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Biochemistry

Human endomembrane H^+ pump strongly resembles the ATP-synthetase of Archaebacteria

(H^+-ATPase/clathrin-coated vesicle/renal acidification/evolution)

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ABSTRACT **Preparations of mammalian H^+ pumps that acidify intracellular vesicles contain eight or nine polypeptides, ranging in size from 116 to 17 kDa. Biochemical analysis indicates that the 70- and 58-kDa polypeptides are subunits critical for ATP hydrolysis. The amino acid sequences of the major catalytic subunits (58 and 70 kDa) of the endomembrane H^+ pump are unknown from animal cells. We report here the complete sequence of the 58-kDa subunit derived from a human kidney cDNA clone and partial sequences of the 70- and 58-kDa subunits purified from clathrin-coated vesicles of bovine brain. The amino acid sequences of both proteins strongly resemble the sequences of the corresponding subunits of the vacuolar H^+ pumps of Archaebacteria, plants, and fungi. The archaebacterial enzyme is believed to use a H^+ gradient to synthesize ATP. Thus, a common ancestral protein has given rise to a H^+ pump that synthesizes ATP in one organism and hydrolyzes it in another and is highly conserved from prokaryotes to humans. The same pump appears to mediate the acidification of intracellular organelles, including coated vesicles, lysosomes, and secretory granules, as well as extracellular fluids such as urine.**

Vacuolar, or endomembrane, H^+-translocating ATPases are responsible for the acidification of a variety of intracellular organelles in plant, yeast, and animal cells. In accord with this distribution, these H^+ pumps serve a diverse set of functions. In eukaryotic cells, the H^+ gradients generated by these enzymes are necessary for lysosomal acid hydrolase activity, processing of endocytosed receptor–ligand complexes, and posttranslational modifications within the Golgi apparatus. Specialized functions in animal cells include a role in the maturation of prohormones within storage granules and in the energization of neurotransmitter uptake in synaptic vesicles. Similar H^+-translocating ATPases are found in the plasma membrane of renal epithelial cells of the distal nephron (cortical and medullary collecting ducts) where they serve to excrete H^+ in compensation for the acid load arising from dietary intake and basal catabolism (reviewed in refs. 1–5).

Because of the diverse functions associated with intraorganelle acidification by the vacuolar H^+ pumps, much attention has recently been directed toward their biochemical and molecular characterization. There is general agreement among numerous groups that structurally these H^+-translocating ATPases are large (300–700 kDa) heterooligomers (6–11). The reconstitutively active enzyme, purified from bovine brain clathrin-coated vesicles, contains eight major polypeptides with apparent molecular masses of 116, 70, 58, 40, 38, 34, 33, and 17 kDa (6, 12). A similar polypeptide composition has been reported for the bovine adrenal chromaffin granule ATPase (10, 11). H^+ pumps with similar enzymatic properties have been purified from plant and fungal vacuolar membranes and shown to contain at least three polypeptides with molecular masses of approximately 70, 58, and 17 kDa (7–9). At present, the subunit composition of endomembrane H^+ pumps is controversial. Through reconstitution of dissociated subunits, selected polypeptides have been proven to be subunits and, in some instances, their roles in pump function have been ascribed.

Generally, these H^+ pumps resemble F_1F_0-type ATPases in that they are functionally and structurally comprised of two main sectors, one responsible for ATP hydrolysis and one that serves to lower the activation energy for transmembranous H^+ movement—i.e., a pore. Biochemical analysis has led to partial definition of both domains. The 17-kDa subunit has been isolated from the clathrin-coated vesicles and was shown to serve as a *N,N'*-dicyclohexylcarbodiimide-sensitive H^+ pore (13). A cDNA encoding the 17-kDa subunit of the chromaffin granule H^+ pump was isolated, and its sequence characteristics were shown to agree well with a function in H^+ transport (14). Partial resolution and reconstitution of the ATP hydrolytic domain of the clathrin-coated vesicle H^+ pump has also been achieved. It appears that the 70- and 58-kDa components constitute the core of the catalytic center and two additional polypeptides, likely the 40- and 33-kDa species, are required to render the reaction center catalytically active (15). The central role of the 70-kDa subunit in catalysis has been emphasized by the finding that ATP, nucleotide analogues, and *N*-ethylmaleimide (an inhibitor of this pump) bind to this component (4, 7–9). Thus, at present, five of the eight major polypeptides (70, 58, 40, 33, and 17 kDa) present in the purified bovine brain clathrin-coated vesicle H^+ pump have been shown to be genuine subunits. The role of the remaining polypeptides (116, 38, and 34 kDa) remains unknown. Of these, the 116-kDa polypeptide is perhaps the most controversial candidate subunit. One preparation of mammalian H^+-ATPase (16) apparently lacks this polypeptide, whereas three other preparations from mammalian membranes have this polypeptide as a constant feature (6, 10, 11). From a biochemical perspective, removal of this polypeptide from the H^+-translocating complex results in a transition in function such that the enzyme can no longer use Mg^{2+} as divalent cation to support ATP hydrolysis; rather, ATPase activity becomes Ca^{2+} dependent. However, attempts at restoring Mg^{2+}-activated ATPase activity by reconstitution of the 116-kDa polypeptide to the 116-kDa-depleted subcomplex have been unsuccessful to date and thus the 116-kDa polypeptide has not been causally related to pump function.

The primary structures of the 70- and/or the 58-kDa subunits of the vacuolar H^+ pump have recently been re-

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Table 1. Amino acid sequences determined from the 58-kDa and 70-kDa subunits of the bovine clathrin-coated vesicle H^+ pump

Peptide	Yield	Residue 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32
58 kDa subunit																																	
T-51	80 pmol	L	T	Y	K																												
T-97	75 pmol	T	P	V	S	E	D	M	L	G	R																						
T-132A	45 pmol	Y	A	E	I	V	H	L	T	L	P	D	G	T	K																		
T-132B	6 pmol	(Q)	L	F	H	P	E	Q	(L)	I	T	G	K	E	D																		
T-206	65 pmol	T	V	Y	E	T	L	D	I	G	W	Q	L	L	R																		
T-210	40 pmol	A	V	V	G	E	E	A	L	T	S	D	D	L	L	Y	L	E	F	L	Q	K	F	E	R								
CNBr2	3 pmol	x	G	I	V	D	G	A	A	P	E	L	P	V	P	T	S	G	P	L	A	G	(S)	V	(E	Q)							
CNBr3	22 pmol	x	G	I	V	D	G	A	A	P	E	L	P	V	P	T	S	G	P	L	A	G	S	(V)	E	Q	A						
CNBr7	11 pmol	(K)	A	V	V	G	E	E	A	L	T	S	D	D	L	L	Y	L	E	F	L	Q	K	F	(E)								
CNBr6	45 pmol	N	S	V	A	L	G	Q	(N	I	P)	I	F	S	A	A	G	(L)															
70 kDa subunit																																	
T-99	120 pmol	W	A	E	A	L	R																										
T-103	130 pmol	L	A	S	F	Y	E	R																									
T-129A	95 pmol	L	A	E	M	P	A	D	S	G	Y	P	A	Y	L	G	A	R															
T-129B	40 pmol	V	G	H	S	E	L	V	G	E	I	I	R																				
T-157	30 pmol	V	G	S	H	I	T	G	G	D	I	Y	G	I	V	N	E	N	S	L	I	K											
T-229	40 pmol	T	G	L	P	L	S	V	(E)	L	G	P	G	I	M	G	A	I	F	D	G	I	Q	(R)	P	L	(S)	D	I	(S	S)	Q	T
T-233	50 pmol	E	G	S	V	S	I	V	G	A	V	S	P	P	G	G	D	F	S	D	P	V	(T	S)	T	T	L	(H)	I	(V	G	T	F)
CNBr-1	2 pmol	x	x	D	S	G	Y	P	A	Y	L	G	A	(R)	L	A	S	F	Y	E	(R)	A	G	X	V	(T)	Q	L	G	N	P		
CNBr-2	7 pmol	(G)	A	I	F	D	G	I	Q	(V)	P	L	(V)	D	I	S	S	Q	T														

Amino acid sequences are given in single-letter code. Unidentifiable residues are denoted by x, and ambiguous residues are in parentheses.

ported from *Neurospora* (17, 18), plants [*Daucus* (19) and *Arabidopsis* (20)], and Archaebacteria [*Sulfolobus* (21, 22)]. These structures demonstrate a high degree of interspecies conservation of the vacuolar H^+ pump and a low, but significant, homology between the 70- and 58-kDa subunits and the catalytic (β and α) subunits of another class of ATP-driven H^+ pumps, the F_1F_0-type ATPases. Up to now, little information has been obtained regarding the structures of the 70- and 58-kDa subunits from animals, and the relationship between the animal H^+ pumps operating in highly specialized organelles and the enzymes from prokaryotes and lower eukaryotes is unknown. We report here information concerning the amino acid sequences of the mammalian 58- and 70-kDa subunits.‖ These sequences show a remarkable degree of conservation with respect to the catalytic subunits of the H^+-pumping ATPases from plants, fungi, and Archaebacteria, suggesting a possible pathway for the evolution of eukaryotic cells from the progenitors of Archaebacteria and Eubacteria.

EXPERIMENTAL PROCEDURES

Protein Purification and Amino Acid Sequencing. The endomembrane H^+ pump was purified from clathrin-coated vesicles of bovine brain as described (6). Individual subunits were purified from the H^+ pump preparation by preparative SDS/PAGE and recovered by electroelution. The NH_2 termini of both the 58- and 70-kDa subunits were found to be blocked to Edman degradation. Two strategies were pursued to obtain internal amino acid sequences (23, 24). First, ≈100 pmol of electroeluted protein was cleaved with CNBr in 70% formic acid. Cleaved protein fragments were separated by SDS/PAGE, electroblotted onto Immobilon membranes (Millipore), stained with Coomassie blue, and subjected to amino acid sequencing directly from the membrane as described by Matsudaira (25). In the second approach, ≈300 pmol of electroeluted protein was digested with trypsin. The protein digest was fractionated by reverse-phase (C18) HPLC, and individual peaks were collected and subjected to automatic amino acid sequencing as described (23, 24). Sequences and yields are summarized in Table 1.

cDNA Cloning and Sequencing. Oligonucleotides designed to be complementary to the peptide sequences T-210 and CNBr7 were used to screen a human kidney cDNA library (kind gift of Graeme Bell, University of Chicago). Hybridization positive clones were plaque purified and analyzed as described (26, 27), and two different clones with large inserts (λPP1a and λPP2a) were selected for study. DNA sequencing of the inserts of these clones was performed on single-stranded DNA as reported (77) by the method of Sanger *et al.* (28) after subcloning into pBluescript and M13 vectors.

Sequence Analysis. Sequence analysis was performed on an IBM-AT computer using the Microgenie Software (Beckman).

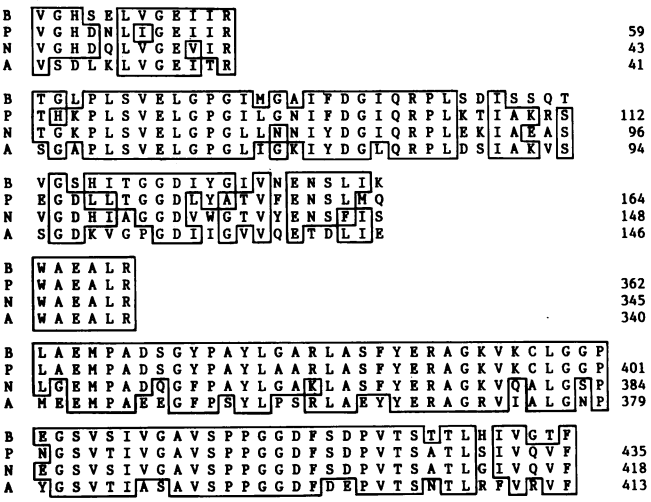

FIG. 1. Alignment of the amino acid sequences (single-letter code) from the bovine 70-kDa subunit of the vacuolar H^+ pump with the corresponding sequences from fungi, plants, and Archaebacteria. Origin of the sequences is indicated on the left: B, bovine (Table 1); P, plant [*Daucus carota* (19)]; N, fungus [*Neurospora* (17)]; A, Archaebacterium [*Sulfolobus* (21)], and residue numbers are shown on the right. Identical residues present in at least two of the four sequences are boxed.

‖The sequence reported in this paper has been deposited in the GenBank data base (accession no. M25809).

Preparation of Antipeptide Antibodies. A synthetic peptide containing a sequence from the bovine 70-kDa subunit (sequence: C S H I T G G D I Y G I V N E N, T-157 in Table 1) was coupled to keyhole limpet hemocyanin and used to raise antibodies in rabbits as described (29). Antibodies were used without further purification for immunoblotting and immunoprecipitation experiments; preimmune sera and blockage of the reactivity with the peptide were used as controls.

RESULTS

Amino Acid Sequences from the 58- and 70-kDa H^+ Pump Subunits from Bovine Brain Clathrin-Coated Vesicles. The amino termini of the purified 58- and 70-kDa H^+ pump subunits were found to be blocked to Edman degradation. We therefore obtained internal amino acid sequences from these proteins after cleavage with CNBr or trypsin. Tryptic digests of the proteins were separated by reverse phase HPLC. Selected peaks were subjected to sequence analysis, and the resulting partial sequences obtained from the bovine 58- and 70-kDa subunits are listed in Table 1, together with the sequences obtained from fragments produced by CNBr cleavage. The initial yields recovered during amino acid sequencing of the different tryptic fragments are of comparable magnitude for all fragments except for the sequence T-132B, which was recovered in much lower yields and upon data bank searches was identified as an α-tubulin contaminant (data not shown).

To confirm that the peptide sequences obtained were indeed derived from the H^+ pump, an anti-peptide antibody was raised against one of the sequences of the 70-kDa subunit. This antibody specifically reacted with the corresponding subunit on immunoblots in both crude and highly purified H^+ pump preparations.

Homologies of the Amino Acid Sequences from the Bovine H^+ Pump with Those of Other Organisms. The primary structures of the 70- and 58-kDa subunits of a vacuolar-type H^+ pump from plant, fungal, and archaebacterial organisms were recently published (17–22). These sequences were aligned with each other and with those we determined from the corresponding bovine subunits. All amino acid sequences of the bovine 70-kDa subunit that we determined (Table 1) could be found in the aligned sequences of the other three species, as shown in Fig. 1. In this alignment, identical residues are boxed, demonstrating the high degree of conservation of the 70-kDa subunit from Archaebacteria to mammals and plants in the regions of the peptide sequences. A similar analysis of the peptide sequences from the 58-kDa subunit also revealed strong conservation, except that not all of the amino acid sequences determined from the purified 58-kDa subunit could be aligned with the other species' sequences (data not shown). To obtain a full-length sequence for the 58-kDa subunit, and to resolve this discrepancy, we decided to molecularly clone its message.

The Complete Primary Structure of the Human 58-kDa Subunit. Oligonucleotides were designed based on the determined peptide sequences and were used to screen several

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TCGCCCAATTCCGGGCTCAGACACTGGGCTCCCAGCTGGGGACTGCTCCATGGCCATGGAGATAGACAGCAGGCCTGGGGGGCTCCCCGGCAGTAGCTGCAACCTAGGTGCAGCCCGAGA  120
                                                 M  A  M  E  I  D  S  R  P  G  G  L  P  G  S  S  C  N  L  G  A  A  R  E    24

ACACATGCAGGCGGTCACCCGAAACTACATCACCCACCCCCGTGTCACCTACAGGACTGTGTGCAGCGTGAACGGGCCCCTGGTGGTGCTGGACCGGGTCAAGTTTGCCCAGTATGCGGA  240
 H  M  Q  A  V  T  R  N  Y  I  T  H  P  R  V  T  Y  R  T  V  C  S  V  N  G  P  L  V  V  L  D  R  V  K  F  A  Q  Y  A  E    64
                                             L  T  Y  K                                                        Y  A  E
                                            └──T-51──┘                                                        └──────

GATCGTCCACTTCACCCTCCCAGATGGGACTCAGAGGAGCGGGCAGGTGCTTGAGGTGGCTGGCACCAAGGCGATTGTTCAGGTGTTTGAAGGGACATCAGGGATCGATGCCAGGAAGAC  360
 I  V  H  F  T  L  P  D  G  T  Q  R  S  G  Q  V  L  E  V  A  G  T  K  A  I  V  Q  V  F  E  G  T  S  G  I  D  A  R  K  T   104
 I  V  H  L  T  L  P  D  G  T  K
────────────T-132A────────────┘

CACTTGCGAATTTACAGGGGACATCCTACGAACTCCGGTGTCAGAGGACATGCTGGGTCGGGTTTTCAATGGCTCCGGCAAGCCCATTGACAAGGGGCCAGTGGTCATGGCGGAGGACTT  480
 T  C  E  F  T  G  D  I  L  R  T  P  V  S  E  D  M  L  G  R  V  F  N  G  S  G  K  P  I  D  K  G  P  V  V  M  A  E  D  F   144
                                T  P  V  S  E  D  M  L  G  R
                               └────────────T-97─────────────┘

TCTGGATATCAATGGCCAGCCCATCAACCCGCACTCCCGCATCTACCCCGAGGAGATGATTCAGACGGGCATTTCTCCTATTGACGTCATGAACAGCATTGCCCGCGGCCAGAAGATCCC  600
 L  D  I  N  G  Q  P  I  N  P  H  S  R  I  Y  P  E  E  M  I  Q  T  G  I  S  P  I  D  V  M  N  S  I  A  R  G  Q  K  I  P   184
                                                                                          N  S  V  A  L  G  Q  N  I  P
                                                                                         └────────────CNBr6────────────

CATCTTCTCAGCAGCCGGGCTCCCCCACAATGAGATTGCCGCTCAGATCTGCCGCCAGGCGGGGCTGGTGAAGAAGTCCAAGGCTGTGCTGGATTACCATGACGACAACTTCGCCATCGT  720
 I  F  S  A  A  G  L  P  H  N  E  I  A  A  Q  I  C  R  Q  A  G  L  V  K  K  S  K  A  V  L  D  Y  H  D  D  N  F  A  I  V   224
 I  F  S  A  A  G  L
─────────────────────┘

CTTTGCAGCCATGGGGGTGAACATGGAGACAGCCAGATTCTTCAAGTCTGACTTTGAGCAGAATGGAACCATGGGGAACGTCTGCCTCTTCCTGAACTTGGCCAATGACCCCACGATCGA  840
 F  A  A  M  G  V  N  M  E  T  A  R  F  F  K  S  D  F  E  Q  N  G  T  M  G  N  V  C  L  F  L  N  L  A  N  D  P  T  I  E   264

GCGGATCATCACCCCGCGCCTGGCGCTGACCACTGCTGAATTCCTTGCCTACCAGTGTGAGAAGCATGTGCTGGTCATACTGACGGACATGAGTTCCTATGCAGAGGCCTTGCGGGAGGT  960
 R  I  I  T  P  R  L  A  L  T  T  A  E  F  L  A  Y  Q  C  E  K  H  V  L  V  I  L  T  D  M  S  S  Y  A  E  A  L  R  E  V   304

CTCTGCTGCTAGAGAGGAGGTGCCTGGGCGCCGAGGGTTTCCTGGATATATGTACACAGACCTGGCCACCATCTACGAGCGGGCGGGCCGTGTGGAGGGTCGGGGAGGATCCATCACACA  1080
 S  A  A  R  E  E  V  P  G  R  R  G  F  P  G  Y  M  Y  T  D  L  A  T  I  Y  E  R  A  G  R  V  E  G  R  G  G  S  I  T  Q   344

GATCCCCATCCTCACCATGCCCAACGACGATATCACCCACCCTATCCCAGACTTGACGGGCTTCATCACAGAGGGACAGATCTACGTGGACAGACAGCTTCACAACAGACAGATCTACCC  1200
 I  P  I  L  T  M  P  N  D  D  I  T  H  P  I  P  D  L  T  G  F  I  T  E  G  Q  I  Y  V  D  R  Q  L  H  N  R  Q  I  Y  P   384

CCCCATCAACGTGCTCCCTTCCCTGTCGCGGCTGATGAAGTCAGCCATTGGGGAAGGCATGACAAGAAAGGACCATGGAGATGTCTCCAACCAGCTGTACGCCTGCTATGCCATCGGGAA  1320
 P  I  N  V  L  P  S  L  S  R  L  M  K  S  A  I  G  E  G  M  T  R  K  D  H  G  D  V  S  N  Q  L  Y  A  C  Y  A  I  G  K   424

GGACGTGCAGGCCATGAAGGCAGTAGTTGGGGAGGAGGCGCTCACCTCTGAGGACCTGCTCTACCTGGAATTCCTGCAGAAGTTTGAGAAGAACTTCATCAATCAGGGCCCCTACGAGAA  1440
 D  V  Q  A  M  K  A  V  V  G  E  E  A  L  T  S  E  D  L  L  Y  L  E  F  L  Q  K  F  E  K  N  F  I  N  Q  G  P  Y  E  N   464
                K  A  V  V  G  E  E  A  L  T  S  D  D  L  L  Y  L  E  F  L  Q  K  F  E  R
                  └─────────────────────────T-210─────────────────────────┘
              └──────────────────────────CNBr7──────────────────────────┘

CCGCTCGATGTTCGAGTCGCTGGACCTTAGCTGGAAGCTGCTGCGCATCTTCCCCAAGGAGATGCTGAAGCGCATTCCGCAGGCCGTGATCGACGAGTTCTATTCCCGCGAGGGGCGGCT  1560
 R  S  M  F  E  S  L  D  L  S  W  K  L  L  R  I  F  P  K  E  M  L  K  R  I  P  Q  A  V  I  D  E  F  Y  S  R  E  G  R  L   504

GCAGGACCTCGCGCCTGACACTGCGCTCTAGCCCCGCGCGCCGTGGCACCCCAACACCGGCAGGAACCTACCCTCGGCTCCCGGGTCTCCCCGTCCCTCGCCACCCCTAACCAGCGGCTT  1680
 Q  D  L  A  P  D  T  A  L  *                                                                                              513

TCGCGCCGCCCTCCGCCCTCCGTGGCTCCGAGGTGGTGGGGGGCGCCGCAGTCATCCCTTTCCTCGCTCGATTCCTTTTCCCGCGCTCCATGCCTCCCCCTCAGCTCCCGGTGCTGCGGA  1800

AGAACTGAAGGTTCATGCCTACTCTGACGGGAGCATCTGTATTTTTTATGTTAAAAGCCCACAAAATAAAAATAAAAATGAACTGAGAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA  1920
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FIG. 2. Nucleotide and derived amino acid sequence (single-letter code) of a cDNA encoding the 58-kDa subunit of the vacuolar H^+ pump from human kidney. The deduced amino acid sequence is shown in single-letter code below the nucleotide sequence and the sequences are numbered on the right. Peptide sequences obtained from the bovine 58-kDa subunit are aligned with the deduced human sequence and numbered as in Fig. 1 and Table 1. Asterisk indicates the stop codon and the two polyadenylylation consensus sites are underlined.

bovine and human libraries. Of these, only a library constructed from human kidney RNA yielded positive clones that hybridized with several independent oligonucleotides. The nucleotide and translated amino acid sequences of a selected clone (λPP2a) are shown in Fig. 2. Positions and sequences of the peptides isolated from the 58-kDa subunit of the H^+ pump from bovine brain clathrin-coated vesicles are given below the translated amino acid sequence. These are almost 100% conserved between bovine and human sequences with a few conservative changes. The translated amino acid sequence predicts a protein of 513 amino acids that has a calculated molecular weight of 56,661. There are no outstanding characteristics in its primary amino acid sequence apart from a comparatively high content of hydrophobic residues (35%) that agrees well with the biochemical observation of a strong hydrophobic interaction between the 70- and 58-kDa subunits of the clathrin-coated vesicle H^+ pump (15).

Two bovine peptide sequences (T-206 and CNBr2/3) that were obtained with a yield comparable to that of the other sequences (Table 1) could not be found in the human sequence, even under low stringency homology search conditions. Since the other bovine sequences were found to be highly conserved in the human sequence, it appears unlikely that these sequences are not conserved and are truly derived from the 58-kDa subunit of the bovine H^+ pump. Careful inspection of the protein profiles in the glycerol gradients used for H^+ pump purification indeed suggests that there is a second 58-kDa protein in coated vesicles that is more broadly distributed across the glycerol gradient than the H^+ pump (data not shown). This other 58-kDa protein probably was the source of the unidentified peptide sequences.

Evolution and Structure of the 58-kDa Subunit. The predicted amino acid sequence of the 58-kDa subunit of the human H^+ pump was aligned with those reported for the corresponding subunits of the H^+ pump from plants [*Arabidopsis thaliana* (20)], fungi [*Neurospora crassa* (18)], and Archaebacteria [*Sulfolobus acidocaldarius* (22)]. In Fig. 3, residues that are shared between at least two of the four sequences are boxed. A high degree of conservation is apparent and most of the substitutions observed are conservative. There are three regions in the protein that are not conserved in the four species: the amino and carboxyl termini and a sequence stretch in the middle of the protein. The most conserved regions of the protein contain up to 16 consecutive residues that are identical between the human and archaebacterial sequences. In total, 47% of all residues are invariant between plants, fungi, Archaebacteria, and humans, and 49% of the residues are identical between the archaebacterial and human sequences.

The 58-kDa subunit sequence was also compared to the sequences of the 70-kDa subunit of the vacuolar H^+ pump from plants, fungi, and Archaebacteria, and to the α and β subunits of the F_1-ATPase from *Escherichia coli*, chloroplasts, and mitochondria. These sequences were aligned with each other in all combinations. In the resulting pairwise comparisons, the percentages of identities observed were found to fall between 23% and 27%, and there seemed to be no two subunits that were significantly more homologous to each other than to any other subunits (data not shown). This finding indicates that although the 70- and 58-kDa subunits of the vacuolar H^+ pump are analogous in stoichiometry and nucleotide binding properties to the α and β subunits of F_1-ATPases, there is no simple evolutionary relationship. Three possible pathways of the evolution of the major catalytic subunits of the H^+-ATPases are diagrammed in Fig. 4. Since the F_1 α and β subunits and the vacuolar 70- and 58-kDa subunits are all significantly related to each other, a common evolutionary precursor can be postulated that duplicated twice to form these four subunits. Did the first duplication of the primordial ATPase result in an evolutionarily stable intermediate that then duplicated again to form the current four subunits? This pathway is diagrammed in Fig. 4 *A* and *B*. This would predict that in pairwise comparisons of the four subunits to each other in two of the possible six comparisons the subunits should be more homol-

FIG. 3. Alignment of the amino acid sequences (single-letter code) of the human 58-kDa subunit with the corresponding sequences from fungi, plants, and Archaebacteria. The origin of the sequences is indicated on the left: H, human (Fig. 3); N, fungus [*Neurospora* (18)]; P, plant [*Arabidopsis* (20)]; A, Archaebacterium [*Sulfolobus* (22)], and sequences are numbered on the right. Identical residues present in at least two of the sequences at a given position are boxed.

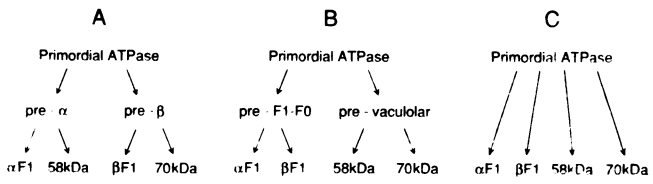

FIG. 4. Schemes for the possible evolution of the catalytic subunits of H^+-pumping ATPases. (*A* and *B*) Models assume the presence of stable evolutionary intermediates. (*C*) Model suggests that the four subunits are derived from a single precursor in two successive duplications that established two independent lineages of ATPases without a stable intermediate.

ogous than in the other four. Since this is not the case, it seems unlikely that the F_1F_0 H^+-ATPases and the vacuolar H^+ pump shared a stable evolutionary precursor. Instead, one has to postulate a third model whereby the hypothetical evolutionary precursor to these four subunits duplicated twice without forming a stable intermediate (Fig. 4*C*). Alternatively, the sequence similarity between the four subunits could be the result of convergent evolution. This explanation cannot be excluded at present but appears to be unlikely in view of the conservation and number of homologous sequences.

DISCUSSION

We have presented structural information about the catalytic subunits of the mammalian vacuolar-type H^+ pump. Partial amino acid sequences of the purified 58- and 70-kDa subunits from bovine brain were obtained, and the full-length amino acid sequence of the human 58-kDa subunit was deduced from the sequence of a cDNA clone. After this study was initiated, the sequences encoding the corresponding subunits from the plant, fungal, and archaebacteria enzymes were published (17–22). We now present the first animal sequences for these major catalytic subunits of the vacuolar H^+ pump. Comparison of the mammalian sequences with those from the other three organisms demonstrates an extraordinary degree of conservation with important evolutionary and structural implications. The 70- and 58-kDa subunits have been demonstrated to participate in ATP hydrolysis and to be tightly bound in a high molecular weight complex that contains three copies of each subunit. The structural constraints on these properties apparently result in an evolutionary conservation that is only paralleled by those of other subunits of high molecular weight complexes such as ribosomes. The strong conservation of the endomembrane H^+ pump in evolution suggests that not only its enzymatic functions but also its assembly into high molecular weight complexes are similar.

In many respects, the vacuolar H^+ pump is structurally similar to the F_1F_0-type H^+-ATPases. Both pumps consist of a high molecular weight heterooligomer that couples a gated H^+-pore to an ATP-hydrolytic or -synthetic site. The α and β subunits of the F_1-ATPase have been compared to the 58- and 70-kDa subunits of the vacuolar H^+ pumps, all of which appear to be present in similar stoichiometries in their respective pumps and to be intimately involved in nucleotide binding and catalysis. The α and β subunits of the F_1-ATPase are weakly related to each other (30), and the 58- and 70-kDa subunits of the vacuolar pumps also show a significant and comparable homology. Interestingly, pairwise comparisons between the four subunit structures in all possible combinations do not demonstrate a significant difference in the relatedness of any given pair as compared to another pair. In Fig. 4, three possible schemes for the evolution of the H^+-ATPases are presented. The lack of preferential relatedness between any pair of subunits provides evidence against a stable intermediate precursor, as suggested by diagrams in Fig. 4 *A* and *B*. It indicates that if the H^+-ATPases are evolutionarily related, the split of the primordial ATPase gene into two subunits must have been followed closely by a further split into four subunits (Fig. 4*C*).

It has been suggested that Archaebacteria do not contain a true F_1F_0-ATPase and use the vacuolar-type ATPase for ATP synthesis (31, 32). This raises the possibility that there exist two prokaryotic lineages, with each containing a different enzymatic system for ATP synthesis. Within each lineage, the enzyme is very highly conserved. With the emergence of eukaryotes, both enzyme systems are incorporated. The low degree of homology between the lineages, on the other hand, agrees well with the different enzymologic properties of the two enzymes. The diversity of functions of mammalian endomembrane H^+ pumps is notable in view of its possible origin as an ATP synthetic prokaryotic enzyme.

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