Available online at www.sciencedirect.com
SCIENCE DIRECT
Biochimica et Biophysica Acta 1717 (2005) 89 – 96
BIOCHIMICA ET BIOPHYSICA ACTA
BBA
http://www.elsevier.com/locate/bba

Proteolipid of vacuolar H^+-ATPase of *Plasmodium falciparum*: cDNA cloning, gene organization and complementation of a yeast null mutant☆

Shouki Yatsushiro [a,*], Shinya Taniguchi [a], Toshihide Mitamura [b], Hiroshi Omote [a], Yoshinori Moriyama [a]

[a] *Department of Biochemistry, Faculty of Pharmaceutical Sciences, Okayama University, Okayama 700-8530, Japan*
[b] *Institute of Microbial Disease, Osaka University, Osaka 565-0871, Japan*

Received 17 January 2005; received in revised form 28 August 2005; accepted 30 August 2005
Available online 15 September 2005

Abstract

Vacuolar H^+-ATPase (V-ATPase), an electrogenic proton pump, is highly expressed in *Plasmodium falciparum*, the human malaria parasite. Although V-ATPase-driven proton transport is involved in various physiological processes in the parasite, the overall features of the V-ATPase of *P. falciparum*, including the gene organization and biogenesis, are far less known. Here, we report cDNA cloning of proteolipid subunit *c* of *P. falciparum*, the smallest and most highly hydrophobic subunit of V-ATPase. RT-PCR analysis as well as Northern blotting indicated expression of the proteolipid gene in the parasite cells. cDNA, which encodes a complete reading frame comprising 165 amino acids, was obtained, and its deduced amino acid sequence exhibits 52 and 57% similarity to the yeast and human counterparts, respectively. Southern blot analysis suggested the presence of a single copy of the proteolipid gene, with 5 exons and 4 introns. Upon transfection of the cDNA into a yeast null mutant, the cells became able to grow at neutral pH, accompanied by vesicular accumulation of quinacrine. In contrast, a mutated proteolipid with replacement of glutamate residue 138 with glutamine did not lead to recovery of the growth ability or vesicular accumulation of quinacrine. These results indicated that the cDNA actually encodes the proteolipid of *P. falciparum* and that the proteolipid is functional in yeast.

Keywords: V-ATPase; Proteolipid; Subunit *c*; Malaria; *Plasmodium falciparum*; Complementation; Yeast null mutant

1. Introduction

V-ATPase is a multisubunit protein complex and is composed of two different sectors, a catalytic V1 sector comprising eight subunits and a membranous V0 sector comprising at least three subunits, in various endomembrane systems in eukaryotes and in the plasma membranes of some bacteria (reviewed in Refs. [1,2]). V-ATPase is an electrogenic proton pump, and forms an electrochemical gradient of protons across membranes at the expense of ATP hydrolysis, which plays important roles in various physiological and pathological processes, such as storage of neurotransmitters in synaptic vesicles, maturation of peptide hormones in secretory granules, digestion of bone matrix by osteoclasts, infection by viruses and so on [1,2].

Plasmodium falciparum is the parasitic unicellular protozoan that causes malaria, one of the most serious infectious diseases for human beings. *P. falciparum* highly expresses V-ATPase in food vacuoles, counterparts of mammalian lysosomes and yeast vacuoles, small clear vesicles and the plasma membrane [reviewed in refs. 3 and 4]. V-ATPase energizes these organelles through active transport of protons, and the resultant acidic pH of the limited area of extracellular space and membrane potential across the membrane are responsible for the digestion of hemoglobin, and accumulation of ions and antimalarial agents in food vacuoles, and uptake of nutrients through the plasma membrane [3–15]. For instance, pantothenate is taken up by the parasite through H^+-coupled active transport [10,11]. The uptake of choline through the plasma

Abbreviations: RT-PCR, reserve transcriptional polymerase chain reaction; V-ATPase, vacuolar H^+-ATPase

☆ The nucleotide sequence data for the *P. falciparum* proteolipid have been submitted to GenBank under accession number AY725216.

* Corresponding author. Present address: Department of Biological Pharmacy, School of Pharmacy, Shujitsu University, Okayama 703-8516, Japan. Tel.: +81 86 271 8407; fax: +81 86 271 8320.
E-mail address: shouki@shujitsu.ac.jp (S. Yatsushiro).

0005-2736/$ - see front matter © 2005 Elsevier B.V. All rights reserved.
doi:10.1016/j.bbamem.2005.08.011

membrane is driven by a membrane potential [12,13]. Although these findings indicate the essential role(s) of V-ATPase in the life of the malaria parasite, little is known about the overall features of the V-ATPase in the malaria parasite. It is unknown whether or not subunits other than subunits *A* and *B* are actually expressed in the malaria parasite [6,7]. The details of the subunit composition, gene organization of the individual subunits, biogenesis and sorting are also unknown.

To elucidate all the features of the V-ATPase in the malaria parasite, we have tried to clone cDNAs of the individual subunits of the V-ATPase from *P. falciparum*. Here, we report cDNA cloning of the proteolipid, the smallest hydrophobic subunit comprising the V0 proton channel, of *P. falciparum*. To our surprise, the cDNA of *P. falciparum* without any codon usage modification complements a yeast null mutant.

2. Materials and methods

2.1. P. falciparum cells

P. falciparum strains CDC 1, which is sensitive to chloroquine, and K1, which is resistant to chloroquine, were obtained, and the cells were cultured in RPMI 1640 medium (Gibco, NY) containing 10% O^+ human serum and 0.2% $NaHCO_3$, at a hematocrit of 5%, according to the established procedure [16]. Erythrocytes exhibiting 6–8% parasitemia were added to each plate in 10 ml of culture medium to give a final hematocrit of 10%. Then, the plates were incubated at 37 °C under 5% CO_2, 5% O2 and 90% N_2 gas. Then, *P. falciparum* cells (about 10^9 cells) were isolated by saponin treatment and washed twice with phosphate-buffered saline [16].

2.2. RT-PCR

Total RNA extracted from parasitized erythrocytes (1 μg each) was transcribed into cDNA in a final volume of 20 μl of reaction buffer containing 0.5 mM dNTP, 10 mM dithiothreitol, 100 pmol of random octamers, and 200 units of Molony murine leukemia virus reverse transcriptase (Amersham). After incubation for 1 h at 42 °C, the reaction was terminated at 90 °C for 5 min. For PCR amplification, the 100-fold diluted synthesized cDNA solution was added to the reaction buffer containing 0.12 mM dNTPs (30 μM each dNTP), 25 pmol of primers, and 1.5 units of Ampli Taq-Gold polymerase (Perkin Elmer). Thirty-five temperature cycles were conducted as follows: denaturation at 94 °C for 30 s, annealing at the temperature specific for each set of primers for 30 s, and extension at 72 °C for 30 s. Based on the known nucleotide sequences for man (GenBank accession number, MN001694), cow (M61709), *Drosophila* (X55979), *S. cerevisiae* (Z98598) and *A. sativa* (M73232) [17–20], the following primers were constructed: sense, 5′-AA(A/T)(G/C)(A/T)AT(A/T)GT(A/T)CC(A/T)GT(A/T)GT(A/T)ATGGC(A/T)GG-3′, and anti sense, 5′-CT(A/T)AC(A/T)CC(A/T)GCATC(A/T)CC(A/T)AC(A/T)AT(A/T) CC(A/T)AT-3′. For amplification of the parasite actin-1 gene, as a control, the following primers were used (GenBank accession number, M22719) [21]: sense, 5′-GCAGCAGGAATCCACACAAC-3′ (bases 1119–1138), and antisense, 5′-GTGGACAATACTTGGTCCTG-3′ (bases 1402–1421).

2.3. Southern and Northern blotting

Genomic DNA (4 μg) isolated from parasitized erythrocytes was digested with the indicated restriction enzymes, and then separated electrophoretically on a TE agarose gel (0.8%) and transferred to a nylon membrane (Amersham). The immobilized genomic DNA was then probed with a fragment of the cDNA of the V-ATPase proteolipid (bases 190–402) amplified as above and labeled with [α-^{32}P]dATP by random priming. After extensive washing, the membrane was subjected to autoradiography on a BAS 1000 imaging plate (Fuji Film Co.). For Northern analysis, total RNA (25 μg) isolated from parasitized erythrocytes was separated on a formaldehyde agarose gel (1%) and then transferred to a nylon membrane (Amersham). The immobilized RNA was probed with the cDNA fragment of the V-ATPase proteolipid as described above. After extensive washing, the membrane was subjected to autoradiography on a BAS 1000 imaging plate (Fuji Film Co.).

2.4. Cloning of cDNA encoding the proteolipid

cDNA encoding the *P. falciparum* V-ATPase proteolipid was screened from a *P. falciparum* cDNA library (MRA-61) kindly donated by MR4 (Malaria Research and Reference Reagent Resource Center, VA), using an amplified DNA fragment (bases 190–402 or 41–238) according to the published procedure [22]. Two positive clones containing a full reading frame were obtained from 10^5 clones, whose nucleotide sequences were found to be identical (see Fig. 2).

RT-PCR products encoding the full reading frame of the proteolipid subunit were amplified from total RNA extracts of strains CDC 1 and K1 using a pair of primers: sense, 5′-AAATGCGACAATGTGATCCTAATTC-3′ (bases 39–63) and antisense, 5′-TACACATTATAGGGTGTACATAATTTTGG-3′ (bases 511–539). The DNA sequence was determined by direct sequencing of these RT-PCR products.

2.5. Gene manipulations and site-directed mutagenesis

*Kpn*1 and *Sac*1 restriction sites were added at the terminals of the cDNA of the *P. falciparum* proteolipid, which was subsequently subcloned into the pYES3/CT yeast expression vector (Invitrogen, CA). Point mutations were introduced into the parasite cDNA by the overlap extension method according to the published procedure [23] with the following oligonucleotide: E138Q: 5′-GATTCTTGTTTTTTCTCAAACCTTAGC-3′. The identity of the mutated cDNAs was confirmed by sequencing.

2.6. Complementation test with yeast cells

S. cerevisiae strain W303-1B (Matα, leu2, his3, ade2, trp1, ura3) was used as the wild type cell strain. W303-1B ΔVma3 (VMA3::URA3), kindly supplied by Dr. N. Nelson (Tel Aviv University, Israel), was used as the V-ATPase proteolipid null strain. Yeast cells were grown in YPD medium containing 1% yeast extract, 2% peptone, and 2% dextrose, pH 5.5, at 30 °C. Transfection of the *P. falciparum* cDNA into yeast cells was carried out by the lithium acetate method [24]. Staining with quinacrine was performed as described [25], and observed under an Olympus FV-300 confocal laser microscope.

2.7. Other procedures

Total RNA and genomic DNA were extracted from the cells with a TRIZOL Reagent and DNAZOL Reagent Kits, respectively, according to the published procedures [22]. The DNA sequences were determined by a standard method using a BigDye Terminator Sequencing Kit (PE Biosystems) [22].

3. Results

3.1. Expression of the V-ATPase proteolipid

A pair of primers was designed to detect the expression of proteolipid subunit *c* in *P. falciparum* cells based on the nucleotide sequences of the bovine, *Drosophila*, yeast and plant genes. We observed that an about 200 bp DNA fragment was specifically amplified on RT-PCR from total extracted RNA of parasitized erythrocytes infected by either the CDC 1 or K1 strain (Fig. 1A). No amplification was

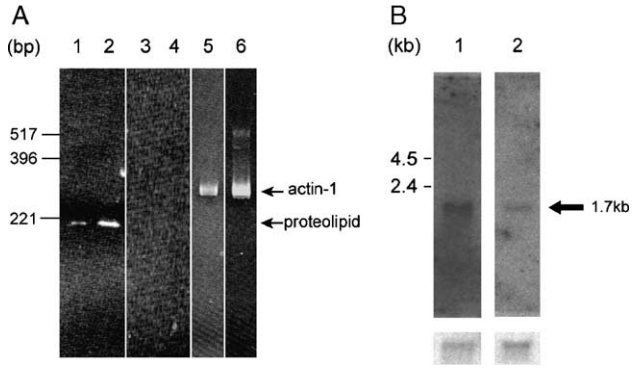


Fig. 1. Expression of the gene of the proteolipid in *P. falciparum*. (A) RT-PCR detection of transcripts for the proteolipid gene in *P. falciparum* CDC 1 (lanes 1, 3 and 5), and the K1 strain (lanes 2, 4 and 6). Actin-1 transcripts were amplified as a control (lanes 5 and 6). No amplified products were obtained without the RT reaction (lanes 3 and 4). The positions of transcripts for the proteolipid and actin-1 are indicated by arrows. (B) Northern blotting indicates expression of the proteolipid gene in *P. falciparum*, CDC 1 strain (lane 1) and K1 strain (lane 2). As a loading control, hybridization of probes specific for actin-1 transcripts was performed on the same RNA blots (lower panel). Size markers were also included.

observed when the RT reaction was omitted (Fig. 1A). Northern blot analysis was carried out using the amplified fragment as a probe. We detected a 1.7-kb fragment in the total RNA extracts of both the CDC 1 and K1 strains (Fig. 1B). These results clearly demonstrate that proteolipid subunit *c* of the V-ATPase is actually expressed in chloroquine-sensitive and resistant *P. falciparum* cells.

3.2. cDNA cloning and Structure of the P. falciparum V-ATPase Proteolipid

Successively, a cDNA clone encoding the entire V-ATPase proteolipid was isolated from the MR4 cDNA library using the 200-bp fragment amplified on RT-PCR as a probe. The deduced amino acid sequence comprises 165 residues of the *P. falciparum* proteolipid subunit (Fig. 2A). The *P. falciparum* proteolipid is a hydrophobic peptide and spans the membrane four times, as indicated by hydropathy plot (Figs. 2B and C). This is a common feature of proteolipid subunit *c* of V-ATPases in all organisms [1,2]. Amino acid alignment with known sequences revealed sequence identity of 68.5% with *Avena*, and slightly less with animals and yeast (Fig. 2D). The glutamate residue in the 4th transmembrane helix is also conserved in the *P. falciparum* proteolipid (Fig. 2C). This glutamate residue acts as a proton acceptor in the proton channel of the V0 complex [1,2]. *N*,*N*′-dicyclohexylcarbodiimide (DCCD) can bind to this glutamate residue and abolishes V-ATPase activity in all organisms so far examined [1,2]. These results suggest that the cloned proteolipid subunit acts as a component of the V-ATPase.

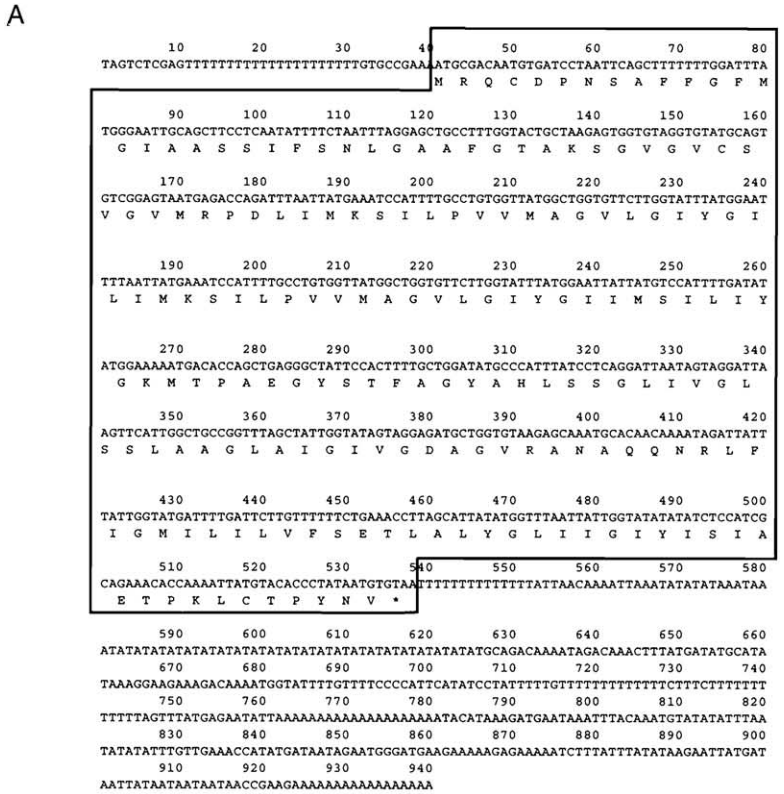
To determine whether or not the proteolipid subunit is involved in the chloroquine sensitivity difference, the DNA sequences of the proteolipid subunits from the CDC 1 and K1 strains were determined. For this purpose, we first amplified the 501-bp fragment by RT-PCR from total RNA extracts of the CDC 1 and K1 strains, which encodes the full length proteolipid subunit (Fig. 2A, boxed region). Then, DNA sequencing of these fragments was performed, they being found to be identical to each other. These results indicated that there is no difference in the proteolipid between the CDC 1 and K1 strains. Identical sequence was also obtained for proteolipid cDNAs between the Dd2 and K1 strains (not shown).

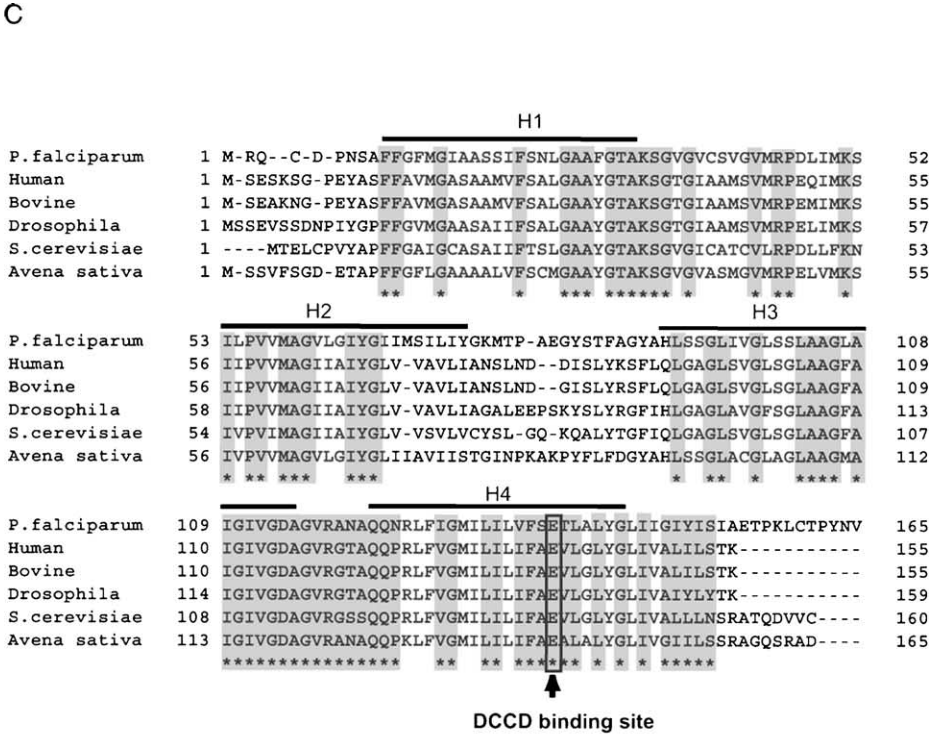
3.3. Gene structure of the P. falciparum V-ATPase proteolipid

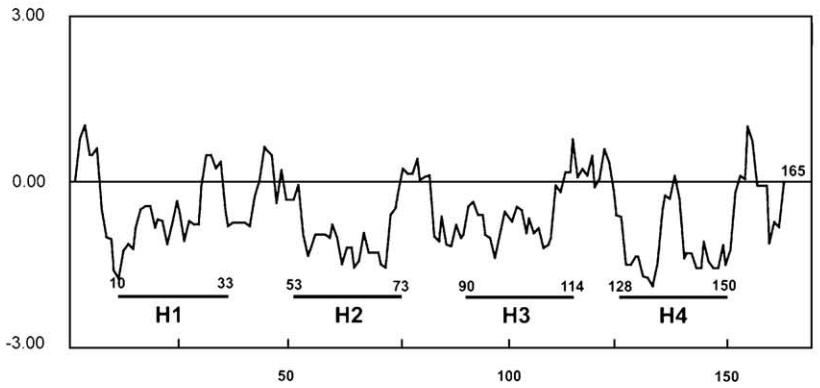
Southern blot analysis of genomic DNA with various restriction enzymes revealed the presence of a single positive DNA band for both CDC 1 and K1 strains (Fig. 3A). The size of these fragments is consistent with the results of genomic sequence analysis, suggesting the presence of a single gene for the proteolipid in CDC 1 and K1 cells. Based on the nucleotide sequences of the genome and cloned cDNA, and the results of Southern blot analysis, the structure of the gene-encoded proteolipid subunit *c* was reconstructed, as shown in Fig. 3B. The proteolipid gene is localized at PFE0965c on chromosome 5, with 5 exons and 4 introns. The exon/intron boundary and overall gene structure are consistent with the gene structure deduced in the genome project [26].

3.4. Functional complementation with a yeast proteolipid null mutant

It would be interesting to determine whether or not *P. falciparum* proteolipid subunit *c* is functional. We examined this by complementation assaying with a yeast proteolipid subunit *c* null mutant. Because the yeast proteolipid is essential for the V-ATPase function, loss of this subunit causes multiple deleterious phenotypes of yeast such as no acidification of the intravacuolar space, high Ca^{2+} sensitivity, pH-dependent growth and an abnormal vacuole morphology [25,27,28]. Thus, these phenotypes could be used as indicators of V-ATPase activity. As shown in Fig. 4A, the yeast null mutant did not grow under neutral pH conditions, consistent with previous observations [25,27,28]. The same strain expressing the authentic proteolipid grew very well (Fig. 4A). Surprisingly, the cDNA clone from *P. falciparum* overcame the deleterious phenotypes of the null mutant despite the high codon usage difference. The mutant yeast cells had small vacuoles compared with those of the wild-type and did not accumulate quinacrine in the vacuoles. The cDNA clone derived from *P. falciparum* restored the vacuole morphology (Fig. 4B, upper panel). Transfection of the cDNA also increased the vesicular accumulation of quinacrine, although the major sites of quinacrine accumulation are small particles, and the level of quinacrine in the vacuoles is not high (Fig. 4B, lower panel). Replacement of the essential glutamate residue with glutamine E138Q, which generates a nonfunctional V-ATPase [25], did not complement the yeast null mutant (Figs. 4A and B). These results indicate that the cDNA clone from *P. falciparum* compliments yeast null mutant, at least in part, and that the *P. falciparum* proteolipid







D

	ORF (%)	Amino acids (%)
Human	54.2	56.6
Bovine	53.5	57.2
Drosophila	56.7	61.8
S.cerevisiae	59.9	51.9
Avena sativa	57.2	68.5

Fig. 2. Cloning of the cDNA-encoded proteolipid of *P. falciparum.* (A) Putative amino acid sequence of the proteolipid. The ORF is boxed. (B) Hydropathy plot of the proteolipid [31]. The four transmembrane helices are denoted as H1, 2, 3 and 4. (C) Alignment of the amino acid sequences of proteolipids from various sources. The amino acid sequence of the proteolipid of *P. falciparum* (top line) is aligned with those of other origins, as indicated. Asterisks indicate conserved amino acid residues. The DCCD-binding glutamate residue is indicated by an arrow. (D) The overall identities of gene sequences and amino acid sequences.

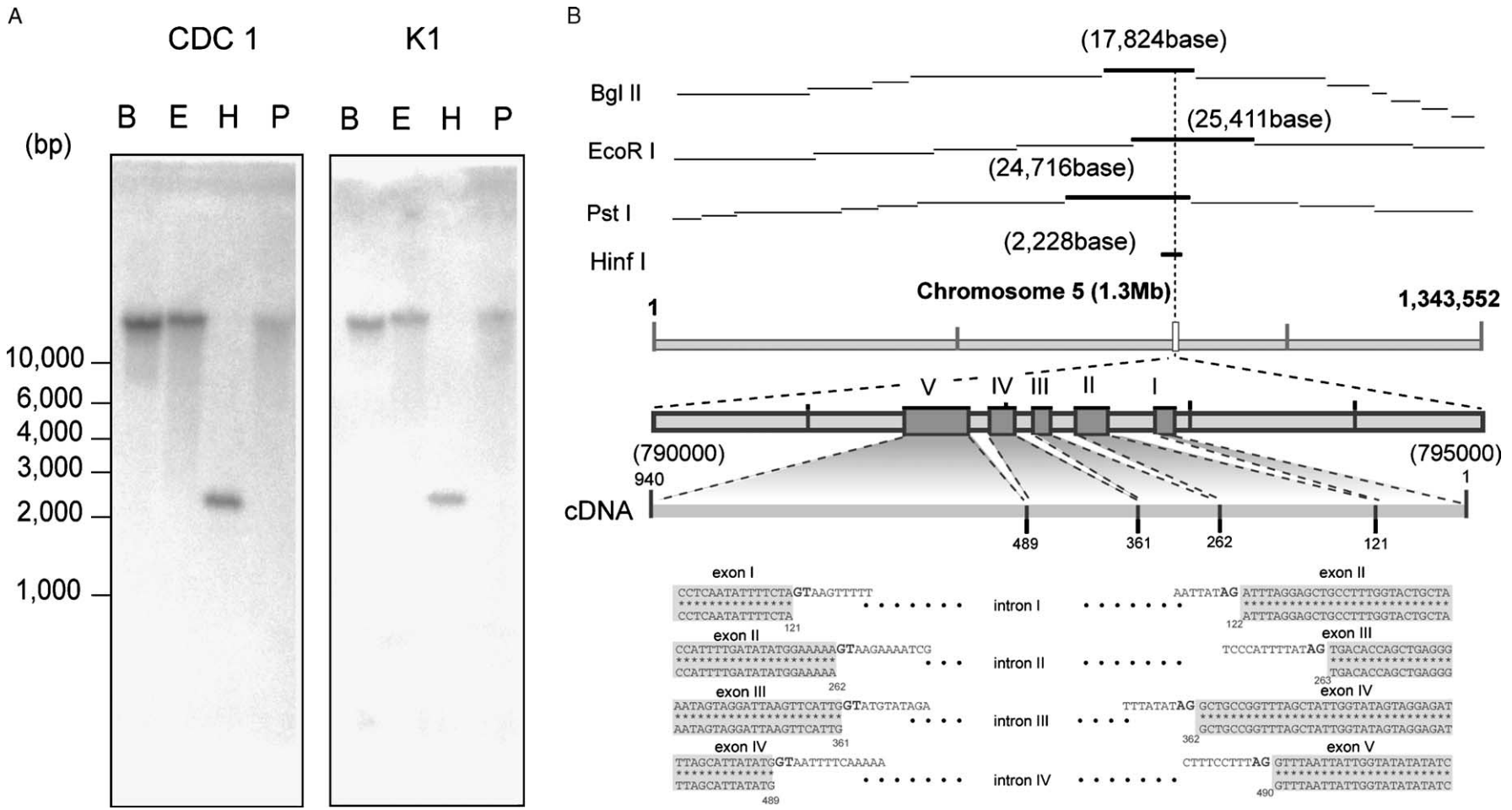


Fig. 3. Gene organization of the *P. falciparum* proteolipid. (A) Southern blotting analysis of *P. falciparum* proteolipid genes. Genomic DNA isolated from CDC 1 and K1 was probed with the amplified product obtained on RT-PCR. The letters of the top of the autoradiograms indicate the restriction enzymes used for digestion of the genomic DNA: B, *Bgl*II; E, *Eco*RI; H, *Hin*fI; and P, *Pst*I. Size markers were also included. (B) Deduced gene structure of the *P. falciparum* proteolipid. The top four lines labeled *Bgl*II, *Eco*RI, *Pst*I and *Hin*fI show fragments of genomic DNA generated with each restriction enzyme. The dashed vertical line indicates the position of the probe. The dark gray boxes on the central horizontal bar are exons. Exon/intron boundaries are indicated in the sequences at the bottom.

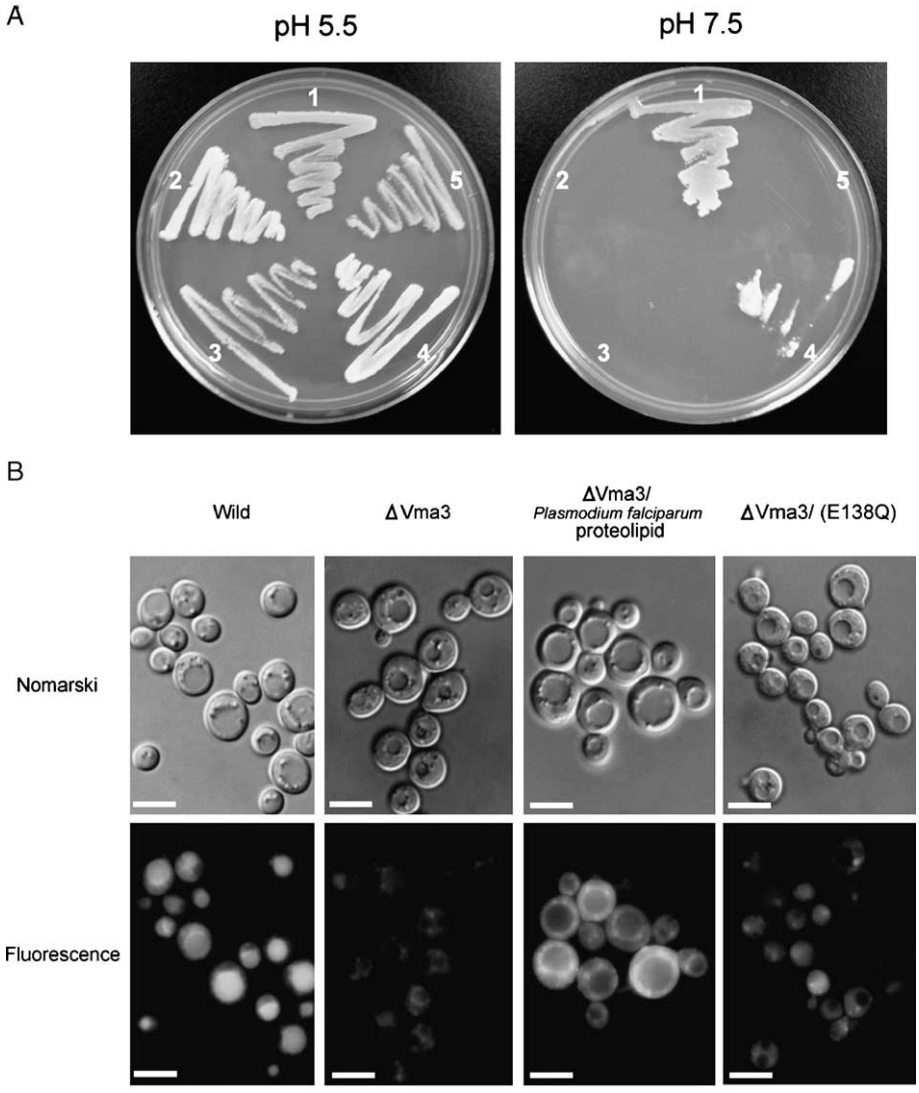


Fig. 4. Complementation of the yeast null mutant. (A) Growth of the wild type and mutant yeast strains at pH 5.5 and 7.5. 1, Wild type (303-1B); 2, yeast null mutant (VMA3::URA3); 3, ΔVma3/pYES3 (no insert); 4, ΔVma3/pYES3+*Plasmodium* proteolipid gene; 5, ΔVma3/pYES3+Pf proteolipid mutant (E138Q) gene. (B) Nomarski images (top panels) and quinacrine fluorescence images (bottom panels) of the wild and mutant yeast strains. Bar=5 μm.

is functional as part of the proton channel of the V-ATPase in yeast.

4. Discussion

Based on the results of the malaria genome project, we know that the *P. falciparum* genome contains genes encoding essentially all subunits of the V1 and V0 sectors [4]. The only exception is the subunit c′ gene, which has been shown to be an essential subunit in yeast and *C. elegans* [29,30], but it was not found in the *Plasmodium* genome. Although this supports the idea that the authentic V-ATPase is functional in the malaria parasite, substantial information on each V-ATPase subunit is still necessary to elucidate the structure, function and physiological and pathological relevance of the V-ATPase complex in the malaria parasite. Such studies, not only on the V-ATPase complex but also almost all other proteins, have always presented difficulties, mainly because the application of various standard biochemical and molecular biological approaches is difficult due to the extremely high AT ratio of the *Plasmodium* genome.

Here, we have reported on cDNA of proteolipid subunit *c* of the V-ATPase of *P. falciparum*. We obtained firm biochemical and molecular biological evidence that the cDNA is actually expressed in *P. falciparum* cells. The deduced amino acid sequences of the individual genes indicated that there is some similarity with counterpart subunits of plant and animal origin, and the similarity is much greater with those of plant origin than those of animal origin. It is noteworthy that there are no changes in the sequences of the coding regions of the genes encoding subunits *A*, *B*, and *c* between a few chloroquine-sensitive strains and chloroquine-resistant strains, suggesting that V-ATPase is not involved in the chloroquine resistance phenotype [[6],[7] and this study].

Subunit c is a major component of V0, the membrane proton channel sector. Genetic and biochemical studies have proven that the other two kinds of subunit c-like proteolipids called c′ and *c″* are independent of each other as to formation of the

holoenzyme moiety and proton transport activity [1,2]. The *P. falciparum* genome project indicated the presence of a gene encoding subunit c″, which is located at MAL13p 1.271 on chromosome 13, with five exons [5,26]. This gene encodes 181 amino acid residues, the similarity being 48.8 and 44.6% to those in yeast and mouse [31,32], respectively. The homology between subunit c″ and subunit c of *P. falciparum* is not high, being 29% based on the deduced amino acid sequence. In contrast, we could not detect a gene encoding the c′ subunit in the genome of *P. falciparum*. Further study is necessary to determine whether *P. falciparum* cells possess a counterpart of the c′ gene product.

The surprising finding in this study was that the cDNA can complement a yeast null mutant: the cDNA of the *P. falciparum* proteolipid partially restored the growth, the morphology of vacuoles, and the accumulation of quinacrine, an indicator of intravesicular acidity, of the yeast null mutant. This indicates that the *Plasmodium* proteolipid is actually expressed in yeast cells, and that the proteolipid constitutes the functional V-ATPase complex. To our knowledge, this is the second case of the expression of a *Plasmodium* protein in yeast cells without any codon usage modification. The drug resistance phenotype of the yeast ste6 gene mutant can be complemented by the pfmdr1 gene, a gene conferring multidrug resistance on *P. falciparum* without any codon usage [33]. It should be stressed, however, that quinacrine accumulation in the restored yeast cells is slightly different from that in the wild type control cells: quinacrine is most densely accumulated in small vesicles, not in large vacuoles (Fig. 4B). Although we do not know the reason for the distinct accumulation of quinacrine in the restored yeast cells, it is possible that the *Plasmodium* proteolipid is processed through a biogenesis pathway different from that for the yeast counterpart.

Replacement of Glu138 with Gln caused loss of the ability of complementation of the yeast null mutant (Fig. 4). This also supports the functional occurrence of the *Plasmodium* proteolipid in yeast, and indicates the possibility that molecular genetical approaches involving yeast can be applied to elucidate the functions of *Plasmodium* V-ATPase. For instance, we can prepare mutants of the *Plasmodium* proteolipid, which is expressed in both parasite cells and the yeast null mutant. Then, the molecular bases of some phenotypes in *P. falciparum* can be investigated to more detail in yeast cells. Studies along these lines are in progress in our laboratory.

In conclusion, we have shown the gene organization and cDNA cloning of the *P. falciparum* proteolipid. The cDNA can complement a yeast null mutant, at least partially, which provides direct biochemical and molecular biological evidence for the functional occurrence of the V-ATPase complex in *P. falciparum*.

Acknowledgements

We are grateful to Drs. Nathan Nelson and Hannah Nelson (Tel Aviv University, Israel) for sending us the yeast cells and cDNA probes. This study was supported in part by a Grant-in-aid for Scientific Research on Priority Areas, Infectious Disease, 16-17264, to Y. M. from the Japanese Ministry of Education, Science, Culture and Sports.

References

[1] T.H. Stevens, M. Forgac, Structure, function and regulation of the vacuolar H+-ATPase, Annu. Rev. Cell Dev. Biol. 13 (1997) 779–808.
[2] N. Nelson, W.R. Harvey, Vacuolar and plasma membrane proton-adenosine-triphosphatases, Physiol. Rev. 79 (1999) 361–385.
[3] K. Halder, Sphingolipid synthesis and membrane formation by Plasmodium, Trends Cell Biol. 6 (1996) 398–405.
[4] H. Ginsburg, Abundant proton pumping in *Plasmodium falciparum*, but why? TRENDS in Parasitol, Trends Parasitol. 18 (2002) 483–486.
[5] Y. Moriyama, M. Hayashi, S. Yatsushiro, A. Yamamoto, Vacuolar proton pumps in malaria parasite cells, J. Bioenerg. Biomembr. 35 (2003) 367–375.
[6] S.R. Karcz, V.R. Herrmann, A.F. Cowman, Cloning and characterization of a vacuolar ATPase A subunit homologue from *Plasmodium falciparum*, Mol. Biochem. Parasitol. 58 (1993) 333–344.
[7] S.R. Karcz, V.R. Herrmann, F. Trottein, A.F. Cowman, Cloning and characterization of the vacuolar ATPase B subunit from *Plasmodium falciparum*, Mol. Biochem. Parasitol. 65 (1994) 123–133.
[8] M. Hayashi, H. Yamada, T. Mitamura, T. Horii, A. Yamamoto, A.Y. Moriyama, Vacuolar H+-ATPase localized in plasma membranes of malaria parasite cells, *Plasmodium falciparum*, is involved in regional acidification of parasitized Erythrocytes, J. Biol. Chem. 275 (2000) 34353–34358.
[9] R.J. Allen, K. Kirk, The membrane potential of the intraerythrocytic malaria parasite *Plasmodium falciparum*, J. Biol. Chem. 279 (2004) 11264–11272.
[10] K.J. Saliba, K. Kirk, H+-coupled pantothenate transport in the intracellular malaria parasite, J. Biol. Chem. 276 (2001) 18115–18121.
[11] G.A. Biagini, E.M. Pasini, R. Hughes, H.P. De Koning, H.J. Vial, P.M. O'Neill, S.A. Ward, P.G. Bray, Characterization of the choline carrier of Plasmodium falciparum: a route for the selective delivery of novel antimalarial drugs, Blood 104 (2004) 3372–3377.
[12] A.M. Lehane, K.J. Saliba, R.J. Allen, K. Kirk, Choline uptake into the malaria parasite is energized by the membrane potential, Biochem. Biophys. Res. Commun. 320 (2004) 311–317.
[13] K.J. Saliba, R.J. Allen, S. Zissis, P.G. Bray, S.A. Ward, K. Kirk, Acidification of the malaria parasite's digestive vacuole by a H+-ATPase and a H+-pyrophosphatase, J. Biol. Chem. 278 (2003) 5605–5612.
[14] K.J. Saliba, K. Kirk, pH regulation in the intracellular malaria parasite, *Plasmodium falciparum*: H+ extrusion via a V-type H+-ATPase, J. Biol. Chem. 274 (1999) 33213–33219.
[15] A. Yayon, Z.I. Cabantchik, H. Ginsburg, Identification of the acidic compartment of *Plasmodium falciparum*-infected human erythrocytes as the target of the antimalarial drug chloroquine, EMBO J. 3 (1984) 2695–2700.
[16] W. Trager, J.B. Jensen, Human malaria parasites in continuous culture, Science 193 (1976) 673–675.
[17] G.A. Gillespie, S. Somlo, G.G. Germino, D. Weinstat-Saslow, S.T. Reeders, CpG island in the region of an autosomal dominant polycystic kidney disease locus defines the 5′ end of a gene encoding a putative proton channel, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 4289–4293.
[18] M. Mandel, Y. Moriyama, J.D. Hulmes, Y.C. Pan, H. Nelson, N. Nelson, cDNA sequence encoding the 16-kDa proteolipid of chromaffin granules implies gene duplication in the evolution of H+-ATPases, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 5521–5524.
[19] L. Meagher, P. McLean, M.E. Finbow, Sequence of a cDNA from *Drosophila* coding for the 16 kD proteolipid component of the vacuolar H+-ATPase, Nucleic Acids Res. 18 (1990) 6712–6713.
[20] S.P. Lai, J.C. Watson, J.N. Hansen, H. Sze, Molecular cloning and sequencing of cDNAs encoding the proteolipid subunit of the vacuolar H+-ATPase from a higher plant, J. Biol. Chem. 266 (1991) 16078–16084.

[21] J.G. Wesseling, P.J. Snijders, P. van Someren, J. Jansen, M.A. Smits, J.G. Schoenmakers, Stage-specific expression and genomic organization of the actin genes of the malaria parasite *Plasmodium falciparum*, Mol. Biochem. Parasitol. 35 (1989) 167–176.

[22] J. Sambrook, D.W. Russell, Molecular Cloning—A Laboratory Manual, 1989.

[23] W. Ito, H. Ishiguro, Y. Kurosawa, A general method for introducing a series of mutations into cloned DNA using the polymerase chain reaction, Gene 102 (1991) 67–70.

[24] F.M. Ausubel, R. Brent, R.E. Kingston, D.D. Moore, J.G. Seidman, J.A. Smith, K. Struhl, Current Protocols in Molecular Biology, 1990.

[25] H. Nelson, N. Nelson, Disruption of genes encoding subunits of yeast vacuolar H+-ATPase causes conditional lethality, Proc. Natl. Acad. Sci. U. S. A. 87 (1990) 3503–3507.

[26] M.J. Gardner, N. Hall, E. Fung, O. White, M. Berriman, R.W. Hyman, J.M. Carlton, K.E. Pain, S. Nelson, I.T. Bowman, K. Paulsen, J.A. James, K. Eisen, S.L. Rutherford, A. Salzberg, S. Craig, M.S. Kyes, V. Chan, S.J. Nene, B. Shallom, J. Suh, S. Peterson, M. Angiuoli, J. Pertea, J. Allen, D. Selengut, M.W. Haft, A.B. Mather, D.M. Vaidya, A.H. Martin, M.J. Fairlamb, D.S. Fraunholz, S.A. Roos, G.I. Ralph, L.M. McFadden, G.M. Cummings, C. Subramanian, J.C. Mungall, D.J. Venter, S.L. Carucci, C. Hoffman, R.W. Newbold, C.M. Davis, B. Fraser, Genome sequence of the human malaria parasite *Plasmodium falciparum*, Nature 419 (2002) 498–511.

[27] T. Noumi, C. Beltran, H. Nelson, N. Nelson, Mutational analysis of yeast vacuolar H+-ATPase, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 1938–1942.

[28] K. Aviezer-Hagai, H. Nelson, N. Nelson, Cloning and expression of cDNAs encoding plant V-ATPase subunits in the corresponding yeast null mutants, Biochim. Biophys. Acta 1459 (2000) 489–498.

[29] N. Umemoto, Y. Ohya, Y. Anraku, VMA11, a novel gene that encodes a putative proteolipid, is indispensable for expression of yeast vacuolar membrane H+-ATPase activity, J. Biol. Chem. 266 (1991) 24526–24832.

[30] T. Oka, R. Yamamoto, M. Futai, Multiple genes for vacuolar-type ATPase proteolipids in *Caenorhabditis elegans*. A new gene, vha-3, has a distinct cell-specific distribution, J. Biol. Chem. 273 (1998) 22570–22576.

[31] M. Apperson, R.E. Jensen, K. Suda, C. Witte, M.P. Yaffe, A yeast protein, homologous to the proteolipid of the chromaffin granule proton-ATPase, is important for cell growth, Biochem. Biophys. Res. Commun. 168 (1990) 574–579.

[32] T. Nishi, S. Nishi-Kawasaki, M. Forgac, Expression and localization of the mouse homologue of the yeast V-ATPase 21-kDa subunit c″ (Vma16p), J. Biol. Chem. 276 (2001) 34122–34130.

[33] S.K. Volkman, A.F. Cowman, D.F. Wirth, Functional complementation of the ste6 gene of *Saccharomyces cerevisiae* with the pfmdr1 gene of Plasmodium falciparum, Proc. Natl. Acad. Sci. U. S. A. 92 (1995) 8921–8925.