cDNA Cloning of the $\beta$-Subunit of the Rat Gastric H,K-ATPase*

(Received for publication, April 23, 1990)

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A cDNA encoding the $\beta$-subunit of the rat gastric H,K-ATPase has been identified using oligonucleotide probes based on the amino acid sequences of two peptides from the pig H,K-ATPase $\beta$-subunit (Hall, K., Perez, G., Anderson, D., Gutierrez, C., Munson, K., Hersey, S. J., Kaplan, J. H., and Sachs, G. (1990) Biochemistry 29, 701-706). The nucleotide sequence of the 1.3-kilobase cDNA has been determined and the primary structure of the protein deduced. The protein consists of 294 amino acids and has an $M_r$ of 33,625. The amino acid sequence of the H,K-ATPase $\beta$-subunit is similar to those of the $\beta_1$ (29% identity) and $\beta_2$ (37% identity) subunits of the Na,K-ATPase. Based on the hydrophy profile it seems to have the same transmembrane organization as the Na,K-ATPase $\beta$-subunit, with a single membrane-spanning domain near the amino terminus. Seven potential N-linked glycosylation sites are located in the putative extracellular regions of the protein. Northern blot analyses of poly(A)$^+$ RNAs from 13 tissues demonstrate that the H,K-ATPase $\beta$-subunit mRNA is expressed at high level in stomach and is not expressed in any of the other tissues.

Secretion of acid into the stomach is mediated by the H,K-ATPase of the gastric mucosa, an ATP-dependent pump that catalyzes the exchange of H$^+$ and K$^+$. Characterization of cDNAs for the catalytic component of the enzyme ($M_r = 114,000$) from rat (1) and pig (2) has shown that it is closely related to the $\alpha$-subunit of the Na,K-ATPase. In addition to the $\alpha$-subunit, the Na,K-ATPase contains a second subunit ($\beta$). Two isoforms of the $\beta$-subunit, $\beta 1$ and $\beta 2$, have been identified and cloned (3-6). Both are glycoproteins containing N-linked carbohydrates and have a protein $M_r$ of approximately 35,000. Although the function of the $\beta$-subunit is unknown, it is essential for enzyme activity (7, 8).

Until recently there was little data supporting the existence of a second subunit for the H,K-ATPase. However, there is now compelling evidence from several laboratories that the H,K-ATPase contains an additional subunit that is related to the $\beta$-subunit of the Na,K-ATPase. Using lectin affinity column chromatography and immunoprecipitation procedures, Okamoto et al. (9) demonstrated that the catalytic component of the enzyme is tightly associated with a glycoprotein of 60-80 kDa. Removal of carbohydrates by treatment with endoglycosidase F resulted in a core protein of 32 kDa, which is approximately the same size as that of the Na,K-ATPase $\beta$-subunit (3, 6). Also, like the Na,K-ATPase $\beta$-subunit, it was shown to be an integral membrane protein and to contain a number of N-linked carbohydrates. This protein was also identified in a separate study, and the amino acid sequences of three proteolytic fragments were determined (10) and shown to be similar to sequences of the $\beta_1$- and $\beta_2$-subunits of the Na,K-ATPase.

The objective of the present study was to isolate and characterize a cDNA for the H,K-ATPase $\beta$-subunit. Oligonucleotide hybridization probes based on amino acid sequences from the peptide fragments reported by Hall et al. (10) were used to identify the corresponding cDNA in a rat stomach cDNA library. The cDNA encodes a 294-amino acid protein that exhibits substantial similarity in its amino acid sequence and general structural organization to the $\beta$-subunit of the Na,K-ATPase. In addition, RNA hybridization analysis of a number of adult rat tissues suggests that expression of the mRNA encoding the H,K-ATPase $\beta$-subunit is restricted to stomach.

EXPERIMENTAL PROCEDURES

Screening of Rat Stomach cDNA Library and Analysis of cDNA—The 75,000 colony rat stomach cDNA library used in this study has been described previously (11). It consisted of size-selected cDNA (1.0-3.2 kb) that was tailed with deoxycytidine and annealed to G-tailed pBR322 plasmid vector. Replica filters were prehybridized overnight at 55°C in 6X SSC, 1X Denhardt's solution, 0.1% SDS, and 100 ng of denatured salmon sperm DNA/ml (see Ref. 12 for composition of SSC and Denhardt's solution) and then hybridized for 22 h at 40°C in the same solution containing two oligonucleotides (1 ng/ml each) that had been 5'-end-labeled using T4 polynucleotide kinase and [gamma-32P]ATP. The oligonucleotides were based on amino acid sequences from peptides B and C of the $\beta$-subunit of the pig H,K-ATPase reported by Hall et al (10). They consisted of preferred anticodons (13) and contained deoxynucleosine in some positions of degeneracy (14). Oligonucleotide 1 (5' TAITGIGGCTGIGCCTTCT- TICCITAIAGGIAITATG 3') was derived from the sequence HYPPYGKQAQPHY of peptide B and oligonucleotide 2 (5' A CTTACATTTTACATTTTATTTA 3') was derived from the sequence FIJKMNHVBF of peptide C. The filters were washed two times for 15 min each in 3X SSC, 0.1% SDS at room temperature and then two times for 60 min each in 1X SSC, 0.1% SDS at 40°C. Following autoradiography, colonies that gave hybridization signals on both replicas were purified. DNA sequence analysis was performed by the chemical cleavage procedure (15). Computer analyses of the nucleotide sequences were performed using the DNANALYZE program (16), and hydrophy analysis was performed using the procedure of Kyte and Doolittle (17).

Northern Blot Hybridization Analysis—Poly(A)$^+$ RNA was isolated from various tissues of adult CD rats, and Northern blots were prepared exactly as described by Hall et al (11). They were hybridized in 20 ml of hybridization fluid with 50 ng of a BstEI-BstEI restriction fragment (nucleotides 255-1247) from the H,K-ATPase $\beta$-subunit cDNA. Solutions, temperatures, and times used for prehybridization, hybridization, and washing of membranes were exactly as described previously (11).

I. The abbreviations used are: SDS, sodium dodecyl sulfate; kb, kilobase(s); $\beta$-HKA, H,K-ATPase $\beta$-subunit; $\beta_1$-NKA, Na,K-ATPase $\beta_1$-subunit; $\beta_2$-NKA, Na,K-ATPase $\beta_2$-subunit.
RESULTS

Isolation and Characterization of H,K-ATPase β-Subunit cDNAs—A rat stomach cDNA library was screened simultaneously with two oligonucleotide hybridization probes based on amino acid sequences of peptides B and C of the β-subunit of the pig H,K-ATPase (10). Twelve of the sixteen colonies identified were purified, and their plasmid DNAs were analyzed by agarose gel electrophoresis after digestion with the restriction endonuclease PstI. Eleven of the clones contained a 1.35-kb insert and exhibited the same restriction pattern. The remaining clone appeared to be a partial cDNA of the same class. Later analysis indicated that this clone was missing part of the 3' end. The nucleotide sequence of clone RS25-1 was determined according to the sequencing strategy illustrated in Fig. 1. The sequence of a second cDNA, RS25-3, was determined in one strand.

The nucleotide sequence of the putative H,K-ATPase β-subunit cDNA and its deduced amino acid sequence are shown in Fig. 2. The cDNA is 1313 nucleotides in length and is followed by a poly(A) tail of over 50 nucleotides. It contains a 294-codon open reading frame, beginning with an ATG triplet at nucleotide 12, and encodes a protein with an Mr of 33,625. Although an in-frame stop codon is not present in the region 5' to the putative translation start site, this ATG triplet coincides precisely with the location of the initiating methionine codon of β2-NKA (see Fig. 3). The two cDNAs analyzed started and ended at the same positions and were identical except for a shorter poly(A) tail in clone RS25-3 and nine nucleotide substitutions (see Fig. 2 legend). Only one of these substitutions resulted in an amino acid change (His→Tyr). Amino acids 380-499 were identical to the sequence reported for peptide B of the pig β-HKA (10), and amino acids 176-190 contained a 12 out of 13 match with the residues determined for peptide C of the pig protein. Amino acids 220-229 were identical to peptide A at 5 out of 10 positions, and of the 4 nonsynonymous residues were conservative changes. Based on these data, it seems that the cDNA encodes the β-subunit of the H,K-ATPase. As discussed below the high degree of similarity to the Na,K-ATPase β-subunit and the mRNA tissue distribution study lends further support to the conclusion that the cDNA encodes the H,K-ATPase β-subunit.

Comparison of H,K-ATPase β-Subunit with the β1- and β2-Subunits of the Na,K-ATPase—A comparison of the amino acid sequences of the β1- and β2-subunits of the Na,K-ATPase and β-HKA is shown in Fig. 3, and a comparison of their hydropathy profiles is shown in Fig. 4. β-HKA exhibits 29% amino acid identity to β1-NKA and 37% identity to β2-NKA. All three proteins have a similar hydropathy profile with a single major hydrophobic domain near the NH2 terminus which is known to traverse the membrane in β1-NKA. Interestingly, β-HKA seems to be more closely related to the β2-subunit than the β1- and β2-subunits are to each other. The initiating methionine residue of β-HKA matches that of the β2-subunit, and both proteins have a similar 39-amino acid NH2-terminal sequence (44% identity) preceding the major hydrophobic domain (residues 40-63). β-HKA has seven potential N-linked glycosylation sites (underlined in Fig. 3), and several of these sites are in similar positions to the potential glycosylation sites of the β1- and/or β2-subunits. β-HKA has 9 cysteine residues, 5 of which are conserved in all three proteins (boxed in Fig. 3). A sixth cysteine (residue 201) is in close proximity to an additional cysteine that is conserved in β1- and β2-NKA.

Tissue Distribution of the H,K-ATPase β-Subunit mRNA—Poly(A)+ RNA from adult rat uterus, skeletal muscle, heart, brain, lung, liver, kidney, spleen, testis, stomach, small intestine, large intestine, and pancreas were analyzed by Northern blot hybridization in order to determine the tissue distribution of the β-HKA mRNA (Fig. 4). Following a 1-day autoradiographic exposure a strong signal was observed in stomach that corresponded to a 1.6-kb mRNA, the expected size of the β-HKA mRNA with its poly(A) tail. After a 1-day autoradiographic exposure additional RNA species were observed in

**Fig. 1.** Restriction map and sequencing strategy for the rat stomach H,K-ATPase β-subunit cDNA clone RS25-1. Dark areas represent the untruncated sequences, and the open area represents the open reading frame. Restriction sites relevant to the sequencing strategy are shown. The direction and extent of sequencing are indicated by arrows (lower arrows, coding strand; upper arrows, noncoding strand).

**Fig. 2.** Nucleotide sequence of rat stomach cDNA RS25-1 and deduced amino acid sequence of the H,K-ATPase β-subunit. Nucleotides are numbered at the right of the sequence; amino acids are numbered below the sequence. The sequence of a second cDNA, RS25-3, was determined in one strand and found to contain the following nucleotide differences: 212, T; 417, T; 902, A; 1118, C; 1212, T; 1271, C.
FIG. 3. Amino acid similarity comparison. The deduced amino acid sequences of the H,K-ATPase \( \beta \)-subunit are compared with those of the rat Na,K-ATPase \( \beta_1 \) (18) and \( \beta_2 \) (4) subunits. Amino acids are numbered at the right. Gaps indicated by dashes were introduced to maintain the alignment. Dots above amino acids of \( \beta_1 \) and \( \beta_2 \) indicate identity with the corresponding residue of \( \beta \)-HKA. The potential transmembrane domain is indicated by a heavy bar. Potential N-linked glycosylation sites, Asn-X-Ser/Thr (19), are underlined, and cysteine residues that are conserved in all three \( \beta \)-subunits are boxed.

FIG. 4. Hydropathy profiles of the H,K-ATPase \( \beta \)-subunit and the \( \beta_1 \) - and \( \beta_2 \)-subunits of the Na,K-ATPase. Hydropathy profiles of rat HKA, rat \( \beta_1 \)-NKA (18), and rat \( \beta_2 \)-NKA (4) were determined according to the procedure of Kyte and Doolittle (17) using a window of 11 amino acids.

stomach that may be processing intermediates of the \( \beta \)-HKA mRNA. The \( \beta \)-HKA mRNA was not detected in any other tissue. A faint hybridization signal at 1.4 kb was detected in uterus after a 3-day exposure (data not shown). However, this mRNA was not detected when the same blot was hybridized with a probe that included only the first 138 codons (data not shown). Thus, it is unlikely to represent a related mRNA.

DISCUSSION

Based on the results of two recent studies (9, 10) it is apparent that the gastric H,K-ATPase contains, in addition to a catalytic subunit that is homologous to the \( \alpha \)-subunit of the Na,K-ATPase, a glycoprotein subunit that is homologous to the \( \beta \)-subunit of the Na,K-ATPase. In order to determine the complete primary structure of the rat gastric H,K-ATPase \( \beta \)-subunit, a cDNA cloning procedure was employed that utilized oligonucleotide hybridization probes based on the amino acid sequences of pig HKA determined by Hall et al. (10). This allowed the isolation of cDNAs encoding a protein that included a 20-amino acid sequence that was identical to peptide A of the pig protein (10). Of the 43 amino acids determined for three peptide fragments of pig HKA, there are only six substitutions in the rat protein. Five of these are conservative changes suggesting that they represent species differences in the same protein. This strongly indicates that the protein encoded by the cDNA is rat HKA. Analyses of the deduced amino acid sequence provided confirmation that the cDNAs identified encode a protein that is homologous to the Na,K-ATPase \( \beta_1 \) - and \( \beta_2 \)-subunits. By analogy to \( \beta_1 \)-NKA it appears that the stomach \( \beta \)-subunit homolog has a short amino-terminal cytoplasmic sequence followed by a single transmembrane domain and an extensive extracellular region (see Refs. 20 and 21 for reviews on the structural organization of \( \beta \)-NKA). Seven potential sites for N-linked glycosylation (19) are present in the putative extracellular region of the stomach protein. Two of these sites, Asn-161 and Asn-193, correspond closely to two of the three N-linked glycosylation sites of \( \beta_1 \)-NKA (22). It is likely that most of these potential glycosylation sites are used since Okamoto et al. (9) have reported evidence for at least five N-linked carbohydrates in HKA. \( \beta_1 \)-NKA has been shown to contain three disulfide bonds, Cys\(^{105} \)-Cys\(^{147} \), Cys\(^{158} \)-Cys\(^{171} \), and Cys\(^{212} \)-Cys\(^{225} \) (23, 24). All six cysteine residues (boxed in Fig. 3) seem to be conserved between \( \beta_1 \)-NKA and \( \beta_2 \)-NKA. Five of these cysteines are conserved in the stomach \( \beta \)-subunit homolog, and a sixth cysteine, residue 201, is shifted by only a few positions from Cys\(^{212} \) of \( \beta_1 \)-NKA, suggesting that the protein contains three disulfide linkages that are similar to those of \( \beta_1 \)-NKA.

It was of interest to determine whether the mRNA for \( \beta \)-HKA is expressed only in stomach or has a broader tissue distribution. Because of the similarity of \( \beta \)-HKA to the \( \beta \)-NKA isoforms, it is conceivable that it could serve as a \( \beta \)-subunit for the Na,K-ATPase. It is also possible that it could serve as a \( \beta \)-subunit for isoforms of the H,K-ATPase expressed in other tissues. For example, a H,K-ATPase has been identified in distal colon (25), and there is immunological evidence for a \( \beta \)-subunit-like protein in the apical membrane of distal colon epithelial cells (26). Northern blot analysis (Fig. 5) of mRNA from a number of adult rat tissues demonstrated that the \( \beta \)-HKA mRNA is expressed at high levels in stomach and is not detected in any of the other tissues examined, even after longer exposure times than those shown in Fig. 5. These results support the conclusion that the \( \beta \)-subunit homolog identified in this study is a subunit of the gastric H,K-ATPase and suggest that it does not serve as a \( \beta \)-subunit for the Na,K-ATPase or for the distal colon H,K ATPose.

Acknowledgments—I am grateful to George Sachs and Michael Reuben for sharing the results of their work on the cloning and

\(^2\) During the preparation of this manuscript, M. Reuben, L. Lasater, and G. Sachs (personal communication) were preparing a manuscript describing similar work on a cDNA encoding the rabbit H,K-ATPase \( \beta \)-subunit. In a comparison between their rabbit sequence and the rat sequence reported here, they determined that the two enzymes are 82% identical and aligned perfectly except for a three-amino acid deletion (amino acids 200-203) in the rabbit sequence that allows a closer alignment of Cys\(^{210} \) of \( \beta \)-HKA with Cys\(^{110} \) of \( \beta_1 \)-NKA.
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characterization of a rabbit H,K-ATPase β-subunit cDNA prior to publication. I also thank Greg Wernke for help with the computer analyses, Mike Hughes for synthesis of the oligonucleotides, Jeannette Greeb for technical assistance, and Jennifer Schroeder for typing the manuscript.

REFERENCES

FIG. 5. Tissue distribution of the H,K-ATPase β-subunit mRNA. Poly(A)+ RNA from adult rat tissues was analyzed by Northern blot hybridization as described under "Experimental Procedures." Upper panel, 1-h autoradiographic exposure time; bottom panel, 24-h exposure. The positions and sizes (in kb) of the RNA markers are shown on the right.