Proposed Function of the Accumulation of Plasma Membrane-Type Ca\(^{2+}\)-ATPase mRNA in Resting Cysts of the Ciliate Sterkiella histriomuscorum

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From an mRNA differential-display analysis of the encystment-excystment cycle of the ciliate Sterkiella histriomuscorum, we have isolated an expressed sequence tag encoding a plasma membrane-type Ca\(^{2+}\)-ATPase (PMCA). PMCAs are located either in the plasma membranes or in the membranes of intracellular organelles, and their function is to pump calcium either out of the cell or into the intracellular calcium stores, respectively. The S. histriomuscorum macronuclear PMCA gene (ShPMCA) and its corresponding cDNA were cloned; it is the first member of the Ca\(^{2+}\)-ATPase family identified in Sterkiella. The predicted protein of 1,065 amino acids exhibits 37% identity with PMCAs of diverse organisms. A phylogenetic analysis showed its relatedness to homologs of two alveolates: the ciliate Paramecium tetraurelia and the apicomplexan Toxoplasma gondii. Overexpression of the protein ShPMCA failed to rescue the wild-type phenotype of three Ca\(^{2+}\)-ATPase-defective mutant strains of Saccharomyces cerevisiae; this failure contrasts with the reported ability of the PMCAs of parasites to complement defects in yeast. ShPMCA mRNA is markedly accumulated during encystment and in resting cysts, suggesting a function during excystment. To address the possibility of a signaling role for calcium at excystment, the capacity of calcium to induce excystment was examined.

In protists, many free-living or parasitic species transform into resting cysts (encyst) in response to unfavorable environmental conditions. These cysts are able to transform back to the vegetative form (excyst) when favorable conditions are restored. The encystment-excystment cycle (E-E cycle) can be viewed as two antagonistic developmental processes during which the cell undergoes profound metabolic and morphological changes. The morphological transformation may be considerable, involving, in some species, the nearly complete dedifferentiation of the encysted cell. This is the case with the E-E cycle (Fig. 1) of the ciliate Sterkiella histriomuscorum (an oxytrichid formerly known as Oxytricha trifallax), whose encystment can be induced by starvation.

Among protists, ciliates display a complex pattern in their vegetative forms. This pattern includes a complex oral apparatus and a clustered body ciliature, which is duplicated in an orderly process during cell division (12). During encystment, Sterkiella disassembles all its cilia and basal bodies, becomes round, surrounds itself with a protective wall, and dehydrates (Fig. 1a to d). The cyst thus appears as a highly dedifferentiated cell (Fig. 1e) and remains stable for months or even years, until food is restored in the medium. During excystment, the cell rehydrates, reenters the cell cycle, and rebuilds its cortical structure by reassembling new basal bodies, which then move to their specific location (Fig. 1f) (13). After the resorption of the cystic wall, the cell swims freely in the medium. The duration of the whole excystment process is 3 to 4 h at room temperature. The E-E cycle of S. histriomuscorum is thus a good model to study cellular differentiation within a single cell. To date, little is known about the molecular events underlying excystment.

In an attempt to get some insight into these events, the mRNA population of excysting cells was analyzed; similar cyst and excystment mRNA profiles were evidenced by mRNA differential display, which demonstrated no thorough change in the mRNA population during excystment (28). We hypothesize that excystment (rather than encystment) triggers a reprogramming of gene expression, including the synthesis of transcripts that are used at the onset of excystment. This hypothesis indeed seems to be true, since the exploration of the encystment phase by an mRNA differential-display analysis led us to isolate a set of transcripts that appear de novo in cysts. One of them, isolated as an expressed sequence tag (EST), shows high sequence similarity at the amino acid level with plasma membrane-type Ca\(^{2+}\)-ATPase (PMCA) proteins. These proteins, which are implicated in the control of the cytosolic Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)), are good candidates to take part during excystment in the recovery of vegetative cellular functions, such as the maintenance of Ca\(^{2+}\) homeostasis. Also and very interestingly, they may be good candidates to be involved during the excystment process as regulators of a putative Ca\(^{2+}\) signaling pathway.

Among the different types of proteins implicated in the control of [Ca\(^{2+}\)]\(_{c}\), PMCAs are structurally and functionally well documented in numerous species. Early studies with animals distinguished two types of Ca\(^{2+}\)-ATPases (PMCA and sarco-/endo)plasmic reticulum Ca\(^{2+}\)-ATPase [SERCA]) based on their cellular localization and regulation. Animal PMCAs are localized in the plasma membrane and are regulated by the binding of calmodulin or by phosphorylation at the C-terminal
Ca\(^{2+}\) are regulated by phospholamban. These two types of Ca\(^{2+}\)-ATPases have also been characterized for plants, fungi, and protists. However, compared to the animal prototype, the PMCA of these organisms show greater structural and functional diversity and may exhibit different localizations and a possible lack of calmodulin regulation. In plants, in addition to having a plasma membrane localization, PMCA are localized in intracellular organelles, such as the vacuole, the chloroplast, and the endoplasmic reticulum, and are regulated by calmodulin binding at the NH\(_2\)-terminal part of the protein (reviewed in reference 25). In Saccharomyces cerevisiae, Pmc1 is found in the vacuole membrane and is not regulated by calmodulin (5). For some protistan PMCA, an absence of calmodulin regulation and an organellar localization have also been observed. Some of these proteins are found mainly in acidic compartments; this is the case for Tca1 of the trypanosomatid Trypanosoma cruzi (15) and TgA1 of the apicomplexan Toxoplasma gondii (16), which are localized in acidicalcisomes, and for Pat1 of the slime mold Dictyostelium discoideum, which is localized in contractile vacuoles (19).

In this study, we report the cloning of the full-length cDNA and the macronuclear gene corresponding to the Ca\(^{2+}\)-ATPase EST of S. histrio\-muscorum. A phylogenetic analysis confirmed its assignment to the PMCA family. Its expression was examined during the E-E cycle, and a functional complementation of Ca\(^{2+}\)-ATPase-defective yeast mutant strains with the encoded ciliate protein was checked. As the function of PMCA is localized in contractile vacuoles (19),

**MATERIALS AND METHODS**

**Ciliate cultures.** S. histrio\-muscorum strain BA was cultured to a density of 1,000 to 2,000 cells/ml at 17°C in commercial mineral water (Volvic, Volvic Puy-de-Dome, France) with Tetrahymena pyriformis or Chlorogonium sp. as food organisms. Protargol staining was performed as previously described (11). The encystment and storage of cysts were performed as described previously (3). Excystment of calcium ionophore A23187-treated cells was tested as follows. Cysts were resuspended in fresh mineral water. One millimolar CaCl\(_2\) was added in the presence (or the absence) of a 10 \(\mu\)M concentration of the calcium ionophore A23187 (Sigma). After a 15-min incubation at room temperature, samples of 100 cysts were washed and resuspended in mineral water. Excysting cells were counted and removed at different intervals through a 24-h incubation of cysts. The viability of the cysts was checked by stimulating a fraction of the untreated cysts to excyst by feeding them with Chlorogonium or a dried-milk solution (0.02%).

**Nucleic acid extraction and hybridization analyses.** S. histrio\-muscorum genomic DNA was extracted using proteinase K and phenol according to standard procedures described by Sambrook et al. (22). S. histrio\-muscorum total RNA was extracted by following the protocol of Chomczynski and Sacchi (4) with some modifications. Prior to precipitation, the RNA extracts were incubated with RNase-free DNase (Promega, Madison, Wis.) at 37°C for 15 min. Before RNA was isolated from cysts, the cystic walls were broken with a mini-head-beater homogenizer (Biospect Products, Bartlesville, Okla.), as described by Villalobo et al. (28).

For Southern blots, S. histrio\-muscorum DNA (8 \(\mu\g) kindly provided by L. Amar, Université Paris-Sud, Orsay, France) were digested overnight at 37°C with restriction enzymes, fractionated on a 1% (wt/vol) agarose gel, and transferred to a Hybond-N nylon membrane during an overnight blotting with 10× SSC buffer (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate). The probe was a fragment of the S. histrio\-muscorum PMCA (ShPMCA) gene amplified with the following pair of primers: 5′-AGAG TATTAGCTGTTGAAAC-3′ (nucleotides 1483 to 1504) and 5′-GACCTGAC TAAACACGAAAGTGTGGA-3′ (nucleotides 3302 to 3277). It was labeled with \([\alpha-32P]dATP\) by use of the Megaprime DNA labeling system (Amersham). Hybridizations were performed overnight at both 65 and 48°C for all samples. At 65°C, the washing solution was 40 mM sodium phosphate, 0.5% sodium dodecyl sulfate (SDS), and 0.08 mM EDYA; whereas at 48°C the washing solution was 1× SSC–1% SDS.

**Inverse PCR.** To amplify the 3.9-kb ShPMCA gene, two rounds of PCR using the Long Expand Template PCR system (Boehringer Mannheim, Mannheim, Germany) were performed according to the manufacturer’s instructions with 10 ng of genomic DNA circularized as described previously (28). The first pair of outward-facing primers consisted of 268F (5′-CAACACGACCAAAAATTAGGAG CCCA-3′) and 268R (5′-GACTGCTGCAAAACACCG ATGATC-3′). The second pair consisted of 28F (5′-CTCATGTCATGGTGATC-3′) and 28R (5′-ACAGTGCAGTGGAGCGCA-3′) (nucleotides 1552 to 1534). The PCR fragments of the expected sizes were cloned in pGEM-T Easy Plasmid (Promega).

**RT-PCR.** Total RNA (2 \(\mu\g) was reverse transcribed in a 20-\(\mu\l reaction volume with a poly(dT) primer (dT\(_11\)VN) as described by Villalobo et al. (28). PCR was carried out in a 25-\(\mu\l reaction volume with 1 U of Q-Bio Taq DNA polymerase (Quantum Appagine, Illkirch, France) and 30 cycles of denaturation at 94°C for 30 s, annealing at 57°C for 30 s, and polymerization at 72°C for 1 min. The primers used were as follows: 28F2 (5′-GCTGCACCTGCTCTTGGGC-3′) and 28R (5′-GGGAAGATGAACCCACG ATGATC-3′) (nucleotides 3314 to 3337), and 28R (5′-GACTGCTGCAAAACACCG ATGATC-3′) and 28R (5′-GGGAAGATGAACCCACG ATGATC-3′) (nucleotides 3302 to 3277). The second pair consisted of 28F (5′-CTCATGTCATGGTGATC-3′) and 28R (5′-ACAGTGCAGTGGAGCGCA-3′) (nucleotides 1552 to 1534). The PCR fragments of the expected sizes were cloned in pGEM-T Easy Plasmid (Promega).

**Phylogenetic analysis.** Alignment and formatting of the sequences for the tree-building programs were carried out using the MUST package. The terminal parts of the sequences and several internal domains which were alignable only between close species were removed, as were all gaps. The alignments were used for phylogenetic analyses based on three programs. Distance and parsimony analyses were performed with version 3.6 (alpha2) of the PHYLIP package of Felsenstein (http://evolution.genetics.washington.edu/phylip.html), using the neighbor-joining (NJ) method, and the PROTPARS program, respectively. Evaluation of the statistical validity of the nodes was performed by applying the bootstrap procedure (100 replicates) to the tree construction method. A maximum likelihood analysis was also performed using TREE-PUZZLE 5.0 (http://www.tree-puzzle.de). This program uses the quartet-puzzling method and estimates support values, which are comparable to bootstrap values, for each internal branch.

**Site-directed mutagenesis.** The following primers were used to change the 11 TAA codons of the ShPMCA coding sequence into CAA codons: 5′-CTTTGTG GTTGGGACAGCAT-3′ (nucleotides 216 to 197), 5′-CTAAATGGCTTGGT GATGTC-3′ (nucleotides 277 to 255), 5′-CTCCTCATATTGTCCTGGTTC-3′ (nucleotides 911 to 891), 5′-AACTTGTCCTGCAATGTTGTC-3′ (nucleotides 1091 to 1068), 5′-CTAAATGGCTTGGTTCATTT-3′ (nucleotides 1584 to 1563), 5′-GTTCAGTCTTGGTGGTAA-3′ (nucleotides 2196 to 2175), 5′-CTTTCTCAATTTTACCTT-3′ (nucleotides 2205 to 2214), 5′-CAA...
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CAACTGCTTTGCTGCCCCCC3’ (nucleotides 2412 to 2391), 5’-CCACACACATTTGGACGAAGCTG-3’ (nucleotides 2702 to 2682), 5’-GAAGATAGCTTTGAGCACAAAT-3’ (nucleotides 2849 to 2829), and 5’-CATTGTATATTGACCTTATC-3’ (nucleotides 2961 to 2940). Based on the protocol of Deng and Nickoloff (8), two other primers were used to change the unique SphI restriction site sequence 5’-GCTAGC-3’, located in the pGEM-T Easy vector (Promega), into a NheI restriction site, 5’-GCTAGC-3’, and vice versa. The primers were SphI (5’-GCCGGACGTGCTGATGCTCCC-CCG-3’) and NheI (5’-GCCGGACGTGCTGATGCTCCC-CCG-3’).

DNA to be mutated was alkaline denatured and then annealed to two or three nucleotide-modifying primers and to the primer changing the restriction site. After elimination of the unmodified plasmid using the SphI or the NheII restriction enzyme, mutS Escherichia coli bacteria (Strategene, La Jolla, Calif.) were transformed with this population of linearized and circularized plasmids. In the resulting culture, a mix of parental and mutant plasmids was recovered. The plasmid mix was restricted with the enzyme that cut the parental plasmid and then used to transform XL1-Blue MRF+ bacteria (Strategene). Colonies carrying correct plasmids were identified by plasmid sequencing.

Functional complementation in yeast. The coding region of the ShPMCA gene was cloned at the NorI site of the yeast expression vector pYES2 (Invitrogen, Cergy Pontoise, France), placing it under the control of the galactose-inducible GAL1 promoter. The mutated strains K609 (pYES1:SHPMCA), K616 (pYES1:SHPMCA pmc1::TRP1 cnb1::LEU2), and K665 (pYES1:TRP1 vscl1::A) of S. cerevisiae strain W303-1A (MATa ade2-1 can1-100 his3-11,15 leu2-3,112 trpl-1 ura1-1) were cultured as previously described (5, 6) and transformed with pYES2-ShPMCA. Transformants were selected on synthetic complete Glu-uracil (SCGlu-ura) plates and then cultured for 10 h in the presence of galactose. Five microliters of serial tenth mants were selected on synthetic complete Glu-uracil (SCGlu-ura) plates and then cultured for 10 h in the presence of galactose. Five microliters of serial tenth dilutions, starting with a concentration for which the optical density at 600 nm was 0.1, were spotted on plates containing SCGal-ura medium supplemented with 10 mM EGTA, 3 mM MnCl2, or 300 mM CaCl2.

Nucleotide sequence accession number. The nucleotide sequence of the macronuclear minichromosome carrying the ShPMCA gene has been deposited in GenBank under accession number AF486142.

RESULTS

Molecular cloning and sequence analysis of ShPMCA. Among 40 different ESTs isolated from an mRNA differential-display analysis of the encysting cells (i.e., precystic cells and newly formed cysts) and mature cysts (i.e., 3-week-old cysts) of S. histriomuscorum (22), 208-long sequence present only in cysts revealed high similarity with a COOH-terminal conserved domain of Ca2+-ATPas from several species (top BLAST hits gave 64% identity to and 84% similarity with most animal PMCA).

In hypotrichous ciliates such as S. histriomuscorum, the macronuclear DNA is organized into minichromosomes ranging from 0.5 to 20 kb. Each macronuclear molecule generally encodes a single transcription unit flanked by 5’ and 3’ noncoding sequences and ended by telomeres. A Southern blot using the cDNA fragment as a probe showed a major band of 4 kb (data not shown). A 4-kb minichromosome should be able to encode such a protein, since all the known Ca2+-ATPase proteins are about 120 kDa, corresponding to mRNAs with lengths of at least 3,000 bp. To clone the corresponding macronuclear gene, we used an inverse-PCR strategy that allowed us to clone the flanking 5’ and 3’ regions up to the telomeres. A 2,304-long PCR product lacking both telomeres and the 5’ end of the putative gene was first obtained using the pair of primers 28F-28R designed from the EST (Fig. 2A). In order to walk forward on the minichromosome, we then designed a new pair of primers (28F1-28R1) corresponding to the ends of this first fragment. A 1,901-long PCR product sharing high similarity with the NH2-terminal part of Ca2+-ATPas and carrying at its 5’ end a single CAGA telomeric repeat provided the 5’ end of the macronuclear gene. The reconstituted minichromosome fragment is 3,827 bp long and lacks parts of the 3’ subtelomeric and telomeric regions (Fig. 2A). Since the expected size of the macronuclear molecule is close to 4,000 bp with telomeres (72 bp) included, the missing subtelomeric stretch is at most 100 bp long.

Examination of the full-length nucleotide sequence indicates a typical hypotrichous minichromosome with a putative open reading frame easily distinguishable from a 5’ leader region (186 bp) that is very AT rich (82.3%), contains putative TATA boxes, and ends with an ATG codon located in a Kozak environment (ATTATGG). The incomplete 3’ trailer region (147 bp) is also AT rich (78.2%) and is located downstream from a TGA codon (in Sterkiella, as in many ciliates, TGA is the unique stop codon, while TAA and TAG encode glutamine). This sequence encodes a Ca2+-ATPase-type protein, but several frameshifts in the 5’ part of the gene suggest the presence of introns. The complete cDNA was recovered by PCR; its sequence encodes a predicted protein of 1,065 amino acids, which fits well with the size of known Ca2+-ATPas and contains 11 glutamine TGA codons. The comparison of the cDNA and genomic sequences identified four introns ranging from 48 to 110 bp (Fig. 2A).

Analysis of the deduced protein ShPMCA. A BLAST search of protein databases showed that the predicted protein is closely related to PMCas of Mus musculus (37% identity and 54% similarity), Arabidopsis thaliana (37% identity and 53% similarity), D. discoideum (36% identity and 54% similarity), and P. tetraurelia (35% identity and 51% similarity). The protein shares several characteristics with eukaryotic Ca2+-ATPas. As typically observed in all the Ca2+-ATPas, the hydrophobic profile of ShPMCA displays 10 transmembrane domains. The presence of several specific and functionally important Ca2+-ATPase domains, namely, the phosphate intermediate region, the calcium transport region, the hinge region, and the ATP-binding region, was revealed by alignment with PMCA amino acid sequences from other species (Fig. 2B). ShPMCA lacks an NH2-terminal extension that is found in the PMCas of plants, as well as a COOH-terminal extension that is found in TgA1 (T. gondii), Pat1 (D. discoideum), and the PMCas of animals. In addition, the alignment did not reveal a calmodulin-binding domain.

Phylogenetic analysis. The 35 eukaryotic sequences included in the analysis comprised 17 SERCAs, 15 PMCas, and 3 PMRIs. The PMRIs correspond to a third type of Ca2+-ATPase, which was first identified in S. cerevisiae (21). PMR1 is located in the Golgi apparatus (2), and homologous proteins have been found in animals and in plants (26, 30). Two bacterial sequences were used as outgroups. The phylogenetic tree (Fig. 3) displays three branches corresponding to three solid clusters representing the SERCA, PMCA, and PMRI subfamilies. The values are close to 100% for the three methods of tree construction used. The mean distance within PMCas is 1.5 times higher than that within SERCAs. For PMR1, animal and fungal sequences are also strongly grouped (100%). A putative Ca2+-ATPase sequence of Plasmodium falciparum emerges as a deep lineage in the tree remote from all other eukaryotic sequences. Each branch comprises a set of taxa whose branching order (animals, fungi, plants, and ciliates, etc.) does not contradict organismal phylogenies. The sequence of S. histriomuscorum is located within the PMCA cluster, confirming its af-
filialation to the PMCA type of Ca\(^{2+}\)-ATPases. Within this cluster, although the bootstrap values are below 70%, the *S. histriomuscorum* protein sequence is consistently grouped with homologs of the ciliate *P. tetraurelia* and the apicomplexan *T. gondii*, which accords with the monophyly of ciliates and, at a higher level, supports the grouping of alveolates. By restricting the analysis to PMCAs, additional domains can be aligned, giving a total of 496 amino acid sites, of which 425 are variable and 383 informative. This leads to significant increases in the branch support values in distance and maximum likelihood analyses (62 and 75%, respectively). In agreement with the monophyly of alveolates, this grouping strengthens the idea of an ancient origin for the Ca\(^{2+}\)-ATPase families, which preceded the emergence of the major eukaryotic groups. In *P. tetraurelia*, four genes encoding complete PMCAs have been cloned and validated, raising the possibility that duplication of the gene has also occurred in *S. histriomuscorum*.

**Evidence for additional PMCAs in *S. histriomuscorum*.** Southern blot analysis was performed at high (65°C) and low (48°C) stringencies, using a large fragment of ShPMCA as a probe to search for additional PMCA-encoding gene sequences (Fig. 4). The probe encompassed all the conserved functional domains depicted in Fig. 1B. Genomic DNA from *S. histriomuscorum* was digested with KpnI and PstI and hybridized in parallel with genomic DNA of *P. tetraurelia* digested with EcoRI. Under both hybridization conditions, very faint bands were observed in DNA of *P. tetraurelia* (not visible in Fig. 4). This was not
FIG. 3. Distance matrix-based phylogenetic tree of Ca\textsuperscript{2+}-ATPase amino acid sequences. Accession numbers are given next to the species names. The domains analyzed correspond to residues 184 to 232, 250 to 278, 286 to 324, 347 to 414, 603 to 659, and 713 to 893 of the ShPMCA amino acid sequence. Of the 387 aligned sites, there were 348 variable and 318 informative sites for the parsimony analysis. The two cyanobacterial sequences serve as outgroups. Boolean distances were calculated. Consensus NJ trees constructed from different matrices (distance estimates) and displaying identical groupings were obtained after 100 bootstrap resamplings. Numbers on the branches are bootstrap proportions obtained from a Dayhoff-Pam matrix-derived NJ tree. The parsimony analysis yielded five trees, and a strict consensus was calculated. The groupings present in this 50% majority rule consensus tree are indicated, with the bootstrap values recorded for 100 resamplings shown below the branches. The maximum likelihood analysis, a tree was constructed using the Mueller-Vingron model of substitution and a uniform rate of heterogeneity. Branches showing a reliability score above 50% are indicated. The PMCA, SERCA, and PMR1 clusters are indicated by brackets. Bar, 12% of site substitutions. P. yoelli, Plasmodium yoelli; O. Cuniculus, Oryctolagus cuniculus; S. mansoni, Schistosoma mansoni; D. melanogaster, Drosophila melanogaster; C. elegans, Caenorhabditis elegans; N. crassa, Neurospora crassa; L. major, Leishmania major; L. amazoniensis, Leishmania amazoniensis; Z. mays, Zea mays; D. bioculata, Dunaliella bioculata; E. histolytica, Entamoeba histolytica; B. oleracea, Brassica oleracea; G. max, Glycine max.

surprising, considering the genetic distance between \textit{P. tetraurelia} and \textit{S. histriomuscorum} PMCAs (Fig. 3). For \textit{S. histriomuscorum}, undigested DNA under both conditions gave a unique band at 4 kb. KpnI-digested DNA exhibited the expected two bands (1.4 and 1.2 kb) when hybridized at 65°C, whereas at 48°C, two additional bands (4 and 2.5 kb) were revealed. With PstI-digested DNA, two bands (3.5 and 4 kb) were also revealed at 48°C in addition to the two homologous bands of 1.8 and 2 kb. The genome of \textit{S. histriomuscorum} thus contains at least two other PMCA-related genes closer to \textit{ShPMCA} than to the PMCA genes of \textit{P. tetraurelia}.

Functional complementation of yeast mutants. It has been reported by several authors that heterologous Ca\textsuperscript{2+}-ATPase genes are able to functionally complement \textit{S. cerevisiae} strains with mutations in their Ca\textsuperscript{2+}-ATPase genes. To ascertain the degree of functional conservation of the \textit{S. histriomuscorum} Ca\textsuperscript{2+}-ATPase, we tested its capacity to substitute for the yeast Ca\textsuperscript{2+}-ATPases (5). The K609 mutant strain is null for the PMR1 gene, which encodes a Ca\textsuperscript{2+}-ATPase located in the Golgi apparatus and is able to transport either calcium or manganese ions. The phenotype of the mutant is the inability to grow in the presence of manganese ions. The K665 mutant strain lacks the PMC1 gene, which encodes a Ca\textsuperscript{2+}-ATPase located in the vacuole. This strain cannot grow in a medium rich in calcium ions. The K616 mutant strain lacks both the PMR1 and PMC1 genes. This mutant is unable to grow in a calcium-depleted medium. The TAA\textsuperscript{3}CAA-altered \textit{ShPMCA} coding region cloned under the control of the galactose-induc-
Yeast strains (K609, K616, and K665) mutant strains, respectively. YPGal medium consists of 10 g of yeast extract/liter, 20 g of Difco Peptone/liter, and 2% galactose.

FIG. 5. Yeast functional-complementation analysis. The different yeast strains (K609 pmr1, K616 pmr1 pmc1, and K665 pmc1) transformed with ShPMCA cDNA in the pYES2 vector (pCa) or the empty vector (pYES2) were grown in SCGal-ura medium; 5 μl of serial tenth dilutions of these cultures were spotted on selective medium, and 3 mM MnCl₂, 10 mM EGTA, and 300 mM CaCl₂ were added to the K609, K616, and K665 mutant strains, respectively. YPGal medium consists of 10 g of yeast extract/liter, 20 g of Difco Peptone/liter, and 2% galactose.

Effect of calcium loading on excystment. Given the function of the PMCs, which is to pump calcium either out of the cell or into the internal calcium stores, it may be possible that ShPMCA is implicated in a primary Ca²⁺ signal that acts at the onset of excystment. To test this hypothesis, we loaded cysts with Ca²⁺ using the calcium ionophore A23187 to determine whether an increase in the [Ca²⁺] in cysts could trigger the excystment process. In the first series of experiments using the same batch of cysts, different incubation times (15, 30, 45, and 60 min), ionophore concentrations (10 and 20 μM), and calcium concentrations (1 and 1.5 mM) were tested. Under the best conditions (15 min of incubation, 10 μM ionophore, and 1 mM CaCl₂), we observed that nearly 90% of cysts excysted within 6 h. By comparison, only 15% of the ionophore-un treated cysts in Volvic mineral water supplemented with 1 mM CaCl₂ could excyst. To confirm this observation, several independent experiments using these conditions (15 min of incubation, 10 μM ionophore, and 1 mM CaCl₂) were performed on cysts of different ages (from 1 week up to several months) and encysted from different cultures (Chlorogonium or Tetrahymena feeding) at different times of the year. A Ca²⁺-induced excystment was observed in 6 of 11 experiments, with a large
variability, ranging from 7 to 60%, in the percentages of excysting cells. In these experiments, untreated cysts in the presence of CaCl2 did not excyst.

**DISCUSSION**

We report the cloning and characterization of a ciliate Ca2+-ATPase gene which belongs to the PMCA subfamily. It is the second ciliate member of this family to be isolated. In the Ca2+-ATPase phylogenetic tree, the good congruence between sequences and species clustering allows us to interpret the gene tree as a species tree and thus to infer the existence of several members of the Ca2+-ATPase family in *Sterkiella*: at least one PMCA, one SERCA, and, perhaps, one PMR1 gene. Indeed, two findings in particular from Southern blot experiments support the existence of other Ca2+-ATPase genes in the genome of *S. histriomuscorum*. First, the identification of two other copies of *ShPMCA* confirms that a duplication of the *PMCA* gene has occurred in ciliates after the divergence of *Sterkiella* and *Paramaecium* (i.e., after the diversification of the group). Second, *ShSERCA* could be detected, but only as very faint bands in Southern blots hybridized with a fragment of *P. tetraurelia* SERCA (data not shown).

Like some of its counterparts in unicellular organisms, *ShPMCA* is devoid of a recognizable plant-like or animal-like calmodulin-binding domain. These domains lead to an autoinhibition of the protein in the absence of calmodulin (7). We can predict from the sequence analysis that *ShPMCA* may be a new example of a non-calmodulin-regulated PMCA which cannot take such an inactive conformation (5, 15, 16, 18). From the observed dichotomy between unicellular and multicellular organisms, it seems that this regulation of PMCAs (via a direct interaction of calmodulin with conserved domains of the enzyme) has been acquired at least two times (in plants and animals) during evolution, following the extension of the proteins.

Given the phylogenetic position of *ShPMCA* within the Ca2+-ATPase tree, the high structural conservation of several Ca2+-ATPase functional domains, and the features common to *ShPMCA* and the yeast PMC1 (similar hydrophobicity profiles and the absence of calmodulin regulation), it might be expected that *ShPMCA* functioned in the yeast. Very likely, a problem of localization could explain this lack of complementation. Protists contain numerous types of calcium storage intracellular organelles, such as the acidicocalcisomes (9), the contractile vacuole (1), and the alveolar sacs (23), which provide many distinct intracellular sites of action for Ca2+-ATPases. Evidence exists for the functional equivalency of *TgA1* (*Toxoplasma gondii*) and *Tc1* (*Trypanosoma cruzi*) with PMC1 (*S. cerevisiae*), demonstrating that pumps localized in acidicocalcisomes can substitute for the vacuolar pumps of the yeast. However, this may not be true for other PMCAs. Indeed, a recent characterization of two PMCAs in *Trypanosoma brucei* (17) showed that *Trypanosoma brucei* PMC1 (*TbPMC1*) and *TbPMC2*, which are localized in the acidicocalcisomes and in the plasma membrane, respectively, do not have the same ability to function in yeast. *TbPMC2* appears much less efficient in restoring *pmc1* mutants to the wild-type level, very likely in relation to the endogenous subcellular location of PMC1 (17).

Based on the available data pertinent to PMCAs in ciliates, this could also be the case in *Sterkiella*; for *Paramaecium*, Wright et al. (29) reported a protein present in the pellicle (plasma membrane plus tightly bound underlying alveolar sacs) having the characteristics of a plasma membrane Ca2+-ATPase, while Elwess and Van Houten isolated a gene encoding a PMCA-type protein that is localized at the surface of the cell or, more precisely, at the bases of cilia in the cortical units (10, 27). We do not yet know where *ShPMCA* is localized, but given the known locations of PMCAs in *Paramaecium*, it is possible that *ShPMCA* is localized in the plasma membrane and/or in alveolar sacs and thus is not properly targeted or able to function in yeast.

We have shown that *ShPMCA* mRNAs disappeared in starved cells and accumulated again in cysts. The fact that 1/10 as much of the RT reaction volume was used in lanes 13 to 15 of the gel shown in Fig. 6A as was used in lanes 10 to 15 (see also data in Fig. 6B) suggests that *ShPMCA* mRNA may actually increase during encystment. This possibility raises the question of the function of the transcript and the role of Ca2+-ATPase either at the end point of encystment or later, at the start of excystment. Since cysts are highly dedifferentiated cells which have lost all cortical structures and organization, such an accumulation in resting cysts would be rather in accordance with the view that, in cysts, a pool of mRNAs is stored to act later during excystment (28). *ShPMCA* may be involved in the functional reorganization of the cell which takes place during excystment.

In all eukaryotic cells, Ca2+-ATPases play a major role in Ca2+ homeostasis. The control of the concentration of free intracellular Ca2+ is essential for cell viability, as [Ca2+]i functions in the regulation of a wide variety of cellular processes. In ciliates, for instance, exocytosis, endocytosis, ciliary beat, and cell contraction have been shown to be regulated by Ca2+ (reviewed in reference 20).

Alternatively, a second attractive and more specific hypothesis is that Ca2+ itself is a key transduction signal at excystment, with *ShPMCA* being involved in the restoration of the calcium homeostasis. Indeed, it has been shown that during fertilization of aquatic oocytes, calcium waves or oscillations occurred following the sperm entry (24). A parallel can be drawn between the events initiated during the oocyte’s fertilization and those begun during the excystment process of *S. histriomuscorum*, since, in both cases, cells have to reenter the cell cycle and to initiate differentiation events. Such an effect of Ca2+ inside excysting cells may exist, since we observed that a prior incubation of cysts with the A23187 calcium ionophore was able to trigger their excystment in the absence of food. The fact that this phenomenon was not systematically observed could be due to the calcium loading in cysts and the difficulty of making Ca2+ enter into the cell. The cystic wall is constituted by several layers whose biochemical composition is largely unknown. Obviously, some as-yet-unknown parameters could play a role in the biochemical properties of the wall, which renders the permeability to the ionophore experimentally uncontrolled. A visualization of intracellular calcium cytosolic influx during a food-triggered excystment is necessary to confirm such excystment-induced calcium signaling.
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