**Schistosoma mansoni** Ca\(^{2+}\)-ATPase SMA2 Restores Viability to Yeast Ca\(^{2+}\)-ATPase-deficient Strains and Functions in Calcineurin-mediated Ca\(^{2+}\) Tolerance*

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The sarco(endo)plasmic reticulum of animal cells contains an ATP-powered Ca\(^{2+}\) pump that belongs to the P-type family of membrane-bound cation-translocating enzymes. In **Schistosoma mansoni**, the sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase (SERCA) is encoded by the SMA1 and SMA2 genes. A full-length SMA2 cDNA clone was isolated, sequenced, and expressed in a yeast Ca\(^{2+}\)-ATPase-deficient strain requiring plasmid-borne SERCA1a for viability. The **S. mansoni** Ca\(^{2+}\)-ATPase supports growth of mutant cells lacking SERCA1a, indicating functional expression in yeast and a role in calcium sequestration. Subcellular fractionation showed that the SMA2 ATPase is localized in yeast internal membranes. SMA2 expression was found to be associated with thapsigargin-sensitive, Ca\(^{2+}\)-dependent ATPase activity. The activity increased 2-fold upon calcineurin inactivation, which correlates with *in vivo* stimulated contribution of SMA2 in calcium tolerance. These results suggest that calcineurin controls calcium homeostasis by inhibiting Ca\(^{2+}\)-ATPase activity in an internal compartment.

**Schistosoma mansoni** is a human parasite trematode causing the chronic debilitating disease schistosomiasis, which affects hundreds of millions of people over the world. Many different aspects of the parasite’s life cycle, such as penetration through the host’s skin, locomotion, feeding, and eggshell formation, are controlled by calcium (1–3).

In animal cells, calcium is stored principally within the sarco(endo)plasmic reticulum, which acts as a storage site for subsequent calcium release in response to environmental signals and as a sink for maintaining cytoplasmic calcium concentrations at submicromolar level (4). The sarco(endo)plasmic reticulum compartment is loaded by an ATP-powered Ca\(^{2+}\) pump (5, 6), a member of the P-ATPase family, which is characterized by the formation of a phosphoenzyme catalytic intermediate during ATP hydrolysis (7). In animal cells, the subfamily of sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPases (SERCAs)\(^1\) comprises six isoforms, which are encoded by three separate genes and each of which has a distinct pattern of tissue-specific and developmentally regulated expression (8, 9).

In **S. mansoni**, there is evidence of at least two SERCA-like pumps, SMA1 and SMA2, in addition to a homolog of the yeast Golgi PMR1 Ca\(^{2+}\)-ATPase (10) and the rat secretory pathway SPCA Ca\(^{2+}\)-ATPase (11) as suggested by PCR analysis (12). Ca\(^{2+}\)-stimulated, Mg\(^{2+}\)-dependent ATPase activity has been found in **S. mansoni** tissue homogenates (13) and microsomal fractions (14), which is coupled with an active transport of calcium (15). However, a correlation between this ATPase activity and specific Ca\(^{2+}\)-ATPase isoforms has not been established.

In this study, we determined the amino acid sequence of the **S. mansoni** SMA2 Ca\(^{2+}\)-ATPase. We used a yeast expression system to determine whether Ca\(^{2+}\)-dependent ATPase activity was associated with the expression of SMA2. The results indicate that the **S. mansoni** Ca\(^{2+}\) pump is present in yeast intracellular membranes and shows kinetic properties and inhibitor sensitivities comparable to those of other SERCA isoforms. In *Saccharomyces cerevisiae*, the PMR1 and PMC1 genes encode for Ca\(^{2+}\)-ATPases located in the Golgi system and the vacuole, respectively (16, 17). A yeast strain lacking both Ca\(^{2+}\)-ATPase functions is not viable (17). However, inactivation of calcineurin, a Ca\(^{2+}\)/calmodulin-activated phosphoprotein phosphatase (18, 19) restores viability to the Ca\(^{2+}\)-ATPase-deficient strain, through post-transcriptional activation of the low affinity vacuolar H\(^+/Ca\(^{2+}\)\) exchanger, VCX1/HU1M1 (20, 21). We found that the **S. mansoni** SMA2 substitutes for loss of both PMR1 and PMC1 Ca\(^{2+}\)-ATPases and restores the calcineurin-dependent Ca\(^{2+}\) tolerance of vacuolar *pmc1* mutants. The physiological relevance of these results for the regulation of calcium homeostasis in yeast is discussed.

**EXPERIMENTAL PROCEDURES**

**Strains, Media, and Growth Conditions**—Adult worms of the Puerto Rican strain of **S. mansoni** were recovered from infected hamsters by portal venous perfusion as described previously (22). The *S. cerevisiae* strains used in this study are listed in Table I. The Ca\(^{2+}\)-ATPase mutant strains K605 (*pmc1Δ*), and K616 (*pmc1Δ pmr1Δ* *exb1Δ*) are isogenic to the parental strain W303–1A and were isolated by Cunningham and Fink (17). The MG10 *pmc1Δ pmr1Δ* double mutant expressing SMA2 from the LEU2-marked pS15SMA2 plasmid (see below) is derived from the MGY10–6B strain.\(^2\) MGY10–6B contains deletions of the *PMR1* and *PMC1* genes and requires rabbit SERCA1a

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\(^1\) The abbreviations used are: SERCA, sarco/endoplasmic reticulum Ca\(^{2+}\)-ATPase; PMCA, plasma membrane Ca\(^{2+}\)-ATPase; PIPES, piperazine-N\(_2\)N’-bis(2-ethanesulfonic acid); MES, 4-morpholineethanesulfonic acid; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; PCR, polymerase chain reaction; kb, kilobase pair(s); bp, base pair(s).

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on a Ura3-marked plasmid for viability. The MG10 strain containing only pS155MA2 was selected on 5-FOA-containing medium that selects for loss of the plasmid-borne SERRCA1a. The MG83–9D pnc1Δ cbin1Δ strain was selected as a Leu+ Trp+ His- meiotic segregant of a diploid strain that was derived from a standard genetic cross between K605 and K616. The vcx1Δ:Kanb null allele was transformed in the MG10 and MG83–3D strains to give MM10DV and MG83DV, respectively. Geneticin-resistant transformants were isolated and correct integration of the Kanb marker at the VCX1 target site was confirmed by PCR amplification, using two oligonucleotides (PNCVX1K1 and PNCVX1K2) that are complementary to the 5′- and 3′-flanking regions of VCX1 gene and oligonucleotide (PKAN) which is located within the Kanb disruption cassette.

Yeast strains were grown on YPD (1% yeast extract, 2% Bacto- peptone, and 2% glucose) medium at 30 °C. The cells (10 g) were washed twice with cold water and then resuspended in 250 ml of 250 mM sorbitol, 1 mM phenylmethylsulfonyl fluoride, 1 mM MgCl₂, 10 mM imidazole, 5 mM dithiothreitol, and 2 μg/ml protease inhibitors. After cell disruption with glass beads, lysates were centrifuged twice at 1500 × g for 5 min to remove cell debris and then at 5000 × g for 5 min to give the Pp3pellet. The S5/5 supernatant fraction was then centrifuged at 15,000 × g for 40 min to obtain a crude membrane fraction, called P1pellet, and a supernatant fraction that was centrifuged at 100,000 × g for 1 h to yield the high speed membrane fraction Pmembrane. In some cases, the 15,000 × g/mrn cin curation step was skipped to recover all membrane proteins, with only a slight decrease in specific SMA2 ATPase activity. The Pm membrane pellet, which was obtained after centrifugation of the S5/5 supernatant at 100,000 × g for 1 h, consisted of intracellular and plasma membranes as well as polysomes. Membrane pellets were resuspended in 1 mg Mgl₂, 10 mg imidazole, pH 7.5, and stored at −70 °C.

For subcellular fractionation experiments, Pm membranes (10 mg of protein) were loaded onto a linear 20–60% sucrose gradient and then centrifuged at 100,000 × g for 16 h at 4 °C. Gradients were fractionated from the top to bottom, manually, in 1-ml aliquots (fractions 1–15) that were stored at −70 °C.

Protein concentration was determined by the Lowry (34) or BCA (Sigma procedure, TPRO562) method, using bovine serum albumin as a standard.

### Isolation of Yeast Membrane Fractions Enriched in SMA2—Pm membranes (16 mg of protein) from the Pm fraction were solubilized in 4 ml of buffer A (100 mM KCl, 100 mM CaCl₂, 1 mM phenylmethylsulfonyl fluoride, 10 mM HEPES, 10 mM PIPES, pH 7.5) containing 0.1%
(w/v) asolectin and 0.32% (w/v) n-dodecyl-b-D-maltoside. After agitation for 30 min at 4 °C, the membrane suspension was centrifuged at 100,000 x g for 1 h to remove the unsolubilized membranes. The resulting membrane extract was concentrated by filtration through an Amicon membrane with a cutoff of 50,000 Da and applied to a Superdex 200 column (Amersham Pharmacia Biotech). Proteins were eluted with buffer B (100 mM CaCl2, 0.052% (w/v) n-dodecyl-b-D-maltoside, 0.01% (w/v) asolectin, 100 mM HEPES, 100 mM PIPES, pH 7.2), and 0.5-ml fractions were collected and stored at -70 °C.

**Western Blot Analysis**—Proteins were incubated 15 min at 56 °C in sample buffer (80 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 1% dithiothreitol, and 0.005% bromophenol blue) and separated on a 7% SDS-polyacrylamide gel. When required, proteins were precipitated by the addition of trichloroacetic acid to 10% and centrifuged for 20 min in an Eppendorf microcentrifuge before resuspension in sample buffer. After electrophoresis, proteins were electrotransferred onto a 0.45-μm nitrocellulose membrane (Schleicher & Schuell) using the Bio-Rad blot system filled with 24.6 mM Tris, 1.56 mM glycine, and 20% methanol.

The filter was saturated in Tris-buffered saline (50 mM Tris-HCl, pH 7.5, 150 mM NaCl) containing 3% (w/v) low fat dried milk and 0.5% (w/v) Tween 80 for 30 min at room temperature and then incubated in Tris-buffered saline containing polyclonal antibodies against rabbit SERCAla (35), PMA1 (36), and SEC63 (37) at 1:5000, 1:6500, and 1:1000 dilutions, respectively. Antigen-antibody complexes were revealed with 125I-labeled protein A or by the enhanced chemiluminescence method (Boehringer Mannheim) using secondary horseradish peroxidase-conjugated anti-rabbit IgG antibodies. Band intensity was quantified using Molecular Analyst® (Bio-Rad) or Image Master (Amersham Pharmacia Biotech) softwares.

**ATPase Activity Assay**—SMA2-dependent ATPase activity was assayed in 10 mM HEPES/PIPES, pH 7.2, 100 mM KCl, 2.5 mM MgCl2, 1 mM MgATP, 100 mM CaCl2, 2 mM phosphoenolpyruvate, 10 μM γ-mercaptoethanol, 50 mM KNO3, and 2.5 mM NaN3. Samples were pre-incubated 5 min at 30 °C before the addition of 5 μl of 25 mM MgATP, pH 7.0. After 2, 4, 6, and 8 min at 30 °C, 20-μl aliquots were withdrawn and the ATPase reaction was stopped by 0.32% (w/v) NH4-molybdate in 1.14 N H2SO4. Inorganic phosphate was measured at 630 nm using the method described in Ref. 38. SMA2-dependent ATPase activity was calculated from the difference between the slopes obtained in the absence or presence of 40 μM thapsigargin. Stock solution of thapsigargin was prepared in dimethyl sulfoxide, which at the highest concentrations used (4.8% (w/v) in the assay) had no effect on ATPase activity. For measurements of apparent Km for MgATP and Vmax, ATPase activity was determined in 50 mM MES/Tris, pH 7.0, using an ATP regenerating system and MgATP concentrations ranging from 0 to 3.5 mM. Free Mg2+ concentrations were kept at 2.5 mM as described in Ref. 39. In experiments involving inhibitors (vanadate, erythrosine B, cyclopiazonic acid), ATP concentration was kept constant at 1 mM MgATP. Measurements of pH optimum were made in 50 mM MES, 50 mM MOPS, 50 mM Tris, 1 mM MgATP, 2.5 mM sodium azide, 50 mM KNO3, adjusted to the indicated pH with HCl or KOH. To determine the effects of calcium concentrations on ATPase activity, the reaction mixture also contained EGTA. Free Ca2+ concentrations were calculated from stability constants for CaEGTA and MgEGTA complexes (40, 41).

The yeast plasma membrane H+ -ATPase activity was measured at pH 6.0 (33) after a pre-incubation of 5 min at 30 °C in the absence of ATP. Mitochondrial ATPase activity was assayed in 25 mM Tris/HCl, pH 9.0, 500 μM vanadate, 50 mM KNO3, in the absence or presence of 10 mM sodium azide.

**Computer Analysis**—The nucleotide sequence was edited using the DNASTAR Software (Lasergene). Homology searches were done using the BLAST program (42) implemented at NCBI (National Center for Biotechnology Information). Amino acid alignments and identity levels were performed using the Pile Up and Bestfit programs (CCG software version 8.0, Genetics Computer Group) available on the Belgian EMBnet Node (BEN).

**Results**—A DNA library of S. mansoni adult worms was screened by DNA hybridization to the PCR product SmII (12) which codes for an internal sequence of SMA2 (for S. mansoni ATPase 2). DNA sequencing analysis of independently isolated clones (clones 2A1 and 1A1 in Fig. 1A) revealed a 5'-truncated open reading frame highly homologous to rabbit SERCA isoforms. The 1-kb EcoRI-BamHI fragment of clone 2A1 was then used to reprop the S. mansoni cDNA library (see "Experimental Procedures"). Lane 1, S. mansoni adult worms; lanes 2 and 3, yeast cells expressing SMA2 from the high copy number p246SMA2 (lane 2) and low copy number p315SMA2 (lane 3) plasmids, respectively.

**RESULTS**

**Isolation of a Complete SMA2 cDNA Clone**—A cDNA library of S. mansoni adult worms was screened by DNA hybridization to the PCR product SmII (12) which codes for an internal sequence of SMA2 (for S. mansoni ATPase 2). DNA sequencing analysis of independently isolated clones (clones 2A1 and 1A1 in Fig. 1A) revealed a 5'-truncated open reading frame highly homologous to rabbit SERCA isoforms. The 1-kb EcoRI-BamHI fragment of clone 2A1 was then used to reprop the S. mansoni cDNA library (see "Experimental Procedures"). Lane 1, S. mansoni adult worms; lanes 2 and 3, yeast cells expressing SMA2 from the high copy number p246SMA2 (lane 2) and low copy number p315SMA2 (lane 3) plasmids, respectively.

**Northern Blot Analysis of S. mansoni SMA2**—The full-length SMA2 cDNA contains an open reading frame of 3033 bp. The 5'- and 3'-untranslated regions are 126 and 989 bp long, respectively. No poly(A) addition site could be identified in the 3'-untranslated region. Northern blot analysis of total RNA extracted from S. mansoni adult worms revealed a unique 4.3-kb band (Fig. 1B, lane 1), consistent with the size predicted from the sequence of the isolated SMA2 cDNA clones. The SMA2 transcript seems not to be subject to alternative splicing control.

**Sequence Analysis of the SMA2 ATPase**—The SMA2 cDNA encodes a 111-kDa polypeptide which shares 70% overall identity with the S. mansoni SMA1 isoform (12). Most amino acid differences are located in the N- and C-terminal regions as well as the region flanking the fluorescein isothiocyanate binding site. Regions typically conserved in the P-type family of transport ATPases (7) are found in SMA2 (Fig. 2). These encompass the hydrophilic head protruding into the cytosol and containing the phosphorylation and ATP-binding domains (43) as well as the fluorescein isothiocyanate binding site (44), the smaller cytosolic loop located in the N-terminal region, the hinge motif (45), and the membrane-spanning segments M1–10 (46).

The M4–6 and M8 transmembrane segments contain amino acids crucial for high affinity Ca2+ binding (47) (double underlined residues in Fig. 2). A close examination of these regions
revealed that SMA2 is more related to Ca\textsuperscript{2+}-ATPases than other P-type ATPases. In particular, the Glu-771 residue in the M5 region of SERCA1a, which is only found in SERCA isoforms, is also conserved in SMA2. The M1 transmembrane segment of SMA2 contains the Lys-Ile-Leu-Leu-Met pentapeptide (residues 63–67), which is identical to the putative sarcoplasmic reticulum retention signal (Lys/Arg-Ile-Leu-Leu-Leu) found in SERCA ATPases (48). This region has been shown to target chimeras of SERCA and plasma membrane Ca\textsuperscript{2+} pumps to the endoplasmic reticulum of animal Cos cells (49). In common with the non-muscle SERCA3 isoform, SMA2 lacks the phospholamban-binding region (50). The C terminus of SMA2 also lacks the calmodulin-binding domain found in plasma membrane Ca\textsuperscript{2+}-ATPases (51).

When full-length sequences are aligned, SMA2 shows 72–74% identity to \textit{Artemia franciscana} Ca\textsuperscript{2+}-ATPase (52) and mammalian SERCA isoforms, 52% identity to \textit{Trypanosoma brucei} endoplasmic reticulum Ca\textsuperscript{2+}-ATPase, TBA1 (53), and less than 37% to human plasma membrane Ca\textsuperscript{2+}-ATPase (54) and yeast Ca\textsuperscript{2+}-ATPases, PMR1 and PMC1 (10, 17). Therefore, sequence analysis strongly suggests that the \textit{S. mansoni} SMA2 ATPase is a new member of the SERCA subfamily of Ca\textsuperscript{2+}-ATPases.

\textbf{Functional Expression of \textit{S. mansoni} SMA2 in Yeast—}We used a yeast expression system to determine whether SMA2-dependent ATPase activity could be detected. The SMA2 cDNA was expressed in \textit{S. cerevisiae} from the strong and constitutive promoter of the plasma membrane H\textsuperscript{+}-ATPase (PMA1) gene (55, 56), cloned in the high copy (2\mu) p426SMA2 or low copy (Cen) p315SMA2 plasmids (see “Experimental Procedures”). Expression of SMA2 was tolerated in the YPH500 wild-type strain although the doubling time increased from 1.5 h (control cells transformed with an empty plasmid) to 2 h (cells with p315SMA2) and 3 h (cells with p426SMA2). Electron microscopic analysis of SMA2-expressing cells showed no accumulation of karmellae or other internal membranes (data not shown) in contrast to the overexpression of yeast hydroxymethylglutaryl-CoA reductase (57), plant plasma membrane H\textsuperscript{+}-ATPase (26), or rabbit SERCA1a. In \textit{S. cerevisiae}, the PMC1 and PMR1 Ca\textsuperscript{2+}-ATPases function together in Ca\textsuperscript{2+} sequestration. They transport Ca\textsuperscript{2+} from the cytosol into the vacuole and the Golgi, respectively. Mutant strains lacking both Ca\textsuperscript{2+}-ATPase functions are not viable on standard rich YPD medium, which contains low (0.18 mM) Ca\textsuperscript{2+} (17). Synthetic lethality of \textit{pmr1}D\textit{pmc1}D double mutants is prevented by inactivation of calcineurin (17), a Ca\textsuperscript{2+}/calmodulin-dependent protein phosphatase (18, 19) that inhibits vacuolar VCX1-dependent H\textsuperscript{+}/Ca2\textsuperscript{2+} exchange. In the absence of calcineurin, VCX1 functions in Ca\textsuperscript{2+} sequestration much more efficiently and substitutes for loss of \textit{PMR1} and \textit{PMC1} (Ref. 20, and strain K616 in Fig. 3).

We showed that expression of SMA2 from the p315SMA2 plasmid restores cell viability to the MG10 \textit{pmr1}D\textit{pmc1}D mutant strain (Fig. 3). In this functional assay, SMA2 was able to replace plasmid-borne rabbit SERCA1a which is required for growth of \textit{pmr1}D\textit{pmc1}D mutants. In separate control experiments, SERCA1a could not be replaced by the empty p315C vector as indicated by the inability of the transformed cells to...
MG10, mutant transformed with the SMA2 expression plasmid, p315SMA2; PMR1 PMC1 CNB1 W303–1A, mainly (92%) found in the crude membrane fraction P15/40. Mobility of SMA2 in SDS-polyacrylamide gel agrees well with wild-type membranes lacking SMA2 (Fig. 4).

FIG. 3. Synthetic lethality of pmr1Δ pmc1Δ double mutants is prevented by expression of S. mansoni SMA2 ATPase. Yeast strains were grown on YPD agar medium supplemented with 0 and 200 mM CaCl₂ and incubated 3 days at 30 °C. Strains, clockwise from top: W303–1A, PMR1 PMC1 CNB1 null mutant transformed with the SMA2 expression plasmid, p315SMA2; MG10, pmr1Δ pmc1Δ double mutant carrying p315SMA2; K616, pmr1Δ pmc1Δ cnb1Δ triple mutant.

Localization of SMA2 in Yeast Internal Membranes—Cell lysates were prepared from the wild-type YPH500 strain transformed with low copy p315SMA2 or high copy p426SMA2 plasmids. Fractionated membrane fractions were isolated by differential centrifugation and assayed for the presence of SMA2 protein by immunoblotting, using antibodies directed against rabbit SERCA1a Ca²⁺-ATPase (see “Experimental Procedures”). Cross-reaction was expected from the high sequence identity shared by the two Ca²⁺ pumps. The antibodies detected a 111-kDa polypeptide, which was not detected in control wild-type membranes lacking SMA2 (Fig. 4A). The apparent mobility of SMA2 in SDS-polyacrylamide gel agrees well with the size predicted from its amino acid sequence. SMA2 is mainly (92%) found in the crude membrane fraction P₄₅/₅₀. However, small amounts of SMA2 were also found in the microsomal fraction P₁₀₀/₇₀. The abundance of SMA2 in P₂₅₀₀, membranes was estimated to be 4.6 μg/mg of total protein. This value is probably an underestimation as rabbit SERCA1α was used for the calibration curve. Higher levels of expression were obtained with the low copy p315SMA2 plasmid than with the high copy p426SMA2 (compare lanes 4 and 5 in Fig. 4A), consistent with Northern blot analysis of SMA2 transcript levels (Fig. 1B, lanes 3 and 2).

To determine the subcellular localization of SMA2, total membranes consisting of P₅₀₄₀₀ and P₁₀₀₀₀₀ membranes were prepared from YPH500 wild-type cells bearing p315SMA2 and fractionated by sucrose gradient density centrifugation. Individual fractions were assayed for the distribution of proteins typical of a particular organelle, by immunoblotting or by measuring marker enzyme activity (see “Experimental procedures”). Fig. 4 (B and C) shows that the marker enzyme for plasma membranes (PMA1) migrated to a density peaking at 45% sucrose and was clearly separated from mitochondrial ATPase and other internal membranes, peaking at sucrose concentrations of 28% and 42%, respectively. The SMA2 protein fractionates predominantly with the endoplasmic reticulum marker, SEC63 (Fig. 4C). The fractions were also assayed for SMA2-dependent thapsigargin-sensitive ATPase activity (see below). Nearly all thapsigargin-sensitive ATPase activity was found in SEC63-enriched internal membranes (fractions 7–9 in Fig. 4B). This ATPase activity was not detected in cells lacking the SMA2 expression plasmid.

Subcellular localization of SMA2 was also analyzed by electron microscopy. Sections of SMA2-expressing cells were incubated in the anti-SERCA1a antiserum followed by incubation in gold-conjugated protein A. However, no specific immunolabeling was detected under these conditions (data not shown).

Thapsigargin-sensitive ATPase Activity Is Associated with SMA2 Expression—SMA2-dependent ATPase activity was measured in solubilized membrane fractions obtained after gel filtration chromatography instead of the sucrose gradient fractions, as the latter showed significant contamination by PMA1 (Fig. 4C).

Yeast P₂₅₀₀ membranes (4 mg/ml) were solubilized with n-dodecyl-β-D-maltoside in a ratio of 0.8 mg of detergent/mg of membrane protein. These conditions, which solubilized 50% of total membrane protein, enabled the complete recovery of SMA2 activity in the supernatant fraction (see “Experimental Procedures”). Addition of 0.1% (w/w) aselectin to the solubilization medium resulted in a 2-fold increase in ATPase activity and in a better SMA2 stabilization at either 4 °C or 30 °C. Among the other detergents tested, Triton X-100, CHAPS, and n-octyl-β-D-glucopyranoside solubilized less than 20% of membrane-bound SMA2 activity, whereas octaethylene glycol mono-n-dodecyl ether (C₁₂E₈), lyssolecithin, and sodium deoxycholate inactivated the ATPase activity (data not shown).

Solubilized membranes were fractionated by gel filtration chromatography, and fractions were assayed for the presence of SMA2 by immunoblotting and measuring thapsigargin-sensitive ATPase activity (see “Experimental Procedures”). Thapsigargin is a plant sesquiterpene lactone, which is a specific inhibitor of SERCAs (58, 59). Fig. 5 shows elution of SMA2 in the presence of 200 mM CaCl₂ (Fig. 3). Moreover, we showed that deletion of VCX₁ was not lethal in MG10DV cells expressing SMA2 (fractions 15–17) and the peak of thapsigargin-sensitive ATPase activity was not detected in cells lacking the SMA2 expression plasmid. There is a one-fraction shift between the fractions enriched by each detergent/protein ratio is no longer optimal, consistent with the observation that 75% of total SMA2 activity was lost after gel filtration chromatography. Gel filtration fractions 14 and 15 were combined to analyze kinetic properties of SMA2. An 8-fold purification and a yield of 11.5% of ATPase activity was achieved in these pooled fractions compared with total membranes. The amount of PMA1 protein in the pooled fractions represented less than 6% (data not shown).

Kinetic Properties of SMA2 ATPase Activity—Thapsigargin-sensitive ATPase activity of solubilized membrane fractions
enriched with SMA2 was measured in the presence of 100 μM free Ca²⁺, 2.5 mM free Mg²⁺ (shown to be required for maximal stimulation of the activity), an ATP regenerating system, and inhibitors of pyrophosphatases, mitochondrial, and vacuolar ATPases (see “Experimental Procedures”). Under these conditions, thapsigargin-sensitive ATPase activity represented up to 75% of total ATPase activity.

In Eadie-Hofstee plots, SMA2 activity shows an apparent Km of 0.25 mM and a Vmax of 0.11 μmol P1/min−1mg−1 (data not shown). An optimum pH of 7–7.4 was detected (data not shown), which is similar to the optimum pH of the SERCA3 isoform (60). For measurement of Ca²⁺ dependence, free Ca²⁺ was varied in the reaction mix using EGTA as described under “Experimental Procedures.” An assay of ATPase activity as a function of calcium concentration revealed a maximum at 10 μM free Ca²⁺, an apparent Kₘ (Ca²⁺ concentration yielding half-maximal activation) of 1 μM free Ca²⁺ (Fig. 5C). This result is very close to that of SERCA1a (60). In a Hill plot representation, the best linear fit of the data indicated a Hill coefficient of 1.29, consistent with the presence of two calcium binding sites showing positive cooperativity.

Inhibitor sensitivities of SMA2 expressed in solubilized yeast membranes are summarized in Table II. Vanadate is a characteristic inhibitor of P-type ATPases. SMA2 activity was completely inhibited at vanadate concentrations above 40 μM. SERCA ATPases are specifically inhibited by nanomolar concentrations of thapsigargin (59) and cyclopiazonic acid (61, 62). In comparison with other SERCA isoforms, the SMA2 enzyme appears less sensitive to inhibition by thapsigargin (IC₅₀ of 18 μM) and cyclopiazonic acid (IC₅₀ of 8 μM). This relative inhibitor insensitivity probably results from membrane solubilization as ATPase activity was completely inhibited by 5 μM thapsigargin in crude yeast membranes. Erythrosine B has been shown to inhibit ATP binding of the yeast plasma membrane H⁺-ATPase, PMA1 (63). We found that SMA2 was likewise sensitive to this inhibitor, with an IC₅₀ of 3 μM. Praziquantel is currently the drug of choice for the treatment of human schistosomiasis (64), causing rapid damages to the parasite’s tegument and a subsequent increase in the intracellular pool of free Ca²⁺ (65–67). However, praziquantel had no effect on SMA2 activity at concentrations up to 300 μM (data not shown), consistent with the observation that Ca²⁺-dependent ATPase activity in S. mansoni membrane fractions is not affected by praziquantel (68).

**SM2 Contributes to Ca²⁺ Tolerance upon Calcineurin Inactivation**—The vacuolar Ca²⁺-ATPase, PMC1, is required for growth at high Ca²⁺ concentrations (17), indicating that, under these conditions, PMC1 provides the largest contribution to Ca²⁺ tolerance, whereas VCX1/HUM1 and PMR1 contribute much less (20, 21).

The growth defects of pmc1Δ mutants in high Ca²⁺ media were not suppressed by the expression of SMA2, consistent with the inability of SMA2 to support cell growth of the MG10 pmr1Δ pnc1Δ mutant on media containing 200 mM CaCl₂ (Figs. 3 and 6A, lines 1 and 2). Interestingly, the role of SMA2 in Ca²⁺ tolerance was increased after calcineurin inactivation, through VCX1/HUM1 independent mechanisms. We first constructed the MGY8–3D pnc1Δ mutant strain which also con-
pressed drug cyclosporin A, in association with the cyclophilin A homolog CPR1 (69). The addition of cyclosporin A prevented the inhibitory effects of high Ca\(^{2+}\) on the growth of pmc1Δ mutants (Fig. 6B, left panel) and the activation of VCX1 after calcineurin inhibition (17). We also found that SMA2 was able to support growth of the MM10DV pmr1Δ pmc1Δ vcx1Δ triple mutant on high Ca\(^{2+}\) media when supplemented with cyclosporin A (Fig. 6B, right panel). Therefore, it seems likely that the function of the SMA2 Ca\(^{2+}\)-ATPase in yeast is inhibited through a calcineurin-mediated regulatory event.

Calcineurin-dependent Stimulation of SMA2 Activity—To determine whether calcineurin regulates, either directly or indirectly, SMA2 activity, total membrane fractions were prepared from the pmc1Δ (K605), pmc1Δ cnb1Δ (MGY8–3D), and pmc1Δ cnb1Δ vcx1Δ (MGY8SDV) strains carrying the SMA2 expression plasmid and assayed for thapsigargin-sensitive ATPase activity (Fig. 7). The ATPase activity of MGY8SDV membranes showed a 2-fold increase (21.6 nmol of P\(_i\) min\(^{-1}\)mg\(^{-1}\)) in comparison with that of K605 membranes (10.7 nmol of P\(_i\) min\(^{-1}\)mg\(^{-1}\)) and was significantly higher than the activity of MGY8–3D membranes (17.5 nmol of P\(_i\) min\(^{-1}\)mg\(^{-1}\)). These findings indicate that inactivation of calcineurin results in stimulated SMA2 activity, consistent with increased contribution to Ca\(^{2+}\) tolerance in vivo.

**DISCUSSION**

In this report, we have described the biochemical and functional properties of *S. mansoni* SMA2, a new sarco(endo)plasmic reticulum Ca\(^{2+}\)-ATPase isomerase. SMA2 was able to support growth of yeast Ca\(^{2+}\)-ATPase-deficient strains. The expression of SMA2 was associated with thapsigargin-sensitive, Ca\(^{2+}\)-dependent ATPase activity in intracellular membranes. This ATPase activity showed kinetic properties and inhibitor sensitivity comparable to that of mammalian SERCA isoforms. We also found that SMA2 contributes to Ca\(^{2+}\) tolerance in yeast strains lacking calcineurin activity through mutations in the CNB1 regulatory subunit or inhibition by cyclosporin A. These results indicate that calcineurin inhibits the activity of Ca\(^{2+}\)-ATPases localized in intracellular compartments to maintain Ca\(^{2+}\) homeostasis.

Four classes of Ca\(^{2+}\)-ATPases have been identified up to the present. The well characterized plasma membrane (PMCA) and SERCA Ca\(^{2+}\) pumps differ from one another in their primary structure, mode of regulation, kinetic properties and cellular localization. The yeast PMR1 Ca\(^{2+}\)-ATPase and its rat homologue SPCA1 belong to the third class of secretory pathway Ca\(^{2+}\) pumps. It has recently been shown that the kinetic characteristics and inhibitor sensitivities of PMR1 are clearly distinct from both SERCA and PMCA pumps (70). The yeast vacuolar PMC1 ATPase belongs to the fourth class consisting of plasma membrane-like Ca\(^{2+}\)-ATPases, which are localized in internal acidic compartments and lack the calmodulin-binding site of PMCA ATPases (70).

High sequence similarity to SERCA pumps as well as comparable inhibition of the ATPase activity by thapsigargin (58, 59) and cyclopiazonic acid (61, 62) clearly indicate that *S. mansoni* SMA2 belongs to the SERCA subfamily of Ca\(^{2+}\)-ATPases. In common with the mammalian non-muscle SERCA3 isofunction (71, 72), SMA2 lacks the phospholamban-binding region (50), has an optimum pH of 7.0–7.4, and is relatively insensitive to inhibition by vanadate (60).

SMA2 was found in intracellular yeast membranes enriched with SEC63, an integral membrane protein in the endoplasmic reticulum. The intracellular distribution of SMA2 differs from the plasma membrane localization of rabbit SERCA1b when expressed in yeast (73). One explanation could be the difference.

**TABLE II**

| Inhibitors        | IC\(_{50}\) (\(\mu\)M) |
|-------------------|------------------------|
| Erythrosine B     | 3.2 ± 0.1              |
| Vanadate          | 4.5 ± 0.6              |
| Thapsigargin      | 18.1 ± 1.6             |
| Cyclopiazonic acid| 8.0 ± 0.9              |

**Fig. 5.** Isolation of solubilized yeast membrane fractions enriched for SMA2 activity. Solubilized P\(_{tot}\) membranes from YPH500 cells carrying p315SMA2 were concentrated by filtration through a Amicon membrane and loaded onto a Superdex 200 gel filtration column as described under "Experimental Procedures." A, Western blot analysis. Rabbit sarcoplasmic reticulum (SR) membranes (50–150 ng), P\(_{tot}\) membranes (10 ng), Amicon concentrate (20 mg), or filtrate (60 mg) and 100-\(\mu\)l samples of fractionated solubilized membranes (fractions 11–20) were analyzed by immunoblotting with anti-SERCA1a antibodies. B, thapsigargin-sensitive ATPase activity of fractions 11–22. C, calcium dependence of SMA2 ATPase activity in pooled gel filtration fractions 14 and 15. The values shown are the average of triplicate experiments (S.E. was less than 4%).

**Inhibitor sensitivity of SMA2 determined in solubilized yeast membrane fractions**

ATPase activity was measured in pooled gel filtration fractions as described under "Experimental Procedures."
SERCA1a was 4-fold higher than that of SMA2. In contrast expression of rabbit SERCA1a. The expression level of 27838
activity. Total membranes (Ptot fractions) from YPH500 (wild-type), K605 pmc1Δ and
were (line 2 and 5) or without (lines 1 and 4) the SMA2 expression p426SMA2 plasmid, and MGY8–3D pmc1Δ cnb1Δ cells (line 3) were spotted onto solid media supplemented with 200 and 400 mM CaCl2 as indicated. B, the K605 pmc1Δ (left panel) and
MM10DV pmc1Δ pmr1Δ vcx1Δ (right panel) strains expressing SMA2 from low copy p315SMA2 (right panel) were grown on a rich medium supplemented by either 200 mM CaCl2 or 200 mM CaCl2 and 100 μg/ml cyclosporin A.

The contribution of SMA2 to Ca2+ tolerance is increased upon calcineurin inactivation, through mutation of regulatory subunit CNB1 (A) or inhibition by cyclosporin A (B). A, serial 10-fold dilutions of saturated cultures (40 x 10^6 cells/ml) of K605 pmc1Δ and MGY8DV pmc1Δ cnb1Δ vcx1Δ cells, with (lines 2 and 5) or without (lines 1 and 4) the SMA2 expression p426SMA2 plasmid, and MGY8–3D pmc1Δ cnb1Δ cells (line 3) were spotted onto solid media supplemented with 200 and 400 mM CaCl2 as indicated. B, the K605 pmc1Δ (left panel) and MM10DV pmc1Δ pmr1Δ vcx1Δ (right panel) strains expressing SMA2 from low copy p315SMA2 (right panel) were grown on a rich medium supplemented by either 200 mM CaCl2 or 200 mM CaCl2 and 100 μg/ml cyclosporin A.

Calcineurin-mediated regulation of SMA2 ATPase activity. Total membranes (Ptot fractions) from YPH500 (wild-type), K605 (pmc1Δ), MGY8–3D (pmc1Δ cnb1Δ), and MGY8DV (pmc1Δ cnb1Δ vcx1Δ) cells expressing SMA2 from the low copy (p315SMA2) or high copy (p426SMA2) plasmids were assayed for thapsigargin-sensitive ATPase activity as described under “Experimental Procedures.” +, wild-type allele. The values shown are the mean plus standard deviation from two independent experiments.

in the yeast expression systems utilized and/or expression levels of the Ca2+ ATPase isoforms. We used the constitutive PMA1 promoter to express SMA2 instead of the inducible GAL1 promoter (73). The low copy number SMA2 expression plasmid was better tolerated than the corresponding high copy number plasmid, which had adverse effects on cell growth and SMA2 expression levels. These results suggest that Ca2+-ATPase overproduction might be limited in yeast by cellular mechanisms that monitor the levels of membrane proteins and compensate for changes in these levels by inducing synthesis of novel internal membrane structures or karmellae (57). In a separate study,2 the PMA1 promoter was also used to direct expression of rabbit SERCA1a. The expression level of SERCA1a was 4-fold higher than that of SMA2. In contrast with SMA2, expression of SERCA1a resulted in an accumulation of endoplasmic reticulum membranes which were found to affect fractionation of plasma membrane and endoplasmic reticulum markers in sucrose density gradients.

In the yeast S. cerevisiae, the PMR1 and Pmc1 Ca2+-ATPases function together in Ca2+ sequestration. Synthetic lethality of pmr1 pmc1 double mutants in low Ca2+ conditions is prevented by expression of SMA2, indicating that the S. mansonii Ca2+ pump substitutes for loss of both PMR1 and PMC1. The effects of pmc1 mutations on cell growth at high Ca2+ concentrations are partially suppressed by the expression of SMA2 but only in strains deficient in calcineurin (and lacking the Vcx1 Ca2+/H+ exchanger). It is unlikely that calcineurin regulates expression levels of SMA2 through the PMA1 promoter and the calcineurin-dependent transcription factor TCN1/CRZ1 (74, 75) as calcineurin inactivation has no effect on the amount of PMA1 in plasma membranes (76). We also tried to determine whether SMA2 could suppress the growth defect of pmr1 mutants in calcium-depleted (0.81 mM Ca2+) growth media. However, we were not able to discriminate between the PMR1 wild-type and the pmr1 mutant strains under these conditions (data not shown).

Ca2+ tolerance of pmc1 mutants is restored by increasing the copy number of the PMR1 or Vcx1 genes (17). These three Ca2+ transporters are therefore fully interchangeable in vivo despite a different subcellular localization. Their contributions to Ca2+ tolerance, however, are differently regulated. In the wild-type strain, PMC1 provides the largest contribution because Vcx1 function is negatively regulated through calcineurin-dependent post-translational mechanisms. Expression of a PMCI reporter gene increases dramatically in response to high Ca2+ concentrations by calcineurin-dependent regulatory processes. In contrast, expression of a Pmr1 reporter gene increases only 2-fold in a process which also seems to require
calcineurin activation (20). By using vacuolar ATPase mutants to analyze the role of calcineurin in intracellular Ca\textsuperscript{2+} homeostasis, it had been suggested that PMR1 sequesters Ca\textsuperscript{2+} into the Golgi under the negative control of calcineurin (77). Our results also indicate that SMA2 activity is inhibited by a calcineurin-dependent regulatory mechanism which remains to be identified.

The simplest explanation of these results is that calcineurin activation by Ca\textsuperscript{2+}/calmodulin diminishes the contribution of PMR1 to Ca\textsuperscript{2+} tolerance, through a direct or indirect inhibitory effect on the ATPase activity. As a result, Ca\textsuperscript{2+} uptake into the Golgi is repressed and cytosolic free Ca\textsuperscript{2+} concentration is increased, leading to further activation of calcineurin and amplification of the Ca\textsuperscript{2+} signal. This would explain why PMR1, if not overexpressed, cannot replace PMC1 for growth under high Ca\textsuperscript{2+} conditions. According to this explanation, the function of PMR1 in Ca\textsuperscript{2+} transport would be derepressed in calcineurin-deficient strains. However, the resulting activation of PMR1 would not be sufficient to compensate for loss of PMC1 at high Ca\textsuperscript{2+} concentrations, as pmc1 cnb1 vcx1 mutants without the SMA2 expression plasmid failed to grow under these conditions. Consistently, the expression level of PMR1 is 2-fold less than that of PMC1 in the presence of 200 mM CaCl\textsubscript{2} (20). Finally, the possibility that the function of PMR1 is also regulated by VCX, through a calcineurin-independent mechanism, is suggested by the increased level of SMA2 ATPase activity in the pmc1 cnb1 vcx1 mutant compared with that seen in the pmc1 cnb1 mutant.

There is no evidence that calcineurin regulates intracellular ion homeostasis in \textit{S. mansoni}. It is therefore difficult to assess the physiological relevance of our results to the regulation of \textit{S. mansoni} Ca\textsuperscript{2+}-ATPases. The mechanisms of calcineurin activation by Ca\textsuperscript{2+}/calmodulin and inhibition by cyclosporin A are well conserved between yeast and animal cells (69, 78). Moreover, the observation that yeast calcineurin inhibits heterologous intracellular Ca\textsuperscript{2+} pumps is consistent with the effects of calcineurin overexpression on decreased levels of PMA1 activity in yeast (76) and with regulation of Na\textsuperscript{+}/K\textsuperscript{+}-ATPase activity by calcineurin in animal cells (79).

In conclusion, our results show that the yeast \textit{S. cerevisiae} is an eminently suitable system for heterologous expression of \textit{S. mansoni} Ca\textsuperscript{2+}-ATPases from parasites. First, expression of \textit{S. mansoni} SMA2 in yeast results in the synthesis of a fully functional Ca\textsuperscript{2+} pump. The enzyme is correctly targeted to the endoplasmic reticulum and exhibits kinetic properties comparable to SERCA3 in mammalian non-muscle tissues. Second, we estimated the amount of SMA2 in yeast membranes as representing 0.5% of total protein. There is no significant background of unrelated Ca\textsuperscript{2+}-dependent ATPase activity in yeast membranes, due to low levels of PMR1 and PMC1 expression. Moreover, the corresponding genes can be replaced by SMA2 with no effect on cell viability under standard growth conditions. Finally, the possibility to study the function of SMA2 in Ca\textsuperscript{2+} tolerance and its regulation \textit{in vitro} expands the usefulness of yeast for structure-function relationships studies on various \textit{S. mansoni} Ca\textsuperscript{2+}-ATPase isoforms.

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