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Short sequence-paper

Molecular cloning and sequencing of the cDNA for vacuolar H^+-pyrophosphatase from *Chara corallina*[1]

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Abstract

We have cloned a cDNA for vacuolar proton-translocating pyrophosphatase of *Chara corallina* that is one of the closest green algae to the land plants. The deduced protein consists of 793 amino acid residues. Its sequence is 71% identical to the H^+-pyrophosphatases of land plants, and is less than 46% identical to those of marine alga and phototrophic bacterium.

Keywords: cDNA; H^+-Pyrophosphatase; Plant; Proton pump; Vacuolar membrane; (*Chara corallina*)

The H^+-translocating inorganic pyrophosphatase (H^+-PPase) is an electrogenic proton pump that acidifies vacuoles in plant cells [1]. The enzyme is a fine model for research on the coupling mechanism between the pyrophosphate hydrolysis and the active proton transport, because the enzyme consists of a single protein and its substrate is also simple. In addition to these characteristics, H^+-PPase is an interesting enzyme with respect to molecular evolution. It may be a key enzyme for studying the origin of plant vacuoles. To our knowledge, the enzymatic activity of the membrane-bound H^+-PPase has been found only in the chromatophores from the photosynthetic bacterium *Rhodospirillum rubrum* [2,3] except for vacuolar membranes of various plant species [4–7]. Information concerning the primary sequences of H^+-PPases of land plants [8–14] is now available, and recently cDNAs for H^+-PPases of *R. rubrum* [15] and a marine alga *Acetabularia acetabulum* [16] have been cloned. At least, the essential sequences should be conserved among the H^+-PPases from various organisms. In the present study, we cloned a cDNA for H^+-PPase of *Chara corallina* to clarify the functional domain and catalytic residues in comparison with H^+-PPases of higher plants.

Chara was grown in a water tank filled with tap water at about 23–27°C. They were illuminated for 16 h a day with two 20 W fluorescent lamps placed 30 cm over the water surface. Vacuolar membranes

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[1] The nucleotide sequence data reported in this paper will appear in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB018529.

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Fig. 1. The nucleotide sequence of cDNA (CPP1) for *Chara* H^+-PPase and the deduced amino acid sequence. The termination codon is marked with an asterisk. Fourteen putative membrane-spanning domains predicted from the hydropathy profile (TMpred program) are underlined.

were isolated from internode cells of *Chara* as described previously [6].

For preparation of peptide antibodies to *Chara* H^+-PPase, a peptide NARTRSRRGGGVGPAFKAAFR that corresponds to a hydrophilic loop (residues 186–206) between the third and fourth membrane domains of the enzyme was synthesized. An antibody was raised in a rabbit by injection of the peptide conjugated to keyhole limpet hemocyanin (about 2 mg of peptide). Antibodies specific to the catalytic domain of H^+-PPase (DVGADLVGKVE) were prepared as reported previously [17]. Immunostaining was carried out by the standard procedures. The antigens on a PVDF membrane were detected with the peroxidase-linked protein A and an ECL western blotting detection kit (Amersham).

Total RNA was isolated from several hundreds of young internode cells of *C. corallina*, and used for construction of a cDNA library. cDNA was synthesized with a cDNA synthesis kit (Gibco), ligated into phage vector (λZAPII, Stratagene), and then packaged. Recombinant phages (1.2×10^5 pfu) from the library were blotted onto nylon membranes and then subjected to hybridization screening by the standard method [18].

Based on the amino acid sequences of H^+-PPase of mung bean [11], barley [14], *Arabidopsis thaliana* [13] and *A. acetabulum* (unicellular marine alga) [16],

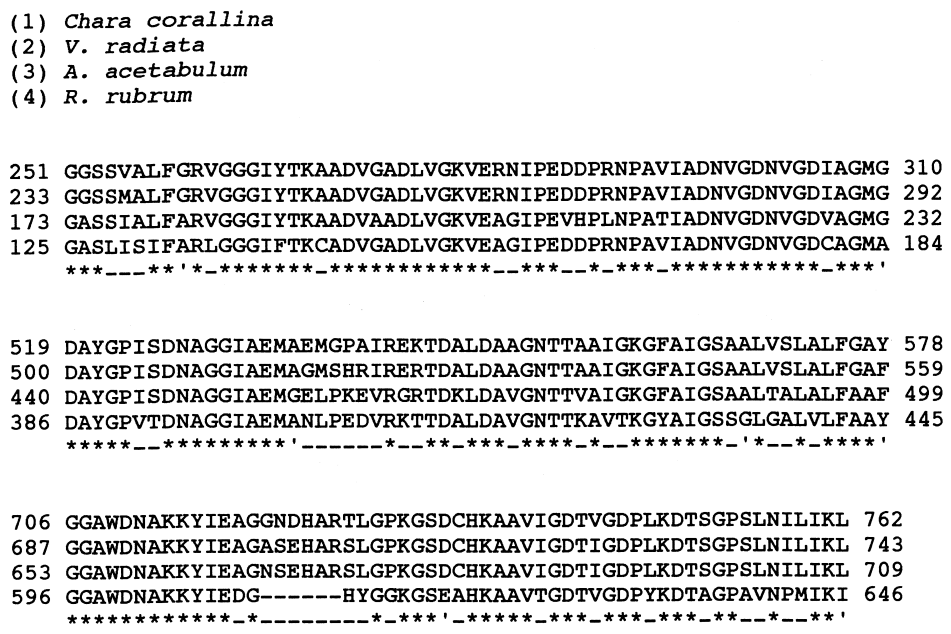

Fig. 2. Alignment among four H^+-PPases of the segments containing the highly conserved motif. H^+-PPases of *C. corallina* (this study), *V. radiata* [11], *A. acetabulum* [16], and *R. rubrum* [15]. The first segment is proposed to be important for PP_i hydrolysis activity. The identical and conserved residues are marked by (*) and (′), respectively.

degenerate oligonucleotide primers were designed for the polymerase chain reaction (PCR) (forward, 5′-GA(CT)AACGC(ACT)GG(ACGT)GG(ACGT)AT-CGC-3′; reverse, 5′-CCGAT(AGT)GC(AG)AA(A-CGT)CCCTT(ACGT)CC-3′). Phage DNA isolated from a cDNA library was used as template. A 120-bp fragment was obtained by PCR with a pair of primers. The reaction mixture contained 2 μM primers, AmpliTaq Gold DNA polymerase (Perkin Elmer Cetus), and 1.5 mM Mg^{2+}. The annealing temperature was gradually decreased by 3°C per three cycles from 65°C to 41°C during 40 cycles (step-down PCR). The obtained PCR product (120 bp) was sequenced, and then used as a DNA probe for hybridization screening. A probe DNA was labeled with DIG-11-dUTP (67 μM, Boehringer). DNA hybridization was carried out at 58°C for 18 h in a hybridization buffer as described previously [11], and then positive plaques were visualized with a DIG detection kit (Boehringer).

DNA sequence analysis was performed by the dideoxy chain-termination method with a Thermo Sequence cycle sequencing kit (Amersham) and a Li-Cor 4000 DNA sequencer (Lincoln, NE). The sequences were aligned using the DNASIS program of the Hitachi Software Engineering (Tokyo, Japan). Hydropathy values were determined by the TMpred program (http://www.isrec.isb-sib.ch/software/TMPRED_form.html) [19].

Total RNA was isolated from *Chara* internodal cells for Northern analysis. RNAs were denatured, electrophoresed in 1.0% agarose gel, and capillary transferred to a nylon membrane (Bio-Rad). A membrane was prehybridized in a hybridization buffer containing 0.02% denatured salmon sperm DNA at 58°C for 1 h and was hybridized with a DIG-labeled probe DNA (10 ng/ml) at 58°C overnight.

Seven positive λZAP II clones were identified by screening the 120 000 phage library with a H^+-PPase cDNA probe. The cDNA probe could be obtained by the step-down PCR. Based on restriction digestion of the insert DNAs and the nucleotide sequences of the DNA fragments, the seven clones appeared to be identical. The longest of the H^+-PPase clones was denoted CPP1, and both of its strands were fully sequenced.

The clone CPP1 consists of 2839 bp upstream of the polyadenylate tail, which includes a 236-bp 5′ leader sequence, followed by an open reading frame of 2382 bp, and finally, a 221-bp 3′-noncoding region. The cDNA has an open reading frame that codes for a protein of 793 amino acids as shown in

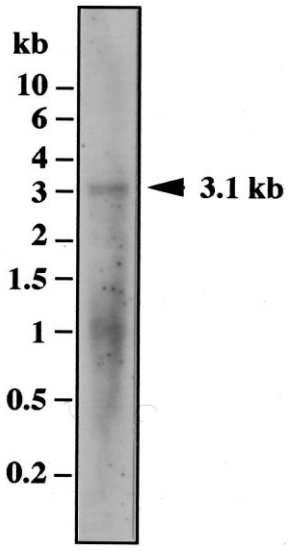

Fig. 3. Northern analysis of *Chara* mRNA with DNA probes for H^+-PPase. The total RNA fraction was prepared from internode cells of *C. corallina*. Then 5 μg of RNA was subjected to Northern analysis. Molecular sizes (kb) of standards are shown on the left.

Fig. 1. Fourteen transmembrane segments were predicted from analysis by the TMPred program. The calculated mass of 82.7 kDa is slightly higher than the H^+-PPases of land plants (about 80 kDa). Especially, the N-terminal part of the *Chara* H^+-PPase is 28 amino acid residues longer than the H^+-PPase of *Vigna radiata* [11]. The amino acid sequence is about 71% identical to that of the H^+-PPases of land plants, such as mung bean [11], *A. thaliana* [13], tobacco [9], rice [12], and barley [14]. In contrast, it is 46% and 35% identical to the H^+-PPases of *A. acetabulum* [16] and *R. rubrum* [15], respectively.

Fig. 2 shows the three segments that are conserved among H^+-PPases of the land plants, *A. acetabulum*, *R. rubrum*, and *C. corallina*. The first conserved segment includes the catalytic domain for the substrate hydrolysis [1,17], and was estimated to be exposed to the cytosol [17]. Indeed, the antibody specific to a peptide DVGADLVGKVE (residues 271–281 for the *Chara* H^+-PPase) that is located in the first conserved segment strongly inhibited the activity of the mung bean H^+-PPase [17]. Interestingly, in this segment there is an NPA sequence that is a common motif in the aquaporin family. Two NPA motifs in a single aquaporin polypeptide are thought to form a filter of water channels, although the physiological function of NPA motif in H^+-PPase is unclear. The second conserved segment is also located in the hydrophilic loop. The third homologous region in the carboxyl-terminal part is also conserved among H^+-PPases of various organisms. This is the most hydrophilic region with a dozen charged residues. The segment is estimated to face the cytosolic side. In our preliminary experiment, the individual replacement of Glu residues in this segment of mung bean H^+-PPase to Ala residues resulted in the loss of the enzymatic activity (Y.N., M.M., unpublished data). Therefore, the C-terminal conserved segment may also play a critical role in the enzymatic function together with the other conserved segments. The recent extensive investigations [20–22] on the structure–function relationship of H^+-PPase may provide information for our understanding of the coupling mechanism of this enzyme between the PP_i hydrolysis and the proton translocation.

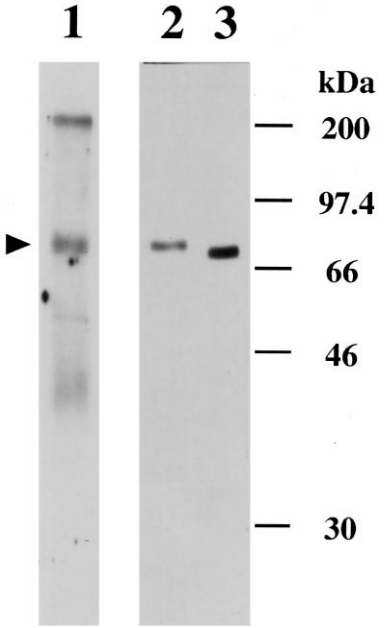

Fig. 4. Immunoblotting of *Chara* H^+-PPase with two antibodies. Vacuolar membranes were prepared from *Chara* internode cells (lanes 1 and 2) and mung bean hypocotyls (lane 3), and then subjected to immunoblot analysis with the two different antibodies. Lane 1, immunoblot with the antibody to a peptide corresponding to the N-terminal part (NARTRSRRGGGVGPAFKAAFR) of *Chara* H^+-PPase. Lanes 2 and 3, immunoblot with the antibodies to a peptide corresponding to the catalytic site of H^+-PPase [17]. The arrowhead indicates the position of *Chara* H^+-PPase of 74 kDa.

In order to examine the expression of CPP1 in *Chara* plants, we prepared the total RNA fraction from young *Chara* plants and then performed northern hybridization analysis. V-PPase mRNA of about 3.1 kb was detected in a gel blot of the RNA fraction (Fig. 3), and this size corresponds to that of the cDNA. The expression of CPP1 was confirmed by immunoblot analysis. We prepared an antibody specific to a peptide that is unique to *Chara* H^+-PPase, and another antibody specific to a peptide that corresponds to the catalytic domain of H^+-PPase (DVGADLVGKVE) [17]. H^+-PPase in the vacuolar membrane from *Chara* internode cells reacted with both antibodies (Fig. 4). In addition to a 74-kDa band of H^+-PPase, two immunostained bands were observed at about 200 and 40 kDa. The corresponding authentic peptide competitively inhibited the reaction of the *Chara* H^+-PPase of 74 kDa, but not that of the two bands of 200 and 40 kDa, with the antibody to *Chara*-enzyme-specific sequence. The results clearly confirm the presence of the translational product of CPP1 in the *Chara* vacuolar membrane.

The molecular sequence data from nuclear-, plastid-, and mitochondrial genes support a sister-group relationship between charophytes and land plants [23,24]. Among green algae, *Chara* is thought to be the closest relative to the land plants. This relationship is supported by the evolution of flavonoid biosynthesis. The sequence of *Chara* H^+-PPase is more identical to the enzymes of land plants rather than that of *A. acetabulum* or *R. rubrum*. The sequence similarity of H^+-PPases indicates that *Chara* is evolutionarily close to the land plants. The prasinophytes have been proposed to be the ancestors of the charophytes [24]. A single-celled alga similar to *Mesostigma viride* (the Prasinophyceae) may have evolved to the charophytes, to the bryophytes and then to the other land plants [24]. Since H^+-PPase is a characteristic enzyme of plant vacuoles, the molecular evolution of this enzyme may be closely related to the evolution of plant vacuoles. Determination of the primary sequences of H^+-PPases of the other organisms, such as *M. viride* and the bryophytes, should provide critical information for the evolution of H^+-PPase. Further research on vacuolar H^+-PPase is needed to clarify the origin and evolution of plant vacuoles.

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References

[1] P.A. Rea, R.J. Poole, Vacuolar H^+-translocating pyrophosphatase, Annu. Rev. Plant Physiol. Plant Mol. Biol. 44 (1993) 157–180.

[2] P. Nyrén, B.F. Nore, A. Strid, Proton-pumping *N,N′*-dicyclohexylcarbodiimide-sensitive inorganic pyrophosphate synthase from *Rhodospirillum rubrum*: Purification, characterization, and reconstitution, Biochemistry 30 (1991) 2883–2887.

[3] B.F. Nore, Y. Sakai-Nore, M. Maeshima, M. Baltscheffsky, P. Nyrén, Immunological cross-reactivity between proton-pumping inorganic pyrophosphatases of widely phylogenic separated species, Biochem. Biophys. Res. Commun. 181 (1991) 962–967.

[4] M. Maeshima, S. Yoshida, Purification and properties of vacuolar membrane proton-translocating inorganic pyrophosphatase from mung bean, J. Biol. Chem. 264 (1989) 20068–20073.

[5] M. Ikeda, S. Satoh, M. Maeshima, Y. Mukohata, C. Moritani, A vacuolar ATPase and pyrophosphatase in *Acetabularia acetabulum*, Biochim. Biophys. Acta 1070 (1991) 77–82.

[6] M. Maeshima, T. Mimura, T. Sato, Distribution of vacuolar H^+-pyrophosphatase and a membrane integral protein in a variety of green plants, Plant Cell Physiol. 35 (1994) 323–328.

[7] K. Takeshige, M. Tazawa, A. Hager, Characterization of the H^+-translocating adenosine triphosphatase and pyrophosphatase of vacuolar membranes isolated by means of a perfusion technique from *Chara corallina*, Plant Physiol. 86 (1988) 1168–1173.

[8] Y. Kim, E.J. Kim, P.A. Rea, Isolation and characterization of cDNAs encoding the vacuolar H^+-pyrophosphatase of *Beta vulgaris*, Plant Physiol. 106 (1994) 375–382.

[9] J. Lerchl, S. König, R. Zrenner, U. Sonnewald, Molecular cloning, characterization and expression analysis of isoforms encoding tonoplast-bound proton-translocating inorganic pyrophosphatase in tobacco, Plant Mol. Biol. 29 (1995) 833–840.

[10] C. Maruyama, Y. Tanaka, N.T. Mitsuda, K. Takeyasu, M. Yoshida, M.H. Sato, Structural studies of the vacuolar H^+-pyrophosphatase: sequence analysis and identification of the residues modified by fluorescent cyclohexylcarbodiimide and maleimide, Plant Cell Physiol. 39 (1998) 1045–1053.

[11] Y. Nakanishi, M. Maeshima, Molecular cloning of vacuolar H^+-pyrophosphatase and its developmental expression in growing hypocotyl of mung bean, Plant Physiol. 116 (1998) 589–597.

[12] Y. Sakakibara, H. Kobayashi, K. Kasamo, Isolation and characterization of cDNAs encoding vacuolar H^+-pyrophosphates isoforms from rice (*Oryza sativa* L.), Plant Mol. Biol. 31 (1996) 1029–1038.
[13] V. Sarafian, Y. Kim, R.J. Poole, P.A. Rea, Molecular cloning and sequence of cDNA encoding the pyrophosphate-energized vacuolar membrane proton pump (H^+-PPase) of *Arabidopsis thaliana*, Proc. Natl. Acad. Sci. USA 89 (1992) 1775–1779.
[14] Y. Tanaka, K. Chiba, M. Maeda, M. Maeshima, Molecular cloning of cDNA for vacuolar membrane proton-translocating inorganic pyrophosphatase in *Hordeum vulgare*, Biochem. Biophys. Res. Commun. 190 (1993) 1110–1114.
[15] M. Baltscheffsky, S. Nadanaciva, A. Schultz, A pyrophosphate synthase gene: Molecular cloning and sequencing of the cDNA encoding the inorganic pyrophosphate synthase from *Rhodospirillum rubrum*, Biochim. Biophys. Acta 1364 (1998) 301–306.
[16] M. Ikeda, M.H. Rahman, C. Moritani, K. Umami, Y. Tanimura, R. Akagi, Y. Tanaka, M. Maeshima, Y. Watanabe, A vacuolar H^+-pyrophosphatase in *Acetabularia acetabulum*: Molecular cloning and comparison with higher plants and a bacterium, J. Exp. Bot. 50 (1999) 139–140.
[17] A. Takasu, Y. Nakanishi, T. Yamauchi, M. Maeshima, Analysis of the substrate binding site and carboxyl terminal region of vacuolar H^+-pyrophosphatase of mung bean with peptide antibodies, J. Biochem. 122 (1997) 883–889.
[18] J. Sambrook, E.F. Fritsch, T. Maniatis, Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, 1989.
[19] K. Hofmann, W. Stoffel, TMbase – A database of membrane spanning protein segments, Biol. Chem. Hoppe-Seyler 347 (1993) 166. (Abstract)
[20] A.R. Façanha, L. de Meis, Reversibility of H^+-ATPase and H^+-pyrophosphatase in tonoplast vesicles from maize coleoptiles and seeds, Plant Physiol. 116 (1998) 1487–1495.
[21] R. Gordon-Weeks, S.H. Steele, R.A. Leigh, The role of magnesium, pyrophosphate, and their complexes as substrates and activators of the vacuolar H^+-pumping inorganic pyrophosphatase, Plant Physiol. 111 (1996) 195–202.
[22] E.J. Kim, R.G. Zhen, P.A. Rea, Site-directed mutagenesis of vacuolar H^+-pyrophosphatase: Necessity of Cys^{634} for inhibition by maleimides but not catalysis, J. Biol. Chem. 270 (1995) 2630–2635.
[23] L.E. Graham, Origin of Land Plants, John Wiley, New York, 1993.
[24] D. Bhattacharya, L. Medlin, Algal phylogeny and the origin of land plants, Plant Physiol. 116 (1998) 9–15.