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Characterization of a β subunit of the gastric H^+/K^+-transporting ATPase

(H^+/K^+-ATPase β subunit/protein sequence/pernicious anemia/cDNA cloning)

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ABSTRACT **The catalytic subunit of the H^+/K^+-transporting ATPase (EC 3.6.1.3) has 62% identity to the α, or catalytic subunit, of the Na^+/K^+-transporting ATPase (EC 3.6.1.37); however, a homologous β subunit was unknown until recently. Removal of the carbohydrate from purified hog H^+/K^+ATPase vesicles reveals a 35-kDa peptide that, when fragmented with protease V8, gives sequences homologous to both β1 and β2 subunits of the Na^+/K^+-ATPase. cDNA clones for a β subunit of the gastric H^+/K^+-ATPase were isolated from a rabbit stomach cDNA library by using degenerate 17-mer oligonucleotide probes made to the protease V8-treated peptides. An open reading frame (54–926) encodes a predicted 291-amino acid peptide with M_r = 33,320, which exhibits 31% and 44% homologies to the Na^+/K^+-ATPase β1 and Na^+/K^+-ATPase β2 proteins, respectively. A Kyte–Doolittle hydropathy plot predicts a single N-terminal transmembrane domain with a small hydrophobic region near the C terminus. The presumed extracytosolic domain contains seven potential N-linked glycosylation sites and six out of nine cysteines. Northern (RNA) blot analysis of stomach RNA with the rabbit H^+/K^+-ATPase β probe identifies a single mRNA of 1.3–1.5 kilobases, similar in concentration to the α subunit mRNA. The presence of a defined gastric H^+/K^+-ATPase β subunit extends the homology between H^+/K^+-ATPase and the Na^+/K^+-ATPase subclass of phosphoenzyme transport ATPases and distinguishes them from the monomeric Ca^{2+} and proton pump subclasses.**

The gastric H^+/K^+-ATPase is an electroneutral pump that catalyzes the countertransport of H^+ and K^+ at the expense of ATP and is responsible for the secretion of acid into the stomach. The H^+/K^+-ATPase is a member of the phosphoenzyme family of ion-pumping, membrane-bound proteins that form an aspartyl phosphate intermediate during the hydrolysis of ATP. This family includes the three known isoforms of the Na^+/K^+-ATPases, several Ca^{2+}-ATPases, and the H^+-ATPases of *Neurospora* and *Saccharomyces*. The H^+/K^+-ATPase is most closely related to the Na^+/K^+-ATPase in terms of sequence, exhibiting 62% sequence homology between the catalytic subunits, as well as catalytic and transport properties (1, 24).

For many years a second subunit, the β subunit, has been known to be associated with the catalytic subunit of the Na^+/K^+-ATPase. Both subunits would appear to be necessary for ion transport and ATPase activity. A Na^+/K^+-ATPase β subunit (Na^+/K^+-β1) was cloned from kidney in 1986 (2), and in 1989 a second Na^+/K^+-β subunit (Na^+/K^+-β2) was cloned from both brain and liver (3). Recently the Na^+/K^+-β2 subunit was shown to have identity with a Ca^{2+}-independent adhesion protein (AMOG) in astrocytes (4), suggesting a more extensive role for this protein.

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Until recently there has been no evidence suggesting the presence of a β subunit for the H^+/K^+-ATPase. In 1989 a H^+/K^+-ATPase β (H^+/K^+-β) peptide sequence was found in hog gastric vesicle preparation labeled with a photolabile, K^+-competitive H^+/K^+-ATPase inhibitor (5). In that study, two radiolabeled peptide fragments obtained from a tryptic digest were isolated and sequenced. One peptide fragment matched a sequence from the luminal domain between transmembrane regions H-5 and H-6 of the α subunit of the H^+/K^+-ATPase. The second peptide had no homology with the H^+/K^+-ATPase α subunit, but instead was homologous with the Na^+/K^+-β1 and -2 subunits. Labeling of both peptide fragments suggested the close proximity of an extracytosolic region of the α subunit and a putative H^+/K^+-β subunit. Additional evidence for a close association between the two subunits is that glutaraldehyde crosslinking of purified soluble gastric membrane fractions yields αβ heterodimers, composed of fluorescein isothiocyanate (FITC)-labeled α subunit and a wheat germ agglutinin-reactive H^+/K^+-β (6, 7).

Wheat germ agglutinin strongly stains a region from 60 to 85 kDa on SDS/PAGE gels of highly purified hog H^+/K^+-ATPase (8, 9). This region is very weakly stained by Coomassie blue, which may explain why the β subunit had previously gone undetected. Treatment of the purified membrane fraction with N-glycosidase F reduced the broad 60- to 85-kDa region to a single 35-kDa Coomassie blue-positive band (8, 9). This result is similar to earlier experiments with the Na^+/K^+-β subunit, except the Na^+/K^+-β clearly stains with Coomassie blue on SDS gels (10), probably due to a lower level of glycosylation than H^+/K^+-β. Digestion of the isolated 35-kDa band with V8 protease, electrophoresis, and transfer of the resulting peptides to poly(vinyl difluoride) (PVDF) membranes, provided sequence information for two distinct peptides (8), one of which overlapped the original sequence obtained from the photolabile, K^+-competitive inhibitor experiment (5).

Regions of these two peptides exhibiting strong, but not complete, identities to the Na^+/K^+-β1 and -2 proteins were used to design mixed, degenerate 17-mer oligonucleotide probes. The probes were prepared in sense and antisense directions to be used for polymerase chain reactions (PCRs) and were successfully used to isolate several positive cDNA clones from a rabbit gastric cDNA library. The deduced protein sequence† obtained from these clones establishes the presence of a β subunit of the H^+/K^+-ATPase distinct in sequence from the β subunits of the Na^+/K^+-ATPase isoforms.

Abbreviations: H^+/K^+-β, H^+/K^+-ATPase β subunit; Na^+/K^+-β, Na^+/K^+-ATPase β subunit; PCR, polymerase chain reaction.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35544).

MATERIALS AND METHODS

Probes. Degenerate oligonucleotide probes were designed from reverse translation of peptide sequence obtained from proteolytic digests of the 35-kDa core deglycosylated H^+/K^+-β protein. The probe HB-5 to peptide sequence IIKMR (in one-letter code)—5′-ATHATHAARATGAAYMG-3′, where H is T or C or A, R is A or G, Y is T or C, and M is A or C, was in the sense direction, and probe HB-6 to peptide sequence YGKKAQ—5′-TGNGCYTTYTTNCCRTA-3′ where N is T or C or G or A, was in the antisense direction. All oligonucleotides were prepared by Dohn Glitz, Department of Biological Chemistry, University of California–Los Angeles.

Cloning and Sequencing. Hog and rabbit stomach >1-kilobase (kb) cDNA libraries in λ Zap II (prepared by Stratagene) were screened using degenerate 17-mer oligonucleotide probes. Hybridization to plaque lifts on nitrocellulose filters (Millipore) was done at 37°C in 6× SSPE (1× SSPE is 150 mM NaCl/5 mM NaH_2PO_4/1 mM EDTA, pH 7.4)/5× Denhardt's solution (1× Denhardt's solution is 0.02% Ficoll/0.02% polyvinylpyrrolidone/0.02% bovine serum albumin)/0.1% SDS/denatured salmon sperm DNA at 0.1 mg/ml. Washing was done at 52°C following the tetramethylammonium chloride protocol (11). Several positive clones were isolated from both libraries, and phage DNA was prepared from plate lysates (12). PCR of two hog cDNA clones was done using the degenerate oligo probes as primers. Twenty micrograms of each primer mixture was added to 60–300 ng of isolated λ phage DNA and 2.5 units of *Thermus aquaticus (Taq)* DNA polymerase (Promega). The PCR reaction conditions were 1 cycle (94°C, 5 min; 48°C, 1 min; 72°C, 2 min), 30 cycles (94°C, 1 min; 48°C, 45 sec; 72°C, 2 min), and a final extension cycle (94°C, 1 min; 48°C, 1 min; 72°C, 10 min). All PCR reactions were done in either an Eppendorf or Hybaid thermal cycler.

Sequencing was performed on double-stranded template by using the dideoxy chain-termination method (13) following the Sequenase protocol supplied by the manufacturer (United States Biochemical). Isolated cDNA inserts were subcloned into pGEM7zf(+) (Promega). Interior sequences were obtained by using synthetic oligonucleotide primers to regions of known sequence to obtain adjacent regions of unknown sequence.

Sequence Analysis. Sequence analysis was done by using either the University of Wisconsin UWGCG program (14) or GENEPRO (Riverside Scientific, Seattle).

Rescreening for 5′-End Clones. Rescreening of the rabbit stomach cDNA library with a 1.3-kb rabbit H^+/K^+-β clone (RB21-2) yielded 133 new clones. To select clones containing the 5′ end of the H^+/K^+-β cDNA, two PCR primers to the 5′ region of the 1.3-kb clone were prepared; PCR1 (5′-AGATGTTGTAGTGGATT) and PCR2 (5′-CATCTTAATGATGAAGC). These primers were used in conjunction with the T7 and T3 sequencing primers (Stratagene) (5′-AATACGACTCACTATAG and 5′-ATTAACCCTCACTAAAG, respectively), to run PCR reactions as a screening protocol. Single positive phage plugs were pulled and placed in 200 μl of SM buffer (50 mM Tris·HCl, pH 7.5/100 mM NaCl/10 mM $MgSO_4$/1 mM EDTA) and shaken a minimum of 4 hr at 4°C. One hundred nanograms of each primer was used with 10 μl of phage-containing supernatant/200 μM NTPs/1× *Taq* buffer (10 mM Tris·HCl, pH 8.4/50 mM KCl/3 mM $MgCl_2$/0.02% gelatin/0.05% Triton X-100/0.05% Tween 20)/2.5 units of *Taq* polymerase. Reaction conditions were 1 cycle (94°C, 5 min; 55°C, 2 min; 72°C, 2 min), 33 cycles (94°C, 1 min; 55°C, 1 min; 72°C, 2 min), and a final extension cycle (94°C, 1 min; 55°C, 1 min; 72°C, 10 min).

RNA Blot Hybridization. RNA was isolated following a described method (15). Ten to twenty micrograms of total RNA was run on a 1% formaldehyde-denaturing gel (12). RNA was blotted onto nitrocellulose paper (MSI) overnight by capillary action. Filters were baked 2 hr at 80°C, prehybridized in 50% (vol/vol) formamide (BRL)/5× SSPE/5× Denhardt's solution/denatured salmon sperm DNA at 10 mg/ml. Probe was added to the prehybridization solution and allowed to incubate overnight at 42°C. Washing was done by using 0.2× SSPE/0.1% SDS at 68°C.

RESULTS AND DISCUSSION

The Sequence of H^+/K^+-β. A screen of hog and rabbit gastric cDNA libraries with mixed degenerate probes to putative H^+/K^+-β peptide fragments yielded several positive clones. Isolated λ phage DNAs from two of the hog cDNA clones were used for a PCR reaction primed with the mixed oligonucleotide probes initially used for screening. A 176-base pair (bp) fragment was generated by the PCR reaction, which would be the predicted size if H^+/K^+-β were homologous to Na^+/K^+-β2 in this region. Na^+/K^+-β1 has an additional 45-bp sequence through this region, when compared with Na^+/K^+-β2. The two partial-length hog H^+/K^+-β cDNA clones (565 and 530 bp in length) were sequenced to confirm the presence of the amino acid sequences obtained from protein sequence data. Exact matches were found to the oligonucleotide probe sequences.

The PCR product generated from the hog H^+/K^+-β clone was isolated from a 1.5% agarose gel and labeled with [^{32}P]dCTP to probe rabbit stomach cDNA clones that had already been processed to the level of tertiary positives by using the degenerate oligonucleotide probes. Seven positive rabbit clones were selected from this screen, and their DNA was isolated from plate lysates. After comparison for insert size, the largest, a 1.3-kb clone (RB21-2), was subcloned into pGEM7zf(+).

RB21-2 was sequenced in both directions and shown to contain a polyadenylylation signal, a poly(A)$^+$ tail, a stop codon located to the same region as both Na^+/K^+-βs and an open reading frame coding for 278 amino acids. This sequence did not have an initiator methionine, and by comparison with the Na^+/K^+-βs was probably 30–45 bp short of the initiator ATG. To select a clone that would provide the full 5′-coding sequence of H^+/K^+-β, the rabbit cDNA library was reprobed with clone RB21-2, and 133 new positive clones were identified. Two PCR primers from near the 5′ end of clone RB21-2 were used in conjunction with the T7 and T3 primers, which are contained in the vector sequence of λ ZAP II to screen these clones rapidly for the presence of the 5′ end. These primers yielded PCR products of 300 and 600 bp with clone RB21-2 as a control template. Using plaque pure phage

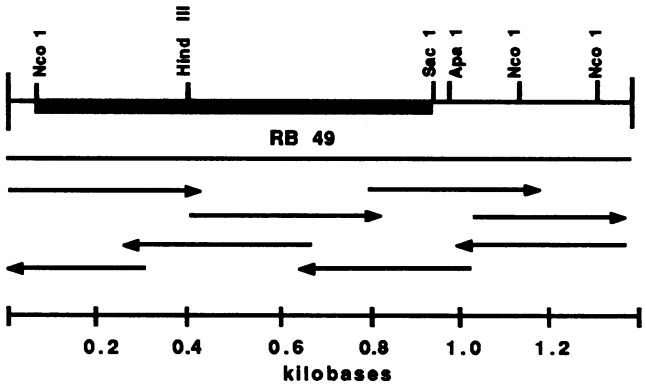

FIG. 1. Restriction mapping and sequencing strategy. The direction and length of each sequence are indicated by an arrow. The open reading frame from 54–926 is shown by the bold line on the restriction map. A scale in kb is indicated at the bottom.

plugs from positives, we found 8 of 34 clones tested yielded PCR products longer than controls. One of these clones (RB49) gave a PCR product of 700 bp with PCR2 and 400 bp with PCR1.

The 1.4-kb insert of clone RB49 was subcloned into pGEM7zf(+) and sequenced in both directions (Fig. 1). The clone extended 95 bp beyond the 5′ end of RB21-2 and was seven adenosine residues shorter at the 3′ end. Fig. 2 shows the DNA sequence from clone RB49, a total of 1367 bp, with the deduced amino acid sequence of 291 amino acids. The predicted M_r of 33,320 agrees closely with the M_r determined by SDS/PAGE (8, 9) for the deglycosylated core protein of H^+/K^+-β. The initiator ATG has a 8/9 identity with the consensus eukaryotic translational start site CCRCCATGG (16). There is a 53-bp 5′-untranslated region and a 441-bp 3′-untranslated region. Because the RNA blot (Fig. 5) indicates a mRNA size of 1.3–1.5 kb, it would appear that the clone approximates the full-length mRNA for the H^+/K^+-β. The sequencing information provides an open reading frame with a proper initiator sequence, a stop codon, and a length comparable to the Na^+/K^+-β2, its most closely related homologue.

Predicted Secondary Structure of the H^+/K^+-β. Fig. 3 provides a comparison of the H^+/K^+-β primary sequence with its Na^+/K^+-β homologues. The H^+/K^+-β is more closely related to the Na^+/K^+-β2 (44%) than β1 (31%), although several identities exist between β1 and H^+/K^+-β that are absent in β2. Surprisingly H^+/K^+-β shares a greater amino acid identity with Na^+/K^+-β2 (39%) than Na^+/K^+-β2 shares with Na^+/K^+-β1 (35%). The evolutionary significance of this is unclear because the H^+/K^+-β appears to contain coding regions from both the Na^+/K^+-βs.

The single transmembrane domain predicted by the Kyte–Doolittle algorithm is indicated by the stippled box. The seven potential glycosylation sites are boxed. It has been concluded from N-glycanase digestion that at least five of these seven sites are involved in asparagine-linked glycosylation (9). Heterogeneous glycosylation is also suggested by the presence of at least two molecular-size classes of glycoproteins centered at 60 and 85 kDa in detergent-treated

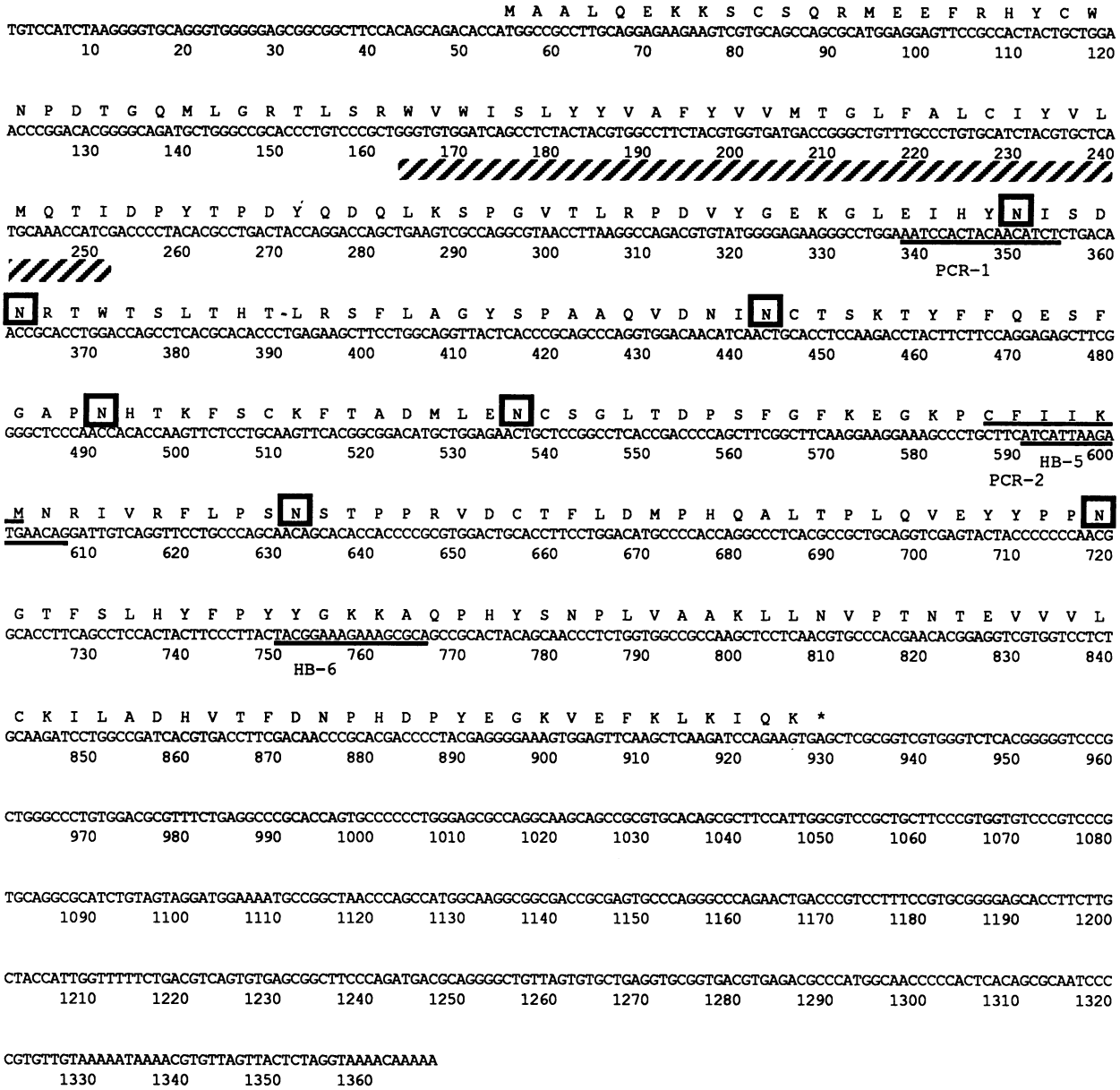

FIG. 2. DNA sequence and deduced amino acid sequence. The predicted membrane-spanning domain is indicated by the stippled bar. The potential N-linked glycosylation sites are boxed. The probes HB-5 and HB-6 and PCR primers PCR1 and PCR2 are underlined.

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Rat Na,K ß2     MVIQKEKKSC GQVVEEWKEF VWNPRTHQFM GRTGTSWAFI LLFYLVFYGF LTAMFTLTMW VMLQTVSDHT PKYQDRLATP GLMIRPK--- TENLDVIVNI SDTESWDQHV
Rabbit H,K ß    MAALQEKKSC SQRMEEFRHY CWNPDTGQML GRTLSRWVWI SLYYVAFYVV MTGLFALCIY VLMQTIDPYT PDYQDQLKSP GVTLRPDVYG EKGLEIHYNI SDNRTWTSLT
Hog Na,K ß1          MARGK AKEEGSWKKF IWNSEKKEFL GRTGGSWFKI LLFYVIFYGC LAGIFIGTIQ VMLLTISEFK PTYQDRVAPP GLTQIPQ--- SQKTEISFRP NDPQSYESYV

Rat Na,K ß2     QKLNKFLEPY NDSIQAQKND VCRPGRYYEQ P-DNGVLNY- --PKRACQFN RTQLGNCSGI GDPTHYGYST GQPCVFIKMN RVINFYAGAN QS-------- -------MNV
Rabbit H,K ß    HTLRSFLAGY SPAAQVD-NI NCTSKTYFFQ E-SFGAPNH- --TKFSCKFT ADMLENCSGL TDPS-FGFKE GKPCFIIKMN RIVRFLPSNS TP-------- -------PRV
Hog Na,K ß1     VSIVRFLEKY KDLAQKDDMI FEDCGNVPSE LKERGEYNNE RGERKVCRSK LEWLGNCSGL NDET-YGYKD GKPCVIIKLN RVLGFKPKPP KNESLETYPV MKYNPYVLPV

Rat Na,K ß2     TCVGKKDED- AENLGHFIMF PANGNID--L MYFPYYGKKF HVNYTQPLVA VKFLNVTPNV EVNVECRINA ANIATDDERD KFAARVAFKL RINKA
Rabbit H,K ß    DCTFLDMPHQ ALTPLQVEYY PPNGTFS--L HYFPYYGKKA QPHYSNPLVA AKLLNVPTNT EVVVLCKILA DHVTFDNPHD PYEGKVEFKL KIQK
Hog Na,K ß1     HCTGKRDED- KEKVGTMEYF GLGGYPGFPL QYYPYYGKLL QPKYLQPLMA VQFTNLTMDT EIRIECKAYG ENIGYSE-KD RFQGRFDVKI EVKS
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FIG. 3. Alignment of H^+/K^+-β, Na^+/K^+-β1, and Na^+/K^+-β2. Amino acid identities between the H^+/K^+-β and the other sequences are marked with a |. Basic alignments were done using University of Wisconsin Genetics Computer Group and GENEPRO software and then adjusted to achieve better alignments where possible.

vesicles labeled with [^{3}H]UDP-galactose and UDP-galactosyltransferase (8).

Cysteine residues are also well conserved between all of the β sequences. Three pairs of disulfide-linked cysteine residues have recently been shown (17) in the luminal domain of Na^+/K^+-β1. These cysteine residues are conserved in Na^+/K^+-β2 and H^+/K^+-β, suggesting a similar disulfide-determined folding pattern for all three proteins in their extracytosolic domain. Unlike the Na^+/K^+-βs, H^+/K^+-β has two cysteine residues at positions 10 and 21, which are on the cytoplasmic side and one cysteine predicted to be in the membrane-spanning domain, a feature found in β1 but not in β2. Omeprazole, a substituted benzimidazole sulfoxide, activates in the acid space of isolated gastric vesicles or secreting parietal cells to form a cationic sulfenamide that reacts only from the luminal surface of the enzyme complex (18). This highly reactive thiol reagent labels only the α subunit in both isolated vesicles and intact stomach. The lack of omeprazole reaction with the β subunit suggests that the luminal cysteines of the H^+/K^+-β are also disulfide linked.

Fig. 4 shows the hydropathy plot of H^+/K^+-β, which predicts a single transmembrane domain from residues 37–66, at the same position as the Na^+/K^+-β1 and -2 predicted transmembrane region. The remaining hydropathy pattern is generally similar to that of the Na^+/K^+-βs, although there are also distinct differences. For example, the residues just before the C terminus are more hydrophobic in the H^+/K^+-β protein, although it is probably not a membrane-spanning domain. Also the H^+/K^+-β, although hydrophilic between residues 66 and 260, is not as hydrophilic as Na^+/K^+-β1 or -2.

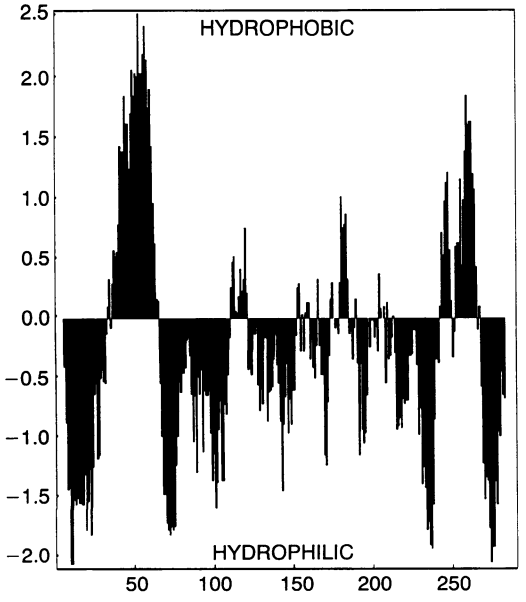

FIG. 4. Hydropathy profile of H^+/K^+-β protein. Plot was done on GENEPRO using a window of 11 residues.

Determination of the exact stoichiometry of H^+/K^+-ATPase α and -β is incomplete. Fig. 5 *a* and *b* qualitatively shows that H^+/K^+-ATPase α and β mRNA are of comparable levels, suggesting but not confirming that there may be comparable protein levels as well.

Functional Aspects of H^+/K^+-β. In contrast to the α subunits, it is difficult to see a motif in the regions of homology between the H^+/K^+- and Na^+/K^+-βs. When regions of H^+/K^+-β are compared with Na^+/K^+-β1 (using the H^+/K^+-β sequence for numbering) there appear to be four regions of greater homology. These are as follows: a portion of the hydrophobic domain, the initial extracytosolic sequence (positions 71–86), a region from 159 to 191 where 18 of 27 amino acids are identical and a region from position 225 to 247 where 14 of 23 amino acids are identical. Com-

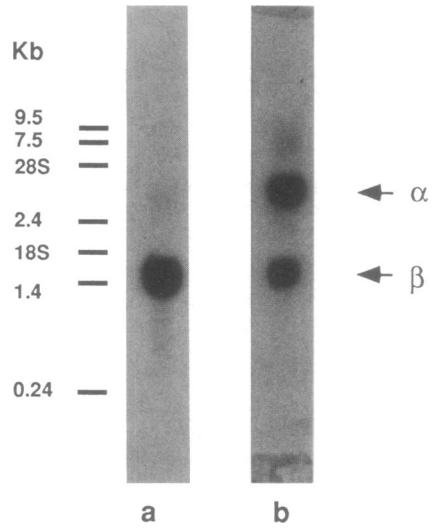

FIG. 5. Northern (RNA) blot analysis of rabbit stomach RNA. (Lane a) Ten micrograms of total rabbit stomach RNA was run on an 0.8% formaldehyde denaturing gel and blotted onto nitrocellulose. The filter was probed with H^+/K^+-β clone RB21-2 (1.5×10^6 cpm/ml) and washed at 68°C in 0.2× SSPE/0.1% SDS and exposed overnight at −70°C. The positions of the 28S and 18S RNAs, as well as RNA size markers, are indicated. (Lane b) The same filter used above was reprobed with a 3.3-kb rabbit H^+/K^+-ATPase (0.8×10^6 cpm/ml) without stripping the filter and processed the same as above.

parison between H^+/K^+-β and Na^+/K^+-$\beta 2$ shows higher than average homology towards the N and C termini, immediately after the membrane-spanning sequence on the extracytosolic side (70–86), and in two regions from positions 159 to 189 (17 of 31 amino acids identical) and from positions 227 to 268 (25 of 41 amino acids identical). From these comparisons, a common theme for retention of sequence appears to reside in the immediate extracytosolic domain and in two regions centered around amino acids 180 and 245. Whether these regions have functional or structural significance remains to be tested.

The functional relationship of the H^+/K^+-β to the H^+/K^+-ATPase catalytic subunit is unknown. Initial experiments, expressing the α subunit of the H^+/K^+-ATPase in the plasma membrane of MDCK cells, were not able to demonstrate function (19). Expression of H^+/K^+-ATPase α subunit with and without H^+/K^+-β in transformed cells may provide the answer to this question and whether the Na^+/K^+-ATPase β subunits could act as surrogates for H^+/K^+-ATPase transport and catalytic function.

Because the Na^+/K^+ and H^+/K^+-ATPases are the only obligate antiporters of the phosphoenzyme type pumps, perhaps one role for the β subunit involves the generation of an extracellular K^+-binding domain. The plasma membrane location of these pumps is shared by the calmodulin-sensitive Ca^{2+} ATPases, which have no β subunits; thus, it does not seem likely that the heavily glycosylated β subunit of the phosphoenzyme countertransport class is necessary for plasma membrane targeting. In studies where Na^+/K^+-ATPase was expressed in the presence of glycosylation inhibitors, it appears that the catalytic subunit was synthesized but did not leave the endoplasmic reticulum (20). Initial studies on expression of the catalytic subunit of the H^+/K^+-ATPase have shown that the ATPase is able to reach the apical plasma membrane of MDCK cells in the absence of expression of H^+/K^+-β (19). Targeting to the apical membrane would seem to reside within the catalytic sequence of the H^+/K^+-ATPase; however, targeting to the plasma membrane could imply the surrogate use of Na^+ pump β subunit.

The antigens of sera of pernicious anemia and autoimmune gastritis patients, who produce autoantibodies specific to parietal cell-surface proteins, have been recently debated in the literature. Several reports have identified the H^+/K^+-ATPase as the major antigen recognized by these autoimmune antibodies from pernicious anemia and atrophic gastritis patients (21, 22). One group has demonstrated that some of their sera inhibit H^+/K^+-ATPase activity (22). Other researchers characterized pernicious anemia sera that recognize an antigen that appears to be H^+/K^+-β (23). In this work, Goldkorn *et al.* (23) have shown a prominent signal on Western (immunologic) blots over the range of 60 to 90 kDa. N-Glycanase treatment of the immunoprecipitated and isolated ^{125}I-labeled 60- to 90-kDa protein produced a deglycosylated protein migrating at 34 kDa on SDS/PAGE, just as has been seen for the H^+/K^+-β. The identification of the H^+/K^+-β subunit as a major antigen recognized by sera from pernicious anemia and atrophic gastritis patients may help facilitate the understanding, early diagnosis, and treatment of these diseases.

Note. Gary E. Shull (University of Cincinnati) has sent us a preprint on his work involving the cDNA cloning of a rat gastric H^+/K^+-β. Comparison between the rabbit and rat amino acid sequences showed 82% identity.

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