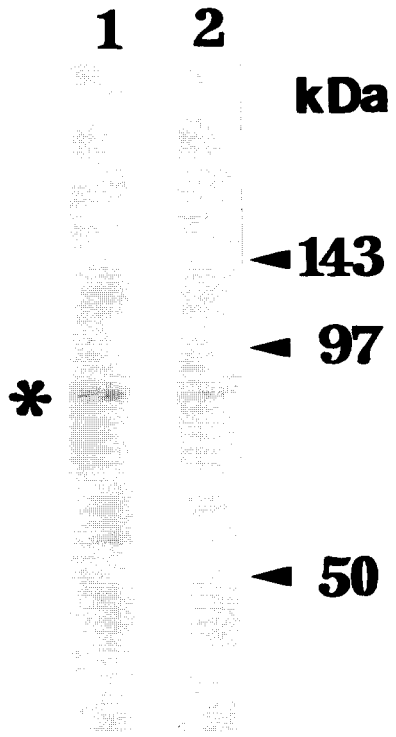

Fig. 5. Western blot showing reactivity of affinity-purified antipeptide antibody to the novel C-terminus of the AE2α in samples of cochlea (lane 1) and tongue (lane 2). An ~89 kDa band (*) is evident in both samples.

System (Millipore, Bedford, MA). With this affinity-purified anti AE2α antibody, raised against the novel C-terminus, an 89 kDa polypeptide (Fig. 5) was detected. This polypeptide has the molecular mass expected for the AE2α cDNA encoded product, providing evidence that such protein is indeed expressed in cochlea and tongue tissues.

In summary, we have isolated a near full length cDNA clone from a guinea pig organ of Corti library, designated AE2α, that encodes a new isoform of the AE2 protein. The deduced polypeptide has a conserved cytoplasmic N-terminal domain and a membrane C-terminal domain with only two of the fourteen membrane spanning regions present in the conventional AE2.

The plasma membrane of auditory hair cells is the site for mechano-electrical as well as electromechanical transduction [7]. Coupling of the pliant plasma membrane to the underlying actin filaments in the stereocilia, cuticular plate or the lateral cortical lattice is required for effective mechanical transduction [7]. The possibility that the anion exchanger activity is completely or partially abolished in AE2α is suggestive of a dedicated structural function that fits the requirements for a plasma membrane-actin filament linking protein in hair cells. Other cell biological studies are being carried on to further characterize this molecule and its function in the plasma membrane of auditory hair cells.

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