Colonic H-K-ATPase β-subunit: identification in apical membranes and regulation by dietary K depletion

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Sangani, Pitchai, Sarah S. Kolla, Vazhaikkurichi M. Rajendran, Michael Kashgarian, and Henry J. Binder. Colonic H-K-ATPase β-subunit: identification in apical membranes and regulation by dietary K depletion. Am. J. Physiol. 276 (Cell Physiol. 45): C350–C360, 1999.—P-type ATPases require both α- and β-subunits for functional activity. Although an α-subunit for colonic apical membrane H-K-ATPase (HKα) has been identified and studied, its β-subunit has not been identified. We cloned putative β-subunit rat colonic H-K-ATPase (HKβ) cDNA that encodes a 279-amino-acid protein with a single transmembrane domain and sequence homology to other rat β-subunits. Northern blot analysis demonstrates that this HKβ is expressed in several rat tissues, including distal and proximal colon, and is highly expressed in testis and lung. HKβ mRNA abundance is upregulated threefold compared with normal in distal colon but not proximal colon, testis, or lung of K-depleted rats. In contrast, Na-K-ATPase β1 mRNA abundance is unaltered in distal colon of K-depleted rats. Na depletion, which also stimulates active K absorption in distal colon, does not increase HKβ mRNA abundance. Western blot analyses using a polyclonal antibody raised to a glutathione S-transferase-HKβ fusion protein established expression of a 45-kDa HKβ protein in both apical and basolateral membranes of rat distal colon, but K depletion increased HKβ protein expression only in apical membranes. Physical association between HKβ and HKα proteins was demonstrated by Western blot analysis performed with HKβ antibody on immunoprecipitate of apical membranes of rat distal colon and HKα antibody. Tissue-specific upregulation of this β-subunit mRNA in response to K depletion, localization of its protein, its upregulation by K depletion in apical membranes of distal colon, and its physical association with HKα protein provide compelling evidence that HKβ is the putative β-subunit of colonic H-K-ATPase.

Active potassium absorption; rat distal colon; hydrogen-potassium-adenosinetriphosphatase α-subunit

Active K absorption and secretion are important transport processes of mammalian large intestine (2). Active K absorption, a unique function of the distal colon, is energized and regulated by H-K-ATPase, an apical membrane P-type ATPase (8, 11). This colonic H-K-ATPase is a member of a gene family of related P-type ATPases that include Na-K-ATPase and gastric H-K-ATPase (7, 14, 35, 36). ATPases in this gene family usually are heterodimers that consist of α- and β-subunits. The α-subunit contains the catalytic function of these ATPases, whereas the specific function of the heavily glycosylated β-subunit is not completely known. Recent studies indicate that important functional properties of β-subunits include an essential role in the stabilization, maturation, and enzymatic activity of both Na-K-ATPase and H-K-ATPase (4).

Colonic K transport is modified by several factors, including changes in dietary K and aldosterone (11, 12). Increases in dietary K induce active K secretion, whereas dietary K depletion enhances active K absorption (12). Aldosterone, either as a result of dietary Na depletion or following its subcutaneous administration, markedly stimulates active K absorption in the rat distal colon (11, 37). The recent cloning of a cDNA that encodes the α-subunit of the rat distal colon H-K-ATPase (HKα) (7, 14) led to studies that have assessed the regulation by which dietary Na and dietary K depletion modify active K absorption and colonic H-K-ATPase activity in the rat distal colon. In contrast, dietary K depletion did not increase the abundance of HKα message or protein in the distal colon (15, 33).

One possible explanation to account for the absence of an upregulation of HKα message by dietary K depletion is that the effect of dietary K depletion on active K absorption is mediated by the β-subunit of the colonic H-K-ATPase, as β-subunits are required for the catalytic activity of P-type ATPases. Although a specific β-subunit for the colonic H-K-ATPase has not been isolated, conflicting observations exist as to whether a colon-specific β-subunit is required for maximal colonic H-K-ATPase activity (5, 6, 20). Thus, although Lee et al. (20) recently expressed HKα in Sf9 cells as ouabain-insensitive H-K-ATPase activity in the apparent absence of any β-subunit, other studies in Xenopus oocytes have demonstrated that the expression of HKα required noncolon β-subunits (5, 6). This requirement for such β-subunits may either represent a promiscuous expression by a related β-subunit, indicating that the colonic β-subunit is closely related, or indicate that colonic H-K-ATPase activity requires a β-subunit that is not colon specific.

To identify a β-subunit for colonic H-K-ATPase, a series of low-stringency Northern blot analyses with gastric H-K-ATPase β-subunit (HKβ) and Na-K-ATPase β1 and β2-subunit (NaKβ1 and NaKβ2) cDNAs were performed. These analyses demonstrated either no hybridization (HKβ, NaKβ1) or only a single band (NaKβ2) with mRNA from rat distal colon. A novel β-subunit cDNA that had been recently cloned from a rat astrocytoma cell line (38) and that had hybridized with guinea pig colon mRNA (Y. Suzuki, personal communication) was also used as a probe in additional Northern blot analyses using mRNA from rat distal colon. Because this cDNA hybridized with mRNA from...
rat distal colon at the size of 1.9 kb, we initiated experiments to clone a rat colon-derived β-subunit using the rat astrocytoma β-subunit cDNA as a probe.

The present study demonstrates the following. 1) A full-length β-subunit cDNA (HKçβ) isolated from a rat colon cDNA library is identical to a cDNA isolated from rat astrocytoma cells (38). In studies using this cDNA, dietary K depletion selectively increases the abundance of HKçβ message in the rat distal colon. 2) A polyclonal antibody to HKçβ protein identifies a protein in apical and basolateral membranes of rat distal colon. 3) This HKçβ protein expression is selectively increased in K depletion in apical but not in basolateral membranes. 4) Coimmunoprecipitation experiments reveal a physical interaction between HKα and HKçβ proteins. As a result, these observations suggest that this cDNA encodes the colonic H-K-ATPase β-subunit.

METHODS

Male Sprague-Dawley rats (200–250 g body wt; Charles River Laboratories, Wilmington, MA) were divided into three experimental groups: the control group was fed normal rat food that contained 4.4 g Na/kg and 9.5 g K/kg. The Na-depleted group was given a Na-free diet for 1 wk. The K-depleted group was given a K-free diet (0.6 mg K/kg) for 3 wk. All animals were allowed free access to water.

At the end of the experimental diet periods, the animals were killed and proximal colon, distal colon, ileum, jejunum, stomach, kidney, brain, lung, testis, liver, spleen, and heart were collected and processed as previously described (33) and all tissues were then homogenized using a Polytron homogenizer in 4 M guanidine isothiocyanate buffer for 60 s and centrifuged at 3,000 rpm for 10 min to remove any unbroken cells. Total RNA was prepared from the supernatant by ultracentrifugation through a buffer containing 2 M guanidine hydrochloride, 0.5% nonfat dry milk, 10 mM dithiothreitol, 10 mM EDTA, and resuspended in the lysis buffer containing 50 mM Tris·HCl (pH 7.4), 100 mM NaCl, 5 mM dithiothreitol, and 1 μg/ml lysozyme, mixed 45°C for 30 min and then 4°C for 15 min. Total RNA was isolated by passing total RNA through a column equilibrated with the lysis buffer. The total RNA was then quantitated by absorbance at 260 nm in an ultraviolet-visible double-beam spectrophotometer (Shimadzu). Northern blot analysis. Northern blot analyses were performed using poly(A)+ mRNA, as previously described (33). A 32P-labeled full-length HKçβ probe (1×106 counts·min−1·μg−1) was added to the membrane for hybridization at 42°C in a Hybond membrane for 18 h. Blots were washed for 15 min in 0.1× SSC (1× SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and 0.1% SDS at 65°C and were exposed to Hyperfilm (Amersham, La Jolla, CA) using a Biolight intensifying screen at 70°C.

cDNA screening and sequencing. A rat colon cDNA library with randomly primed cDNA inserts >1.0 kb in length was cloned in pDNAI (Invitrogen, San Diego, CA) and was used in the cloning of the β-subunit; 300,000 colonies were plated onto 20 Luria-Bertani [LB, containing 50 μg/ml ampicillin and 7 μg/ml tetracycline (amp/tet)] plates 150 mm in diameter. A microwave colony-screening protocol from Invitrogen was strictly followed. Briefly, colonies were transferred to nylon membrane (UV Duralon membrane, Stratagene, La Jolla, CA) after nylon membrane had been placed on top of the colonies in the plate. Membrane filters were lifted carefully and were laid on a fresh LB (amp/tet) agar plate by facing the colony side up; the plates were incubated with filters at 37°C for 1 h to allow colonies to grow. To regrow the colonies on the agar plates that were lifted, plates were incubated at 37°C for about 6 h and then stored at 4°C. The filters were carefully removed from the agar plate and placed colony side up on a Whatman 3 filter paper pretreated in lysis buffer (2× SSC-5% SDS, pH 7.0). The filter paper and the membrane with colonies were placed in a turntable microwave oven and heated at high power for 6 min. The filters were then placed in prehybridization solution containing 2× SSC (pH 7.0), 1% SDS, and 0.5% nonfat dry milk at 65°C for 1 h. The filters were removed from the prehybridization solution; any cellular debris was removed. The filters were then hybridized in buffer containing 6× SSC, 1% SDS, 0.5% nonfat dry milk, 100 μg denatured salmon sperm DNA, and 1×106 cpm/ml 32P-labeled full-length β-subunit cDNA probe from a rat astrocytoma cell line (38) labeled by random primer method (32) at 65°C for at least 16 h. The filters were then washed at room temperature for 10 min by gentle shaking in a buffer containing 2× SSC (pH 7.0) and 1% SDS, followed by 1× SSC (pH 7.0) and 1% SDS; the filters were transferred to prewarmed buffer (45°C) containing 0.1× SSC (pH 7.0) and 1% SDS and washed by gentle shaking at 45°C for 15 min. The filters were air dried, covered with Saran wrap, and exposed to Hyperfilm (Amersham) using a Biolight intensifying screen for 18 h. After development of the film, positive colonies were identified and screened two more times to obtain positive clones. Plasmids were prepared according to the method of Morel (27) using 2 ml of "terrific broth" (32), and the plasmid DNA containing inserts was sequenced in both strands using an automated fluorescence sequencing machine at the Yale Sequencing Facility.

Computer analysis of the nucleotide sequences was performed using Blast search [Genetics Computer Group (GCG) program, University of Wisconsin]. Hydrophy analysis was performed by the procedure of Kyte and Doolittle (19). Multiple sequence alignment of β-subunits was performed by the Pileup program (GCG software) (9).

Antibody production. The cDNA sequence (165 bp) corresponding to the amino acid sequence between Pro-87 and Ser-142 of HKçβ was amplified using the full-length colon-derived HKçβ as a template with a sense primer (5’-GGGGGGATCCCCACGAGTCTGGACCCTCCTG-3’) and an antisense primer (5’-GGGGGAAATTCGACGAGGCCGCTCTCGT-3’) to which an EcoRI site was appended at the 5’ end and an antiserum (5’-GGGGGGGAATTCGACGAGGCCGCTCTCGT-3’) to which an EcoRI site was appended at the 5’ end.

The expected size 165-bp fragment was gel purified, digested with BamH I and EcoRI, and ligated using T4 DNA ligase into PGEK-KG vector that had previously been digested with BamH I and EcoRI and dephosphorylated with calf intestine phosphatase. The ligated DNA was transformed into XL-1 blue Escherichia coli cells and spread onto LB-ampicillin plates. Plasmid DNAs were prepared from recombinant colonies, digested with BamH I and EcoRI, and electrophoresed onto 1.5% agarose gels. Positive clones containing the 165-nucleotide HKçβ fragment were selected and sequenced. Clones with correct reading frame and with correct cDNA sequence were selected to prepare glutathione S-transferase (GST)-HKçβ fusion protein. The recombinant colonies were grown in LB-ampicillin medium to an absorbance of 0.8 at 600 nm; the GST-HKçβ fusion proteins were then overexpressed after induction with 1.0 mM isopropyl β-d-thiogalactopyranoside at 37°C for 3 h. After induction, the cells were harvested, washed with 50 mM Tris-HCl (pH 7.4) containing 10 mM EDTA, and resuspended in the lysis buffer containing 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 5 mM dithiothreitol, 2 mM EDTA, 2 mM EGTA, and 1 mg/ml lysozyme, mixed 45°C for 10 min. The resulting mixture was centrifuged at 18,000 × g for 30 min, and the supernatant was used to carry out the GST pull-down assay. A GST-HKçβ fusion protein (20 μg) was incubated with glutathione-Sepharose 4B (Pharmacia, Piscataway, NJ) bound to an indicator and washed extensively with buffer containing 1× SSC-5% SDS, pH 7.0. The GST-HKçβ fusion protein was then eluted using 2× sample buffer, resolved on an SDS-6% polyacrylamide gel, and analyzed using a fluorography kit (Amersham).
min at 4°C, frozen at 80°C, and thawed. Then 1% Triton X-100, 10 mM MgCl₂, and 0.1 mg/ml DNase I were added and incubated at 4°C for an additional 30 min. After centrifugation, the GST-HKcβ fusion protein was purified from the supernatant by passage through a glutathione-agarose column; after the column was washed separately with 1× PBS and 1× PBS containing 1% Triton X-100 and with 50 mM Tris·HCl (pH 8.0) containing 1 mM EDTA, the GST-HKcβ fusion protein was eluted from the column using 10 mM reduced glutathione (pH 8.0) by incubating at 4°C with gentle nutation. The eluted GST-HKcβ fusion protein was dialyzed against 1× PBS at 4°C and concentrated using Centriprep 10. At this stage, the purified GST-HKcβ fusion proteins were run on SDS-PAGE gels and analyzed for homogeneity. The homogeneous preparation of GST-HKcβ fusion proteins was used to inject rabbits to raise polyclonal antibodies.

Antibodies were produced in a New Zealand White rabbit following primary subcutaneous injection of purified GST-HKcβ fusion protein in complete Freund’s adjuvant; subsequent boosts of fusion protein were injected in incomplete Freund’s adjuvant. The animal was killed and exsanguinated on day 11 after the last boost. Serum IgG was affinity purified using a protein A column. Antibodies to GST proteins were also removed by glutathione-Sepharose affinity chromatography. The purified antibody to HKcβ protein was used for Western blot analyses.

Specificity of HKcβ antibody. The specificity of the HKcβ antibody was established by the following experiments. 1) In an immunodepletion experiment, GST-HKcβ fusion protein and HKcβ antibody were mixed and incubated at room temperature. The antigen-antibody complex mixture was then used as an antibody source for the Western blot analysis, which contained apical membranes, basolateral membranes, or the immunoprecipitate of apical membranes by HKα antibody. 2) HKcβ cDNA and NaKβ1 cDNA were subcloned into pcDNA 3.1 (+), which was transfected into COS-7 cells (13). The expressed HKcβ and NaKβ1 proteins were used for Western blot analysis with HKcβ antibody. 3) Highly purified Na-K-ATPase from rabbit kidney (18) was run on SDS-PAGE gels and transferred to nitrocellulose membranes, and Western blot analysis was performed using HKcβ or NaKβ1 antibodies.

Isolation of apical and basolateral membranes. Apical and basolateral membranes were isolated by methods previously described in detail (29, 30).

Western blot analysis. Western blot analyses were performed with HKα, HKcβ, and NaKβ1 antibodies using previously described methods (20, 33). Apical or basolateral membrane proteins (50 µg) were electrophoresed on SDS-PAGE gels and transferred to nitrocellulose membranes. Western blot analysis was then performed with dilution of 1:1,000 HK-cβ antibody in Tris-buffered saline-Tween containing 5% nonfat dry milk; anti-rabbit IgG horseradish peroxidase conjugate (1:5,000 dilution) was used as the secondary antibody. HKcβ antibody-specific protein bands were visualized by the enhanced chemiluminescence procedure.

Immunoprecipitation. One hundred micrograms of apical membrane proteins of rat distal colon were resuspended in 1 ml of immunoprecipitation buffer containing 10 mM Tris·HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1% Triton X-100. One microliter of HKα antibody was added and incubated at room temperature for 2 h in a nutor. To the above mixture, 50 µl of protein A-Sepharose (50% suspension) were added to bind antigen-antibody complex and incubated at room temperature for 1 h in a nutor. After a 1-min centrifugation, the precipitate was washed three times for 5 min each in the above immunoprecipitation buffer containing 5% nonfat dry milk. The precipitate was then washed three times with immunoprecipitation buffer. Finally, the precipitate was resuspended in 2× SDS sample buffer containing 2-mercaptoethanol, boiled for 5 min, and centrifuged briefly, and the supernatant was loaded onto SDS-PAGE gels. After electrophoresis, proteins were transferred onto a nitrocellulose membrane and Western blot analysis was performed with HKcβ antibody diluted to 1:1,000.

RESULTS
Isolation and characterization of HKcβ cDNA clones. We screened a rat colon cDNA library using a full-length novel β-subunit cDNA isolated from a rat astrocytoma cell line (38) as a probe and obtained positive clones that were sequenced in both directions. The full-length cDNA of HKcβ consists of 1,728 nucleotides with an open reading frame encoding 279 amino acids, an initiation codon ATG, an in-frame termination codon (TAG), a 78-nucleotide 5’ untranslated region, and an 810-nucleotide 3’ untranslated sequence. The sequence of this cDNA is identical to that previously cloned by Watanabe et al. (38) from a rat astrocytoma cell line.

A comparison of the predicted amino acid sequence of the rat HKcβ with those of the rat NaKβ1, NaKβ2, and HKα subunits is shown in Fig. 1. The predicted amino acid sequence of HKcβ exhibits 100% identity to the astrocytoma β-subunit probe we used for screening (38), 36.4% identity to rat HKcβ (34), 36.5% identity to rat NaKβ2 (26), and 49% identity to rat NaKβ2 (23), all previously characterized rat β-subunits. In addition, the HKcβ coding region has 80% identity to human NaKβ2 and 100% identity to the recently cloned partial NaKβ3 cDNA from rat placenta (22). The Asn residues at positions Asn-124 and Asn-158 are potential sites of N-linked glycosylation that are not conserved with the other rat β-subunits. The six Cys residues (at positions Cys-128, Cys-144, Cys-154, Cys-170, Cys-191, and Cys-250) that might interact with an α-subunit are present in the extracellular domain of the HKcβ subunit and are highly conserved with rat HKβ, NaKβ1, and NaKβ2 subunits. Hydropathy analysis (not shown) by the Kyte and Doolittle (19) algorithm predicts that HKcβ contains a charged cytoplasmic amino terminus, a single transmembrane domain, and a large extracellular carboxy-terminal domain. The predicted secondary structure of HKcβ is similar to those of rat HKβ, NaKβ1, and NaKβ2 subunits. The hydrophobic residue-rich region of HKcβ is between amino acids Trp-36 and Leu-62. These observations establish substantial structural similarities between HKcβ and other P-type ATPase β-subunits.

Expression of HKcβ subunit mRNA in rat tissues. Expression of HKcβ mRNA was analyzed in several tissues by Northern blot analysis (Fig. 2). HKcβ cDNA probe hybridized with a transcript of ~1.9 kb in distal colon, proximal colon, ileum, jejunum, stomach, liver, lung, kidney, brain, testes, spleen, and heart. It should be noted that HKcβ cDNA that was cloned from colon is ~1.73 kb. HKcβ message was highly expressed in testis and lung; the message was slightly smaller in size in testis than those identified in other tissues.
Regulation of HKcβ expression. The identification of a colon-derived putative β-subunit isoform permitted studies to evaluate the role of HKcβ in the transport function of the rat distal colon. Because HKcβ is expressed in rat distal colon, it could be associated with the regulation of K transport and/or Na transport, which are closely linked to colonic H-K-ATPase and Na-K-ATPase, respectively (2). Expression of HKcβ was analyzed by Northern blot with mRNA isolated from distal colon of normal, dietary Na-depleted, and dietary...
K-depleted rats (Fig. 3). Na depletion stimulates Na and K absorption and K secretion (2, 37), whereas K depletion enhances only K absorption in distal colon (12). Figure 3A demonstrates that HKcβ mRNA expression is increased in distal colon of K-depleted rats compared with normal rats. HKcβ message expression was normalized to glyceraldehyde-3-phosphate dehydrogenase expression and quantitated by densitometric analysis. K depletion resulted in a 3.5-fold increase in HKcβ message expression compared with controls (Fig. 3). In contrast, HKcβ mRNA abundance in Na-depleted rats was not significantly altered (Fig. 3), although both dietary K depletion and Na depletion induce comparable increases in active K absorption in the rat distal colon (11, 12). In parallel studies with mRNA isolated from proximal colon, an organ in which active K absorption is not present (11), dietary K depletion did not increase HKcβ mRNA abundance (Fig. 4).

As shown in Fig. 2, HKcβ mRNA expression was highest in testis and lung. Therefore, additional Northern blot analyses were performed to determine whether dietary K depletion increased HKcβ mRNA abundance in these two organs. Figure 5 demonstrates that dietary K depletion did not alter HKcβ mRNA expression in either lung or testis. In both tissues, two distinct bands with equal intensity were identified; the smaller bands may represent alternatively spliced products of HKcβ mRNA. It should be noted that HKcβ cDNA probe hybridizes with slightly smaller mRNA species in testis than in lung. Therefore, the selective increase in HKcβ mRNA expression in distal colon of K-depleted rats (Fig. 3) suggests that this β-subunit may be associated with the colonic H-K-ATPase α-subunit.

To assess the significance of the increase in HKcβ mRNA by K depletion, the expression of NaKβ1 mRNA abundance in distal and proximal colon of normal, Na-depleted, and K-depleted rats was also analyzed by Northern blot; these results are presented in Fig. 6. Expression of NaKβ1 mRNA in distal colon of K-depleted rats did not change compared with control animals. In contrast, NaKβ1 mRNA abundance was modestly increased in Na-depleted rats, an observation consistent with the stimulation of active Na absorption in the distal colon by Na depletion (37). Northern blot analyses were performed to determine the presence of other β-subunit mRNAs in rat distal colon. Neither HKgβ nor NaKβ1 cDNA probes hybridized with mRNA prepared from rat distal colon (data not shown).

Expression of HKcβ in apical and basolateral membranes. The results of the Northern blot analyses (Fig. 3) are consistent with the thesis that HKcβ is the β-subunit required for colonic H-K-ATPase function. HKcβ has significant (80%) homology to a recently
identified human β-subunit that was designated NaKβ3 (18). This assignment of NaKβ3 was based on chromosomal localization and sequence relatedness. As a consequence, additional studies were performed with HKcβ to establish whether HKcβ is present in apical and/or basolateral membranes and whether HKcβ is associated with HKα.

Several studies were performed that established the specificity of HKcβ antibody to HKcβ protein without evidence of cross-reactivity to NaKβ1 protein. 1) The specificity of HKcβ antibody was initially confirmed by Western blot analysis against the GST-HKcβ fusion protein (data not shown). 2) Immunodepletion experiments were performed in which GST-HKcβ fusion protein and HKcβ antibody complex mixture was used as an antibody source for Western blot analysis. Figure 7A demonstrates that no protein was identified in apical (lane 1) or in basolateral (lane 2) membranes, but the immunoglobulin band (lane 3) that was present in the original blot (Fig. 8B, lane 3) was identified. 3) Expression studies of HKcβ cDNA in COS-7 cells demonstrated that HKcβ protein was identified by the HKcβ antibody only in cells transfected with HKcβ cDNA (Fig. 7B, lane 3). HKcβ antibody did not cross-react with NaKβ1 protein in cells transfected with NaKβ1 cDNA (Fig. 7B, lane 4). 4) Highly purified Na-K-ATPase protein from rabbit renal medulla that consists of NaKα1 and NaKβ1 proteins was not identified by HKcβ antibody (Fig. 7C) but was identified by NaKβ1 antibody (Fig. 7D). These several observations establish the specificity of HKcβ antibody to HKcβ protein.

Figure 8 presents a Western blot analysis of apical and basolateral membranes using antibodies to HKα, HKcβ, and NaKβ1 and preimmune serum. HKα was
Fig. 8. Western blot and coimmunoprecipitation analyses: apical and basolateral membranes were used for Western blot analysis. A: Western blot analysis using HKcβ antibody (20). Lane 1, apical membrane; lane 2, basolateral membrane; lane 3, a Western blot analysis was performed with HKcβ antibody for apical membrane proteins immunoprecipitated with HKcα antibody. HKcβ protein band is shown by arrow at left. *Immunoglobulin band that was bound to protein A-Sepharose during immunoprecipitation experiments. Additional band seen in lane 1 may represent a nonglycosylated form of HKcβ protein or a degradation product of HKcβ. B: Western blot analysis using HKcβ antibody (35). Blot from B was stripped and used to perform Western blot analysis using NaKβ1 antibody. D: Western blot analysis using preimmune serum. Blot from C was stripped to perform Western blot analysis using preimmune serum. Specific protein bands that had been previously identified by HKcβ and NaKβ1 antibodies in B and C, respectively, were not identified by preimmune serum, except for immunoglobulin band in lane 3.

predominantly expressed in apical membranes (Fig. 8A, lane 1), confirming previous studies of the localization of HKcα protein to the apical membrane of surface cells of rat distal colon (20). The very low expression of HKcα protein in basolateral membrane (Fig. 8A, lane 2) probably reflects minimal contamination of the basolateral membrane preparation by apical membranes. Figure 8B demonstrates that HKcβ protein was expressed in both apical (lane 1) and basolateral (lane 2) membranes in approximately equal amounts. In contrast, NaKβ1 protein was expressed only in basolateral membranes (Fig. 8C, lane 2). The presence of HKcβ and not NaKβ1 in apical membrane supports a possible role of HKcβ as the β-subunit for HKcα. The specificity of HKcβ protein expression in rat colonic membranes was provided by the immunodepletion experiment shown in Fig. 7A. This observation confirms that the protein band at 45 kDa in apical and basolateral membranes is specific to HKcβ antibody.

Additional Western blot analyses were performed on apical and basolateral membranes from normal and K-depleted rats using HKcβ antibody. Figure 9 presents the results of two of the three separate preparations of apical and basolateral membranes. Each preparation was prepared from at least six normal or K-depleted rats. HKcβ protein expression was significantly increased by 216% in apical membranes but was reduced by 30% in basolateral membranes (Fig. 9). The results presented in Figs. 3 and 9 suggest that K depletion regulates HKcβ mRNA and protein.

Identification of physical interaction of HKcβ and HKcα. Experiments were designed to establish whether HKcβ protein was associated with HKcα protein in the apical membrane of distal colon. Therefore, apical membrane proteins from rat distal colon were immunoprecipitated with HKcα antibody (see Coimmunoprecipitation). The resulting antigen-antibody complex was analyzed by Western blot using HKcβ or NaKβ1 antibodies. Figure 8B (lane 3) demonstrates the presence of HKcβ protein in the immunoprecipitate, demonstrating the physical interaction of HKcα and HKcβ proteins. In contrast, NaKβ1 was not identified in the immunoprecipitate (Fig. 8C, lane 3). The specificity of the bands of the immunoprecipitate was confirmed by
performing a Western blot analysis of the protein blot in Fig. 8C using preimmune serum following stripping. The preimmune serum identified only the immunoglobulin, as shown in Fig. 8D, lane 3. In addition, the immunodepletion experiments (Fig. 7A) established the specificity of HK\(c\) antibody to HK\(c\) protein. These observations are consistent with HK\(c\) functioning as the \(\beta\)-subunit for the apical membrane localized \(\alpha\)-subunit of colonic H-K-ATPase.

**DISCUSSION**

Active K absorption and an apical membrane H-K-ATPase have been studied extensively in the rat large intestine. Active K absorption is restricted to the distal colon and is upregulated both by dietary K depletion and by aldosterone and Na depletion (11, 12). It is generally accepted that active K absorption is energized by apical membrane H-K-ATPase, the \(\alpha\)-subunit of which (HK\(\alpha\)) was recently cloned (7, 14) and has been studied extensively (5, 6, 15, 20, 33). HK\(\alpha\) message is restricted to surface (and the upper 20% of the crypt) cells of the distal colon, whereas its protein is localized to the apical membrane of surface cells of the rat distal colon (14, 20). Although several \(\beta\)-subunit isoforms of both Na-K-ATPase and noncolonic H-K-ATPase have been isolated, a colonic H-K-ATPase \(\beta\)-subunit had not previously been identified.

Recent studies have established that dietary Na depletion upregulates HK\(\alpha\) message and protein abundance and apical membrane H-K-ATPase activity in the rat distal colon but does not alter HK\(\alpha\) message and protein expression in the kidney (15, 33). In contrast to the effect of Na depletion on HK\(\alpha\) expression in the colon, dietary K depletion did not affect HK\(\alpha\) message and protein expression in the rat distal colon (33). Of potential importance, HK\(\alpha\) protein expression was enhanced in the principal cell in the kidney of dietary K-depleted rats compared with control rats. Thus the mechanism by which dietary K depletion increases active K absorption in the rat distal colon is not known. Lescalle-Matys et al. (21) demonstrated in LLC-PK\(_1\) cells that a decrease in extracellular K concentration was associated with an increase in NaK\(\beta\) message and protein but not in NaK\(\alpha\) message and protein. Their study concluded that regulation of Na-K-ATPase activity by K concentration was pretranslational and that \(\beta\)-subunit synthesis was rate limiting. These observations with LLC-PK\(_1\) cells parallel the present results with rat distal colon. Dietary K depletion enhances active K absorption but is associated with an increase in HK\(c\) (Fig. 3) but not HK\(\alpha\) mRNA abundance (33). As a result, we speculate that the mechanism of regulation of H-K-ATPase function by dietary K depletion is mediated via HK\(c\) and not via HK\(\alpha\).

The data presented in Fig. 3 are consistent with the role of HK\(c\) mRNA in the regulation of H-K-ATPase by dietary K depletion. These Northern blot analyses using the newly cloned colon-derived \(\beta\)-subunit establish that HK\(c\) mRNA abundance was enhanced in dietary K-depleted rats. Specificity of this observation is provided by four important experiments. First, dietary K depletion did not increase the mRNA abundance of the NaK\(\beta\) subunit (Fig. 6), indicating that dietary K depletion does not result in a nonspecific increase in message abundance of all \(\beta\)-subunits. Second, HK\(c\) mRNA abundance was not increased in Na-depleted rats (Fig. 3), indicating that the increase in HK\(c\) message was specific and was not merely secondary to an increase in active K absorption. Third, although HK\(c\) message is present in the proximal colon (Fig. 4), a tissue in which active K absorption is not present (11), HK\(c\) mRNA abundance was not increased in dietary K-depleted animals in proximal colon. Fourth, expression of HK\(c\) mRNA abundance was not altered in lung and testis of K-depleted rats compared with normal rats (Fig. 5). Thus the effect of K depletion on HK\(c\) mRNA is tissue specific, as HK\(c\) mRNA is upregulated in the distal but not in the proximal colon, testis, or lung of K-depleted rats. These observations provide compelling evidence that HK\(c\) is closely linked to the stimulation of active K absorption by dietary K depletion in the rat distal colon.

Controversy exists regarding the role of a \(\beta\)-subunit in the functional expression of HK\(\alpha\). Although HK\(\alpha\) was expressed as ouabain-insensitive H-K-ATPase in
Sf9 cells without the apparent presence of a β-subunit (20), the expression of HKα in oocytes requires the coinjection of cRNAs of a Bufo marinus urinary bladder β-subunit (6) or of HKβ or NaKβ (5). It is of interest that the amphibian β-subunit (17) that induced 86Rb uptake in oocytes when coinfected with HKα (6) has significant homology (52.3%) to HKβ. The demonstration that dietary K depletion resulted in an increase in HKβ mRNA abundance but not an enhancement of HKα mRNA abundance strongly suggests that the regulation of active K absorption by dietary K depletion is mediated by this β-subunit and not by HKα. Because Na depletion stimulates HKα but not HKβ message abundance in the rat distal colon (Refs. 15, 33 and Fig. 3), differential regulation of HKα and HKβ subunits plays a significant role in the stimulation of active K absorption in rat distal colon by K depletion and Na depletion. In addition, because dietary K depletion increased the abundance of HKβ message but not that of NaKβ, these observations suggest that HKβ may be the specific β-subunit required for optimal H-K-ATPase function in the distal colon.

The close identity between HKβ and the recently reported human putative NaKβ (2, 22) requires comment. The latter cDNA sequence was identified from the human-expressed sequence tag data bank and was designated NaKβ based solely on chromosomal localization and sequence relatedness, but without demonstration of membrane localization or physical association with an α-subunit. In addition, this human putative NaKβ has an amino acid sequence with a 55% identity to a recently cloned β-subunit from amphibian bladder (17), which, when coexpressed in Xenopus oocytes with an amphibian bladder H-K-ATPase α-subunit, stimulated H/K and not Na/K function (16). Because HKβ protein has 52% identity to the amphibian bladder β-subunit, HKβ may be the rat homologue of this amphibian bladder β-subunit. Thus it is possible that the human putative NaKβ may also manifest H-K-ATPase function.

The identification of HKβ protein in basolateral membranes (see Fig. 8B, lane 2) raised the possibility that HKβ might also function with α-subunits of Na-K-ATPase and that therefore the upregulation of HKβ mRNA by dietary K depletion (Fig. 3) was causally related to an increase in Na-K-ATPase activity by dietary K depletion. Such a possibility would be consistent with the previous observations that dietary K depletion and incubation of LLC-PK1 cells in a low-K medium are associated with increases in NaKβ subunit and/or Na-K-ATPase activity (21). Therefore, additional experiments were performed to determine whether dietary K depletion was associated with an increase in Na-K-ATPase activity in rat distal colon. These studies demonstrated that Na-K-ATPase activity in basolateral membranes isolated from rat distal colon was not altered by dietary K depletion (unpublished observations). Thus the observed increase in HKβ mRNA abundance in dietary K depletion cannot be responsible for an increase in Na-K-ATPase. Therefore, HKβ is uniquely regulated by dietary K depletion in the distal colon and likely is the β-subunit required for H-K-ATPase in the rat distal colon.

H-K-ATPase is localized to the apical membrane in the distal colon (8), in contrast to the localization of Na-K-ATPase to the basolateral membrane. The Western blot studies presented in Fig. 8 present three important observations that indicate HKβ is the β-subunit for the colonic H-K-ATPase. First, HKβ protein was expressed in apical membrane (Fig. 8B, lane 1), the site at which HKα protein is selectively expressed (see Fig. 8A, lane 1). Second, although HKβ protein was identified in both apical and basolateral membranes (Fig. 8B, lanes 1 and 2), K depletion resulted in an increase in HKβ protein expression only in apical membranes (Fig. 9). The selective increase in HKβ protein in the apical membrane requires comment. It is possible that the protein recognized by the HKβ antibody in the basolateral membrane is not HKβ protein but a closely related β-subunit that was recognized by the HKβ antibody but not regulated by K depletion. The mechanism for the 30% decrease of HKβ protein in basolateral membrane of K-depleted rats is not known. Third, coimmunoprecipitation experiments demonstrated the physical association between HKβ and HKα proteins in the apical membrane (Fig. 8B, lane 3). In addition, the preimmune serum (Fig. 8D, lane 3) and immunodepletion experiment (Fig. 7A, lane 3) did not identify any protein bands except immunoglobulins. These observations confirm that HKβ protein bands are specific, and the two additional low-molecular weight protein bands in the immunoprecipitate may be a HKβ degradation product. In contrast, an antibody to NaKβ identified protein in basolateral but not in apical membranes (Fig. 8C, lane 2). As a result, it is unlikely that NaKβ protein is associated with HKα protein or is the β-subunit for H-K-ATPase. The demonstration of the selective increase in HKβ protein expression in apical membrane of rat distal colon and the association of HKβ protein with HKα protein in apical membrane (Fig. 8B, lane 3) strongly support the possibility that HKβ is most likely the β-subunit for colonic H-K-ATPase α-subunit.

Although dietary K depletion unequivocally stimulates active K absorption in the large intestine of both rat (12) and mouse (25), enhancement of H-K-ATPase in dietary K depletion has been inconsistent. It is generally believed that active K absorption in the distal colon is a result of an H/K exchange energized by an apical membrane H-K-ATPase (2), but the effect of dietary K depletion on colonic active K absorption and H-K-ATPase activity is complex. Confounding variables of the presumed stimulation of H-K-ATPase by dietary K depletion include its duration and the presence of two H-K-ATPase isoforms and two components of active K absorption. The two H-K-ATPases have different sensitivities to ouabain and spatial distributions (8, 31), and the two active K absorptive processes also have differ-
ent sensitivities to ouabain, as well as different responsiveness to aldosterone (28). Additionally, direct demonstration of an H/K exchange in colonic apical membranes has not as yet been established, and Feldman and Ickes (10) presented evidence of a dissociation between active K absorption and protein secretion.

In conclusion, we have identified a β-subunit from rat distal colon, and its mRNA is upregulated in a tissue-specific manner in the distal colon of K-depleted but not Na-depleted rats. This β-subunit protein localized in the apical membrane, physically associated with HKα protein. Its protein expression is selectively increased in the apical membrane of K-depleted rats. These observations are all consistent with the probability that HKα is the putative β-subunit for colonic H-K-ATPase.

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