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The guinea pig cochlear AE2 anion exchanger: cDNA cloning and in situ localization within the cochlea

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Abstract

This study has characterized the repertoire of the anion exchanger (AE) family members expressed within the guinea pig organ of Corti, the auditory neuroepithelia. Both AE2 and AE3 cDNAs were present, but AE1 cDNA was not detected. The more abundant AE2 was sequenced and its expression characterized in the cochlea. The 3888 base pairs (bp) AE2 sequence, compiled from multiple clones, includes 150 bp of upstream non-coding sequence and 3717 bp of open reading frame encoding a protein of 1238 amino acids. Immunoblot of cochlear homogenate revealed a single AE2-immunoreactive band of M_r 180 kDa. In situ hybridization and immunohistochemical analysis localized AE2 expression to several tissues and cell types within the guinea pig inner ear, including superior half of the spiral ligament and within the interdental cells lining the spiral limbus. However, AE2 was not clearly detected in the outer hair cells (OHC) of the organ of Corti by either immunohistochemistry or in situ hybridization. The results of these studies imply a physiologic role of AE2 in the cochlear homeostasis, but do not support its role as a potential 'motor protein' in mediating the in vitro-observed voltage-gated, ATP-independent OHC motility.

Keywords: Anion exchanger (AE); AE2; Organ of Corti; OHC motility

1. Introduction

The outer hair cells (OHC) and the inner hair cells (IHC) are the two subtypes of mechanosensory cells within the mammalian cochlea. Although, structurally very similar, several differences have been identified between the two cell types that reflect their distinct in vivo function. A particular in vitro characterized OHC trait is its electromotility. Current injection into isolated OHC can change cell length up to 4% [1,2]. This contractile or motile response is unique to the OHC; it is not observed in the IHC or in the hair cells of the vestibular apparatus responsible for our sense of balance. The motility is Ca^{2+} and ATP-independent [3,4], and thus not likely to involve conventional motor proteins, such as actin and myosin, that have been associated with cell movement. The voltage-gated electromotility, also referred to as electromechanical transduction, is postulated to augment the vibrations of the basilar membrane immediately beneath the IHC and OHC, and thereby to amplify the input signal [2,4–6].

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Physiological/biochemical studies investigating this contractile apparatus suggest that the putative motor protein resides within the OHC plasma membrane. The critical experiments supporting this hypothesis include demonstration of OHC motility in isolated OHC plasma membrane, free from its cytoskeletal framework and underlying substructure [7] and its persistence, postintracellular trypsin digestion [8]. Furthermore, it is postulated that changes in cell length are brought about through voltage-gated conformational changes in an abundant transmembrane protein. It is estimated that there exists in each OHC approximately 1.2×10^6 such molecules [7,9,10] whose voltage-gated movement, transverse to the plane of the membrane, is responsible for the 'gating current' that accompanies the OHC motile response.

It is inferred that the force generated in the lateral wall of the plasma membrane needs to be linked with the underlying cytoskeletal framework to effect change in cell shape. Ultrastructural studies of the OHC membrane have demonstrated the presence of a lattice-like structure directly beneath the plasma membrane. Ultrastructural and molecular analysis of the cortical lattice has revealed an architecture and components similar to those identified in the cytoskeleton of the erythrocyte [11]. Similar to the erythrocyte cytoskeleton, the cortical lattice includes actin and spectrin isoforms [12–14]. The spectrin–actin complex of the erythrocyte cytoskeleton is linked to the band 3 protein or anion exchanger 1 (AE1), the major integral plasma membrane protein of the erythrocyte, via the linkage proteins, ankyrin I, protein 4.2, and protein 4.1. Proteins recognized by antibodies raised against erythroid AE1 and protein 4.1 have been detected within the OHC of guinea pigs [15] and gerbils [16]. The relevance of these putative cytoskeletal components of OHC to its motility stems from the recognized importance of actin, spectrin, ankyrin I, protein 4.1 and AE1 to the mechanical stability of the erythrocyte.

AE1, an anion exchanger that facilitates exchange of Cl/HCO_3 across the plasma membrane, is one of a family of related proteins considered to be an important component in the regulation of intracellular pH and volume (for reviews see Kopito [17], Alper [18], and Alper et al. [19]). The anion transport function of AE1 is inhibited by 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid (DIDS), a stilbene disulfonate agent that also exerts inhibitory effect upon OHC electromotility. These data, taken together, suggest the possibility that an AE-like anchor protein could link the OHC cytoskeleton to its plasma membrane, perhaps to mediate or modulate the contractile response, and so functioning as or contributing to the molecular motor in the OHC.

The AE gene family consists of at least three members, AE1, AE2 and AE3, whose cDNAs have been cloned in several species from different tissues or cell types. AE1 (band 3) was the first of the AE anion exchangers to be cloned and characterized [20]. This anion carrier is highly abundant in the red cell membrane, comprising ~25% of total membrane protein, and up to 50% of integral membrane protein, and present in 1–1.2 million copies per human red cell. The role of AE1 in the erythrocyte is to facilitate carriage of CO_2 away from respiring tissues, and to stabilize the cytoskeleton. Genetic deficiency of AE1 has been associated with autosomal dominant forms of spherocytosis [21] and of distal renal tubular acidosis [22,23].

Other members of the AE gene family have been cloned from cDNA libraries probed with AE1 coding sequence under low stringency hybridization. Alternative exon usage has been found to generate multiple mRNA transcripts from each of the three AE anion exchanger genes [24–26]. Physiological roles for the polypeptide products of these alternative transcripts from the AE2 and AE3 genes remain to be determined in intact animals. Outside the red cell, AE1 or related protein has been found in acid-secreting renal intercalated cells, and in cardiac myocytes. In contrast, AE2 is widely expressed in epithelial and non-epithelial cells, with high levels found in choroid plexus and gastric parietal cells [27]. AE3 is expressed at highest levels in excitable tissues including neurons and muscle, as well as in the gastrointestinal tract.

Proteolytic cleavage studies have defined two major structural domains of AE1 and AE2 [28]. The hydrophilic aminoterminal domain of the AE polypeptides is located on the cytosolic side of the membrane, and interacts with several cytoplasmic proteins including ankyrin, that links the plasma membrane with the underlying membrane skeleton. Ankyrin-1 has a binding site for AE1 [29] and for bAE3, but not for AE2 [30]. The ion transport function of the AE proteins is mediated by their carbox-

yl-terminal membrane-spanning domains. Presence of the N-terminal domain from the AE polypeptides is not required for expression of basal anion exchange activity [17]. The highest degree of sequence conservation is in the carboxy-terminal domain, while the amino-terminal domain displays greater variability. Topography experiments on AE1, together with hydropathy analysis of the deduced polypeptide sequences of all the AE polypeptides, suggest that the highly conserved carboxy-terminal domain traverses the lipid bilayer 12 or more times.

An initial step towards assessing a possible role of AEs in OHC motility is to identify and characterize expression of its family members at the RNA and protein level within the OHCs. A candidate gene product for the role of OHC motor protein should meet several criteria: it should be present within the OHC (but not necessarily exclusively), and localized along the length of the lateral OHC membranes [10,31], likely excluding the apical and basal membranes since the force is generated longitudinally. Such candidate motor proteins could then be characterized physiologically for their roles in OHC motility.

Due to the limited number of outer hair cells present within the auditory neuroepithelia and to the relative difficulty of their extraction in an intact and, homogeneous form, we have chosen to assess their genetic repertoire in a cDNA library derived from the entire guinea pig (gp) organ of Corti. This library, generated by Wilcox and Fex [32], represents one of the richest sources of hair cell cDNA. The hair cells represent approximately 5% of the total cells within the organ of Corti. This library has been used previously by Negrini et al. [33] to isolate a novel AE2 cDNA clone. AE2α encodes a truncated AE2 polypeptide of M_r 89 kDa secondary to deletion in the cDNA of 83 bp near the beginning of the membrane-spanning domain, and was detected by RT-PCR in every tissue examined. The predicted 89 kDa polypeptide was detected in cochlear homogenate by immunoblot [33], and antibody to the novel sequence of AE2α immunostained stereocilia and the lateral wall of the OHC, as well as stereocilia of the IHC [34].

In this study, we have screened, at low stringency, the organ of Corti cDNA library to detect members of the AE gene family. AE2 clones were found to be most abundant members of the AE family in the library. The complete coding sequence of gp AE2, together with flanking regions, was determined. Expression of mRNA encoding AE2, but not AE2α, was confirmed in gp organ of Corti by RT-PCR. Expression of AE2 polypeptide was characterized in cochlear homogenate by immunoblot, and in situ by immunohistochemistry and in situ hybridization. The results of this study are discussed in the context of the hypothesis that an anion exchanger of the AE gene family may mediate or contribute to OHC electromotility.

2. Materials and methods

2.1. Materials

Plasmids, pMAE1, pMAE2 and pMAE3 were obtained from R. Kopito (Stanford University). The coding sequence of these AE cDNAs were used to screen a gp organ of Corti cDNA library obtained from E. Wilcox (NIH) [32]. The AE2 antibody raised against mouse AE2 1224–1237 [27]. Specificity of this antibody for immunocytochemistry has been previously characterized in multiple tissues [19,27,35,36].

2.2. Methods

2.2.1. Library screen

Approximately 10^6 clones from an amplified organ of Corti cDNA library were screened with ^{32}P labeled, random primed probe generated from equimolar amounts of AE1, AE2 and AE3 full length coding sequences. Hybridization (42°C) and post-hybridization washes (45°C at 1×SSC) were carried out under low stringency conditions as described by Church and Gilbert [37]. Positive clones were then characterized by restriction digest and sequence analysis of the 5′ and 3′ ends of the inserts.

2.2.2. DNA sequencing and analysis

Individual clones from the library were sequenced using the ABI automated sequencer. T7, SP6, M13 forward and reverse primers and numerous custom made primers were used for sequencing reactions on purified plasmids. In addition, RT-PCR products derived from organ of Corti cDNA using AE2 specific

Fig. 1. Guinea pig AE2 sequence. The 3888 bp sequence consists of 150 nt of 5′ untranslated sequence and an open reading frame (ORF) of 3717 nt that extends from nt 151 to 3870.

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GAGGCAGCACCTACACCGGGGCACGGCAACACAAGCTCAGCTGAGCAGGCTGGAGTGGAG 2820
 Glu  Ala  Ala  Pro  Thr  Pro  Gly  His  Gly  Asn  Thr  Ser  Ser  Ala  Glu  Gln  Ala  Gly  Val  Glu
AGACCCCAGGGCCAGCCCAACACAGCCCTGCTGTCGTTGGTGCTCATGGCTGGCACCTTC 2880
 Arg  Pro  Gln  Gly  Gln  Pro  Asn  Thr  Ala  Leu  Leu  Ser  Leu  Val  Leu  Met  Ala  Gly  Thr  Phe
TTCATTGCCTTCTTCTTGCGAAAATTTAAGAACAGCCGGTTTTTCCCTGGCCGGATCCGG 2940
 Phe  Ile  Ala  Phe  Phe  Leu  Arg  Lys  Phe  Lys  Asn  Ser  Arg  Phe  Phe  Pro  Gly  Arg  Ile  Arg
CGGGTGATTGGGGACTTTGGAGTGCCCATCGCCATCCTCATCATGGTGCTCGTGGATTAC 3000
 Arg  Val  Ile  Gly  Asp  Phe  Gly  Val  Pro  Ile  Ala  Ile  Leu  Ile  Met  Val  Leu  Val  Asp  Tyr
AGTATTCAAGACACCTACACGCAGAAGCTGAGTGTGCCCAGTGGATTCTCTGTGACAGCC 3060
 Ser  Ile  Gln  Asp  Thr  Tyr  Thr  Gln  Lys  Leu  Ser  Val  Pro  Ser  Gly  Phe  Ser  Val  Thr  Ala
CCTGAAAAGCGGGGCTGGATCATCAACCCTCTGGGGGAAGAAGAGCCCTTTCCCGTGTGG 3120
 Pro  Glu  Lys  Arg  Gly  Trp  Ile  Ile  Asn  Pro  Leu  Gly  Glu  Glu  Glu  Pro  Phe  Pro  Val  Trp
ATGATGGTGGCCAGCCTGCTACCCGCCATCTTGGTCTTCATCCTCATCTTCATGGAGACA 3180
 Met  Met  Val  Ala  Ser  Leu  Leu  Pro  Ala  Ile  Leu  Val  Phe  Ile  Leu  Ile  Phe  Met  Glu  Thr
CAGATCACCACGCTGATCATCTCCAAAAAGGAGCGTATGCTGCAGAAGGGCTCTGGCTTT 3240
 Gln  Ile  Thr  Thr  Leu  Ile  Ile  Ser  Lys  Lys  Glu  Arg  Met  Leu  Gln  Lys  Gly  Ser  Gly  Phe
CACCTGGACCTGCTGCTCATCGTGGCCATGGGTGGCATCTGTGCCCTCTTTGGCCTCCTC 3300
 His  Leu  Asp  Leu  Leu  Leu  Ile  Val  Ala  Met  Gly  Gly  Ile  Cys  Ala  Leu  Phe  Gly  Leu  Leu
TGGTTGGCTGCTGCTACCGTCCGCTCAGTCACTCATGCCAACGCGCTCACTGTCATGAGC 3360
 Trp  Leu  Ala  Ala  Ala  Thr  Val  Arg  Ser  Val  Thr  His  Ala  Asn  Ala  Leu  Thr  Val  Met  Ser
AAGGCTGTGGCACCCGGGGATAAGCCCAAGATTCAGGAGGTCAAGGAGCAACGGGTGACA 3420
 Lys  Ala  Val  Ala  Pro  Gly  Asp  Lys  Pro  Lys  Ile  Gln  Glu  Val  Lys  Glu  Gln  Arg  Val  Thr
GGACTGCTGGTAGCCCTGCTCGTGGGACTCTCCTTAGTTATCGGGGATCTACTCCGGCAA 3480
 Gly  Leu  Leu  Val  Ala  Leu  Leu  Val  Gly  Leu  Ser  Leu  Val  Ile  Gly  Asp  Leu  Leu  Arg  Gln
ATCCCCTTAGCTGTGCTCTTTGGAATTTTCCTATACATGGGGGTGACCTCCCTAAACGGA 3540
 Ile  Pro  Leu  Ala  Val  Leu  Phe  Gly  Ile  Phe  Leu  Tyr  Met  Gly  Val  Thr  Ser  Leu  Asn  Gly
ATCCAATTCTATGAGCGGCTACACCTGCTGCTCATGCCACCCAAACATCACCCAGATGTA 3600
 Ile  Gln  Phe  Tyr  Glu  Arg  Leu  His  Leu  Leu  Leu  Met  Pro  Pro  Lys  His  His  Pro  Asp  Val
ATGTATGTAAAGAAGGTCCGCACAATGCGCATGCACCTTTTCAAAGCCCTGCAGCTGCTC 3660
 Met  Tyr  Val  Lys  Lys  Val  Arg  Thr  Met  Arg  Met  His  Leu  Phe  Lys  Ala  Leu  Gln  Leu  Leu
TGCCTGGCCCTGCTCTGGGCTGTCATGTCCACGGCGGCCTCCCTGGCCTTTCCATTCATC 3720
 Cys  Leu  Ala  Leu  Leu  Trp  Ala  Val  Met  Ser  Thr  Ala  Ala  Ser  Leu  Ala  Phe  Pro  Phe  Ile
CTCATCCTCACAGTGCCACTGCGCATGGTGGTGCTCACCCGCATCTTCACAGAGCGAGAG 3780
 Leu  Ile  Leu  Thr  Val  Pro  Leu  Arg  Met  Val  Val  Leu  Thr  Arg  Ile  Phe  Thr  Glu  Arg  Glu
ATGAAATGTTTGGATGCTAATGAGGCAGAACCCGTGTTTGATGAGCGTGAGGGCGTGGAC 3840
 Met  Lys  Cys  Leu  Asp  Ala  Asn  Glu  Ala  Glu  Pro  Val  Phe  Asp  Glu  Arg  Glu  Gly  Val  Asp
GAGTACAACGAGATGCCCATGCCTGTGTAGCTGCAACCTGCAGGGACAGCTGAGGGCCCA 3900
 Glu  Tyr  Asn  Glu  Met  Pro  Met  Pro  Val  •
GTGGGCAGAGGGATTGGGGGCTGGTGGGATGGGCCTCCCCCTCCCCCTGCCTGTTTTTAT 3960
TTAAGTGAATAATTTAAAGTCCCCTTCTCCCCACCCCCACAGTAAAGTGCTACAGCCCCC 4020
ACCAAAAAAAAAAAAAAAAAAA 4042
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Fig 1 (continued).

primers were also sequenced directly. The complete AE2 sequence was compiled from a contig of 17 different AE2 clones of varying lengths as well as the AE2 specific RT-PCR products from organ of Corti cDNA. Both, Sequencher and DNASTAR sequence analysis programs were used for sequence alignments.

2.2.3. *RNA isolation*

RNA was isolated (Trizol, BRL) from gp organ of Corti (microdissected from six cochleae) as well as rat cochleae from which the bony labyrinth was removed.

2.2.4. *Protein extraction and Western blot analysis*

Tissue samples (stomach and cochleae), harvested from adult Hartley gp, were snap frozen in liquid nitrogen, pulverized in a Bio-Pulverizer and then immediately resuspended in cell lysis/gel loading buffer (50 mM Tris-Cl, pH 6.8, 100 mM DTT, 2% SDS, 10% glycerol). The homogenate was passed through 26 gauge needle to shear genomic DNA, then resolved on 1.0 mm thick 6% Tris-glycine mini-gel (Novex precast mini gel) via electrophoreses at 125 V for 90 min in Laemmli running buffer. Electrophoretic transfer to nitrocellulose membrane (Novex) was then performed at 25 V for 120 min using Tris-glycine transfer buffer (Tris base 12 mM, glycine 96 mM, methanol 20%). The membrane was treated with 5% non-fat dry milk powder in 0.05% PBT at 4°C overnight to block non-specific binding sites. The blot was then incubated with the affininty purified anti-AE2 antibody [19,27,38] diluted 1/10 000 in 0.05% PBT at RT for 1 h. The blot was then washed

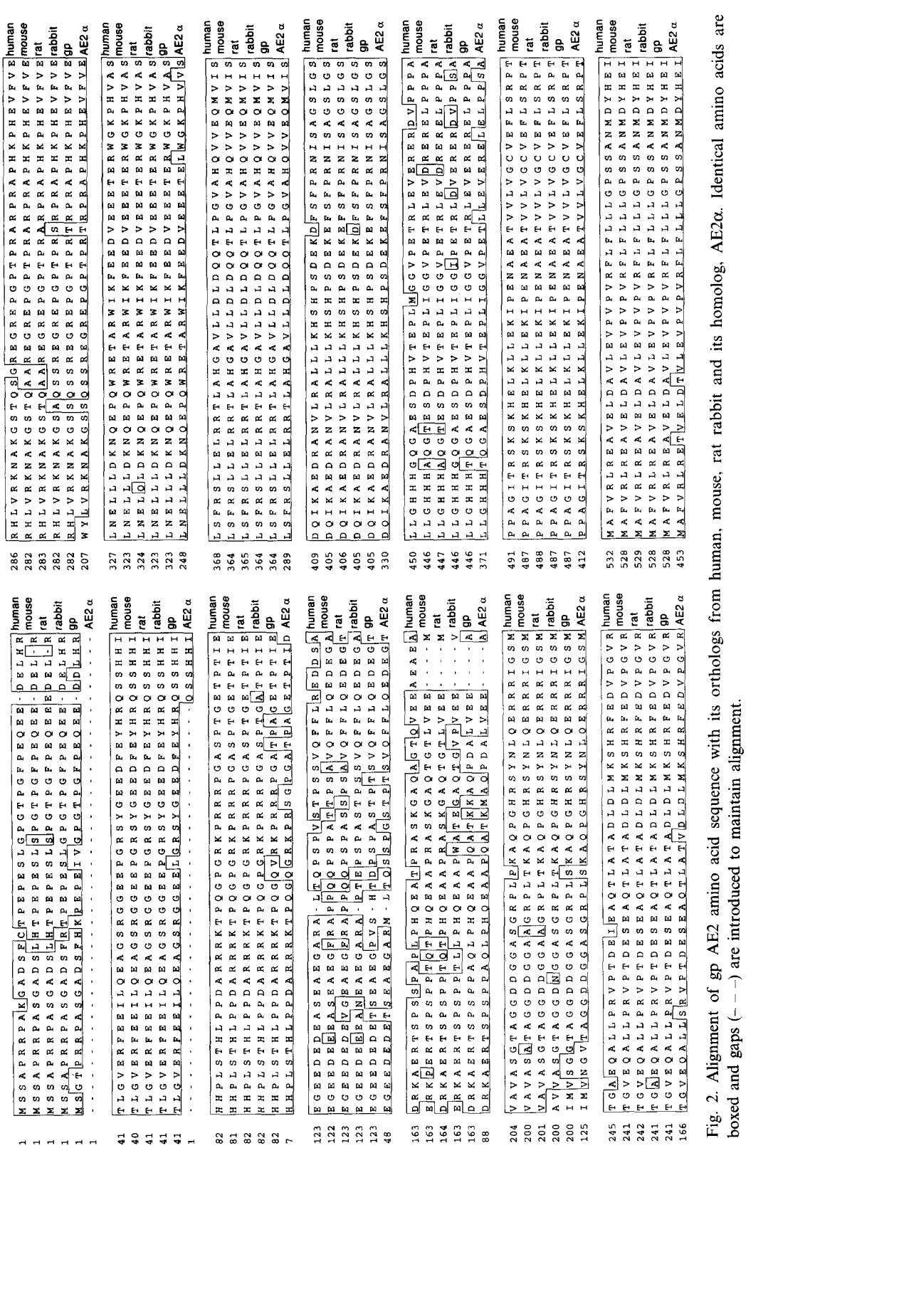

Fig. 2. Alignment of gp AE2 amino acid sequence with its orthologs from human, mouse, rat rabbit and its homolog, AE2α. Identical amino acids are boxed and gaps (– – –) are introduced to maintain alignment.

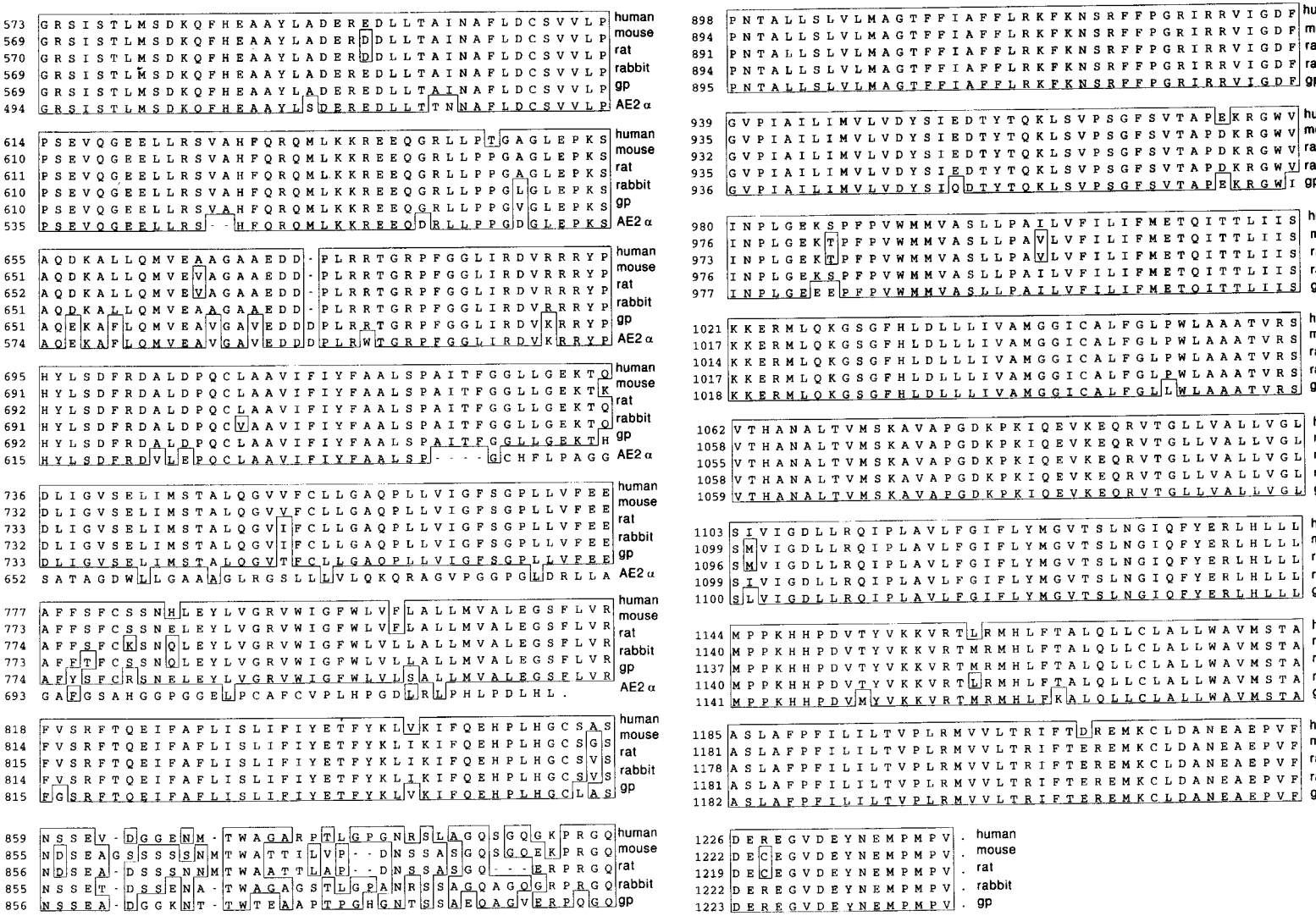

Fig 2 (continued).

in PBS followed by incubation with HRP conjugated, goat anti-rabbit IgG (Amersham) for another 1 h. After washing in PBS, the immunoblot was then developed using the ECL kit from Amersham.

2.2.5. Tissue processing

Animals were sacrificed with an intraperitoneal overdose of sodium pentobarbital (250 mg/kg) and bilateral thoracotomy. Temporal bones were harvested from both sides of the head. Each bulla was opened using rongeurs to expose the cochlea. The stapes was removed and the cochlea fixed by perfusion of 4.5% paraformaldehyde through the round window. The cochlea was then removed from the remaining temporal bone and immersed in 4.0% paraformaldehyde overnight at 4°C. After complete fixation, specimens were decalcified in 0.2 M EDTA/1×PBS/4.0% paraformaldehyde for 2–3 weeks, with at least three solution changes. Following decalcification, the specimens were placed in 0.9% saline, dehydrated through a graded alcohol series and then xylenes. Specimens were embedded in paraffin and sectioned at 6 μm on a microtome (Leica RM2035).

2.2.6. Immunohistochemistry

The paraffin-embedded cochlear sections were dewaxed, hydrated, blocked with 10% NHS, 0.1% Tween 20 in PBS and then hybridized overnight with rabbit antibody against mouse AE2 (1224–1237) [27] diluted 1:2000 to 1:5000. The sections were washed to remove unbound antibody and then hybridized with a secondary antibody (biotin-labeled anti-rabbit IgG monoclonal antibody). The bound label was then amplified using biotinylated tyramine and ABC reagent (Vector), as described by Adams [39], then developed with DAB. Specificity of this antibody for immunocytochemistry has been previously characterized in multiple tissues [19,27,38] and does not recognize AE3 in the conditions used. In the current experiments, antibody specificity was assessed by incubating the primary antibody in presence of a competing peptide, 12 μg/ml, against which the antibody was raised. Sections were examined under low and high power magnification.

2.2.7. In situ hybridization

Paraffin-embedded cochlear sections were dewaxed, hydrated, incubated in 0.2 M HCl for 10 min, rinsed in PBS, digested with proteinase K (10 μg/ml), fixed with 4% paraformaldehyde and then treated with 0.1 M triethanolamine and 0.25% acetic anhydride. The slides were rinsed in 2×SSC and then hybridized with in vitro transcribed, biotin labeled gp AE2 cRNA probe, 10–50 ng/ml in hybridization buffer (50% formamide, 4×SSC, 5×Denhardt's solution and 200 μg/ml of yeast tRNA) overnight at 50°C in a sealed chamber. The sections were washed under high stringency (50% formamide/2×SSC at 50°C) to remove unbound label. The bound label was then amplified using biotinylated tyramine and ABC reagent (Vector, Burlingame, CA), as described by Adams [39], then developed with DAB. The AE2 cRNA probe used in in situ hybridization is transcribed from *Xba*I linearized pGEM7Z-AE (2169–3010) via SP6 polymerase. The sense probe was transcribed from *Bam*HI linearized template with T7 RNA polymerase. pGEM7Z-AE (2169–3010) was constructed by ligating an 841 bp AE2 fragment, amplified from a pSPORT1-AE2 clone using primers (P1, ACTGGTCAAGATCTTCCA; P2, GTCACCCCCATGTAT) spanning nucleotides (nt) 2169–3010 of the gp AE2 cDNA, into the *Sma*I site within the pGEM7Z polylinker.

3. Results

3.1. Relative abundance of AE cDNAs in the gp organ of Corti cDNA library

A total of 37 'positive' clones were purified. Of these, 23 were selected for sequence analysis after elimination of duplicates, determined from insert size and restriction fingerprints. These positives were sequenced from both the 5′ and 3′ ends of the insert. The nt sequence of the positive clones was compared (aligned) with sequence of AE1, AE2 and AE3 to determine their identity. Of the 23 clones 17 were identified as AE2 clones, ranging in size from 4.0 to 0.7 kb, while four were AE3 clones of sizes 2.7 to 1.0 kb. No AE1 cDNA clone was identified, and no cDNAs encoding novel AE genes were identified by the screen. AE2 cDNAs were chosen for further study.

3.2. Sequence analysis of gp cochlear AE2

The longest AE2 clone sequenced was nearly 4.0 kb in size. This clone was sequenced in both directions and integrated with partial 5′ sequences from 16 additional AE2 clones of varying size and AE2 specific RT-PCR products from organ of Corti cDNA. The compiled AE2 cDNA sequence and its encoded polypeptide sequence are shown in Fig. 1. The complete cDNA sequence is 3888 bp in length, including 150 bp of 5′ untranslated sequence and an open reading frame (ORF) of 3717 bp that extends from nt 151 to 3870. The ORF encodes the AE2 polypeptide consisting of 1238 amino acids and with calculated apoprotein molecular weight of 137 kDa.

The gp AE2 coding sequence was further characterized by alignment with AE2 polypeptides from other species, including human, mouse, rat and rabbit, as well as the gp AE2α sequence [33]. The sequence identity of gp AE2α with its orthologs from other species ranged from 90 to 92%. The rat and the mouse AE2 sequence displayed the highest degree of sequence identity (97%) between species. The gp AE2α sequence differs from the conventional gp AE2 sequence by the 83 bp deletion spanning nt 2158–2240 of gp AE2. In addition, the current gp AE2 sequence differs in 35 amino acid residues from the sequence presented by Negrini et al. (GeneBank accession no. U20524) [33]. Among those 35 amino acid differences, 23 represent residues for which the present gp AE2 sequence shares identity with human, mouse, rat and rabbit sequences, and four represent residues identical to those of three of the four other species. These differences between gp AE2 and AE2α are illustrated in Fig. 2.

We did not find AE2α among any of the AE2 cDNA clones picked from the organ of Corti cDNA library. Pooled cDNA from the organ of Corti cDNA library as well as organ of Corti cDNA was also characterized for presence of clones containing the AE2α transcript via PCR analysis using primers flanking the 83 bp deletion/splice site reported within the variant by Negrini et al. [33]. A single uniform product was consistently obtained from this reaction corresponding to the length of gp AE2, but no shorter fragment corresponding to the gp AE2α sequence was ever observed (data not shown).

Also included amongst the 'positives' from the cDNA library, identified as AE2 clones, were a single clone with an insertion of 133 bases and a single clone with a deletion of 155 bases (distinct from the AE2α variant). However, these alterations were not detected by RT-PCR analysis in the organ of Corti cDNA or in cDNA from brain and kidney, and were concluded to be library artifacts.

3.3. Western blot analysis of AE2 expression in the gp cochlea

AE2 expression within the cochlea was also characterized by immunoblot analysis, as illustrated in Fig. 3. The anti-AE2 antibody revealed a single immunoreactive band of M_r 180 kDa in gp cochlear homogenate. Stomach homogenate revealed two distinct bands of M_r 165 and 125 kDa, consistent with previous results. The immunoreactive bands from both the cochlear and stomach homogenates were absent in presence of synthetic peptide antigen corresponding to mouse AE2 amino acid residues 1224–1237.

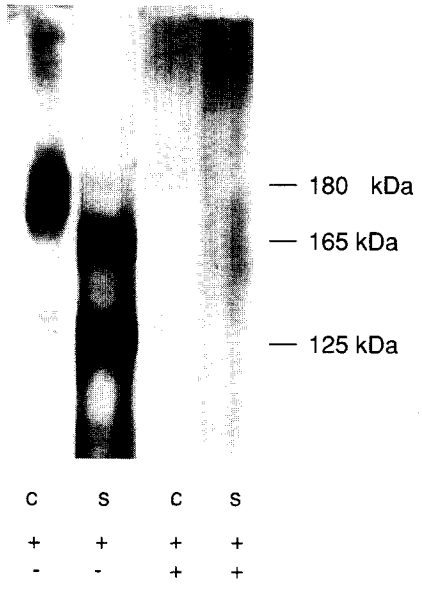

Fig. 3. Western blot analysis of AE2 expression in the gp cochlea. Homogenate from gp tissues including cochleae (c) was resolved on a 6% SDS-PAGE, transferred to nitrocellulose membrane. The Western blot was probed with anti-AE2 Ab and then developed using the ECL kit from Amersham. A single immunoreactive band, M_r 180 kDa, is observed in the cochlear homogenate while two major bands, M_r 165 and 125 kDa, are seen in the stomach (s) homogenate. The immunoreactive bands are eliminated in presence of a synthetic immunogenic peptide.

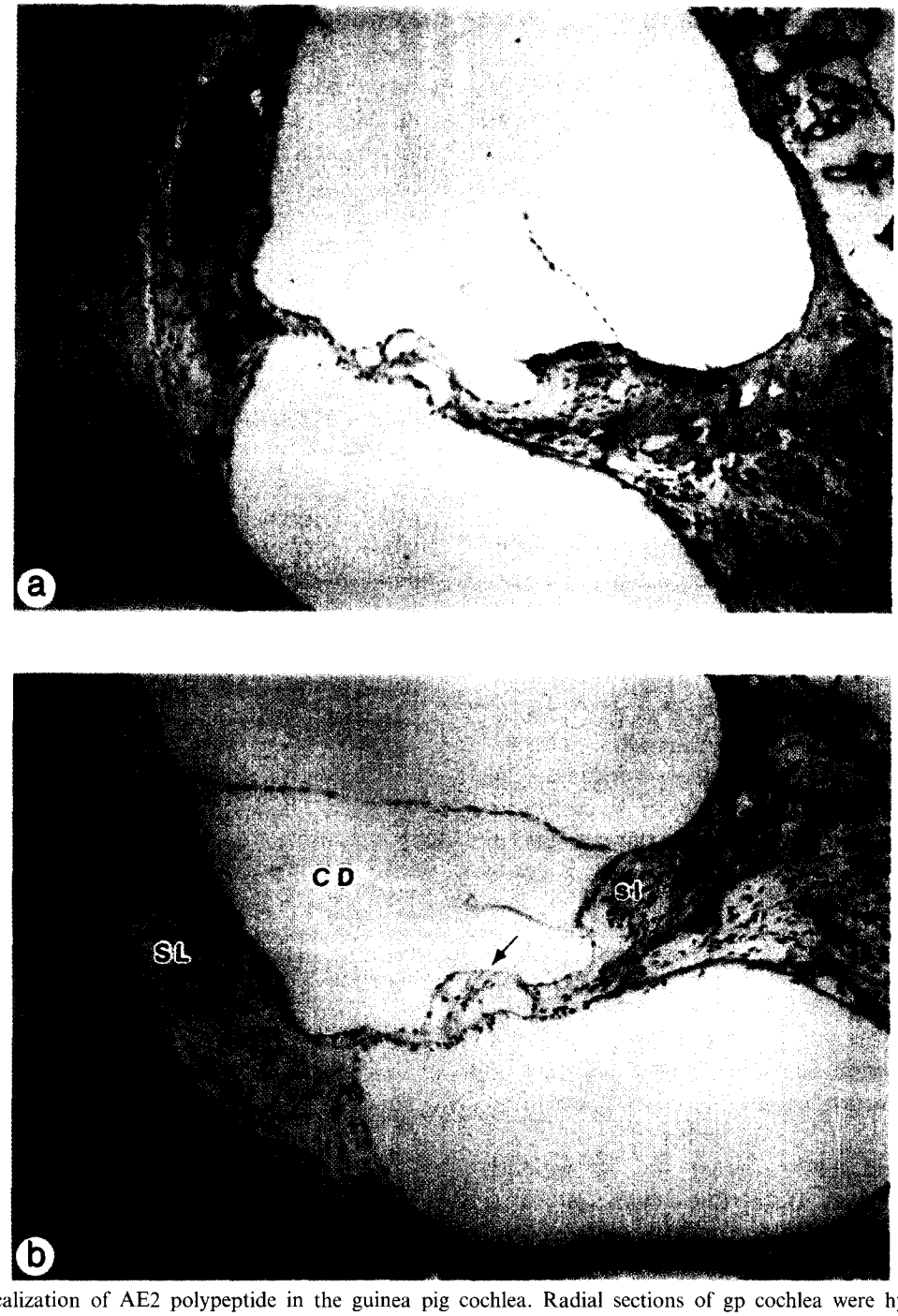

Fig. 4. Immunolocalization of AE2 polypeptide in the guinea pig cochlea. Radial sections of gp cochlea were hybridized with anti-AE2 antibody and then developed using biotin labeled anti rabbit 2° antibody, biotin amplification system and then stained with DAB. Several regions surrounding the cochlear duct (CD) are immunoreactive with the anti AE2 antibody (a). These include the superior half of the spiral ligament (SL), the interdental cells in the spiral limbus (sl), several cell types within the organ of Corti and the vasculature. Region surrounding the cochlear duct (CD), including the sensory cells within organ of Corti (arrow), is unstained when the antibody is hybridized in presence of competitive peptide (b). A higher power view of the organ of Corti from (a) reveals weak AE2 immunoreactivity in or near the OHC (c), while the cell bodies of the spiral ganglia are unstained (d).

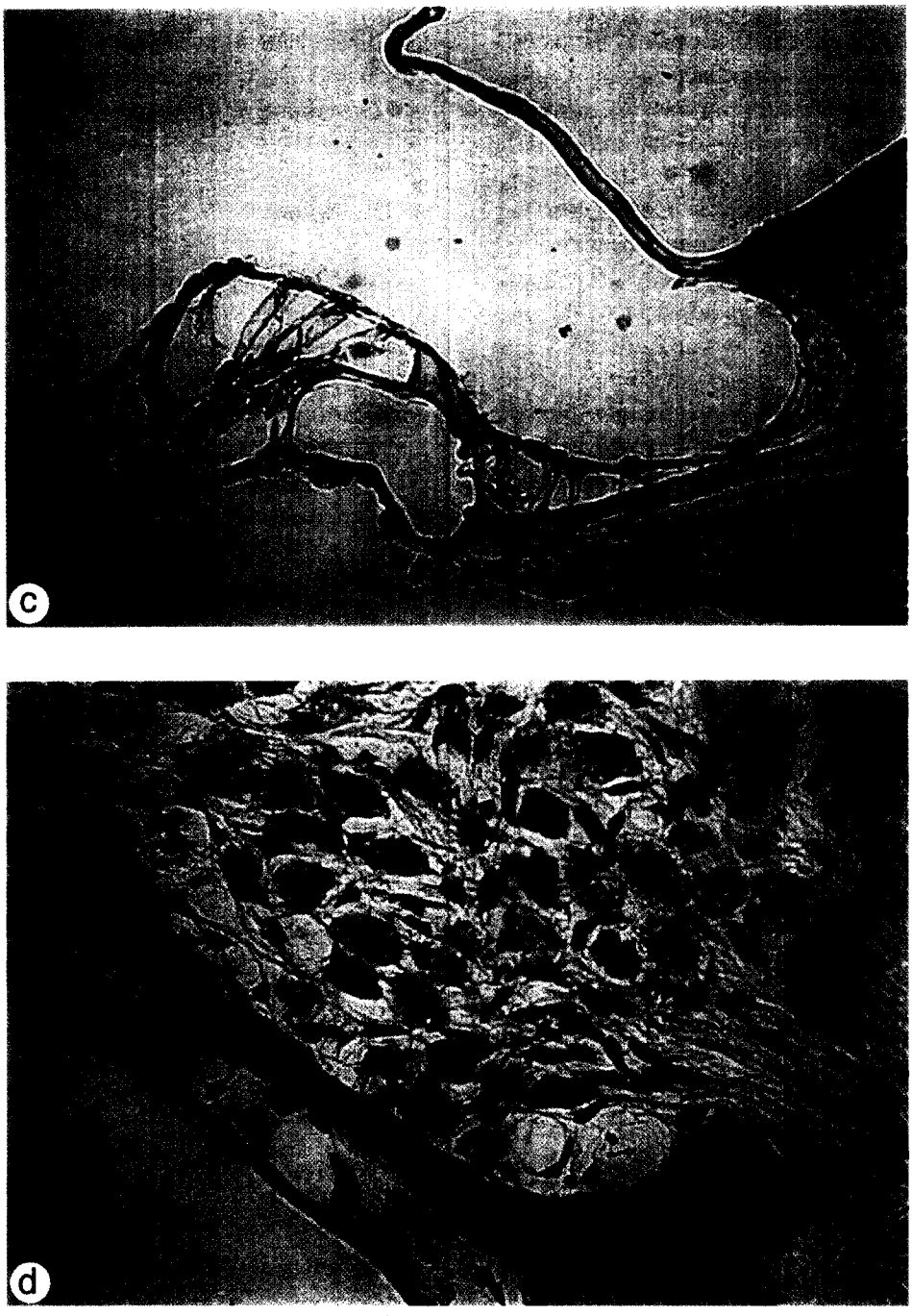

Fig. 4 (continued).

3.4. *In situ expression analysis of AE2 in the gp cochlea*

Localization of AE2 expression within the gp cochlea was determined via immunohistochemistry and in situ hybridization. Cochlear sections stained with the anti-mouse AE2 antibody showed immunostaining signal in several regions as illustrated in Fig. 4a. The immunoreactive regions include the superior half of the spiral ligament (the lateral wall of the cochlear duct), the interdental cells of the spiral limbus, the hinge region between the spiral ligament and

the organ of Corti, within the organ of Corti, and in vascular smooth muscle. Stained red cells as observed in Fig. 4a represent crossreactivity of the antibody with AE1. Immunostaining was operationally specific, as defined by abolition of staining in the presence of excess peptide antigen (Fig. 4b). A higher magnification view of the organ of Corti from Fig. 3a shows light staining of several cell types within the organ of Corti-Hensen cells, the Deiters cells, the outer phalangeal cells, as well as adjacent to the outer hair cells (Fig. 4c). The DAB signal intensity and the magnification, however, did not suffice to permit definitive localization of immunoreactivity within the OHC. A prominent tissue type unstained by the AE2 antibodies is the spiral ganglion (Fig. 4d). Absence of signal in the neuronal bodies contrasts with immunoreactivity (AE1 crossreactivity) of blood cells and the smooth muscle cells of the vasculature that traverses the spiral ganglion.

Immunohistochemical analysis of AE2 expression was supplemented with in situ hybridization. Similar to immunohistochemical detection, the antisense AE2 cRNA probe localized the AE2 transcript in the superior half of the spiral ligament as well as in the interdental cells of the spiral limbus (Fig. 5). The additional sites that are stained in the immunohistochemistry assay may reflect differing sensitivities of the two assays in this tissue preparation.

4. Discussion

Study of the role of anion exchangers within the OHC was initiated by characterizing expression of its family members within the organ of Corti cDNA library. cDNAs encoding two members of the AE gene family, AE2 and AE3 were cloned from this library, and were present in a 4:1 ratio. Neither, AE1 nor any other AE homologs were detected through the screen. Subsequent characterization focused on the more abundant AE2 cDNAs.

Complete gp AE2 coding sequence was obtained and compared with its orthologs from other species. The sequence alignment analysis did not identify unique or substantially altered sequence motifs within the gp AE2 relative to its orthologs from other species. Thus the AE2 clones obtained from the organ of Corti are representative of the predominant

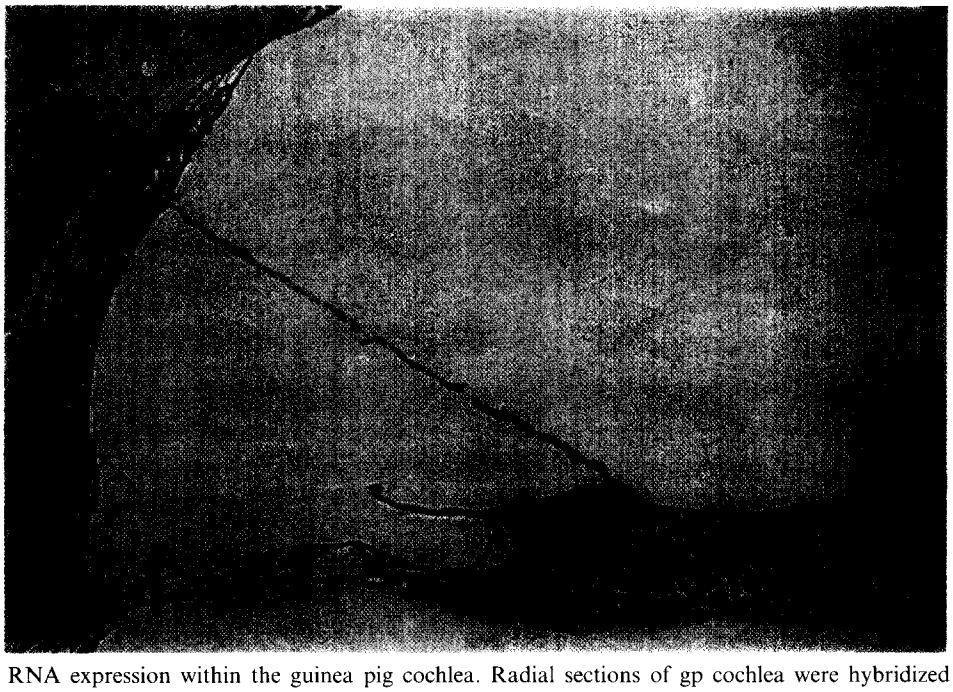

Fig. 5. In situ AE2 RNA expression within the guinea pig cochlea. Radial sections of gp cochlea were hybridized with biotin labeled antisense gp AE2 RNA, developed using biotin amplification system and then stained with DAB. The superior half of the spiral ligament lining the cochlear duct and the interdental cells in the spiral limbus display AE2 hybridization signal.

form of AE2 that has been cloned from other tissue types, including kidney and colon, in different species [26,35,36].

The nt sequence of gp AE2 was also compared with gp AE2α, the AE2 isoform [33] (data not shown). In addition to revealing the codons that underlie the amino acid differences, the alignment of the two sequences also identified the nt that flank the putative 'splice' junction in the AE2α, 'CA' on the 5′ side and 'GG1' on the 3′ side. These flanking nt differ from the invariant consensus nt pairs found at splice junctions, 'AG' on the 5′ side and 'GT' on the 3′ side. Although the AE2α isoform was isolated from the same cDNA library as that used in the present experiments, a thorough search of all our isolated AE2 clones whose 5′ ends extended beyond the AE2α splice point [33] did not reveal presence of the AE2α isoform. Interestingly, Abs raised against the AE2α yield a protein band of the size expected from the length of its polypeptide [33,34], indicating absence of post-translational modifications such as *N*-glycosylation present in other cloned AE2 polypeptides. [27,36].

Identification and sequence analysis of gp AE2 cDNA within the gp organ of Corti cDNA library was supplemented with characterization of the gp cochlear homogenate for AE2 polypeptide expression. Immunoblot analysis using an anti-AE2 Ab, raised against the C-terminus peptide [27] identified a single immunoreactive band of M_r 180 kDa Immunoreactivity of this protein band against the anti-AE2 Ab was competed by synthetic peptide antigen. The 180 kDa polypeptide band identified by the anti-AE2 Ab is larger than the 165 and 125 kDa AE2 polypeptides detected in gp stomach homogenate, also competed by synthetic peptide antigen. The 165 kDa band corresponds to with the observed size for rat and murine AE2 [27,36]. The predicted molecular weight of gp AE2 is 137 kDa. Thus, the increased band size observed on SDS-PAGE likely reflects *N*-glycosylation of AE2 [28]. The 125 kDa band represents either an N-terminal proteolytic product, an immature glycosylation product, or may represent the AE2c isoform [24].

The AE2 Ab used in this study has also yielded an AE2 immunoreactive smear extending from 165 upward to 185 kDa in the rat aorta and a 180/185 kDa band in rat kidney (Haller and Alper, unpublished data). Polyclonal Ab to a conserved C-terminal peptide was shown to recognize a single major 185 kDa band in a immunoblot of a membrane fraction from rabbit stomach. Similarly, a 180 kDa band was identified in immunoblot of membrane fraction from human kidney probed with a polyclonal Ab raised against a C-terminal fragment of human AE1 [40]. Neither of these immunoreactive bands identified in the rabbit stomach or the human kidney were detected when probed with AE1-specific Abs directed towards the variant N-terminus.

Thus, the presence of a single AE2-immunoreactive protein species within the cochlear homogenate, its operational immunospecificity, and the correspondence of its size with those previously reported for AE2 in several tissues supports its identity as cochlear AE2.

Immunohistochemical analysis and in situ hybridization identified two distinct regions of AE2 expression within the gp cochlea. These included the superior half of the lateral wall of the cochlear duct and the interdental cells within the spiral limbus. These regions yielded positive signal for both in-situ hybridization and immunohistochemical assays. Curiously, neither of these regions interfaces directly with a lumen. One of the stained regions, the spiral ligament, is located underneath the stria vascularis that lines the cochlear duct, though the strial cells themselves were unstained. The interdental cells within the spiral limbus, the other stained region, are underneath a portion of the tectorial membrane that reaches out across the organ of Corti. The cells bordering the inner sulcus, adjacent to the interdental cells and directly in contact with the endolymph, were unstained. The presence of AE2 polypeptide and mRNA within the cells of the spiral ligament (superior half/peripheral region) and the interdental cells strongly suggests that they express Cl/HCO_3 exchange function.

The immunohistochemical assay also yielded a signal in several other sites that were absent in the in situ hybridization assay. These included the hinge region between the spiral ligament and the organ of Corti as well as the blood cells and smooth muscle of the vasculature. Several cell types within the organ of Corti were stained weakly in the immunohistochemical assay, but not via in situ hybridization. These cell types include the Deiters cells, phalangeal cells

and the pillar cells that surround the inner and outer hair cells. The partially overlapping staining patterns of the two assays may reflect differences in their specificity and/or sensitivity, or regional differences in relative abundance of AE2 mRNA and protein.

The peptide sequence against which the AE2 Ab was raised, corresponding to mouse AE2 amino acid residues 1224–1237, is fully conserved in the rat and displays over 90% identity (13/14) in both human and gp AE2 (the lone difference is substitution of C_{1225} with R, Fig. 2). The conservation of this antigen sequence is reduced to 64 and 71% with cross-reactive mouse and rat AE1, respectively. The AE2 peptide antigen sequence is less well conserved in AE3 and the anti-AE2 antibody displays marginal or no crossreactivity with overexpressed recombinant AE3 and no crossreactivity with native AE3 in stomach, heart, and brain (Alper and Stuart-Tilley, unpublished results). Accordingly, the neurons of the spiral ganglion of the cochlea are unstained, consistent not only with absence of AE2 expression, but with likely expression of AE3.

The relative specificity or crossreactivity of the AE2 cRNA probe used in the in situ hybridization assay may be similarly assessed by comparative sequence analysis with its homologs. The cRNA probe used in the assay is 841 bp long derived from sequence within the 3′-half of the gp AE2 cDNA. This sequence corresponds to amino acids 838–1133 within the mouse AE2. This probe, although located within the relatively conserved C-terminus, does not overlap the peptide sequence to which the AE2 antibody was raised. If the motor protein, inferred to be an abundantly expressed transmembrane protein (one million copies per OHC), is AE2, then both in situ hybridization and immunohistochemistry should have yielded an unambiguous signal in OHC. This was not found thus negating a role for AE2 in mediating OHC electromotility.

The human genes encoding AE1, AE2 and AE3 have been mapped. AE1 to 17q21–q22, AE2 to 7q35–q36 [41], and AE3 to 2q36. Mutations in human AE1 have been linked to 25% of dominant hereditary spherocytosis [21,42], and to some forms of dominant distal renal tubular acidosis without hearing impairment [22,23]. Human mutations within AE2 or AE3 have not been reported. Although the presence of AE2 anion exchanger within the inner ear implies a physiological role in ionic homeostasis of the cochlea, no hereditary syndromic or non-syndromic hearing loss loci map within the chromosomal regions of the AE anion exchanger genes [43].

In summary, we have cloned the AE2 cDNA from gp organ of corti, detected AE2 polypeptide by immunoblot of gp cochlea and localized AE2 mRNA and AE2 polypeptide in the gp cochlea. Though AE2 is expressed in gp cochlea, it was not detected in hair cells. Thus, the present results suggest that AE2 may not be considered as a candidate motor protein of the outer hair cells.

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