Identification of the Mammalian Na,K-ATPase β3 Subunit*

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We have isolated and characterized cDNA clones encoding the human and rat Na,K-ATPase β3 subunit isoform. The human cDNA encodes a polypeptide of 279 amino acids that exhibits primary sequence and secondary structure similarities to Na,K-ATPase amino acids that exhibit primary sequence and second- ary structure similarities to Na,K-ATPase β subunit isoforms. Sequence comparisons showed that the human β3 subunit closely resembles the β3 subunit of Xenopus laevis (59% amino acid identity) and is less similar to the human Na,K-ATPase β1 and β2 subunits (38% and 48% amino acid identity, respectively). By analyzing the segregation of restriction fragment length polymorphisms among recombinant inbred strains of mice, we localized the β3 subunit gene to murine chromosome 7. Northern blot analysis revealed that the β3 subunit gene encodes two transcripts that are expressed in a variety of rat tissues including testis, brain, kidney, lung, stomach, small intestine, colon, spleen, and liver. Identification of the mammalian β3 subunit suggests an even greater potential for Na,K-ATPase isoenzyme diversity than previously realized.

Na,K-ATPase is a membrane-associated enzyme responsible for the active transport of Na⁺ and K⁺ in most animal cells. By coupling the hydrolysis of ATP to the movement of Na⁺ and K⁺ ions across the plasma membrane, the enzyme produces the electrochemical gradients that are the primary energy source for the active transport of nutrients, the action potential of excitable tissues, and the regulation of cell volume (1, 2).

In all tissues from which Na,K-ATPase has been isolated, the enzyme has been found to consist of two subunits present in equimolar amounts. The α subunit is a polypeptide of ~100 kilodaltons (kDa) which contains the binding sites for ATP and cardiac glycosides such as digoxin and ouabain (3). The β subunit is a glycosylated polypeptide of molecular mass ~50–60 kDa (2). The function of the β subunit has yet to be elucidated.

The α and β subunits of Na,K-ATPase are each encoded by multigene families. Three α subunit and two β subunit genes have been localized to different chromosomes in the mouse (4, 5), and cDNA clones encoding three rat α (α1, α2, α3) and two β (β1, β2) subunit isoforms have been characterized (6–9). Substantial differences in the tissue and cell specificity of expression have been found for each α and β subunit. α1 subunit polypeptides have been detected in virtually all rat tissues (10). In contrast, α2 subunits are expressed predominantly in brain, heart, lung (10), and skeletal muscle (11), while α3 subunits are abundant in tissues of neural origin (10, 12, 13). Expression of β1 subunit polypeptides has been detected in brain, heart, and kidney (10), whereas β2 subunits are expressed predominantly in brain (14), pineal gland (12), and photoreceptor cells (13). Within the central nervous system, expression of the α3 subunit is restricted exclusively to neurons, while β2 subunit expression appears to be astrocyte-specific (15, 16). The identification of multiple α and β subunit isoforms has raised questions regarding the extent of Na,K-ATPase isoenzyme complexity. RNA hybridization (15) and immunoblotting analyses (16) suggest that α and β subunit association is promiscuous, and that all six αβ subunit combinations are likely to exist.

Several lines of evidence have suggested the potential existence of additional β subunit isoforms. Antibody probes have failed to detect expression of β1 or β2 subunits in several rat tissues, despite the presence of α subunits in these tissues (12, 14). A putative β3 subunit isoform has been identified in the toad Xenopus laevis (17). However, in the absence of identifiable mammalian β3 and Xenopus β2 subunit sequences, it is unclear whether the Xenopus β3 subunit actually represents the amphibian homolog of the mammalian β2 subunit.

In a search of the GenBank™ EST database, we identified a cohort of cDNA clones that encode the human Na,K-ATPase β3 subunit. The β3 subunit gene has been localized to mouse chromosome 7. This gene is expressed in a wide variety of rat tissues including brain, kidney, and liver. The identification of a mammalian β3 subunit suggests the possible existence of 9 distinct Na,K-ATPase isoenzymes.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of cDNA Clones—Two human ESTs with sequence homology to the Xenopus Na,K-ATPase β3 subunit were identified in a search of the GenBank™ EST data base. BLAST analysis (18) identified additional human ESTs (I.M.A.G.E. Consortium, Lawrence Livermore National Laboratory) with overlapping sequence homology. One of these clones (I.M.A.G.E. Consortium clone 139611) was obtained from Research Genetics (Huntsville, AL), sequenced in its entirety, and found to encode a full-length β3 subunit. A cDNA fragment encoding a portion of the β3 subunit was isolated from rat placental RNA by reverse transcriptase-mediated polymerase chain reaction (PCR). First strand cDNA was synthesized from 1 μg of total rat placental RNA. A (dT)₇ adapter (50 pmol) served as primer for Moloney murine leukemia virus reverse transcriptase (Stratagene) as described previously (19). PCR was performed using the following primer mixtures.

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1 The abbreviations used are: EST, expressed sequence tag; PCR, polymerase chain reaction; RFLP, restriction fragment length polymorphism; RI, recombinant inbred; kb, kilobase(s); cM, centimorgan.
5’-GGGAATTCCGGIGA(A/G)TT(C/T)(C/T)TIGGI(A/C)GIACIGC-3’, corresponding to amino acids 26–33 of the human β3 polypeptide, and 5’-GGGAATTCCCIATIATIC(G/T)(A/G)TTCAT(C/T)TTIAC-3’, corresponding to amino acids 173–180 of the human β3 polypeptide. In the primer sequence, I specifies inosine. PCR was carried out with Taq polymerase (Perkin-Elmer) for 40 cycles (1 min at 95°C, 1 min at 37°C, and 2 min at 72°C) using buffers supplied by the distributor. Buffers contained 1.5 mM magnesium. PCR products were inserted into the EcoRI site of Bluescript (Stratagene). Both the rat and human cDNAs were sequenced by dideoxynucleotide chain termination sequencing using a Sequenase kit (United States Biochemicals). DNA sequences were analyzed using the programs from the University of Wisconsin Genetics Computer Group (20).

RNA Blot Hybridization—RNA was extracted from rat tissues using Trizol Reagent (Life Technologies, Inc.) according to the method described by Chomczynski and Sacchi (21). Conditions for electrophoresis, transfer, and hybridization were as described previously (19). A 320-base pair segment of the rat reverse transcriptase-mediated PCR fragment was radiolabeled by the random priming method (22) using a High Prime DNA Labeling Kit (Boehringer Manheim) and used as probe. Blots were washed to a final stringency of 0.13 SSC (13 SSC is 0.15 M NaCl, 15 mM sodium citrate), 0.1% SDS at 72°C with an intensifying screen.

Chromosomal Localization—DNA samples from recombinant inbred (RI) strains of mice (A/J, C57BL/6J) were purchased from the Jackson Laboratory (Bar Harbor, ME). Genomic DNA was digested to completion with MspI, separated on a 1% agarose gel, transferred to a nylon filter membrane (Hybond-N, Amersham), and hybridized as described previously (19). Blots were washed to a final stringency of 0.2 × SSC, 0.5% SDS at 65°C for 45 min, then exposed to Kodak X-AR film at 280°C with an intensifying screen. The strain distribution pattern of the MspI polymorphism in RI strains derived from A/J × C57B1/6J crosses (AXB and BXA) was determined as described previously (5, 19) and is presented in Table II.

RESULTS

Identification and Characterization of β3 Subunit cDNA Clones—In a search of the GenBank™ EST data base, we identified several human cDNAs having homology to Na+,K+ATPase β3 subunits. Both strands of a full-length human cDNA (I.M.A.G.E. Consortium clone 138611) were subjected to dideoxynucleotide sequencing. The complete nucleotide se-
Fig. 2. Comparison of the human β3 and β2 subunits. The deduced amino acid sequence of the human β3 subunit is shown above the human β2 subunit. Alignments are optimal for amino acid insertions/deletions. Asterisks denote asparagine residues which are possible sites of N-linked glycosylation. The putative transmembrane-spanning domains are underlined. Identical residues are shaded. Conserved cysteine are indicated by arrowsheads. Amino acids are numbered above the sequence.

The human β3 cDNA has a 27-nucleotide 5'-untranslated region, an 840-nucleotide open reading frame, and a 507-nucleotide 3'-untranslated sequence. The nucleotide sequence of the coding region of the human cDNA exhibits 40% homology with the human Na,K-ATPase β2 subunit (9). The open reading frame encodes a 279-amino acid polypeptide with a molecular mass of 31,639 daltons. As shown in Table I, the amino acid sequence of the β3 polypeptide exhibits 59% identity with the β3 subunit of X. laevis (17), 38% identity with the human Na,K-ATPase β1 subunit (23), and 48% identity with the human β2 subunit (9). We therefore conclude that the human cDNA encodes a mammalian Na,K-ATPase β3 subunit isoform.

PCR was used to generate a portion of the corresponding rat cDNA. The nucleotide and deduced amino acid sequence of the rat PCR product is presented in Fig. 1. The rat β3 subunit PCR product corresponds to amino acids 26 through 180 of the human polypeptide. In the region between the PCR primers (amino acids 34–172), the rat and human β3 subunits exhibit 83% nucleotide and 80% amino acid sequence identity, indicating that the PCR product is likely to represent a segment of the rat β3 subunit.2 The extent of amino acid sequence divergence observed for the corresponding region of the β3 subunit compared to seven in the β2 (97% identity) subunits. Se-quence comparisons indicate that the Na,K-ATPase β3 subunit is more divergent among species than either the β1 or β2 subunits.

Structure of the Human Na,K-ATPase β3 Subunit—A comparison of the amino acid sequence of the human β3 subunit with the human β2 subunit is shown in Fig. 2. The human β3 subunit consists of 279 amino acids, whereas the human β2 subunit is composed of 290 amino acid residues. Of the 279 residues compared, 48% positions were occupied by identical residues while 18% were occupied by favored substitutions. The asparagine residues marked with asterisks represents potential sites of N-linked glycosylation. There are two such sites in the human β3 subunit compared to seven in the β2 subunit. The positions of the N-linked glycosylation sites in the human β3 subunit are not strictly conserved with any of the N-linked glycosylation sites in the human β1, human β2, or Xenopus β3 subunit isoforms. There are six cysteine residues (positions 128, 144, 154, 170, 191, and 250) within the proposed extracellular domain of the β3 subunit. In the computer-aligned sequences, these cysteine residues appear to be highly conserved among Na,K-ATPase and H,K-ATPase β subunits. A putative transmembrane segment is located between residues 39 and 66. Of the 28 amino acid residues in this region, 21 are identical and 6 are conservative substitutions between the β3 and β2 subunits.

Hydropathy profiles of the human Na,K-ATPase β1, β2, and β3 subunit sequences obtained by using the algorithm of Kyte and Doolittle (24) are shown in Fig. 3. This analysis predicts that all three β subunit isoforms contain a highly charged cytoplasmic amino terminus followed by a single hydrophobic transmembrane segment of 28 amino acids and a large extracellular carboxyl-terminal domain. A comparison of the amino acid sequence of the human β3 subunit with other Na,K-ATPase β subunits is presented in Fig. 4 and Table I. A computer-assisted analysis of sequence similarities (Fig. 4) shows that the human β3 subunit clusters with the β3 subunits of Xenopus and Bufo marinus (25). These sequences are clearly distinct from clusters containing Na,K-ATPase β1 and β2 subunit isoforms. Interestingly, the human β3 subunit sequence is most closely related to that of the chicken β3 subunit described as β2 (61% amino acid sequence identity) (26). The fact that the chicken β2 subunit sequence clusters with β3 subunit sequences (Fig. 4) suggests the possibility that the chicken sequence actually represents the avian β3 subunit.

Expression of β3 Subunit mRNA in Rat Tissues—A panel of adult rat tissues was examined for the presence of Na,K-ATPase β3 subunit mRNA. The pattern of expression of β3 subunit mRNA is shown in Fig. 5. The Na,K-ATPase β3 subunit gene encodes two transcripts, ~1.6 and ~1.8 kb in size. Of the rat tissues analyzed, β3 subunit mRNA was detected at highest levels in testis. Transcripts of the β3 subunit gene were present in much lower abundance in brain, kidney spleen, and lung, and at even lower levels in stomach, colon, and liver. No β3 subunit transcripts were detectable in heart. Transcripts of the β3 subunit gene were also present in placenta and mammary gland of pregnant rats and in the brain of 19-day-old fetal rats (data not shown).

Chromosomal Localization of the Na,K-ATPase β3 Subunit Gene—We have used segregation of restriction fragment length polymorphisms (RFLPs) among recombinant inbred (RI) strains of mice to identify the chromosomal location of the mouse gene encoding the Na,K-ATPase β3 subunit (Atp1b3).3 Mouse genomic DNA was identified by hybridizing Southern blots to radiolabeled full-length human β3 subunit cDNA. As shown in Fig. 6, this probe hybridized to three major genomic fragments, 4.3, 3, and 1.5 kb long, in MspI-digested

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2 While this manuscript was under review, the full-length sequence of the rat β3 subunit was deposited in the GenBank™ data base (Watanabe et al., accession number D84450). The partial rat sequence published here shows 100% nucleotide identity to the corresponding region of the full-length β3 clone.

3 The mouse gene encoding the Na,K-ATPase β3 subunit has been named according to the rules for mouse gene nomenclature (33).
**Na,K-ATPase β3 Subunit**

**FIG. 3.** Hydropathy profiles of human β1, β2, and β3 subunits. Hydropathy values were obtained by using the algorithm and hydropathy values of Kyte and Doolittle (24). Hydrophobic regions are above the center line and hydrophilic regions are below.

**FIG. 4.** Sequence relatedness of Na,K-ATPase β subunit isoforms. Dendrogram analysis of Na,K-ATPase amino acid sequences was obtained using the PileUp program of the GCG software package (20). The scale at the bottom indicates percent identity between β subunit isoforms. The dotted line represents the partial rat β3 sequence obtained by reverse transcriptase-mediated PCR. The following sequences were obtained from the SwissProt or GenBank sequence data bases: rat β1 (atnb_rat), human β1 (atnb_human), chicken β1 (atnb_chick), X. laevis β1 (U17061), B. marinus β1 (atnb_bufma), eel Anguilla anguilla β1 (X76109), rat HKβ (atnb_rat), chicken HKβ (L08047), rat β2 (atnb_rat), mouse β2 (atmc_mouse), human β2 (atmc_human), B. marinus β (bladder) (Z35812), chicken β2 (atmc_chick), X. laevis β3 (atnd_xen), B. marinus β3 (atnd_bufma), zebrafish β (X89722).

**FIG. 5.** Expression of β3 subunit mRNAs in rat tissues. A, total cellular RNA was prepared from various adult rat tissues, fractionated by electrophoresis through a formaldehyde-containing 1% agarose gel (20 μg/lane), transferred to a Zetabind filter, and hybridized with the rat β3 cDNA probe. The positions of the 28 S and 18 S markers are indicated at the left. B, ethidium bromide-stained gel in the region of 18 S RNA to indicate approximately equal loading.

**FIG. 6.** Restriction fragment length polymorphism for the mouse Na,K-ATPase β3 subunit gene. Genomic DNA from A/J (A) and C57BL/6J (B) inbred mouse strains was digested to completion with MspI and hybridized with a full-length human β3 subunit cDNA probe. Arrow indicates the position of the polymorphic hybridizing fragment. Sizes of the hybridizing fragments (in kb) are shown at left.

**DISCUSSION**

We have identified cDNAs and genomic sequences encoding a third mammalian member of the Na,K-ATPase β subunit gene family. The genes for the Na,K-ATPase β1 and β2 subunits have previously been localized to mouse chromosomes 1 and 11, respectively (4, 5). Here we have mapped the β3 subunit gene to mouse chromosome 7. The fact that each β subunit gene is located on a different mouse chromosome suggests that correction events such as gene conversion did not participate in the evolution of the β subunit gene family.

The classification of Na,K-ATPase β subunits as β2 or β3 has been difficult to resolve, primarily because in all species so far examined, only two β subunit isoforms have been identified. The putative Xenopus β3 subunit (17) exhibits greater sequence divergence from the mammalian β2 subunit than do the mammalian and Xenopus β1 subunits. The same is true for the chicken non-β1-like subunit, which was classified as β2 based on its tissue-specific expression pattern (26). The identification of a mammalian β3 subunit helps clarify the evolutionary relationships among β subunit isoforms. Sequence comparisons among Na,K-ATPase and H,K-ATPase β subunits show three distinct clusters containing Na,K-ATPase β1, β2, or β3 subunit isoforms, and a fourth representing the H,K-ATPase β subunit. This type of analysis predicts that the chicken β2 subunit is actually a β3 subunit, as it clusters with the family of Na,K-ATPase β3 subunits. In this context, it should be noted that a recently cloned β subunit from B. marinus bladder (30) clusters with Na,K-ATPase β2 subunit isoforms. It will clearly be interesting to determine whether a β2 subunit homolog can also be identified in Xenopus and avian species.
Northern blot analysis indicates that β3 subunit mRNA sequences are expressed in a variety of rat tissues including testis, brain, kidney, spleen, stomach, small intestine, colon, lung, and liver. Highest expression levels were detected in testis, whereas substantially lower levels of β3 subunit transcripts were present in brain. The low level of β3 subunit expression in rat brain is somewhat surprising since the *Xenopus* β3 (17), *Bufo* β3 (25), and chicken β2 (the apparent avian β3 subunit homolog) (26) subunits appear to be expressed predominantly in neural tissue. In contrast, β2 subunits are abundant in mammalian brain (9, 14), while β2 subunit homologs have not yet been detected in amphibian or avian brain. β1 subunits are also expressed in mammalian and non-mammalian brain (10, 25). Thus, it is possible that in non-mammalian species, β1 and β3 are the predominant β subunits of brain, whereas in mammalian species, β1 and β2 subunits are the predominant neural isoforms. In mammalian brain, β1 subunits are localized exclusively in neurons whereas β2 subunits are glia-specific (16). It will be interesting to determine whether β3 subunit expression patterns within brain exhibit regional or cellular differences among species. It is noteworthy that we detect β2 subunit mRNA in kidney, a tissue thought to express only α1 and β1 subunit isoforms (10). Our results raise the possibility that kidney may also contain Na,K-ATPase isoenzymes composed of α1 and β1 subunits. The development of β3 isoform-specific antisera will allow us to determine whether β3 subunit polypeptides are present in kidney and whether β3-containing isoenzymes exhibit differences in cellular distribution compared with β1-containing isoenzymes in this tissue. A further issue that should be raised in this context is the recent identification of a putative fourth Na,K-ATPase α subunit isoform (31). This α subunit isoform is expressed primarily in testis, raising the possibility that the predominant Na,K-ATPase of testis may be an α3/β3-containing isoform.

As a step toward our goal of elucidating the molecular evolution of the cation-motive transporter β subunit gene family, we determined the chromosomal position of the mouse gene encoding the Na,K-ATPase β3 subunit. By analyzing the segregation of RFLPs in RI strains of mice, the β3 subunit gene (*Atp1b3*) was found to segregate with markers (*Gpi1* and *Tam1*) localized to the proximal region of mouse chromosome 7. These markers constitute part of a highly conserved linkage group on mouse chromosome 7 and human chromosome 19. We therefore speculate that the human β3 subunit gene is likely to be located on human chromosome 19. In the mouse, the Na,K-ATPase β1, β2, and β3 subunits are encoded by separate genes located on three different chromosomes (4, 5). Chromosomal dispersion and tissue-specific expression of the β subunit genes suggests that each gene may encode a β subunit with distinct functional properties.

Several lines of evidence suggest that Na,K-ATPase α/β subunit interaction is promiscuous and that six possible α/β subunit-containing isoenzymes are likely to be formed. Cellular localization studies have provided evidence for the existence of isoenzymes composed of α1/β1 and α3/β1 subunit combinations in neurons and α2/β1 and α2/β2 subunit combinations in astroglia (16). Isoenzymes composed of α3/β2 subunit combinations have been detected in pineal gland (12) and photoreceptors (13), while α2/β2-containing isoenzymes appear to be expressed in skeletal muscle (32). The identification of the mammalian Na,K-ATPase β3 subunit raises the possibility of nine potential α/β subunit-containing isoenzymes. It will now be important to determine whether different combinations of α and β subunits may be related to specific functional roles of the Na,K-ATPase in different tissues and cell types.
