Mutation of an unusual mitochondrial targeting sequence of SODB2 produces multiple targeting fates in *Toxoplasma gondii*

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Summary

Proteins destined for the mitochondria travel an intricate pathway through two membranes, each with its own receptors and channels. These proteins interact with receptors via N-terminal presequences that form amphipathic helices. Generally, these helices contain abundant positive charges on one face and hydrophobic residues on the other, but share little primary sequence homology. While extensive research on mitochondrial import has been done in yeast and mammalian cells, little is known about import or contents of the single mitochondrion of *Toxoplasma gondii*, a parasite in the phylum Apicomplexa. We describe here the characterization of TgSODB2, a novel, mitochondrial superoxide dismutase in *T. gondii* with an unusual targeting sequence consisting of a hydrophobic segment resembling a signal peptide, followed by a presequence. We show that although the hydrophobic segment is competent to target a reporter protein to the secretory system, it is prevented from directing ER translocation when coupled with the presequence. When we mutated the only charged residue in the hydrophobic sequence, ER translocation is restored and the reporter targeted to the apicoplast, a chloroplast-like organelle found in most apicomplexans. The presequence that follows is predicted to form an amphipathic helix, but targeted the cytoplasm when the hydrophobic peptide is removed. In addition to having an unusual targeting sequence, TgSODB2 is only the second mitochondrially imported, iron-containing SOD to be described.

Key words: Apicomplexa, *Toxoplasma gondii*, Mitochondrion, Apicoplast, Targeting, Presequence

Introduction

Protein trafficking in eukaryotic cells is a complex process that involves many different signal motifs. Proteins destined for the nucleus, ER, chloroplasts, mitochondria and other organelles all travel along distinct pathways. In particular, proteins targeted to the mitochondria or chloroplasts have an especially complicated route in that they must cross at least two membranes, each with its own set of receptors and channels. Most of these proteins contain cleavable N-terminal targeting sequences that are removed upon import (reviewed by Pfanner, 2000).

Proteins destined for the mitochondria are synthesized in the cytosol and imported directly, without ER involvement (Pfanner, 2000). Mitochondrial presequences contain several positively charged residues interspersed with hydrophobic amino acids, allowing the presequence to form an amphipathic helix (Roise and Schatz, 1988; von Heijne et al., 1989). The presequence binds to the Tom20 (transporter of outer membrane) receptor via its hydrophobic face before it transfers to the Tom22 receptor, where it interacts via its positively charged face (Brix et al., 1997). Following translocation through the TOM channel into the inter membrane space, the basic residues of the presequence mediate interaction with the highly acidic TIM (transporter of inner membrane) complex for further importation of matrix-destined proteins (Abe et al., 2000). While this is the general rule for mitochondrial targeting, there is wide variation in presequence length and amino acid composition, suggesting that there is considerable plasticity in the import system.

Most studies of mitochondrial import and presequence requirements have been done on mammalian cells, yeast, or the fungus *Neurospora crassa* (reviewed by Neupert, 1997). While all cells with membrane-bound organelles face the problem of targeting proteins correctly, apicomplexan parasites such as *Toxoplasma gondii* contain further levels of complexity. In addition to the typical eukaryotic organelles, *T. gondii* cells also contain three types of regulated secretory organelles and a chloroplast-like organelle called the apicoplast, which is delimited by four membranes (Kohler et al., 1997; Waller et al., 1998). Transport to the apicoplast requires a signal peptide, which first targets the protein to the endoplasmic reticulum (ER), and a transit peptide, which directs import into the apicoplast (Jelenska et al., 2001; Waller et al., 1998).

Little work has been done to study the single mitochondrion of *T. gondii*. The mitochondrion appears active, in that it maintains a membrane potential both in extracellular parasites and in replicating intracellular parasites (Melo et al., 2000). *T. gondii* heat shock protein 70 (TgHSP70), the only mitochondrially imported protein characterized to date (Toursel

et al., 2000), contains a typical presequence, indicating that at least some of the machinery and importing requirements studied in other systems may also be found in *T. gondii*.

We describe here the discovery of a second mitochondrially targeted protein, an iron-containing superoxide dismutase (TgSODB2). SODs scavenge oxygen radicals by converting them to molecular oxygen and hydrogen peroxide (McCord and Fridovich, 1969). They are found in virtually all organisms and can be classified into three main groups, based on their metal cofactors (Keele et al., 1970; McCord and Fridovich, 1969; Yost and Fridovich, 1973). Cu/ZnSODs (SODA) are found mainly in the cytosol of higher eukaryotes, FeSODs (SODB) exist mainly in prokaryotes, protists and chloroplasts, while MnSODs (SODC) are generally found in mitochondria and chloroplasts (Bannister et al., 1987; Fridovich, 1995; Fridovich, 1997). Of these, mitochondrially targeted SODs are the main enzymes responsible for neutralizing the abundant superoxide radicals generated by cellular respiration (Beyer et al., 1991). Underscoring the importance of mitochondrial SODs, *Drosophila*, *Escherichia coli*, and yeast mutants lacking mitochondrial SOD activity are highly sensitive to oxidative stress (Balzan et al., 1995; Carlioz and Touati, 1986; van Loon et al., 1986).

TgSODB2 is unusual in two ways. First, it is an iron-containing SOD targeted to the mitochondrion, a characteristic shared with only one other SODB (PmSOD1) found in the related parasite, *Perkinsus marinus* (Schott and Vasta, 2003). Unlike PmSOD1, which contains a standard N-terminal presequence, TgSODB2 is additionally notable because it contains an unusual presequence consisting of a hydrophobic N-terminal sequence resembling a signal peptide, followed by a more conventional amphipathic mitochondrial presequence. We demonstrate that both elements are necessary for correct targeting to the mitochondrion. Removing the hydrophobic sequence resulted in targeting to the cytoplasm, and deleting the presequence led to secretory targeting. Furthermore, mutating the only charged residue in the hydrophobic sequence resulted in a third mistargeting event, to the apicoplast.

Materials and Methods

Parasite propagation and harvest

T. gondii strain 2F was propagated in human foreskin fibroblast (HFF) cells grown in Dulbecco's modified Eagle's medium (DMEM, BioWhittaker) supplemented with 10% fetal calf serum (BioWhittaker), 2 mM glutamine (Sigma), 5000 IU/ml penicillin, and 5 mg/ml streptomycin (Mediatech). Cells and parasites were grown at 37°C in 5% CO_2 and passaged every 48 hours. Parasites were harvested by passing twice through 20 and 23 gauge needles and then filtering through a 3 μm filter to remove host cell debris.

EST database search

Database searches (ParaDB.cis.upenn.edu/toxo/index.html) for secreted *T. gondii* proteins using the cDNA sequence of *T. gondii SODB* (Odberg-Ferragut et al., 2000) revealed a single expressed sequence tag (EST). The bacterial clone (TgEST32h05) containing this cDNA was obtained from L. D. Sibley (Washington University, St Louis, MO) and sequenced in both directions.

Recombinant protein production

Two constructs were made to produce histidine-tagged recombinant protein. The *SOD* homology region alone (*TgSODB2*$^{255\text{-}864}$) or the *SOD* region plus the presequence (*TgSODB2*$^{75\text{-}864}$) (numbers denote nucleotide positions) were cloned into the vector pQE30 (Qiagen). *TgSODB2*$^{75\text{-}864}$ was PCR-amplified from clone TgEST32h05 with primers *TgSOD*.75.*Bam*HI.f (GATC**GGATCC**CTGGAGACGGCTGCTCTC) and *TgSOD*.864.*Kpn*I.r (GATC**GGTACC**GTTGCTTTCAAGTGC) (restriction sites in bold). *TgSODB2*$^{255\text{-}864}$ was amplified with primers *TgSOD*.255.*Bam*HI.f (GATC**GGATCC**GCTTTCACACTGCCTCCC) and *TgSOD*.864.*Kpn*I.r. Thermocycling conditions were as follows: 94°C 3 minutes; 5 cycles of 94°C 1 minute, 48°C 1 minute and 68°C 1.5 minutes; 25 cycles of 94°C 1 minute, 52°C 1 minute and 68°C 1.5 minutes; followed by 7 minutes at 68°C. PCR products were first sub-cloned into pGEM vector (Promega) according to the manufacturer's protocol and then cloned into pQE30, creating constructs pQE30-*TgSODB2*$^{75\text{-}864}$ and pQE30-*TgSODB2*$^{255\text{-}864}$.

For large-scale purification of histidine-tagged recombinant protein, constructs pQE30-*TgSODB2*$^{75\text{-}864}$ and pQE30-*TgSODB2*$^{255\text{-}864}$ were electroporated into *E. coli* strain M15(pREP4) (Qiagen). Expression of his-rSOD$^{85\text{-}287}$ and his-rSOD$^{26\text{-}287}$ (numbers denote amino acid positions) was induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). Recombinant protein (confirmed by western blotting with anti-histidine tag antibodies) was affinity-purified with Ni-NTA agarose according to the manufacturer's protocol (Qiagen). The purity (>95%) of the eluted recombinant protein was assessed by Coomassie Blue staining of sodium dodecyl sulfate polyacrylamide (SDS-PAGE) gels. Protein concentration was measured using the micro-BCA assay (Pierce).

Production of antisera

Seven 10-week-old female BALB/c mice were injected intraperitoneally with 30 μg his-rSODB2$^{85\text{-}287}$ emulsified in Freund's complete adjuvant (Sigma) and boosted twice at two-week intervals with 20 μg his-rSODB2$^{85\text{-}287}$ in Freund's incomplete adjuvant (Sigma). Antibodies were tested for specificity and sensitivity by western blotting and enzyme-linked immunosorbant assay (ELISA).

Western blotting and ELISAs

Parasite lysates were made by resuspending pellets of freshly lysed tachyzoites in boiling SDS-PAGE sample buffer containing 2% β-mercaptoethanol. Parasite lysates of 3×10^6 tachyzoites/lane were electrophoresed on 12.5% SDS-PAGE gels and transferred to nitrocellulose (Schleicher and Schuell) for 40 minutes at 16 V using a Transblot SD semi-dry transfer cell (BioRad). Western blots were developed as described previously (Wan et al., 1997). ELISAs to titrate antibodies were done according to a standard protocol (Harlow and Lane, 1988). Antibodies are referred to as mαrSODB2.

Superoxide dismutase activity assays

To visualize SOD activity, a modified in-gel protocol was used (Beauchamp and Fridovich, 1971; Ismail et al., 1997). Briefly, *E. coli* lysates and purified his-rSODB2$^{85\text{-}287}$ were run on native 12.5% PAGE gels and stained with 2 mg/ml nitroblue tetrazolium (Sigma) for 15 minutes in the dark. Gels were rinsed briefly with water and incubated in 10 μg/ml riboflavin (Sigma) in the dark. Gels were exposed to fluorescent light for 2 hours and regions of SOD activity appeared as clear areas on a uniformly blue background. To define units of his-rSODB2$^{85\text{-}287}$, a modified version of a xanthine oxidase assay was used (Huang et al., 2000; Ukeda et al., 1997). Briefly, varying amounts of his-rSODB2$^{85\text{-}287}$ were added to a mixture of 5.6 mM $NaHCO_3$, pH 10.2; 0.1 mM EDTA, 0.1 mM xanthine, and 0.03 mM 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide (XTT) in plastic cuvettes (Fisher). The reaction was started by adding 32 μg xanthine oxidase in 25 μl 5 mM $NaHCO_3$,

pH 10.2. One unit of dismutase activity is defined as the amount of his-rSOD$^{85\text{-}287}$ needed to reduce oxidation of XTT by 50%.

Metal ion cofactor determination

Iron content in his-rSOD$^{85\text{-}287}$ was determined by atomic absorption spectroscopy on an AAnalyst 600 instrument (Perkin Elmer). Recombinant protein was first dialyzed against PBS. Iron content of PBS was also measured as a background control.

Intracellular localization constructs

To determine the subcellular localization of TgSODB2, constructs (termed p*TgSODB2*$^{1\text{-}287}$*/GFP*, and p*TgSODB2*$^{1\text{-}84}$*/GFP*) were made of the open reading frame (ORF) or the N-terminal extension alone linked C-terminally to green fluorescent protein (GFP), respectively. Briefly, primers *TgSODB2*.1.*Bgl*II.f (GATC**AGATCT**ATGTCCATCACAGCTGTC) and T*gSODB2*.861.*Avr*II.r (GATC**CCTAGG**GTTGCTTTCAAGTGCTTTC) and *TgSODB2*.1.*Bgl*II.f and *TgSODB2*.258.*Avr*II.r (GATC**CCTAGG**AGCACAGGCGTTTGTGG), respectively, were used to PCR amplify the two regions with the cycling parameters described above. The two regions were ligated into the plasmid p*GFP-sagCATsag* (courtesy of D. Roos, University of Pennsylvania), creating *pTgSODB2*$^{1\text{-}287}$*/GFP* and p*TgSODB2*$^{1\text{-}84}$*/GFP*. Qiagen Endotoxin-free plasmid MAXI kits were used to isolate and sterilize plasmids. *SOD* regions were confirmed correct by DNA sequencing.

Mutant constructs

To determine the function of the two parts of the N-terminal extension of TgSODB2 and to determine what role arginine^{12} plays, three constructs were made. *TgSODB2*$^{\Delta 1\text{-}25}$, the full-length ORF without the hydrophobic N terminus, was PCR amplified with primers *TgSODB2*.75.*Bgl*II.*2*.f (GATC**AGATCT**ATGGTCCTGGAGACGGCTGCTCTC) (contains an extra met and ala residues 3′ to the restriction site for expression in *T. gondii*) and *TgSODB2*.861.*Avr*II.r. *TgSODB2*$^{\Delta 26\text{-}84}$, the full-length ORF without the presequence, and *TgSODB2*^{R12A}, the complete ORF with arginine^{12} mutated to alanine, were amplified in two PCR steps. For *TgSODB2*$^{\Delta 26\text{-}84}$, the hydrophobic sequence was first amplified with primers *TgSODB2*.1.*Bgl*II.f and *TgSODB2*.271.r (**CGCGGCTAACCGGAGCGT**ACACGAAAGTGTGACGG), while the SOD enzymatic region was amplified with primers *TgSODB2*.58.f (**GCGCCGATTGGCCTCGCA**TGTGCTTTCACACTGGGTC) and *TgSODB2*.861.*Avr*II.r (primer regions of overlap in bold). Full-length *TgSODB2*$^{\Delta 26\text{-}84}$ was then amplified in a fusion PCR step in which the initial PCR products, which had compatible 3′ and 5′ ends, were mixed with primers *TgSODB2*.1.*Bgl*II.f and *TgSOB2*.861.*Avr*II.r, respectively. For *TgSODB2*^{R12A}, construct p*TgSODB2*$^{1\text{-}287}$*/GFP* was used as template and primers *GFPsagCATsag*.2502.f (GATC**TCGCGA**AAGGCATCACGAAC (anneals within the untranslated sequence of the template upstream of the *SOD* insert and contains an endogenous *Nru*I site in bold) and *TgSODB*2.48.r (**CTAGGGCAGCCGAAAGGGCGAGTGCTGGCAC**, mutated residues underlined) were used to amplify one half of the insert, while primers *TgSODB2*.22.f (**GTGCCAGCACTCGCCCTTTCGGCTGCCCTAG**) and *TgSODB2*.861.*Avr*II.r were used to amplify the second half. The full product was obtained using primers *GFPsagCATsag*.2502.f and *TgSODB2*.861.*Avr*II.r. Thermocycling conditions for all three constructs were as follows: 94°C 3 minutes, 30 cycles of 94°C 1 minute, 52°C 1 minute, and 68°C 1.5 minutes; followed by 7 minutes at 68°C. All three amplicons were cloned into p*GFP-sagCATsag*.

Endoplasmic reticulum localization constructs

Four constructs expressing TgSODB2 regions fused to GFP with an ER localization signal (HDEL) added to the C terminus were made. Briefly, two PCR reactions, the first using primers *GFP*.1.*Avr*II.f (GATC**CCTAGG**ATGAGTAAAGGAGAAGAAC) and *DHFR*.18.HDEL.r (**GGCTGCAGGTCTGGACATTTACAGCTCATCATGTTTGTATAGTTCATCCATGC**) and the second with primers *GFP*.699.HDEL.f (**GCATGGATGAACTATACAAACATGATGAGCTGTAAATGTCCAGACCTGCAGCC**) and *DHFR*.790.*Not*I.r (GATC**GCGGCCGC**TCTAGAACTAGTGGATCC) (regions of overlap in bold), were used in fusion PCR with primers *GFP*.1.*Avr*II.f and *DHFR*.790.*Not*I.r to add the HDEL sequence (underlined) to the 3′ end of *GFP*. The *GFP* sequence in constructs p*TgSODB2*^{R12A}*/GFP*, p*TgSODB2*$^{\Delta 26\text{-}84}$*/GFP*, p*TgSODB2*$^{\Delta 1\text{-}25}$*/GFP* and p*TgSODB2*$^{1\text{-}287}$*/GFP* was replaced with *GFP-HDEL*, creating constructs p*TgSODB2*$^{\Delta 1\text{-}25}$*/GFP-HDEL*, p*TgSODB2*$^{1\text{-}287}$*/GFP-HDEL*, p*TgSODB2*$^{\Delta 26\text{-}84}$*/GFP-HDEL* and p*TgSODB2*^{R12A}*/GFP-HDEL*.

SOD domain with GRA8 signal peptide construct

Construct p*GRA8(s)TgSODB2*$^{26\text{-}287}$*/GFP*, in which the hydrophobic sequence of *TgSODB2* is replaced with the signal peptide of the secreted protein GRA8, was created as follows. Primers *TgSODB2*.75.*Nhe*I.f (GATCGCTAGCCTGGAGACGGCTGCTCTC) and *TgSODB2*.861.*Avr*II.r were used to amplify *TgSODB2*$^{26\text{-}287}$ under the conditions above. This amplicon was then ligated into plasmid p*GRA8(s)ftsZ(t)GFP* (courtesy of D. Roos), replacing *ftsZ*.

Electroporation and GFP visualization

100 μg of all *GFP, GFP-HDEL* constructs, and p*FNR(l)-dsRED* construct were each resuspended in 100 μl sterile cytomix (2 mM EDTA, 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM Hepes, 5 mM $MgCl_2$-$6H_2O$, pH 7.6) and added to 600 μl tachyzoite suspension at 1.7×10^7/ml in cytomix. Parasites were electroporated in 0.4 cm gap cuvettes with a BioRad Gene Pulsar® II electroporater with voltage set to 1.5 kV, capacitance at 25 μF and no resistance. For intracellular growth, 10 μl of each electroporation was added to HFF monolayers on Lab-Tek chamber slides (Nunc, Inc.) and parasites were grown overnight at 37°C. The remainder of the transfected parasites were centrifuged, resuspended in 500 μl medium, and added to a fresh HFF monolayer for overnight growth. To visualize extracellular parasites, monolayers were scraped and syringed and 2×10^6 parasites in 100 μl were added to 900 μl medium containing 0.1 nM Mitotracker-Red CMXRos™ (8-(4′-chloromethyl)phenyl-2,3,5,6,11,12,14,15-octahydro-1H,4H,10H,13H-diquino lizino-8H-xanthylium chloride; Molecular Probes). Parasite mitochondria were stained for 30 minutes at 37°C and mounted on poly-L-lysine-coated glass slides for viewing. For visualization of mitochondria in intracellular parasites, HFF cells infected for 24 hours on chamber slides were stained with Mitotracker™ as above. Monolayers were fixed with 4% formaldehyde and 0.02% glutaraldehyde and examined for mitochondria expression and Mitotracker™ staining. Digital micrographs of all slides were acquired on a Nikon Eclipse E800 microscope equipped with a Spot RT slider CCD camera. Images were deconvolved using SimplePCI program (Compix Inc.) with the following settings: 1.3 numerical aperture, 1.515 refractive index, 520 nm emission wavelength, 90% haze removal, and a Z kernel width of 5.

Selection of stable transfectants

T. gondii populations stably expressing construct p*TgSODB2*$^{1\text{-}287}$*/GFP* were selected by adding chloramphenicol to the growth medium at a final concentration of 20 μM 18-24 hours post-transfection. Populations were cultured continuously in the presence of chloramphenicol and tested for expression of TgSODB2$^{1\text{-}287}$/GFP protein by western blot with mαrSODB2 and mouse monoclonal

αGFP (Clontech) after approximately 2 weeks of selection. Positive populations were maintained under selection for the duration of the study.

Computer-based analysis

Analyses of DNA sequences were performed with the DNAStar software suite. The signal peptidase cleavage site was predicted with SignalP (http://www.cbs.dtu.dk/services/SignalP/). Mitochondrial and chloroplast/apicoplast targeting signals were predicted by iPSORT (http://www.HypothesisCreator.net/iPSORT/predict.cgi). Phylogenetic analyses were done using BLAST (http://www.ncbi.nlm.nih.gov/BLAST/).

Results

A search of the *T. gondii* tachyzoite EST database with the cDNA sequence of a previously identified *T. gondii SODB* [termed *TgSODB* (Odberg-Ferragut et al., 2000)] yielded a single unique hit for a second putative *SODB* that we termed *TgSODB2*. The *TgSODB2* EST (TgEST32h05) contained a complete ORF of 864 nucleotides predicting a 287 amino acid

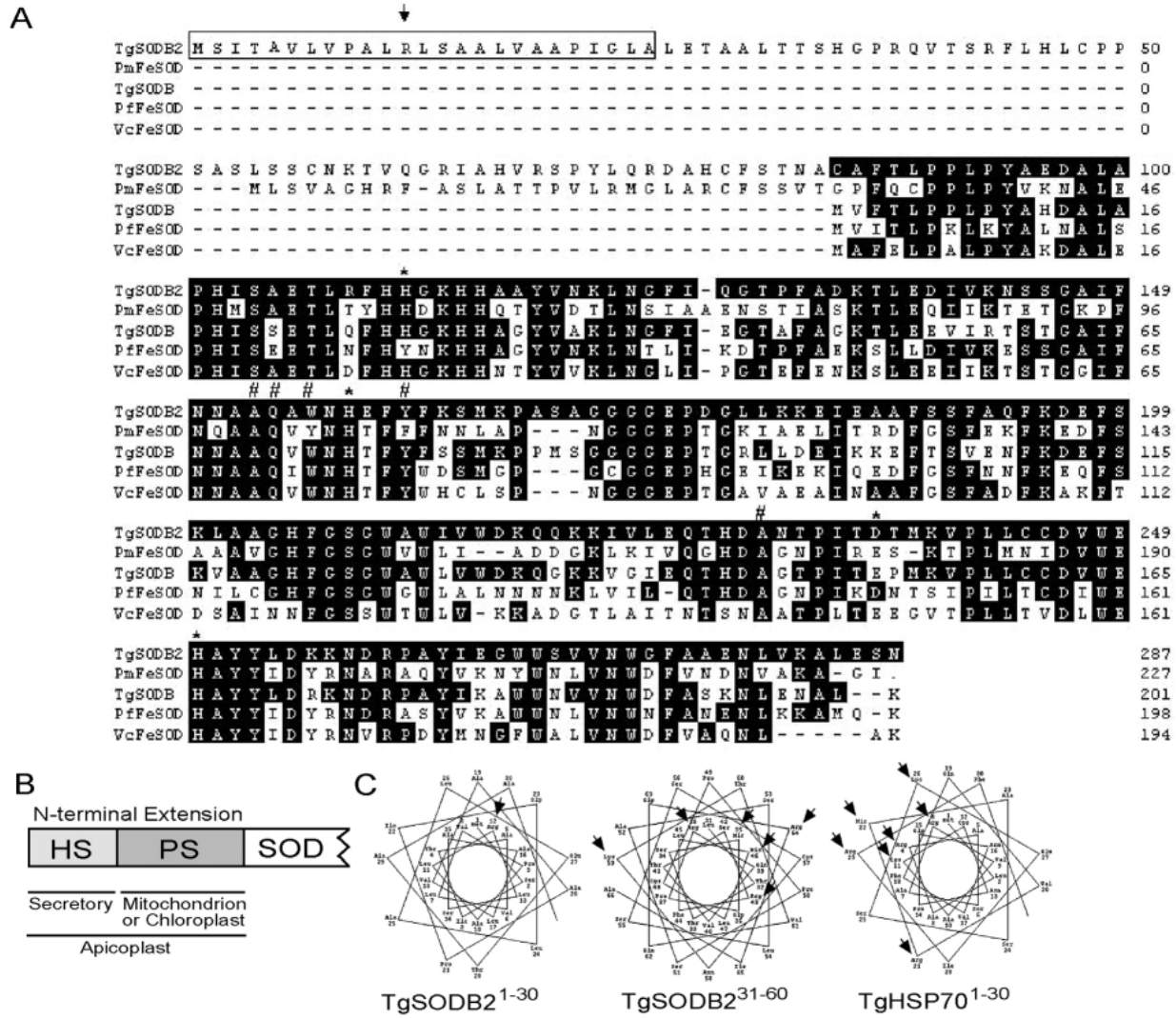

Fig. 1. Structural properties of TgSODB2. (A) Comparison of amino acid sequences of TgSODB2 (accession no. AY176062), *Perkinsus marinus* FeSOD (PmSOD1, AY095212), TgSODB (AAC63943), *Plasmodium falciparum* FeSOD (PfFeSODB, CAA89971) and *Vibrio cholerae* FeSOD (VcFeSOD, NP_231679). Protein sequences were aligned using the ClustalW program (DNAStar-Megalign) and identical residues among sequences are shaded. TgSODB2 contains all residues deemed important for enzyme function (marked with #) and metal ion binding (marked with *) and shows extensive similarity with TgSODB and other FeSODs. The N-terminal hydrophobic sequence of TgSODB2 is boxed and the single basic residue (arg^{12}) within this region is indicated with an arrow. (B) Schematic representation of the TgSODB2 N-terminal extension containing a hydrophobic sequence (HS) and presequence (PS). The HS is predicted by SignalP to be a signal peptide for targeting to the secretory pathway, the PS is predicted by iPSORT to be a mitochondrial or chloroplast targeting sequence, and the entire N-terminal extension is predicted to be an apicoplast targeting sequence. (C) Helical wheel projections (Webgenetics; http://webgenetics.com/cgi-bin/wg?form=wheel&ID=0) of TgSODB2 amino acids 1 to 30 ($TgSODB2^{1-30}$) and 31 to 60 ($TgSODB2^{31-60}$) and TgHSP70 amino acids 1-30 ($TgHSP70^{1-30}$). Arrows denote charged residues, all of which are basic amino acids. Note that $TgSODB2^{1-30}$ is predicted to form a hydrophobic helix with only one charged residue (arg^{12}) whereas $TgSODB2^{31-60}$ exhibits the properties of an amphipathic helix that is similar to the mitochondrial import presequence of TgHSP70.

protein 74% identical to TgSODB (Fig. 1A). Residues 85-287 of TgSODB2 showed extensive homology with TgSODB and other SODBs. Furthermore, this region contains all the residues previously shown to be important for SODB enzymatic activity (namely, ala^{67}, gln^{68}, trp^{70}, tyr^{75} and ala^{144}) and metal binding (his^{25}, his^{72}, asp^{150} and his^{164}; numbered from the beginning of the SOD enzymatic region) (Odberg-Ferragut et al., 2000; Tannich et al., 1991; Viscogliosi et al., 1998). However, unlike TgSODB, TgSODB2 possesses an 84 amino acid N-terminal extension. The first 25 amino acids of this extension are almost exclusively hydrophobic and are predicted by the SignalP algorithm to constitute a secretory signal peptide with a cleavage site between ala^{25} and leu^{26} (Fig. 1B). Based on this, we anticipated that TgSODB2 might be a secretory protein discharged from the dense granules into the parasitophorous vacuole (PV) in which the parasite replicates intracellularly (dense granules are the default secretory pathway in *T. gondii*) (Roos et al., 1999; Waller et al., 2000). However, the N-terminal extension without the hydrophobic sequence is predicted by iPSORT to be a chloroplast or mitochondrial targeting presequence. Since *T. gondii* contains an apicoplast, which uses a bipartite targeting sequence consisting of a signal peptide followed by a chloroplast targeting sequence (McFadden et al., 1996), the chloroplast presequence prediction was feasible. Although the region following the hydrophobic sequence was strongly predicted to be a mitochondrial or chloroplast import signal, such signals are not generally preceded by hydrophobic regions. Helical wheel projections indicate that residues 26-84 form an amphipathic alpha helix similar to the presequence of TgHSP70 (Fig. 1C). However, unlike the TgHSP70 amphipathic helix, which is at the extreme N terminus, the TgSODB2 amphipathic helix begins more than 25 amino acids from the N terminus, after the hydrophobic sequence.

To analyze the basic characteristics of TgSODB2, we expressed two recombinant, histidine-tagged forms of TgSODB2: his-rSOD$^{26\text{-}287}$ and his-rSOD$^{85\text{-}287}$, corresponding to the enzyme with or without the presequence, respectively. His-rSOD$^{26\text{-}287}$ (30 kDa) was expressed at low levels and was exclusively in the insoluble fraction. However, the soluble fraction contained a strongly induced 24 kDa band (Fig. 2B) that failed to react with anti-6-histidine antibodies (data not shown). Based on this we concluded that the N-terminal extension and histidine tag were removed from his-rSOD$^{26\text{-}287}$ by an *E. coli* protease, thereby producing an untagged, soluble recombinant protein the same size as his-rSOD$^{85\text{-}287}$. Supporting this notion, the induced soluble band of his-rSOD$^{26\text{-}287}$ showed strong SOD activity in native gels (data not shown). Only one major cleavage product was observed, suggesting that there could be a hypersensitive site adjacent to the catalytic domain. Since the enzymatically active cleavage product could not be easily purified, no further work was done with this construct. In contrast, his-rSOD$^{85\text{-}287}$, consisting of the SOD domain without the presequence, expressed well in a soluble form and reacted with anti-6-histidine antibodies (not shown), indicating an intact N terminus. This form was affinity-purified to near homogeneity and used for preparing antibodies and for testing enzymatic activity.

To determine if his-rSOD$^{85\text{-}287}$ possessed SOD activity, we used a modified in-gel assay (Beauchamp and Fridovich, 1971; Ismail et al., 1997), which takes advantage of the tendency of riboflavin to oxidize on exposure to light and air, producing oxygen radicals. These radicals then oxidize the dye component of the assay, generating a blue product everywhere on the gel except in areas of SOD activity. Similar to the positive control (*E. coli* SODB), his-rSOD$^{85\text{-}287}$ produced a large clear zone when run on a native gel, indicating ability to dismutate radicals (Fig. 2C). No clear zone was observed around rMIC5, which was included as a negative control. To measure enzymatic units for his-rSOD$^{85\text{-}287}$, we used a quantitative xanthine-xanthine-oxidase assay (Huang et al., 2000; Ukeda et al., 1997). In this assay, his-rSOD$^{85\text{-}287}$ exhibited 2441 units/mg protein (average of 5 experiments). Atomic absorption experiments showed that his-rSOD$^{85\text{-}287}$ contains 0.87 atoms of iron per molecule of protein, confirming that TgSODB2 is a SODB. Results from non-denaturing protein gel sizing (data not shown) indicate that the protein probably exists as a dimer, which is a common property of SODBs (Becuwe et al., 1996; Kanematsu and Asada, 1979; Salin and Bridges, 1980). Therefore, each dimer of TgSODB2 would contain on average 1.8 atoms of iron, similar to other

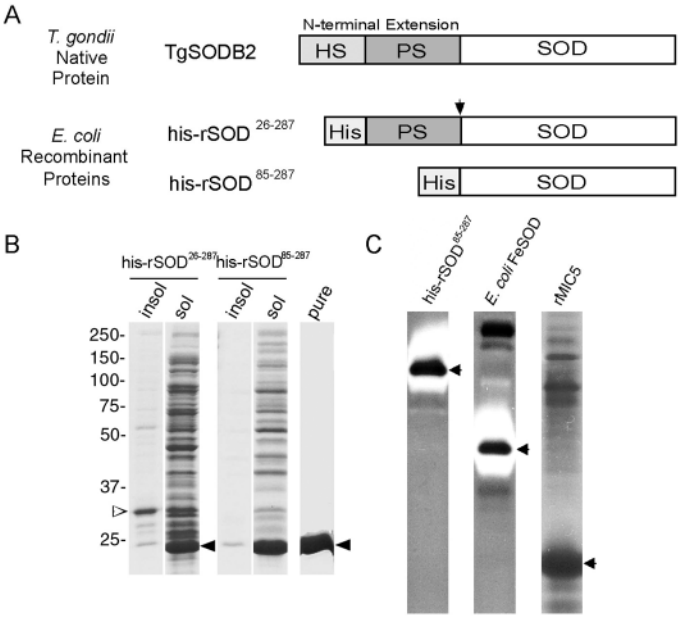

Fig. 2. Expression and analysis of recombinant TgSODB2. (A) Schematic of constructs used in this study including two *E. coli* N-terminally histidine-tagged (His) expression constructs: his-rSOD$^{26\text{-}287}$ contains the presequence (PS) and the SOD domain, and his-rSOD$^{85\text{-}287}$ consists of the SOD domain alone. An arrow denotes the approximate position of cleavage of his-rSOD$^{26\text{-}287}$ by an endogenous *E. coli* protease. (B) Coomassie Blue-stained SDS-PAGE gels showing insoluble (insol) and soluble (sol) fractions of *E. coli* expressing his-rSOD$^{26\text{-}287}$ and his-rSOD$^{85\text{-}287}$ and final purification of his-rSOD$^{85\text{-}287}$ (pure). The open arrowhead denotes the position of his-rSOD$^{26\text{-}287}$ in the insoluble fraction; closed arrows denote the position of his-rSOD$^{85\text{-}287}$ in the soluble fraction of induced cells. (C) SOD activity assay using native gel electrophoresis. A 12.5% polyacrylamide gel was first stained for SOD activity (see Materials and Methods) and then stained with Coomassie Blue. Light areas indicate regions of SOD activity: 10 μg *E. coli* FeSOD (positive control), 10 μg purified rSOD$^{85\text{-}287}$ showing strong dismutase activity, and 15 μg purified recombinant *T. gondii* MIC5 (negative control).

SODB dimers (Kanematsu and Asada, 1979; Le Trant et al., 1983; Odberg-Ferragut et al., 2000; Salin and Bridges, 1980).

As mentioned above, because TgSODB is nearly the same size as TgSODB2 and shares extensive sequence homology, it is likely they are at least partially antigenically cross-reactive. Accordingly, immunofluorescence assays with antibodies to TgSODB2 were inconclusive. To avoid the complication of antibody cross-reactivity, we created GFP fusions to define the subcellular localization of TgSODB2. Constructs p*TgSODB2*$^{1\text{-}287}$*/GFP* and p*TgSODB2*$^{1\text{-}84}$*/GFP* were created to express fusions of green fluorescent protein (GFP) with the full-length protein or the N-terminal extension alone, respectively. As a control for apicoplast targeting, we also tested a plasmid expressing acyl carrier protein fused to GFP (p*TgACP/GFP*) (Waller et al., 1998). All three constructs were transiently transfected into *T. gondii* and examined for fluorescence in intracellular parasites. While parasites transfected with p*TgACP/GFP* each showed discrete foci of GFP fluorescence indicative of the apicoplast, parasites transfected with the two *SOD* constructs instead showed staining of a long, twisting structure that extended throughout the cell (Fig. 3). No evidence of secretion into the PV was seen. In some cases a single tract of intracellular fluorescence was observed, while in other cases multiple, connected tracts were seen. These patterns are highly indicative of mitochondrial staining (Seeber et al., 1998).

To determine if the TgSODB2 was localized to the parasite mitochondrion, we stained parasites transfected with p*TgSODB2*$^{1\text{-}287}$*/GFP* and p*TgSODB2*$^{1\text{-}84}$*/GFP* with Mitotracker^{TM}, a dye that preferentially accumulates in the mitochondria (Poot et al., 1996). Intracellular parasites showed colocalization of the GFP signal with Mitotracker^{TM} staining, indicating that TgSODB2 associates with the mitochondrion. TgSODB2 did not colocalize with Mitotracker^{TM} in the host cell mitochondria surrounding the PV, demonstrating the specificity of targeting (Fig. 4A). To corroborate these results, we also stained extracellular parasites with Mitotracker^{TM} after transfection with p*TgSODB2*$^{1\text{-}287}$ and again saw colocalization, which was clearer in the absence of host mitochondria (Fig. 4B). In addition, parasites expressing the N-terminal extension alone fused to GFP (p*TgSODB2*$^{1\text{-}84}$*/GFP*) showed similar patterns, indicating that the N-terminal extension is sufficient to target a heterologous protein to the mitochondrion, a phenomenon seen with other mitochondrial presequences (Neupert, 1997; Schatz and Dobberstein, 1996; Voos et al., 1999). Collectively, these results demonstrated that despite having a hydrophobic, signal-like peptide at its N terminus, TgSODB2 is exclusively targeted to the parasite mitochondrion.

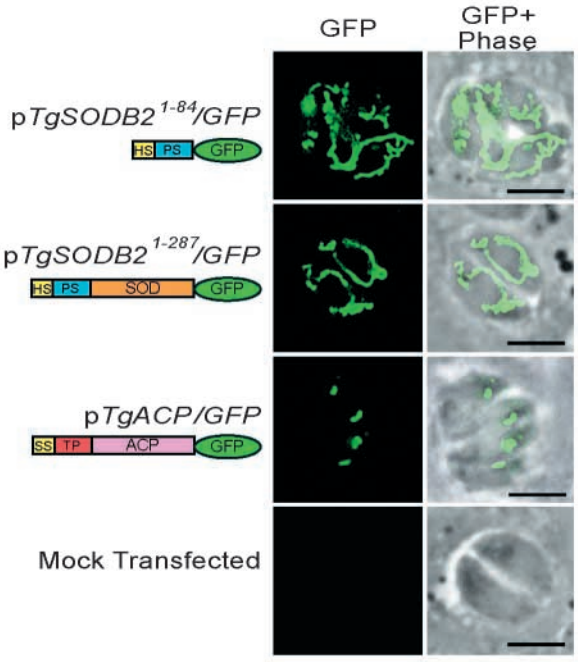

Fig. 3. Comparison of GFP fluorescence in intracellular parasites transiently expressing TgSODB2$^{1\text{-}84}$/GFP, TgSODB2$^{1\text{-}287}$/GFP, or TgACP/GFP. The apicoplast is delineated as a single, discrete dot of GFP fluorescence in p*TgACP/GFP* transfected cells (third row, four-cell stage), while parasites transfected with the *SOD* constructs show a twisting pattern of GFP fluorescence characteristic of the parasite mitochondrion (first and second rows, four- and two-cell stages, respectively). Mock transfected parasites showed no fluorescence when photographed with the same exposure time. Scale bar: 5 μm.

Since the presequences of mitochondrially imported proteins are generally cleaved upon entry (Neupert, 1997), the presence of two bands in parasite lysate corresponding to the complete protein and the enzymatic region suggests that TgSODB2 is imported into the mitochondrion. However, because TgSODB2 and TgSODB are potentially cross-reactive, it remained possible that the lower band is actually TgSODB and the upper band is full-length TgSODB2 that is associated with the mitochondrion without being imported. To rule out this possibility, we made stable populations of *T. gondii* expressing TgSODB2$^{1\text{-}287}$/GFP, allowing us to specifically detect the recombinant protein by immunoblotting. Lysates of these populations showed four bands reacting with mαrSODB2: 24 and 32 kDa bands seen in lysate from untransfected parasites, corresponding to endogenous mature and proTgSODB2, respectively, and two other bands at 60 and 50 kDa. The 60 kDa band was faint and was the size expected for TgSODB2$^{1\text{-}287}$/GFP, whereas the much more abundant 50 kDa band corresponded to TgSODB2$^{85\text{-}287}$/GFP (N-terminal extension removed) (Fig. 5). Only the 50 and 60 kDa bands reacted with αGFP. Some additional faint bands were seen in the TgSODB2$^{1\text{-}287}$/GFP lysate and may represent degradation products of the fusion protein, as they are recognized by both mαrSODB2 and αGFP. Since TgSODB2$^{1\text{-}287}$/GFP is cleaved, it is probably imported into the mitochondrion, suggesting that endogenous TgSODB2 is also imported and cleaved.

To determine whether both parts of the N-terminal extension were necessary for targeting the mitochondrion, we tested two additional constructs, p*TgSODB2*$^{\Delta 26\text{-}84}$*/GFP* and p*TgSODB2*$^{\Delta 1\text{-}25}$*/GFP*, consisting of the *SOD* ORF without the amphipathic presequence or hydrophobic sequence, respectively. p*TgSODB2*$^{\Delta 26\text{-}84}$*/GFP* transfected tachyzoites showed GFP fluorescence in the PV when examined inside HFF cells (Fig. 6) and in a punctate pattern in extracellular parasites (data not shown). When cells were stained with antibodies to GRA4, a protein known to target the dense granules and PV, extensive colocalization was seen. Therefore, in the absence of the mitochondrial presequence, the hydrophobic N terminus acts as a signal peptide and directs the protein to the secretory system and subsequently the PV. In contrast, parasites expressing TgSODB2$^{\Delta 1\text{-}25}$/GFP showed

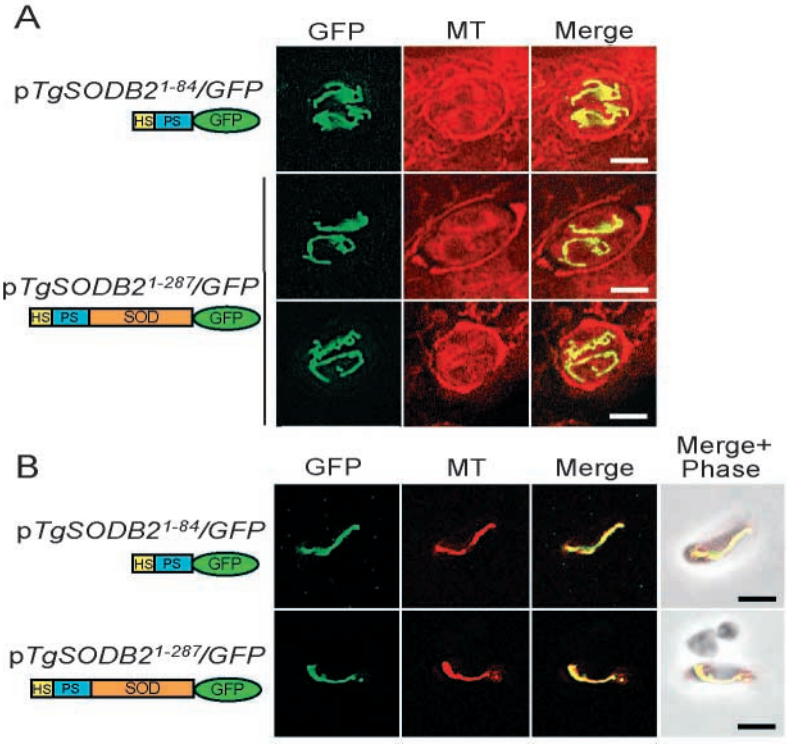

Fig. 4. TgSODB2 localizes to the parasite mitochondrion. Parasites were transiently transfected with p*TgSODB2*$^{1-84}$*/GFP* and p*TgSODB2*$^{1-287}$*/GFP* and imaged for GFP and Mitotracker-red^{TM} fluorescence. (A) Deconvolved images of intracellular parasites (two-cell stage), showing extensive Mitotracker^{TM} staining of host mitochondria, as well as colocalization of GFP and Mitotracker^{TM} in the parasite mitochondrion. (B) Extracellular parasites, showing colocalization of GFP and Mitotracker^{TM}. Scale bar, 5 μm.

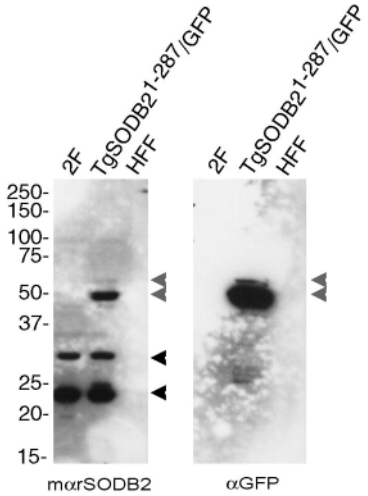

Fig. 5. TgSODB2 is imported into the mitochondrion. A western blot of lysate from parasites stably expressing TgSODB2$^{1-287}$/GFP shows two bands (grey arrows) in addition to the endogenous TgSODB2$^{1-287}$ and TgSODB2$^{85-287}$ bands (black arrows) seen in 2F lysate: a faint band at about 60 kDa corresponding in size to TgSODB2$^{1-287}$/GFP and a much more abundant product at about 50 kDa corresponding to TgSODB2$^{85-287}$/GFP. mαrSODB2 recognized all four bands, whereas αGFP recognized only the 50 and 60 kDa, GFP-containing products. HFF cell lysate is included as a negative control.

GFP fluorescence throughout the cell, a pattern consistent with cytoplasmic localization. Collectively, these results demonstrated that the amphipathic presequence alone is incapable of targeting the protein correctly. Rather, the complete N-terminal extension is necessary and sufficient for proper targeting.

To determine whether the only charged residue (arg^{12}) in the hydrophobic sequence plays a role in targeting, we also expressed p*TgSODB2*^{R12A}*/GFP*, a construct consisting of the complete ORF with arg^{12} mutated to ala to render residues 1-25 completely hydrophobic. Surprisingly, parasites expressing this construct showed single, bright foci of GFP fluorescence consistent with apicoplast localization. When cells were cotransfected with a known apicoplast marker (p*FNR(l)-dsRED*) (Striepen et al., 2000), colocalization was observed. This demonstrates that the arg^{12} is critically important for correct targeting and that without it, the N-terminal extension is able to act as a bipartite apicoplast-targeting sequence, directing the protein first to the ER and then to the apicoplast.

Since the hydrophobic N terminus is competent for directing TgSODB2$^{\Delta 26-84}$/GFP to the secretory system, we then asked whether TgSODB2 is targeted to the mitochondrion via the ER and secretory pathway. This would be a highly unusual circumstance since mitochondrial proteins normally traffic directly from the cytosol. We tested this possibility in two ways.

First, we predicted that if the hydrophobic N terminus of TgSODB2 targets it to the mitochondrion via the secretory pathway, then substituting it for another signal sequence should still direct it to the mitochondrion. To test this we made construct p*GRA8(s)TgSODB2*$^{26-287}$*/GFP*, which replaced the hydrophobic N-terminus of TgSODB2 with the signal sequence of GRA8. When transiently expressed in *T. gondii*, apicoplast localization was observed (data not shown), indicating that a heterologous signal peptide is not capable of substituting for the hydrophobic N-terminal sequence of TgSODB2.

Second, we engineered the addition of a *T. gondii* ER retention signal (HDEL) (Hager et al., 1999) to the C terminus of GFP in construct p*TgSODB2*$^{1-287}$*/GFP-HDEL*. If TgSODB2$^{1-287}$/GFP-HDEL is imported into the ER, the retention signal should prevent it from trafficking to the mitochondrion and trap it in the ER lumen. As positive controls for ER retention, we also created constructs p*TgSODB*$^{2\Delta 26-84}$*/ GFP-HDEL* and p*TgSODB2*^{R12A}*/GFP-HDEL*, which normally traffic through the ER en route to the PV and apicoplast, respectively. As a negative control, we made construct p*TgSODB2*$^{\Delta 1-25}$*/GFP-HDEL*, which should remain cytoplasmic. *T. gondii* transiently expressing TgSODB2$^{\Delta 26-84}$/ GFP-HDEL showed GFP fluorescence in a cup-shaped region around the nucleus and no vacuolar staining, indicating complete ER retention (Fig. 7). As expected, parasites expressing TgSODB2^{R12A}/GFP-HDEL also showed ER staining, as well as some labeling of the apicoplast. Also as expected, TgSODB2$^{\Delta 1-25}$/GFP-HDEL showed cytoplasmic localization, indicating that the HDEL only acts to retain proteins in the ER once imported.

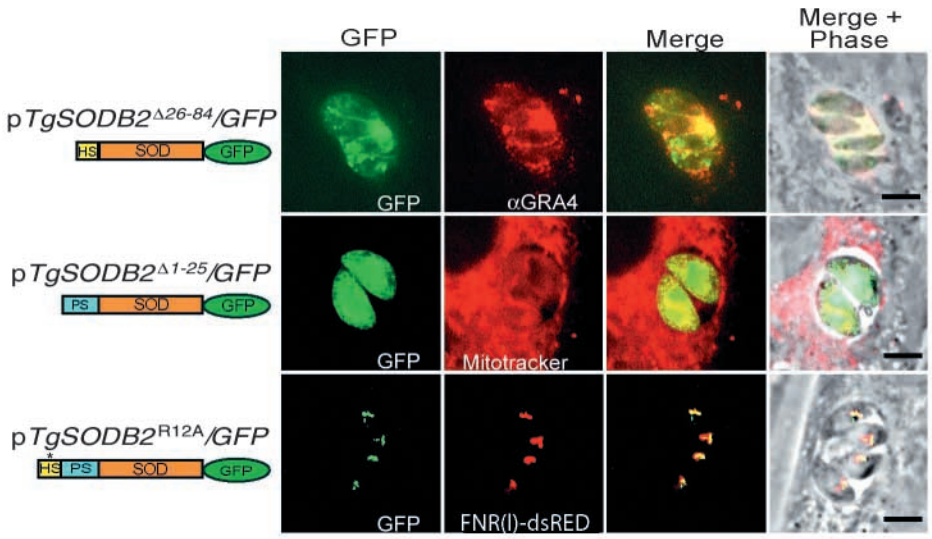

Fig. 6. Mutating the N-terminal extension results in mistargeting phenotypes. Parasites transiently transfected with p*TgSODB2*$^{\Delta 26\text{-}84}$*/GFP*, which contains a deletion of the presequence (PS), showed GFP fluorescence in a punctate pattern within the parasites (four-cell stage). GFP signal was also detected in the PV, where it colocalized with GRA4. Transfection with p*TgSODB2*$^{\Delta 1\text{-}25}$*/GFP* bearing a deletion of the hydrophobic sequence (HS) resulted in diffuse localization within the parasite cytoplasm in a pattern distinct from the mitochondrion, visualized by Mitotracker-red^{TM} staining. Transfection with p*TgSODB2*^{R12A}*/GFP*, in which arg^{12} was replaced by an ala (asterisk), showed a discrete localization pattern that colocalized with an apicoplast targeted protein, FNR(l)-dsRed. Scale bar, 5 μm.

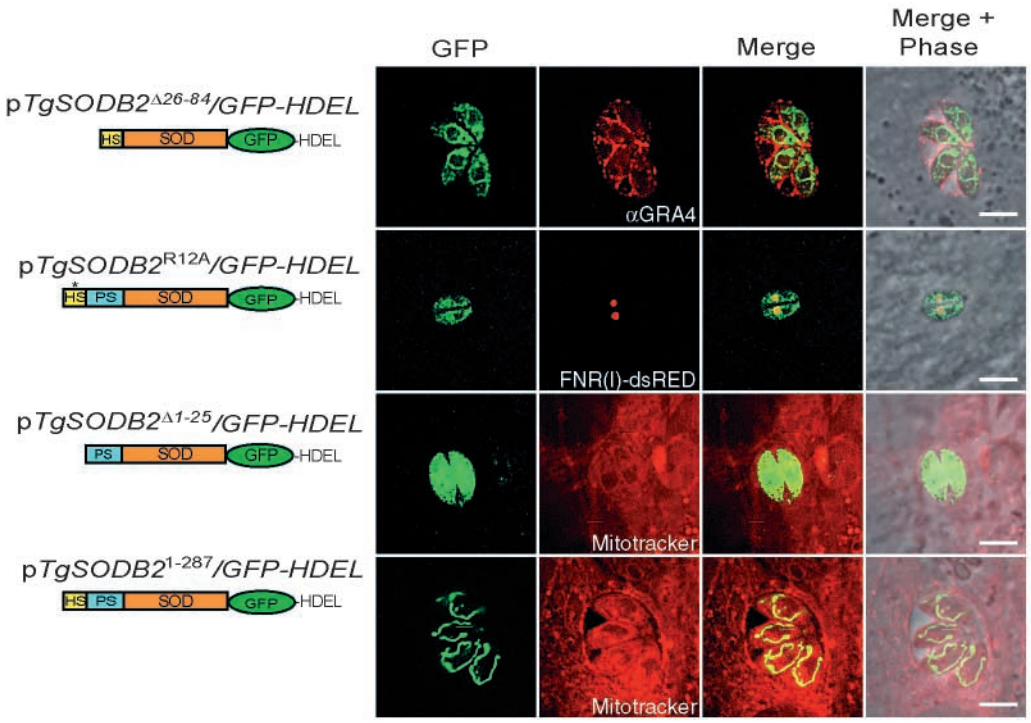

Fig. 7. TgSODB2 does not target to the mitochondrion via the ER. Adding an ER retention signal (HDEL) to dense granule and apicoplast targeting constructs to create p*TgSODB2*$^{\Delta 26\text{-}84}$*/GFP-HDEL* and p*TgSODB2*^{R12A}*/GFP-HDEL*, respectively, caused ER retention of GFP in a cup-shaped pattern. Retention was complete for parasites expressing TgSODB2$^{\Delta 26\text{-}84}$/GFP-HDEL, with no vacuolar staining overlapping with GRA4. Some apicoplast staining that colocalized with FNR(l)-dsRED was seen for p*TgSODB2*^{R12A}*/GFP-HDEL* transfected parasites, with extensive smearing and ER involvement. Addition of HDEL had no effect on targeting of cytoplasmic fusion protein TgSODB2$^{\Delta 1\text{-}25}$/GFP, indicating that the HDEL signal cannot misdirect proteins not destined for the secretory system. No ER signal was seen for TgSODB2$^{1\text{-}287}$/GFP-HDEL, which colocalized perfectly with Mitotracker^{TM}, implying that TgSODB2 does not enter the ER and probably traffics from the cytosol. Scale bar: 5 μm.

In contrast to the ER localized constructs, mitochondrial localization was clearly evident for cells expressing TgSODB2$^{1\text{-}287}$/GFP-HDEL, with no ER involvement, indicating that TgSODB2 probably follows the normal cytosolic path for import into the mitochondrion. Thus the presequence of TgSODB2 is capable of overriding the tendency of the hydrophobic sequence to target the protein to the secretory pathway.

Discussion

Protein import into the mitochondrion is a complex process involving sequential interaction with several receptors and channels. The process is initiated when the amphipathic presequence binds to Tom20, a receptor anchored in the mitochondrial outer membrane with most of its sequence extending into the cytosol (Pfanner, 2000). Tom20 contains a

shallow, hydrophobic groove made up of four alpha helices with negatively charged residues on the periphery. The presequence binds the groove via its hydrophobic side while its positively charged side interacts with the peripheral, negative residues (Abe et al., 2000). Tom20 then transfers the presequence to the Tom22 receptor, its small subunit Tom5, and the associated channel made up of Tom40. Tom22 is highly negatively charged and thus interacts with the positive face of the helix. Once within the inter membrane space, proteins destined for the matrix must pass through the TIM complex, which is made up of the Tim44 receptor and channel-forming proteins Tim23 and Tim17 (Pfanner, 2000). The driving force for this transfer comes primarily from an electrophoretic effect acting on the positively charged presequence, drawing it into the more negative matrix. In addition, HSP70 in the inter membrane space binds the presequence and further promotes translocation (Schatz, 1997; Jensen and Johnson, 1999; Pfanner, 2000). Although each individual TOM and TIM component binds presequences with low affinity, the sum of all the involved interactions is thought to promote the high specificity of import.

The unusually hydrophobic N terminus of the presequence of TgSODB2 raises the issue of just how this protein is correctly targeted and imported into the mitochondrion. As shown, the hydrophobic sequence is capable of functioning as a signal peptide, directing a reporter protein to the secretory pathway and into the PV via the dense granules. Therefore, it might be expected that the signal recognition particle (SRP) could bind residues 1-25 in the endogenous, intact protein and feed the protein into the ER. A few examples of mitochondrial presequences preceded by signal peptides have been found in mammalian cells, but in these cases the signal sequence overrides the presequence and the protein is targeted to the ER. Mitochondrially targeted versions of these proteins are only made when the signal sequence is absent owing to an alternate transcription initiation site or alternate splicing (Bhagwat et al., 1999; Huang et al., 1999; Ma and Taylor, 2002). In these examples, regulated inclusion of a signal peptide provides a mechanism for differentially targeting a single protein to either the mitochondrion or the ER. However, the situation is different for TgSODB2 because we show that the signal-like peptide is incapable of overriding the amphipathic presequence and both elements are necessary for mitochondrial targeting. Thus, the hydrophobic sequence is an integral part of the mitochondrial targeting sequence.

Since residues 1-25 are capable of directing GFP to the secretory system, SRP should bind the hydrophobic sequence co-translationally. If SRP does bind the hydrophobic sequence in context with the presequence, TgSODB2 would reach the mitochondrion via the ER, an unprecedented phenomenon. This highly unusual possibility was finally ruled out by experiments with the *T. gondii* ER retention signal. Thus, the presequence must in some way prevent SRP interaction, perhaps by pairing with and shielding the hydrophobic sequence.

It remains unclear how the atypical presequence of TgSODB2 interacts with mitochondrial import machinery since the hydrophobic sequence is probably too long to fit in the shallow Tom20 groove (Abe et al., 2000). One possibility is that the presequence could interact as an amphipathic helix with Tom20, while the hydrophobic sequence is largely excluded. In this scenario, the hydrophobic sequence would be available to interact with another TOM receptor such as Tom70 or Tom5 or with the outer membrane of the mitochondrion itself. Alternatively, since both parts of the presequence are necessary for proper targeting, the Tom20 homologue in *T. gondii* may have a unique binding pocket that is capable of accommodating presequences with both hydrophobic and amphipathic elements. Future identification and structural modeling of the *T. gondii* TOM receptors should help distinguish between these possibilities.

The critical importance of arg^{12} for TgSODB2 mitochondrial targeting may reflect an uncommonly high level of specificity for organellar targeting pathways in the Apicomplexa, since presequences in other organisms can be extensively mutated while still retaining their targeting capabilities. Net positive charge appears to be more important in targeting than individual residues (Abe et al., 2000; Pfanner, 2000; Ma and Taylor, 2002). Since *T. gondii* contains an additional organelle (the apicoplast) that uses a targeting element (transit peptide) similar to a mitochondrial presequence, but with a signal peptide in front, this organism may have more refined sequence requirements. This could complicate using bioinformatic strategies to predict where presequence-containing proteins are targeted in *T. gondii*, an issue that will become especially acute when the ongoing effort to sequence the *T. gondii* genome is completed. Indeed, predictions for TgSODB2 localization were complicated, not only by the disagreement of different prediction algorithms, but also by the anticipation that SODB would have plausible functions in each of the predicted destinations: the PV, the apicoplast, or the mitochondrion. In the PV, SOD activity would detoxify the potential flood of oxygen radicals coming from host mitochondria that cluster around the PV (Sinai et al., 1997). In chloroplasts, from which the apicoplast is derived, SODB scavenges radicals generated by photosynthesis. While the apicoplast has lost photosynthetic capability, it is possible it retained a SODB to protect its contents against radicals originating outside the organelle. Finally, the parasite mitochondrion has been shown to maintain a membrane potential and thus is likely to produce large amounts of oxygen radicals during respiration, again necessitating SOD activity.

While SOD activity in mitochondria is well documented, mitochondrial SODs are generally manganese-containing enzymes (SODC). This constitutes the second definitive report of a mitochondrial iron-containing SOD and the first such report in the Apicomplexa. Interestingly, the first mitochondrial, iron-containing SOD was found in the facultative intracellular parasite *Perkinsus marinus* (Schott and Vasta, 2003). *P. marinus* was previously classified as an apicomplexan, but has since been reclassified into a basal phylum, Perkinsozoa (Noren et al., 1999). Other SODB sequences containing presequence-like elements at their N-termini have been identified in *Leishmania chagasi* (Paramchuk et al., 1997), *Trypanosoma cruzi* (Ismail et al., 1997), and *Plasmodium vivax*, though localization has not been shown. These findings may indicate that a mitochondrial SODB was present in the ancestor of all these parasites and selectively lost, perhaps to be replaced by SODC.

It is possible that TgSODB2 was once an apicoplast-targeted enzyme, but switched localizations when a hydrophobic residue at position 12 mutated to arg. In this event, it is

expected that TgSODB2 would be most closely related to plant chloroplast SODB. However, phylogenetic analyses do not support this contention since TgSODB2 is most closely related to prokaryotic SODBs, outside of other apicomplexan SODBs. Thus, TgSODB2 probably originated from the prokaryotic endosymbiont that was the precursor to the mitochondrion and the gene was transferred to the nucleus along with other mitochondrial genes.

TgSODB2 may be of critical importance to the parasite because of its ability to provide front-line defense against radical-mediated damage resulting from respiration. Although other apicomplexans (e.g., *Plasmodium, Cryptosporidium)* are well known for causing disease, *T. gondii* can also be highly pathogenic, causing life-threatening encephalitis in the immune-compromised and is responsible for about 10% of deaths in these patients (Luft and Remington, 1988; Luft and Remington, 1992). Treatment options are limited and the standard therapy (pyrimethamine and sulfadiazine) is often poorly tolerated by patients (Haverkos, 1987). Novel drug targets are therefore of significant importance, especially as the global population of immune-compromised individuals continues to grow. Since SODB is not found in vertebrates and is the only form of SOD documented in *T. gondii,* as well as *Trypanosoma* (Ismail et al., 1997), *Leishmania* (Paramchuk et al., 1997), *Plasmodium* (Becuwe et al., 1996), and *Entamoeba* (Tannich et al., 1991), it presents a potential new therapeutic target for parasitic infections. Indeed, efforts are underway (Meshnick et al., 1985) to find specific SODB inhibitors and these may lead to future, less toxic treatments. In addition, studies on TgSODB2 orthologs in other apicomplexans should be conducted to determine definitively whether the N-terminal extension containing SODBs found in *Plasmodium, Leishmania* and *Trypanosoma* are truly mitochondrially localized. Such studies would elucidate whether potential therapeutic compounds will be broad-spectrum, making them useful for treating a variety of parasitic infections.

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