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cDNA CLONING OF THE ß-SUBUNIT OF THE HUMAN GASTRIC H,K-ATPase[1]

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SUMMARY: A full-length cDNA clone encoding the human gastric H,K-ATPase (EC 3. 6. 1. 36) ß-subunit was isolated from a human gastric mucosal λgt10 library using oligonucleotide probes which were based on the cDNA sequence from rat and rabbit H,K-ATPase ß-subunits. The insert was 1407 bp in length and encoded a polypeptide of 291 amino acids with a MW = 33,367 Da. It exhibited 84.2%, 85.6% and 81.3% identity to the H,K-ATPase ß-subunits of rabbit, pig and rat, respectively.

The gastric H,K-ATPase (EC 3. 6. 1. 36) is an ATP-dependent proton pump and a member of the cation-transporting ATPase family (1). The primary structure of the α-subunit of this enzyme is highly homologous with other cation-transporting ATPases, e.g. the Na,K-ATPase (63%). In addition to the α-subunit, both of these enzymes also contain a ß-subunit. The ß-subunit of the H,K-ATPase has been shown to be a 60-80 kDa glycoprotein with a 35 kDa protein core. The ß-subunit is tightly associated with the α-subunit as shown by lectin affinity chromatography, immunoprecipitation and immunogold electron microscopy (2-6). Both the α- and ß-subunits of the H,K-ATPase have been reported as major autoantigens in autoimmune gastritis associated with pernicious anaemia (4,7,8). The nucleotide sequences of the α-subunit of several species, including human have been reported (9), as well as the sequences

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from rat, rabbit and pig ß-subunits (10,11,6), but not from human. The present investigation describes the cDNA sequence of the ß-subunit of the human H,K-ATPase.

MATERIALS AND METHODS

Construction of cDNA library Human gastric mucosal cells were prepared by consecutive digestions with pronase and collagenase (12) of resection specimens of human stomachs obtained from the University Hospital of Uppsala (approved by the Ethical Committee of the Medical Faculty, University of Uppsala). Total RNA was isolated from these cells by acid guanidinium thiocyanate phenol chloroform extraction (13). Polyadenylated RNA was purified by oligo(dT) cellulose chromatography. The cDNA was synthesized using a cDNA synthesis kit from Pharmacia LKB (Uppsala, Sweden). The frist strand of cDNA was synthesized from 14 µg of polyadenylated RNA using oligo $d(T)_{12-18}$ primer and Moloney Murine Leukemia Virus (MMLV) reverse transcriptase. Synthesis of the second strand involved a modification of the procedure of Gubler and Hoffman (14), in which RNase H nicked the RNA strand of the RNA:cDNA duplex, and DNA polymerase I used these nicks to replace RNA with DNA by nick translation. Klenow fragment was added to ensure that the ends of the cDNA were blunt. To prepare the blunt-ended cDNA for insertion into the *Eco*R I site of the λgt10 vector, an *Eco*R I / *Not* I adaptor was ligated to each end of the double stranded cDNA, and then ligated to λgt10. The ligation mixture was packaged in vitro according to the procedure of Packagene from Promega (Madison, U.S.A.).

Screening of cDNA library To select clones containing H,K-ATPase ß-subunit cDNA, the cDNA library was screened at a density of approximately 1300 plaques on 9 cm diameter plates. The plaques were transferred to nitrocellulose filters followed by denaturation of the DNA in alkaline solution (0.5 M NaOH, 1.5 M NaCl) for 1 min, neutralization in 1.5 M NaCl, 1 M Tris-HCl buffer, pH 7.5 for 5 min, and washing in 2 X SSC (1 X SSC = 0.15 M NaCl in 0.015 M sodium citrate buffer, pH 7.0) for 5 min. Duplicate filters were prehybridized in 6 X SSC, 5 X Denhardt's solution (1 X Denhardt's solution = 0.02% bovine serum albumin, 0.02% Ficoll and 0.02% polyvinylpyrrolidone) and 50 µg of denatured salmon sperm DNA/ml at 58°C for 2 h and then hybridized in the same solution containing two oligonucleotide probes (1ng/ml each) that had been 5'-end-labeled using T4 polynucleotide kinase and [γ-^{32}P]ATP at 58°C overnight. The probes, in the antisense direction, were 5' CATGCGCTGGCTGCAT GACTTCTTCTCCTGCA 3' and 5' CATGCGCTGGCTGCACGACTTCTTCTCCTGCA 3'. They were based on published nucleotide sequences at the amino terminal region of the ß-subunit of H,K-ATPase from rat (10) and rabbit (11), respectively. After hybridization, the filters were washed twice in 6 X SSC and 0.1% SDS for 10 min at room temperature and then washed in the same solution for 1 min at 58°C. Following autoradiography, the plaques which were positive on both replicas were picked up, plated out at a density of approximately 100 plaques and rescreened to ensure that only single, positive plaques were selected. Phages were prepared by the liquid culture method and the DNA was extracted by standard methods (15). The full length inserts obtained after digestion of positive clones with *Not* I were checked by electrophoresis in 1% agarose gel and subcloned into the phagemid pBluescript II SK+ for sequence analysis.

Nucleotide sequence determination In order to determine the full length sequence of the ß-subunit, unidirectional deletions were generated with Erase-a-Base system from Promega (Madison, U.S.A.). The recombinant pBluescript II SK+ was doubly digested with *Xba* I and *Kpn* I to leave 5'-overhang and 3'-overhang, respectively, between the end of the insert to be deleted and the sequencing primer binding site. Exonuclease III (Exo III) was used to

specifically digest DNA from the 5′-overhang end into the insert at 25°C. Samples were removed at 1 min intervals to 14 eppendorf tubes containing S1 nuclease, which removed the single-stranded tails remaining after Exo III digestion. After neutralization and heat inactivation of the S1 nuclease, Klenow DNA polymerase was added to make the ends flush. The ends were then ligated to recircularize the deleted molecules. The ligation mixtures were used directly to transform competent cells DH 5™ from BRL (Gaithersburg, MD, U.S.A.). A number of subclones from each time point were then screened to select for appropriate intervals between deletions. The nucleotide sequence was determined and verified on both strands in pBluescript II SK+, using the dideoxy-chain-termination reaction with T7 primer and T7 DNA polymerase (16).

Sequence analysis Sequence analysis was done using the Genetis Computer Group (GCG) program and hydropathy analysis was performed according to the procedure of the Kyte and Doolittle (17) using an interval of 7 amino acids.

Northern blot analysis Total RNA (10 μg) from human gastric mucosal cells and from human thyroid tissue was separated in a 1% agarose gel containing 2.2 M formaldehyde (15) and transferred onto Pall Biodyne A Transfer membrane (Biosupport, England) overnight. The membrane was baked at 80°C for 2 h and fixed by UV irradiation. Prehybridization was performed in 10 ml of prehybridization solution, which contained 10% dextran sulphate sodium salt, 0.5 M NaCl, 50 mM Na-phosphate, pH 6.5, 5 X Dehnhardt's solution, 40% formamide, 1% SDS and 100 μg/ml single stranded salmon sperm DNA for 2 h at 42°C. The probe was added to the prehybridization solution and allowed to incubate overnight at 42°C. Purified full length ß-subunit of human H,K-ATPase was labeled by random priming (18). Autoradiography on X-ray film was performed overnight at -70°C.

RESULTS AND DISCUSSION

Isolation and characterization of human H,K-ATPase ß-subunit cDNA clones A human gastric mucosal cell cDNA library was screened simultaneously with two oligonucleotide hybridization probes based on the published H,K-ATPase of rat (10) and rabbit (11) ß-subunit cDNA sequences. From 6000 screened clones, one positive clone was identified and subcloned. The λgt10 phage DNA was extracted and digested with the restriction endonucleoase *Not* I. This clone contained a 1.4-kb insert as judged after electrophoresis in a 1% agarose gel. The 1.4-kb insert clone was subcloned into phagemid (pBluescript II SK+) for sequencing. The nucleotide sequence of the H,K-ATPase ß-subunit cDNA and its deduced amino acid sequence are shown in Fig. 1. The full-length cDNA sequence was 1407 nucleotides in length which ended by a poly(A)+ tail of 10 nucleotides; the poly(A)+ signal, ATTAAA, appeared 24 bp upstream from the poly(A)+ tail. The cDNA comprised a 17-bp 5′-untranslated region, a 873-bp coding region (ATG triplet at nucleotides 18-20) followed by a 517-bp untranslated region. The open reading frame encoded a protein of 33,367 Da comprising 291 amino acids. The molecular weight is in good agreement with that of the core protein after N-Glycanase treatment of the native ß-subunit (35,000 Da) (2-5). Fig. 2 shows the hydropathy plot of human H,K-ATPase ß-subunit, which predicts a single transmembrane domain from residues 38 to 66.

```
                M  A  A  L  Q  E  K  K  T  C  G  Q  R  M  E 15
ATCTCAGGCCAGGGACGATGGCGGCTCTGCAGGAGAAGAAGACGTGTGGCCAGCGCATGG 60

  E  F  Q  R  Y  C  W  N  P  D  T  G  Q  M  L  G  R  T  L  S 35
AGGAGTTCCAGCGTTACTGCTGGAACCCGGACACGGGGCAGATGCTGGGCCGCACCCTGT 120

  R  W  V  W  I  S  L  Y  Y  V  A  F  Y  V  V  M  T  G  L  F 55
CCCGGTGGGTGTGGATCAGCCTGTACTACGTGGCCTTCTACGTGGTGATGACTGGGCTCT 180

  A  L  C  L  Y  V  L  M  Q  T  V  D  P  Y  T  P  D  Y  Q  D 75
TCGCCCTGTGCCTCTATGTGCTGATGCAGACAGTGGACCCGTACACACCGGACTACCAAG 240

  Q  L  R  S  P  G  V  T  L  R  P  D  V  Y  G  E  K  G  L  E 95
ACCAGCTACGGTCACCAGGGGTAACCTTAAGGCCGGATGTTTACGGGGAGAAAGGCCTGG 300

  I  V  Y  N  V  S  D  N  R  T  W  A  D  L  T  Q  T  L  H  A 115
AAATTGTCTACAACGTCTCTGATAACAGAACCTGGGCAGACCTCACACAGACTCTCCACG 360

  F  L  A  G  Y  S  P  A  A  Q  E  D  S  I  N  C  T  S  E  Q 135
CCTTCCTAGCAGGCTACTCTCCAGCAGCCCAGGAGGACAGCATCAACTGCACCTCCGAGC 420

  Y  F  F  Q  E  S  F  R  A  P  N  H  T  K  F  S  C  K  F  T 155
AGTACTTCTTCCAGGAGAGTTTCCGCGCTCCCAACCACACCAAGTTCTCCTGCAAGTTCA 480

  A  D  M  L  Q  N  C  S  G  L  A  D  P  N  F  G  F  E  E  G 175
CGGCAGATATGCTGCAGAACTGCTCAGGCCTGGCGGATCCCAACTTCGGCTTTGAAGAAG 540

  K  P  C  F  I  I  K  M  N  R  I  V  K  F  L  P  S  N  G  S 195
GAAAGCCATGTTTTATTATTAAAATGAACAGGATCGTCAAGTTCCTCCCCAGCAACGGCT 600

  A  P  R  V  D  C  A  F  L  D  Q  P  R  E  L  G  Q  P  L  Q 215
CGGCCCCCAGAGTGGACTGCGCCTTCCTGGACCAGCCCCGCGAGCTCGGCCAGCCGCTGC 660

  V  K  Y  Y  P  P  N  G  T  F  S  L  H  Y  F  P  Y  Y  G  K 235
AGGTCAAGTACTACCCTCCCAACGGCACCTTCAGTCTGCACTACTTCCCTTATTACGGGA 720

  K  A  Q  P  H  Y  S  N  P  L  V  A  A  K  L  L  N  I  P  R 255
AGAAAGCCCAGCCCCACTACAGCAACCCCCTGGTGGCAGCGAAGCTCCTCAACATCCCCA 780

  N  A  E  V  A  I  V  C  K  V  M  A  E  H  V  T  F  N  N  P 275
GGAACGCTGAGGTCGCCATCGTGTGCAAGGTCATGGCAGAGCACGTGACCTTCAACAATC 840

  H  D  P  Y  E  G  K  V  E  F  K  L  K  I  E  K             291
CCCACGACCCGTATGAAGGGAAAGTGGAGTTCAAACTCAAGATTGAGAAGTGAAACGTTT 900

GCGCAGGGGTCCTGGGCACGCCTGCGGGGTCGCTCAAGGACACCCTCCTGGTTGGGCTTA 960
CCTTGCCCGTCAGTTCCCTGCCAAATCATCCCCAAAGTGGTTTGGAGCAACGGTGTTGTC 1020
AGTGTGCGAACTCCAGAGAAGCGCCCACATCTGAAGGACCTGCTCGCGAGTATCAGTTCT 1080
TCCTTGTTGAATTCTTACAGTTTTTAGATGGAATTTGCTGCTATAAGAATGTCCAGCTAC 1140
CATGGGAACGCAAGGCAGCAACTCTCTAATTAACCAGGTCATAAAAACGATTCGTCTTCT 1200
ATGTAGACATCACTTTCTTACTATAATTTATTTTTCTACACTTCAATATGAACTGCCCCC 1260
CCCACATTAATATAAAAACTACTAATGCACTGATATGAAACACGGCTTACACTAATGACA 1320
TTCTGAATTCTTGCTTTTAAAATTGCAATTCCTAAGTTGTAAACATAAAATATATTAAAG 1380
TTACTCTTATTGTATGTAAAAAAAAAA                                   1407
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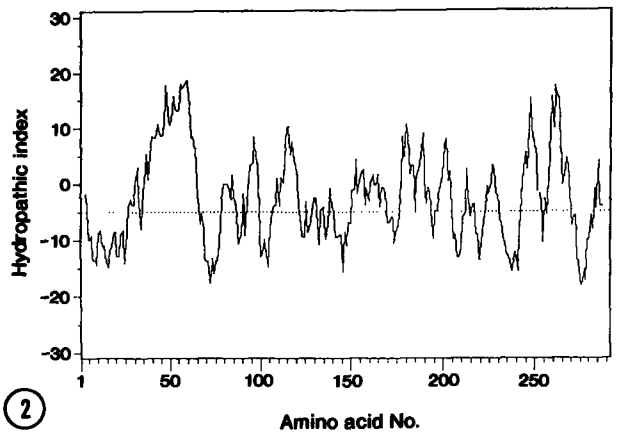
Fig. 1. cDNA nucleotide sequence and deduced amino acid sequence of human H,K-ATPase ß-subunit. Nucleotides and amino acids are numbered at the right of the sequences. The poly(A)+ signal, ATTAAA, is underlined. The transmemberane domain is also underlined (amino acid residues 38 to 66). Seven potential N-linked glycosylation sites are indicated in bold.

Northern blot analysis The 1.4-kb *Not* I fragment of human H,K-ATPase ß-subunit cDNA was used as a hybridization probe in Northern blot analysis to characterize the mRNA transcripts in human gastric mucosa (Fig. 3). Ten micrograms per lane of total RNA from



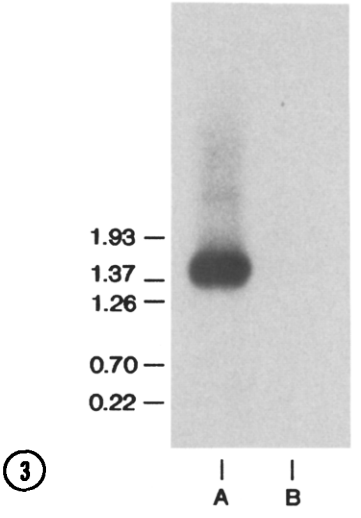


Fig. 2. Hydropathy profiles of the human H,K-ATPase ß-subunit.

Fig. 3. Northern blot analysis of total RNA from human gastric mucosa (lane A) and human thyroid (lane B). A total of 10 µg of RNA was analyzed in a denaturing agarose gel; the probe was labeled by random priming with [γ-^{32}P]CTP. The size of RNA markers are shown in kilobases.

human gastric mucosa yielded a 1.4-kb hybridization signal which suggested that the isolated nucleotide sequence was full length (lane A). The probe did not bind to the RNA from thyroid gland (lane B). The results indicated that the human H,K-ATPase ß-subunit mRNA is specifically expressed in the stomach.

Comparison of human, rabbit, pig and rat H,K-ATPase ß-subunit sequence Fig. 4 shows a comparison of the amino acid sequences of human, rabbit pig and rat H,K-ATP ß-subunits. The amino acid sequence of the H,K-ATPase ß-subunit of human displays 84.2%, 85.6% and 81.3% identity to the ß-subunit of rabbit, pig and rat (11,6,10), respectively, and 53% to the Na,K-ATPase ß-subunit of human (19, data not shown). Human, rabbit, pig and rat ß-subunits consist of 291, 291, 290 and 294 amino acids, respectively. Seven potential sites for N-linked glycosylation in the rat and rabbit ß-subunit sequences are all conserved in the human ß-subunit (only one site is different in the pig).

The α-subunit of the H,K-ATPase has been reported as a major antigen in pernicious anaemia. However, the parietal cell antibodies also recognize a 60-90 kDa protein (4,5,7,8,20). Recently this protein was suggested to be the ß-subunit of the H,K-ATPase based on the following evidences: the protein was co-immunoprecipitated and co-purified with the α-subunit; cDNA cloning of the protein from rat, rabbit and pig showed at least 30% homology with the ß-subunit of the Na,K-ATPase (10,11,6). Antibody binding to the 60-90

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human     MAALQEKKTCGQRMEEFQRYCWNPDTGQMLGRTLSRWVWISLYYVAFYVV   50
rabbit    MAALQEKKSCSQRMEEFRHYCWNPDTGQMLGRTLSRWVWISLYYVAFYVV   50
pig       MAALQEKKSCSQRMEEFQRYCWNPDTGQMLGRTLSRWVWISLYYVAFYVV   50
rat       MAALQEKKSCSQRMAEFRQYCWNPDTGQMLGRTPARWVWISLYYAAFYVV   50
          ********.*.***.**..************** .*********.*****

human     MTGLFALCLYVLMQTVDPYTPDYQDQLRSPGVTLRPDVYGEKGLEIVYNV   100
rabbit    MTGLFALCIYVLMQTIDPYTPDYQDQLKSPGVTLRPDVYGEKGLEIHYNI   100
pig       MSGIFALCIYVLMRTIDPYTPDYQDQLKSPGVTLRPDVYGEKGLDISYNV   100
rat       MTGLFALCIYVLMQTIDPYTPDYQDQLKSPGVTLRPDVYGERGLQISYNI   100
          *.*.****.****.*.***********.*************.**.* **.

human     SDNRTWADLTQTLHAFLAGYSPAAQEDSINCTSEQYFFQESFRAPNHTKF   150
rabbot    SDNRTWTSLTHTLRSFLAGYSPAAQVDNINCTSKTYFFQESFGAPNHTKF   150
pig       SDSTTWAGLAHTLHRFLAGYSPAAQEGSINCTSEKYFFQESFLAPNHTKF   150
rat       SENSSWAGLTHTLHSFLAGYTPASQQDSINCSSEKHFFQETFSAPNHTKF   150
          *.. .*..*..**. *****.**.* ..***.*. .****.* *******

human     SCKFTADMLQNCSGLADPNFGFEEGKPCFIIKMNRIVKFLPSNGSAPRVD   200
rabbit    SCKFTADMLENCSGLTDPSFGFKEGKPCFIIKMNRIVRFLPSNSTPPRVD   200
pig       SCKFTADMLQNCSGRPDPTFGFAEGKPCFIIKMNRIVKFLPGNSTAPRVD   200
rat       SCKFTADMLQNCSGLVDPSFGFEEGKPCFIIKMNRIVKFLPSNNTAPRVD   200
          *********.****  **.*** **************.***.*...****

human     CAFLDQP---RELGQPLQVKYYPPNGTFSLHYFPYYGKKAQPHYSNPLVA   247
rabbit    CTFLDMP---HQALTPLQVEYYPPNGTFSLHYFPYYGKKAQPHYSNPLVA   247
pig       CAFLDQP---RD-GPPLQVEYFPANGTYSLHYFPYYGKKAQPHYSNPLVA   246
rat       CTFQDDPQKPRKDIEPLQVQYYPPNGTFSLHYFPYYGKKAQPHYSNPLVA   250
          *.* * *   ..   ****.*.*.***.**********************

human     AKLLNIPRNAEVAIVCKVMAEHVTFNNPHDPYEGKVEFKLKIEK   291
rabbit    AKLLNVPTNTEVVVLCKILADHVTFDNPHDPYEGKVEFKLKIQK   291
pig       AKLLNVPRNRDVVIVCKILAEHVSFDNPHDPYEGKVEFKLKIQK   290
rat       AKFLNVPKNTQVLIVCKIMADHVTFDNPHDPYEGKVEFKLTIQK   294
          **.**.* * .* ..**..*.**.*.**************.*.*
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Fig. 4. Comparison of human, rabbit, pig and rat H,K-ATPase ß-subunits. Asterisks show that a position in the alignment is perfectly conserved; points show that a position is well conserved.

kDa antigen was eliminated on removal of the N-linked oligosaccharides, indicating their participation in antibody binding (4). The function of the 60-90 kDa protein in the parietal cell is not known, but the availability of the human H,K-ATPase ß-subunit cDNA clone will facilitate the expression of the 60-90 kDa protein in eukaryotic systems, which may provide a suitable antigen for early diagnosis of pernicious anaemia and gastric mucosal atrophy (8) in the future.

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