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The 60- to 90-kDa parietal cell autoantigen associated with autoimmune gastritis is a β subunit of the gastric H^+/K^+-ATPase (proton pump)

(acid secretion/organ-specific autoimmunity/pernicious anemia)

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ABSTRACT **Autoantibodies in the sera of patients with pernicious anemia recognize, in addition to the α subunit of the gastric H^+/K^+-ATPase, an abundant gastric microsomal glycoprotein of apparent M_r 60,000–90,000. Herein we have colocalized the glycoprotein and the α subunit of the gastric H^+/K^+-ATPase to the tubulovesicular membranes of the parietal cell by immunogold electron microscopy. Moreover, the glycoprotein and the α subunit were coimmunoprecipitated, and copurified by immunoaffinity chromatography, with an anti-glycoprotein monoclonal antibody. The pig glycoprotein was purified by chromatography on tomato lectin-Sepharose, and five tryptic peptides from the purified glycoprotein were partially sequenced. The complete amino acid sequence, deduced from the nucleotide sequence of overlapping cDNA clones, showed 33% similarity to the sequence of the β subunit of the pig kidney Na^+/K^+-ATPase. We therefore propose that the 60- to 90-kDa glycoprotein autoantigen is the β subunit of the gastric H^+/K^+-ATPase and that the α and β subunits of the proton pump are major targets for autoimmunization in autoimmune gastritis.**

The ATPases are a highly conserved family of proteins responsible for the ATP-dependent transport of ions across the membranes of mammalian cells (1). The gastric H^+/K^+-ATPase, or proton pump, which is responsible for acid production in the stomach (2), was initially thought to contain a single subunit of apparent M_r 95,000–100,000 (3). In contrast, the Na^+/K^+-ATPase from the kidneys of many species contains equimolar amounts of a related α subunit and a smaller glycosylated β subunit (4). The complete sequences of both subunits of the pig kidney Na^+/K^+-ATPase have been determined from cDNA clones (5). Recent experiments with a yeast expression system have demonstrated that both α and β subunits of the Na^+/K^+-ATPase are required for ATPase activity and binding of the inhibitor ouabain (6).

Autoimmune gastritis associated with pernicious anemia is a paradigm of the organ-specific autoimmune diseases that also include Hashimoto thyroiditis and type I diabetes mellitus. Pernicious anemia is the final stage of autoimmune gastritis and the resultant gastric lesion shows severe mucosal atrophy with ensuing failure of intrinsic factor secretion and vitamin B_{12} absorption (7). The disease is characterized by circulating parietal cell autoantibodies in >90% of patients (8). One major parietal cell autoantigen recognized by the human autoantibodies is the α subunit of the gastric H^+/K^+-ATPase (9, 10). A second major parietal cell autoantigen targeted by the human autoantibodies is a highly conserved 60- to 90-kDa glycoprotein (gp60–90) (11, 12). As the protein cores of gp60–90 and the β subunit of the Na^+/K^+-ATPase are similar in size (12–15) and as peptide sequences from a 35-kDa protein presumed to be the gp60–90 core are related to the β subunit of the Na^+/K^+-ATPase (15), gp60–90 may be the equivalent subunit of the H^+/K^+-ATPase. We show here that gp60–90 coimmunoprecipitates and copurifies with the α subunit of the gastric H^+/K^+-ATPase and is similar in sequence‖ to the β subunit of the Na^+/K^+-ATPase (5).

MATERIALS AND METHODS

Antibodies. Monoclonal antibodies (mAbs) 3A6 and 4F11 were produced by fusion of myeloma cells Sp2/0-Ag 14 with splenocytes of BALB/c mice immunized with 100 μg of dog gastric microsomes emulsified in Freund's complete adjuvant. mAbs 2B6 and 1H9 were derived from the splenocytes of mice with autoimmune gastritis induced by neonatal thymectomy (16). mAbs 3A6 and 4F11 bound only to dog parietal cells whereas mAb 1H9 also reacted with pig, mouse, and human parietal cells. Sera positive for parietal-cell autoantibodies were from patients attending the Alfred Hospital, Melbourne. Control sera were from healthy individuals. A rabbit anti-α subunit antibody was prepared by immunization with 100 μg of a 70-kDa fusion protein containing the hydrophilic domain (residues 507–961) of the α subunit of the rat H^+/K^+-ATPase (17) at the carboxyl terminus of glutathione *S*-transferase.

Immunofluorescence and Electron Microscopy. Immunofluorescence and immunogold electron microscopy were carried out as described (13). For double-labeling studies, the mouse mAb was labeled with fluorescein isothiocyanate-conjugated sheep anti-mouse immunoglobulin and the human serum containing parietal-cell autoantibody was labeled with biotinylated sheep anti-human immunoglobulins (Amersham) followed by Texas red-labeled streptavidin (Amersham).

Immunoaffinity Chromatography with mAb 3A6-Sepharose. Dog gastric tubulovesicular membranes (13) showed 10-fold enrichment for the gastric H^+/K^+-ATPase, as as-

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Abbreviations: gp60–90, 60- to 90-kDa parietal cell glycoprotein autoantigen; mAb, monoclonal antibody; PCR, polymerase chain reaction.

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‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M35497).

sessed by K^+-dependent *p*-nitrophenyl phosphatase activity. Membranes were solubilized in 1% Triton X-100 (18) and 150 μg of protein was applied to mAb 3A6-Sepharose 4B (10 mg of γ-globulin fraction of ascites fluid per 1 ml of gel) at 4°C. The unbound fraction was collected and recycled a further four times through the column. The column was washed with 50 mM Hepes, pH 7.6/0.1% Triton X-100/0.15 M NaCl and the bound material was eluted with 0.1 M glycine hydrochloride, pH 2.5/0.1% Triton X-100 and immediately neutralized.

Purification and Amino Acid Sequence of gp60–90. gp60–90 was purified from detergent extracts of pig gastric tubulovesicules by tomato lectin chromatography (J.M.C., B.-H.T., and P.A.G., unpublished data). Samples of the tomato lectin-bound material were treated with N-Glycanase (peptide:*N*-glycosidase F; Genzyme) and subjected to preparative $NaDodSO_4$/PAGE, and the 35-kDa core protein was electroeluted from the gels (13, 19). Tryptic peptides from digests of reduced and carboxymethylated samples were purified by microbore reversed-phase HPLC (19). Automated Edman degradation of tryptic peptides was performed using Applied Biosystems sequencers (models 470A and 477A) equipped with on-line phenylthiohydantoin amino acid analyzers (model 120A).

Isolation of Clones Encoding gp60–90. Total poly(A)$^+$ mRNA was isolated from the mucosa of pig gastric corpus and used to prepare a cDNA library of 5×10^6 independent clones in the vector λgt10 (20). Synthetic oligonucleotides 10R and 11R (see Fig. 1), encoding peptides 3 and 5, respectively, were designed on the basis of human codon frequency tables. Both peptides were initially isolated and sequenced from tryptic digests of a gastrin-binding protein complex isolated from pig gastric mucosa (18) and subsequently from digests of gp60–90. The oligonucleotides were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase and used to screen 5×10^5 recombinant phage as described (21). A single positive clone (STO 1) was obtained.

First-strand oligo(dT)-primed cDNA was prepared from total poly(A)$^+$ mRNA isolated from the mucosa of pig gastric corpus (22). Conventional polymerase chain reaction (PCR) (23) between oligonucleotide 9 and oligo(dT) was performed for 50 cycles with 5 units of *Thermus aquaticus* polymerase (Perkin–Elmer/Cetus) according to the manufacturer's instructions. Cycle times were 95°C, 1.5 min; 37°C, 2 min; 50°C, 3 min. The anchored PCR (24) was performed on first-strand cDNA specifically primed with oligonucleotide 19 at either 42°C (ANC1) or 55°C (ANC2 and ANC3), with the nested primers indicated in Fig. 4. The higher temperature was chosen because it appeared that mRNA secondary structure was preventing the synthesis of full-length cDNA. The PCR products were digested with appropriate restriction enzymes and subcloned into pGEM-3Z (Promega).

Nucleotide Sequencing. Inserts from pGEM-3Z were subcloned into m13mp18 or -mp19 and sequenced by the dideoxynucleotide method using Sequenase DNA polymerase (United States Biochemical) according to the manufacturer's instructions.

RESULTS

gp60–90 Is Associated with the α Subunit of the H^+/K^+-ATPase. To determine whether gp60–90 was associated with the α subunit of the H^+/K^+-ATPase, we obtained a panel of monospecific reagents to the two molecules (Fig. 1). The dog-specific mAbs 3A6 and 4F11 and the interspecies-specific mAb 2B6 (16) were directed against gp60–90, which was the major glycosylated component of gastric tubulovesicles (Fig. 1). All three mAbs recognized tomato lectin-bound and autoantibody-purified gp60–90 and the 35-kDa core protein of gp60–90 in immunoblots (Fig. 1). A rabbit anti-α subunit antibody and mAb 1H9 (16) recognized the α subunit of the H^+/K^+-ATPase from gastric tubulovesicular membranes in immunoblots (Fig. 1).

Immunofluorescence revealed that mAbs 3A6 and 4F11 and the rabbit anti-α subunit antibody bound specifically to internal components of parietal cells in gastric mucosa (Fig. 2). Immunogold electron microscopy revealed that gp60–90 resided with the α subunit in the parietal cell tubulovesicles. Moreover, mAbs 2B6 and 1H9 have been shown (16) to bind these same structures. Evidently, gp60–90 and the α subunit of the gastric H^+/K^+-ATPase colocalized.

Immunoprecipitation from detergent extracts of dog or pig tubulovesicular membranes with the anti-α subunit antibodies resulted in coprecipitation of gp60–90. Conversely, immunoprecipitation of gp60–90 with mAbs 3A6 and 2B6 led to the coprecipitation of the α subunit (data not shown). The extent of association between the two components was quantified by immunoaffinity chromatography of a detergent extract of dog gastric tubulovesicular membranes on mAb 3A6-Sepharose 4B (Fig. 3). gp60–90 was recovered only in the bound fraction with the majority of the α subunit. Therefore, the α subunit of the H^+/K^+-ATPase and gp60–90 were physically associated, suggesting that gp60–90 was a subunit of the proton pump.

Purification and Partial Amino Acid Sequence of gp60–90. gp60–90 was purified from detergent extracts of pig gastric tubulovesicular membranes in a single step by chromatography on tomato lectin-Sepharose. Pig, rather than dog, gastric mucosa was chosen due to the ready availability of pig tissue. Six peptide sequences were obtained from a tryptic digest of the lectin-bound material. The amino acid sequences of three peptides (data not shown) were identical to regions of the α subunit of the pig gastric H^+/K^+-ATPase. The other three peptide sequences (Table 1) were similar to the β subunit of the Na^+/K^+-ATPase. To confirm that these latter sequences were derived from the protein core of gp60–90, two additional peptide sequences were obtained from the deglycosylated

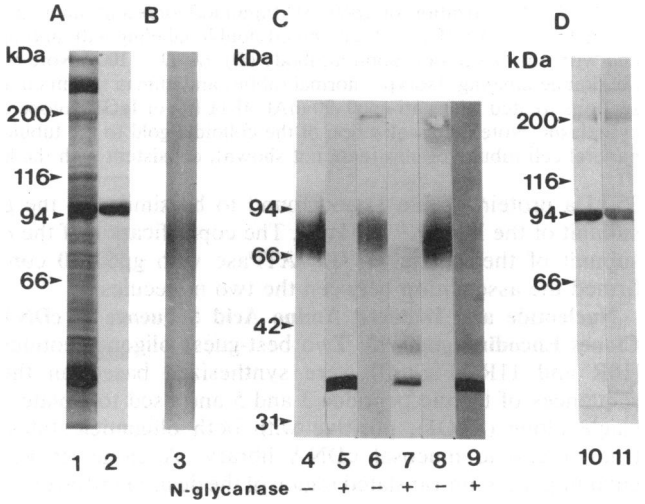

FIG. 1. Antibody reactivity with α subunit and gp60–90 transferred to nitrocellulose membranes after $NaDodSO_4$/PAGE. (*A*) Dog gastric membranes (lane 1) and tubulovesicular membranes (lane 2) stained for protein with Coomassie blue. (*B*) Tubulovesicular membranes stained for carbohydrate (13) (lane 3). (*C*) Reactivity of mAb 3A6 with gastric tubulovesicular membranes before (–, lane 4) and after (+, lane 5) N-Glycanase treatment; bound fraction from a tomato lectin-Sepharose 4B column, before (–, lane 6) and after (+, lane 7) N-Glycanase treatment and the bound fraction from a parietal cell autoantibody-Sepharose 4B column before (–, lane 8) and after (+, lane 9) N-Glycanase treatment. mAbs 4F11 and 2B6 gave results identical to 3A6. (*D*) Dog gastric tubulovesicular membranes treated with mAb 1H9 (lane 10) and with rabbit anti-α subunit antibody (lane 11). Isotype and normal rabbit serum controls showed no reactivity.

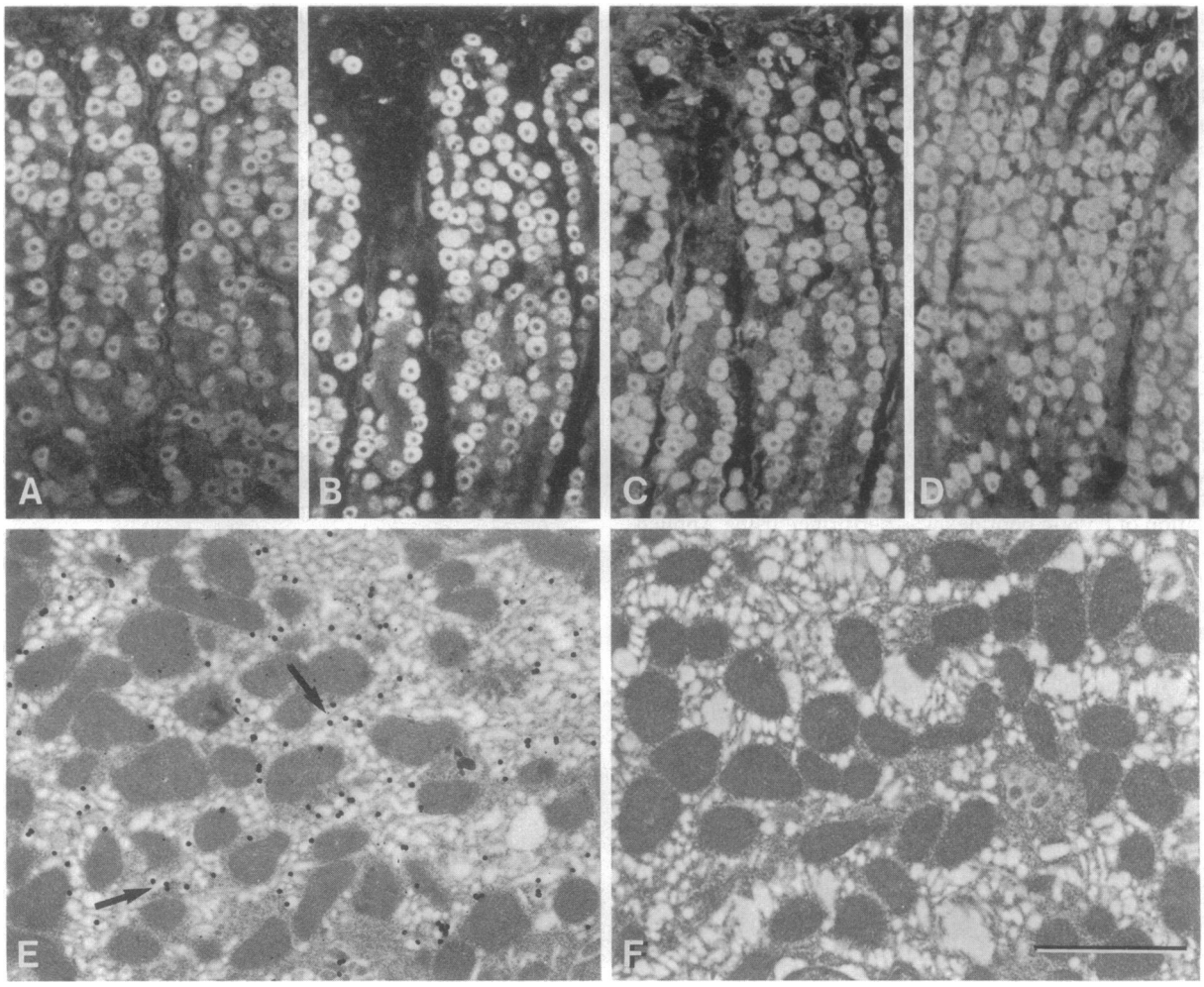

FIG. 2. Localization of gp60–90. Immunofluorescent staining of formalin-fixed paraffin-embedded dog stomach sections incubated with anti-gp60–90 mAb 4F11 (*A*); combined double-labeling with anti-gp60–90 mAb 3A6 (*B*) and with parietal cell autoantibody-positive sera (*C*); and with anti-α-subunit rabbit antibody (*D*). (*A–D*, ×100.) Note the identical internal binding of the large parietal cells by both antibodies in the double-labeling. Isotype, normal rabbit, and human serum controls showed no staining. Immunogold electron microscopy of dog stomach sections treated with anti-gp60–90 mAb 4F11 (*E*) or IgG1 isotope control (*F*). (*E* and *F*, bar = 1 μm.) The micrographs show the parietal cell cytoplasm. Note the localization of the colloidal gold to the tubulovesicular membranes. The anti-α-subunit antibody gave similar binding to parietal cell tubulovesicles (data not shown), consistent with the known localization of the α subunit.

35-kDa protein (Table 1) and found to be similar to the β subunit of the Na^+/K^+-ATPase. The copurification of the α subunit of the gastric H^+/K^+-ATPase with gp60–90 confirmed the association between the two molecules.

Nucleotide and Deduced Amino Acid Sequence of cDNA Clones Encoding gp60–90. Two best-guess oligonucleotides [10R and 11R (Fig. 4)] were synthesized based on the sequences of tryptic peptides 3 and 5 and used to isolate a single clone (STO1), positive with both oligonucleotides, from a gastric mucosal cDNA library. A clone (PCR1) encoding the 3′ untranslated region of the desired mRNA was then generated by conventional PCR between oligonucleotide 9 and oligo(dT) primers (Fig. 4). The 5′ untranslated and amino-terminal coding regions of the desired mRNA were generated by anchored PCR. The composite nucleotide sequence derived from clones STO1, PCR1, ANC1, ANC2, and ANC3 (Fig. 5) was 1073 base pairs (bp) long and was comprised of a 21-bp 5′ untranslated region, an 870-bp coding region, and a 182-bp 3′ untranslated region. The observation of a single 1-kilobase band in Northern blots of total poly(A)$^+$ mRNA from gastric corpus (data not shown) suggested that the composite nucleotide sequence was probably full length. No mRNA was detected in gastric forestomach, gastric antrum, or liver (data not shown).

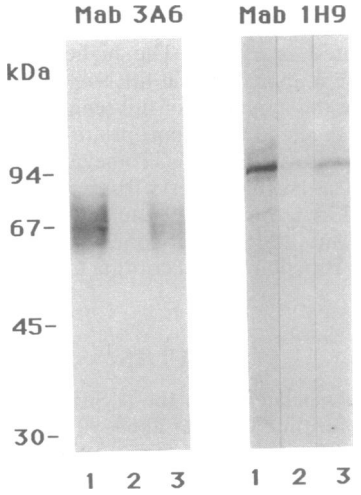

FIG. 3. Recovery by immunoaffinity chromatography on a mAb 3A6-Sepharose 4B column of gp60–90 and the α subunit from a dog gastric tubulovesicular membrane extract. Immunoblot analysis of equal amounts of total (lanes 1), unbound (lanes 2), and bound (lanes 3) fractions reacted with anti-gp60–90 mAb 3A6 or with anti-α-subunit mAb 1H9, as indicated. Neither component bound to a control column of Sepharose 4B.

Table 1. Sequences of peptides obtained from tomato lectin-bound gp60–90

Peptide	Position	Sequence
1	65	tIDPYTPDYQDqLK
2	225	SLHYFPYYGK
3	236	AQPHYSNPLVAAK
4	93	GLdISYXVSXSTt
5	209	DGPPLQVEYFPADGT

Samples of the tomato lectin-bound material from detergent extracts of pig tubulovesicles (peptides 1–3) and of the 35-kDa protein electroeluted from preparative gels of the same material after treatment with N-Glycanase (peptides 4 and 5) were reduced, carboxymethylated, and digested with trypsin. Tryptic peptides were purified by microbore reversed-phase HPLC and sequenced by automated Edman degradation. Lowercase letters represent uncertain amino acid assignments.

The nucleotide sequence encoded a protein of 290 amino acids (Fig. 5) whose sequence was in complete agreement with the five tryptic peptide sequences obtained from gp60–90. Comparison with the three peptide sequences obtained from a 35-kDa protein isolated from N-Glycanase-treated gastric vesicular membranes by Sachs and coworkers (15) revealed a single discrepancy (arginine at position 185 instead of histidine). The calculated molecular weight of the protein was 33,038, in good agreement with the apparent molecular weight (35,000) of the core protein after N-Glycanase treatment (12–15). Most of the six potential N-glycosylation sites (Fig. 5) presumably have carbohydrate attached, since four or five deglycosylated products were observed during N-Glycanase treatment (12, 14). Asn-221 was clearly glycosylated since after cleavage with N-Glycanase, which converts glycosylated asparagine to aspartic acid (25), aspartic acid was observed in the corresponding position of peptide 5. Hydropathy analysis predicted a single transmembrane domain located near the amino terminus of the protein.

The predicted protein sequence of gp60–90 was 33% similar to the β subunit of the pig kidney Na^+/K^+-ATPase and 41% similar to the β subunit of the Na^+/K^+-ATPase from human tissues other than kidney (Fig. 6). The conservation of the six cysteine residues that form the three disulfide bonds of the kidney β subunit (28) suggested that the folding of the protein was also conserved. Since gp60–90 coimmunoprecipitated and copurified with the α subunit of the gastric H^+/K^+-ATPase and showed extensive similarity to the β subunits of Na^+/K^+-ATPase, it was concluded that gp60–90 was the β subunit of the gastric H^+/K^+-ATPase.

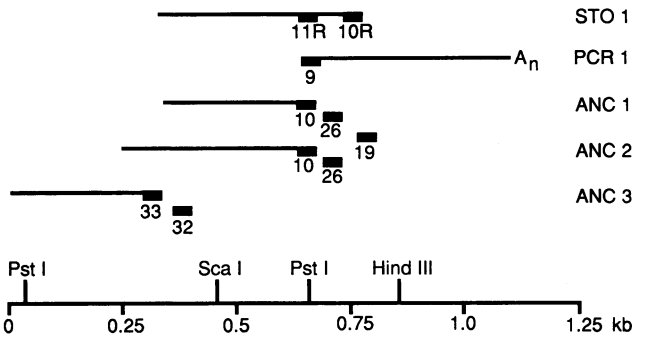

FIG. 4. Alignment of clones encoding gp60–90. Clone STO1 was isolated by screening a pig gastric mucosal cDNA library with oligonucleotides 10R and 11R. Clone PCR1 was isolated by conventional PCR with oligonucleotide 9 and oligo(dT) as primers and oligo(dT)-primed pig gastric mucosal cDNA as template. Clones ANC1–3 were isolated by an anchored PCR with oligonucleotides 26 and 10 (ANC1 and 2) or 32 and 33 (ANC3), as nested primers and oligonucleotide 19-primed porcine gastric mucosal cDNA as template. Oligonucleotides used for library screening or for PCR are shown as solid boxes. A partial restriction map of the composite gp60–90 cDNA is indicated. kb, Kilobase(s).

DISCUSSION

The molecular targets of most organ-specific autoimmune diseases remain largely unknown. Prior to this study, the identity of the target antigens in autoimmune gastritis and pernicious anemia was also unresolved. The gastric parietal cell appears to be the principal cell targeted in this disease, and it has been shown (11–13, 16) that parietal cell autoantibodies immunoprecipitate molecules of 60–90 kDa and 92 kDa. The 92-kDa autoantigen has been identified as the α subunit of the gastric H^+/K^+-ATPase (9, 10).

The α subunits of the gastric proton pump and the Na^+/K^+-ATPase show a high level of sequence similarity. The Na^+/K^+-ATPase was considered unique in also having a β subunit comprising a 55-kDa glycoprotein with a 35-kDa protein core. However, since gp60–90 colocalizes, coimmunoprecipitates, and copurifies with the catalytic subunit of the H^+/K^+-ATPase and shows sequence similarity to the β subunits of the Na^+/K^+-ATPases, we conclude that gp60–90

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                                M  A  A  L  Q  E  K  K  S  C  S  Q  R
AGGCTTTGGCAGGAGCAGGCGATGGCGGCTCTGCAGGAGAAGAAGTCGTGCAGCCAGCGC
14                                                              60
 M  E  E  F  Q  R  Y  C  W  N  P  D  T  G  Q  M  L  G  R  T
ATGGAGGAGTTCCAGCGCTACTGCTGGAACCCAGACACCGGGCAGATGCTGGGCCGGACG
34                                                             120
 L  S  R  W  V  W  I  S  L  Y  Y  V  A  F  Y  V  V  M  S  G
CTGTCCCGGTGGGTGTGGATCAGCCTCTACTACGTGGCCTTCTACGTGGTCATGAGCGGG
54                                                             180
 I  F  A  L  C  I  Y  V  L  M  R [T  I  D  P  Y  T  P  D  Y]
ATCTTCGCGCTCTGCATCTACGTGCTGATGCGCACCATCGACCCCTACACGCCCGACTAC
74                                                             240
[Q  D  Q  L  K] S  P  G  V  T  L  R  P  D  V  Y  G  E  K [G]
CAGGACCAGCTCAAGTCGCCAGGGGTGACCCTGAGGCCGGACGTCTACGGGGAGAAAGGC
94                 *                                           300
[L  D  I  S  Y  N  V  S  D  S  T  T] W  A  G  L  A  H  T  L
CTGGACATTTCCTACAACGTGTCTGACAGCACGACCTGGGCGGGGCTGGCCCACACCCTG
114                                                    *       360
 H  R  F  L  A  G  Y  S  P  A  A  Q  E  G  S  I  N  C  T  S
CACCGCTTCCTGGCAGGCTACTCGCCGGCTGCCCAGGAGGGCAGCATCAACTGCACATCG
134                                    *                       420
 E  K  Y  F  F  Q  E  S  F  L  A  P  N  H  T  K  F  S  C  K
GAGAAGTACTTCTTCCAGGAGAGCTTCCTGGCGCCGAACCACACCAAGTTCTCCTGCAAG
154                    *                                       480
 F  T  A  D  M  L  Q  N  C  S  G  R  P  D  P  T  F  G  F  A
TTCACCGCAGACATGCTACAAAACTGCTCCGGCCGGCCCGACCCCACCTTCGGCTTCGCG
174                                                            *
 E  G  K  P  C  F  I  I  K  M  N  R  I  V  K  F  L  P  G  N
GAGGGAAAGCCGTGTTTCATCATCAAGATGAACCGGATCGTCAAGTTCCTCCCGGGTAAC
194                                                            600
 S  T  A  P  R  V  D  C  A  F  L  D  Q  P  R [D  G  P  P  L]
AGCACAGCCCCCAGGGTGGACTGCGCCTTCCTGGACCAGCCCCGGGACGGCCCACCCCTG
214                    *                                       660
[Q  V  E  Y  F  P  A  N  G  T] Y [S  L  H  Y  F  P  Y  Y  G]
CAGGTGGAATACTTCCCTGCCAACGGCACCTACAGTCTGCACTACTTCCCCTATTACGGC
234                                                            720
[K] K [A  Q  P  H  Y  S  N  P  L  V  A  A  K] L  L  N  V  P
AAGAAAGCCCAGCCCCACTATAGCAACCCGCTGGTGGCCGCCAAGCTCCTCAACGTCCCC
254                                                            780
 R  N  R  D  V  V  I  V  C  K  I  L  A  E  H  V  S  F  D  N
AGGAACAGGGACGTGGTCATCGTGTGCAAGATCCTGGCGGAGCACGTGAGCTTCGACAAC
274                                                            840
 P  H  D  P  Y  E  G  K  V  E  F  K  L  K  I  Q  K
CCCCACGACCCCTATGAAGGGAAGGTGGAGTTCAAGCTTAAGATCCAGAAGTAGAATTTG
                                                               900
CAGCCGTGACGCCCACGCGGGTCCCTAAGGCAGCAGCGGTCCAACCAACAAGACCCTAAA
                                                               960
GGACTCACCTCTTCCATGTGGGCATCACCCTTCTTGCTGTAACTTATTTTTCTGTCCGTT
                                                              1020
AATAGAAAGACTAGCAGTGCGTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA
                                                              1073
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FIG. 5. Nucleotide sequence and predicted amino acid sequence of gp60–90. Nucleotides are numbered on the right and amino acids on the left. The putative polyadenylylation recognition sequence is underlined, and the asparagines of the six potential N-glycosylation sites are marked by asterisks. Peptides isolated from gp60–90 are boxed.

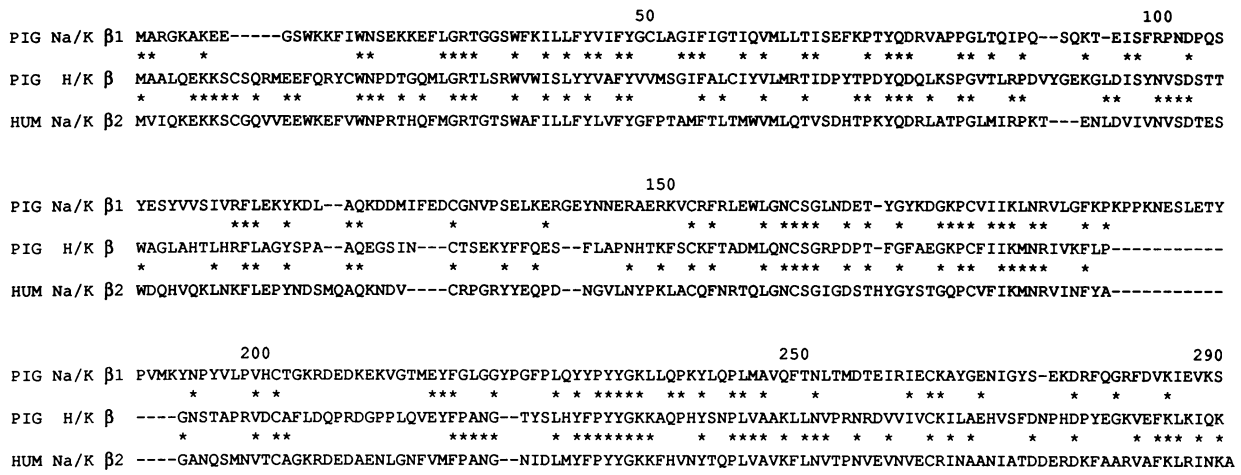

FIG. 6. Alignment of the predicted amino acid sequence of gp60–90 with the sequences of the β subunit of the pig kidney Na^+/K^+-ATPase (5) and of a related β subunit present in several other human tissues (26). Optimal alignments were generated with the program ALIGN, using the mutation data matrix, a matrix bias of +6, and a break penalty of 6 (27). Amino acids in common with the sequence of gp60–90 are marked with an asterisk. Gaps were introduced to maximize similarity and are indicated by hyphens.

is the β subunit of the gastric H^+/K^+-ATPase. By analogy with the Na^+/K^+-ATPase, whose β subunit has been shown to be essential for ATPase activity and for binding of the inhibitor ouabain (6), the β subunit of the gastric H^+/K^+-ATPase presumably plays an essential role in proton transport across the tubulovesicular membrane of the gastric parietal cell.

We have also shown in the present study that mAbs, derived from mice with autoimmune gastritis induced by neonatal thymectomy, react exclusively with both α and β subunits of the gastric proton pump. Thus these findings provide compelling evidence for a unifying model of the proton pump as a major target for autoimmunization in human and murine diseases. Furthermore, these findings suggest that the murine disease is an excellent model for studies of (*i*) the role of the proton pump in the immunopathogenesis of the gastric lesion of autoimmune gastritis and (*ii*) the role of the thymus in the maintenance of tolerance to these tissue-specific molecules.

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