The obligate intracellular parasite *Toxoplasma gondii* secretes a soluble phosphatidylserine decarboxylase

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Running head: *Toxoplasma gondii* secretes a soluble PtdSer decarboxylase

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**Background:** Many genes and enzymes of lipid metabolism in *T. gondii* remain uncharacterized.

**Results:** The parasite secretes a soluble phosphatidylserine decarboxylase (*TgPSD1*), which acts on biological membranes.

**Conclusion:** *T. gondii* uses its secretory apparatus to modify lipids in the parasitophorous vacuole membrane and host cell membranes.

**Significance:** Secreted *TgPSD1* reduces externalized phosphatidylserine on host cells, enabling evasion of phagocytosis.

*Toxoplasma gondii* is an obligate intracellular parasite capable of causing fatal infections in immunocompromised individuals and neonates. Examination of the phosphatidylserine (PtdSer) metabolism of *T. gondii* reveals that the parasite secretes a soluble form of PtdSer decarboxylase (*TgPSD1*), which preferentially decarboxylates liposomal PtdSer with an apparent $K_m$ of 67 $\mu$M. The specific enzyme activity increases by 3-fold during the replication of *T. gondii*, and soluble PSD accounts for ~20% of the total PSD, prior to the parasite egress from host cells. Extracellular *T. gondii* secreted ~20% of its total PSD activity at 37°C, and the intracellular Ca$^{2+}$ chelator BAPTA-AM inhibited the process by 50%. Cycloheximide, Brefeldin A, ionic composition of the medium and exogenous PtdSer did not modulate the enzyme secretion, which suggests a constitutive discharge of a presynthesized pool of PSD in axenic *T. gondii*. *TgPSD1* consists of 968 residues with a 26-amino acid hydrophobic peptide at the N-terminus, and no predicted membrane-domain. Parasites over-expressing *TgPSD1-HA* secreted 10-fold more activity compared to the parental strain. Exposure of apoptotic Jurkat cells to transgenic parasites demonstrated interfacial catalysis by secreted *TgPSD1* that reduced host cell surface expression of PtdSer. Immuno-localization experiments revealed that *TgPSD1* resides in the dense granules of *T. gondii* and is also found in the parasitophorous vacuole of replicating parasites. Together, these findings demonstrate novel features of the parasite enzyme since a secreted, soluble and interfacially active form of PSD has not been previously described for any organism.

**INTRODUCTION**

*Toxoplasma gondii* is an obligate intracellular parasite of the phylum *Apicomplexa*, which also includes the human and animal parasites, *Plasmodium, Eimeria, Neospora* and *Babesia* (1,2). *Toxoplasma* causes an opportunistic disease, toxoplasmosis, in individuals with immune dysfunction, and in developing fetuses and neonates. The tachyzoite stage of *T. gondii* infects host cells and replicates to high numbers, ultimately lysing the cell, prior to the next round of invasion. Successful intracellular infection by *T. gondii* tachyzoites depends on its ability to modify the external micro-milieu by secreting a variety of factors (3,4). *Toxoplasma* harbors at least three distinct secretory organelles, which contribute to the parasite invasion, and formation and modification of an intracellular parasitophorous vacuole (PV). Micronemes and rhoptries, located at the apical end of *T. gondii*, discharge their contents at the time of invasion...
and formation of the nascent PV (3,4). The dense granules are released into the vacuolar space after invasion is complete. A coordinated secretion of these organelles is a prerequisite for successful invasion and replication of *T. gondii* within its host cell.

Phosphatidylserine decarboxylase catalyzes the synthesis of phosphatidylethanolamine (PtdEtn) from phosphatidylserine (PtdSer), a crucial process required for membrane biogenesis by numerous prokaryotic and eukaryotic organisms (5,6). The PSD enzymes belong to an interesting subgroup of decarboxylases that contain a pyruvoyl prosthetic group constituting an essential part of the catalytic site. The mature enzyme is a heterodimer composed of a large, membrane-associated β-subunit and a small, pyruvoyl-containing α-subunit (6,7). The mature form is generated by auto-proteolytic cleavage of a single polypeptide producing α- and β-subunits, and covalent attachment of the pyruvoyl group in a concerted reaction. The bacterial enzyme is an integral protein present in the cytoplasmic membrane of *E. coli* (7). Two distinct PSDs are expressed in *Saccharomyces cerevisiae*, one located in inner mitochondrial membrane (*ScPsd1p*) and the other in Golgi and vacuolar compartments (*ScPsd2p*) (6,8). In mammals, only one PSD associated with the mitochondrial membrane has been described (8). Only two parasite PSD enzymes have been studied in any detail, so far (9,10).

Our previous studies demonstrated the major routes of phospholipid synthesis in *T. gondii* (11). The parasite can acquire the precursors, serine, ethanolamine, and choline from its environment and use them for the synthesis of its major phospholipids, PtdSer, PtdEtn and PtdCho, respectively (11). These precursor utilization studies in conjunction with enzyme assays identified the presence of the Kennedy pathways for synthesis of PtdEtn and PtdCho (8,12,13), the base-exchange pathway for generation of PtdSer (8,14), and a PtdSer decarboxylase route for formation of additional PtdEtn (7,8,15) in *T. gondii* (11). Surprisingly, the PSD activity (214 nmol per hr per mg protein) was 10-fold higher than that observed in extracts prepared from other eukaryotes such as yeast (16) and mammalian cells (17). This report focuses on *TgPSD1*, one of two detectable PSD activities, expressed in *T. gondii* tachyzoites. *TgPSD1* is secreted to the vacuole of intracellular parasites, and into the external environment of host-free (axenic) parasites. *TgPSD1* secreted by extracellular parasites is a soluble protein, which acts on liposomal and host cell PtdSer.

**MATERIALS AND METHODS**

*Biological reagents*

Dulbecco's modified Eagle's medium (DMEM), MEM amino acids and vitamins were purchased from Invitrogen. The L-[U-14C]serine and L-[1-14C]serine were obtained from ICN Radiochemicals Inc. Brefeldin A, colchicines, cytochalasin D and cycloheximide were obtained from Sigma. The intracellular calcium chelator BAPTA-AM was purchased from Molecular Probes. Lipids were procured from Avanti Lipids. Silica gel H and Sil60 plates for thin layer chromatography (TLC) were from Analtech and Merck. Sephacryl 200 gel filtration matrix was from GE Healthcare. The *Escherichia coli* strain XL-1 Blue (Stratagene) was used for molecular cloning and vector amplification. Parasite RNA isolation, cDNA synthesis and plasmid preparations were performed with Invitrogen kits. All DNA-modifying enzymes and primers were obtained from NEB and Invitrogen. Primary (anti-HA) and secondary (Alexa488, Alexa594) antibodies were from Invitrogen. The *TaTi* strain of *T. gondii* was a kind donation of Dominique Soldati-Favre (University of Geneva, Switzerland). Transgenic *T. gondii* tachyzoites expressing red fluorescent protein and the pNTP3 expression vector were kindly provided by Isabelle Coppens (Johns Hopkins Bloomberg School of Public Health, Baltimore). Anti-*TgGra1*, *TgGra3* and *TgGra5* antibodies were gifts of Marie-France Cesbron-Delauw (CNRS, Grenoble, France). Jurkat cells were kindly provided by Carsten G. Lüder (Georg-August-University, Göttingen, Germany).

**Parasite culture and preparation of homogenates**

Human foreskin fibroblasts (HFF) obtained from the American Type Culture Collection were...
cultured in DMEM supplemented with 10% fetal calf serum (FCS), 2 mM glutamine, MEM non-essential amino acids, 100 units/ml penicillin, and 100 µg/ml streptomycin at 37°C, in a humidified incubator, with a 10% CO₂ atmosphere (11). The HFF were passaged by trypsinization at least once a week, and used up to the 10th passage. *T. gondii* tachyzoites of the RH strain were routinely propagated in vitro by serial passage in HFF monolayers at a multiplicity of infection (MOI) of 4, unless stated otherwise. To purify the axenic parasites, supernatants from freshly lysed monolayers were collected and centrifuged at 2000 x g x 10 min followed by three subsequent washings with cold phosphate-buffered saline (PBS). For some preparations, infected but unlysed HFF monolayers were passed twice through 20- and 22-gauge needles to release tachyzoites. The parasites were used immediately after isolation. Although *T. gondii* tachyzoites are unable to replicate outside of host cells, they remain viable long enough (t1/2 ~ 10 hrs) in an extracellular environment to permit experimentation. In the early stages of these studies we compared parasites purified on Nycodenz gradients (18) with those prepared by simple centrifugation and PBS washing. We observed no significant differences between the two methods and hence used the simple centrifugation and washing procedure for the experimental manipulations described in this report. Parasite homogenates were prepared from a suspension containing 1 – 2 x 10⁸ tachyzoites/ml, by probe sonication at 0°C using five 30-s bursts at 50 watts with 30-s cooling intervals between bursts. The homogenates were kept on ice prior to initiating enzyme reactions by pre-mixing all assay components at 0°C and then shifting to 37°C. Heat-inactivated (95°C for 10 min) enzyme extract and/or reactions lacking enzyme were included as negative controls for catalysis in each assay.

*Phosphatidylserine decarboxylase (PSD) assay*

PSD activity was measured by trapping ¹⁴CO₂ released from Ptd[U-¹⁴C]Ser, or Ptd[1-¹⁴C]Ser, on filter paper impregnated with 2 M KOH (7). Dioleoyl- Ptd[U-¹⁴C]Ser and Ptd[1-¹⁴C]Ser were synthesized from L-[U-¹⁴C]serine or L-[1-¹⁴C]serine and dioleoyl-CDP-diacylglycerol using PtdSer synthase. The PtdSer synthase was purified from *E. coli* strain JA-200 harboring the plasmid pPS3155 as described previously (19). The reactions were performed in 16x100-mm borosilicate glass tubes sealed with an air-tight rubber septum, to which was attached a well holding the 2 M KOH-saturated paper. The parasite extract was prepared in 50 mM potassium phosphate buffer (pH 6.8), 0.25 M sucrose, 0.5 mM phenylmethylsulfonyl fluoride (PMSF) and 3 mM EDTA. The 0.8-ml assay mixture contained 60 mM potassium phosphate (pH 6.8), 0.17 M sucrose, 0.35 mM PMSF, 2 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.5 mM dioleoyl Ptd[U-¹⁴C]Ser (0.1 µCi/µmol), 0.1% (w/v) Triton X-100 and 0.2 ml of parasite extract. In studies using liposomal forms of Ptd[U-¹⁴C]Ser, the detergent was omitted. The liposomes were freshly prepared using a Liposofast (Avestin) and 100 nm filters. The enzyme reactions were terminated at the indicated times by the addition of 0.5 ml of 0.25 M H₂SO₄, introduced through the rubber septum using a hypodermic needle. The emitted ¹⁴CO₂ was trapped for 30 min prior to recovering the filter paper for liquid scintillation counting.

To measure the PSD expression during intracellular parasite growth, the HFF were infected at a multiplicity of 3. The infected cells were washed with cold PBS and harvested by scraping at 8, 16, 24, 32, 40 and 48 hrs post-infection. The crude extract and reactions were prepared as described above. The 0.5-ml reactions contained 50 mM potassium phosphate (pH 6.8), 0.125 M sucrose, 0.25 mM PMSF, 1.5 mM EDTA, 0.5 mM 2-mercaptoethanol, 0.25-0.5 mM (0.4-0.8 µCi/µmol) of dioleoyl Ptd[U-¹⁴C]Ser and 0.2 ml of parasite homogenate, and were conducted for 1 hr at 37°C.

*PtdSer metabolism by *T. gondii* tachyzoites*

Aliquots of 1x10⁸ parasites were incubated with 1–100 µM (0.1 mCi/reaction) dioleoyl Ptd[U-¹⁴C]Ser in 1 ml of intracellular-type medium (ICM) in 16x100-mm borosilicate glass tubes at 37 °C for 1–6 hrs with shaking. The ICM contained 20 mM HEPES, 140 mM KCl, 10 mM NaCl, 2.5 mM MgCl₂, 5 mM glucose, 0.1 µM CaCl₂, 1 mM sodium pyruvate, MEM vitamin solution lacking choline, MEM amino acids, and...
serine-free nonessential amino acids (pH 7.4). The nonessential amino acid mix contained 200 µg/ml of Ala, Asp, Glu, Gly, Pro and Asn. The lipid precursors, serine, choline, ethanolamine and inositol (20 µM each) and ATP-Mg (1 mM) were also included.

**Phosphatidylserine decarboxylase secretion**

Typically, 1–1.3x10⁸ parasites were incubated at 37°C for 0.25–4 hrs in 1.3 ml of ICM supplemented with 20 mM each of serine, choline, ethanolamine and inositol, and 0.75 mM freshly-prepared ATP-Mg. ATP-depletion experiments were performed in glucose- and pyruvate-free ICM, lacking ATP and supplemented with 7.5 mM NaN₃, 7.5 mM NaF, 384 mM NaVO₄, 153 mM dinitrophenol and 7.5 mM Oligomycin A. The incubation conditions for ¹⁴CO₂ trapping and measurements were the same as those described above. A low speed supernatant (LSS) was prepared by three centrifugations (1500 g x 15 min). Subsequently, a high speed supernatant (HSS) was prepared by centrifuging the LSS at 150000 g x 45 min. An aliquot of 900 ml HSS was mixed with 100 ml of 1% Triton X-100-solubilized, or liposomal Ptd[U⁻¹⁴C]Ser. The enzyme reaction was incubated for 1–6 hrs at 37°C, and then terminated by the addition of 0.5 ml of 0.25 M H₂SO₄. The ¹⁴CO₂ was trapped for 30-60 min in the sealed reaction tubes prior to recovering the filter paper for liquid scintillation spectrometry.

**Extraction and analysis of lipids**

Lipids were extracted using the Bligh and Dyer method (20). Each 1-ml PSD reaction was terminated by addition of 1.1 ml CH₃OH and 1.1 ml CHCl₃, followed by vigorous mixing and centrifugation. The resultant chloroform phase was washed 3 times with 2.1 ml of CH₃OH/PBS/CHCl₃ (10:9:1.5, v/v). The final chloroform phase containing lipids was dried, and the radioactivity was quantified by liquid scintillation spectrometry. Alternatively, lipids were dried under N₂ and suspended in 50–100 µl of CHCl₃/CH₃OH/2-propanol/KCl (0.25%/triethylamine (90:28:75:18:54, v/v) or by 2-dimensional TLC on Sil60 plates (1st dimension in CHCl₃/CH₃OH/NH₄OH (65:35:5, v/v), and the 2nd in CHCl₃/CH₃COOH/CH₃OH/H₂O (75:25:5:2.2, v/v)). Lipids were visualized by spraying TLC plates with 0.2% (w/v) anilino-1-naphthalene sulfonic acid and exposure to UV light, or by iodine staining, or by autoradiography. All lipids were identified based on their co-migration with authentic standards.

**Cloning of the PtdSer decarboxylase cDNA and expression in T. gondii**

HFFs were infected with T. gondii tachyzoites at an MOI of 3 and cultured for 48 hrs. Parasites in the early stage of lysis were harvested from the medium and liberated from host cells by 2 passages through 22- and 27-gauge needles. The parasites were washed by three centrifugations in diethylpyrocarbamate-treated PBS. RNA was isolated using Trizol method (Invitrogen) and transcribed into first-strand cDNA. The TgPSD1 cDNA was amplified using the forward (CTCGATATCATGGCTAAGGTTATGAGGTTATC) and reverse (CTCTTAATTAATCAAGCGTAATCTGGAA CATCGATGGGTAGATCCCATCGTATGGGTAGAGAC) primers and Pfu-Ultra FusionII Polymerase (Stratagene). The cDNA was cloned into the pNTP3 vector using EcoRV and PacI restriction enzyme sites. Purified tachyzoites (1x10⁷) of the TaTi strain (21) were transfected with 50 µg of the TgPSD1 construct using the BTX630 instrument (2 kV, 50 Ohm, 25 µF, 250 µs). The drug-resistant parasites were selected with 1 µM pyrimethamine as described before (22), and clonal transgenic tachyzoites were analyzed for PSD expression.

**Indirect Immuno-fluorescence analysis (IFA)**

The IFA of intracellular and extracellular T. gondii tachyzoites was performed as described previously (23). In brief, the parasitized HFF monolayers grown on glass coverslips for 24-36 hrs were washed with PBS and fixed with 4% paraformaldehyde for 10 min, followed by neutralization in 0.1 M Glycine/PBS (5 min). Cells were permeabilized in 0.2% Triton-X-100/PBS for 20 min, and non-specific binding was blocked with 2% BSA in 0.2% Triton-X-100/PBS. Samples were stained with primary antibodies (rabbit anti-HA 1:1500; mouse anti-HA 1:1000; mouse anti-TgGra1 1:500; rabbit
anti-TgGra3 1:500; mouse anti-TgGra5 1:500), followed by three washes with 0.2% Triton X-100 in PBS. The corresponding secondary antibodies (mouse or rabbit Alexa488, or Alexa594) were applied (1:3000) for 45 min, and after three PBS washes, the slides were mounted in DAPI-Fluoromount G for fluorescent imaging (Apotome, Carl Zeiss, Germany). For immunostaining of the extracellular stage, the parasites were allowed to settle on poly-L-lysine-coated coverslips before fixation by paraformaldehyde (4%) for 10 min at room temperature. Samples were stained and imaged as described above.

**Annexin V Binding to apoptotic Jurkat T-cells**

Jurkat cells (E6.1 T-lymphocyte derived cells) were grown in RPMI medium supplemented with 10% FCS and penicillin (100U/ml)/streptomycin (100µg/ml) at 37°C and 5% CO2 in a humidified incubator. For induction of apoptosis 1x10⁶ cells were resuspended in 1 ml of fresh RPMI medium containing 0.1% DMSO either with, or without 1 µM staurosporine (Invitrogen), for 2 hrs in a 12-well plate. Cells were harvested by centrifugation (400 x g for 10 min at room temperature) and washed twice with fresh medium. Subsequently, the cells were incubated with wild type or a transgenic *T. gondii* strain over-expressing TgPSD1-HA (MOI ~30) or with the medium control for 2 hrs. The cells were resuspended in 100 µl of annexin V-FLUOS labeling reagent (Roche), stained for 15 min in the dark, and then fixed with 2% paraformaldehyde for 15 min. Cells were subsequently washed and resuspended in 200 µl HEPES buffer for FACS analyses or seeded on poly-L-lysine-coated coverslips for microscopy. Samples were examined for annexin V staining on LSRFortessa cell analyzer (BD Biosciences). The parasites and Jurkat cells were fractionated prior to analyzing the results using FlowJo suite.

**RESULTS**

**Axenic *T. gondii* secretes a soluble PSD**

As an intracellular parasite *T. gondii* imports numerous classes of molecules to support its growth. To investigate whether *T. gondii* can transport and metabolize exogenous phospholipid, we incubated the axenic parasites with unilamellar liposomes of dioleoyl PtdSer, and measured its decarboxylation. Surprisingly, parasites exhibited a high rate of concentration and time-dependent decarboxylation (Figure 1A, B). The presence of high PSD activity in the living parasite is consistent with previous studies conducted with the parasite homogenates (11). Initially, these assays suggested import of PtdSer by the parasite and decarboxylation in *T. gondii*. However, further analyses did not support this interpretation of the data.

We next sought to determine where liposomal PtdSer was being decarboxylated. For these experiments, a defined number of parasites were incubated in ICM followed by preparation of cell free supernatants. Surprisingly, we observed a time-dependent increase in PSD activity in the high speed supernatant (HSS) indicating the presence of an enzyme that appeared to be secreted by the parasite (Figure 2A). The secretion was rapid in the first hour and gradually reached a plateau over the next 3 hrs. The unexpected presence of PSD in the HSS after parasite incubation demonstrated the soluble nature of the enzyme. Our assays using extracellular-type (high Na+/K+) media yielded no differences in secretion of PSD compared to ICM (not shown). To exclude the prospect of parasite lysis during culture, we performed similar experiments with transgenic *T. gondii* expressing red fluorescent protein and measured its release into the medium. We did not find any measurable release of red fluorescent protein that might be attributed to cellular disintegration. Moreover, the number of intact parasites prior to and after incubation was the same. The observed behavior of the enzyme is consistent with this secreted PSD being a soluble protein.

To further establish the nature of the parasite-secreted enzyme as PSD, we examined the product of Ptd[U-14C]Ser metabolism by tachyzoite-derived HSS. As shown in Figure 2B, the major product of PSD was Ptd[U-14C]Etn as deduced by thin layer chromatography and radioactive imaging of the phospholipid products. As expected, we also observed an intense band of PtdSer, which represents the substrate excess. Our simultaneous measurement of PtdSer decarboxylation rates, deduced by the release of 14CO2, and of Ptd[U-14C]Etn formation
detected by TLC, matched the theoretical yields of the two products in a ratio of ~1:2, suggesting that no other significant product was formed during the HSS-mediated catalysis (Figure 2C). These data confirm that the catalytic function of this novel PSD is not different from other reported PSD proteins.

**Secreted and soluble PSD exhibits a high catalytic activity with liposomal PtdSer**

Based on our conjecture that a soluble PSD may act at membrane interfaces, we compared the catalytic behavior of PSD using detergent-solubilized and liposomal PtdSer (Figure 3A). The secreted PSD of *T. gondii* consistently exhibited nearly 2-fold higher activity with liposomal PtdSer compared to a detergent-dispersed substrate. However, the total PSD activity in the parasite homogenates was 1.5-fold higher with the detergent-dispersed substrate compared to the liposomal PtdSer. The activity of the secreted, soluble PSD with liposomal PtdSer was linear for 2 hrs (Figure 3B). The secreted PSD decarboxylated PtdSer in a concentration-dependent manner, and about 5% of the substrate was consumed at 200 µM PtdSer. Kinetic analysis indicated an apparent *Kₘ* value of 67 µM with liposomal PtdSer. Taken together, these results indicate a relatively stable, secreted *T. gondii* PSD, with an increased affinity for liposomal PtdSer. In addition, these data suggest the presence of a second membrane-bound PSD in *T. gondii*, which is consistent with the parasite database (ToxoDB) predicting two PSD genes.

**PSD secretion is a temperature-, ATP- and calcium-dependent process**

To determine some of the general characteristics of PSD secretion, we examined the temperature-, ATP- and calcium- dependence of the process, as shown in Figure 4. Under routine conditions at 37°C, the extracellular parasites secreted about ~20% of their total PSD activity (Figure 4A). The enzyme secretion was inhibited by almost 90% when the incubation temperature was reduced to 0°C. As anticipated, the secretory process was ATP-dependent and we observed ~92% reduction in *T. gondii*-secreted soluble PSD activity, when the parasites were pre-incubated with metabolic inhibitors depleting the intracellular ATP pool (Figure 4B). When the parasites were exposed to the intracellular Ca²⁺ chelator BAPTA-AM (Figure 4C), the secretion of PSD was inhibited by approximately 50%. We were unable to reverse the effect of BAPTA-AM by simultaneous addition of calcium (1 mM). The addition of the calcium ionophore, ionomycin, did not affect the enzyme secretion by free parasites (not shown). Together, the data show a partial dependence of the PSD secretion on intracellular calcium stores in free parasites. The use of ATP inhibitors and BAPTA-AM did not have any direct effect on the PSD activity (not shown). The dependence of PSD secretion on temperature, ATP and intracellular calcium is consistent with enzyme release from intracellular stores.

**Toxoplasma contains a soluble pool of secretable PSD throughout its replication**

We examined the expression of PSD during the intracellular growth of the parasite in human fibroblasts and discovered an increase in specific activity of PSD as the parasite number increases. As shown in Figure 5A, the total PSD activity in cultures of infected fibroblasts increase in a linear manner from 8 hrs after infection until 32 hrs, prior to the host-cell lysis and parasite egress. Between infection and parasite-induced lysis of the host cells, we observed a 4-fold increase in the specific activity of PSD. In contrast, the PSD activity in uninfected host cells was very low and remained constant between 8–32 hrs. We also measured the specific activity of the soluble and membrane-associated PSD pools in HSS and high-speed pellets in parasites mechanically liberated from host cells at 16 and 30 hrs after infection. The results in Figure 5B demonstrate the PSD activity was distributed in the pellet as well as in the soluble fraction and the latter comprised ~20% of the total enzyme activity at 16 and 30 hrs of infection. Just prior to egression (~30 hrs), the soluble PSD specific activity was 3-fold higher than that of 16 hrs post-infection. Our data strongly suggest the presence of two different variants of PSD in the parasite, one of which is membrane-associated, and the other soluble. In addition, the data demonstrate that the soluble pool of PSD occurs at a fixed percentage of the total enzyme pool regardless of whether the parasite is within the parasite.
host cell or outside the host cell. This latter result also suggests that the soluble pool of PSD is likely secreted from the parasite during and/or after egress from the host.

To determine if there is a soluble PSD pool stored in host cell free axenic *T. gondii* tachyzoites, we conducted secretion assays in the presence of cycloheximide, an inhibitor of protein synthesis in *T. gondii* (24). The cycloheximide treatment failed to inhibit PSD secretion. We also examined the effect of brefeldin A, an inhibitor of Golgi-mediated secretory function, upon release of PSD. This drug treatment failed to block PSD secretion, consistent with the idea that the parasite stores significant quantities of the enzyme in prepackaged secretory organelles. Treatment of axenic parasites with colchicine or cytochalasin D also did not affect PSD secretion, indicating that the process is likely not directed by microtubule or microfilament function.

**TgPSD1 encodes a 968-amino acid protein containing an N-terminal hydrophobic peptide**

Inspection of the *T. gondii* database revealed two putative PSD genes. Our experimental annotation of *TgPSD1* identified a protein containing 968 amino acids with a likely signal peptide at the N-terminus, as shown in Figure 6A. The SignalP suite predicted a cleavage site between positions V26 and Q27 (score: 0.742) of *TgPSD1*, consistent with the observed secretory behavior. A PtdSer decarboxylase homology domain encompassing residues 419 to 720 is conserved in the *TgPSD1*. Analysis of the secondary structure of *TgPSD1*, devoid of the signal peptide did not reveal any hydrophobic transmembrane peptides. The catalytic site of *TgPSD1* is predicted within a 681FGST684 motif, which conforms to a consensus subunit cleavage site reported for other PSD enzymes (5,6). Autocatalytic processing of the *TgPSD1* pro-enzyme (~108-kDa) should yield a pyruvoyl-containing α-subunit (~32-kDa) derived from the C-terminus and a β-subunit (~76-kDa) derived from the N-terminus.

Bioinformatic analyses of *TgPSD1* revealed two putative N-glycosylation sites at positions N94 and N820. Numerous sites for O-glycosylation, C-mannosylation and phosphorylation are also predicted, but their significance remains to be determined. Phylogenetic analyses showed that *TgPSD1* might be distantly related to type-I PSD enzymes from mammals, fungi and plants, whereas type-II and bacterial PSD proteins form their own clade as depicted schematically in Figure 6B. Our attempts to express an active form of *TgPSD1* in *E. coli* and *S. cerevisiae* were unsuccessful. However, *TgPSD1* could be overexpressed in its active form in *T. gondii* tachyzoites as described below.

**TgPSD1 is localized within the parasitophorous vacuole of *T. gondii***

To examine the subcellular location of the *TgPSD1* protein in parasites, we expressed this protein containing a C-terminal HA epitope (*TgPSD1-HA*) in *T. gondii* tachyzoites under the control of the p*TgNTP3* promoter (Figure 7). Immuno-staining of the transgenic parasites present within the host cell revealed that *TgPSD1* was secreted into the parasitophorous vacuole, which separates the entire population of replicating parasites from the host cytosol. Figure 7 demonstrates that the secreted *TgPSD1* co-localized with *TgGra1*, *TgGra3* and *TgGra5* (Figure 7A), which are *bona fide* proteins of the parasite dense granules that are secreted into the vacuole (25). *TgGra1* remains soluble within the vacuolar space, whereas *TgGra3* and *TgGra5* are associated with the vacuolar membranes. When the focal plane was selected to demonstrate the distribution of *TgGra1*, there is significant co-localization of this protein with *TgPSD1*. When the focal plane was chosen to accentuate the localization of *TgGra3* and *TgGra5*, *TgPSD1* was also detectable within the dense granules, and at the vacuolar membrane. Fluorescence images of parasites after egress from host cells (Figure 7B) revealed co-localization of *TgPSD1* with *TgGra1* and *TgGra3*. These results confirm that *TgPSD1* resides in the dense granules of *T. gondii*, prior to its secretion into the vacuole by replicating parasites. Consistent with our secretion studies, these findings also show that dense granule pools of *TgPSD1* are present in axenic parasites.

**TgPSD1-HA is secreted as a soluble protein by axenic *T. gondii***
We next examined the function of TgPSD1 by determining the catalytic activity and secretory nature of the recombinant protein. The TgPSD1 ORF with a C-terminal HA epitope (TgPSD1-HA) was over-expressed in transgenic parasites. Immunoblot analysis confirmed the presence of TgPSD1-HA in parasite homogenates, HSS fractions, and in high speed pellet (HSP) fractions containing membranes and dense granules. As shown in Figure 8A, TgPSD1-HA was detectable almost exclusively as the processed, mature form of the ~32-kDa α-subunit, indicating that nearly all of the proenzyme was efficiently processed to the mature enzyme.

We quantified the over-expression of TgPSD1 by measuring the enzyme activity in subcellular fractions isolated from parasitized HFFs (Figure 8B). The transgenic parasites over-expressing TgPSD1-HA had ~10-fold more PSD activity in homogenates compared to the parental strain; and ~20% of the enzymatic activity was detectable in a soluble form present in HSS. Secretions studies were also performed using the axenic transgenic parasites. As shown in Figure 8C, the transgenic strain of T. gondii secreted approximately 10 times more soluble TgPSD1 than the parental strain, and neither strain released a significant amount of TgPSD1-HA at low temperature. Collectively, the data confirm the activity of TgPSD1-HA and the soluble and secreted properties of the enzyme.

**Secreted TgPSD1 can perform interfacial catalysis at host cell plasma membranes**

In an effort to understand the biological role of TgPSD1, we examined the ability of the secreted enzyme to act upon PtdSer exposed on cell surfaces. In these experiments we compared the amounts of externalized PtdSer present on mammalian cells treated with staurosporine, in either the absence, or presence of axenic parasites secreting TgPSD1. Staurosporine elicits an apoptotic host cell response that induces PtdSer externalization at the plasma membrane (26). The exposed PtdSer was detected by measuring fluorescent annexin binding (27). Cultures of DMSO-treated control Jurkat cells contained a minor population of cells that stained with fluorescent annexin V, detected by microscopy and cell sorting (Figure 9A and 9B). Treatment of Jurkat cells with staurosporine elicited fluorescent annexin staining in nearly the whole population (Figure 9A). When the drug-treated cells were exposed to wild type parasites for a period of 2 hrs, the annexin binding of the cells remained unchanged (parental vs. medium in Figure 9B). In contrast, a co-incubation with transgenic T. gondii over-expressing TgPSD1 caused a significant 34% reduction in surface intensity of staurosporine-treated cells. These experiments reveal that under the appropriate conditions, the TgPSD1 can reduce the amount of surface exposed PtdSer present on host cells. From a biochemical perspective, the data show that the interfacial catalysis performed by PSD1 with a liposomal substrate is recapitulated with PtdSer present in the exofacial leaflet of plasma membranes.

**DISCUSSION**

This study provides evidence for the presence of a novel phosphatidylserine decarboxylase expressed by T. gondii that is soluble and secreted from the parasite into the parasitophorous vacuole (PV) during the intracellular phase of parasite growth. During the axenic phase of the parasite life cycle the TgPSD1 is secreted into the external medium. Although some soluble forms of PSD have been reported in bacteria (28), and as a subpopulation of a Plasmodium transgene encoded protein expressed in yeast (10), no secreted forms of the enzyme have been reported previously from any organism. Our results also suggest a second membrane-associated PSD is present in the parasite.

Unlike homologs from other organisms residing in either the inner mitochondrial membrane, or in Golgi/vacuolar/ endosomal membranes (5,6); TgPSD1 harbors a putative secretory signal peptide with a predicted proteolytic cleavage site at its N-terminus, but no predicted transmembrane domains. The secretion of PSD by free T. gondii tachyzoites is time-, temperature- and ATP-dependent; and appears to be independent of the exogenous PtdSer concentration, Na+ or K+ gradients across the plasma membrane, active protein synthesis, trafficking from endoplasmic reticulum to Golgi.
network, and cytoskeletal integrity. These features are common to proteins secreted from parasite dense granules (25,29). Immunolocalization of TgPSD1 in the dense granules and in the PV of parasites further supports the secretory nature of TgPSD1 in axenic parasites. Because dense granule release is not directional, as in other T. gondii secretory organelles, such as micronemes and rhoptries, there is no specific requirement for a cytoskeleton-dependent transport of vesicles to the apical end of T. gondii for PSD secretion.

The dense granules in T. gondii constitute an unusual secretory pathway that allows soluble export of many membrane-associated proteins (e.g. TgGra proteins) to the PV via information encoded within the N-terminal domain (25,29). These proteins insert into the PV membranes after initially being secreted as soluble proteins into the vacuole. We show that intracellular parasites release TgPSD1 into the PV, where the enzyme may bind to membranes to support the biogenesis of an expanding vacuole, and ensure a faithful parasite replication. Moreover, PtdEtn can promote protein conformational changes, and influence lateral movement and activity of the membrane-bound proteins (30,31). The association of TgPSD1 with the PVM, also has the potential to regulate sequestration of host-derived endocytic vesicles (32), or provide an optimal bilayer environment for vacuolar proteins. Finally, TgPSD1-mediated PtdEtn production can also influence membrane curvatures that may dictate the fusion and fission events in the PV. To this end, the catalytic action of TgPSD1 could facilitate escape of the parasite by altering the integrity of the vacuolar and host membranes.

Unlike many membrane-bound enzymes of lipid metabolism, TgPSD1 does not require detergents to interact with its substrate, and shows nearly twice the activity with liposomal PtdSer when compared to detergent-dispersed substrate. These findings are consistent properties for an enzyme that works at a membrane interface. Our studies with transgenic parasites and apoptotic cells show that the overexpressed and secreted TgPSD1 can reduce the levels of PtdSer exposed on apoptotic cells. Although this same result was not obtained with parasites expressing wild type levels of TgPSD1, this finding may be due to the differences between the catalysis by TgPSD1 in a relatively large cell free volume in tissue culture, as compared to the concentrated foci of infected cells in solid tissues. Typically, 64-128 parasites are released from a parasitized host cell and the egressed population of parasites acts very locally in time and space to infect the adjacent cells. In such a microenvironment, the wild type levels of TgPSD1 are expected to be sufficient for reducing the amount of externalized PtdSer on host cells.

Externalized plasma membrane PtdSer is a potent signal that stimulates phagocytic cells to recognize, engulf and destroy host cells with this surface property (33). In vivo, the catalytic action of TgPSD1 may serve to suppress this PtdSer signal on host cells, generated either in response to parasite invasion, or following neighboring host cell lysis. A reduction of surface PtdSer should function to reduce, or even prevent the cells from detection and phagocytosis by macrophages, and thereby promote persistence of the parasite. In brief, the extracellular TgPSD1 may enhance the ability of T. gondii to evade detection by the innate immune system.

In summary, our studies identify an unusual PSD family member expressed by T. gondii that is soluble and secreted, and capable of catalysis at membrane interfaces. TgPSD1 can reduce the exposed PtdSer content on apoptotic cells. These findings identify unexpected new aspects of the phospholipid metabolism of T. gondii.

REFERENCE


**FOOTNOTES**

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**ABBREVIATIONS**

CFE, cell-free extract; HFF, human foreskin fibroblasts; HSS, high speed supernatant; ICM, Intracellular-type medium; IFA, indirect immuno-fluorescence assay; LSS, low speed supernatant; ORF, open reading frame; PtdCho, phosphatidylcholine; PtdEtn, phosphatidylethanolamine; PtdSer, phosphatidylserine; PSD,
phosphatidylserine decarboxylase; PV, parasitophorous vacuole; RFP, red fluorescent protein; TLC, thin layer chromatography.

FIGURE LEGENDS

Fig 1: Axenic T. gondii decarboxylates exogenous PtdSer in a concentration- and time-dependent manner. (A) Purified T. gondii tachyzoites of the RH strain (0.7–1x10^8) were incubated at 37°C with probe-sonicated dioleoyl Ptd[U-14C]Ser (100 nCi/reaction), at the indicated concentrations for 4 hrs. (B) The same conditions described for panel A were used to follow the decarboxylation of 45 µM dioleoyl PtdSer (2.2 nCi/nmol) for the indicated times. The incubation of parasites was performed in 1 ml of intracellular-type medium containing 20 µM each of serine, choline, ethanolamine and inositol, and 1 mM ATP-Mg. The ^14CO_2 was trapped on filter paper and quantified by liquid scintillation spectrometry. Values are means ± S.E. for three experiments.

Fig 2: Extracellular T. gondii secretes a soluble PtdSer decarboxylase. (A) Purified T. gondii tachyzoites of the RH strain (1–1.1x10^8) were incubated for the indicated times in intracellular-type medium containing serine, choline, ethanolamine and inositol (20 µM each), and ATP-Mg (0.75 mM) followed by high speed supernatant (HSS) preparation. The HSS was assayed for the PSD activity with 50 µM (2 nCi/nmol) liposomal dioleoyl Ptd[U-14C]Ser. (B) Purified tachyzoites (1.3x10^8) were incubated (37°C, 2 hrs), and the HSS was assayed with 100 µM (1 nCi/nmol) of liposomal dioleoyl Ptd[U-14C]Ser. Lipids were extracted and subjected to two-dimensional thin layer chromatography on a silica gel 60 plate and visualized by autoradiography at –80°C for 40 hrs. (C) Phospholipids resolved in panel B were scraped and quantified by liquid scintillation spectrometry. To monitor the relative proportion of the catalytic products formed, we performed simultaneous quantifications of the TLC-resolved lipids and of ^14CO_2 production from the same reaction. Values are means ± S.E. for three experiments.

Fig 3: The secreted and soluble PtdSer decarboxylase displays higher catalytic activity with liposomal PtdSer. (A) Purified T. gondii tachyzoites of the RH strain (1–1.3x10^8) were incubated at 37°C for 2 hrs, and cell free extracts (CFE) and HSS fractions were prepared for measuring PSD activity. The parasite CFE was prepared by probe sonication. The PSD activity in HSS, or CFE was measured at 37°C for 4 hrs with 0.5 mM (0.2 nCi/nmol) liposomal dioleoyl Ptd[U-14C]Ser. (B) The time dependence of the PSD reaction was examined using HSS as the enzyme source and liposomal dioleoyl Ptd[U-14C]Ser (0.2 mM PtdSer, 1 nCi/nmol), as the substrate. (C) The substrate concentration dependence of the PSD reaction (solid line) was examined using the indicated concentrations of liposomal dioleoyl Ptd[U-14C]Ser (100 nCi/reaction) and an incubation time of 4 hrs. The K_m value was determined by fitting the data (dotted line) to the Michaelis-Menten equation. Values are means ± S.E. for three experiments.

Fig 4: The secretion of PtdSer decarboxylase is a temperature, ATP and calcium-dependent process. Purified T. gondii tachyzoites of the RH strain (1–1.3x10^8) were incubated for 2 hrs at 37°C (A, B, C) or at 0°C (A) in intracellular-type medium with 20 µM each of serine, choline, ethanolamine and inositol, and 0.75 mM ATP-Mg. (B) ATP synthesis inhibitors (7.5 mM NaN_3, 7.5 mM NaF, 384 µM NaVO_4, 153 µM DNP and 7.5 µM oligomycin A in glucose-, pyruvate- and ATP-free ICM) were added as indicated. (C) Calcium chelator BAPTA-AM (38 µM in Ca^{++}-free ICM) was added where indicated. The high-speed supernatant (HSS) was assayed with 50–100 µM (1–2 nCi/nmol) liposomal dioleoyl Ptd[U-14C]Ser at 37°C for 4 hrs. Equivalent amount of T. gondii cell-free extract (CFE) was used as a control and represents the total cellular PSD activity before secretion. Values are means ± S.E. for three experiments.

Fig 5: PSD activity increases during the intracellular growth of T. gondii, and freshly released parasites contain 20% of their PSD in soluble form. (A) Human foreskin fibroblast (HFF) cultures infected with T. gondii tachyzoites (MOI = 4) were harvested at different stages of intracellular parasite
growth. The cell-free extract was prepared and assayed for PSD activity with 0.25 mM (0.8 nCi/nmol) of liposomal dioleoyl-Ptd[U-14C]Ser for 1 hr at 37°C. Uninfected healthy HFF were used as a control. (B) T. gondii tachyzoites isolated from infected HFF, were fractionated into a soluble fraction and a particulate fraction by centrifugation, and assayed for PSD with 0.5 mM (0.4 nCi/nmol) liposomal dioleoyl Ptd[U-14C]Ser for 1 hr at 37°C.

Fig 6: The TgPSD1 sequence and its phylogenetic comparison with other PSD enzymes. (A) The amino acid sequence of TgPSD1. The SignalP-predicted signal peptide appears on a dark gray background. The light gray shows a core PSD domain as predicted by the NCBI-BLAST program, where the post-translational proteolytic cleavage site (FGST) is boxed. The stop codon of TgPSD1 is indicated as an asterisk. (B) Phylogenetic presentation of TgPSD1 in comparison with its orthologs. Protein sequence comparison, alignment curation, construction and visualization of phylogenetic tree were performed using MUSCLE, Gblocks, PhyML and TreeDyn algorithms, respectively. The numbers on the tree represent bootstrap support values for individual branches. The NCBI accession for PSD sequences are as follows: AtPSD1, Arabidopsis thaliana (NM_117771); AtPSD2, Arabidopsis thaliana (NM_125101); AtPSD3, Arabidopsis thaliana (NM_118730); CaPSD1, Candida albicans (XM_713812); CaPSD2, Candida albicans (XM_716770); CgPSD, Cricetulus griseus (P27465); EcPSD, Escherichia coli (NP_418584); HsPSD, Homo sapiens (BC001482); LePSD, Lycopersicon esculentum (AY093689); MmPSD, Mus musculus (BC094594); PaPSD, Pseudomonas aeruginosa (NP_253644); PjPSD, Plasmodium falciparum (AF312489); PpPSD1, Pichia pastoris (CAG36934); PpPSD2, Pichia pastoris (CAG39010); ScPSD1, Saccharomyces cerevisiae (NP_014230); ScPSD2, Saccharomyces cerevisiae (NP_011686); and TgPSD1, Toxoplasma gondii (JN003619). EK, Eukaryotic; PK, Prokaryotic.

Fig 7: TgPSD1 is stored in the dense granules, and secreted into the parasitophorous vacuole of T. gondii tachyzoites. Purified tachyzoites of the TaTi strain (1x10⁶) were transfected with TgPSD1-HA expression construct under the control of the pNTP3 promoter. Stable pyrimethamine-resistant transgenic lines were generated prior to immuno-fluorescence detection using the indicated antibodies. In panel A, a schematic version of the TgPSD1 construct is shown with the predicted signal peptide (SP), core enzyme (TgPSD1) and epitope tag (HA) domains highlighted. The immunofluorescence-staining images of intracellular parasites using TgGra1, TgGra3 and TgGra5 antibodies and anti-HA antibodies, as well as the DIC images are shown beneath the schematic. The white arrows in the third row of panel A indicate the parasitophorous vacuole membrane (PVM) and PV space. In panel B, additional immunofluorescence and DIC images are shown for axenic parasites. The white arrows in the first row of panel B show the apex and posterior of an extracellular crescent-shaped tachyzoite.

Fig 8: T. gondii tachyzoites harboring transgenic TgPSD1-HA over-express the recombinant protein and secrete it into the extracellular milieu. A stable transgenic parasite line expressing TgPSD1-HA, under the control of the pNTP3 promoter was generated using tachyzoites of the TaTi strain. In Panel (A) cell free extracts (CFE) were prepared from uninfected HFF, or HFF infected with parental or transgenic parasites, as indicated. Parasite homogenates were fractionated by centrifugation to produce HSS and HSP. Immunoblot analysis of the CFE, HSS and HSP fractions was performed using anti-HA (1:500) and anti-TgActin (1:1000) antibodies. Primary antibodies were detected using HRP-conjugated polyclonal secondary antibodies. In panel (B), uninfected HFF, or purified parasites were used as sources of PSD enzyme activity measured in homogenates (CFE), or subcellular fractions (HSS and HSP). The PSD activity was quantified using [14C]-dioleoyl-PtdSer (2 nCi/nmol, 0.2 mM solubilized in 0.1% Triton X-100). In panel (C), fresh extracellular parasites were incubated for 2 hrs in ICM and secreted fractions were used to prepare the HSS. The PSD activity was assayed for 2 hrs at 37°C in ICM using [14C]-dioleoyl-PtdSer (2 nCi/nmol, 0.2 mM solubilized in 0.1% Triton X-100).

Fig 9: Parasite-secreted TgPSD1 decarboxylates PtdSer exposed on the surface of Jurkat cells undergoing apoptosis. (A) Microscopic imaging of Jurkat cells following induction of apoptosis by 1 µM
staurosporine for 2 hrs at 37°C. DMSO (carrier) treatment was included as a control. PtdSer exposed on the cell surface was visualized by staining with annexin V-FLUOS labeling reagent. (B) Quantification of the annexin binding to the cell surface by FACS analysis. Jurkat cells were treated with staurosporine or DMSO and co-incubated with axenic *T. gondii* tachyzoites (parental and over-expressing *TgPSD1-HA*), as indicated, followed by staining with annexin V-FLUOS. The fluorescent intensity of fractionated Jurkat cells was determined using FlowJo software. Statistical significance was determined by one-way ANOVA test. Cells were cultured and processed as described in the *materials and methods*. Values are means ± S.E. for three experiments. *** indicates a p< 0.05.
Figure 1

A

![Graph A: CO₂ production versus PtdSer concentration.](image)

B

![Graph B: CO₂ production versus time.](image)
**Figure 4**

A. **Temperature-Dependence**

- CFE
- HSS @ 0°C
- HSS @ 37°C

B. **ATP-Dependence**

- HSS (Control)
- HSS (ATP-Inhibited)

C. **Calcium-Dependence**

- HSS (Control)
- HSS (BAPTA-AM)
Figure 5

A. Graph showing the PSD (nmol/hr/mg protein) over hours post-infection for parasitized HFF and healthy HFF.

B. Bar graph comparing PSD (nmol/hr/mg protein) in the soluble and particulate fractions 16 and 30 hours post-infection.
Figure 6

MAKVMRLLIFVCVALVAISVPAASSVQSSQERIRPGFRQQLPSSIRPFSAFRRRG
QEASDSSVYLYLVRGLTRQCATGELTVVVHGGKALNNATLGVDVQTSLTTRFAS
SLKEPSSTLRWKSDDLFMLMLTRARVTGTGGRSTPQYVAKQRLFFVV
RKPTPTSSCRARALDPFLYGVPKGVQDKYTLHSMVLGMLREPLEHQQEEEP
GPASVSTYAAAQAILGDASSFLGAFGSAKNLQISMYASGALEKQVTPTTS
QLALFLPSKTFFGVEVTSVDGTFRIPVSAVLVPFSAQMTGSSQTHKLTQTG
MNVEKRHKGKEPSEGAEVLNLALLARKPVGVEFQDPTVVLQLWTREGSE
SWQETPLMWEFPDTLEAIEDAGEYRSGLSIIANTRIIGKMGWLASRSFSRRF
IRTLIRLNIDDETEAFMSMDSKRRHSAADFQRSVEFFTRPINHYVRMDPRASI
MAPASLQINYITIPDFGEISHPIIPIQVKSTSFNLREFLYQBARQVPPLQLQSP
SNRLFVSLYLAHSVHRCPADAVTSQTYIPGCPVSVRNLEADGDLHRYE
RTALIGHWDPEKNGQOLFFSTMDVAAMFVGLRSLWEEELPGAAMRLGRCTYTE
SYEKQVDVLCASQIGARFWGSTDVMIFEAPEFDMDTSVQGCSHVAAGQAPAGYL
GQQRERPLQRCAFRCNFESPFHWKKLKTLKQNTLAPAQRNWEPGR
VWAELVLRATERGLLYGFLASHHLYKRATGENGNLIELVLQGPEVLQRNQINSDSV
IREGRFCFAAKDKQIRLQMSGRQSQVSTATVPDEQFLQHPFGYCVGDEKLG
KLVRGIDATWILLPERAVLLLTKVSTGSKQEDRVRVATKIEVQTEPCQGGWE
ESRVTGTSTTCRITYKREESISEGVTLNGDL*
The obligate intracellular parasite *Toxoplasma gondii* secretes a soluble phosphatidylserine decarboxylase

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