H-K-ATPase in the RCCT-28A rabbit cortical collecting duct cell line

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Campbell, W. Grady, I. David Weiner, Charles S. Wingo, and Brian D. Cain. H-K-ATPase in the RCCT-28A rabbit cortical collecting duct cell line. Am. J. Physiol. 276 (Renal Physiol. 45): F237–F245, 1999.—In the present study, we demonstrate that the rabbit cortical collecting duct cell line RCCT-28A possesses three distinct H-K-ATPase catalytic subunits (HKα). Intracellular measurements of RCCT-28A cells using the pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) indicated that the mechanism accounting for recovery from an acid load exhibited both K+ dependence and sensitivity to Sch-28080 characteristic of H-K-ATPases. Recovery rates were 0.022 ± 0.005 pH units/min in the presence of K+, 0.004 ± 0.002 in the absence of K+, and 0.002 ± 0.002 in the presence of Sch-28080. The mRNAs encoding the HKα1 subunit and the H-K-ATPase β-subunit (HKβ) were detected by RT-PCR. In addition, two HKα2 species were found by RT-PCR and 5' rapid amplification of cDNA ends (5'-RACE) in the rabbit renal cortex. One was homologous to HKα2 cDNAs generated from other species, and the second was novel. The latter, referred to as HKα2x, encoded an apparent 61-residue amino-terminal extension that bore no homology to reported sequences. Antipeptide antibodies were designed on the basis of this extension, and these antibodies recognized a protein of the appropriate mass in both rabbit renal tissue samples and RCCT-28A cells. Such findings constitute very strong evidence for expression of the HKα2x subunit in vivo. The results suggest that the rabbit kidney and RCCT-28A cells express at least three distinct H-K-ATPases.

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complete cDNA for rabbit cortex HKα2a, a putative alternatively spliced variant was found. This rabbit variant differed from the HKα2a alternatively spliced variant reported for rat (29), and we have designated the new subunit HKα2c. RT-PCR detected mRNAs for H-K-ATPase subunits, including HKα1, HKα2a, HKα2c, and HKβ in both kidney tissues and RCC-28A cells. An antipeptide antibody specific for the HKα2c isoform was used to detect the subunit. The results establish that multiple isoforms of H-K-ATPase, including the novel HKα2c, reside within a single clonal cell line.

METHODS

Cell culture The RCC-28A cell line was derived from immunodissected rabbit renal CCD (4). These cells were the kind gift of Dr. William Spiegelman, and experiments were performed using cells between passages 11 and 31. Cells were grown in DMEM media supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. All media were bubbled overnight with a mixture of 5% CO2-21% O2-74% N2, then filtered sterilized by use of a syringe-mounted filter. Cells were maintained in culture in tissue culture flasks at 37°C in a 5% CO2 atmosphere and were plated on Corning Costar Transwell collagen-coated semi-permeable inserts for experiments. Cells were plated at a density of 2 x 10^5/cm^2, grown for 2 days in media containing 10% FBS, and then shifted to 0.1% FBS for a period of 24 h prior to the experiment.

Measurement of pHr. The fluorescent, pH-sensitive dye 2',7'-bis(2-carboxyethyl)-5(6)-carboxyfluorescein (BCECF) was used to measure pHr (44). Cells were incubated at room temperature for a period of 30 min in solution 1 (Table 1) with the acetoxyethyl ester of BCECF (BCECF-AM; Molecular Probes, Eugene, OR) at a final concentration of 5 µM. A minimum of 5 min perfusion with solution 1 delivered at 37°C rinsed away BCECF-AM at the beginning of each experiment. Cells were excited at 440 and 490 nm, and emission was measured at 530 nm. Measurements were made at 30-s intervals. Fluorescence was detected by a Videomac model KS-1381 image intensifier coupled to a Dage model 72 charge-coupled device camera. Images were digitized and stored to the hard disk of a personal computer using commercially available equipment (Image 1/FL software package; Universal Imaging, Westchester, PA), allowing subsequent analysis of single cells.

Table 1. Solutions

<table>
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All values are in mM. Osmolality was adjusted to 290 ± 5 mosmol/kgH2O by addition of the principal salt. pH was adjusted to 7.40 ± 0.05 by addition of tetramethylammonium-OH. Solutions were bubbled with 100% O2.

During measurements, cells were constantly perfused at a rate of ~10 ml/min by HEPES-buffered solutions (Table 1) that were continuously bubbled with O2 and preheated to be delivered at a temperature of 37°C. Apical and basolateral sides were perfused simultaneously with identical solutions. Inhibitors were diluted into solutions immediately before experiments were begun. Cells were acid loaded using the NH4Cl prepulse technique. Briefly, cells were incubated with 20 mM ammonium chloride (equimolar substitution for NaCl) for 5 min, then ammonium chloride was removed from the perfused solution. Addition of inhibitors or K+ removal was done at the beginning of the NH4Cl prepulse. At the end of each protocol, ethylisopropylamiloride (EIPA) was removed from the perfusing solutions, and an increased rate of pHr recovery was used as a sign of cell viability. The EIPA stock solution was 1 mM dissolved in DMSO, and Sch-28080 was 10 mM dissolved in DMSO. EIPA was obtained from Research Biochemicals International (Natick, MA), and Sch-28080 was the kind gift of Dr. James Kaminski at Schering (Bloomfield, NJ).

Calibration of the pHr measured by BCECF fluorescence was carried out by the high potassium-nigericin calibration technique of Thomas et al. (42). Buffer capacities were calculated according to the method of Boyarsky et al. (8). Determination of total RNA. New Zealand White rabbits were killed by decapitation, and the kidneys were removed immediately. Kidneys were sliced coronally, and cortex and medulla were separated. Tissues were Dounce homogenized, and total RNA was prepared from each tissue by the acid phenol method (12).

RT-PCR. RT-PCR was carried out as described by Davis et al. (19). Template for the RT-PCR reaction was 1 µg total RNA from rabbit renal cortex or RCC-28A cells using random hexamers to prime the reverse transcription. PCR reactions were primed with pairs of oligonucleotides (10 µM) synthesized by the University of Florida Interdisciplinary Center for Biotechnology Research (UF ICBR) DNA Synthesis Core (Table 2). For amplification from rabbit renal cortex using degenerate oligonucleotides, thermal parameters of the PCR reactions included a 5-min 94°C preincubation followed by denaturation at 94°C for 40 s, anneal at 55°C for 1 min, and extension at 72°C for 2 min, for a total of 30 cycles followed by a final extension of 5 min. For amplification from RCC-28A cell RNA, thermal parameters of the reactions included a 2-min 94°C preincube followed by 40 cycles of denaturation at 94°C for 30 s and anneal/extension at 68°C for 1 min, with a final extension of 5 min. PCR products were cloned into the pCRII vector utilizing the TA Cloning Kit (Invitrogen, San Diego, CA). Sequencing of plasmids was carried out by the UF ICBR DNA Sequencing Core.

Rapid amplification of cDNA ends. 5'-RACE was carried out as described by Davis et al. (19). mRNA was prepared from rabbit renal cortex total RNA using the PolyATtract System (Promega, Madison, WI). Complementary strand was synthesized using the sense primer (TGCGGAAACTCTTCTCATCAGG, nucleotides 3079–3098 of the rabbit HKα2a sequence). The PCR was performed as before for 40 cycles, and the products were cloned. The library of clones produced in this manner was screened using an oligonucleotide (CTCTACCCCTGGCACCTGGTG, nucleotides 3099–3118 of the rabbit HKα2a sequence) 5'-labeled using an enhanced chemiluminescence kit (ECL; Amersham, Arlington Heights, IL).

5'-RACE was performed using the Marathon cDNA Amplification Kit (Clontech, Palo Alto, CA) following manufacturer's instructions except as follows. RT reactions were carried out using 5.5 µg total RNA of rabbit renal cortex, incubated with the rabbit HKα2 gene-specific primer TTGCCATCTGC-CCTCCTTT (nucleotides 121–102) for 30 min at 50°C, then

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Table 2. PCR primer pairs

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<td>CGGAGAAAAGGCTGACTGGAGGCT</td>
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<td>304–873</td>
<td>TAAGGCAGACGTUTATGG</td>
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Notation is that of the International Union of Biochemistry Nomenclature Committee, i.e., N = any base, R = A or G, M = A or C, W = A or T, and Y = C or T.

at 55°C for 15 min. PCR reactions were carried out using the anchor primer included in the Marathon kit paired with gene-specific primer TATCTGTAGCTGACATGGGCCAC (nucleotides 93–69).

Northern analysis. Northern blots were carried out following the procedures of Davis et al. (19). mRNA (1 µg per lane) underwent electrophoresis in a 1% agarose, 0.22 M formaldehyde denaturing gel. Following capillary transfer to nylon membrane (Hybond N, Amersham), membranes were probed with 32P-labeled probes prepared using the MegaPrime kit (Amersham). The rabbit HKα2-specific probe corresponded to nucleotides 7–93 of the HKα2 sequence (Table 2). The HKα2-specific probe corresponded to nucleotides 24–377 of HKα2. Following hybridization, three 20-min washes were done in 0.2× SSC (0.03 M NaCl, 3 mM sodium citrate), 0.1% SDS at 55°C. Autoradiography was carried out for 6 days using BioMax MS film and screens (Kodak, Rochester, NY).

Western analysis. Antipeptide antibodies were raised in chickens by Lofstrand Laboratories (Bethesda, MD). Peptides were synthesized and conjugated to keyhole limpet hemocyanin (Amersham). The rabbit HKα2-specific antibody was used at a dilution of 1:10,000. Detections of HKα2 proteins were performed using the ECL system (Amersham) by allowing the reaction to run for 6 days using BioMax MS film and screens (Kodak, Rochester, NY).

RESULTS

K+-dependent pHi regulation. Prior to engaging in the molecular analysis of the H-K-ATPase activity present in the cells. We examined the extent to which an H-K-ATPase contributed to pHi, recovery from an acid load by BCECF fluorescence microscopy with digital video image analysis. An H-K-ATPase activity had been reported previously by other methods (7). The large contribution of the Na+/H+ exchanger to H+ extrusion normally obscures recovery by other mechanisms, so the potent amiloride analog EIPA was used to inhibit the Na+/H+ exchanger in these experiments.

To detect H-K-ATPase activity in RCCT-28A cells, K+-dependent recovery from an acid load was determined. A representative tracing of a cell acid loaded by NH4Cl withdrawal. Data were collected for independent experiments involving separate passages of cells. pHi recovery rates for each experimental condition are presented as the means of the rates determined for the individual experiments ± SE. Cells without Na+/H+ exchange activity, defined as an increase in pHi, recovery rate with EIPA removal, were classified as nonviable and excluded from analysis. P < 0.05 by Student’s t-test was taken as significant.
pHi recovery rates are influenced by the degree of intracellular acid loading and by buffer capacity. Actual nadir pHi values observed in these experiments in the presence and absence of K⁺ were not significantly different (6.64 ± 0.06 and 6.59 ± 0.08, respectively; P = not significant (NS)). No significant difference in buffer capacities in the presence of K⁺ and absence of K⁺ was seen (15 ± 1 and 16 ± 1 meq H⁺·pHi unit⁻¹·liter cell volume⁻¹, respectively; P = NS). Consequently, differences in pHi recovery rates found in our experiments indicated differences in H⁺ extrusion.

To test the hypothesis that K⁺-dependent pHi recovery from an acid load resulted from H-K-ATPase activity, the effect of the classic H-K-ATPase inhibitor Sch-28080 (10 µM) on RCCT-28A cell pHi regulation was examined (Fig. 2C). Using this protocol, 109 cells from 4 separate cell preparations had a mean recovery rate of 0.002 ± 0.002 pH units/min (P < 0.05 vs. 5 mM K⁺, P = NS vs. 0 mM K⁺) in the presence of 10 µM Sch-28080. The buffer capacity of 16 ± 2 meq H⁺·pHi unit⁻¹·liter cell volume⁻¹ and the nadir pHi of 6.54 ± 0.03 were not significantly different from those measured in the absence of Sch-28080 (P = NS).

In summary, either the presence of the H-K-ATPase inhibitor Sch-28080 or the absence of K⁺ virtually abolished EIPA-insensitive pHi recovery from an acid load in RCCT-28A cells (Fig. 3). These results confirm the presence of H-K-ATPase activity in RCCT-28A cells.

Generation of a rabbit HKα₂ cDNA. To define the molecular identity of HKα isoforms present in RCCT-28A cells, it was first necessary to find the nucleotide sequence of those isoforms present in rabbit renal cortex. Although a complete sequence for the rabbit HKα₂ cDNA was available (5), only a partial nucleotide sequence has been reported for the rabbit HKα₂ cDNA (22). Degenerate oligonucleotides designed to prime amplification reactions at sites that are highly conserved among P-type ATPases were used to obtain a cDNA fragment of HKα₂ from rabbit renal cortex RNA. These oligonucleotides were specifically designed to yield RT-PCR products from any HKα subunit mRNA present in the rabbit renal cortex. Only products repre-

![Fig. 2. Intracellular pH (pHi) recovery from an acid load by RCCT-28A cells. A: pHi recovery from an acid load by a K⁺-dependent, ethylisopropylamiloride (EIPA)-insensitive mechanism. Recovery toward physiological pH is seen in presence of K⁺ in a representative tracing of pHi in an individual cell. Periods during which solutions contain 20 mM NH₄Cl and 1 µM EIPA are indicated. B: pHi recovery from an acid load by a K⁺-independent, EIPA-insensitive mechanism. Negligible recovery toward physiological pH is seen in absence of K⁺ in a representative tracing of pHi in an individual cell. C: pHi recovery from an acid load by a Sch-28080-sensitive, EIPA-insensitive mechanism. Negligible recovery toward physiological pH is seen in presence of the H-K-ATPase inhibitor Sch-28080 in a representative tracing of pHi in an individual cell.](http://ajprenal.physiology.org/)

![Fig. 3. Summary of the rates of pH recovery from an acid load. Mean rates of recovery are indicated by open (presence of K⁺), solid (absence of K⁺), and stippled (in presence of Sch-28080 with K⁺ present) bars. *P < 0.01 vs. 5 mM K⁺, **P < 0.05 vs. 5 mM K⁺, P = NS vs. 0 K⁺.](http://ajprenal.physiology.org/)
senting Na-K-ATPase α₁- and HKα₂-like messages were found. Thus our next goal was to determine the complete coding sequence for the rabbit HKα₂ cDNA. Using the sequence information found in this manner, subsequent RT-PCR reactions were carried out in which one primer of a pair was gene specific and the other was a degenerate primer designed using conserved regions of P-type ATPases. Outside the coding region, conservation between members of the P-type ATPase gene family declines, so 3'- and 5'-RACE was carried out to extend the sequence to the 3' and 5' ends.

Two rabbit renal HKα₂ cDNA sequences were found in this manner, having 4,035 bases in common at the 3' end but different at the 5' end. One of the 5' ends had a high degree of homology to the human and rat HKα₂ cDNAs, and this clone was designated as the rabbit HKα₂a. The other was a novel sequence named HKα₂c. These sequences have been submitted to GenBank (accession nos. AF 023128 and AF 023129). A segment of the shared 3' portion of the sequences was identical to the 1,456-bp sequence obtained by Fejes-Toth et al. (22) except for two single base mismatches (C→T at nucleotide 2927, which does not change the primary protein sequence, and G→T at nucleotide 3259, in the 3'-untranslated region). The rabbit HKα₂a nucleotide sequence was 86% identical to human ATP1AL1 and 83% to rat HKα₂c cDNAs, whereas identity of HKα₂a to rabbit HKα₁ was only 67%. The deduced amino acid sequence shared 93% similarity with human ATP1AL1, 93% similarity with rat HKα₂, but merely 80% with rabbit HKα₁.

However, the 5' end of the rabbit HKα₂c differed dramatically from the rat HKα₂a and HKα₂b sequences (29). Rabbit HKα₂c cDNA lacked the start ATG codon corresponding to that present in the rat and human HKα₂ sequences. The new probable start codon was located upstream in frame with the HKα₂ open-reading frame. Thus the deduced amino acid sequence of the HKα₂c appeared to be 61 amino acids longer at the amino terminal end than the rabbit HKα₂a subunit (Fig. 1). This extension and the 5'-untranslated region bore no homology to the comparable segments from the HKα₂ cDNA from rat or to any GenBank sequence. It may represent an alternative splicing product; the sequence homology diverges from the human HKα₂ sequence at a point known in the human ATP1AL1 gene to be a splice junction (nucleotide 177 of ATP1AL1) (41).

Detection of H-K-ATPase mRNAs in RCCT-28A cells. We examined whether the RCCT-28A cells possessed mRNA for the H-K-ATPase α- and β-subunits. RT-PCR products were generated and sequenced using RCCT-28A cell total RNA as a template. The presence of HKβ subunit mRNA was observed by amplification of a 570-bp cDNA corresponding to nucleotides 304–873 of the sequence of rabbit gastric HKβ (37) (Fig. 4). Nucleotide sequencing of the RCCT-28A product confirmed that the amplified product was identical to the rabbit HKβ subunit mRNA.

A similar strategy was employed to show that HKα₁ was present in the RCCT-28A cells. Primers were designed to amplify a 611-bp region of HKα₁ mRNA (nucleotides 2537–3147), and again a product of the expected size was observed (Fig. 5). The nucleotide sequence was identical to that reported by Bamberg et al. (5) except for two single base mismatches (G→A at nucleotide 2567 and G→C at nucleotide 3089; neither affects the deduced amino acid sequence).

For HKα₂ mRNA, a primer pair yielded the anticipated product of 306 bp, which was identical to nucleotides 1264–1569 of the HKα₂ sequence, contained in the region common to HKα₂a and HKα₂c (Fig. 6A). To
Since RCCT-28A cells are a clonal cell line, individual cells appear to express at least three different H-K-ATPases.

Detection of H-K-ATPase mRNAs in rabbit distal colon and renal cortex. Northern analysis was used to detect the presence of HK α mRNAs in rabbit tissues. A probe specific for the 5′-untranslated region of HK α subunit hybridized at high level to distal colonic mRNA and to mRNA derived from renal cortex at a much lower level (Fig. 7A). In fact, it was necessary to overexpose the blot with respect to the colon tissue mRNA lanes to detect any signal in the kidney mRNA lanes. Another probe specific to the HK α subunit 5′-untranslated region hybridized to mRNA isolated from distal colon, but any hybridization to mRNA from renal cortex was apparently below detectable levels (Fig. 7B). As shown above (Fig. 6), HK α mRNA was detectable by RT-PCR using renal cortex as template.

Detection of the HK α2c subunit. Although the HK α2c cDNA indicated a continuous open-reading frame including the amino-terminal extension, the possibility remained that translation might be initiated at the ATG codon homologous to that reported for the rat HK α2c (29). Therefore, to determine whether the upstream ATG served as a translational start site, antipeptide antibodies were generated for peptides corresponding to amino acids 13–25 and 79–98 of the HK α2c subunit. The former (antibody LLC27) was HK α2c specific, whereas the latter (antibody LLC25) recognized a segment common to both HK α2a and HK α2c subunits. Western analyses of rabbit kidney tissues using antibody LLC25 revealed a doublet migrating at an apparent molecular mass of ∼90 kDa (Fig. 8A). Experiments using the HK α2c-specific antibody LLC27 indicated a single band with a migration comparable to the upper band of the doublet (Fig. 8B). Although we have not yet achieved separation of HK α2a and HK α2c proteins on immunoblots of membrane proteins from RCCT-28A cells, both antibodies recognize a protein of the correct mobility (Fig. 9). This provides strong evidence that the HK α2c subunit containing the amino-terminal extension was indeed present in both rabbit renal tissue and RCCT-28A cells.

DISCUSSION

We have generated complete cDNAs for both the rabbit renal HK α2a and the novel HK α2c subunits. mRNAs corresponding to these cDNAs were detected in rabbit distal colon and renal cortex. Proteins corresponding to both HK α2 isoforms were detected by immunoblot analysis, indicating that the novel HK α2c has the predicted amino-terminal extension. HK α1 protein has been previously detected by immunohistochemistry in CCD intercalated cells (6, 47). We also demonstrated that RCCT-28A cells express the HK β subunit, the HK α1 subunit, and both isoforms of the HK α2 subunit. The presence of multiple H-K-ATPase isoforms in a clonal cell line derived from CCD is the first evidence that several isozymes of H-K-ATPase may contribute to net activity in a single cell.
The finding that the three forms of the catalytic subunit are all present in a clonal cell line derived from CCD suggests that multiple H-K-ATPase isoforms exist in a single cell type in the CCD. Presence of mRNA for HKα1, HKα2a, and HKα2c isoforms in the RCCT-28A cell line is consistent with previous studies finding the presence of multiple H-K-ATPase catalytic subunit isoforms in the kidney (1–3, 18, 21, 22, 25, 32, 34). Expression of multiple HKα subunits within a cell suggests the possibility of greater complexity associated with formation of hybrid H-K-ATPases containing two different catalytic subunits. Moreover, an additional level of complexity appears with the consideration that HKα2 subunits are capable of forming stable complexes with both the HKβ and the Na-K-ATPase β1-subunits (15).

Our results demonstrate the presence of Sch-28080-sensitive H-K-ATPase activity in RCCT-28A cells. These observations are consistent with the findings of Bello-Reuss (7). Although Sch-28080 inhibits the gastric H-K-ATPase, the inhibition observed in this study using very low concentrations of Sch-28080 does not necessarily indicate that HKα1-containing H-K-ATPases account solely for the activity in RCCT-28A cells. The reported pharmacological properties of the non-gastric H-K-ATPases containing the rat HKα2a and human ATP1AL1 subunits vary widely. For example, studies in heterologous expression systems have reported that the HKα2a-containing H-K-ATPase is insensitive to Sch-28080 (15, 16), whereas the ATP1AL1 pump is sensitive to high concentrations of the inhibitor (35), and both have been reported to be Sch-28080 sensitive at much lower concentrations of Sch-28080 (23, 33). These same studies also report differing results using ouabain. Since the rabbit HKα2a sequence reported here has essentially equivalent homology to both rat HKα2a and human ATP1AL1 cDNAs, there is no basis for predicting the pharmacological properties of the pump in RCCT-28A cells. An interesting study by Buffin-Meyer et al. (9) reported a decrease in sensitivity of H-K-ATPase activity to Sch-28080 in the CCD between rats fed a normal diet and K+-depleted rats.

Fig. 7. HKα2 subunit mRNA. mRNA isolated from rabbit distal colon and renal cortex hybridized by probes specific for HKα2a (A) and HKα2c (B). No hybridization was observed when renal cortex mRNA was probed with the HKα2c-specific fragment.

Fig. 8. HKα2 subunit protein in renal cortex. Western analysis of membrane protein fractions derived from rabbit renal cortex was carried out by running all samples on the same gel for optimal comparison. After electrophoresis, the membrane was cut, and A and B were each probed with the antibodies indicated. A: antibody to HKα2 proteins (LLC25) detects a doublet indicating the two expected species of HKα2 protein. B: antibody to HKα2c amino-terminal extension (LLC27) detects a single reactive protein at the apparent mass as the larger species shown in A. Additional bands were visualized at lower molecular weights. No degradation products were consistently observed with different preparations of antibodies. No reactivity was seen to preimmune serum. Preabsorption with immunizing peptide blocked antibody reactivity.

Fig. 9. HKα2 subunit protein in RCCT-28A cells. Western analysis of membrane protein fractions derived from RCCT-28A cells is shown. A: antibody to HKα2 proteins (LLC25) detects a protein near the predicted mass for HKα2 proteins. B: antibody to HKα2c amino-terminal extension (LLC27) detects a reactive protein at the same apparent mass as the HKα2 common antibody (LLC25).
Whether this reflects expression of differing isoforms of H-K-ATPase in the K\(^+-\)depleted state or mobilization of existing pumps has not yet been defined.

The rabbit renal cortex HK\(_{\alpha2}\) cDNAs reported here have high homology to rat HK\(_{\alpha2}\) and human ATP1AL1 sequences (18, 24). The level of homology is relatively low compared with the homology typically found when comparing HK\(_{\alpha1}\) or Na-K-ATPase \(\alpha\)-subunits across mammalian species. However, it is much higher than the homology between the rat HK\(_{\alpha2}\) and HK\(_{\alpha2}\) cDNAs (18) or among the different Na-K-ATPase isoforms within a species. If the rat HK\(_{\alpha2}\) isoforms, the human ATP1AL1 protein, and the rabbit HK\(_{\alpha2}\) subunits are products of homologous genes in the different species, then the genes appear to have undergone greater evolutionary divergence than HK\(_{\alpha1}\) or the Na-K-ATPase catalytic isoforms.

Like the rat, the rabbit appears to have alternatively spliced transcripts of HK\(_{\alpha2}\) in the kidney. The organization of the rat alternatively spliced HK\(_{\alpha2b}\) cDNA (29) omitted the exon containing the start codon of the sequence previously reported for rat distal colon HK\(_{\alpha2a}\) cDNA (18), giving rise to a protein truncated by 108 amino acids at the amino-terminal end. The rabbit HK\(_{\alpha2e}\) sequence also omits the start codon present in the HK\(_{\alpha2a}\) sequence. However, an upstream 5' ATG codon lies in the same uninterrupted reading frame as the coding sequence for HK\(_{\alpha2}\), so initiation of translation at this position yields a subunit having an extended amino terminus 61 amino acids longer than the HK\(_{\alpha2a}\) protein. Western analysis demonstrated that a protein having this extension is present in rabbit kidney and the RCCT-28A cell line.

The HK\(_{\alpha2e}\) extension is hydrophilic in nature and lacks any conspicuous membrane-spanning domains. Because the amino terminus of H-K-ATPase catalytic subunits are cytosolic (40), the extension can be expected to have a cytosolic location. Chou-Fasman calculations predict this segment to be predominantly \(\alpha\)-helical with a turn in a region containing seven prolines between amino acids 26-40. A similarly proline-rich hinge region is found in the band 3 protein, and an ankyrin-binding site has been localized to that site within band 3 (45). The extended portion of the HK\(_{\alpha2e}\) protein contains a casein kinase II phosphorylation motif at Thr\(^{12}\), and a CAMP-dependent protein kinase phosphorylation motif at Thr\(^{53}\). These sites impart a potential for differential regulation of H-K-ATPases containing the HK\(_{\alpha2a}\) and HK\(_{\alpha2e}\) subunits.

Why are there three different H-K-ATPase isoforms in a cell line derived from an intercalated cell from the CCD? One possibility is to provide a means to respond to different signals and signal transduction systems governing H\(^+\) and K\(^+\) homeostasis. These adjustments might occur at the level of transcription of the HK\(_{\alpha2}\) gene, by intracellular trafficking of the pumps or by differential modification of the HK\(_{\alpha2a}\) and HK\(_{\alpha2e}\) containing H-K-ATPases.

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