THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 273, No. 14, Issue of April 3, pp. 7894–7899, 1998
© 1998 by The American Society for Biochemistry and Molecular Biology, Inc.
Printed in U.S.A.

The α-Subunit of the Colonic H^+,K^+-ATPase Assembles with β_1-Na^+,K^+-ATPase in Kidney and Distal Colon*

(Received for publication, September 5, 1997, and in revised form, December 10, 1997)

Juan Codina, Juan T. Delmas-Mata, and Thomas D. DuBose, Jr.‡

From the Division of Renal Diseases and Hypertension, Department of Internal Medicine, University of Texas, Houston Medical School, Houston, Texas 77030

Previous experiments from our laboratory (Codina, J., Kone, B. C., Delmas-Mata, J. T., and DuBose, T. D., Jr. (1996) *J. Biol. Chem.* 271, 29759–29763) demonstrated that the α-subunit of the colonic H^+,K^+-ATPase ($HK\alpha_2$) requires coexpression with a β-subunit to support H^+/K^+ transport in a heterologous expression system (*Xenopus laevis* oocytes). In these studies, $HK\alpha_2$ formed stable and functional α·β complexes when coexpressed with either the rat β_1-subunit of the Na^+,K^+-ATPase or the β-subunit of the gastric H^+,K^+-ATPase, suggesting that different β-subunits may interact with $HK\alpha_2$. The present studies tested this hypothesis by development and application of a specific antibody against $HK\alpha_2$ peptide. Subsequently, immunoprecipitation experiments were performed to determine if $HK\alpha_2$ co-precipitates with the same β-subunit in organs known to express $HK\alpha_2$ protein. The data demonstrate that $HK\alpha_2$ assembles with β_1-Na^+,K^+-ATPase in the renal medulla and in distal colon.

It is assumed that all members of the X^+,K^+-ATPase family, including the gastric H^+,K^+-ATPase isoform, exist as dimers of α- and β-subunits (1, 2). The α- or catalytic subunit of the H^+,K^+-ATPase has a molecular mass of approximately 100 kDa, contains the binding site for specific inhibitors such as Sch-28080 and omeprazole, and is responsible for internalizing K^+ and secreting H^+. The catalytic subunit for those X^+,K^+-ATPases characterized thus far requires, for biological activity, a glycosylated β-subunit. Nevertheless, the absolute requirement of the colonic α H^+,K^+-ATPase subunit ($HK\alpha_2$)[1] for a β-subunit for functionality, has been called into question (3).

Lee and associates (3) reported that when the rat $HK\alpha_2$ is expressed in a baculovirus expression system, in the absence of exogenous β-subunit, the $HK\alpha_2$ migrated to the plasma membrane and exhibited K^+-ATPase activity that was partially sensitive to Sch-28080 and totally insensitive to ouabain. In contrast a different pharmacologic profile was observed when $HK\alpha_2$ was expressed in oocytes from *Xenopus laevis* (4, 5). Cougnon and associates (4) employed the β-subunit from toad bladder, for which there is no known mammalian counterpart. Our laboratory, on the other hand, used two different β-subunits as follows: the β-subunit of the rat gastric H^+,K^+-ATPase ($\beta_{HK\alpha1}$) and the β_1-subunit of the rat Na^+,K^+-ATPase (β_1) (5). Both studies reported an enzymatic activity, measured as $^{86}Rb^+$ uptake, that was totally dependent on the presence of a β-subunit. Also in contrast with the report of Lee and associates (3), this activity was insensitive to Sch-28080 and was partially sensitive to ouabain.

Although the findings of Lee and associates (3) suggested that under certain conditions $HK\alpha_2$ may function in the absence of a β-subunit, the study by Cougnon and associates (4) and that from our laboratory (5), cited above, demonstrated, nevertheless, that under certain physiological conditions different β-subunits may interact with $HK\alpha_2$. The interaction of $HK\alpha_2$ with various β-subunits observed in heterologous systems does not necessarily reveal what may pertain in intact tissues, however. Therefore, the purpose of this study was to analyze those interactions in the two tissues known to express $HK\alpha_2$ protein (the rat distal colon and rat renal medulla) (6–8). Our results demonstrate that $HK\alpha_2$ assembles with β_1 in both organs.

EXPERIMENTAL PROCEDURES

Experimental Animals—Chronic hypokalemia (LK) was accomplished in male Sprague-Dawley rats (135–175 g) by dietary K^+ restriction (9). Rats in the LK group received tap water to drink and were maintained for 14 days on a customized vitamin-fortified nominally K^+-free diet that contained 0.001 nmol/g potassium (ICN Biochemicals, Cleveland, OH, lot 960189). Control rats were pair fed each day with the same diet, to which KCl (0.03 mmol/g) was added. (ICN Biochemicals, P5411). Tap water was consumed *ad libitum*. The dietary modification was well tolerated and generated a stable and reproducible model of chronic K^+ depletion. The rats were sacrificed in sets of two rats (one from each experimental group) to minimize bias during preparation.

Membrane Preparation—To prepare membranes, the organs to be studied were homogenized with a Polytron (Brinkmann, model PT 10/35) in the presence of 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 1 mM PMSF, 3 mM benzamidine, and 1 μg/ml soybean trypsin inhibitor (buffer A) containing 27% sucrose (w/v). Nuclei were removed by centrifugation at 2,000 × *g* for 4 min at 4 °C, and the supernatant was applied to the top of 45% (w/w) sucrose in buffer A and centrifuged at 200,000 × *g* for 45 min at 4 °C (10). The membranes in the interphase 27/45% sucrose were diluted in buffer A and collected by centrifugation at 25,000 × *g*. The final protein concentration was measured using the Lowry method (11).

Anti-$HK\alpha_2$ Antibody—A synthetic peptide (TPEQLDELLTNYQ) (12) that extends from amino acid 686 to 698 of the published sequence of $HK\alpha_2$ (13) was synthesized (Genosys, The Woodlands, TX). The peptide was cross-linked to keyhole limpet hemocyanin. Two rabbits were injected and bled according to standard protocols. The peptide was tested for similarity to different X^+,K^+-ATPases, and the maximum identity (69%) of the peptide was with the human ATP1AL1 (or $HK\alpha_4$) protein which is known to be expressed in brain, skin, and kidney (14), whereas the most divergence was with rat $HK\alpha_1$ (23% identity) (15).

The sensitivity of the antibodies against $HK\alpha_2$ were tested by Western blot analysis of $HK\alpha_2$ synthesized "*in vitro*" using rabbit reticulocyte lysate as described previously by our laboratory (16). The specific-

* This work was supported in part by NIDDKD Grant DO-30603-16 from the National Institutes of Health (to T. D. D., Jr.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom correspondence should be addressed: Division of Renal Diseases and Hypertension, University of Texas-Houston Medical School, 6431 Fannin St., Room 4.148, Houston, TX 77030. Tel.: 713-500-6868; Fax: 713-500-6882; E-mail: tdubose@heart.med.uth.tmc.edu.

[1] The abbreviations used are: $HK\alpha_2$, α-subunit of the colonic H^+,K^+-ATPase; $HK\alpha_1$, α-subunit of the gastric H^+,K^+-ATPase; $\beta_{HK\alpha1}$, β-subunit of the gastric H^+,K^+-ATPase; β_1, β-subunit of the Na^+,K^+-ATPase; PAGE, polyacrylamide gel electrophoresis; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; PMSF, phenylmethylsulfonyl fluoride.

This paper is available on line at http://www.jbc.org

This is an Open Access article under the CC BY license.

ity was tested using membranes prepared from rat stomach (enriched in $HK\alpha_1$) (1), total rat kidney (enriched in α_1-Na^+,K^+-ATPase) (17), or rat distal colon (enriched in $HK\alpha_2$) (6–8).

Immunoblots—Membranes, prepared as described above, were separated on SDS-PAGE as described previously by our laboratory (18). The proteins were transferred overnight at 30 V to a nitrocellulose membrane (Schleicher and Schuell, BA85) in the presence of Tris/glycine buffer (25 mM Trizma (Tris base), 250 mM glycine, and 20% (v/v) methanol). The nonspecific binding sites of the nitrocellulose membrane were blocked in the presence of 5% nonfat dry milk in phosphate-buffered saline/Tween (10 mM sodium phosphate, pH 7.5, 150 mM NaCl, 0.05% Tween 20). This was followed by 2-h incubation with the primary antibody diluted 1:1000–10,000 in phosphate-buffered saline/Tween. After extensive washing, the membranes were incubated with a peroxidase-bound donkey anti-rabbit IgG, and the reacted antibody was detected using the ECL system (Amersham Pharmacia Biotech, RPN2108) following the manufacturer's instructions.

Synthesis of Recombinant Proteins—The complete open reading frame of $HK\alpha_1$ (15), $HK\alpha_2$ (13), α_1-Na^+,K^+-ATPase (19), β_1 (20), β_2 (21), and $\beta_{HK\alpha}$ (22), all them from rat origin, were subcloned into the plasmid pAGA#2 as described previously by our laboratory (16). The *in vitro* synthesis of the recombinant proteins was accomplished using the TnT system (Promega, Madison, WI, catalog number L4610) in the presence of [^{35}S]methionine following the instructions of the manufacturer. The quality of the synthesized proteins was verified by separating the synthesized proteins on SDS-PAGE, followed by autoradiography of the dried gel. The quantity of the synthesized protein was monitored by trichloroacetic acid precipitation of the ^{35}S-synthesized recombinant protein (16).

Immunoprecipitation—Subunit assembly was detected as coimmunoprecipitation of $HK\alpha_2$ and a β-subunit using an anti-$HK\alpha_2$ antibody. Immune serum (150 μl) was incubated overnight at 4 °C with 1 mg (1 mg/ml) of membranes. The membranes were rinsed twice with 1 ml of buffer B (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM PMSF, 3 mM benzamidine, and 1 μg/μl soybean trypsin inhibitor). Membrane proteins were extracted with 500 μl of buffer B containing 1% CHAPS for 1 h at 4 °C. The insoluble material was removed by centrifugation, and the $HK\alpha_2$/anti-$HK\alpha_2$ antibody complex was precipitated by addition of 40 μl of protein A/G Plus agarose (Santa Cruz Biotechnology, Santa Cruz, CA, catalog number sc-2003) for 2 h at 4 °C (5). The resin was extensively washed, and the bound protein was extracted with Laemmli buffer (23) and separated in SDS-PAGE. The presence of a β_1-subunit was detected with a monoclonal antibody (Upstate Biotechnology, Lake Placid, NY, catalog number 05-382).

Detection of Immunoprecipitated $HK\alpha_2$ from Distal Colon Membranes—After immunoprecipitation of $HK\alpha_2$ from the distal colon with anti-$HK\alpha_2$ antibody, the precipitated material was treated with Laemmli sample buffer containing 10% β-mercaptoethanol and heated at 65 °C for 30 min. This treatment induces a total reduction of the dimer heavy/light chain of the IgGs that are similar in mobility to $HK\alpha_2$. We found that about 60–70% of the $HK\alpha_2$ is lost in this step at 65 °C.

Affinity of Anti-$HK\alpha_2$ Antibody for Renal and Colonic $HK\alpha_2$—The interaction between anti-$HK\alpha_2$ antibody and the native $HK\alpha_2$ from different membranes was verified by incubating anti-$HK\alpha_2$ antibody overnight at 4 °C in increasing concentrations of distal colon, renal medulla, and renal cortex membranes from both control and LK rats, in the presence of buffer B. The membranes were centrifuged, and the supernatant was used as a source of anti-$HK\alpha_2$ antibody to detect $HK\alpha_2$ in membranes prepared from distal colon (30 μg).

N-Deglycosylation of β_1 with PNGase F—To remove *N*-linked carbohydrates from the β_1-subunit, membranes were incubated at 4 °C for 1 h in the presence of buffer B containing 1% CHAPS. The insoluble material was removed by centrifugation at 10,000 × *g* for 10 min at 4 °C, and deglycosylation was accomplished in the presence of 25,000 units/ml PNGase F (24) (New England Biolabs, Beverly, MA, catalog number 704L) for 1 h at 37 °C in the presence of 50 mM sodium phosphate, pH 8.0, 1 mM PMSF, 3 mM benzamidine, 1 μg/ml soybean trypsin inhibitor, and 1% CHAPS. The reaction was stopped by addition of Laemmli buffer (23); proteins were resolved on SDS-PAGE, and the immunoblots were performed as described above. When necessary, *N*-deglycosylation was performed using immunoprecipitated samples. In this case, deglycosylation was accomplished before the addition of Laemmli sample buffer to the Protein A/G Plus agarose, and the sample was incubated as described above for 60 min at 37 °C.

Deglycosylation of β_1 Coimmunoprecipitated with $HK\alpha_2$ from the Distal Colon—The immunoprecipitated $HK\alpha_2/\beta$ complex from the distal colon was incubated for 3 h at 37 °C in the presence of 20 mM sodium phosphate, pH 6.7, 1 mM PMSF, 3 mM benzamidine, 1 μg/ml soybean trypsin inhibitor, 1% CHAPS, 0.03 units/ml endo-α-*N*-acetylgalactosaminidase (Sigma, catalog number E2391) (25), 0.07 units/ml neuraminidase (Sigma, catalog number N7885) (26), 0.05 units/ml α-L-fucosidase (Sigma, catalog number F7753) (27), and 25,000 units/ml PNGase F (New England Biolabs, Beverly, MA, catalog number 704L) (24). Incubation was stopped by addition of Laemmli sample buffer (23). The proteins were separated on SDS-PAGE and transferred to a nitrocellulose membrane, and β_1 was detected by immunoblotting with the anti-β_1 antibody exactly as described above. In some experiments the presence of either endo-α-*N*-acetylgalactosaminidase or PNGase F, or both were omitted during the deglycosylation (see figure legends).

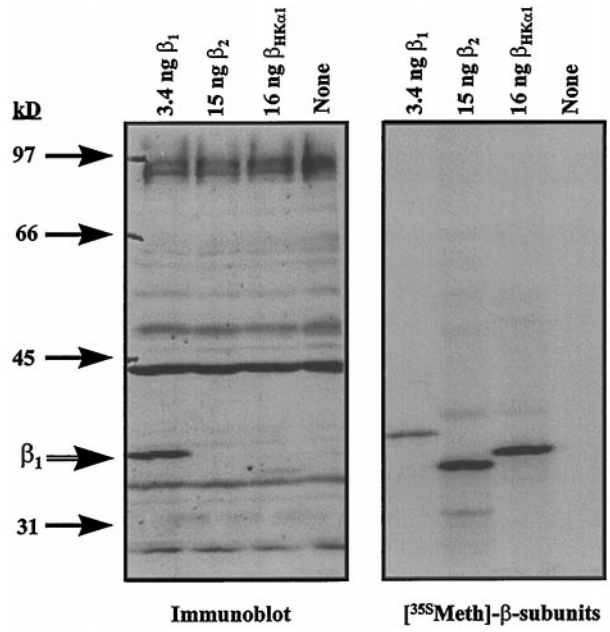

FIG. 1. **Monoclonal anti-β_1-Na^+,K^+-ATPase is specific for β_1.** *Left panel*, β_1, β_2, and $\beta_{HK\alpha1}$ were synthesized in rabbit reticulocyte in presence of [^{35}S]methionine. The synthesized proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane, and probed with the anti-β_1-Na^+,K^+-ATPase antibody (1 μg/ml). *Right panel*, the ECL signal was allowed to decay for 24 h, and the nitrocellulose membrane was exposed overnight at room temperature. The symbols are as follows: β_1, β_1-subunit of the Na^+,K^+-ATPase; β_2, β_2-subunit of the Na^+,K^+-ATPase; $\beta_{HK\alpha1}$, β-subunit of the gastric H^+,K^+-ATPase.

Additional Reagents—The monoclonal antibody against the β_1-Na^+,K^+-ATPase was purchased from Upstate Biotechnology (Lake Placid, NY, catalog number 05-382). Anti-α_1-Na^+,K^+-ATPase (LEAVE) was a gift from Dr. T. Pressley (Texas Tech, Lubbock, TX) (28).

RESULTS

Antibody Specificity—The specificity and sensitivity of the monoclonal β_1-Na^+,K^+-ATPase antibody was verified using recombinant β_1, β_2, and $\beta_{HK\alpha1}$ synthesized *in vitro* (rabbit reticulocyte lysate) in the presence of [^{35}S]methionine as described under "Experimental Procedures." The *left panel* of Fig. 1 demonstrates that anti-β_1 antibody reacted with recombinant β_1 (3.4 ng). Signal was not detected when either β_2 (15 ng) or $\beta_{HK\alpha1}$ (16 ng) was applied to the SDS-PAGE. The ECL signal of the nitrocellulose membrane was allowed to decay for 24 h and exposed again overnight to detect [^{35}S]methionine-labeled proteins. Fig. 1 (*right panel*) demonstrates that all three subunits ran as a single band of the expected mobility.

The specificity of anti-$HK\alpha_2$ antibody was tested by immunoblot using rat plasma membranes from different organs as follows: distal colon membranes (10 μg) (enriched in $HK\alpha_2$), whole kidney membranes (100 μg) (enriched in α_1-Na^+,K^+-ATPase), and stomach membranes (100 μg) (enriched in $HK\alpha_1$). The results of one of these experiments are shown in Fig. 2, *left panel*. Specific antibody reactivity was detected in colon membranes (10 μg) but was not observed in kidney or stomach (up to 100 μg). This signal was blocked by preincuba-

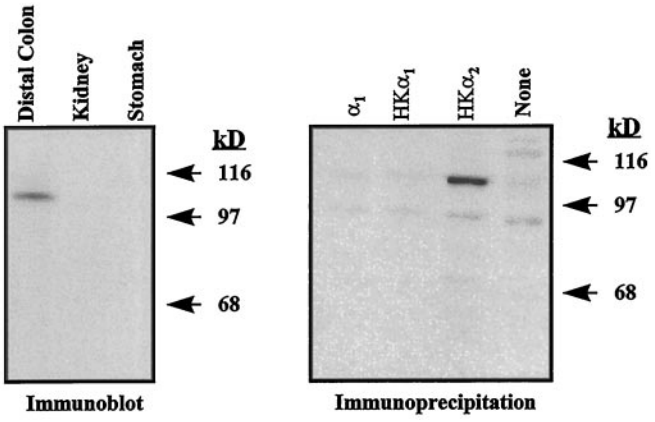

FIG. 2. **Anti-HKα_2 antibody is specific for HKα_2.** *Left panel*, membranes from distal colon (5 μg), total kidney (100 μg), and stomach (100 μg) were resolved on SDS-PAGE, transferred to nitrocellulose membrane, and probed with the anti-HKα_2 antibody, diluted 1:1000. *Right panel*, α_1, HKα_1, and HKα_2 were synthesized *in vitro* in the presence of [^{35}S]methionine using rabbit reticulocyte lysate. The synthesized protein was immunoprecipitated with 5 μl of immune serum. The immunoprecipitated samples were resolved on SDS-PAGE. The gel was dried and exposed overnight at room temperature. The symbols used are as follows: α_1, α_1-subunit of the Na^+,K^+-ATPase; HKα_1, α-subunit of the gastric H^+,K^+-ATPase; HKα_2, α-subunit of the colonic H^+,K^+-ATPase.

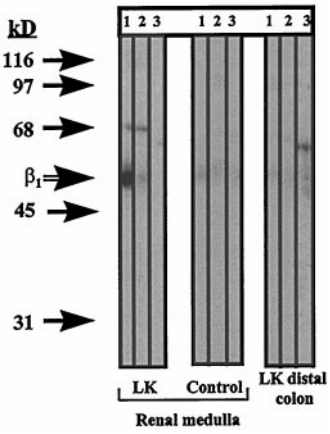

FIG. 3. **Heterogeneity of the β_1-subunit in the renal medulla and in the distal colon.** HKα_2 was immunoprecipitated from 1 mg of membranes prepared as described under "Experimental Procedures." The presence of β_1 in the immunoprecipitated sample was verified by immunoblot using a monoclonal antibody against the β_1-subunit of the Na^+,K^+-ATPase. *Left panel*, membranes prepared from renal medulla of rats with chronic dietary K^+ depletion (*LK*). *Central panel*, membranes prepared from renal cortex of control rats. *Right panel*, membranes prepared from distal colon of LK rats. *Lane 1* of each panel: the immunoprecipitation was performed in the presence of the anti-HKα_2 antibody (150 μl). *Lane 2* of each panel: the immunoprecipitation was performed with anti-HKα_2 antibody that had been preincubated in the presence of immunizing peptide (500 μM for 1 h at 4 °C). *Lane 3* of each panel: the immunoprecipitation was performed in the absence of anti-HKα_2 antibody and in the absence of immune serum.

tion (1 h at 4 °C) with the immunizing peptide (250 μM) (data not shown). By using recombinant protein as standard, the anti-HKα_2 antibody achieved a lower limit of detection equal to 2–5 ng. The specificity of the immunoprecipitation was established by immunoprecipitation of recombinant protein. The results of one experiment is shown in Fig. 2, *right panel*. Only HKα_2 was immunoprecipitated by the addition of anti-HKα_2 antibody, and the immunoprecipitation was blocked by incubating the antibody with immunizing peptide (data not shown).

Heterogeneity of the β_1-Subunit in the Renal Medulla and in the Distal Colon—Our laboratory has reported previously that HKα_2 assembles stably and functionally in a heterologous expression system with two different rat β-subunits ($\beta_{HK\alpha1}$ and β_1) (5). To explore the relevance of this observation *in vivo*, membranes (1 mg) were prepared from the renal medulla of rats after chronic dietary K^+ depletion (chronic hypokalemia), a condition known to maximize H^+,K^+-ATPase expression in kidney (6, 7). These membranes were incubated overnight with anti-HKα_2 antibody (150 μl) (see "Experimental Procedures"). The immunoprecipitated proteins were separated on SDS-PAGE, transferred to nitrocellulose membrane, and blotted against a monoclonal antibody to the β_1-subunit of the rat Na^+,K^+-ATPase (Fig. 3, *left panel*). The anti-β_1 monoclonal antibody detected a band at approximately 50–55 kDa (*lane 1*). This band was not present when the immunoprecipitation was performed with anti-HKα_2 antibody that was preincubated with the synthetic immunizing peptide (*lane 2*) or with preparations that did not contain membranes (*lane 3*). The *central panel* of Fig. 3 demonstrates that β_1 was not detected from 1 mg of membranes prepared from the renal medulla of control rats. However, β_1 was detected in the immunoprecipitated sample of renal medulla from control rats when as much as 35 mg of proteins were used in the immunoprecipitation experiment (data not shown). This observation agrees with data reported previously by our laboratory (6) and others (7, 8) that the abundance of HKα_2 is very low in renal medulla of control rats. Surprisingly, a β_1-subunit was not detected, as well, when the immunoprecipitation experiments were performed using up to 1 mg of membranes from control distal colon or the distal colon harvested from rats with chronic hypokalemia (Fig. 3, *right panel*).

To verify that the distal colon contains a β_1-subunit detectable by our monoclonal antibody, we performed the experiment displayed in Fig. 4. Distal colon membranes (50 μg) were separated on SDS-PAGE and transferred to a nitrocellulose membrane. The β_1-subunit was detected by immunoblot using the monoclonal β_1 antibody. The antibody recognized a wide band in the region expanding from 40 to 80 kDa. This wide band "disappeared" upon deglycosylation with PNGase F (which remove the *N*-linked carbohydrates), generating a distinct band at 33 kDa (the expected mobility for β_1). A band of the same mobility was generated upon deglycosylation of membranes from renal medulla (50 μg). The β_1-subunit from both organs when deglycosylated displayed the same mobility as the β_1-subunit synthesized *in vitro* using rabbit reticulocyte lysate (data not shown). Furthermore, when variable amounts of membranes were deglycosylated, it could be ascertained that there was approximately 2-fold more *N*-glycosylated β_1 in the renal medulla as compared with the distal colon.

Anti-HKα_2 Antibody Recognizes Native HKα_2 in Distal Colon—The observation that the β_1-subunit was not immunoprecipitated from the distal colon using the anti-HKα_2 antibodies can be explained if HKα_2 were to undergo secondary modification in the distal colon which could render HKα_2 inaccessible to the antibody.

To test this possibility we performed two experiments. Increasing concentrations of distal colon membranes (from 0 to 2000 μg) were incubated overnight at 4 °C with anti-HKα_2 antibody. Membranes with bound antibody were then centrifuged, and the supernatant was used as a source of anti-HKα_2 antibody to detect HKα_2 by immunoblot in membranes prepared from distal colon. As expected, increasing concentrations of membranes decreased the intensity of the signal in the immunoblot, indicating that anti-HKα_2 antibody interacted with the native HKα_2 in the distal colon membranes (Fig. 5). In contrast, when 2000 μg of renal cortex from control rats were incubated with anti-HKα_2 antibody, the intensity of the band in the immunoblot did not decrease. This finding is in agreement with previous observations from our laboratory indicat-

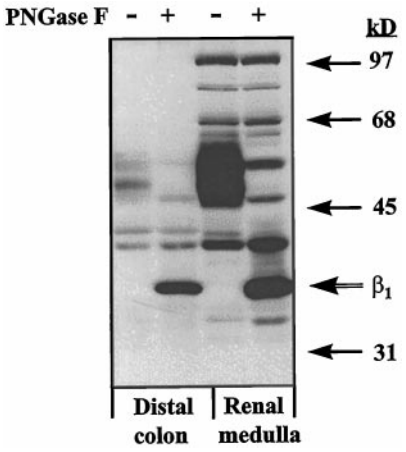

FIG. 4. **β_1 is present in distal colon membranes.** Membranes from distal colon (50 μg) or from renal medulla (50 μg) were solubilized in the presence of CHAPS, and *N*-linked carbohydrates were removed with PNGase F as described under "Experimental Procedures." The presence of the β_1-subunit was detected by immunoblot with the monoclonal antibody against the β_1-Na^+,K^+-ATPase (1 μg/ml).

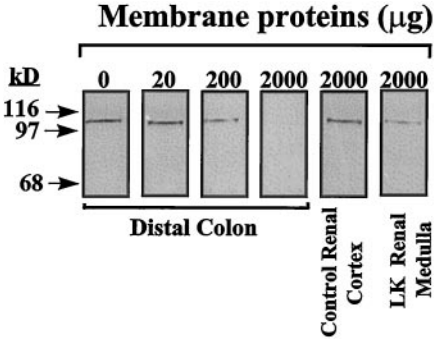

FIG. 5. **Anti-HKα_2 antibody does not discriminate between the HKα_2 from distal colon and renal medulla.** The indicated quantities of membranes were dissolved in 1 ml of buffer B (see "Experimental Procedures") and incubated overnight at 4 °C with immune serum (1 μl). The membranes were centrifuged, and the supernatant was used to detect HKα_2 in 30 μg of distal colon membranes. By using this assay the distal colon contains about 20-fold more HKα_2 than membranes prepared from renal medulla of rats with chronic dietary K^+ depletion. These data are consistent with the ratio observed when levels of HKα_2 are quantified by direct immunoblotting in both types of membranes.

ing that the levels of HKα_2 are either very low or absent from the renal cortex of normal rats (6). Finally, when the antibody was incubated overnight with 2000 μg of membranes prepared from renal medulla of chronically hypokalemic rats (condition shown to enhance the expression of HKα_2 in these membranes), there was a decrease in the intensity of the band on the immunoblot. This finding indicates that HKα_2 is present in membranes from the renal medulla during hypokalemia but, as expected, in less abundance than in the distal colon (6).

In the second experiment, distal colon plasma membranes (1 mg) were immunoprecipitated with anti-HKα_2 antibody. The pellet was dissolved in Laemmli sample buffer containing 10% β-mercaptoethanol and incubated at 65 °C for 30 min. The sample was separated on SDS-PAGE, and the presence of HKα_2 was detected by immunoblot using the anti-HKα_2 antibody. Fig. 6 shows a band which comigrated with HKα_2 from distal colon membranes (5 μg). The mixture of anti-HKα_2 and anti-α_1 antibodies recognized a "doublet" in the membranes from distal colon, whereas a band corresponding to the mobility of HKα_2 was detected in the immunoprecipitated sample (data not shown). These findings verify that the immunoprecipitated α-subunit is not the α_1-Na^+,K^+-ATPase.

Taken together, the two experiments described above indicate that anti-HKα_2 antibody does not discriminate between HKα_2 from renal medulla and distal colon, and the differences in the β_1-subunit detected in the immunoprecipitation shown in Fig. 3 reside in the β-subunit *per se*.

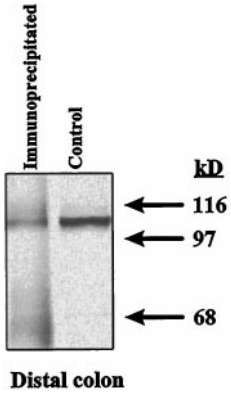

FIG. 6. **Anti-HKα_2 antibody immunoprecipitates HKα_2 from the distal colon.** HKα_2 from the distal colon was immunoprecipitated as described under "Experimental Procedures." The pellet was resuspended in Laemmli sample buffer containing 10% β-mercaptoethanol. The sample was heated for 30 min at 65 °C and run on a SDS-PAGE, transferred to a nitrocellulose membrane, and HK_2 detected by immunoblot.

The β_1 Associated with HKα_2 Is an N-Linked Carbohydrate in the Renal Medulla—Fig. 4 reveals that it was more difficult for the β_1 monoclonal antibody to detect the glycosylated as opposed to the deglycosylated form of β_1 in distal colon. To investigate the possibility that β_1 might be associated with HKα_2 in the distal colon, but could not be detected by Western analysis, we performed the following experiment. The anti-HKα_2 antibody was used to immunoprecipitate the HKα_2/β complex from distal colon and renal medulla from rats after chronic dietary K^+ depletion. The immunoprecipitated protein was *N*-deglycosylated in the presence of PNGase F and applied to SDS-PAGE and transferred to a nitrocellulose membrane. The presence of β_1-subunit was tested with the monoclonal antibody against β_1. The results shown in Fig. 7 demonstrate that the antibody detected β_1 in the immunoprecipitate from renal medulla but not in the immunoprecipitate prepared from distal colon membranes.

We have estimated, using recombinant HKα_2 as a standard (16), that each milligram of membranes from renal medulla contains approximately 30 ng of native HKα_2 (which would be predicted to associate with 10–12 ng of β_1). By using recombinant β_1 as standard, we detected that the total β_1 immunoprecipitated from 1 mg of membranes from the renal medulla was approximately 10 ng. This finding suggests that in the renal medulla of the chronically hypokalemic rat, β_1 may represent the only β-subunit that associates with HKα_2. Comparison of the quantities of HKα_2 present in membranes from both renal medulla and distal colon indicates that the distal colon contains 20-fold more HKα_2 than the renal medulla. Nevertheless, β_1 could not be detected in distal colon if deglycosylation was confined to conventional *N*-deglycosylation.

The previous observation that β_1 did not immunoprecipitate with HKα_2 in the distal colon could be interpreted as follows: (*a*) in the distal colon, the β_1 associated with HKα_2 is an *O*-linked carbohydrate; (*b*) in the distal colon, the β_1 associated with the HKα_2 is an *N*-linked carbohydrate, but the glycosylated protein is not accessible to PNGase F; or (*c*) the β_1-subunit does not associate with HKα_2 in the distal colon. As displayed in Fig. 8, the immunoprecipitated sample from the distal colon was deglycosylated with a mixture of enzymes to remove sialic acid, fucose, and *O*- and *N*-linked carbohydrates as described under "Experimental Procedures." The sample

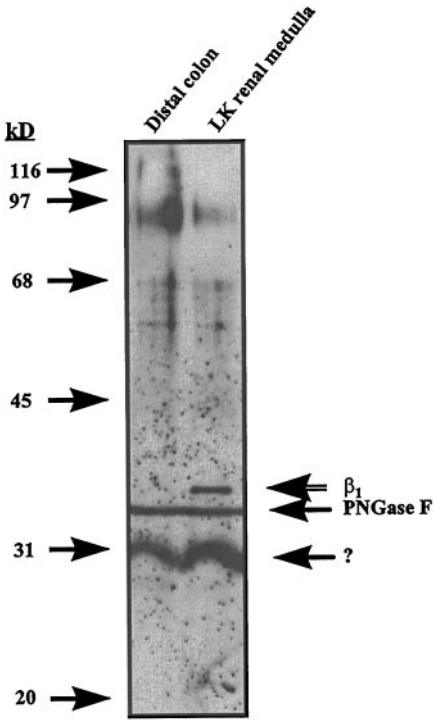

FIG. 7. **The β_1 associated with HKα_2 is an *N*-linked carbohydrate in the renal medulla.** Immunoprecipitation of HKα_2 from membranes (1 mg) prepared from renal medulla of LK rats and deglycosylation with PNGase F generated a sharp β_1 band, which was not present when the same immunoprecipitation was performed using distal colon membranes (1 mg).

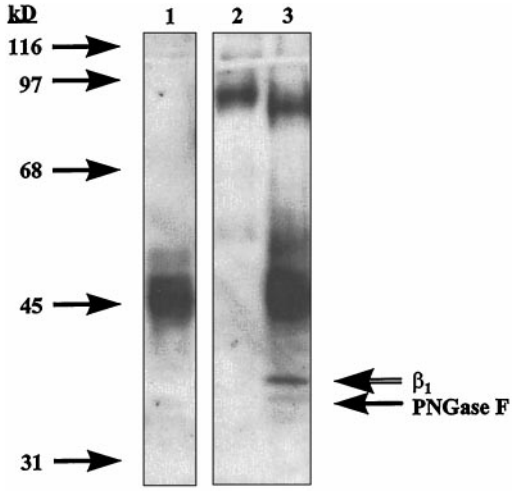

FIG. 8. **β_1 associates with HKα_2 in the distal colon.** HKα_2 from 1 mg of distal colon membranes was immunoprecipitated with anti-HKα_2 antibody. The immunoprecipitated HKα_2·β complex was deglycosylated with a mixture of neuraminidase, α-L-fucosidase, endo-α-*N*-acetylgalactosaminidase, and PNGase F (see "Experimental Procedures"), and β_1 was detected with the anti-β_1 antibody. *Left lane*, no immunoprecipitated sample (enzymes alone); *central lane*, the immunoprecipitated sample that was not deglycosylated; *right lane*, the sample was deglycosylated with the enzyme mixture.

was separated on SDS-PAGE and transferred to a nitrocellulose membrane, and the presence of β_1 was detected with anti-β_1 monoclonal antibody. Fig. 8 shows that β_1 was detected in the immunoprecipitated sample and, as in the renal medulla, displayed a mobility which was identical to the mobility of β_1-Na^+,K^+-ATPase synthesized *in vitro* using rabbit reticulocyte lysate (data not shown).

To determine if the β_1-Na^+,K^+-ATPase associated with

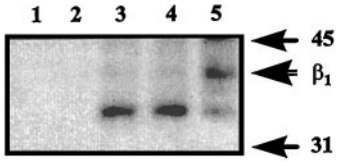

FIG. 9. **The β_1 associated with HKα_2 is an *N*-linked carbohydrate in the distal colon.** HKα_2 from 1 mg of distal colon membranes was immunoprecipitated with anti-HKα_2 antibody. The immunoprecipitated sample was deglycosylated with endo-α-*N*-acetylgalactosaminidase (*lane 1*), PNGase F (*lane 2*), neuraminidase and α-L-fucosidase (*lane 3*), neuraminidase, α-L-fucosidase, and endo-α-*N*-acetylgalactosaminidase (*lane 4*), and neuraminidase, α-L-fucosidase, endo-α-*N*-acetylgalactosaminidase, and PNGase F (*lane 5*). The *arrow* labeled "β_1" indicates the predicted mobility of β_1-subunit synthesized in rabbit reticulocyte lysate. (*Numbers* to the *right* indicate the molecular mass in kilodaltons.)

HKα_2 in the distal colon contained *N*- or *O*-linked carbohydrate, we performed the following experiment. The immunoprecipitated HKα_2/β_1 complex was deglycosylated with PNGase F or endo-α-*N*-acetylgalactosaminidase to remove *N*- or *O*-linked carbohydrates, respectively. Proteins were separated on SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with the monoclonal antibody against the β_1-subunit. The results demonstrated that β_1 was not detected (Fig. 9, *lanes 1* and *2*). However, removal of the sialic acid and fucose with a mixture of neuraminidase and α-L-fucosidase generated a band that was recognized by the anti-β_1-monoclonal antibody. This band displayed a mobility of 32 kDa (*lane 3*). Addition of endo-α-*N*-acetylgalactosaminidase to the mixture of neuraminidase and α-L-fucosidase, to remove the *O*-linked carbohydrates (*lane 4*), did not alter the mobility of the β_1-subunit recognized by the monoclonal antibody. However, addition of PNGase F to the mixture of neuraminidase and α-L-fucosidase (*lane 5*) rendered a β_1-subunit with the same mobility of the β_1-subunit synthesized *in vitro* using rabbit reticulocyte lysate (data not shown), and this band also displayed a mobility that was identical to the band generated from the renal medulla after PNGase F treatment.

DISCUSSION

Our results clearly demonstrate that under physiological conditions HKα_2 associates with β_1-Na^+,K^+-ATPase in both organs, the renal medulla and the distal colon. The present study employed immunoprecipitation techniques to investigate the specificity of association of HKα_2 with β-subunits in the kidney and in the distal colon. Specifically, two questions were explored as follows: does HKα_2 associate with a β-subunit in tissues in which HKα_2 protein is known to be expressed, and does HKα_2 associate with the same β-subunit at these locations? Our data demonstrate that HKα_2 consistently co-precipitated with the β_1-Na^+,K^+-ATPase subunit in the renal medulla and in the distal colon. The difference between both organs was in the complexity of the glycosylation, particularly in the distal colon, which necessitated complete deglycosylation removing sialic acid and/or fucose bound to the β_1 to achieve deglycosylation by PNGase F.

The highly complex pattern of glycosylation of the β_1-subunit coimmunoprecipitated with HKα_2 in the distal colon was the most likely explanation for our inability to detect the β_1-subunit from immunoprecipitated samples from distal colon prior to more complete deglycosylation (Fig. 3, *right panel, lane 1*).

Lee and associates (3) have expressed HKα_2 in a baculovirus expression system in the absence of an exogenous β-subunit. These investigators reported a K^+-ATPase that was resistant to ouabain and partially sensitive to Sch-28080. These data raised the possibility that, under certain conditions, HKα_2 may be biologically active in the absence of a β-subunit. Our data

from distal colon and renal medulla do not support this hypothesis, since β_1 was consistently coimmunoprecipitated with HKα_2 from both tissues.

Both, β_1-Na^+,K^+-ATPase (29) and HKα_2 (3) have been localized to the apical membrane of distal colonocytes. Furthermore, our laboratory has demonstrated, using oocytes from *X. laevis,* that β_1 assembles stably with HKα_2. The functionality of HKα_2/β_1 was substantiated by $^{86}Rb^+(K^+)$ uptake (5). These data suggested that in the distal colon β_1 could represent the β-subunit that associated with HKα_2. The findings of the present study establish that β_1 and HKα_2 assemble stably and functionally in a plasma vesicle fraction from distal colon. More importantly, by establishing that the Na^+,K^+-ATPase interacts with HKα_2 in the renal medulla and in the distal colon, our data suggest additionally that a unique "colonic" β-subunit may not exist. Factors that regulate α_1-Na^+,K^+-ATPase/β_1 and HKα_2/β_1 association in cells containing both HKα_2 and α_1-Na^+,K^+-ATPase remain to be defined.

Acknowledgments—We thank Thomas A. Pressley (Texas Tech, Lubbock, TX) and Carlos H. Pedemonte (University of Houston, Houston) for carefully reading the manuscript and for their suggestions during the course of this study.

REFERENCES

1. Sachs, G., Shin, J. M., Briving, C., Wallmark, B., and Hersey, S. (1995) *Annu. Rev. Pharmacol. Toxicol.* **35,** 277–305
2. Kone, B. C. (1996) *Miner. Electrolyte Metab.* **22,** 349–365
3. Lee, J., Rajendran, V. M., Mann, A. S., Kashgarian, M., and Binder, H. J. (1995) *J. Clin. Invest.* **96,** 2002–2008
4. Cougnon, M., Planelles, G., Crowson, M. S., Shull, G. E., Rossier, B. C. and Jaisser, F. (1996) *J. Biol. Chem.* **271,** 7277–7280
5. Codina, J., Kone, B. C., Delmas-Mata, J. T., and DuBose, T. D., Jr. (1996) *J. Biol. Chem.* **271,** 29759–29763
6. Codina, J., Delmas-Mata, J. T., and DuBose, T. D., Jr. (1997) *J. Am. Soc. Nephrol.* **8,** 31
7. Kraut, J. A., Hiura, J., Besancon, M., Smolka, A., Sachs, G., and Scott, D. (1997) *Am. J. Physiol.* **272,** F744–F750
8. Sangan, P., Rajendran, V. M., Mann, A. S., Kashgarian, M., and Binder, H. J. (1997) *Am. J. Physiol.* **272,** C685–C696
9. DuBose, T. D., Jr., Codina, J., Burges, A., and Pressley, T. A. (1995) *Am. J. Physiol.* **269,** F500–F507
10. Scherer, N. M., Toro, M. J., Entman, M. L., and Birnbaumer, L. (1987) *Arch. Biochem. Biophys.* **259,** 431–440
11. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275
12. Kraut, J., Hiura, J., Scott, D., Besancon, M., Shang, A., Smolka, A., and Sachs, G. (1995) *10th International Congress of Nephrology, July 2–6*, p. 43, Madrid, Spain
13. Crowson, M. S., and Shull, G. E. (1992) *J. Biol. Chem.* **267,** 13740–13748
14. Grishin, A. V., Sverdlov, V. E., Kostina, M. B., and Modyanov, N. N. (1994) *FEBS Lett.* **349,** 144–150
15. Shull, G. E., and Lingrel, J. B (1986) *J. Biol. Chem.* **261,** 16788–16791
16. Sanford, J., Codina, J., and Birnbaumer, L. (l990) *J. Biol. Chem.* **266,** 9570–9579
17. Shull, G. E., Schwartz, A., and Lingrel, J. B. (1985) *Nature* **316,** 691–705
18. Hsu, W. H., Rudolph, U., Sanford, J., Bertrand, P., Olate, J., Nelson, C., Moss, L. G., Boyd, A. E., Codina, J., and Birnbaumer, L. (l990) *J. Biol. Chem.* **265,** 11220–11226
19. Shull, G. E., Greeb, J., and Lingrel, J. B. (1986) *Biochemistry* **25,** 8125–8132
20. Young, R. M., Shull, G. E., and Lingrel, J. B (1987) *J. Biol. Chem.* **262,** 4905–4910
21. Martin-Vasallo, P., Dackowski, W., Emanuel, J. R., and Levenson, R. (1989) *J. Biol. Chem.* **264,** 4613–4618
22. Shull, G. E. (1990) *J. Biol. Chem.* **265,** 12123–12126
23. Laemmli, U. K. (1970) *Nature* **227,** 680–685
24. Tarentino, A., Plummer, T. H., Jr., and Maley, F. (1970) *J. Biol. Chem.* **245,** 4150–4157
25. Umemoto, J., Bhavanandan, V. P., and Davidson, E. A. (1977) *J. Biol. Chem.* **252,** 8609–8614
26. Chavira, R., Jr., Burnett, T. J., and Hageman, J. H. (1984) *Anal. Biochem.* **136,** 446–450
27. Parekh, R. B., Tse, A. G., Dwek, R. A., Williams, A. F., and Rademacher, T. W. (1987) *EMBO J.* **6,** 1233–1244
28. Pressley, T. A. (1992) *Am. J. Physiol.* **262,** C743–C751
29. Marxer, A., Stieger, B., Quaroni, A., Kashgarian, M., and Hauri, H. P. (1989) *J. Cell Biol.* **109,** 1057–1069