TeSCA Complements Yeast Mutants Defective in Ca\textsuperscript{2+} Pumps and Encodes a Ca\textsuperscript{2+}-ATPase That Localizes to the Endoplasmic Reticulum of Trypanosoma cruzi*

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Intracellular Ca\textsuperscript{2+} in Trypanosoma cruzi is mainly located in an acidic compartment named the acidocalcisome, which among other pumps and exchangers possesses a plasma membrane-type Ca\textsuperscript{2+}-ATPase. Evidence for an endoplasmic reticulum-located Ca\textsuperscript{2+} uptake has been more elusive and based on indirect results. Here we report the cloning and sequencing of a gene encoding a sarcoplasmic-endoplasmic reticulum-type Ca\textsuperscript{2+}-ATPase from *T. cruzi*. The protein (TeSCA) predicted from the nucleotide sequence of the gene has 1006 amino acids and a molecular mass of 108.7 kDa. Several sequence motifs found in sarcoplasmic-endoplasmic reticulum-type Ca\textsuperscript{2+}-ATPases were present in TeSCA. Expression of TeSCA in yeast mutants deficient in the Golgi and vacuolar Ca\textsuperscript{2+} pumps (*pmr1* *pmc1 cnb1*) restored growth on EGTA. Membranes were isolated from the *pmr1* *pmc1* *cnb1* mutant transformed with TeSCA, and it was found that the TeSCA polypeptide formed a Ca\textsuperscript{2+}-dependent and hydroxylamine-sensitive \textsuperscript{32}P-labeled phosphoprotein of 110 kDa in the presence of [\(\gamma\textsuperscript{32}P\)]ATP. Cyclopiazonic acid, but not thapsigargin, blocked this phosphoprotein formation. Transgenic parasites expressing constructs of TeSCA with green fluorescent protein exhibited co-localization of TeSCA with the endoplasmic reticulum proteins BiP and calreticulin. An endoplasmic reticulum location was also found in amastigotes and trypomastigotes using a polyclonal antibody against a COOH-terminal region of the protein. The ability of TeSCA to restore growth of mutant *pmr1* *pmc1* *cnb1* on medium containing Mn\textsuperscript{2+} suggests that TeSCA may also regulate Mn\textsuperscript{2+} homeostasis by pumping Mn\textsuperscript{2+} into the endoplasmic reticulum of *T. cruzi*.

Trypanosoma cruzi, the etiologic agent of Chagas’ disease, is a parasitic protozoan that invades mammalian cells and develops intracellularly as amastigotes. Invasion of cells by *T. cruzi* is dependent upon an elevation in the concentration of cytosolic free calcium in the invading trypomastigote (1, 2). Unlike mammalian cells, *T. cruzi* possesses most of its intracellular Ca\textsuperscript{2+} in an acidic compartment named the acidocalcisome (3–8). The molecular and biochemical characterization of this organelle has provided evidence that it has an orthovanadate-sensitive plasma membrane-type Ca\textsuperscript{2+} ATPase for Ca\textsuperscript{2+} uptake (3, 5–7). Evidence for non-mitochondrial and endoplasmic reticulum-located Ca\textsuperscript{2+} uptake has been more elusive and based on the presence of a low capacity and high affinity orthovanadate-sensitive Ca\textsuperscript{2+} uptake in permeabilized cells and the ability of these cells to buffer [Ca\textsuperscript{2+}] in the range 0.05–0.1 \(\mu\text{M}\) (9), features in common with the sarcoplasmic/endoplasmic reticulum Ca\textsuperscript{2+}-ATPases (SERCA)\textsuperscript{3} of animal cells (10). In addition, like mammalian cells (11), calcium is needed for the correct folding and assembly of proteins in the endoplasmic reticulum of *T. cruzi*, which depends on chaperones such as the Ca\textsuperscript{2+}-binding protein, calreticulin (12).

Biochemical distinction of the different Ca\textsuperscript{2+} pumps present in *T. cruzi* has been hampered by the lack of distinguishing features such as specific inhibitor sensitivity. Orthovanadate inhibits all types of Ca\textsuperscript{2+} ATPases (10), whereas thapsigargin, a specific inhibitor of animal SERCA-type Ca\textsuperscript{2+}-ATPases (13), is ineffective in inhibiting Ca\textsuperscript{2+} uptake in permeabilized *T. cruzi* (14, 15). Thus, a molecular approach to studying individual pumps is necessary. Expression of genes identified as encoding Ca\textsuperscript{2+}-ATPases and localization of the corresponding proteins is necessary, as the plasma membrane-type Ca\textsuperscript{2+} ATPase/SERCA paradigm does not necessarily apply in non-animal cells; a SERCA-type gene in tomato is expressed in different parts of the cell (16). Also, a *Tca1* gene has been identified in *T. cruzi* (6), which encodes a protein with homology to mammalian plasma membrane Ca\textsuperscript{2+}-ATPases but with characteristics that place it in a novel category of Ca\textsuperscript{2+}-ATPases described in Saccharomyces cerevisiae (17), Dictyostelium discoideum (18), Entamoeba histolytica (19), and Toxoplasma gondii (20). The gene is expressed at a high level in the amastigote stage and is localized to acidocalcisomes and the plasma membrane of the parasite (6). Here we demonstrate that a gene from *T. cruzi* (TeSCA) complemented yeast mutants defective in Ca\textsuperscript{2+} pumps by restoring their growth in EGTA. The protein encoded by the TeSCA gene localizes to the endoplasmic reticulum of different stages of the parasite and, in contrast to the acidocalciosomal Ca\textsuperscript{2+}-ATPase (6), it is expressed at similar levels in the different developmental stages of the parasite.

\textsuperscript{3}The abbreviations used are: SERCA, sarcoplasmic-endoplasmic reticulum-type Ca\textsuperscript{2+}-ATPase; TeSCA, *T. cruzi* SERCA-type Ca\textsuperscript{2+}-ATPase; PPR, polymerase chain reaction; bp, base pairs; ORF, open reading frame; GFP, green fluorescent protein; Mops, 4-morpholinepropanesulfonic acid; SC-URA, synthetic complete medium minus uracil; ER, endoplasmic reticulum.
Fig. 1. Alignment of different SERCA-type Ca\(^{2+}\)-ATPases. CLUSTALW alignment of Ca\(^{2+}\)-ATPases from T. cruzi (GenBank™ accession number AF093566), T. brucei (AAA30227; TBA1), L. m. amazonensis (U70620; Lmaa1), Homo sapiens SERCA 1 (1586563; SERCA1), and Arabidopsis thaliana RCA3 (AJ132388; RCA3). Identical residues are in black, and similar residues are shaded. Amino acid residues not present...
EXPERIMENTAL PROCEDURES

Culture Methods—*T. cruzi* amastigotes and trypomastigotes (Y strain) were obtained from the culture medium of L6E9 myoblasts by a modification of the method of Schmatz and Murray (21), as we have described before (14, 15). The contamination of trypanosomes with amastigotes and intermediate forms of or amastigotes with trypanosomes or intermediate forms was always less than 5% unless otherwise stated. Trypanosomes (Y strain) were grown at 28 °C in liver infusion tryptose medium (22) supplemented with 10% newborn calf serum. *S. cerevisiae* strain K616 (MATa trp1::HIS3 pnc1::TRP1 cnb1::LEU2, ura3) (23) was kindly provided by Kyle W. Cunningham, Department of Biology, The Johns Hopkins University, Baltimore, MD and was maintained in YPD agar plates (1% Difco yeast extract, 2% Bacto Peptone, 2% dextrose, and 2% agar).

Chemicals—Restriction enzymes and protease inhibitor mixture (P-8340) were purchased from Sigma. Yeast media were bought from Bio 101 (Vista, CA). Trizol reagent, reverse transcribease, and Taq polymerase were from Life Technologies, Inc. (Eugene, OR). The pGEM-T Easy vector, Riboprobe in vitro transcription system and Prime-a-Gene labeling system were from Promega (Madison, WI). The *PfuTurbo* DNA polymerase, the AZAP-Express phage and pBluescript KSI— vectors were from Stratagene (La Jolla, CA). [γ-32P]ATP, [α-32P]dCTP, and [α-32P]UTP were from Amersham Pharmacia Biotech. The primers were purchased from Genosys Biotechnologies Inc. (Woodlands, TX). Plasmid pYES2 and used to transform yeast cells. To obtain the vector for green fluorescent protein (GFP)-tagged *TcSCA*, a *BamHI/HindIII* fragment that contained the entire *TcSCA* ORF was ligated into the *BamHI/HindIII* site of pBluescript KSI—. Site-directed mutagenesis was carried out to introduce a unique *NheI* site in the ORF to insert GFP into the loop region of *TcSCA*. For this purpose, a pair of primers (5'-GAGGAGAATTTAGCTGATCCGGATCCGTAAC-3' and 5'-GGATTACCCTAGCTGATCCGGATCCGTAAC-3') that correspond to a part of the loop region (amino acids 371–381) containing a *NheI* site and complementary to each other were used to amplify the whole plasmid with the mutation by using *PfuTurbo* DNA polymerase. The DNA product was then digested by *DpnI* to eliminate the methylated, non-mutated parental DNA template and used to transform Escherichia coli. The GFP fragment with *NheI* sites was amplified by PCR with a primer pair (5'-GGAGGAGAATTTAGCTGATCCGGATCCGTAAC-3' and 5'-GGATTACCCTAGCTGATCCGGATCCGTAAC-3') and the GFP-containing plasmid pGFP-2* (28) was digested by *NheI* and ligated into the *NheI* site of the *TcSCA* ORF in pBluescript KSI—. The *BamHI/HindIII* fragment containing the entire *TcSCA* ORF with the GFP insertion was ligated into the *BamHI/HindIII* site of *T. cruzi* expression vector pTEX (29). The resulting vector was named *TcSCA*-GFP/pTEX and was used to transform 101 strain of *T. cruzi* as described above.

Nucleic Acids Blotting—For Southern blotting, total DNA from *epimastigotes* (10 μg/lane) was digested with *BamHI*, *BglI*, *HindIII*, *SalI*, *SphI*, and *EcoRI*, separated on 1.0% agarose with TAE (40 mM Tris, 20 mM acetic acid, 1 mM EDTA (pH 8.0)) buffer, and transferred to Zeta-Probe GT nylon membranes. The blot was probed with [α-32P]dCTP-labeled *TcSCA* DNA. DNA markers and standard procedures (24) for the Northern blot analysis total RNA was isolated from trypomastigotes, epimastigotes, and amastigotes of *T. cruzi* with Trizol reagent according to the manufacturer’s instructions. RNA was electrophoresed in 1.0% agarose gels with 2.2 mM formaldehyde, 20 mM Mops (pH 7.0), 8 mM sodium acetate, and 1 mM EDTA and transferred to Zeta-Probe GT nylon membranes. DNA probes were prepared using random hexamer primers and a Klenow fragment of DNA polymerase I (Prime-a-Gene labeling system) and [α-32P]dCTP. RNA probes were prepared from linearized double-stranded DNA templates with either T3 or T7 promoter sequences upstream of the probe sequence using T3 or T7 RNA polymerase (Riboprobe in vitro transcription system) and [α-32P]dUTP. The *TcP0* (*T. cruzi* ribosomal protein 1; Ref. 30) fragment was used as a control in the Northern blots was obtained by amplifying *T. cruzi* genomic DNA by PCR, with primers corresponding to nucleotides 3–54 and 918–936 of the sequence of the *TcP0* gene (30). Densitometric analyses of Northern blots were done with an ISI-1000 digital imaging system (Alpha Inotech Corp.) and standardized using the intensity of *TcP0* transcripts and assessing a similar level of expression of this gene in all stages (31). Similar results were obtained when the densitometric values were compared by taking into account the amount of RNA added to each lane in three different experiments.

Production of Polyclonal Antibody against *TcSCA*—A DNA fragment encoding the 518-amino acid, COOH-terminal domain of *TcSCA* protein was generated by polymerase chain reaction using *T. cruzi* genomic DNA as a template. A 5′ primer (primer 1) containing an *NheI* site (5'-GTCAGGCGACTCCGCGTGATGCTGACTG-3') and a 3′ primer (primer 2) encoding an *HindIII* site (5'-AGACTCAAGCGCGCCGCTAC-3') were designed. The product was subcloned into the *NheI* and *HindIII* sites of the pET-28a+ expression vector, resulting in a construct that encoded the protein fused next to a six-histidine tag that allowed its purification on nickel-agarose columns. This plasmid was checked by DNA sequencing to ensure that the correct construct had
been obtained. The recombinant plasmid was transfected into the BL21(DE3) strain of E. coli, and cells were grown in LB medium. Gene expression was induced by adding isopropyl-1-thio-galactopyranoside at a final concentration of 1 mM when the cell density reached an A600 of 0.6. The cells were harvested after a 4-h incubation at 37 °C, sonicated (30 s in intervals at 4 °C) in 5 mM imidazole, 500 mM NaCl, and 20 mM Tris-HCl (pH 7.9), and centrifuged at 21,000 × g at 4 °C for 20 min for separation into pellet and supernatant fractions. The pellet, which contained inclusion bodies, was used to extract TeSca for antibody production following the instructions outlined for inclusion body purification in the His-Bind® kit (Novagen, WI). The protein was renatured by dialysis and concentrated to one-tenth volume.

Rabbits received 250 μg of fusion protein injected subcutaneously with Freund’s complete adjuvant (Difco). Subsequent injections were performed at 3-week intervals using 250 μg of fusion protein in incomplete Freund’s adjuvant (Difco). Serum was collected before the initial injection (preimmune serum) (via the ear nick method) and 1 week after every immunization. Once the desired specific antibody titer had been achieved, the rabbit received a final booster injection and was terminated by exsanguination 1 week later. The antisera was aliquoted and stored at −70 °C.

**Immunoblot Methods—**Aliquots of sonicated lysates of different stages of T. cruzi containing 10 μg of total protein were mixed with an equal amount of nonreducing 2× SDS buffer (125 mM Tris-HCl (pH 6.8), 20% glycerol (v/v), 8% SDS (w/v), and 0.005% (w/v) bromphenol blue) and boiled for 5 min before application of SDS-polyacrylamide gels. Proteins were separated using 7.5% Ready Gels (Bio-Rad) and blotted onto nitrocellulose (NitroPure, MSI, Westbrook, MA) using a Bio-Rad transblot apparatus by standard techniques. Subsequent processing steps were done in Dulbecco’s phosphate-buffered saline containing 0.1% Tween 20. Blots were blocked overnight at 4 °C with 5% nonfat dry milk, washed three times, and incubated with primary antibody (1:5,000) for 1 h at room temperature. Blots were then washed three times, incubated for 1 h with horseradish peroxidase-conjugated anti-rabbit IgG antibody (1:10,000), washed three times, and processed for chemiluminescence detection following the instructions of the manufacturer (Amersham Pharmacia Biotech). photographic exposures of 5 to 1 min were made.

**Fluorescence Microscopy—**After washing three times with phosphate-buffered saline, parasites were fixed with 4% formaldehyde in phosphate-buffered saline for 1 h at room temperature and allowed to adhere to poly-L-lysine-coated glass slides (Sigma) for 10 min. After permeabilization with 0.5% Triton X-100 for 3 min and blocking with 3% bovine serum albumin in phosphate-buffered saline for 1 h, the sections were incubated with a 1:100 dilution of the antibody (anti-TeSca) against the 55.3-kDa expressed protein followed by 1:100 of a fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulin G (IgG) secondary antibody, both at room temperature. Control preparations were incubated with preimmune serum (1:50) or without the primary antibody. Immunofluorescence images were obtained with an Olympus BX-60 fluorescence microscope digital image system (G, 32).

For Western blotting with GFP-tagged TcSca, the same methods were used, except that the primary antibodies against calreticulin, BiP, or T. cruzi TeCa1Ca ++-ATPase were used at a 1:150 dilution, and an Alexafluor 584-conjugated goat anti-rabbit IgG secondary antibody was used at 1:300.

**Cell Transformation and Growth—**Yeast strain KG16 was transformed with TcSca/pYES2 or the vector alone by the lithium acetate method (33). Transformants were selected by plating them on synthetic complete medium minus uracil (SC-URA) (23). Cells obtained from a single colony were grown overnight in SC-URA liquid medium with 2% galactose at 30 °C. The resulting cell suspension was used to inoculate the same medium to an initial A600 of 0.01. Either CaCl₂ or EGTA was added to the medium at various concentrations to change free-Ca ++ levels. Growth at 30 °C was monitored by measuring A600 after 24 or 48 h. For the assay of Mn ++ sensitivity, yeast grown in SC-URA plus 2% glucose was inoculated into glucose or galactose medium at an initial A600 of 0.4, and the A600 was measured at daily intervals for 4 days. Epimastigotes of T. cruzi (Y strain) were transformed with TcSca/GFP/ pTEX or vector alone by electroporation. Cells at the logarithmic phase of growth were mixed with Zapalo Medium (152 mM NaCl, 8 mM KCl, 8 mM NaHPO₄, 1.5 mM KH₂PO₄, 0.5 mM MgCl₂, 1 mM EDTA, 10 mM benzamidine, 5 mM dithiothreitol, 1.5% (v/v) protease inhibitor mixture (pH 7.5), and 12 mL of 0.5-mm glass beads (Biospec, Bartlesville, OK) in the chamber of a Biospec Bead Beater, and vortexed for 3 min, which resulted in 80–90% cell lysis. The beads were washed by gravity with YL buffer, and the supernatant fractions were centrifuged at 3000 × g for 5 min. The supernatants from this centrifugation were layered on top of a step gradient of 25%/45% sucrose in 25 mM K-Hepes, 2 mM MgCl₂, 10 mM benzamidine, 5 mM dithiothreitol (pH 7.5) and centrifuged in a Beckman SW28 rotor at 108,000 × g for 2 h. The 25%/45% interface was diluted 5-fold in 25 mM Na-Hepes (pH 7.2), 1 mM dithiothreitol, 2 mM MgCl₂, and centrifuged at 105,000 × g for 50 min. The microsome pellet was resuspended in 125 mM sucrose, 20 mM K-Hepes, 65 mM KCl, 2 mM MgCl₂ (pH 7.2). Protein was assayed with the Bio-Rad protein assay.

**Formation of Phosphoantigen—**32P-labeled phosphoprotein formation was assayed according to Schatzmann and Burgin (34) with some modifications. The reaction mixture (150 μl) contained 150 mM KCl, 1 mM EDTA, 0.02 mM MgCl₂, 75 mM K-Hepes (pH 7.0), and 15 μg of microsomal protein. Where indicated, 0.5 mM LaCl₃ was added to prevent nonspecific phosphorylation of the protein. To test catalytic dependence, CaCl₂ was added to a total concentration of 1.232 mM, resulting in a final free Ca ++ concentration of 220 μM. The reaction was started by adding [γ-32P]ATP (2 μCi/reaction; 3,000 Ci/mmol) to a final concentration of 19 μM and terminated after 30 s at 0 °C by adding 0.2 ml of 50 mM NaH₂PO₄, 2 mM ATP, and 5% trichloroacetic acid followed by centrifugation. Where indicated, 0.8 mM hydrogen peroxide in 0.6 sodium acetate (pH 5.3) added to the pellet and incubated for 15 min at room temperature. After two washes with trichloroacetic acid solution, the pellet was suspended in 20 μl of sample buffer and subjected to acidic SDS/polyacrylamide gel electrophoresis (35) and autoradiography.

**RESULTS**

**Cloning and Characterization of a SERCA-type Ca ++-ATPase Gene—**To clone the SERCA-type Ca ++-ATPase gene of T. cruzi, a region containing a conserved Ca ++-ATPase sequence was amplified from T. cruzi genomic DNA. To design degenerate oligonucleotide primers, amino acid sequences of SERCA-type Ca ++-ATPases of different species were retrieved from GenBank™, and regions with the highest similarity were located. Two primers (F4 and R2) were selected according to these domains, and the PCR was carried out with T. cruzi genomic DNA as a template. One of the PCR products (~650 bp) reacted strongly with a DNA probe from the Leishmania mexicana amazonensis putative SERCA-type Ca ++-ATPase (LmAal1) (32) by Southern blotting, and it was cloned into pGEM-T-easy. The deduced amino acid sequence of this PCR fragment was 77.6 and 84.2% identical to the sequences of the TcP0 gene from T. cruzi (22) and T. brucei (32) by Southern blotting, and it was cloned into pGEM-T-easy. The deduced amino acid sequence of this PCR fragment was 77.6 and 84.2% identical to the sequences of the TcP0 gene from T. cruzi (22) and T. brucei (32) by Southern blotting, and it was cloned into pGEM-T-easy.
A SERCA-type Ca\(^{2+}\)-ATPase from *T. cruzi*

Sequence Analysis of TcSCA—The TcSCA amino acid sequence is 73% identical to the *T. brucei* SERCA-type Ca\(^{2+}\)-ATPase sequence (TBA1 (36, 37)) and 61% identical to the *L. m. amazonensis* putative SERCA-type Ca\(^{2+}\)-ATPase sequence (*Lmaa1* (32)) (Fig. 1). It has about 46–48% identity to SERCA-type Ca\(^{2+}\)-ATPase sequences of non-trypanosomatids and only 24.1–24.8% identity to plasma membrane-type Ca\(^{2+}\)-ATPases from different species. Analysis of the TcSCA amino acid sequence showed that this gene product contains all the conserved subdomains and invariant residues found in other P-type ATPases such as the phosphorylation and ATP binding domains (38, 39). Hydropathy analysis of the deduced amino acid sequence revealed a profile very similar to those of other calcium pumps containing 10 transmembrane domains (M1-M10) (Fig. 1). This is in line with structures obtained from crystals of a mammalian SERCA-type Ca\(^{2+}\)-ATPase at 8 Å of resolution (40). TcSCA also contains all the residues (Glu\(^{315}\), Glu\(^{370}\), Asn\(^{790}\), Thr\(^{793}\), Asp\(^{794}\), and Glu\(^{895}\), indicated by asterisks above the alignment in Fig. 1) that were previously identified as the high affinity Ca\(^{2+}\)–binding sites in the center of the putative transmembrane domains M4, M5, M6, and M8 (41). As occurs with other SERCA-type pumps, TcSCA lacks the conserved amino acid sequence associated with calmodulin binding found near the COOH terminus of mammalian plasma membrane-type Ca\(^{2+}\)-ATPase isoforms (42). The amino acid sequence Lys-Asp-Asp-Lys-Pro-Val\(^{402}\), which was found to be critical for the functional association of the Ca\(^{2+}\)-ATPase of cardiac sarcoplasmic reticulum with phospholamban (42), is absent in TcSCA. Interestingly, the residues located in transmembrane segment 3 important for thapsigargin binding of SERCA Ca\(^{2+}\)-ATPases (43) are different in TcSCA (10 of 20 residues in segment 3 are different as compared with SERCA pumps). In agreement with these results we were unable to detect any significant increase in [Ca\(^{2+}\)]\(_i\) in fura 2-loaded cells in the presence of low concentrations of thapsigargin (0.1–4 \(\mu\)M) (14, 15). These results were also confirmed in experiments with the enzyme expressed in yeast (see below). Interestingly, TcSCA has three potential cAMP-dependent protein kinase phosphorylation sites, which are common to all three putative SERCA-type Ca\(^{2+}\)-ATPases of kinetoplastid parasites but are
not present in those of other species (Fig. 1). A genomic Southern blot probed by TcSCA DNA showed a single hybridizing band in each lane except for EcoRI (results not shown), suggesting that TcSCA is a single-copy gene. The two DNA bands observed by EcoRI digestion are due to the presence of an internal EcoRI site in the TcSCA sequence (nucleotide 1957).

Expression of TcSCA in Different Stages of T. cruzi—Northern blot analysis showed the presence of a single TcSCA transcript of approximately 5 kilobases in each of the three life cycle stages of T. cruzi (Fig. 2, upper panel). Analysis of the 5-kilobase band by densitometry indicated that the TcSCA gene is expressed at similar levels in all stages of T. cruzi. This is in contrast with lmaa1, the gene for the putative L. m. amazonensis SERCA-type Ca\textsuperscript{2+}/H\textsubscript{11001} pump that is developmentally regulated and more abundantly expressed in intracellular amastigotes (32).

Localization of TcSCA—To determine the localization of TcSCA, the sequence of the GFP of Aequorea victoria was fused to the TcSCA ORF and ligated into the T. cruzi expression vector pTEX (28, 29). The loop region in TcSCA was chosen for the site of insertion of GFP to avoid any possible interference with a potential targeting signal sequence. T. cruzi epimastigotes were transfected with this TcSCA-GFP plasmid, and the stable transfectants were observed by fluorescence microscopy. Transgenic parasites expressing the TcSCA-GFP construct exhibited GFP fluorescence as a ring surrounding the nucleus and in a network extending from its periphery (Fig. 3, A–C), suggesting an endoplasmic reticulum (ER) localization. To further confirm these results, transgenic cells were also stained with antibodies against BiP and calreticulin, two ER chaperones involved in the control of protein folding (44). Both BiP (Fig. 3D) and calreticulin (Fig. 3E) co-localized with TcSCA (Figs. 3, A and B), thus confirming the ER localization of TcSCA. In contrast, incubation of transgenic cells with antibodies against the acidocalcisomal...
Ca\(^{2+}\)-ATPase of *T. cruzi* (6) resulted in a punctate staining (Fig. 3F), clearly different from the perinuclear and reticular localization of TcSCA (Fig. 3C).

We also investigated the localization of TcSCA in other developmental stages of *T. cruzi*. Total lysates from different stages of *T. cruzi* were subjected to Western blotting analysis with antibodies against a 518-amino acid, COOH-terminal domain of TcSCA. These antibodies detected a single band of ~110 kDa (Fig. 4, inset), close to the predicted molecular mass of TcSCA, in epimastigotes (E), trypomastigote (T), and amastigote (A) lysates. No band was detected when using preimmune serum (data not shown). In indirect immunofluorescence assays using the anti-TcSCA antiserum, TcSCA was detected (Fig. 4) in epimastigotes (A), trypomastigotes (B), and amastigotes (C) as a ring surrounding the nucleus and in a network extending from its periphery, similar to the GFP protein labeling (Fig. 3). No detectable signal was observed when the preimmune serum was used (Fig. 4D).

Functional Complementation by TcSCA of the Ca\(^{2+}\)-ATPase-deficient *S. cerevisiae* Strain K616—To test the function of TcSCA, 3018 bp of the TcSCA ORF were subcloned into a yeast expression vector, pYES2, under the control of a galactose-inducible promoter, and the resulting construct or the vector alone was used to complement yeast mutant K616. The yeast triple mutant K616 is defective in both the Golgi pumps and also lacks calcineurin (CNN1) function. This mutant provides an extremely valuable expression system for determining the nature of individual Ca\(^{2+}\) pumps from other eukaryotes (23, 45). The triple mutant transformed with vector alone grew poorly on a medium containing low Ca\(^{2+}\) (2–10 mM EGTA) (Fig. 5). This effect was noticeable even at low concentrations of EGTA during the initial 24 h of growth (Fig. 5A) and decreased after 48 h (Fig. 5B). However, the triple mutant transformed with TcSCA became tolerant of EGTA, supporting the idea that TcSCA encodes a functional divalent cation pump. The likely transport of Ca\(^{2+}\) was supported by results on phosphoenzyme formation (below). Transport of Mn\(^{2+}\) was indicated by complementation of the Mn\(^{2+}\) sensitivity of the triple mutant (Fig. 6). The mutant with or without TcSCA grew equally well in glucose medium, where the gene is not induced (results not shown). In galactose medium, though, there was little growth of the vector control strain in the presence of 1–3 mM MnCl\(_2\), whereas the TcSCA-transformed (and induced) strain showed significant growth (Fig. 6).

Effect of Inhibitors on the Formation of a Ca\(^{2+}\)-dependent Phosphoenzyme Intermediate—To test if TcSCA was able to form a phosphorylated intermediate like other P-type Ca\(^{2+}\)-ATPases (46, 47), we isolated microsomes from TcSCA-transformed K616 and incubated them with \([\gamma^32P]ATP\) under various conditions. A major phosphoprotein of 110 kDa was formed in membranes isolated from TcSCA-transformed K616 (Fig. 7A, lane 7) but was absent in yeast transformed with vector alone (Fig. 7A, lane 3). Phosphorylation was dependent on the presence of Ca\(^{2+}\). La\(^{3+}\) enhanced the steady-state level of the phosphoprotein severalfold (Fig. 7A, lane 8). The denatured phosphoprotein was sensitive to hydroxylamine (Fig. 7A, lane 9), indicating the hydrolysis of an acyl phosphate bond (48) probably formed by Asp\(^{357}\) (Fig. 1). Together, these results provide compelling evidence that TcSCA is a P-type Ca\(^{2+}\)-dependent ATPase.

Cyclopiazonic acid, an inhibitor of animal SERCA pumps, inhibited the phosphorylation of TcSCA (Fig. 7B, lanes 6 and 7), whereas thapsigargin had no effect at low concentrations (Fig. 7B, lanes 2 and 3). The phosphorylation of TcSCA was also inhibited by erythrosin B (Fig. 7B, lanes 4 and 5), a halogenated derivative of fluorescein that binds to nucleotide-binding sites with high affinity and specificity (49).

**DISCUSSION**

Using yeast as a heterologous expression system (23), we provide the first direct evidence that a cloned SERCA-type *T. cruzi* gene encodes a functional Ca\(^{2+}\)-ATPase. Expression of the *T. cruzi* TcSCA gene restored the growth in a medium containing submicromolar levels of Ca\(^{2+}\) of a yeast mutant (K616) defective in Ca\(^{2+}\) pumps (Fig. 4). Several lines of evidence indicate that TcSCA encodes an ER Ca\(^{2+}\) pump; 1) its amino acid sequence shares 46–48% identity with SERCA pumps and less identity (24.1–24.8%) with plasma membrane-type Ca\(^{2+}\)-ATPases; 2) TcSCA contains ER retention motifs (50), KXXK-stop at the COOH terminus and RILL in the first transmembrane domain (Fig. 1); 3) TcSCA is mainly localized to the nuclear membrane and to a reticular structure in different stages of *T. cruzi* (Figs. 3 and 4); 4) TcSCA co-localizes with calreticulin and BiP, two well known endoplasmic reticulum chaperones (Fig. 3).

As occurs with plant SERCA-type Ca\(^{2+}\)-ATPases (48), TcSCA appears to be insensitive to thapsigargin (14, 15). Residues located in the third transmembrane segment (M\(_3\)) and in the stalk segment (S\(_3\)) have been postulated to be important for thapsigargin binding and are conserved in all SERCA Ca\(^{2+}\)-ATPases (43, 51). Since the *T. brucei* SERCA pump TBA1 was reportedly sensitive to thapsigargin (37), whereas *L. m. amazonensis* putative SERCA pump LMA1 was not (32), it was proposed that two amino acid substitutions present in LMA1 could account for their different sensitivity: a Gly\(^{271}\) in LMA1 that replaces Lys\(^{261}\) in TBA1...
and a Phe\textsuperscript{279} in LMAA1 that replaces Val\textsuperscript{269} in TBA1 (32). However, one of these amino acids is also present in \textit{T. cruzi} SERCA pump TeSCA; a Lys\textsuperscript{259} is in the same position in TeSCA as Lys\textsuperscript{261} in TBA1. In addition, instead of a Val\textsuperscript{269} in TBA1, there is an Ala\textsuperscript{269} in TeSCA, which is a conserved substitution. A comparison of the sequence of this transmembrane segment M\textsubscript{3} in TeSCA with those of other Ca\textsuperscript{2+}-ATPases indicates that TeSCA shares 12 out of 20 residues with SERCA pumps and 15 out of 20 residues with \textit{T. brucei} Ca\textsuperscript{2+}-ATPase TBA1 (37). (See Scheme I, showing the alignment of TeSCA with TBA1, and LMAA1; the \textit{colons} and \textit{dots} indicate identical and similar amino acid residues, respectively).

Other residues that are different in TeSCA as compared with TBA1 are conserved substitutions and possibly could not account for the differences in sensitivity to thapsigargin, except for a Thr\textsuperscript{266} that replaces an Ile in TBA1 and LMAA1. There is only one difference in the S\textsubscript{3} segment; a Met\textsuperscript{257} replaces a Val in TBA1 and LMAA1. Our conclusion is that these differences in transmembrane segment M\textsubscript{3} and S\textsubscript{3} could account for the differences in sensitivity to thapsigargin of these pumps.

In mammalian cells, SERCAs are important in refilling ER calcium stores used in signaling. Ca\textsuperscript{2+} is released from the ER by inositol 1,4,5-trisphosphate (IP\textsubscript{3}), cyclic ADP-ribose, or nicotinic acid adenine dinucleotide phosphate acting upon IP\textsubscript{3} or ryanodine receptors (52, 53). Whether the same applies to \textit{T. cruzi} is uncertain. The isolated ER has not been studied in trypanosomatids, but the most likely alternative store of calcium for signaling, the acidocalcisome (8), is not sensitive to calcium-releasing metabolites after isolation from trypanosomatids, but the most likely alternative store of calcium is the yeast Golgi PMR1 protein (61). Therefore, in \textit{T. cruzi}, the TeSCA transporter may be important in the maintenance of luminal Ca\textsuperscript{2+} and/or Mn\textsuperscript{2+} required for proper trafficking and modification (glycosylation) of new proteins during differentiation and, particularly (from the point of view of infectivity), cell surface proteins involved in interactions with host cells.

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