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Identification of the β-subunit for nongastric H-K-ATPase in rat anterior prostate

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Pestov, Nikolay B., Tatyana V. Korneenko, Rossen Radkov, Hao Zhao, Mikhail I. Shakhparonov, and Nikolai N. Modyanov. Identification of the β-subunit for nongastric H-K-ATPase in rat anterior prostate. *Am J Physiol Cell Physiol* 286: C1229–C1237, 2004. First published January 28, 2004; 10.1152/ajpcell.00393.2003.—The structural organization of nongastric H-K-ATPase, unlike that of closely related Na-K-ATPase and gastric H-K-ATPase, is not well characterized. Recently, we demonstrated that nongastric H-K-ATPase α-subunit (α_{ng}) is expressed in apical membranes of rodent prostate. Its highest level, as well as relative abundance, with respect to α_1-isoform of Na-K-ATPase, was observed in anterior lobe. Here, we aimed to determine the subunit composition of nongastric H-K-ATPase through the detailed analysis of the expression of all known X-K-ATPase β-subunits in rat anterior prostate (AP). RT-PCR detects transcripts of β-subunits of Na-K-ATPase only. Measurement of absolute protein content of these three β-subunit isoforms, with the use of quantitative Western blotting of AP membrane proteins, indicates that the abundance order is $\beta_1 > \beta_3 \gg \beta_2$. Immunohistochemical experiments demonstrate that β_1 is present predominantly in apical membranes, coinciding with α_{ng}, whereas β_3 is localized in the basolateral compartment, coinciding with α_1. This is the first direct demonstration of the α_{ng}-β_1 colocalization in situ indicating that, in rat AP, α_{ng} associates only with β_1. The existence of α_{ng}-β_1 complex has been confirmed by immunoprecipitation experiments. These results indicate that β_1-isoform functions as the authentic subunit of Na-K-ATPase and nongastric H-K-ATPase. Putatively, the intracellular polarization of X-K-ATPase isoforms depends on interaction with other proteins.

ATP1AL1; *ATP12A*; *ATP1B1*; X-potassium-adenosine triphosphatase; hydrogen-potassium-adenosine triphosphatase; sodium-potassium-adenosine triphosphatase; male accessory glands; potassium transport

NONGASTRIC H-K-ATPASE IS A member of the X-K-ATPase family that also includes Na-K-ATPase and gastric H-K-ATPase. X-K-ATPases are the most closely related among the various P-ATPases (1, 8, 29, 30, 41, 55). These ion pumps are located in plasma membranes and function as cation pumps that transport K^+ into the cell in exchange for Na^+ and/or H^+. Catalytic α-subunits of X-K-ATPases are large polytopic proteins (~100 kDa) that perform ATP hydrolysis and ion translocation. Six isoforms of these subunits can be classified into three groups: Na-K-ATPase (α_1, α_2, α_3, α_4), gastric H-K-ATPase (α_g), and nongastric H-K-ATPase (α_{ng}); all of the three groups are equally related to each other in terms of structure (~64% identical amino acid residues) (29, 30). The domain architecture of all X-K-ATPase α-subunits is thought to share essential elements of well-defined molecular structure of sarcoplasmic reticulum Ca-ATPase (55). However, X-K-ATPases are unique with respect to quaternary structure because they are the only animal P-ATPases that contain a second component, glycosylated β-subunit of ~35 kDa, which has no analogs among subunits of other P-ATPases (1, 8, 13, 24, 30).

The β-subunit plays a crucial role in the structural and functional maturation of the functionally active X-K-ATPase molecule (13, 20, 24) and modulation of the enzymes' affinities for cations (13, 20, 24). Five closely related genes encoding β-subunits have been identified in mammals encoding three Na-K-ATPase β-isoforms (β_1, β_2, β_3), gastric H-K-ATPase β-subunit (β_g), and muscle-specific (β_m) protein. All five members of the family share a common transmembrane structure of type II membrane proteins but exhibit a much lower degree of sequence similarity (~32–48%) than α-subunits (13, 24, 44).

An important issue is the tissue specificity of expression of these genes, suggesting that the existence of multiple X-K-ATPase isoforms is not a consequence of a redundant gene duplication but a means to finely tune the specific features of ion homeostasis in various cell types. Indeed, there is a substantial variability of expression level of all subunits and hence a significant variability of different isoform combinations. The α_1-β_1 complex is a ubiquitous one but has a rather variable level of expression, being especially abundant in brain and in some ion-transporting tissues like kidney. Particular combinations are characteristic for some tissues: $\alpha_3\beta_1$ for neurons, $\alpha_3\beta_2$ for retina, $\alpha_2\beta_2$ for glia, and $\alpha_1\beta_2$ for stria vascularis of the inner ear. Some other subunits have a very strict tissue specificity: α_4, only in male germ cells; β_m, only in striated muscle; α_g and β_g, primarily in parietal cells of stomach mucosa [there are also reports on detection of gastric H-K-ATPase subunits in kidney (3) (conflicting with Ref. 11), epididymis (4), inner ear and choroid plexus (36), and heart (7, 47)]. The β_3 is expressed in many tissues at relatively low levels, being somewhat more abundant in lung, testis, adrenal, brain, and colon (5, 8, 24, 47).

The catalytic α_{ng} is encoded by gene *ATP12A* (alternative name *ATP1AL1*). Mammalian *ATP12A* genes have been known for a long time to be expressed in distal colon, skin, and kidney (30, 48). A broad screening of tissues has revealed that the gene is also expressed in other tissues, like preputial gland (rat) and placenta (human) (48) and, at the highest level, in rodent prostate (46), especially in the anterior lobe. The cellu-

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lar location of α_{ng} in prostate epithelium, distal colon, and kidney was shown to be the apical membranes (23, 33, 46, 49, 58).

Unlike long-known and extensively studied Na-K-ATPase and gastric H-K-ATPase (8, 53), the nongastric H-K-ATPase is not yet sufficiently characterized with respect to structural organization and functional properties. For many years, it remained unclear whether one of the known β-subunits or a hitherto unidentified member of the X-K-ATPase β-subunit family is the authentic subunit of nongastric H-K-ATPase. No unique β-subunit specific only for nongastric H-K-ATPase has been identified, despite intense efforts of our group and others. Published experimental data on this subject were controversial, and it was not clear whether one or several isoforms can function as the β-subunit for nongastric H-K-ATPase. For rat distal colon and kidney, it was reported to be either β_1 (14, 34) or, in sharp contrast, β_3 (52). On the other hand, in heterologous expression systems, the functional expression of nongastric H-K-ATPase can be supported by various X-K-ATPase β-subunit isoforms (1, 2, 6, 15, 18, 21, 24, 27, 30, 31, 40).

In studies reported here, we took advantage of anterior prostate (AP) as the richest source of α_{ng} (46) and have aimed to determine the subunit composition of nongastric H-K-ATPase through the detailed analysis of the expression of all known X-K-ATPase β-subunits in rodent prostate complex. Our findings provide strong evidence that, in rat AP epithelium, X-K-ATPase β_1-isoform serves as an authentic subunit of nongastric H-K-ATPase, assembling preferentially with α_{ng}, whereas β_3 appears to be selective for Na-K-ATPase α-subunit. A preliminary account of this work has been presented (40).

MATERIALS AND METHODS

Animals and tissues. Male Sprague-Dawley rats (60–90 days old) were killed by CO_2 inhalation, and APs (coagulating glands) were dissected as soon as possible.

RT-PCR and cDNA cloning. Conditions of RT-PCR and primer sequences for tissue expression studies for β_1, β_2, and β_3 were essentially as described before (46, 48). Primers used for β_g were GFBE (gcrtctatgtgctgatgcag) and GBBE (gaggaacttgacgatcctgttc). Agarose gels were stained with ethidium bromide and imaged with the help of a Typhoon 8600 laser scanner (Amersham Pharmacia, Piscataway, NJ).

To produce recombinant ectodomains of rat β-subunits, the following primers were used: RB1-F (gcttagatctagtgagctgaaacccacgt) and RB1-B (cttgtgattagctcttaacttca) for β_1; RB2-F (gctgagatctgtctctgaccatacccccaag) and RB2-B (aaggaagcttaggctttgttgattcgaagc) for β_2; and RB3-F (gctcagatctctgaatgacgaggttc) and RB3-B (agacaagcttctcttaggcatgtgctatgact) for β_3. The fragments were amplified from rat brain cDNA, digested with *Bgl*II and *Hin*dIII (except for β_1), and cloned at *Bam*HI/*Hin*dIII sites of pQE30 expression vector (Qiagen, Valencia, CA). The β_1 fragment was blunted with T4 DNA polymerase, digested with *Bgl*II, and cloned at *Bam*HI/*Sma*I sites of the pQE vector.

Antibodies. Recombinant protein expression in *Escherichia coli*, purification by immobilized metal affinity chromatography, and immunization of rabbits were achieved essentially as described before (45). Antibodies were affinity purified by using the antigens absorbed on polyvinylidene difluoride membrane, according to the method of Rucklidge et al. (51).

Mouse monoclonal antibody α6F against α_1 (56) was obtained from the Developmental Studies Hybridoma Bank (Iowa City, IA). Rabbit polyclonal antibodies against α_{ng} have been described before (46). Monoclonal antibody against gastric H-K-ATPase β-subunit was purchased from Affinity Bioreagents (Golden, CO). Monoclonal antibodies IEC 1/48 (37) and MAb 13 against β_1 and 3PE anti-human β_3 (12) were generous gifts from Andrea Quaroni, Michael J. Caplan, and Watchara Kasinrerk. Mouse monoclonal antibodies F10 against rat β_3 and 2C8 against secretory pathway Ca-ATPase were from Ruslan Dmitriev (unpublished observations). Rabbit polyclonal antibodies against human β_1, β_2, and β_3 ectodomains (59) and antibodies against NH_2 terminus of rat β_3 (5) were kindly provided by Pablo Martin-Vasallo and by Kathleen J. Sweadner.

Immunohistochemistry. Tissues were frozen in isopentane/liquid nitrogen and cut at 10-μm thickness. The sections were incubated in 5:3 methanol-acetone at −15°C for 30 min, air dried, and stored. The sections were treated with chloroform for 5 min at room temperature, air dried, incubated with 5% pig serum in PBS, and then immunolabeled by subsequent incubations with primary antibodies and anti-host antibodies conjugated with either Alexa Fluor-498 or Alexa Fluor-594 (Molecular Probes, Eugene, OR). For peroxidase fluorescent labeling, the sections were incubated with 3% hydrogen peroxide in PBS for 1 h, anti-guinea pig peroxidase-conjugated antibodies (Sigma), and tyramide-595 substrate (Molecular Probes). Labeled sections were mounted in SlowFade (Molecular Probes). To label nuclei, 100,000× SYBR Gold (Molecular Probes) or 0.5 μg/ml ethidium bromide plus 10 μg/ml RNase A were added to the mounting medium. Images were collected by using a Nikon Optiphot-2 fluorescent microscope equipped with a SPOT digital camera (Diagnostic Instruments, Sterling Heights, MI) with automatic exposure control. Confocal images were obtained by using an Olympus fluorescent microscope equipped with Radiance XR laser scanning head (Bio-Rad, Hercules, CA).

Preparation of membranes and deglycosylation. Membranes were prepared from rat prostate essentially as described before (46). For deglycosylation, the membranes were incubated for 1 h at 37°C in 0.05 M sodium phosphate, pH 7.4, 0.2% SDS, 2% octyl glucoside, 100 mM dithiothreitol (DTT), 1:50 protease inhibitor cocktail (Sigma), and 5 U/μg peptide N-glycosidase F (New England Biolabs). Treatment with endoglycosidase H (Endo H_f; New England Biolabs) was performed in the same conditions, except that 0.05 M sodium citrate (pH 5.5) were substituted for sodium phosphate.

Immunoprecipitation. Membrane suspension (100 μl, 200 μg protein) in 10 mM HEPES-Na, pH 7.0, 5 mM Na-EDTA, and 0.25 M sucrose was kept on ice, diluted with water 1:1, and made subsequently 0.3 M NaCl, 0.1% β-mercaptoethanol, 1% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) (crystallized from methanol), and 0.02% benzyldimethylhexadecylammonium chloride (Sigma; from 2% solution kept at 50°C before use). The membranes were incubated with 1 μl ascites on a rotary mixer for 8 h at 4°C and centrifuged for 10 min at 16,000 *g*. The supernatant was diluted 1:1 with PBS and incubated with 3 μl protein A/G agarose (Novagene, Madison, WI) for 2 h at 4°C. The sorbent was spinned down and washed with PBS containing 0.2% CHAPS. The bound proteins were eluted by incubation for 5 min at 70°C in a modified electrophoresis sample-loading buffer (5% SDS, 8 M urea, 100 mM DTT, 25 mM glycylglycine, 100 mM Tris·HCl, pH 6.8, 2% β-mercaptoethanol, 1 mM Na-EDTA, 2 mM benzamidine, 1:200 protease inhibitor cocktail) before analysis by Western blotting.

Western blotting. Membranes were dissolved in the SDS sample loading buffer, and protein concentration was measured by a modification of the Bradford procedure that includes coprecipitation of proteins with calcium phosphate (43). Proteins (10 μg per well, or 1:10 of immunoprecipitates) were electrophoresed in polyacrylamide gels (12% for analysis of β-subunits, 8% for α-subunits) and blotted onto polyvinylidene difluoride membrane (Amersham-Pharmacia). The membrane was washed in methanol and stained in 50% methanol, 1% acetic acid, and 0.03% Coomassie brilliant blue G-250 followed by washes with 50% methanol. Then the membrane was cut and destained in methanol, followed by washing with 50% methanol. The membrane was incubated in 50 mM Tris, pH 6.8, 100 mM mercap-

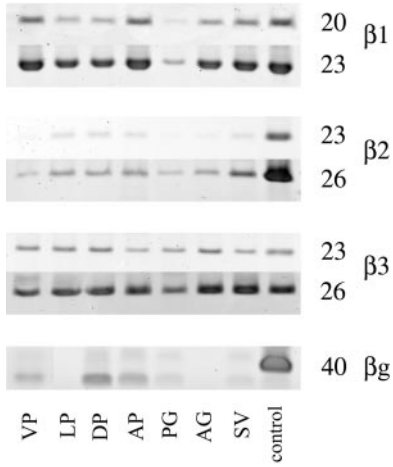

Fig. 1. RT-PCR analysis of lobe-specific expression of X-K-ATPase β-subunit isoforms in rat prostate. No. of cycles used are indicated on the *right*. β_1, β_2, and β_3: amplification products of mRNAs of Na-K-ATPase β_1-, β_2-, and β_3-isoforms, respectively; β_g, amplification products of the gastric H-K-ATPase β mRNA; control, rat brain cDNA for β_1, β_2, and β_3 and rat stomach cDNA for β_g; VP, ventral prostate; LP, lateral prostate; DP, dorsal prostate; AP, anterior prostate; PG, preputial gland; AG, ampullary gland; SV, seminal vesicles.

toethanol, and 2% SDS for 15 min at room temperature; blocked in Tris-buffered saline containing 0.1% Tween-20 and 5% nonfat milk; and incubated with primary antibodies and then with peroxidase-conjugated anti-rabbit antibodies (Zymed) or peroxidase-conjugated anti-guinea pig antibodies (Sigma) for 1 h each with thorough washes in Tris-buffered saline, containing 0.1% Tween-20, between incubations. The immunoblots were visualized with a chemiluminescent substrate (ECL+Plus, Amersham Pharmacia). For quantitative determination, series of standard protein dilutions were prepared in SDS sample loading buffer supplemented with 100 mM DTT and run in parallel. Densitometry of the immunoblot films was performed by using a Bio-Rad model GS-690 imaging densitometer and Molecular Analyst software (Bio-Rad Laboratories).

RESULTS

Analysis of lobe-specific expression of the X-K-ATPase β-subunits in rat prostate. Figure 1 illustrates results of RT-PCR detection of four different X-K-ATPase β-subunits in lobes of rat prostate, as well as in some other male accessory glands that were not studied before. Transcripts of all known Na-K-ATPase β-subunits (β_1, β_2, and β_3) are readily detectable, whereas no signal can be observed in the case of β_g. The absence of β_m in these glands has been reported before (44). Considering the number of cycles, it appears that β_1 transcripts are more abundant than β_2 or β_3 in all tissues studied, and β_3 is more abundant than β_2. The expression levels of β_2 and β_3 are very similar between the tissues studied. In contrast, β_1 transcripts are more abundant in ventral and anterior lobes of the prostate than in dorsal and lateral lobes. Of note also is the fact that the preputial gland has a remarkably lower level of β_1 than other tissues.

Detection of X-K-ATPase β-subunits in rat prostate membranes by Western blotting. Initial experiments on immunochemical detection of β-subunit proteins by Western blotting of rat AP using available rabbit antibodies against ectodomains of human β_1, β_2, and β_3 showed that antibodies against β_1 and β_2 are suitable for this purpose, whereas anti-human β_3 produced no specific bands (Fig. 2*A*). Antibodies against a synthetic peptide comprising an NH_2-terminal fragment of rat β_3 (5), on the other hand, produced a strong band of ~38 kDa that showed no shift on deglycosylation, probably representing a nonspecific interaction with a prostate-specific protein (results not shown). To improve the method of detection, we have expressed recombinant proteins comprising rat β_1, β_2, and β_3 ectodomains NH_2-terminally fused with the hexahistidine tag (Fig. 3) and obtained rabbit and guinea pig polyclonal antibodies against them. For unknown reasons, rabbits produced better anti-β_1 antibodies, whereas guinea pigs produced better anti-β_3. Anti-rat β_2 antibodies from both species were of insufficient quality (results not shown), and, therefore, anti-human β_2 rabbit polyclonal antibodies developed by Pablo Martin-Vasallo were used. Crude sera can be successfully used for the detection of deglycosylated β-subunits (as shown for β_3 in Fig. 2*B*), whereas detection sensitivity of the glyco forms was not suitable because of band smearing. Both β_1 and β_2 core proteins (after removal of N-glycans by glycopeptidase F) from AP have electrophoretic mobilities corresponding to apparent molecular masses slightly higher than the predicted values. The β_3 has a significantly higher apparent molecular mass (36 kDa instead of theoretical 32 kDa). The same behavior of rat β_3 was observed previously in many different tissues (5) but is difficult to understand because β_3 sequence lacks unusual features. All recombinant β-subunit ectodomains have apparent molecular masses somewhat larger than expected (33.9 vs. 29.8 kDa for β_1 and 30.7 vs. 27.1 kDa for β_2), but, in the case of β_3, this effect is much more significant (31.5 vs. 25.8 kDa). Because the ectodomain comprises the majority of the molecule's mass, it is clear that abnormally apparent molecular mass of rat β_3 core protein from native membranes can be fully explained by the abnormal mobility of its ectodomain.

Concentrations of different isoforms of β-subunits in microsomes from AP and several other tissues were measured by quantitative Western blotting by using known quantities of recombinant proteins as standards (Table 1). The β_1 in most tissues and β_2 in brain are easy to determine due to relatively high content. The β_3 has a somewhat lower expression level, whereas β_2 in AP has a very low level, which is sufficient for qualitative detection (Fig. 2*A*) but not for accurate quantitative measurements. For this reason, the data for β_2 are presented as

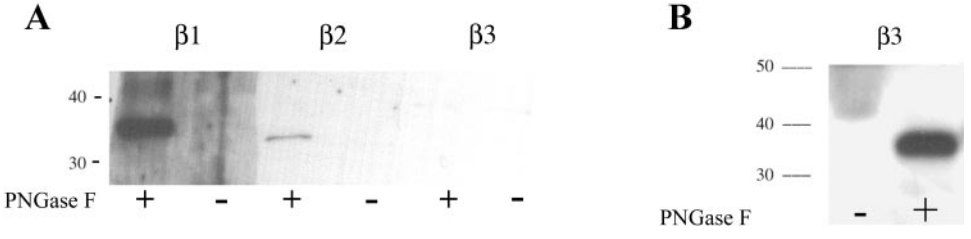

Fig. 2. Immunoblotting of membrane proteins from rat AP with antibodies against X-K-ATPase β_1-, β_2-, and β_3-subunits. *A*: immunoblotting with rabbit antibodies against recombinant ectodomains of human β-subunit isoforms β_1, β_2, and β_3 (59). *B*: immunoblotting with guinea pig antibodies against recombinant protein comprising ectodomain of rat β_3-isoform. PNGase F, peptide N-glycosidase F.

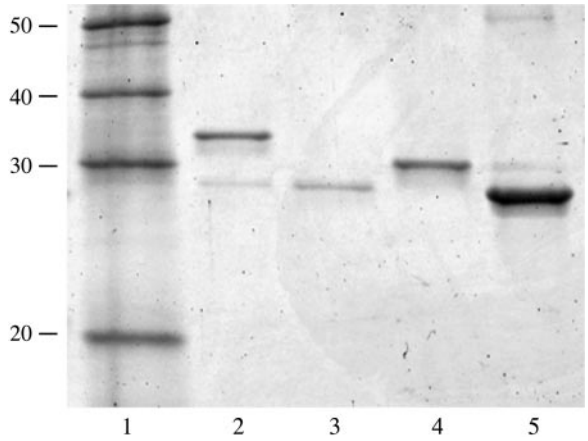

Fig. 3. Electrophoretic analysis of recombinant proteins comprising ectodomains of rat Na-K-ATPase β-subunit isoforms. *Lane 1*, molecular mass standards; *lane 2*, β_1; *lane 3*, β_2; *lane 4*, β_3; *lane 5*, mouse muscle-specific protein (β_m).

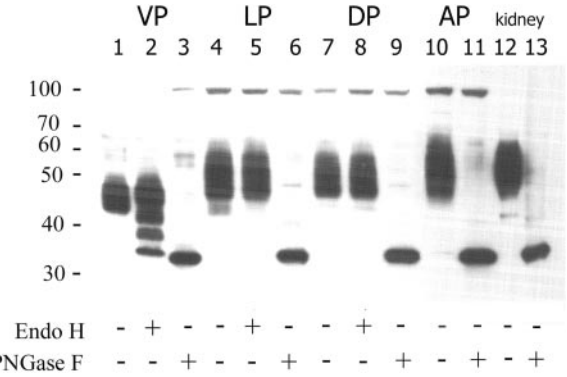

Fig. 4. Immunoblotting of membrane proteins from different lobes of rat prostate with antibodies against X-K-ATPase β_1-subunit. Affinity-purified rabbit polyclonal antibodies against recombinant ectodomain of rat β_1 were used.

an upper threshold. It was of interest to check if our determinations give correct, unbiased values. The contents of β_1- and β_2-subunits in rat brain microsomes were determined previously as 227 ± 23 and 151 ± 32 pmol/mg for β_1 and β_2, respectively, by Lavoie et al. (35). These values are 1.5 and 1.2 times higher than ours for β_1 and β_2, respectively. In fact, this difference seems to be very little and could be explained by variations in experimental procedures. Because β_1 has a 2.5-fold higher level than β_3, it is reasonable to hypothesize that β_1 is the predominant isoform in nongastric H-K-ATPase.

If β_1 is the real partner of α_{ng}, then may β_1 from AP or any of the other prostate lobes be different from that in tissues studied previously? May the β_1-isoform interacting with α_{ng} be a product of alternative splicing or have different modifications, for example, N-glycosylation? This possibility was tested by treatment with glycopeptidase F or endoglycosidase H, the latter being active mostly against high-mannose chains. Figure 4 presents results of such an analysis and demonstrates that β_1 from AP as well as from other α_{ng}-containing lobes (47) is glycosylated to a similar extent as the reference β_1 of kidney, and the amount of high-mannose sugars is negligible. The electrophoretic mobilities of core β_1 proteins from kidney and AP are equal. Very surprisingly, β_1 from ventral prostate was found to have a different N-glycosylation pattern: the amount of sugars is obviously lower, and significant sensitivity to endoglycosidase H indicates that β_1 in ventral prostate has high-mannose chains. In contrast to the distal colon, where the existence of unusual peptide N-glycosidase F-resistant N-glycans in the β_1 associated with α_{ng} has been suggested (14), we did not observe any unusual or unexpected features of β_1.

Table 1. *Quantification of β-subunit proteins*

Tissue Protein	Anterior Prostate	Brain	Testes	Liver
β_1	394±65 (n=7)	4,418±700 (n=4)	220±42 (n=2)	70±36 (n=2)
β_2	<10 (n=2)	3,647±270 (n=2)	<10 (n=2)	<10 (n=2)
β_3	149±43 (n=7)	208±65 (n=3)	520±195 (n=3)	no data

Values are presented as picograms of full-length core β-subunit per milligram of total membrane protein ± SE; n, no. of proteins. The content of X-K-ATPase β-subunit proteins was measured by using quantitative Western blotting of membrane proteins deglycosylated with glycopeptidase F. The polyclonal antibodies against recombinant ectodomains of the β-subunits were used {rabbit polyclonal against rat β_1, rabbit polyclonal against human β_2 [provided by Dr. Martin-Vasallo (59)], and guinea pig polyclonal against rat β_3}. Purified recombinant ectodomain proteins were used as standards.

Immunohistochemical localization of the X-K-ATPase β-subunits in rat AP. In agreement with a previous report (46), anti-α_1 antibodies produce basolateral labeling, and anti-α_{ng} antibodies label apical membranes (Fig. 5*A*). Affinity-purified polyclonal anti-β_1 antibodies and monoclonal antibody MAb 13 against dog β_1 showed weak labeling of apical and none of basolateral membranes (Fig. 5, *B* and *C*). However, another anti-β_1 monoclonal antibody, IEC 1/48 (37), known for its high-detection sensitivity in immunochemical experiments, produced a very bright labeling of apical membranes and a weak labeling of the basolateral compartment (Fig. 5, *D–F*). Anti-β_3 antibodies produced a weak basolateral labeling. Its intensity was increased by the use of tyramide fluorogenic substrate (Fig. 5*G*). No significant labeling of apical membranes with anti-β_3 antibodies was observed. Anti-β_2 antibodies did not produce any significant labeling above nonspecific background (results not shown).

Thus it is obvious that β_3 is polarized laterally, whereas β_1 is polarized mostly apically with detectable levels in basolateral membranes. On the basis of these data, it is possible to conclude that the α_{ng}-β_1 pair is transported to the apical side, whereas both α_1-β_1 and α_1-β_3 are transported to basolateral membranes. Importantly, these results demonstrate that, in AP epithelial cells, α_{ng} binds β_1 preferentially and does not associate with β_3. These conclusions are confirmed by double-labeling experiments (α_{ng} + β_1 in Fig. 5, *H–J*, and α_1 + β_3 in Fig. 5, *K–M*).

Immunoprecipitation. Interestingly, both nongastric H-K-ATPase and Na-K-ATPase in AP membranes are resistant to solubilization under mild conditions with common nonionic or zwitterionic detergents, such as $C_{12}E_8$, digitonin, octyl glucoside, Triton X-100, CHAPS, myristyl sulfobetaine, etc. (results not shown). Such a phenomenon may be explained by a strong interaction of membrane proteins with cytoskeleton. The resistance to mild solubilization creates a strong obstacle against successful immunoprecipitation of the ATPases for direct demonstration of particular αβ interactions. We were not able to immunoprecipitate α_{ng} according to Codina et al. (14) or β_1 according to Marxer et al. (37) by reproducing previously

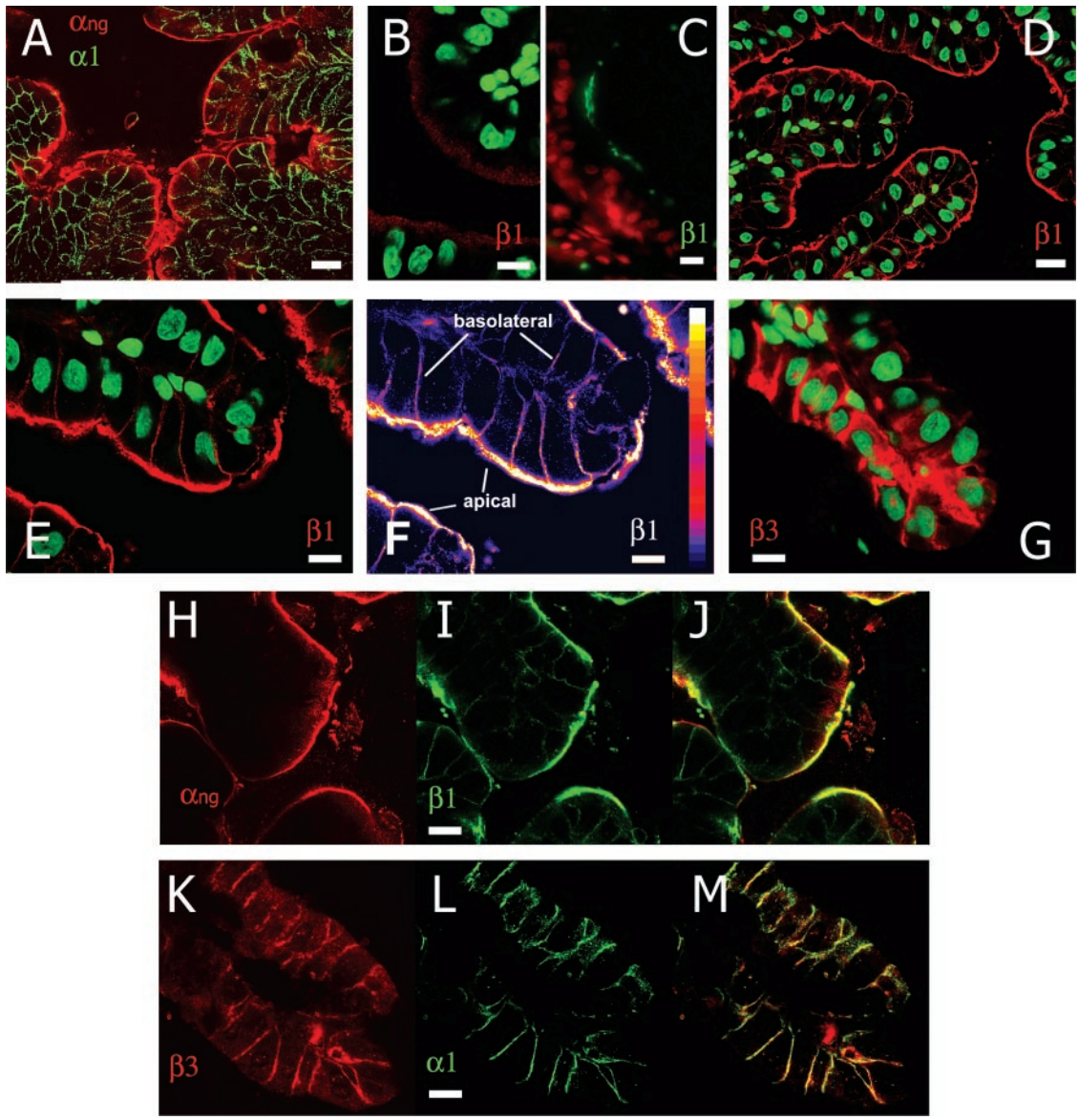

Fig. 5. Immunohistochemical detection of α- and β-subunit isoforms of X-K-ATPases in rat AP. *A*: double labeling with rabbit anti-nongastric H-K-ATPase subunit (α_{ng}) antibodies (red) and mouse monoclonal anti-α_1 (green). *B*: labeling with anti-β_1 monoclonal antibody 13 (red). *C*: labeling with anti-β_1 affinity-purified polyclonal rabbit antibodies with tyramide-488 (green). *D–F*: labeling with anti-β_1 monoclonal antibody IEC 1/48 (red). *F*: false color image of red fluorescence in *E* (increasing intensity form the *bottom* to the *top*, according to the look-up table). *G*: labeling with anti-β_3 affinity-purified rabbit polyclonal antibodies and tyramide-595 (red). *H–J*: colocalization of α_{ng} (rabbit antibodies, red fluorescence) and β_1 (IEC 1/48, green fluorescence). *K–M*: colocalization of β_3 (guinea pig antibodies, tyramide-595, red fluorescence) and α_1 (α6F monoclonal antibody, green fluorescence). *A*, *B*, and *D–M*: confocal images; *C*: conventional microscope images. *A*, *B*, *D*, *E*, and *G*: nuclei stained in green with SYBR Gold; *C*: nuclei stained in red with ethidium bromide. Bars: 20 μm (*A*, *C*, and *D*) and 10 μm (*B* and *E–M*).

used conditions for distal colon membranes (CHAPS or Triton X-100) (results not shown). Here it should be stressed that the intersubunit interactions in X-K-ATPases are not very strong in solubilized form, for example, γ-subunit retains in the complex solubilized with CHAPS and separates even in nonionic detergents, such as 1% octyl glucoside or Triton X-100 (38). It is impossible to use harsh solubilization conditions (e.g., SDS). For this reason, we tried to use mixtures of ionic detergents with nonionic or zwitterionic ones, as previously found excellent for immunoaffinity chromatography (32). As expected, additions of some ionic detergents improve solubilization, especially in the presence of salt (Fig. 6). However, the immunoprecipitation requires not only solubilization but also preservation of the interactions between the subunits, antigen-antibody, and immunoglobulin-protein A/G. For this reason, we canceled attempts for step-by-step optimization and performed a screening for the precipitation conditions using simultaneous solubilization and antibody binding (results not shown). It was found that a significant part of α_{ng} can be co-immunoprecipitated by anti-β_1 monoclonal antibody IEC 1/48 by using CHAPS supplemented with salt and a low concentration of benzyldimethylhexadecylammonium chloride, a cationic detergent. A significant portion of the total nongastric H-K-ATPase α-subunit has been detected in the

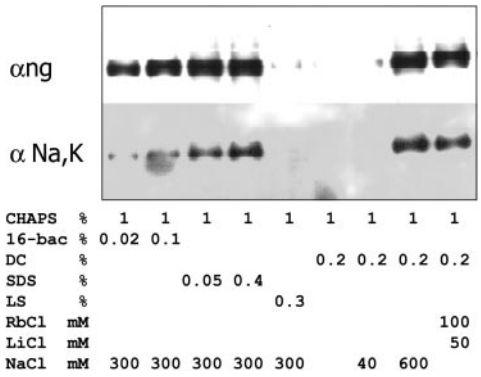

Fig. 6. Solubilization of nongastric H-K-ATPase and Na-K-ATPase from rat AP membranes with 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS) supplemented with other detergents and salt. The membranes were treated for 2 h at 0°C with detergents and salts indicated below and centrifuged for 15 min at 16,000 *g*, and then supernatants were immunoblotted with anti-α_{ng} rabbit polyclonal antibodies (*top*; α_{ng}) or anti-Na-K-ATPase α-subunit rabbit polyclonal antibodies (*bottom*; α Na-K). 16-BAC, benzyldimethylhexadecylammonium chloride; DC, sodium deoxycholate; LS, sodium laurylsarcosine.

fraction precipitated with anti-β_1 monoclonal antibody IEC 1/48 (Fig. 7*A*). These results directly demonstrate that α_{ng} is capable of interacting with β_1 in the AP membranes.

A comparison of the signal intensities in the solubilizates and eluates indicates that the anti-β_1 antibody precipitates α_{ng} much more efficiently than Na-K-ATPase α-subunit (Fig. 7*B*). This may be interpreted either as that β_1 is initially associated with α_{ng} predominantly or that the affinity of the β_1-α_{ng} interaction is higher than that of β_1-α_1. The first explanation is in line with immunohistochemical data and strongly suggests that β_1 associates primarily with α_{ng} and, to a much lesser extent, with α_1. These results also provide indirect evidence that α_1 is complexed mainly with β_3.

DISCUSSION

AP has three Na-K-ATPase β-subunit isoforms in the apparent order of their abundance $\beta_1 > \beta_3 > \beta_2$, whereas other X-K-ATPase β-subunits are absent. No new β-subunit can exist because of the lack of any new genes in sequenced mammalian genomes. Also, both β_m and β_g transcripts are absent in the rat prostate. For these reasons, the search for authentic nongastric H-K-ATPase β-subunit can be narrowed to three candidates: β_1, β_2, and β_3. Results of immunological detection of different β-subunit isoforms by Western blotting of AP membranes indicate that β_1 is the most abundant isoform. Content of β_1 is higher than β_3, and this indirectly indicates that β_1 is more likely than β_3 to be the predominant isoform of nongastric H-K-ATPase. Compared with β_1- and β_3-, β_2-isoform has a very low content, and its participation may be dismissed from consideration. The most interesting result was the comparison of immunohistochemical labeling of AP epithelium, which demonstrates that β_1 is localized predominantly apically, whereas β_3 is limited to basolateral membranes. These observations demonstrate directly that association of α_{ng} with β_3 does not occur in AP cells and suggest that, in these cells, nongastric H-K-ATPase exists as α_{ng}-β_1 complex, whereas Na-K-ATPase may consist of both α_1-β_1 and α_1-β_3 complexes. Importantly, results of co-immunoprecipitation of α_{ng} and α_1 with anti-β_1 antibodies support these conclusions.

Thus our findings provide strong evidence that X-K-ATPase β_1-subunit is not only a subunit of the Na-K-ATPase, but also serves as an authentic subunit of nongastric H-K-ATPase, a real counterpart of the α_{ng} in apical membrane of rat AP epithelium. In these cells, a much higher portion of β_1 appears to be associated with α_{ng} than that with α_1.

Our data resolve the previous contradictions on which β-subunit may be the authentic one in case of nongastric H-K-ATPase. Previously, β_1 antibodies were shown to recognize a protein in immunoprecipitate of α_{ng} from solubilized rat distal colon membranes (14) and in rat kidney membrane vesicles from immunoaffinity isolated with α_{ng} antibodies (34). The strongest argument for β_3 was that it is detected in apical membranes from rat distal colon (52). It should be noted that

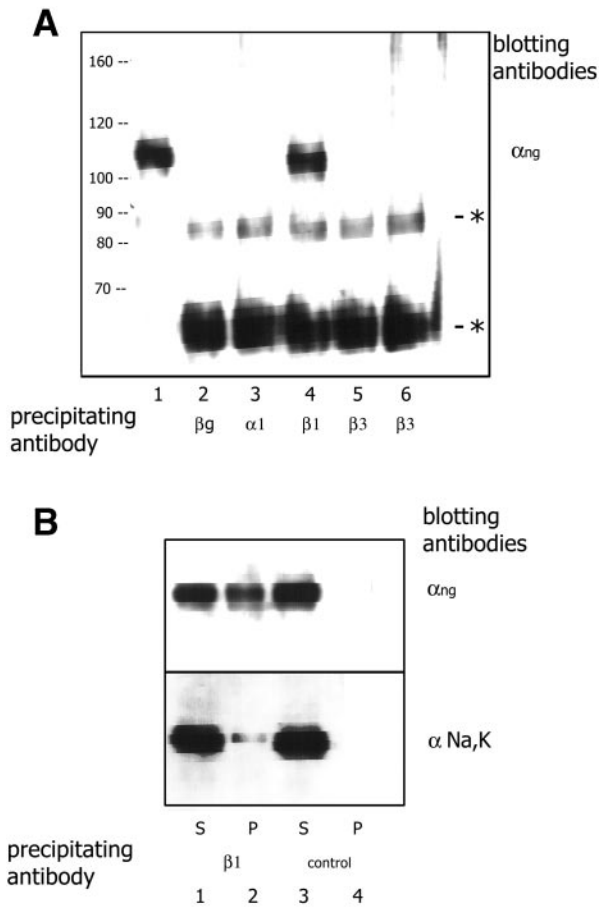

Fig. 7. Co-immunoprecipitation of α_{ng} from rat AP membranes with monoclonal antibody against β_1. *A*: immunoprecipitation of α_{ng} with monoclonal antibodies using simultaneous solubilization by CHAPS/16-BAC/NaCl as described in MATERIALS AND METHODS. *Lane 1*, AP membranes; *lane 2*, anti-β_g; *lane 3*, anti-α_1 (α6F); *lane 4*, anti-β_1 (IEC 1/48); *lane 5*, anti-rat β_3 (F10); *lane 6*, anti-human β_3 (3PE). *Reaction of secondary antibodies with the antibodies used for the immunoprecipitation. *B*: comparison of efficiency of co-immunoprecipitation of α_{ng} and Na-K-ATPase α-subunit by anti-β_1 monoclonal antibody IEC 1/48. Equal parts of immunoprecipitates (P) and remaining solubilizates (S) were analyzed by Western blotting with rabbit polyclonal antibodies against α_{ng} (*top*) or Na-K-ATPase α-subunit (*bottom*; α Na-K). *Lanes 1* and *2*, anti-β_1 (IEC 1/48), remaining solubilizate (S), and resulting precipitate (P), respectively; *lanes 3* and *4*, negative control [solubilizate (S) and precipitate (P) with an unrelated monoclonal antibody 2C8]. Note that a long exposure was used to detect α Na-K in the immunoprecipitate.

the detection of association with one isoform could not rule out the possibility of association with others. Hence the question remained open: whether β_1 (14, 34) or β_3 (52) is characteristic for the nongastric H-K-ATPase or α_{ng} can associate with any β-subunit isoform nonselectively. Our data provide strong evidence that $\alpha_{ng}\beta_1$ exists in AP and, more importantly, that there is a significant selectivity of αβ-subunit interactions. Indeed, association between α_{ng} and β_3 does not occur in AP. On the other hand, it is impossible to completely exclude the possibility that a minor portion of α_{ng} may be associated with β-subunits other than β_1, especially in tissues different from AP.

Interestingly, although our results indicate that, in vivo, there is a strong selectivity of subunit interactions between α_{ng} and β-subunits, the situation in vitro is more complicated. The α_{ng} protein has been coexpressed in various heterologous systems, together with different X-K-ATPase β-subunits (1, 2, 6, 15, 17, 18, 21, 24, 26–28, 30, 31, 39, 50). These studies demonstrated that several X-K-ATPase β-subunits, including the β_1 (6, 15, 21), the analog of mammalian β_2 from *Bufo* bladder (18, 21), *Torpedo* Na-K-ATPase β (6), as well as the β_g (1, 15, 21, 24, 27, 30, 31, 39), can support proper folding of α_{ng} and formation of functionally active nongastric H-K-ATPases. However, detailed comparison of the capability of each of the known X-K-ATPase β-subunits to form a functionally active ATPase complex with the α_{ng} on coexpression in *Xenopus* oocytes revealed that β_g and β_2-like *Bufo* bladder β are able to associate with α_{ng} much more efficiently than its real counterpart, β_1 (21, 26). Formation of the active ATPase complex of α_{ng} with β_g, but not with β_1 or β_3, was observed in baculovirus expression system (1, 2). It is logical to assume that the phenomenon of preferential association of the α_{ng} with β_g and *Bufo* bladder β in heterologous expression systems is based on intrinsic structural features of these particular β-subunits, which are designed by nature to resist the harsh environmental conditions in mammalian stomach or in frog urinary bladder and, therefore, exhibit a greater ability to survive in heterologous expression systems. Native β_g was found to be much less susceptible to digestion with trypsin and other proteases than the native β_1 (57). Therefore, one can suggest that heavy glycosylation [seven or eight N-linked carbohydrate chains in β_g and *Bufo* bladder β vs. three oligosaccharides in β_1 (13)] is an essential feature determining more efficient formation of the recombinant α_{ng}-β complexes.

The relatively weak association of α_{ng} with its authentic counterpart β_1 in *Xenopus* oocytes (21) and the absence of α_{ng}-β_1 association in Sf-21 insect cells (1, 2) may, in fact, indicate that these cells do not contain other subunits or proteins that facilitate or participate in the α_{ng}-β_1 assembly in vivo, as our laboratory suggested previously (26). This idea is supported by recent observations that α_{ng}, in contrast to α_1, is unable to assemble with endogenous β_1 on expression in mammalian human embryonic kidney 293, Madin-Darby canine kidney, and LLC-PK_1 cells (6, 50).

There is evidence that subunit composition and polarization of X-K-ATPases are linked. For example, abnormal expression of β_2 in the kidney results in apical localization of some of the Na-K-ATPase pumps with a concomitant disease (60). It was also demonstrated that the apical localization and trafficking of the gastric H-K-ATPase in tubulovesicles is dependent on its β-subunit (19). On the other hand, the data presented here, as well as results of other studies (10, 22), demonstrate that signals of membrane sorting are encoded in the X-K-ATPase α-subunits. To explain the apparent discrepancy of the above data, it is reasonable to suggest that, at least in some tissues, the efficient formation and intracellular trafficking of a particular combination of α- and β-subunit isoforms of the X-K-ATPases requires interaction with other proteins.

What kind of other proteins may associate with α_{ng}? One class of potential candidates is especially interesting: small transmembrane proteins known as members of the FXYD family, which includes γ-subunit of Na-K-ATPase (25, 54). In the distal colon, FXYD2 interacts only with Na-K-ATPase (16). However, seven members of the FXYD family are known (25, 54), and it is possible that some of them can interact and modulate properties of nongastric H-K-ATPase. An interesting example of putative interaction with unrelated proteins is the observation that the polarity of Na-K-ATPase in retinal pigment epithelium differs in cells with different levels of junctional E-/P-cadherin (9). Thus it is quite feasible that interactions with other proteins may be responsible for the observed specificity of αβ assembly and cellular polarization of X-K-ATPases (10, 42).

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