Identification of Gene Encoding *Plasmodium knowlesi* Phosphatidylserine Decarboxylase by Genetic Complementation in Yeast and Characterization of *in Vitro* Maturation of Encoded Enzyme*

Received for publication, October 13, 2011, and in revised form, November 3, 2011 Published, JBC Papers in Press, November 4, 2011 DOI 10.1074/jbc.M111.313676

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**Background:** Functional screening of malaria (*P. falciparum*) genes is a problem because of A + T content.

**Results:** Use of a *P. knowlesi* cDNA library and a yeast mutant identifies an important parasite cDNA/gene and reveals new regulation of lipid synthesis.

**Conclusion:** The *P. knowlesi* library will enable extrapolation to *P. falciparum.*

**Significance:** Functional screening of malaria genes is now greatly enhanced.

The 23-megabase genome of *Plasmodium falciparum,* the causative agent of severe human malaria, contains ~5300 genes, most of unknown function or lacking homologs in other organisms. Identification of these gene functions will help in the discovery of novel targets for the development of antimalarial drugs and vaccines. The *P. falciparum* genome is unusually A + T-rich, which hampers cloning and expressing these genes in heterologous systems for functional analysis. The large repertoire of genetic tools available for *Saccharomyces cerevisiae* makes this yeast an ideal system for large scale functional complementation analyses of parasite genes. Here, we report the construction of a cDNA library from *P. knowlesi,* which has a lower A + T content compared with *P. falciparum.* This library was applied in a yeast complementation assay to identify malaria genes involved in the decarboxylation of phosphatidylserine. Transformation of a *psd1Δpsd2Δdpl1Δ* yeast strain, defective in phosphatidylethanolamine synthesis, with the *P. knowlesi* library led to the identification of a new parasite phosphatidylserine decarboxylase (PkPSD). Unlike phosphatidylserine decarboxylase enzymes from other eukaryotes that are tightly associated with membranes, the PkPSD enzyme expressed in yeast was equally distributed between membrane and soluble fractions. In vitro studies reveal that truncated forms of PkPSD are soluble and undergo auto-endoproteolytic maturation in a phosphatidylserine-dependent reaction that is inhibited by other anionic phospholipids. This study defines a new system for probing the function of *Plasmodium* genes by library-based genetic complementation and its usefulness in revealing new biochemical properties of encoded proteins.

Malaria, a serious infectious disease responsible for over 800,000 deaths annually, is caused by intraerythrocytic protozoan parasites of the genus *Plasmodium.* *Plasmodium falciparum* is the major cause of human parasitic fatalities. The worldwide emergence of drug-resistant *Plasmodium* strains has made treatment of malaria increasingly difficult, thus emphasizing the need for new chemotherapeutic strategies to combat this disease (1). Following invasion of red blood cells, malarial parasites must increase lipid synthesis for membrane biogenesis and cell division. Inhibition of membrane lipid synthesis provides an attractive target for antimalarial chemotherapy (2, 3). Lipid analysis of *P. falciparum* demonstrates a relatively high content (up to 35% of total phospholipid) of phosphatidylethanolamine (*PtdEtn*)$^2$ (4), which often functions as a nonbilayer hexagonal phase lipid (5, 6).

PtdEtn synthesis in *Plasmodium* occurs through two major pathways, the serine decarboxylase–CDP-ethanolamine pathway and the phosphatidylserine (*PtdSer*) decarboxylation (PSD) pathway (Fig. 1). In the serine decarboxylase–CDP-ethanolamine pathway, serine is decarboxylated by a parasite-specific serine decarboxylase (*PfSD*) to form ethanolamine (*Etn*) (7, 8). Serine decarboxylation is unique to malarial parasites and plants (9). Ethanolamine, either formed through this reaction or directly taken up from the host erythrocyte (8), is then sequentially converted into phosphoethanolamine (Etn–P), CDP-ethanolamine, and PtdEtn by three parasite-specific enzymes, catalyzing an ethanolamine kinase (PfEtk), an ethanolamine cytidylyltransferase (PfECT), and a CDP-choline/ethanolamine phosphotransferase (PfCEPT) reaction. P-Etn can also be methylated to form phosphocholine (P–Cho), which can be further metabolized to phosphatidylcholine (PtdCho) via a CDP–Cho intermediate. In the PtdSer decarboxylation pathway, serine is incorporated into phospholipid by a parasite...

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*This work was supported, in whole or in part, by National Institutes of Health Grant 5R37 GM32543 (to D. R. V.). This work was also supported by the Burroughs Welcome Fund (to C. B. M.).

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‡The abbreviations used are: PtdEtn, phosphatidylethanolamine; Etn, ethanolamine; Pf, *P. falciparum*; Pk, *P. knowlesi*; Cho, choline; PtdSer, phosphatidylserine; PtdCho, phosphatidylcholine; ER, endoplasmic reticulum; PSD, PtdSer decarboxylase; CDP, cytidine diphosphate; DOPS, dioleoyl phosphatidylserine; DOPC, dioleoyl phosphatidylcholine; DOPG, dioleoyl phosphatidylglycerol; DOPA, dioleoyl phosphatidic acid; DOPE, dioleoyl phosphatidylethanolamine; PI, phosphatidylinositol.

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This report demonstrates successful library-based complementation of yeast using the cDNA library and reveals new details about the properties and maturation of PkPSD.

**EXPERIMENTAL PROCEDURES**

**Materials**—All chemicals, including amino acids for yeast media, were purchased from either Sigma or Fisher. Other components for yeast growth media were purchased from Difco. Phospholipids were obtained from Avanti Polar Lipids. Silica gel H plates and Silica gel 60 plates were purchased from Analtech Corp. and EMD, respectively. Radioactive 1-[^3]H]serine was from PerkinElmer Life Sciences. Reagents for protein determination were from Bio-Rad. Pre-cast SDS-polyacrylamide gels were purchased from Invitrogen. Mouse monoclonal antibodies against the V5 and His6 epitope tags of the PkPSD fusion protein were obtained from Invitrogen and Clontech, respectively. Other reagents used for ligand blotting were obtained from Bio-Rad and Sigma.

cDNA Library—The cDNAs were generated from *P. knowlesi* mRNAs and inserted by directional cloning into a modified pBEVY vector (20), pBEVY-DS, containing a single SfiI site. The vector is a *URA3*-based multicopy *Escherichia coli* yeast shuttle vector in which the cloned Pk-cDNAs are under the regulation of ADH1 promoter.

**Pk-cDNA Library Screening**—Pk-cDNA library plasmids were transformed into an Etn auxotrophic strain, HKY44 (MATα *psd1-Δ1::TRP1 psd2-Δ1::HIS3 dpl1Δ::KanMX trp1 ura3 his3 lys2 leu2) (21). Transformants that stably acquired a plasmid from the library were isolated based on the growth on minimal glucose, uracil dropout (SC-U) medium, supplemented with 2 mM Etn. Etn protrophic strains were isolated by replica plating transformants onto SC-U medium with no Etn supplementation.

**Cell Growth**—*psd1Δpsd2Δdpl1Δ* strains harboring the *PkPSD* gene were cultured in SC-U or synthetic lactate uracil dropout (SL-U) medium (22) supplemented with 2 mM Etn. After washing twice with water, serial 5-fold dilutions of cells were plated onto the medium with or without 2 mM Etn. Plates were incubated at 30 °C for 2–4 days.

**Whole Cell Radiolabeling and Phospholipid Analysis**—*psd1Δpsd2Δdpl1Δ* strains harboring the predicted *PkPSD* sequence and *psd1Δpsd2Δdpl1Δ* strains with empty vector were grown in synthetic complete medium plus 2 mM Etn with glucose as a carbon source (SC). Cells in mid-log phase were harvested by centrifugation and washed twice by resuspension in water and recentrifugation. The cells were suspended in SC medium at an *A*_{600} of 0.35 in a volume of 2 ml. Radiolabeling was initiated by adding 10 μCi/ml [1-[^3]H]serine, and growth was continued at 30 °C for 2 h with vigorous shaking. Labeled phospholipids were extracted as described previously (14, 23), and the lipid classes were resolved by thin layer chromatography, and radioactivity was quantified by liquid scintillation spectrometry.

**Measurement of PSD Activities**—Cell-free extracts, membrane fractions, and soluble fractions were isolated from *psd1Δpsd2Δdpl1Δ* strains harboring the predicted *PkPSD* cDNA or empty vector. Endogenous yeast PSD1 activity was measured using MSY30 (*PSD1DPL1psd2Δ*) (24). Strains were
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homogenized by glass bead beating, and subcellular fractions were prepared by differential centrifugation. The assay for PSD activity utilized Ptd[1-14C]Ser as the substrate, and the reaction product was trapped as 14CO2 on 2 M KOH-impregnated filter paper, as described previously (25). PSD activities in psd1Δpsd2Δpsd1Δ strains harboring the predicted PkPSD cDNA are presented as enzyme-specific activity (nmol/mg-protein/45 min), or as the percentage of activity relative to the activity of yeast PSDL1 wild type strains (100%), or as volume-normalized activity (nmol/ml/45 min) in TnT reactions. All enzyme reactions were performed at substrate (0.2 mM) and protein concentrations that produced a linear response with the amount of enzyme added to the reaction (usually 4–20 μg). In the most active preparations, the maximum substrate conversion to product did not exceed 10%.

Lipid Phosphorus Measurement—Yeast strains were cultured in SC-U medium and grown to mid-log phase at 30 °C. The cells were harvested by centrifugation and washed twice with water. The lipids were extracted, as described previously (26). The phospholipids were separated by two-dimensional thin layer chromatography (TLC) on Silica 60 plates using chloroform/methanol/ammonium hydroxide (65:35:5 v/v) followed by chloroform/acetate/methanol/water (75:25:5:2.2 v/v). Lipids were visualized with iodine vapor and quantified by measuring phosphorus (27). The results are shown as the percentage of total lipid phosphorus in each phospholipid fraction.

DATA WRE ARE MEANS ± S.D. FOR THREE OR FOUR INDEPENDENT EXPERIMENTS.

In Vitro Expression of PkPSD—A TnT quick-coupled transcription/translation system (Promega Corp.) was used to express PkPSD proteins in vitro. Reactions were initiated with 0.5 μl of 1 mM methionine and 2 μl of plasmid (0.5 μg/μl) harboring PkPSD with an N- or C-terminal His6 tag, added to 20 μl of TnT quick master mix kit, which contained rabbit reticulocyte lysate, an amino acid mixture without methionine, T7 RNA polymerase, nucleotides, salts, and RNasin ribonuclease inhibitor. To study the effect of phospholipids on the expression and processing of the PkPSD protein, 2 μl of liposomes composed of dioleoyl phosphatidylserine (DOPS), dioleoyl phosphatidylethanolamine (DOPE), dioleoyl phosphatidylcholine (DOPC), dioleoyl phosphatidylglycerol (DOPG), dioleoyl phosphatidic acid (DOPA), or phosphatidylinositol (PI) from bovine liver or soybean were added to the TnT reaction and incubated for the indicated times at 30 °C. Liposomes were prepared fresh for each experiment. To prepare liposomes, phospholipid in chloroform was transferred into an Eppendorf tube and dried under nitrogen gas and chased with methanol. The lipid pellets were resuspended in 20 μl of methanol and dried using centrifugation under vacuum for 30 min to completely remove all organic solvents. The lipid pellets were resuspended in 0.1 M KCl, 10 mM Tris-Cl, pH 7.5, at 1 mg/ml, hydrated at 37 °C for 30 min, and then mixed with a vortex mixer to create multilamellar liposomes. To create unilamellar liposomes, the suspension was bath-sonicated for 20 min. Defined size unilamellar liposomes were made by passing multilamellar liposomes through an Avanti Liposofast with 50-, 200-, or 1000-nm filter sets. When the in vitro transcription/translation step was temporally separated from processing steps, 0.2 mM cycloheximide was added to the TnT reaction to arrest translation, and the reactions were further incubated with liposomes. The expression and processing of PkPSD were monitored by Western blot analysis using anti-His6 antibody and by PSD enzyme assay as described above.

Construction of Vectors to Express PkPSD in E. coli or Yeast—E. coli vectors harboring plasmids for PkPSD-His6 or N-terminal 34-amino acid deleted PkPSD-His6 (PkPSDΔ34-His6) were created using a pET-45b(+) vector. Briefly, specific primers for the individual constructs were generated and used to amplify DNAs from a template cDNA harboring PkPSD using PCR with Pfu-DNA polymerase (Stratagene). The PkPSD and PkPSDΔ34 constructs containing a 5’ SpeI site and a 3’ Xhol site were purified by agarose gel electrophoresis. The pET-45b(+) E. coli expression vector was digested with XbaI (compatible to SpeI ligation) and Xhol restriction enzymes, and the resultant DNA fragment was purified by electrophoresis. Appropriate ligation reactions yielded pET45-PkPSD-His6 and pET45-PkPSDΔ34-His6. The plasmids were introduced in BL21 and Rosetta strain to express the constructs. The pET-45b(+) vector was also utilized to create pET45-His6-PkPSDΔ34, which encodes an N-terminal His6 epitope linked to PkPSDΔ34. The PCR construct, PkPSDΔ34, was digested with KpnI at the 5’ end and Xhol at the 3’ end and subsequently ligated into the linearized pET-45b(+) vector, also cut with KpnI and Xhol restriction enzymes. The construct was introduced into BL21 and Rosetta strains to express the enzyme. The pET45-His6-PkPSDΔ34 construct was also used in vitro transcription/translation using a TnT kit.

A pYES2.1-V5 vector was used to express the proteins PkPSD, PkPSDΔ34, and PkPSD with an N-terminal 55-amino acid deletion (PkPSDΔ55) in yeast. Specific primers were generated so that proteins could be expressed as untagged or the C-terminal V5 fusion strains harboring the predicted PkPSD proteins, PTY44 (urA3 his3 lys2 leu2). Sequencing of these plasmids revealed the presence of the ORF PKH_072580. The sequence was previously annotated as a putative phosphatidylserine decarboxylase. Specific primers were designed for amplification of the PkPSD gene. The primers were used to create pET45-His6-PkPSDΔ34, which encodes an N-terminal His6 epitope linked to PkPSDΔ34. The PCR construct, PkPSDΔ34, was digested with XbaI and subsequently ligated into the linearized pET-45b(+) vector, also cut with KpnI and Xhol restriction enzymes. The construct was introduced into BL21 and Rosetta strains to express the enzyme. The pET45-His6-PkPSDΔ34 construct was also used in vitro transcription/translation using a TnT kit.

RESULTS

Identification of P. knowlesi Phosphatidylserine Decarboxylase—An Etn auxotrophic mutant yeast strain devoid of PSD activity and incapable of forming P-Etn from sphingolipids, HKY44 (psd1Δ::TRP1 psd2Δ::HIS3 trp1 ura3 his3 lys2 leu2), was utilized to screen a multicopy Pk-cDNA library inserted into the vector pBEVY-DS. Approximately 650,000 uracil prototrophic pBEVY-DS library transformants were screened for Etn prototrophy, and 127 transformants were identified. Plasmids from nine uracil/ethanolamine yeast prototrophs were recovered using antibiotic resistance in E. coli. Sequencing of these plasmids revealed the presence of the ORF PKH_072580. The sequence was previously annotated as a putative phosphatidylserine decarboxylase. The remaining Etn prototrophs were also positive for PkPSD sequences when screened by PCR.

The putative PkPSD cDNA encodes a polypeptide of 354 amino acid residues with a deduced molecular weight of 41,524. The PkPSD protein shares strong sequence homology with the
Previously characterized PfPSD from *P. falciparum* with amino acid sequence identity of 72.5% and similarity of 88.2%. An MGSS sequence located at positions 306–309 in the C terminus corresponds to a VGSS sequence located at positions 314–317 of the PfPSD and appears related to the LGST motif, which is the endoproteolytic cleavage site of the *E. coli* and *S. cerevisiae* PSD1 proenzymes (Fig. 2) (14, 28). The cleavage converts the inactive proenzyme to an active form containing a small subunit with a pyruvoyl prosthetic group essential for catalysis and a large subunit (29).

Next, we tested whether the presence of the *PkPSD* cDNA could support growth of the mutant strain under respiratory conditions in minimal medium with lactate as the carbon source, where PtdEtn synthesis is required for normal mitochondrial function. Fig. 3c shows that *psd1*/*psd2*/dpl1 strains harboring *PkPSD* grew well under respiratory conditions in the absence of Etn. The ability of the *PkPSD* enzyme to support yeast growth under respiratory conditions indicates that the enzyme has access to pools of PtdSer located at the outer mitochondrial membrane. Collectively, the above data demonstrate that the *Plasmodium* *PSD* cDNA suppresses the growth defect of *psd1*/*psd2*/dpl1 strains under both fermentative and respiratory conditions.

Characterization of *PkPSD* Expressed in Yeast—To characterize the *PkPSD* enzyme, catalytic activities were assayed both in vivo and in vitro. Yeast strains were labeled with [3H]serine in minimal glucose medium, and the incorporation of the radio-label into aminophospholipids was analyzed by thin layer chromatography (TLC) of the lipid extracts prepared from the cells (Fig. 4a). The mutant psd1Δpsd2Δdpl1Δ strain harboring an...
empty vector failed to generate significant levels of radiolabeled PtdEtn because of the lack of PtdSer decarboxylases. The mutant psd1Δ psd2Δ dpl1Δ strain expressing the PkPSD, and yeast strains lacking PSD2 but containing the PSD1 gene, expressed from either single copy or multicopy plasmids, readily produced radiolabeled PtdEtn at normal levels. This indicates that nascent PtdSer is efficiently converted to PtdEtn by the PkPSD enzyme at levels comparable with that produced by the chromosomal copy of yeast PSD1.

Next, the enzyme activity was measured in cell extracts. Whole cell extracts, membrane fractions, and soluble fractions were incubated with Ptd[1-14C]serine substrate, and the decarboxylase activity was determined by measuring 14CO2 production. As shown in Fig. 4b, high enzyme activity was detected in the cell-free extracts of the psd1Δ psd2Δ dpl1Δ strain harboring a PkPSD cDNA, whereas no activity was found in the psd1Δ psd2Δ dpl1Δ strain harboring the empty vector. The levels of PkPSD expression produced nearly three times the catalytic activity of the endogenous yeast PSD1 gene. Interestingly, significant levels (49%) of PkPSD enzyme activity were detected in the soluble fractions prepared from cell extracts (Fig. 4c). Thus far, all eukaryotic PSD enzymes have been localized to membrane compartments (30–32), and these findings suggest unusual properties of the malarial enzyme. The relatively large soluble population of the PkPSD protein could be an intrinsic feature of the enzyme that renders the molecule amphitropic.

The phospholipid compositions of the psd1Δ psd2Δ dpl1Δ strains with the PkPSD cDNA were analyzed and compared with that of a psd2Δ strain that contains wild type PSD1 and DPL1 genes. Lipids extracted from the cells grown on medium in the absence of Etn were separated by two-dimensional TLC and visualized by iodine staining. The appearance of a PtdEtn band and visualized by iodine staining. The appearance of a PtdEtn band indicates that the lipid was synthesized by the action of PkPSD enzyme (Fig. 5a). Fig. 5b shows the quantification of each lipid extracted from the TLC plates. There was a slight increase in PtdEtn level and a 42% reduction in PtdSer in the psd1Δ psd2Δ dpl1Δ strains expressing the PkPSD cDNA compared with that in the psd2Δ strain. Taken together, the results from Figs. 4 and 5 demonstrate that the PkPSD enzyme expressed in the psd1Δ psd2Δ dpl1Δ strains was fully functional and complemented the biochemical defect of the mutant strain.

**N-terminal Deletion of PkPSD Yields Active Forms of Enzyme**

The N-terminal 137 amino acids of yeast PSD1 contain mitochondrial targeting and inner membrane sorting sequences. The sequence is not only required for the mitochondrial targeting but also for the correct processing into β and α fragments (33). Previously, plant PSD sequences from tomato and Arabidopsis were identified and expressed in the yeast psd1Δ psd2Δ double mutant strain (10). The N-terminal mitochondrial targeting sequence of plant PSD protein was inhibitory to its functional expression in yeast. A deletion construct that removed the plant-specific mitochondrial targeting sequence and a chimeric construct in which the yeast PSD1 targeting sequence replaced the plant sequence could readily suppress the Etn auxotrophy of the yeast double mutant strain.

The N-terminal 55-amino acid sequence of the PkPSD protein does not show any significant amino acid homology with any other PSDs. When the sequence was analyzed with TMpred software, a trans-membrane domain was predicted at positions 18–34. To investigate the role of the N-terminal sequence, two truncated PkPSD cDNAs were constructed to encode proteins with deletions of residues 2–34 (PkPSDΔ34) and 2–55 (PkPSDΔ55) (Fig. 6a). The constructs were placed under control of the GAL1 promoter in an episomal vector (pYES2.1, Invitrogen) and introduced into the psd1Δ psd2Δ double mutant strain. Enzyme assay showed that the PkPSD with a 34-amino acid deletion produced robust activity, whereas the PkPSD with a 55-amino acid deletion produced very weak activity (Fig. 6b). The catalytic activity of the PkPSD with the 34-amino acid deletion was also evenly distributed between membrane and soluble fractions (data not shown). This indicates that the PkPSD residues between 2 and 34 were not required for enzyme maturation, catalysis, or membrane association in the yeast expression system. In contrast, the amino acids between positions 35 and 55 appear crucial for the functional activity of PkPSD.

**Expression of PkPSD Fusion Proteins in Bacteria and Yeast**

To further characterize the activity and processing of PkPSD, we generated PkPSD-His6 and PkPSD-V5 fusion proteins expressed in E. coli and yeast, respectively. A PkPSD-His6 fusion protein was expressed in BL21 and Rosetta strains of E. coli. Protein extracts were separated on SDS-PAGE, and the fusion protein was detected with anti-His6 antibody. As seen in
The fusion protein was detected only with the Rosetta host strain, which provides six tRNAs for codons that are rarely used in E. coli but common in eukaryotic organisms, thus enabling the efficient translation of DNA from P. knowlesi. Two immunoreactive bands were detected with the anti-His6 antibody. The upper 43-kDa band corresponds to the unprocessed proenzyme and the 7-kDa lower band corresponds to the /H9251 subunit containing the active site of the enzyme (28). The majority of the E. coli-expressed PkPSD was in the form of proenzyme, which indicates that the endoproteolytic processing of the PkPSD fusion protein was not efficient. The Rosetta strain expresses its endogenous PSD, but the total catalytic activity in cell extracts increased 2-fold with PkPSD cDNA expression (Fig. 7b).

A multicopy yeast vector encoding a PkPSD-V5 fusion protein under control of the GAL1 promoter was constructed and transformed into a yeast psd1/H9004 psd2/H9004 strain. Fig. 8a shows the growth of the psd1Δpsd2Δ strains harboring either an empty vector, a multicopy vector with the yeast PSD1, or a multicopy vector containing the PkPSD-V5 cDNA fusion construct. As expected, the psd1Δpsd2Δ strains harboring empty vector failed to grow in the absence of Etn, because the strains had no PSD enzymes. Expression of the PkPSD fusion protein readily suppressed the growth defect of the mutant strain in the absence of Etn. Extracts from the yeast cells were analyzed by SDS-PAGE followed by Western blotting using an anti-V5 antibody (Fig. 8b). Two protein bands, corresponding to the proenzyme and the active form of the /H9251 subunit, were detected demonstrating the maturation of the enzyme in vivo. These findings...
with the fusion proteins demonstrated that epitope-tagged versions of PkPSD could undergo processing in vivo and maturation to active enzyme.

In Vitro Processing of PkPSD Demonstrates PtdSer Post-translationally Enhances Enzyme Maturation—The retention of enzyme activity by PkPSD/H900434 (see Fig. 6b) indicated that the predicted membrane binding N terminus of the protein was not required for processing of the proenzyme to its mature form, and it raised the possibility that processing of a soluble form of the enzyme could be examined in vitro using epitope-tagged versions of the enzyme. We utilized a coupled in vitro transcription-translation (TNT) system to examine nascent enzyme formation and its subsequent processing to mature enzyme. We empirically determined that an N-terminal His6 tag appended to PkPSD/H900434 produced the most robust catalytic activity from the in vitro TNT reaction. In our initial experiments, we observed significant production of the PkPSD/H900434 proenzyme (39 kDa), with modest processing to the mature enzyme (seen as the 33-kDa/H9252 subunit), detected by both immunoblotting and measurement of catalytic activity (see Fig. 9, a and b). We tested whether the presence of a membrane compartment, canine pancreatic microsomes, could augment processing of the enzyme, and these experiments did not demonstrate any influence of added membranes upon the process (Fig. 9, a and b).

Because several pyruvoyl enzymes have been reported to be stabilized via a Schiff base conjugate with their substrates and reaction products (34), we first tested whether inclusion of the substrate (PtdSer) for the reaction might be required to promote proenzyme processing. As detailed in Fig. 10a, the inclu-
FIGURE 9. In vitro expression of His<sub>6</sub>-PkPSDΔ34 fusion protein produces an active PSD enzyme. A TNT reaction containing pET45 vector only, 1 µl of canine pancreatic microsomal membranes (Promega), pET45-His<sub>6</sub>-PkPSDΔ34, pET45-His<sub>6</sub>-PkPSDΔ34 + 0.5 µl of canine pancreatic microsomal membranes, or pET45-His<sub>6</sub>-PkPSDΔ34 + 1 µl of canine pancreatic microsomal membranes was performed for 90 min at 30 °C. Upon completion of the TNT reaction, aliquots were used for PSD assays and Western blot analysis. a, Western blot analysis was conducted to detect the His<sub>6</sub> epitope present in the nascent enzyme generated in the TNT reaction. Lane numbers correspond to plasmids and microsome additions to the TNT reaction. Lane 1, empty vector; lane 2, no vector + microsomes; lane 3, pET45-His<sub>6</sub>-PkPSDΔ34; lane 4, pET-His<sub>6</sub>-PkPSDΔ34 + 0.5 µl of microsomes; lane 5, pET45-His<sub>6</sub>-PkPSDΔ34 + 1 µl of microsomes. b, PSD enzyme assay with the samples from the reactions was performed with Ptd[(1-14)C]Ser as the substrate. Data are means ± S.E. for three experiments each performed in duplicate. Micros, microsomal.

FIGURE 10. Endoproteolytic maturation of His<sub>6</sub>-PkPSDΔ34 is enhanced by PtdSer. TNT reactions were conducted for 90 min at 30 °C in either the absence or presence of lipids. Upon the completion of the TNT reactions, aliquots were analyzed by Western blot or PSD assay. a, Western blot analysis was conducted to detect the His<sub>6</sub> epitope present in the nascent and mature proteins produced in the TNT reaction. Lane numbers correspond to the plasmid and lipid additions to the TNT reactions. Lane 1, pET45 empty vector; lane 2, pET45-His<sub>6</sub>-PkPSDΔ34 + no lipid; lane 3, pET45-His<sub>6</sub>-PkPSDΔ34 + 50 µg/ml DOPS and 50 µg/ml DOPC; lane 4, pET45-His<sub>6</sub>-PkPSDΔ34 + 100 µg/ml DOPS; lane 5, pET45-His<sub>6</sub>-PkPSDΔ34 + 200 µg/ml DOPS. b, PSD assays were performed on replicate samples corresponding to the conditions in lanes 1–5 of a. Lane 1, empty vector; lane 2, no lipid; lane 3, DOPS 0.5× + DOPC 0.5×; lane 4, DOPS 1×; lane 5, DOPS 2×. Values are means ± S.D. for three experiments each performed in duplicate. c, Western blot analysis was conducted to detect the His<sub>6</sub> epitope present in proteins appearing as a function of time following translational arrest with 0.2 mM cycloheximide at the end of a 60-min TNT reaction. Following the addition of cycloheximide, the samples were further incubated for the indicated times up to 80 min, either in the absence (− DOPS), or presence (+ DOPS) of lipid. d, Western blot analysis was performed to examine the effects of liposome size upon PkPSD maturation. The TNT reaction was conducted as described for c using pET45-His<sub>6</sub>-PkPSD as the plasmid template and a 60-min reaction that was terminated with 0.2 mM cycloheximide. Following translational arrest, the maturation of the PkPSD was allowed to proceed for 40 min in the presence of either no added lipid (lane 1) or 200 µg/ml DOPS in the form of 50 nm vesicles (lane 2), or 200 nm vesicles (lane 3), 1000 nm vesicles (lane 4), or large multilamellar vesicles (lane 5).

The expression of increasing concentrations of DOPS in the TNT reaction, greatly enhanced the processing of the PkPSD enzyme to its mature form as shown by the reduction in the proenzyme content and increased appearance of the β subunit, detected by immunoblotting. The enhanced processing of the proenzyme was also accompanied by significant quantitative increases (3–4-fold) in the amount of detectable catalytic activity as shown in Fig. 10b.

Next, we examined if enzyme processing is a co-translational event or occurs in a time-dependent manner after translation. First, in vitro synthesis of PkPSD was conducted for 60 min in the absence of DOPS. Subsequently, the translation reactions were halted by addition of cycloheximide, and the reaction aliquots were incubated further to allow processing of PkPSD in the absence or presence of DOPS. The reaction aliquots were removed at increasing times of incubation after translation arrest and analyzed as shown in Fig. 10c. The data reveal that during the initial 60-min translation reaction, significant precursor PkPSDs were made, but with very low levels of processed β subunits. With increasing time after translational arrest, some mature forms were produced even in the absence of DOPS, indicating that PkPSD processing did not need to be co-translational. The lipid supplementation after translational arrest revealed that DOPS significantly enhanced processing of the newly synthesized proenzyme, which resulted in a time-dependent decline in the proenzyme content and an increase in the mature β subunit content.

The DOPS used to stimulate PkPSD processing was prepared as a sonicated suspension of unilamellar vesicles. We also examined whether the diameter of the vesicles affected the processing reaction by preparing liposomes of various sizes, using extrusion through polycarbonate filters. Liposomes of 50, 200, and 1000 nm diameter all produced similar levels of
concomitant reduction of contrast, the anionic lipids, DOPA and DOPG, exerted a strong mixture had only a modest stimulatory effect on the process. In vitro the TNT reactions that used pET45-His6-PkPSDΔ34 as a template. a, TNT reactions were performed for 90 min at 30 °C in the presence of empty vector, or plasmid harboring His6-PkPSDΔ34 in the absence of lipids (No lipid), or the presence of 100 μg/ml DOPC, or 50 μg/ml DOPC + 50 μg/ml DOPE, or 100 μg/ml DOPG, or 100 μg/ml DOPA. Following these incubations, aliquots of the reactions were analyzed by Western blotting. a also contains incubations that included soybean PI. b, same reactions were performed as shown in a with the corresponding plasmid and lipid additions indicated in the figure and then added to PSD assays, and the enzyme activity was quantified. c, TNT reaction using a pET45-His6-PkPSDΔ34 template was performed for 60 min and then arrested by the addition of 0.2 mM cycloheximide. Subsequently, the maturation reaction was conducted at 30 °C for an additional 40 min in either the absence of lipid or the presence of DOPA, DOPC, DOPG, PI, or DOPS. Aliquots from the maturation reaction were examined by Western blotting.

Processing of PkPSD Is Down-regulated by the Anionic Lipids, DOPA, DOPG, and PI—We next investigated if regulation of PkPSD processing is PtdSer-specific. PtdSer is an anionic phospholipid, and we compared its activity with other anionic phospholipids, including DOPA, DOPG, and bovine liver PI. Comparisons were also made with the zwitterionic phospholipids, DOPE, and DOPC. Fig. 11a shows that supplementation of the in vitro processing reactions with DOPC or a DOPC/DOPE mixture had only a modest stimulatory effect on the process. In contrast, the anionic lipids, DOPA and DOPG, exerted a strong inhibitory effect upon maturation of PkPSD, which resulted in concomitant reduction of β subunit formation and catalytic activity. Strong inhibition of the processing of the proenzyme by DOPC and DOPA resulted in reduced PSD activities (90 and 85% reduction by DOPG and DOPA, respectively) as seen in Fig. 11b. DOPC and a DOPC/DOPE mixture show only mild increases in the catalytic activities consistent with their mild stimulatory effects on the processing of the proenzyme. Interestingly, although other lipids did not affect overall synthesis of the PkPSD proenzyme, PI strongly inhibited it, indicating disruption of either transcription or translation by the in vitro reaction.

To bypass the inhibitory effect on expression of precursor PkPSDs by PI, the reaction was conducted for 40 min in the absence of lipid, followed by translational arrest with cycloheximide and further incubation with the various lipids. The Western blot analysis showed that DOPE and DOPC did not alter the basal level of processing, but PI along with DOPA and DOPG strongly inhibited processing (Fig. 11c). Taken together, the data demonstrate that anionic lipids, other than PtdSer, have a strong inhibitory effect on the post-translational processing of the PkPSD precursor. These findings suggest that membrane lipid composition plays an important regulatory role in PSD processing.

Regulating Lipids Do Not Bind to PkPSD Precursors with High Affinity—Because there was strong activation/inhibition by anionic phospholipids of the processing of the PkPSD precursor, we investigated whether there was any stable physical association between lipids and the proenzyme. Using multilamellar liposomes, we tested if the precursor PkPSDs generated in the TnT reactions could be sedimented with the lipid by centrifugation. Following the maturation reaction, aliquots were shifted to 0 °C and either unprocessed (R) or centrifuged at 13 × 10⁴ g for 20 min to recover liposomes (P). Volume normalized aliquots of the total reaction and the liposome pellet were analyzed by Western blotting.

FIGURE 11. Anionic phospholipids inhibit maturation of PkPSD. The influence of multiple phospholipids upon the maturation of PkPSD was examined by Western blotting, and enzyme assay was performed in conjunction with TnT reactions that used pET45-His6-PkPSDΔ34 as a template. a, TnT reactions were performed for 90 min at 30 °C in the presence of empty vector, or plasmid harboring His6-PkPSDΔ34 in the absence of lipids (No lipid), or the presence of 100 μg/ml DOPC, or 50 μg/ml DOPC + 50 μg/ml DOPE, or 100 μg/ml DOPG, or 100 μg/ml DOPA. Following these incubations, aliquots of the reactions were analyzed by Western blotting. a also contains incubations that included soybean PI. b, same reactions were performed as shown in a with the corresponding plasmid and lipid additions indicated in the figure and then added to PSD assays, and the enzyme activity was quantified. c, TnT reaction using a pET45-His6-PkPSDΔ34 template was performed for 60 min and then arrested by the addition of 0.2 mM cycloheximide. Subsequently, the maturation reaction was conducted at 30 °C for an additional 40 min in either the absence of lipid or the presence of DOPA, DOPC, DOPG, PI, or DOPS. Aliquots from the maturation reaction were examined by Western blotting.

FIGURE 12. Regulatory lipids do not bind to the His6-PkPSDΔ34 precursor with high affinity. The TnT reaction for in vitro expression of His6-PkPSDΔ34 was carried out for 40 min at 30 °C. Translation was arrested by addition of 0.2 mM cycloheximide, and maturation reactions were continued for an additional 40 min in either the absence (None) or presence of 100 μg/ml lipids (DOPS, DOPC, PI, and DOPG) as indicated. The lipids were in the form of multilamellar liposomes that could be sedimented by centrifugation. Following the maturation reaction, aliquots were shifted to 0 °C and either unprocessed (R) or centrifuged at 13 × 10⁴ g for 20 min to recover liposomes (P). Volume normalized aliquots of the total reaction and the liposome pellet were analyzed by Western blotting.

DISCUSSION

The successful cloning of the PkPSD cDNA using a library-based genetic complementation approach in yeast demonstrates the utility of this system for assigning function to numerous genes within the Plasmodium genus. We screened ~130 P. knowlesi genome equivalents and identified 127 isolates of PkPSD. These data indicate that the library will likely cover very rare cDNAs expressed at very low levels. The boot-
strapping approach of functional genetic screening with PkPSD libraries and cross-correlating to the P. falciparum genome effectively bypasses the problem of the A + T-rich genome of P. falciparum. We foresee this work as an important stepping stone toward large scale functional annotation of the P. falciparum genome. In addition, our approach has immediately yielded important new insights into the biochemistry of the parasite PSD, which have not otherwise been tractable with other eukaryotic PSD enzymes. More detailed understanding of the mechanism of PkPSD processing has the potential to reveal new and unsuspected vulnerabilities of the parasite to disruption of membrane biogenesis.

Sequence alignment between PkPSD and E. coli PSD reveals that the PkPSD sequence starting at position 56 shares homology with E. coli sequence at position 16 (Fig. 2). The sequence homology between PkPSD and ScPSD1 begins at position 67 of the parasite protein and position 138 of the yeast protein. The N-terminal 137 amino acids of yeast PSD1 contain mitochondrial targeting and inner membrane sorting sequences. The truncated yeast PSD1 with a 137-amino acid deletion was reported as a nonfunctional enzyme because the encoded construct failed to be processed to functional subunits α and β fragments (33). Unlike PSDs from other organisms, including E. coli, yeast, mammals, and plants, that contain a strong hydrophobic transmembrane domain in the C terminus of their β fragments, there is no trans-membrane domain in the corresponding region of PkPSD. Instead, PkPSD contains a predicted trans-membrane domain at the N terminus at positions 18–34. When the PkPSD cDNA was expressed in yeast, nearly half of the enzyme activity was recovered in the soluble fraction. Western blot analysis of epitope-tagged versions of the full-length proenzyme did not reveal any discernible size differences between the soluble and membrane-associated proteins, suggesting that the protein was amphitropic. The amphitropic properties of the enzyme are likely to allow access to its substrate in multiple membranes, including the outer mitochondrial membrane. Decarboxylation of PtdSer at the outer mitochondrial membrane is the likely reason that expression of PkPSD in yeast enables strain growth under respiratory conditions. The factors that dictate membrane association remain unidentified.

Truncated versions of the PkPSD lacking the N terminus and predicted transmembrane domain (amino acids 2–34) were expressed in yeast, and the PkPSDΔ34 version resulted in a highly active form of the enzyme. Epitope-tagged versions of PkPSD also resulted in catalytically active forms of the enzyme in both bacteria and yeast. Epitope-tagged forms of PkPSDΔ34 provided a convenient variant of the enzyme for examination of processing events in vitro, and the His6-PkPSDΔ34 cDNA was transcribed and translated with a TnT system that contains reticulocyte lysate components for protein synthesis. In the absence of lipid supplementation, a modest level of the nascent enzyme is processed to the mature form. Inclusion of DOPS in the transcription-translation reaction increases the processing of proenzyme 3–4-fold, as evidenced by the diminution in the amount of unprocessed form, and a concomitant increase in the amount of mature β subunit. The increased processing observed on Western blotting correlates with definitive quantitative increases in catalytic activity of the enzyme detected in PSD assays. The action of DOPS appears specific, insofar as DOPA and DOPC have little or no effect upon enzyme maturation, whereas DOPG, DOPA, and PI are inhibitory. The observed low level of maturation of the PkPSD in the absence of lipid addition may be a consequence of endogenous levels of phosphatidylserine present in the reticulocyte lysate.

The application of the TnT system also enabled us to determine that the P. knowlesi PSD undergoes processing after translation is complete. This timing may be of important regulatory value insofar as it provides the nascent proenzyme with a time window for sampling membrane content of PtdSer and other anionic phospholipids. Membrane environments rich in PtdSer will promote processing, whereas those with high content of other anionic phospholipids will inhibit processing. A schematic summary of lipid modulation of PkPSD proenzyme processing appears in Fig. 13.

The maturation of PSD enzymes broadly appears to take place in three steps (35). In the first step, the hydroxyl group of a critical serine, which is destined to form the active site pyruvyl group, attacks the proximal (N-terminally located) peptide bond, effecting serinolytic cleavage that results in the β subunit being attached to the α subunit by an ester bond. In the second step, the β subunit is liberated by an elimination reaction, which also yields an α subunit harboring an N-terminal dehydroalanine. In the third step, the reactive dehydroalanine undergoes water addition and ammonia elimination to form the pyruvyl prosthetic group on the α subunit. The mechanism of action of DOPS in promoting proenzyme processing is not yet known, but two possibilities seem likely. One mechanism could involve DOPS forming a Schiff base with the newly formed carbonyl moiety of the α subunit immediately as it is formed, which would function to shift the equilibrium of the processing reaction in the direction of mature enzyme. A second mechanism might involve DOPS binding to the β subunit as an allosteric enhancer of the auto-endoproteolytic reaction. The availability of soluble versions of PkPSD should enable testing of these two mechanisms in the future.
Acknowledgment—We thank Dr. Manoj Duraisingh for providing P. knowlesi RNA for library construction.

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