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Identification of calcium-transporting ATPases of *Entamoeba histolytica* and cellular localization of the putative SERCA

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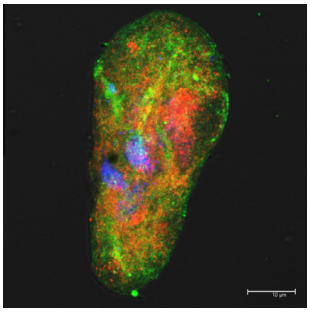
HIGHLIGHTS

- *Entamoeba histolytica* contains five genes encoding putative Ca^{2+}-ATPases.
- Three of the Ca^{2+}-ATPases are grouped with plasma membrane elements (PMCA) and two with organellar members (SERCA and SPCA).
- All Ca^{2+}-ATPases are expressed in *E. histolytica* trophozoites.
- The putative EhSERCA contains several of the structural domains of the rabbit SERCA1a.
- The putative EhSERCA is located in a continuous cytoplasmic network and co-localized with calreticulin.

GRAPHICAL ABSTRACT



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ABSTRACT

Calcium has an important role on signaling of different cellular processes in the protozoa parasite *Entamoeba histolytica*, including development and pathogenesis. However, the systems that control calcium responses in this parasite are incompletely understood. Calcium-ATPases (Ca^{2+}-ATPases) are proteins that play an important role in calcium homeostasis by catalyzing the active efflux of this ion from cytoplasm and are essential to the correct functioning of the cell machinery. Here, we reported the identification of five *E. histolytica* genes encoding putative Ca^{2+}-ATPases, three related to PMCA, and two related to organellar ATPases. RT-PCR assays showed that all those genes are expressed in trophozoites and specific antibodies against the SERCA-like member located this protein in a continuous cytoplasmic network, supporting the hypothesis that it corresponds to the Ca^{2+}-ATPase responsible to sequester calcium in the endoplasmic reticulum of this parasite.

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1. Introduction

Changes in cytosolic free calcium concentrations are induced by many extracellular signals, and various physiological responses are associated with these changes (Brini and Carafoli, 2009). Thus, the maintenance of optimal cytosolic concentrations of calcium is necessary to allow this ion to function as a signaling molecule. High affinity calcium-ATPases (Ca^{2+}-ATPases) play a central role in this regulation by catalyzing the active efflux of calcium from cytoplasm. These Ca^{2+}-pumping ATPases have been classified in three major classes: (i) plasma membrane Ca^{2+}-ATPases (PMCA); (ii) sarco/endoplasmic reticulum Ca^{2+}-ATPases (SERCA); and (iii) Golgi Ca^{2+}-ATPases (SPCA/PMR1) (Brini and Carafoli, 2009). PMCA and SERCA have the highest affinity for calcium mobilization from the cytoplasm and together maintain the resting calcium cytoplasm concentrations. Interestingly, the SPCA of the Golgi membranes also transports Mn^{2+}, which is a cofactor of a number of enzymes in the lumen of the Golgi compartment (Brini and Carafoli, 2009). All three Ca^{2+} pumps belong to the family of P-type ATPases, which is characterized by the temporary conservation of ATP energy in the form of a phosphorylated enzyme intermediate (hence P-type) (Brini and Carafoli, 2009).

SERCA is an intracellular membrane-bound ATPase that sequesters calcium into the sarco/endo plasmic reticulum (intracellular stores) and is known to play an important role in various cellular processes. In mammals, three genes encode three main SERCA proteins, and each transcript undergoes tissue-dependent alternative splicing, increasing the number of pump variants (Brini and Carafoli, 2009).

Entamoeba histolytica is the protozoa parasite causative of human amoebiasis, disease that affects to 50 million individuals worldwide and it is responsible for up to 100,000 deaths annually (Haque et al., 2003). The cytopathogenic mechanism of this parasite has been divided in three main steps: adherence, contact-dependent cytolysis, and phagocytosis (Ravdin et al., 1980) and it has been demonstrated that calcium signaling is involved in these events (Carbajal et al., 1996; Jain et al., 2008; Munoz Mde et al., 1991; Ravdin and Guerrant, 1981; Ravdin et al., 1985). Calcium signaling is also implicated in growth and development of *E. histolytica* and *Entamoeba invadens*, a related reptilian parasite (Makioka et al., 2002, 2001). Increase of intracellular calcium concentration also induced changes in mRNA level of several genes (Debnath et al., 2005; Gilchrist et al., 2003; Leon-Avila et al., 2006) and in the promoter occupancy of the transcription factor EhURE3BP (Gilchrist et al., 2003). Moreover, the analysis of the *E. histolytica* genome showed that this parasite encodes a large number of calcium-binding proteins (Bhattacharya et al., 2006). All these results indicate that this parasite may possess extensive Ca^{2+} signaling pathways. However, the systems that control calcium responses in *E. histolytica* are incompletely understood.

Earlier researches on *E. histolytica* showed that Ca^{2+}-ATPase activities are associated with both the plasma membrane and the inner surface of internal vesicles (Aley et al., 1980; Bakker-Grunwald and Parduhn, 1993; Kobayashi et al., 1982; McLaughlin and Muller, 1981). Indeed, Ghosh et al. (2000) cloned an *E. histolytica* gene encoding a putative PMCA, but the overexpression of the recombinant protein with a chitinase repeat epitope-tag showed that this Ca^{2+}-ATPase is located in cytoplasmic vacuoles. Nevertheless, to date is unknown both the number of Ca^{2+}-ATPases that possess *E. histolytica* and their role in calcium homeostasis.

Here, we reported the identification of five *E. histolytica* genes encoding putative Ca^{2+}-ATPases, three related to PMCA, and two related to organellar ATPases (SERCA and SPCA/PMR1). RT-PCR assays showed that all those genes are expressed in trophozoites and specific antibodies against the SERCA-like member located this Ca^{2+}-ATPase in a continuous cytoplasmic network, supporting the hypothesis that it corresponds to the Ca^{2+}-ATPase responsible to sequester calcium in the endoplasmic reticulum of this parasite.

2. Materials and methods

2.1. Entamoeba histolytica cultures

Trophozoites of *E. histolytica* clone A (strain HM1:IMSS) (Orozco et al., 1983) were axenically cultured in TYI-S-33 medium and harvested as described (Diamond et al., 1978).

2.2. Identification and in silico characterization of E. histolytica genes encoding putative Ca2+-ATPases

To identify putative Ca^{2+}-ATPases encoding genes in *E. histolytica* we performed an exploration of genes labeled as putative calcium-transporting ATPases on the database of the *E. histolytica* genome <http://pathema.jcvi.org/cgi-bin/Entamoeba/PathemaHomePage.cgi>. Then, predicted proteins were characterized in silico using the software deposited in the Expasy Bioinformatics Resource Portal <http://expasy.org> and the NCBI Home Page <http://www.ncbi.nlm.nih.gov>. Phylogenetic analysis was performed using the MEGA 5.05 software package (Tamura et al., 2011). Bootstrapping was performed for 1000 replicates.

2.3. RT-PCR assays

Total *E. histolytica* RNA was obtained using TRIAZOL reagent (Gibco BRL) according to the manufacturer's recommendations. cDNA was synthesized using oligo-dT primer and the superscript II reverse transcriptase (Invitrogen Life Technologies, Grand Island, NY, USA) and then, PCR amplifications were carried out using specific primers corresponding to the putative Ca^{2+}-ATPases encoding genes (Table 1). Reactions were performed in 50 μl volume reactions using a Select cycler (Select BioProducts, Edison NJ, USA). Reaction mixtures contained 100 ng of cDNA, 1 μM of each primer, dNTPs 1.5 mM, $MgCl_2$ 2 mM, and 1 U of recombinant Taq DNA polymerase (Invitrogen Life Technologies, Grand Island, NY, USA). For amplification, cycles comprised: (i) 1 min of denaturing step at 95 °C; (ii) 1 min of annealing step at 52 °C or 55.5 °C; and (iii) 3 min elongation step at 72 °C. The accuracy of amplified sequences was confirmed by sequencing.

2.4. Production of specific antibodies against the putative EhSERCA and Western blot assays

For antibody production against the polypeptide encoded by the EHI_027710 sequence (the putative EhSERCA), the amino acid sequence located in position 959–974 (MSNIMERKPRDPNSNI) of the predicted protein was synthesized. Then, eight week-old New Zaland rabbits were intradermally immunized twice, in an interval of seven days, with 100 μg of the peptide suspended in 0.15 ml of PBS and emulsified in the same volume of TiterMax Classic Adjuvant (Sigma Aldrich, St. Louis, MO, USA) (Hancock and Evan, 1998). The experimental protocol was approved by the institutional committee for animal care and provided all technical specifications for the production, care and use of laboratory animals (NOM-062-ZOO-1999).

For Western blot assays, total *E. histolytica* proteins were separated in 10% SDS–PAGE, transferred to nitrocellulose (Towbin et al., 1979) and probed with the antibodies anti-EhSERCA (1:1,000). Then, membranes were incubated with peroxidase-coupled goat-rabbit secondary antibodies (1:5000) (ZYMED, San Francisco, CA,

Table 1
Primers used for RT-PCR assays.

Primer	Forward sequence (5′-3′)	Backward sequence (5′-3′)
EHI_027710	ATGCTTATTATTGTTATTGTGTCTAAT	CCCTTCTACAACTGGTAAATT
EHI_065670	ATGATGTTAATTATGTTATTTATTTTTGAAG	AGGTTCAGTACAAAAGATAAATTG
EHI_054830	GCTATTTTAATTGCAGTTCTTGCG	
EHI_030830	AACCAAGATAAACCAAGACCA	TTCACATGCCTTTAAATGTCTTAC
EHI_016480	CCAGCTGATGGAGTTTTTGTT	

USA), and finally, reaction was developed by chemioluminescence (ECL Plus, Amersham, Piscataway, NJ, USA).

2.5. Immunofluorescence and confocal microscopy

Trophozoites grown on coverslides were fixed with 4%-paraformaldehyde for 30 min at 37 °C and permeabilized with 0.5%-Triton X-100 for 30 min at room temperature. Then, cells were incubated for 1 h at 37 °C with the rabbit antibody against EhSERCA (1:50 dilution) and a mouse antibody against calreticulin of *E. histolytica* (EhCRT) (Gonzalez et al., 2011) (1:50 dilution). Goat anti-rabbit IgG conjugated to fluorescein and goat anti-mouse IgG conjugated to rhodamine (ZYMED, San Francisco, CA, USA) were used as secondary antibodies (1:200 dilution). Finally, samples were observed through a confocal microscope (Leica TCS SP2, Leica Microsystems Inc. Bannockburn, IL, USA). Observations were performed in 13 planes from the top to the bottom of each sample; the distance between scanning planes was 1 μm.

2.6. Immunoelectron microscopy

The immunoelectron microscopy assays were performed as described (Segovia-Gamboa et al., 2011). *E. histolytica* trophozoites were fixed in 4%-paraformaldehyde and 0.1%-glutaraldehyde in serum-free DMEM for 1 h at room temperature. Samples were embedded in LR White and polymerized under UV at 4 °C overnight. Sections were obtained and mounted on formvar-covered nickel grids. Later they were incubated with antibodies against EhSERCA and against EhCRT (1:20 dilution). Goat anti-rabbit IgG conjugated to 30 nm gold particles and goat anti-mouse IgG conjugated to 15 nm gold particles (Ted Pella Inc., Redding, CA, USA) were used as secondary antibodies (1:60 dilution) to detect EhSERCA and EhCRT, respectively. Immunolabeling was carried out at room temperature. Thin sections were observed with a Morgani 268 D Philips transmission electron microscope (FEI Company, Hillsboro, OR, USA).

3. Results

3.1. Identification, in silico characterization, and expression of E. histolytica genes encoding putative Ca^{2+}-ATPases

To identify putative Ca^{2+}-ATPases encoding genes in *E. histolytica* we performed an exploration of the genes labeled as putative calcium-transporting ATPases on the database of the *E. histolytica* genome <http://pathema.jcvi.org/cgi-bin/Entamoeba/PathemaHomePage.cgi>. By this strategy, eight genomic sequences were identified (EHI_001150, EHI_016480, EHI_027710, EHI_030830, EHI_054830, EHI_065670, EHI_136610, and EHI_159040). No one of these sequences showed the presence of introns. Besides, we performed a search by Blast homology using as a probe the consensus sequence of the E1–E2 domain for mammalian Ca^{2+}-ATPases. This search displayed six of the eight sequences previously found; suggesting that this parasite possibly contains up to eight Ca^{2+}-ATPases.

In silico analysis of the identified sequences showed that they have 1884–3414 nucleotides, encoding polypeptides of 627–1137 amino acid residues. Predicted polypeptides contain several transmembranal sequences (five to ten) and they showed several domains present in the cation transporters of P-type ATPases (Fig. 1A). Five of these polypeptides include towards their amino-terminus a cation ATPase-N domain (Fig. 1A), a sequence found in several classes of cation-transporting P-type ATPases that by reversible phosphorylation induces conformational changes that may be important for regulate the function of these ATPases (Fujitani et al., 2003). Six polypeptides showed the E1–E2 ATPase motif (Fig. 1A) that is distinctive of the p-type ATPases, which form an aspartyl phosphate intermediate in the course of ATP hydrolysis (Brini and Carafoli, 2009). The E1–E2 motif was not found in the predicted polypeptides from EHI_001150 and EHI_159040 sequences (Fig. 1A). Additionally, all polypeptides, excluding that encoded for the EHI_136610 sequence, have a hydrolase domain and a cation ATPase-C domain (Fig. 1A). In addition, hydropathy analysis revealed that five of these predicted polypeptides, like Ca^{2+}-ATPases from different organisms, contain 10 transmembranal sequences (Fig. 1A). Thus, five *E. histolytica* polypeptides (encoded by EHI_016480, EHI_027710, EHI_030830, EHI_054830, and EHI_065670) contain all motifs that are present in bona fide Ca^{2+}-ATPases (Fig. 1A), suggesting that these polypeptides could be playing an important role in calcium homeostasis in this parasite. These polypeptides displayed 31.4–90.1% similarity among them (Table 2), and 30.7–50.8%, 32.8–42.3%, and 33.8–45.1% similarity to human PMCA, SERCA and SPCA, respectively.

To analyze the expression of the five putative Ca^{2+}-ATPases of *E. histolytica* we performed RT-PCR assays using specific primers for each gene (Table 1). These experiments showed the amplification of DNA fragments with the expected size for each putative Ca^{2+}-ATPase when cDNA was used as template, whereas no amplification was obtained in reactions using RNA as template or in reactions without template (Fig. 1B). The accuracy of amplified sequences was confirmed by sequencing. All these results suggest that polypeptides encoded by EHI_016480, EHI_027710, EHI_030830, EHI_054830, and EHI_065670 may be involved in regulate the cytoplasmic concentration of calcium in *E. histolytica*.

3.2. Identification of the putative SERCA of E. histolytica (EhSERCA)

In eukaryotes there are three general types of Ca^{2+}-ATPases: PMCA, located in plasma membrane, SERCA, situated in endoplasmic reticulum, and SPCA/PMR1, which is found in the Golgi apparatus (Brini and Carafoli, 2009). To determine the relationship of each putative Ca^{2+}-ATPase of *E. histolytica* with the different kind of eukaryotic Ca^{2+}-ATPases we aligned the predicted polypeptides with representative members of each type of eukaryotic Ca^{2+}-ATPases. Based on this alignment, we constructed a phylogenetic tree that revealed that polypeptides encoded by EHI_016480, EHI_030830, and EHI_054830 are related to PMCA of different organisms (Fig. 2A), whereas the polypeptide encoded by EHI_027710 is grouped with SERCA pumps of several organisms (Fig. 2A), and the polypeptide encoded by EHI_065670 is classified into the branch containing the PMR1 members of *Saccharomyces*

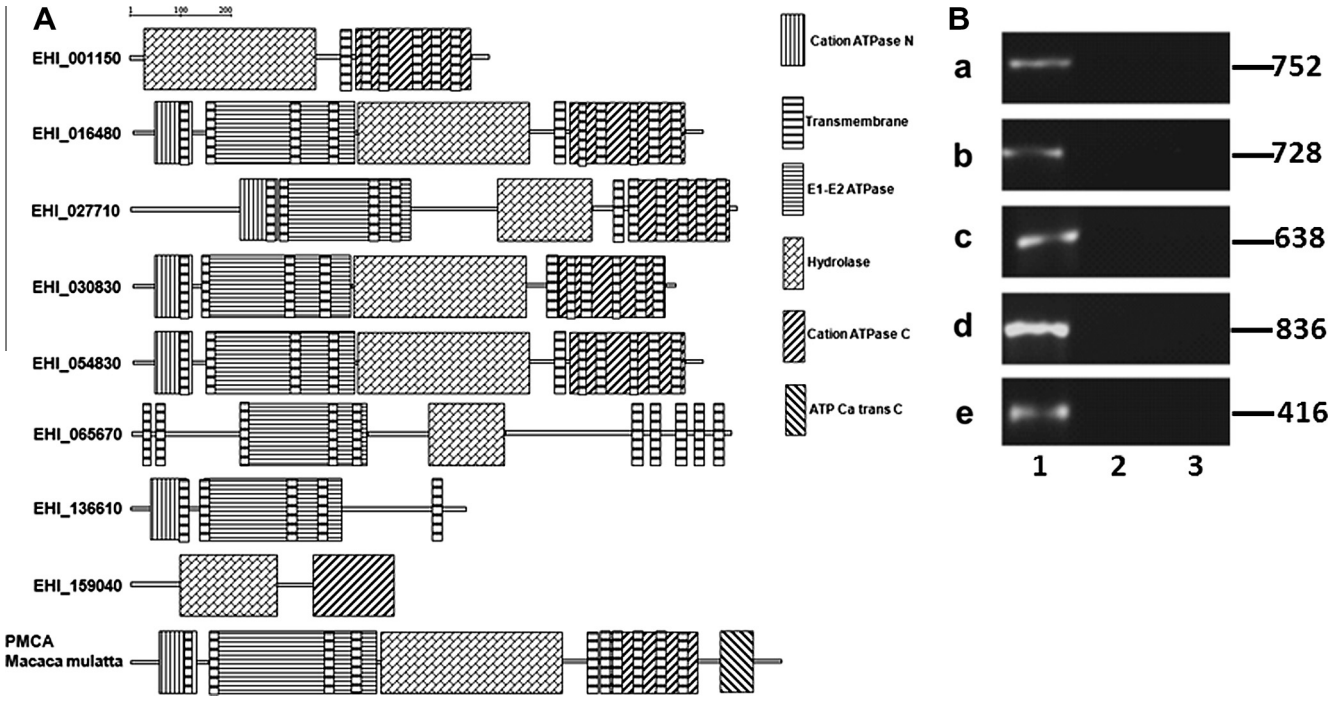

Fig. 1. In silico characterization and expression of putative Ca^{2+}-ATPases of *E. histolytica*. (A) Schematic representation of the predicted polypeptides. We performed an exploration of genes labeled as putative calcium-transporting ATPases on the database of the *E. histolytica* genome and the predicted polypeptides were in silico analyzed. (B) RT-PCR of each putative Ca^{2+}-ATPase gene. *E. histolytica* RNA was reverse transcribed using superscript II and an oligo-dT primer. Then, PCR assays were performed using the cDNA and specific primers for each Ca^{2+}-ATPase gene. Lane 1, PCR using cDNA as template. Lane 2, PCR control using RNA as template (RT enzyme was omitted). Lane 3, PCR control without template.

Table 2
Percentage of similarity between the putative calcium ATPases of *E. histolytica*.

Similarity (%)	EHI_027710	EHI_065670	EHI_054830	EHI_030830	EHI_016480
EHI_027710	–				
EHI_065670	36.6	–			
EHI_054830	39.0	31.4	–		
EHI_030830	39.0	33.1	68.1	–	
EHI_016480	40.8	31.5	90.1	69.8	–

cerevisiae and *Schizosaccharomyces pombe* (Fig. 2A). Interestingly, the organellar-related ATPases of *E. histolytica* have a very large N-terminal sequence before the E1–E2 domain (Fig. 1A), which could be involved in their location or in their functional regulation.

SERCA is an ATPase that sequesters Ca^{2+} into the endoplasmic reticulum and is known to play an important role in various cellular processes (Brini and Carafoli, 2009). The phylogenetic tree showed that the polypeptide encoding for the EHI_027710 sequence is more related to SERCA-type ATPases. In addition, polypeptide encoding by EHI_027710 displayed more similarity to mammalian SERCA (40.4–41.5%) than to PMCA (35.3–36%). These results suggest that this protein could be the putative SERCA of this parasite (EhSERCA). Interestingly, this polypeptide contains in its carboxy-terminus the sequence HDDL (Supplementary Fig. 1), which is very similar to the endoplasmic reticulum-retention signal in *S. cerevisiae* (HDEL) (Pelham et al., 1988), supporting the proposal that this protein could be the SERCA orthologue in *E. histolytica*.

Of the three families of calcium-ATPases, only SERCA pumps translocate two Ca^{2+} ions and hydrolyze one ATP for each catalytic turnover. Thus, SERCA members possess two Ca^{2+}-transport sites: site I and site II; the numbers specify the sequence of filling of the respective sites. The single Ca^{2+}-binding site of the SPCA and PMCA pumps structurally corresponds to site II of SERCA (Toyoshima, 2009). Several residues of the two calcium-binding sites of the rabbit SERCA1a are conserved in the putative EhSERCA (Val-490, Ile-493, Glu- 495, Asn-914, Glu-917, Asn-942, and Glu-946), whereas three of these residues are not conserved (Fig. 2B). Interestingly, residues Ile-493 and Glu-917 (Ile-307 and Glu-800 in rabbit SERCA) are not present in the corresponding regions of PMCA members. The *E. histolytica* protein shared conserved sequences in other regions: LTGE (positions 367–370) in the transduction domain; ICTDKTGTLT (positions 534–543) in the phosphorylation domain; KGACE (positions 669–673), DPPR (positions 747–750) and IITGDH (positions 769–774) in the ATP-binding domain; and TGDGVNDAPAIK (position 847–858) in the hinge domain (Fig. 2B and Supplementary Fig. 1). In addition, in the putative EhSERCA protein lacks the calmodulin-binding sites found in the C-terminus of PMCA members.

3.3. Localization of EhSERCA in trophozoites

To localize the putative EhSERCA in *E. histolytica* trophozoites, we generated a specific antiserum against a 16-amino acid sequence situated in position 959–974. By Western blot assays using total extracts of trophozoites, the antibody against EhSERCA recognized a single band of about 115-kDa, whereas, the preimmune serum did not identify any band (Fig. 3A). The estimated molecular weight of EhSERCA is approximately of 127 kDa. The different molecular weight observed in our assays could be due to posttrans-

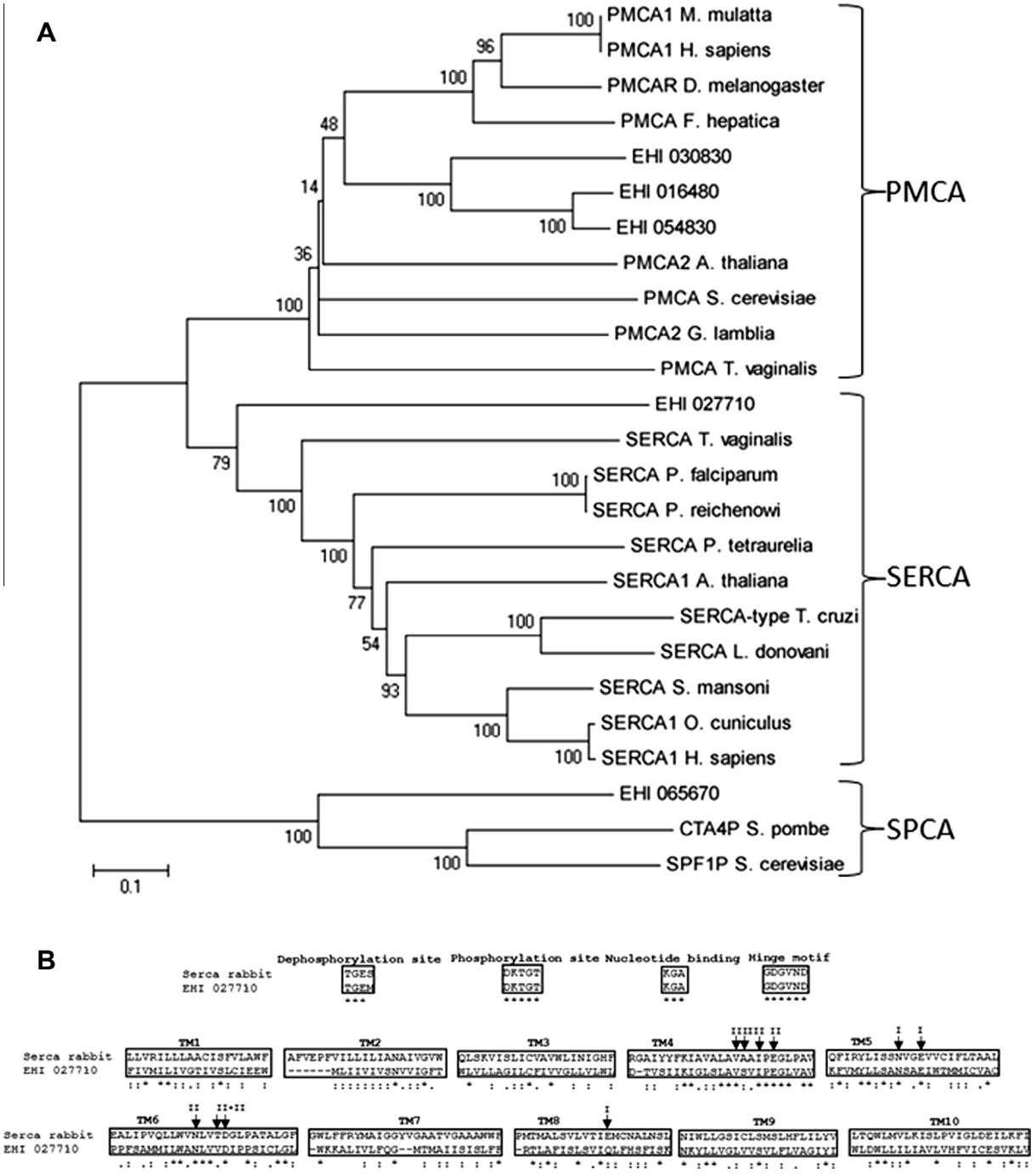

Fig. 2. Phylogenetic analysis and identification of the calcium-binding domains in the putative EhSERCA. (A) Phylogenetic relationship of the Ca^{2+}-ATPases of *E. histolytica*. The predicted amino acid (aa) sequences of the putative Ca^{2+}-ATPases of *E. histolytica* were aligned with sequences of Ca^{2+}-ATPases of other organisms by MUSCLE and data were submitted to phylogenetic analysis by the minimum evolution method using MEGA version 5.05. The Ca^{2+}-ATPases of other organisms used for this analysis were: (i) PMCA: PMCA2 of *Arabidopsis thaliana* (O81108.1), PMCAR of *Drosophila melanogaster* (NP_001188517.1), PMCA of *Fasciola hepatica* (AFM84633.1), PMCA2 of *Giardia lamblia* (EFO61349.1), PMCA of *Homo sapiens* (NP_001673.2), PMCA1 of *Macaca mulatta* (XP_001102118.1), and PMCA of *Trichomonas vaginalis* (XP_001326864.1); (ii) SERCA: SERCA1 of *Arabidopsis thaliana* (P92939.2), SERCA4 of *Arabidopsis thaliana* (AAD29957.1), SERCA1 of *Homo sapiens* (O14983.1), SERCA of *Leishmania donovani* (AAV65111.1), SERCA1 of *Oryctolagus cuniculus* (NP_001082787.1), SERCA of *Paramecium tetraurelia* (CAA76764.1), SERCA of *Plasmodium falciparum* (AFJ67936.1), SERCA of *Plasmodium reichenowi* (BAD73969.1), SERCA of *Schistosoma mansoni* (XP_002574127.1), SERCA-type of *Trypanosoma cruzi* (AAD08694.1), and SERCA of *Trichomonas vaginalis* (EAY14503.1); and (iii) PMR1: SPF1P of *Saccharomyces cerevisiae* (NP_010883.3), and CTA4P of *Schizosaccharomyces pombe* (O14072.1). Bootstrap values based on 1000 replicates are shown above each branch. (B) Comparison of structural domains between rabbit SERCA1a and putative EhSERCA. Upper, dephosphorylation and phosphorylation sites, nucleotide-binding site and hinge domain. Lower, calcium-binding sites and transmembranal domains. Arrows indicate the residues in calcium-binding sites 1 and 2 of rabbit SERCA1a. The structural elements of transmembranal helices 1 to 10 (TM1–TM10) of rabbit SERCA1a are represented by boxes.

lational modifications that produces the 115 kDa band. We suggest that the recognition of the antibody is specific against EhSERCA because the BLAST analysis on the genome database of *E. histolytica* using as a probe the sequence corresponding to the peptide used to generate the antibody only matches with the putative EhSERCA polypeptide.

Then, the anti-EhSERCA antibody was used for immunofluorescence and confocal microscopy (Fig. 3B). In these experiments EhS-

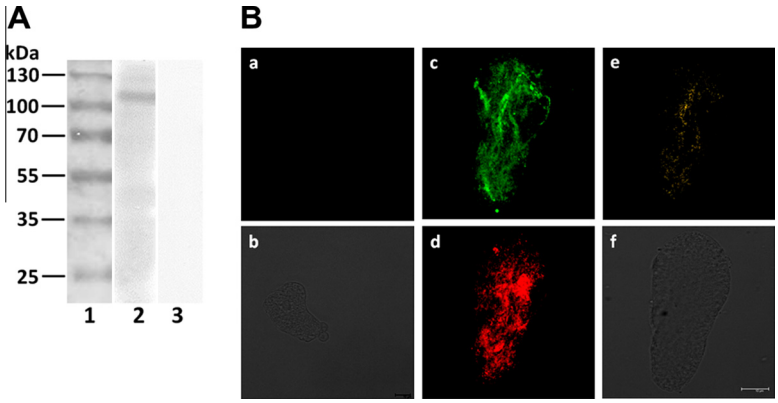

Fig. 3. Identification and cellular localization of a putative EhSERCA. (A) Western blot. A peptide of the putative EhSERCA, located in position 959–974 (MSNIMERKPRDPNSNI), was synthesized and immunized in rabbits. Then, antibodies (anti-EhSERCA) were used in Western blot assays on total extracts of *E. histolytica* trophozoites. Line 1, Molecular weight markers. Line 2, anti-EhSERCA antibodies. Line 3, preimmune serum. (B) Cellular localization of EhSERCA by confocal microscopy. *E. histolytica* trophozoites were fixed, permeabilized and incubated with rabbit anti-SERCA and mouse anti-EhCRT antibodies, and then, with secondary anti-rabbit-fluoresceinated and anti-mouse-rhodamineted antibodies. After that, cells were observed through confocal microscopy. a. Negative control using preimmune serum. b. Phase contrast of control. c. Green channel sowing the localization of EhSERCA. d. Red channel showing the localization of calreticulin. e. Merge. f. Phase contrast of colocalization.

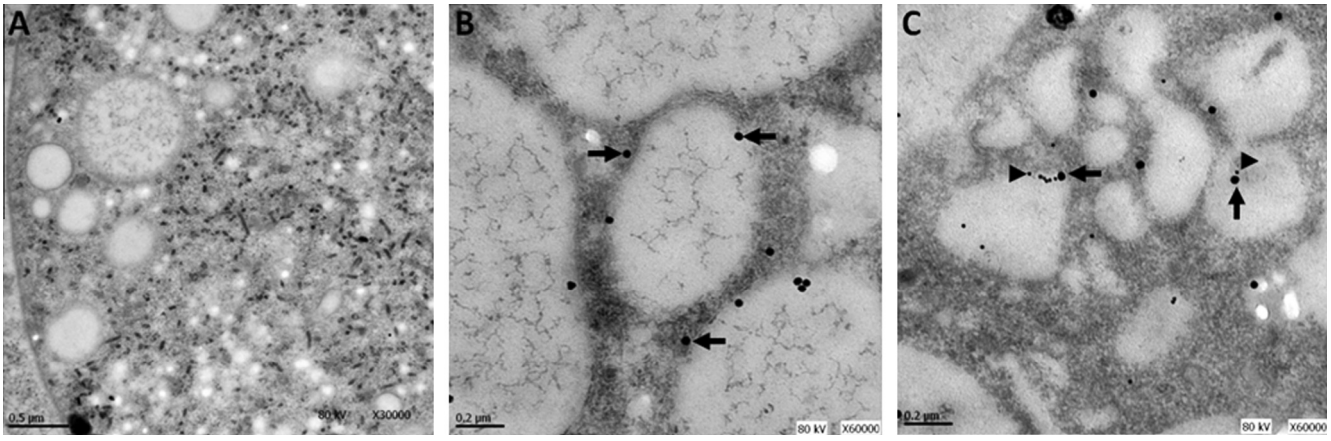

Fig. 4. Immunoelectron microscopy. Sections of trophozoites were incubated with antibodies against EhSERCA and against EhCRT and then, with anti-rabbit IgG conjugated to 30 m gold particles and anti-mouse IgG conjugated to 15 nm gold particles. Then, samples were analyzed by transmission electron microscopy. (A) Control omitting the primary antibodies. (B) Immunoelectron detection of EhSERCA. (C) Immunelectron detection of EhSERCA and EhCRT. Arrows showed the detection of EhSERCA. Arrowheads indicate the detection of EhCRT.

ERCA was detected within an apparently continuous reticular component in the cytoplasm of trophozoites (Fig. 3Bc). This structure is similar to that described as the endoplasmic reticulum of *E. histolytica* (Teixeira and Huston, 2008). As control, we used an antibody against calreticulin of *E. histolytica* (anti-EhCRT) (Gonzalez et al., 2011), a protein mainly located in endoplasmic reticulum, although it may be situated also in the outer cell surface of a variety of cell types, in the cytosol, and in the extracellular matrix (Gold et al., 2010). Indeed, in *E. histolytica* trophozoites, calreticulin has been detected in endoplasmic reticulum (Girard-Misguich et al., 2008; Gonzalez et al., 2011), as well as on the surface following either capping of surface antigens with concanavalin A or interaction with host cells (Girard-Misguich et al., 2008; Marquay Markiewicz et al., 2011; Vaithilingam et al., 2012). In our assays, the anti-EhCRT antibody also recognized a continuous reticular component in the trophozoites (Fig. 3Bd). Interestingly, EhSERCA and EhCRT showed a high level of co-localization (Fig. 3Be).

In addition, we carried out the localization of EhSERCA by immunoelectron microscopy. In these assays, EhSERCA was detected in the internal membranes of some cytoplasmic vesicles (Fig. 4B) and no label was detected in the plasma membrane. Inside of some vesicles labeled by anti-EhSERCA we also detected the presence of calreticulin, identified by the anti-EhCRT antibody (Fig. 4C). All these results demonstrate that polypeptide encoded by the EHI_027710 sequence corresponds to an intracellular Ca^{2+}-ATPase, which probably could corresponds to the orthologue of SERCA in *E. histolytica*.

4. Discussion

The participation of calcium in signaling on many cell processes demands its efficient control. This is possible by the function of membrane calcium transporters and channels that move this ion across membrane boundaries. These proteins belong to several subclasses that differ in transport mechanism, affinity for Ca^{2+}, and total transport capacity (Brini and Carafoli, 2009). Among these proteins, Ca^{2+}-ATPases are key actors in the regulation of calcium in eukaryotic cells and are thus essential to the correct functioning of the cell machinery.

Several reports suggest that calcium also has an important role on signaling of different cellular processes in *E. histolytica*, including development and pathogenesis (Bhattacharya et al., 2006; Carbajal et al., 1996; Debnath et al., 2005; Gilchrist et al., 2003; Jain

et al., 2008; Leon-Avila et al., 2006; Munoz Mde et al., 1991; Ravdin et al., 1980, 1985). However, the search of the *E. histolytica* genome database failed to identify genes encoding intracellular and plasma membrane calcium channel homologues, such as voltage-gated, TPC, TRP, or CRAC (Prole and Taylor, 2011), suggesting that other kind of transport proteins are involved in the flux of calcium in this parasite. Here, we identified five putative Ca^{2+}-ATPases, which could be important in the regulation of the cytoplasmic concentration of calcium in *E. histolytica*.

Ca^{2+}-ATPases have been classified in three major classes, PMCA. SERCA, and SPCA/PMR1. In higher eukaryotes each class is encoded by multigenic families and their diversity increased by the presence of several isoforms produced by alternative splicing (Brini and Carafoli, 2009). In contrast, information about Ca^{2+}-ATPases of protozoa parasites is restricted. A Ca^{2+}-ATPase of *Trypanosoma brucei* (TBCA2) essential for survival and cation homeostasis was identified and localized in the pericellular membrane and the flagelar pocket of bloodstream forms (Ramey et al., 2009). Genome analyses in Apicomplexa (*Plasmodium* spp., *Cryptosporidium* spp., and *Toxoplasma gondii*), showed that these parasites contain a single ortholgue of the SERCA ATPase (Kimura et al., 1993), an orthologue of the yeast PMR1 transporter (Nagamune and Sibley, 2006) that localize in the plasma membrane of asexual stages (Dyer et al., 1996), and several additional Golgi-type Ca^{2+} ATPases (Nagamune and Sibley, 2006).

The phylogenetic analysis of the putative Ca^{2+}-ATPases of *E. histolytica* suggested that PMCA- and organellar-type pumps are present in this parasite, and our results showed that all of them are expressed in trophozoites. Our bioinformatic analysis suggests that three of these putative proteins are associated with plasma membrane elements and possible they could have a redundant function. Interestingly, the PMCA-like polypeptides encoded by EHI_030830 and EHI_016480 were detected among the surface proteins present in a uropod extruded fraction during the exposure of trophozoites to serum of humans suffering amoebiasis (Marquay Markiewicz et al., 2011), supporting the hypothesis that these proteins are located in the plasma membrane. On the other hand, a previous report showed that the polypeptide encoded by EHI_054830, the other PMCA-like protein, is located in cytoplasmic vesicles situated towards the periphery of the parasites (Ghosh et al., 2000). Similarly, a PMCA-type protein of *T. gondii* was located in the acidocalcisomes (Luo et al., 2001). Thus, the precise roles played by the Ca^{2+}-ATPases of *E. histolytica* in calcium homeostasis and signaling remains to be investigated by functional studies.

The SERCA pump is known to be present in most eukaryotes, which delineates this pump as a versatile, evolutionarily conserved protein. Although its primary function is to control cytosolic calcium, it plays a vital role in many cellular functions including cell growth and differentiation (Brini and Carafoli, 2009). In vertebrates there are three distinct genes encoding SERCA that produces by alternative splicing more than ten isoforms, while in invertebrates this pump is encoded by a single gene (Periasamy and Kalyanasundaram, 2007). Here, we identified an *E. histolytica* gene (EHI_027710) that probably encodes a SERCA orthologue. This assumption was based on: (i) the polypeptide contains all the motifs that distinguish to the P-type Ca^{2+}-ATPases and most of the residues of the two calcium-binding sites of rabbit SERCA; (ii) the phylogenetic analysis showed that it is more related to SERCA than to PMCA or SPCA pumps; (iii) the sequence HDDL in its carboxy-terminus is very similar to the reticulum endoplasmic retention signal of *S. cerevisiae* (HDEL); and (iv) the polypeptide was located in reticular structures inside the trophozoites and showed co-localization with calreticulin. However, more experiments are needed to confirm that this polypeptide is the SERCA orthologue in *E. histolytica*.

Thapsigargin, a sesquiterpene lactone produced by the plant *Thapsia garganica*, blocks the activity of SERCA by locking the protein in the E2 form, which has a lower affinity for calcium (Sagara et al., 1992). Carbajal et al. (1996) showed that thapsigargin induced a transient increase in the cytoplasmic concentration of calcium in *E. histolytica*. Artemisinin is also a sesquiterpene lactone that is produced by the plant *Artemisia annua*, which show potent and selective activity against Apicomplexa parasites, making them useful for the treatment against these microorganisms. While the mechanism of action is uncertain, previous studies have suggested that artemisinin may inhibit SERCA, thus disrupting calcium homeostasis in *Plasmodium* spp. and *T. gondii* (Eckstein-Ludwig et al., 2003; Nagamune et al., 2007). Artemisinins also disrupt the growth of *Trypanosoma cruzi* and inhibit calcium-dependent ATPase activity in membrane fractions from the parasite (Mishina et al., 2007). These results support the idea that Ca^{2+}-ATPases are potential drug targets against parasites. Our studies showed that viability of *E. histolytica* trophozoites was not significant altered by the presence of thapsigargin or artemisinin (data not shown), indicating that the orthologue of SERCA in this parasite has structural differences with mammalian and Apicomplexa pumps that make it insensitive to those drugs. Interestingly, the Glu-255 residue of rabbit SERCA and the corresponding Leu-263 of *P. falciparum* orthologue, which are involved in thapsigargin and artemisinin sensitivity, respectively (Uhlemann et al., 2005), is replaced by an asparagine residue in the corresponding region of EhSERCA (Asn-442) (Supplementary Fig. 1). Thus, is possible to suppose that other sesquiterpene lactones could distinctively block EhSERCA and they possibly will be anti-amoebic specific drugs.

In conclusion, in this work we identified five *E. histolytica* genes encoding for putative Ca^{2+}-ATPases, three of them seem to be similar to plasma membrane elements, while the other two belongs to organellar members. Specific antibodies against the polypeptide encoded by the EHI_027710 sequence located this pump within an apparently continuous reticular element in the cytoplasm of trophozoites, strongly suggesting that this polypeptide may possibly be the SERCA orthologue in *E. histolytica*.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.exppara.2013.06.004.

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