The mouse Na\textsuperscript{+}-K\textsuperscript{+}-ATPase γ-subunit gene (Fxyd2) encodes three developmentally regulated transcripts

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METHODS

Southern blotting. Genomic DNA was isolated from fresh, adult mouse liver. Approximately 10 μg of genomic DNA was
digested with restriction enzymes (with BamHI, EcoRI, or HindIII) and processed for Southern blotting (15° C in Hybrid-N nylon membrane (Amersham Pharmacia Biotech, Baie d’Urfé, Quebec, Canada). Blots were probed under conditions of varying stringency (high stringency, 68°C, 0.1× SSC; low stringency, 62°C, 0.5× SSC). The membranes were developed for detection of the hybridized digoxigenin (DIG)-labeled probe using the DIG system for filter hybridization (Roche Diagnostics, Laval, Quebec). The probe was generated from γ-subunit clone AA162918, which contains 518 bp of γ-subunit cDNA isolated from Barstead mouse kidney dbEST library and obtained from American Type Tissue Collection (Rockville, MD).

Library screening and determination of genomic sequence. A phage 129/Sv mouse genomic library was screened using the same DIG-labeled probe as above. Positive plaques were isolated, and the genomic DNA was subcloned into Bluescript vector (Stratagene, La Jolla, CA) for sequencing and characterization. Clones were submitted for automated sequencing at the Robarts Research Institute (RRI) Sequencing Facility (London, Ontario). Sequence information was processed using the Baylor College of Medicine (BCM) Search Launcher (http://searchlauncher.bcm.tmc.edu) to convert file formats and align sequences to those known in GenBank.

RNA isolation and reverse transcription. RNA from 15 mg of mouse tissue was isolated using Trizol reagent (Life Technologies, Burlington, Ontario). Organs from three mice were homogenized separately in 0.9 ml of Trizol and added to 0.2 ml of chloroform before centrifugation for 15 min at 10,000 g. Escherichia coli RNA (20 μg) was added to the supernatant as carrier, and total RNA was precipitated with isopropanol. The pellet was rinsed with ethanol and resuspended in water for RQ1 DNase treatment (Promega, Madison, WI). RNA was isolated from mouse embryos as described by MacPhee et al. (8). Purified RNA preparations were reverse transcribed into cDNA with Superscript II reverse transcriptase after preincubation with oligo(dT) primer (both reagents from Life Technologies).

RACE generation of full-length Fxyd2 cDNAs. Poly(A)⁺ RNA was isolated from mouse kidney, and a Marathon cDNA amplification kit (Clontech, Palo Alto, CA) was used to generate full-length Fxyd2 cDNA clones. 5’-RACE (i.e., “rapid amplification of cDNA ends”) PCR reactions were executed on 1:250 diluted mouse kidney cDNA using adapter primer 1 (AP1) and Fxyd2 3’ primer (5’-TGACCTGCGCTATGTTTCT-3’) in a Perkin-Elmer GeneAmp 2400 PCR machine. Products were visualized on a 1.5% agarose gel, and fragments were isolated with a NucleoTrap DNA purification kit (Clontech) for cloning. 3’-RACE reactions were performed identically, except that 5’ primers were designed using Oligo Primer Analysis Software (Molecular Biology Insights, Cascade, CO) to amplify the three different Fxyd2 transcripts: 2c (5’-GGGATTTCTCGGCTTTAG-3’), 2b (5’-TGACCTGCGCTATGTT-3’), and 2a (5’-AGGAAGACCGTGACCTGGAG-3’). Full-length Fxyd2c sequence was also confirmed by RACE of Marathon-Ready mouse kidney (Amersham Pharmacia Biotech) or PCR2.1-TOPO (Invitrogen) vector and sequenced in both directions. Sequences were processed and aligned using BCM Search Launcher.

Polymerase chain reaction. For Fxyd2 cDNA amplification, the upstream primer was as previously described for each mRNA variant, and the downstream primer was 5’-TGACCTGCGCTATGTTTCT-3’. These primers were designed to amplify distinct fragments for each cDNA (2c = 342 bp, 2b = 217 bp, and 2a = 234 bp). A “touchdown protocol” was used in which a 30-s denaturation step at 94°C was followed by a 30-s annealing step in which the annealing temperature was successively reduced from 63°C to 60°C over the first 12 cycles, then kept at 58°C for 28 cycles, followed by 30 s of primer extension at 72°C, for a total of 40 cycles. An initial 5-min preincubation at 94°C and a final 7-min 72°C extension was also included for each reaction. Each PCR was repeated at least three times to verify expression. The amplicons were analyzed on agarose gels containing 0.75 μg/ml ethidium bromide, and the identity of the amplicons was confirmed by restriction enzyme digestion (2c = Bpm1, 2b = Kpn1, and 2a = Bgl2) and by sequencing. Predicted fragment sizes were as follows: 2c = 81 + 342 bp; 2b = 46 + 171 bp; 2a = 44 + 190 bp.

For amplification of human cDNA (Clontech), primers (5’-ACTGTGGGGGAGGTGGGTCAAATG-3’ and 5’-GATGCCAC-CTGGGTTACATTG-3’) were designed from the human Na-K-ATPase γ-subunit gene (FXYD2; accession number AF816896). These primers were designed to amplify a 887-bp fragment of the human FXYD2 cDNA that spans the novel 5’ sequence, the common transmembrane and COOH-terminal encoding sequence, and 169 bp of the 3’-untranslated region (3’-UTR). Each PCR was run for 35 cycles of 94°C (30 s), 60°C (30 s), and 72°C (45 s). An initial preincubation at 94°C and a final 7-min 72°C extension was also included for each reaction. Each PCR was repeated at least three times to verify expression. The amplicons were analyzed on agarose gels, and the identity of the amplicons was confirmed by sequencing the cloned products from each positive tissue.

RESULTS

Three γ-subunit mRNA variants are encoded by a single gene. A Southern blot of mouse genomic DNA probed with a cDNA encoding the transmembrane and COOH-terminal domains of the γ-subunit shows a single band in each lane (Fig. 1), suggesting the presence of a single copy gene, Fxyd2 (17), encoding the γ-subunit. Lowering the stringency of hybridization did not reveal any additional bands. When a genomic library was screened with the same probe, two clones were isolated and, after characterization with restriction enzymes, were determined to be identical. Restriction mapping and sequencing of the genomic clones revealed three putative NH2-terminal encoding exons and a separate exon encoding the transmembrane domain of the protein. The COOH terminus is encoded by two small exons, and the sequence for the 3’-UTR of the mRNA is contained within its own exon (Fig. 2).
comparison with cDNAs and expressed sequence tags (ESTs) in GenBank, it became apparent that each of the NH2-terminal encoding exons is spliced into the common transmembrane encoding exon, which in turn splices into the COOH-terminal encoding exons and 3’-UTR to make the three distinct mRNAs that are found in the databases. Intervening intron lengths are noted in Fig. 2B and splice sites follow the 5’gt...ag 3’ rule.

Amplification of the three full-length mRNA sequences (Fig. 3) from mouse kidney (and also from

Fig. 2. The γ-subunit (Fxyd2) gene encodes three variants differing in their NH2 termini. A: a block diagram of the γ-subunit genomic sequence shows the relative positions of coding exons. Alternate NH2-terminal (NT) domain exons (a, b, and c) each splice into the same transmembrane (TM) domain exon, which in turn splices into the two COOH-terminal (CT) domain exons and a common 3’-untranslated region (UTR) sequence. B: the cloned Fxyd2 gene contains several small exons (upper case) that encode three separate NH2-terminal domains (NTc, NTb, NTa), a common transmembrane (TM) domain, two common COOH-terminal (CT) domains and a common UTR sequence (all splice junctions follow the 5’gt...ag 3’ rule). Only the first and last 10 base pairs of each intron (lower case) are shown, with the number of omitted base pairs indicated. Exons are labeled as NTa, NTb, and NTc to correspond to the previously reported Fxyd2a and Fxyd2b cDNAs (16), and the proposed translation start site for each variant is shown in boldface. Underscored portions of the sequence refer to the exons that are shared by the three mRNA variants. The polyadenylation site is also shown in boldface. The complete genomic sequence has been submitted to GenBank under accession number AY035583.
Fig. 3. RACE generates three distinct cDNA clones for Fxyd2. A: primers designed from the Fxyd2 genomic sequence allowed the generation of three different cDNA clones (Fxyd2a, Fxyd2b, and Fxyd2c). Each clone was verified by sequencing to match g-subunit cDNA clones deposited in GenBank. The predicted translational start and polyadenylation sites are shown in boldface. B: an alignment of the proposed amino acid sequences shows that, while the NH2 termini of the three variants are distinct, the sequences are identical commencing just prior to the transmembrane domain (shown as dark highlight). C: novel sequence for a possible human FXYD2c mRNA variant obtained from cloned PCR amplicons in brain, spleen, and testis.

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testis for Fxyd2c) was accomplished using a RACE procedure with the amplicon identities being confirmed by cleavage with appropriate restriction enzymes. RACE experiments were repeated on mouse kidney and testis cDNA for confirmation of Fxyd2c sequence, and at least three separate clones were sequenced in both directions. The sequences were compared with ESTs already present in GenBank. The novel cDNA we are reporting, Fxyd2c, corresponds to two ESTs deposited in GenBank (AL892737 and AA106912, both from clone 518773), whereas Fxyd2a and Fxyd2b are described by Sweadner and Rael (17). The three cDNAs encode γ-subunit variants with identical transmembrane and COOH-terminal sequences but different NH2-terminal, or extracellular, domains.

Although Sweadner et al. (18) did not identify a third 5′ exon in human DNA that would encode a γc variant, we were able to find such a sequence by aligning the mouse Fxyd2c 5′ exon to the human gene in the database (accession no. AF316896). This exon shares 82% identity at the nucleotide level with the mouse Fxyd2c 5′ exon, although the alignment shows several sequence gaps. This novel 5′ exon encoding a possible human γc variant is 3,828 bp upstream of the γb exon and 9,193 bp from the first transmembrane encoding domain (18). The proposed human FXYD2c cDNA (Fig. 3C) is longer than the cDNAs reported for human FXYD2a (552 bp) and 2b (567 bp; Ref. 17); its greater length (887 bp) is due to the length of the 5′ exon. This exon, however, does not appear to encode an initiator methionine.

The Fxyd2 transcripts have partially overlapping expression patterns. RNA isolated and reversed transcribed from a variety of mouse organs was used to investigate the expression pattern of each Fxyd2 transcript. Figure 4 shows a gel of PCR amplification products using primers distinct for each mRNA. Both Fxyd2b and Fxyd2a mRNAs are present in most organs, but Fxyd2c mRNA is more restricted in its distribution, being absent from adult liver, spleen, and lung. Fxyd2a mRNA is also absent from the adult lung, leaving that organ with only Fxyd2b expression.

Examination of the developmental expression of Fxyd2 transcripts in the fetal mouse kidney revealed that the mRNAs encoding all three variants are present at days 13.5 and 15.5 (Fig. 5); however, Fxyd2c mRNA was not consistently detected at day 17.5, suggesting very low abundance. In addition, the variants are differentially expressed during preimplantation development (Fig. 6), where Fxyd2a and Fxyd2b mRNAs are clearly present but Fxyd2c mRNA was not detectable.

The expression pattern of human FXYD2c appears to be much more restricted than in the mouse, with PCR amplicons detected only in the brain, spleen, and testis (Fig. 7).

**DISCUSSION**

Our evidence indicates that the γ-subunit, unlike the α- and β-subunits, is encoded by a single gene. Recently, Kuster et al. (7) extracted and purified two anti-γ-immunoreactive polypeptides from an SDS-PAGE gel for peptide sequencing and found that these proteins have different NH2-terminal domains. With our cloning of the γ-subunit gene (Fxyd2), it has become apparent that there are at least three variants that differ in their NH2 termini but have common transmembrane and COOH-terminal domains. Sweadner and Rael (17) reported only two Fxyd2 cDNAs from their search of database deposits, yet GenBank clone 518773 from mouse kidney, in addition to our genomic and RACE cDNA sequence data, clearly indicates the presence of at least one more transcript that encodes the γ-subunit. Although neither clone 518773 nor genomic clone AZ447067 from the Genomic Survey Sequences (GSS) database contains a predicted translation start site, one is predicted from our RACE sequencing and genomic data. Importantly, the human FXYD2 gene also contains a possible γc exon, and a transcript that contains the novel NH2-terminal encoding region fused with the common transmembrane and COOH-terminal encoding regions can be amplified (Fig. 3). Since no initiator methionine can be found in this novel 5′ exon, it may mean that the human γc exon is actually a pseudo-exon but corresponds to an exon that is functional in the mouse. Other pseudo-exons have been reported in the human FXYD2 gene (18).

The genomic structure of mouse Fxyd2 mirrors that reported for the human gene (18), apart from the fusion of the two exons encoding the transmembrane domain.
of the human protein into one exon in the mouse. Both species share the presence of extended repeat elements in the introns, although the functional significance of these sequences is unknown.

The possibility that a third subunit of the sodium pump, γ, may somehow regulate the workings of the more extensively characterized α- and β-subunits of the holoenzyme has only recently been investigated. Reported functional effects of the γ-subunit include an increase in the affinity of the enzyme for ATP (14, 19), the favoring of the E1 conformation (20), and an influence on the voltage sensitivity of K1 activation (3). The γ-subunit also affects cation affinities, although the results of studies with γ transfection into different cell lines remain somewhat puzzling. Thus Arystarkhova et al. (1) reported that γa decreases the apparent affinity of the pump for both Na+ and K+, but with effects on Na+ affinity affected by post translational modification. More recently, Pu et al. (14) reported that the γa and γb variants have similar modulatory effects on pump kinetics, causing a decrease in sodium affinity due to cytoplasmic competition of K+ and Na+. The γ-subunit variants appear to differ in their distribution along the nephron in rat kidneys (although they overlap in the thick ascending limb) suggesting cell-specific functions of the Na+-K+-ATPase rather than different modulatory roles (14). Interestingly, the proposed human FXYD2c mRNA variant may not actually encode a functional peptide due to the absence of an initiator methionine, raising the possibility that the corresponding variant in the mouse may not have an essential function. Clearly, functional analyses of each variant will be required to elucidate the physiological roles of the γ-subunit.

The expression patterns of the mRNAs encoding the three variants indicate that they are partially tissue specific (Fig. 4), which may relate to their proposed ability to perform different regulatory roles or may point to differing associations with the various isozymes of the pump. In the lung, for example, Fxyd2b appears to be the only mRNA expressed. Since this mRNA was detected in all other organs assayed, it may be that this variant plays a more generalized role. In contrast, Fxyd2c mRNA is restricted to certain organs (mouse kidney, heart, brain, ovary, and testis in our study). Fxyd2c mRNA also appears to be developmentally regulated in the kidney (Fig. 5). Such variation in mRNA expression indicates independent transcription of the exons encoding the three variants; however, we
cannot at this point rule out the possibility that these transcripts arise from alternate splicing from a single undiscovered upstream exon.

The expression pattern of the human FXYD2c variant appears to be even more restricted than that of the mouse, with mRNA being detected only in the brain, spleen, and testis (Fig. 7). This limited distribution, perhaps coupled with low abundance, could explain the lack of FXYD2c representation in EST databases (17, 18).

Previously, we had reported that embryonic expression of γ-subunits is required for blastocoe formation in preimplantation embryos, a process driven by basolaterally localized sodium pumps (6). In those experiments, antisense oligonucleotides designed to be complementary to the common transmembrane-encoding portion of the mRNAs were employed to impede synthesis of γ-subunits. Since Fxyd2c mRNA is uniquely absent from preimplantation embryos (Fig. 6), the effect of the antisense treatment (delayed blastocoe formation) was caused by reduction in FXYD2a and/or FXYD2b expression. Experiments in progress are designed to determine whether these two variants are equally important for preimplantation development.

The existence of multiple γ-subunit variants raises many questions as to how this small protein may act in conjunction with the sodium pump, or perhaps on its own, to regulate cation transport in mammalian cells. With the cloning of the gene that encodes this regulatory protein, we hope that further studies will elucidate the mechanism of action of all three of the proposed variants.

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