Plasmodium falciparum Phospholipase C Hydrolyzing Sphingomyelin and Lysocholinephospholipids Is a Possible Target for Malaria Chemotherapy

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Abstract

Sphingomyelinase (SMase) is one of the principal enzymes in sphingomyelin (SM) metabolism. Here, we identified a *Plasmodium falciparum* gene (*PfNSM*) encoding a 46-kD protein, the amino acid sequence of which is ~25% identical to that of bacteria SMases. Biochemical analyses of the recombinant protein GST-PfNSM, a fusion protein of the *PfNSM* product with glutathione-S-transferase, reveal that this enzyme retained similar characteristics in various aspects to SMase detected in *P. falciparum*–infected erythrocytes and isolated parasites. In addition, the recombinant protein retains hydrolyzing activity not only of SM but also of lysocholinephospholipids (LCPL) including lysophosphatidylcholine and lysoplatelet-activating factor, indicating that *PfNSM* encodes SM/LCPL-phospholipase C (PLC). Scyphostatin inhibited SM/LCPL-PLC activities of the *PfNSM* product as well as the intraerythrocytic proliferation of *P. falciparum* in a dose-dependent manner with ID_{50} values for SM/LCPL-PLC activities and the parasite growth at 3–5 μM and ~7 μM, respectively. Morphological analysis demonstrated most severe impairment in the intraerythrocytic development with the addition of scyphostatin at trophozoite stage than at ring or schizont stages, suggesting its effect specifically on the stage progression from trophozoite to schizont, coinciding with the active transcription of *PfNSM* gene.

Key words: lysophosphatidylcholine • lysoplatelet-activating factor • sphingosylphosphocholine • sphingomyelinase • intraerythrocytic stage

Introduction

Malaria remains a devastating disease worldwide, especially in the tropics. *Plasmodium falciparum*, the deadliest among four species of malaria parasites that infect humans, is responsible for more than a million deaths annually. Emergence and spread of resistant parasites to agents such as chloroquine and pyrimethamine/sulfadoxine highlights the need to develop new drugs against this disease. In this quest, elucidating the differences in metabolisms between host and parasite at the molecular level would provide novel targets for malaria chemotherapy.

Lipid metabolism, which is almost nonfunctional in uninfected erythrocytes (1), rises drastically during intraerythrocytic development of malaria parasites (2, 3) contributing mainly to membrane biogenesis. Membrane biogenesis includes not only organelle membranes of proliferating parasites but also the tubovesicular membrane network in the cytoplasm of infected erythrocytes (2, 4). The parasites meet the demand for the necessary lipid species by synthesizing through de novo pathways as well as acquiring from extracellular sources (2, 5, 6). Parasite cells, therefore, appear to develop unique features in lipid metabolism for survival in the intraerythrocytic environ-

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ment, prompting an increase in attention on lipid metabolism to provide rational targets for malaria chemotherapy (7–10).

Sphingolipids, which are widely distributed in eukaryotes, play important roles in the growth of cells (11–13). Sphingomyelin (SM)* is the most abundant of the mammalian sphingolipids, and degradation of SM to produce ceramide appears to be responsible for the modulation of various cellular events including proliferation, differentiation, and apoptosis (14–16). SM is also associated with the plasmodial parasites (2, 17); however, SM metabolism in parasite cells is poorly understood.

Sphingomyelinase (SMase) is a principal enzyme catalyzing the hydrolysis of SM to ceramide and phosphocholine (18). In mammalian cells, there are two well-known types of SMase. One is termed acid SMase, with an optimum pH at around 4.8; further subclassified to two isoforms, an endosomal/lysosomal acid SMase and a secretory Zn^{2+}-dependent SMase. The other type of mammalian SMase, termed neutral SMase, is a membrane-bound and Mg^{2+}-dependent SMase, with an optimum pH at around 7.5. One isoform of neutral SMase has been recently demonstrated to have substantial phospholipase C (PLC) activity toward lysophosphatidylcholine (lysoPtdCho) and lysoplatelet-activating factor (lysoPAF; reference 19). Some bacteria produce extracellular Mg^{2+}-dependent neutral SMases (18) that are presumably implicated in degradation of environmental SM for nutritional use or for infection to vertebrate hosts. Several cDNAs encoding acid and neutral SMases (18, 20–23) and genes for bacterial SMase (24–26) have been identified so far. There has been no report, however, of a plasmodial SMase gene.

We have recently demonstrated that *P. falciparum*–infected human erythrocytes but not uninfected erythrocytes retain the activity of a neutral SMase (27). Here, we identify and characterize the *P. falciparum* gene encoding a neutral SMase. The enzyme encoded by the plasmodial gene retains phospholipase C activity toward lysocholinephospholipids (LCPL) as well as SM, and this PLC activity was inhibited by scyphostatin. In addition, the effect of scyphostatin on in vitro culture of *P. falciparum* was investigated to implicate this enzyme with the intraerythrocytic development of parasite cells.

Materials and Methods

Materials. Bovine brain SM, sphingosylphosphocholine, phosphatidylserine (PtdSer), PAF, lysoPAF, 1-monopalmitoyl-*rac*-glycerol, 1-octadecyl-*rac*-glycerol, intact BSA (product no. A-1933), and fatty acid–free BSA (product no. A-7511) were purchased from Sigma-Aldrich. Fatty acid–free BSA was used as lipid-free BSA (6). Egg lysoPtdCho was from Avanti Polar Lipids, Inc., and 1-hexadecyl-2-acetyl-*sn*-glycerol from Biomol Research Laboratories Inc. [*Choline-methyl*-^{14}C]SM (55 mCi/mmol) and [*choline-methyl*-^{14}C]PtdCho l-α-dipalmitoyl (55 mCi/mmol) were purchased from American Radiolabeled Chemicals Inc. 1-*O*-[^{3}H]*octadecyl* PAF (163 Ci/mmol), 1-*O*-[^{3}H]*octadecyl* lysoPAF (163 Ci/mmol), and [8-^{3}H]-hypoxanthine (18–27 Ci/mmol) were from Amersham Pharmacia Biotech, and [*palmitoyl*-1-^{14}C]lysoPtdCho l-1-monopalmitoyl (55 mCi/mmol) from NEN Life Science Products. Scyphostatin was generously provided from Sankyo Co. and d, l-*threo*-1-phenyl-2-hexadecanoidylamino-3-morphiolino-1-propanol (PPMP) was from Matreya, Inc. Scyphostatin and PPMP were dissolved in DMSO at 10 mM and stored at –20°C until use. PF1350, a *P. falciparum* expression sequence tag (EST) clone, was generously provided by Dr. Debopam Chakrabarti (University of Central Florida, Orlando, FL).

Parasite Culture. *P. falciparum* parasite lines used are 3D7, Honduras-1 (6), Dd2 (28), HB3 (28), and FCR3 (29). 3D7 line was a generous gift from Dr. Masatsugu Kimura (Osaka City University, Osaka, Japan). HB3 and Dd2 lines were provided by Dr. Thomas E. Wellems (National Institutes of Health, Bethesda, MD). Parasite cells were routinely maintained as described previously (6, 27).

5′-Rapid Amplification of cDNA Ends. Parasite cells were isolated from the asynchronous culture of 3D7 line through 0.075% saponin/PBS (wt/vol) treatment. Total RNA was extracted from isolated parasites with Trizol (GIBCO BRL). First strand cDNA was synthesized from 200 ng total RNA with SMART™ 5′-Rapid Amplification of cDNA Ends (RACE) cDNA Amplification Kit (CLONTECH Laboratories, Inc.) by using the Superscript II reverse transcriptase (GIBCO BRL). PCR was conducted with Platinum™ Taq polymerase High Fidelity (GIBCO BRL) using the following conditions in a Peltier Thermal Cycler-200 (MJ Research, Inc.): one initial denaturation cycle at 94°C for 1 min, 10 cycles of denaturation at 94°C for 20 s, annealing and extension at 62°C for 5 min, 1 cycle of extension at 72°C for 10 min proceeded by 35 cycles at 91°C for 30 s, and at 70°C for 5 min, with a final extension cycle at 68°C for 10 min. The gene specific primer, 5′-GGCGCTTGTAATTGCGACCTATTAC-3′, was used together with universal primers provided in SMART™ RACE cDNA Amplification Kit.

Northern Blotting. Total RNA was extracted from parasite cells isolated from asynchronous cultures of 3D7, Honduras-1 and Dd2, or synchronized culture of HB3. For the harvest of ring-rich or trophozoite- and schizont-rich parasite cultures, tightly synchronized culture of HB3 line within 4 h life span (6) was further incubated for 12 or 27 h, respectively, and parasite cells were then isolated by saponin treatment. Extracted total RNA was fractionated on a 1.2% agarose/formaldehyde gel and transferred onto Nytran membrane (Schleicher & Schuell). The membrane was probed with a 744-bp PCR product encoding the internal region of *PfNSM* open reading frame (ORF), exposed to Fujifilm BAS imaging plate, and analyzed with MacBAS 1500 (Fuji Film Co.). For probe preparation, 5′-CCACACAAATGTAATAGGTCGC-3′ and 5′-GTGTTTATAATTATAATCTCTTTCG-3′ were used for PCR as forward and reverse primer, respectively.

Reverse Transcription PCR. Total RNA was extracted from parasite cells isolated from a synchronized culture of Honduras-1 line. Tightly synchronized culture of Honduras-1 line within 4 h life span was further incubated for 1, 20, 25, 30, or 36 h before saponin treatment. Reverse transcription (RT)-PCR was conducted with GIBCO BRL Superscript™ First-strand Synthesis System for RT-PCR using 50 ng total RNA. Target cDNA was

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Abbreviations used in this paper: EST, expression sequence tag; GST, glutathione-S-transferase; K_m, Michaelis constant; LCPL, lysocholinephospholipids; ORF, open reading frame; PAF, platelet-activating factor; PLC, phospholipase C; PPMP, d, l-*threo*-1-phenyl-2-hexadecanoidylamino-3-morphiolino-1-propanol; PtdCho, phosphatidylcholine; PtdSer, phosphatidylserine; RACE, rapid amplification of cDNA ends; RT, reverse transcription; SM, sphingomyelin; SMase, sphingomyelinase.

amplified by the same set of primers used for probe preparation. For control, primer sets that annealed to the conserved block 3 and 5 of merozoite surface antigen-1 gene (30) was used: 5′-TTCGTGCAAATGAATTAGACGTAC-3′ (forward primer) and 5′-GGATCAGTAAATAAACTATCAATGT-3′ (reverse primer). RNA samples from the different stages that were not treated with reverse transcriptase gave no PCR products.

Plasmid Constructs. PF1350C is a *P. falciparum* cDNA cloned in pBluescript® SK (31). For construction of *PfNSM*, ATGCCAAGAATAG sequence was added to 5′ terminus of PF1350C by PCR with PF1350C as template DNA, 5′-TAAGGATCCGCCACCATGCCAAGAATAGAAATATCAAAAGATGTAAAGC-3′ as forward primer, and 5′-TAAATGTACTAGGAATTCC-3′ as reverse primer. The amplified fragment was digested with BamHI and EcoRI, and the resulting 0.8 kbp fragment was ligated to a BamHI- and EcoRI-digested 3.7 kbp fragment from the PF1350C on pBluescript® SK.

The pGEX-PfNSM was constructed from a BamHI- and XhoI-digested 1.7 kbp fragment from *PfNSM*, transferred to the multi-cloning sites of pGEX-6P-2 (Amersham Pharmacia Biotech), with the resulting plasmid encoding a fusion protein of PfNSMase linked to glutathione-S-transferase (GST). To construct pGEX-PfNSM*d*(2/68), the sequence encoding the 2–68th amino acid residues of PfNSMase was deleted from pGEX-PfNSM.

Preparation of Cytosol and Membrane Fractions of Escherichia coli Cells Transfected with pGEX-PfNSM or pGEX-6P-2. Logarithmically growing cells of *E. coli* XL1-Blue strain (Stratagene) transfected with pGEX-PfNSM or pGEX-6P-2 were incubated in 250 ml Terrific broth containing 50 μg/ml ampicillin and 1 mM isopropyl-1-thio-β-d-galactopyranoside for 90 min at 33°C. Hereafter, manipulations were performed at 4°C or on ice. Cells harvested by centrifugation (1,500 *g*, 15 min) were washed with 20 ml of 10 mM Tris-HCl (pH 8.0) containing 0.1 M NaCl and 1 mM EDTA, and suspended with 10 ml of 25 mM Tris-HCl (pH 8.0) containing 50 mM glucose and 10 mM EDTA. Egg white lysozyme was added to the cell suspension at a final concentration of 100 μg/ml, and the mixture was incubated for 10 min. After addition of 10 ml HSEI buffer (10 mM Hepes-NaOH [pH 7.5] containing 0.25 M sucrose, 1 mM EDTA, and a protease inhibitor cocktail [EDTA-free Complete™ Protease Inhibitor; Roche]), the resulting mixture was sonicated five times with a probe-type sonicator at 20 W for 10 s The sonicated sample was centrifuged (1500 *g*, 15 min), and the supernatant fluid was recovered as cell lysate. Cell lysate was centrifuged (10^5 *g*, 1 h), and the recovered supernatant and precipitated fractions were designated as cytosol and membrane fractions, respectively. The precipitated membranes were suspended in HSEI buffer. Fractions were stored at −80°C.

Assays of PLC Activities toward Various Phospholipids. Assay of neutral SMase dependent on Mg^{2+} and PtdSer was routinely performed under detergent-mixed micelle conditions as described previously (27). In brief, enzyme sources were incubated in 50 μl of 50 mM Hepes-NaOH (pH 7.5) containing 10 mM MgCl$_2$, 1 mM bovine brain PtdSer, 0.1% Triton X-100, 0.2% β-octylglucoside, and 10 μM [*choline-methyl*-^{14}C]SM at 37°C for 30 min. Reaction was stopped by addition of 0.8 ml chloroform/methanol (2/1, vol/vol). After phase separation, the radioactivity partitioned to the upper aqueous phase was measured. For PtdCho-PLC assay, 10 μM [*choline-methyl*-^{14}C]PtdCho in place of [*choline-methyl*-^{14}C]SM was used as a substrate. Under detergent-free assay condition, radioactive SM or PtdCho was dispersed in deionized water with a probe-type sonicator before use.

For other PLC assays, enzymatic reaction was proceeded under detergent-free condition using 10 μM corresponding radioactive substrate, l-1-[*palmitoyl*-1-^{14}C]lysoPtdCho, 1-*O*-[^{3}H]*octadecyl* PAF or 1-*O*-[^{3}H]*octadecyl* lysoPAF. During the phase separation, 20 μl monopalmitoylglycerol, 1-hexadecyl-2-acetyl-glycerol or 1-octadecyl-*rac*-glycerol (1 mg/ml each in chloroform) was added as a carrier. Lipids recovered in organic phase were developed on TLC with a solvent system of diethylether/acetic acid (200/1, vol/vol), and separated radioactive lipids were analyzed with a BAS2000 or BAS1800 image analyzer, followed by radioactive measurements of corresponding product with liquid scintillation counter. In all PLC assays described above, background activities from the enzyme-free controls, in which vehicle buffers instead of the enzyme sources were added, were routinely subtracted from the activities of samples containing enzyme sources.

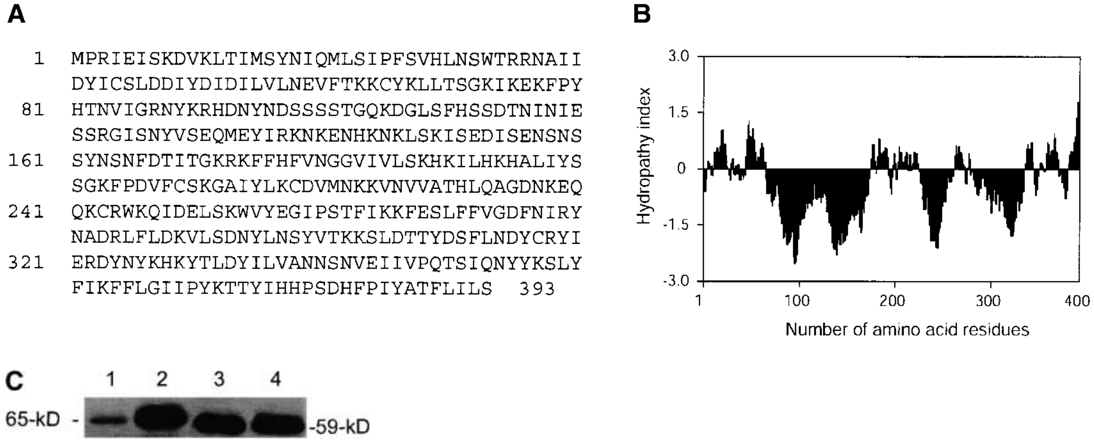
Growth Inhibition of P. falciparum Cell Lines. In vitro susceptibility of *P. falciparum* lines to compounds tested was determined by [^{3}H]hypoxanthine uptake assay (32) using either a modified standard medium or a serum-free medium supplemented with various concentrations of the test compounds. The modified standard medium is a basic medium (27) without hypoxanthine containing 10% human serum, whereas the serum-free medium was prepared as follows: lipid-free BSA powder was dissolved in a hypoxanthine-free basic medium to adjust to 30 μM, and then supplemented with 7.4 mM hypoxanthine and 600 μM intact BSA solutions (6) at 0.1% and 10% (vol/vol), respectively. Parasite cultures synchronized to ring stage with 5% d-sorbitol (33) was adjusted to ∼0.5% parasitemia at 3% hematocrit in 100 μl volume per well. After 24 h incubation, 25 μl of either medium containing 20 μCi [^{3}H]hypoxanthine was added into each well and the culture was incubated for another 24 h before harvesting. The parasitemia was also determined microscopically (6).

Effect of Compounds on the Intraerythrocytic Development of P. falciparum. A tightly synchronized culture of *P. falciparum* Honduras-1 line within 4 h life span was prepared, and adjusted to ∼1% rings at 3% hematocrit in the modified serum-free medium with 370 μM hypoxanthine and 0.1% DMSO. At the time indicated, the medium was replaced with prewarmed medium containing either 1 μM scyphostatin or 5 μM PPMP in place of 0.1% DMSO and the parasite culture was maintained until 52 h. Giemsa-stained thin-blood smears were prepared from the culture at various times. To prevent nutrient deprivation, the medium was changed every 12 h.

Miscellaneous. Anti-PfNSMase antiserum was prepared by immunization of rabbits with synthetic peptides corresponding to the 134–152th amino acid residues of PfNSMase which were conjugated with KLH, and then the anti-PfNSMase antibodies were purified by affinity chromatography with an antigen peptide-linked resin. Western blotting was performed using the rabbit anti-PfNSMase antibodies as the primary antibody and horseradish peroxidase-conjugated goat anti–rabbit IgG (Pierce Chemical Co.) as the secondary antibody, and visualized with an ECL Kit (Amersham Pharmacia Biotech). Membrane fraction from the *P. falciparum* parasite and bovine brain was prepared as described previously (27). Protein concentrations were determined by the method of Lowry et al. (34), using BSA as the standard.

Results

Identification of PfNSM, a Plasmodial Gene Putatively Encoding an SMase. To identify the gene for plasmodial SMase, in silico sequence analysis was performed by searching the NCBI Malaria Genetics and Genomics databases (http://www.ncbi.nlm.nih.gov/Malaria) through the BLAST program with the amino acid sequence of *Staphylococcus aureus* SMase (GenBank/EMBL/DDBJ accession no. X13404) as a query. One EST clone PF1350C (GenBank/EMBL/DDBJ accession no. N97823) and DNA contig fragment (GenBank/EMBL/DDBJ accession no. AC005505) in chromosome 12 of *P. falciparum* was found to encode a candidate gene for SMase. DNA sequence of the 1.7 kbp PF1350C clone matches the corresponding genomic sequence perfectly except for the poly(A) tail. However, comparison of the genome and PF1350C sequences suggested that the longest ORF encoded in PF1350C was not full-length, because there is a Met codon in the genomic sequence at 13 bp upstream of the 5′ terminus of PF1350C in the same frame of the ORF. To know the structure of the full-length ORF, we performed 5′-RACE experiment by using the 3D7 line of *P. falciparum* as RNA source. The nucleotide sequence analysis of the DNA fragment obtained from 5′-RACE reveals a ~450 base noncoding sequence before the first Met codon of the putative ORF. Taken together, the 2.18 kbp cDNA sequence reconstituted from PF1350C and the 5′-RACE product was regarded as a cDNA encoding the full-length ORF (GenBank/EMBL/DDBJ accession no. AF323591). For simplicity, we refer to the locus in the genomic DNA encoding this cDNA as the *PfNSM*. Sequence analysis of *PfNSM* and its cDNA predicts that *PfNSM* is an intronless gene



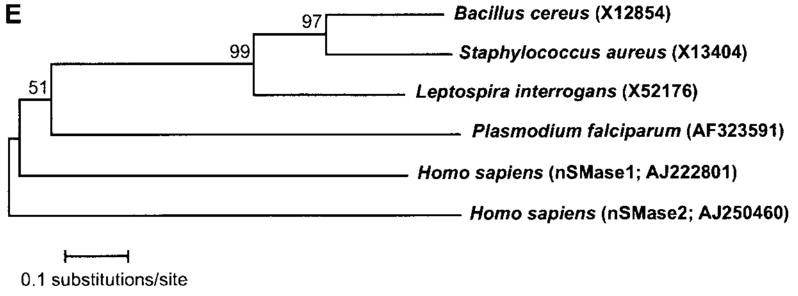


Figure 1. Primary structure of *P. falciparum* SM/LCPL-PLC. (A) Deduced amino acid sequence of *P. falciparum* SM/LCPL-PLC. (B) Hydropathy profile of the deduced *PfNSM* product obtained using Kite-Doolittle algorithm with a window size of nineteen (reference 35). (C) Intracellular distribution of GST-PfNSMase expressed in *E. coli*. After centrifugation at 10^5 *g* for 1 h, *E. coli* cell lysates transfected with pGEX-PfNSM (lanes 1 and 2) or pGEX-PfNSM*d*(2/68) (lanes 3 and 4), were analyzed by Western blotting using anti-PfNSMase antibodies. Lanes 1 and 3, supernatant; lanes 2 and 4, pellet fraction. 2 μg protein was loaded in each lane. (D) Multiple sequence alignment of the SMases from different species. Sequence alignments were performed with CLUSTAL W (reference 47) and refined with GeneAlign program (reference 48) using amino acid sequences in the regions where significant homology was observed through the dotplot analysis. The regions in each amino acid sequence used are PFAL (*P. falciparum*), 182–278; BCER (*B. cereus*), 129–225; SAUR (*S. aureus*), 134–230; LINT (*L. interrogans*), 179–275; HSAP1 (*Homo sapiens*), 81–181; and HSAP2 (*Homo sapiens*), 406–513. Amino acid residues conserved in all species and more than four species are highlighted in black and gray, respectively. (E) Phylogenetic tree of SMases. The multiple sequence alignment shown in D was used to make the phylogenetic tree through neighbor-joining algorithm with MEGA version 2.2 (reference 38). Scale bar indicates the number of substitutions per site. Bootstrap values are percentages of 1,000 replications and are shown at the nodes. GenBank/EMBL/DDBJ accession no. for each SMase is in parentheses. UPGMA also gave similar phylogenetic tree with similar topology.

that encodes a protein of 393 amino acid residues (Fig. 1 A) with a molecular mass of 46,013 and has 80% A/T content typical of plasmodial genes (31).

Although SMase activity detected in isolated parasites is membrane-bound (27), hydropathy profile (35) predicts that neither signal sequence nor transmembrane region was present in the deduced *PfNSM* product (Fig. 1 B). Western blot analysis showed that the *PfNSM* product was largely associated to membrane fraction in *E. coli* cells when expressed as a fusion protein with GST, but that its membrane-bound nature was significantly abrogated by deletion of the 2–68th amino acid residues of the *PfNSM* product (Fig. 1 C). These results suggest that a moderately hydrophobic region encompassing 12–60 amino acid residues of the *PfNSM* product (Fig. 1 B) plays a role in the binding of this protein to membranes, although other regions might also be involved in membrane binding. Motif analysis programs, PSORT (36) and SignalP (37), predict that the *PfNSM* product has no cleavable signal peptide sequence. Phylogenetic analysis conducted using MEGA version 2.2 (38) with the multiple sequence alignment (Fig. 1 D) suggests that the *PfNSM* sequence is more similar to SMase sequences of bacteria than those of human (Fig. 1 E), although *P. falciparum* is a eukaryote.

Stage-specific Expression of PfNSM. Northern blotting experiment with three different parasite lines (Honduras-1, 3D7, and Dd2) showed a single hybridized signal at 2.2 kb (Fig. 2 A), indicating that the *PfNSM* is indeed transcribed in intraerythrocytic parasite cells, and ubiquitously expressed in various *P. falciparum* strains. The 2.2 kb size detected in Northern blotting is consistent with that of the *PfNSM* cDNA described above.

Using a synchronized HB3 parasite line, a 2.2 kb hybridized signal was likewise detected specifically in trophozoite- and schizont-stage rich sample (Fig. 2 B) in a separate Northern blot. However, no distinct band was detected in ring-stage rich sample, though the amount of total RNA loaded was increased from 4 to 10 μg (Fig. 2 B). To further

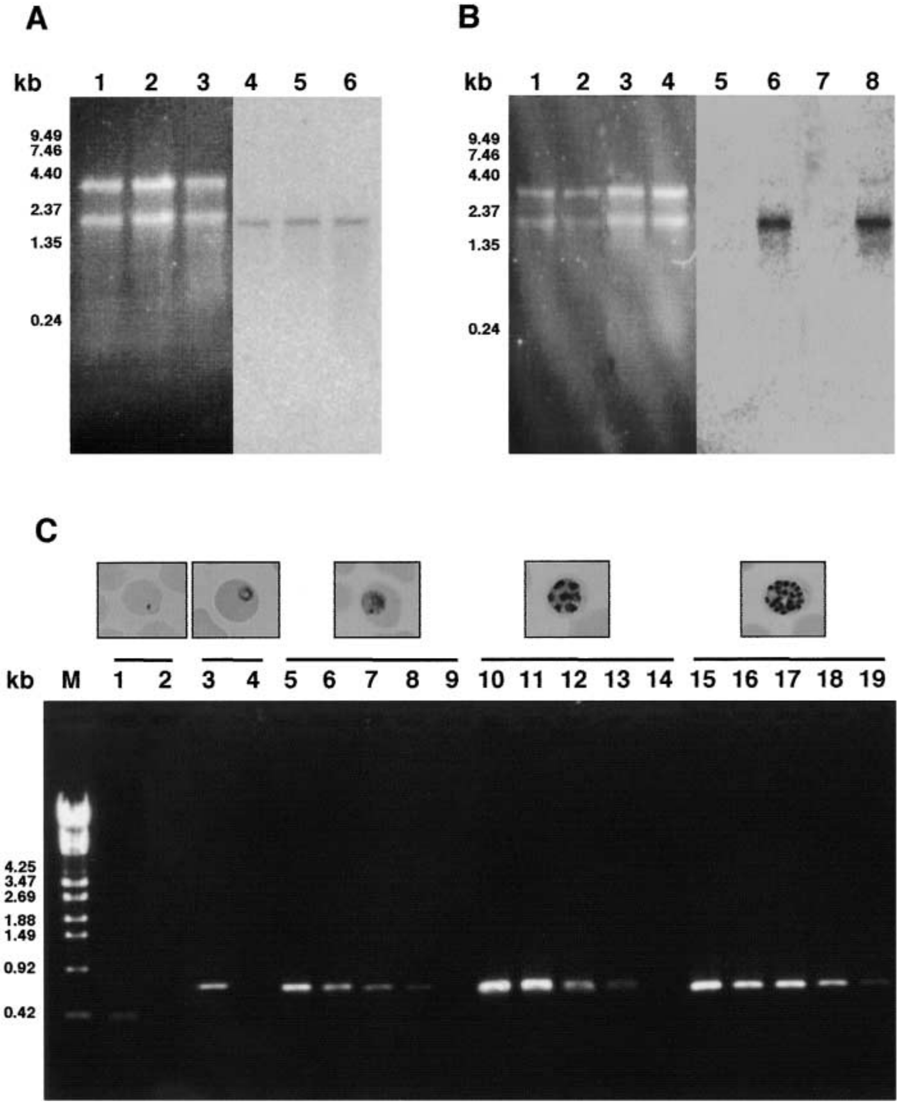


Figure 2. Stage-specific transcription of *PfNSMase* in the intraerythrocytic parasite *P. falciparum*. (A) Northern blotting of asynchronous parasite culture. 10 μg of total RNA prepared from asynchronous cultures of three *P. falciparum* lines was loaded in each lane. The ethidium bromide-stained gel (lanes 1–3) shows the comparable loadings of RNA. Lane 1 and 4, 3D7; lane 2 and 5, Honduras-1; lane 3 and 6, Dd2. The position of the standard RNA marker (GIBCO BRL) is shown at the left. The stage distribution for each line is indicated: 3D7, 63% ring, 26% trophozoite, 11% schizont; Honduras-1, 71% ring, 22% trophozoite, 7% schizont; and Dd2, 48% ring, 26% trophozoite, 26% schizont. (B) Northern blotting of synchronous parasite culture. 4 μg (lanes 1, 2, 5, and 6) and 10 μg (lanes 3, 4, 7, and 8) of total RNA prepared from different stages of tightly synchronized culture of HB3 line was loaded. The ethidium bromide–stained gel (lanes 1–4) indicates the comparable loadings of RNA from the different stages at two different concentrations. Lanes 1, 3, 5, and 7, ring-rich culture (99% ring, 1% trophozoite, 0% schizont); lanes 2, 4, 6, and 8, trophozoite- and schizont-rich culture (0% ring, 86% trophozoite, 14% schizont). The position of the standard RNA marker is shown at the left. (C) RT-PCR experiment. PCR products obtained from different concentrations of first strand cDNA from various stages of tightly synchronized parasite culture of Honduras-1 line were analyzed in 0.8% agarose gel. Lanes 1 and 2, 3 and 4, 5–9, 10–14, and 15–19 are products obtained from ring, young trophozoite, mature trophozoite, schizont, and segmented-schizont, respectively. Parasite morphology at each stage used is shown on top. Dilution factors of the first strand cDNA solution are as follows: lanes 1, 2, 3, 5, 10, and 15, no dilution; lanes 4, 6, 11, and 16, 10-fold; lanes 7, 12, and 17, 100-fold; lanes 8, 13, and 18, 1,000-fold; lanes 9, 14, and 19, 10,000-fold.

specify the stage when the transcription of *PfNSM* occurs, we performed RT-PCR by using the total RNA from ring, young and mature trophozoite, schizont, and segmented schizont stage parasite cultures. A distinct PCR product of the expected size could be detected in the sample starting from young trophozoite stage to the later stages (Fig. 2 C). In contrast, no PCR product could be detected in the ring stage sample, although the quality and quantity of the RNA sample used was enough to amplify a control PCR product corresponding to the conserved region of merozoite surface protein-1 gene (Fig. 2 C, lane 1). The level of the PCR product for *PfNSM* increased as intraerythrocytic development proceeded reaching a maximum at segmented schizont stage.

Expression of PfNSM Gene in E. coli. To determine whether *PfNSM* encodes an SMase, we constructed a recombinant plasmid designated pGEX-PfNSM, in which the coding sequence of *PfNSM* gene was linked to GST sequence proceeded by a bacterial expression unit. Sonicated lysates of *E. coli* cells transfected with pGEX-PfNSM or the empty vector pGEX-6P-2 were assayed for the activity of SM hydrolysis under various conditions (Table I). When assayed under acidic conditions, no activity was detected even in the presence of Zn^{2+}, which activates an isoform of mammalian acid SMase (20). In contrast, under assay conditions for a plasmodial neutral SMase dependent on Mg^{2+} and anionic phospholipids (27), substantial activity was detected in the lysate from cells transfected with pGEX-PfNSM, but not with the empty vector. The activity of the pGEX-PfNSM-transfected cell lysate was reduced to around 1/100 when the exogenous PtdSer was omitted from the assay. Water-soluble radioactivity liberated from [*choline-methyl*-^{14}C]SM comigrated with a standard phosphocholine in TLC (data not shown), indicating that *PfNSM* encodes an SMase belonging to the PLC type. For simplicity, we tentatively refer to the specific products of *PfNSM* and pGEX-PfNSM as PfNSMase and GST-PfNSMase, respectively.

Table I. *SM Hydrolysis in Lysates of E. coli Cells Transfected with pGEX-PfNSM or the Empty Vector*

Assay conditions	SM hydrolyzed pGEX-PfNSM	 Empty vector
	nmol/mg protein/h	
pH 4.8	<0.05	<0.05
pH 4.8, 10 mM $ZnCl_2$	<0.05	<0.05
pH 7.5, 10 mM $MgCl_2$	0.061 ± 0.023	<0.05
pH 7.5, 10 mM $MgCl_2$, 1 mM PtdSer	5.69 ± 0.29	<0.05

Sonicated lysates (25 μg protein) of *E. coli* cells transfected with pGEX-PfNSM or the empty vector pGEX-6P-2 were incubated with [*choline-methyl*-^{14}C]SM in 0.1 M sodium acetate buffer (pH 4.8) containing 0.1% Triton X-100 in the presence or absence of 10 mM $ZnCl_2$, or in 50 mM Hepes-NaOH buffer (pH 7.5) containing 0.1% Triton X-100 and 10 mM $MgCl_2$ in the presence or absence of 1 mM bovine brain PtdSer at 37°C for 30 min. The water-soluble radioactivity released from [^{14}C]SM was measured as described under Materials and Methods. The mean values ± SD from triplicate experiments are shown.

Almost 100% SMase activity in lysate of *E. coli* cells transfected with pGEX-PfNSM was precipitated at high-speed centrifugation. It is likely that the sedimentation of the SMase activity was due to the membrane-bound nature of this enzyme, rather than the formation of an insoluble aggregation ("inclusion body") in *E. coli*, because SMase activity in the precipitate fraction was efficiently solubilized by 1% Triton X-100, a nonionic mild detergent (data not shown). When the membrane fraction of *E. coli* expressing GST-PfNSMase was used as the enzyme source, the SMase activity was linear for at least 30 min, was directly proportional to protein concentrations up to 0.2 mg/ml, showed an optimum pH at around 7.5, had an apparent Michaelis constant (K_m) value for SM of ~90 μM, and was Mg^{2+} dependent with a half saturation concentration of ~3 mM (data not shown).

GST-PfNSMase Is Capable of Hydrolyzing LysoPtdCho and LysoPAF under Detergent-Free Conditions. When assayed in the presence of 0.1% Triton X-100, hydrolysis of [^{14}C]lysoPtdCho by GST-PfNSMase was negligible (data not shown). However, under detergent-free assay conditions, pronounced activity to release radioactive monopalmitoylglycerol from [*palmitoyl*-1-^{14}C]lysoPtdCho was detected in the membrane fraction of *E. coli* cells transfected with pGEX-PfNSM, but not with the empty vector (Fig. 3 A). The lysoPtdCho-PLC activity was Mg^{2+} dependent like SMase activity. The K_m value for lysoPtdCho was determined to be ~25 μM based on double reciprocal plots (data not shown). Surprisingly, the lysoPtdCho-PLC activity in intact membranes did not require the addition of exogenous PtdSer. LysoPtdCho-PLC activity was also detected in the membrane fraction from parasite cells under detergent-free conditions, but not under detergent-mixed micelle conditions (data not shown).

Hydrolysis of 10 μM [*palmitoyl*-1-^{14}C]lysoPtdCho was strongly competed by sphingosylphosphocholine ("lysoSM") as well as nonradioactive lysoPtdCho (Fig. 3 B), suggesting that PfNSMase also recognized sphingosylphosphocholine. The activity was moderately competed by PAF and lysoPAF, but unaffected by lysoPtdSer or lysophosphatidylinositol up to 50 μM (Fig. 3 B). Under detergent-free conditions, the membrane fraction expressing GST-PfNSMase hydrolyzed lysoPAF, but not PAF (Fig. 3 A). No PLC activity toward PtdCho or phosphatidylinositol was observed regardless of the presence of 0.1% Triton X-100 in the assay buffer (unpublished data).

Susceptibility of GST-PfNSMase to Scyphostatin. Previously we have shown that neutral SMase activity associated with the membrane fraction from isolated *P. falciparum* parasite was inhibited by scyphostatin (27), a compound that was originally found as an inhibitor of mammalian neutral SMase (39, 40). The *PfNSM* encodes the enzyme exhibiting PLC activity toward SM and LCPL. We tested

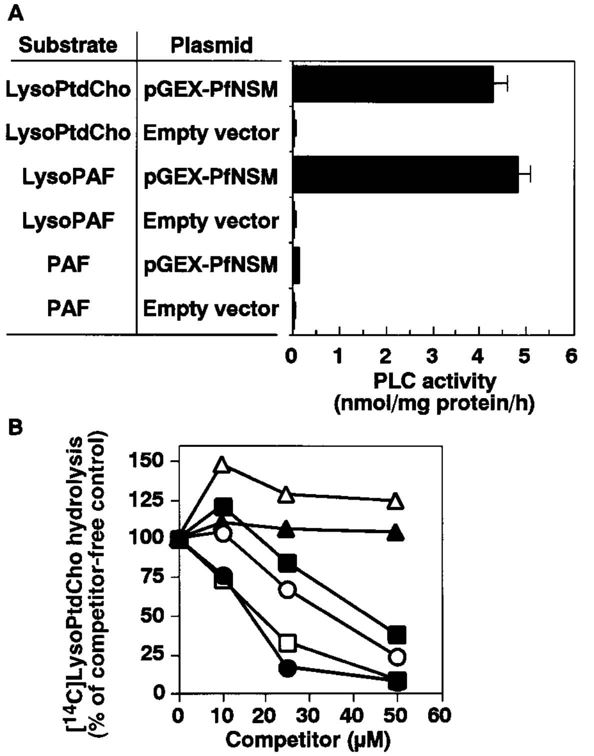


Figure 3. LCPL-PLC activity in the membrane fraction of *E. coli* cells transfected with pGEX-PfNSM. (A) LysoPtdCho-, lysoPAF-, and PAF-PLC activities. Membranes (5 µg protein) from *E. coli* cells transfected with the indicated plasmids were incubated in 50 µl HM buffer (50 mM Hepes-NaOH, pH 7.5, and 10 mM $MgCl_2$) containing 10 µM of various radioactive substrates at 37°C for 30 min. The amount of hydrolyzed substrates was determined as described under Materials and Methods. The data are means ± SD from three experiments. (B) Competition of lysoPtdCho-PLC activity with various lipids. The membrane fraction from pGEX-PfNSM-transfected *E. coli* (5 µg protein) was incubated in 50 µl of 50 mM Hepes-NaOH (pH 7.5) containing 10 mM $MgCl_2$, 10 µM [*palmitoyl-1-*^{14}C]lysoPtdCho, and various concentrations of nonradioactive competitors at 37°C for 30 min. The radioactivity of monopalmitoylglycerol produced was determined as described under Materials and Methods. The data shown are the percentages of the mean activity determined in the absence of competitors. Filled circles, lysoPtdCho; open circles, lysoPAF, filled squares, PAF; open squares, sphingosylphosphocholine; filled triangles, lysoPtdSer; open triangles, lysophosphatidylinositol.

if the PLC activity of this enzyme was inhibited by scyphostatin. Scyphostatin inhibited PLC activity toward SM in a dose-dependent manner with an ID_{50} value of ~3 µM (Fig. 4), similar to the SMase activity detected in isolated parasite membranes (27). Likewise, scyphostatin inhibited PLC activities toward lysoPtdCho and lysoPAF with ID_{50}s of 3–5 µM (Fig. 4). The ID_{50} value of scyphostatin to neutral SMase activity of bovine brain membrane was ~15 µM (Fig. 4), which is ~5-fold higher than that of plasmodial SMase.

Scyphostatin Inhibits the Intraerythrocytic Proliferation of P. falciparum. We then examined the effect of scyphostatin on the in vitro culture of *P. falciparum* to implicate the PLC activities associated with *PfNSM* product to the intraerythrocytic proliferation of parasite cells. For this, three parasite lines were used: FCR3 line resistant to chloroquine, Honduras-1 line resistant to pyrimethamine and cycloguanyl, and 3D7 line sensitive to those conventional antimalarial drugs. As shown in Fig. 5 A, the growth of all parasite lines tested was inhibited by scyphostatin in a dose-dependent manner with 50% inhibition observed at 8, 6, and 6.5 µM for 3D7, Honduras-1, and FCR3, respectively. ID_{50}s for the parasite growth are close to those for SM/LCPL-PLC activities (Fig. 4). PPMP, a ceramide-related compound that inhibits the SM synthase activity as well as intraerythrocytic proliferation of *P. falciparum* (7, 27), showed 50% growth inhibition at ~6 µM (data not shown). Difference in ID_{50} values from the previous report (7) may be partly due to the purity of PPMP and/or the culture condition used.

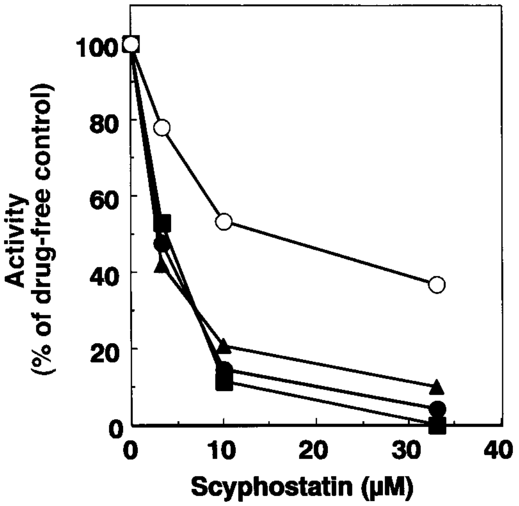


Figure 4. Effect of scyphostatin on SM/LCPL-LPC activity. Membrane fraction prepared from GST-PfNSMase-expressing *E. coli* and bovine brain (0.5 mg protein/ml each) was incubated with various concentrations of scyphostatin in HSEI buffer for 30 min on ice. SMase activity of plasmodial (filled circles) and mammalian enzyme (open circles) was determined in the presence of 0.1% Triton X-100, and activities of lysoPtdCho-PLC (filled squares) and lysoPAF-PLC (filled triangles) were determined under detergent-free conditions as described under Materials and Methods. The values of the activity are shown as the percentage of the control activity determined in the absence of the drug.

Selective Effect of Scyphostatin on the Intraerythrocytic Development of P. falciparum. To examine the effect of scyphostatin on the intraerythrocytic development of *P. falciparum*, we monitored the morphological changes of tightly synchronized parasites within 4 h life span, which were treated with scyphostatin at different stages. PPMP was used for comparison. Honduras-1 line was chosen as the representative parasite since all parasite lines tested so far showed similar ID_{50} values. A serum-free medium supplemented with 1 µM scyphostatin or 5 µM PPMP (~ID_{90}s from the microscopic assay in Fig. 5 B) was used to obtain a reproducible intraerythrocytic development. As shown in Fig. 6, in an inhibitor-free control culture medium containing vehicle solvent (0.1% DMSO), the parasite developed normally and entered the next cycle at 52 h with ~5.6% parasitemia of newly formed ring (lane 1). When 1 µM scyphostatin was added at the initial ring stage (0 h), parasite cells devel-

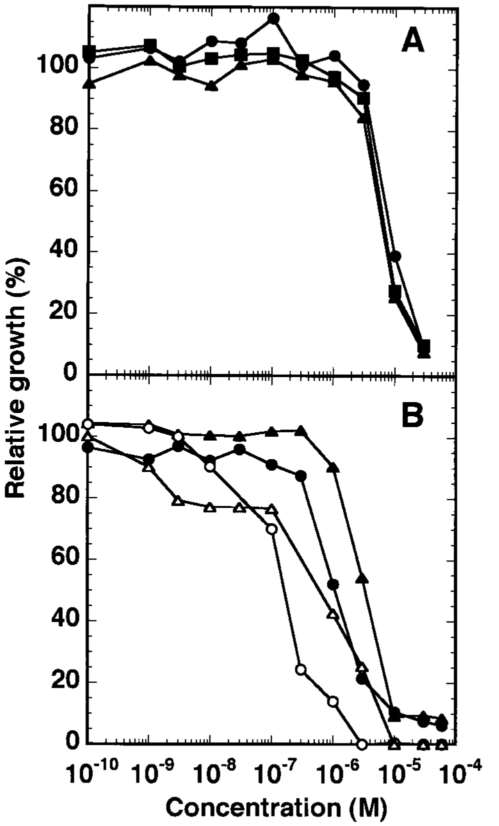


Figure 5. Parasite growth inhibition by scyphostatin. (A) In vitro susceptibility of parasite lines to scyphostatin in a standard medium. The values are expressed as the percentage of the [^{3}H]hypoxanthine incorporation into parasites treated with scyphostatin over those without treatment. The DMSO content in the assay media did not exceed 0.6%, which did not show any effect on the [^{3}H]hypoxanthine incorporation into parasites. The mean values of triplicates from two independent experiments were used for each plot. Filled circles, 3D7; filled triangle, Honduras-1; filled squares, FCR3. (B) In vitro susceptibility of Honduras-1 line to scyphostatin (circles) or PPMP (triangles) in a serum-free medium was examined through either [^{3}H]hypoxanthine incorporation assay (filled symbols) or microscopic assay (open symbols).

oped to mature trophozoites, but could not develop to schizonts, exhibiting a slightly bigger but less stained trophozoite-like morphology. These trophozoite-like structures neither developed further nor formed new ring stage parasites even after 52 h cultivation (lane 2). A similar effect on parasite morphology was observed when the scyphostatin was added at the early-trophozoite stage (24 h; lane 4). Addition of scyphostatin at the mid- and late-trophozoite stages (28 and 32 h) resulted in an increase in the parasitemia of newly formed rings to 1–3% (lanes 6 and 8). Conversely, when added at the schizont (36 h) and segmented schizont (40 h) stages, the parasitemia of rings at 52 h reached the level comparable to that of the inhibitor-free control (lanes 10 and 12). These results indicate that 1 μM scyphostatin has no or little effect on the stage progression from ring to trophozoite as well as that from schizont to the next ring stage, but impaired specifically the maturation of trophozoite into schizont. Interestingly, the time for scyphostatin to start exerting the effect on the intraerythrocytic development is consistent with the time to start transcribing the *PfNSM* (Figs. 2 C and 6).

Similar to scyphostatin, PPMP impaired the stage progression from trophozoite to schizont, though, in this culture condition, the period PPMP exerts its effect was slightly broader than previously reported (7). The parasitemia of newly formed ring was severely affected when PPMP was added from 24–36 h (lanes 5, 7, 9, and 11). Unlike scyphostatin, the typical hemozoin formation was not observed in parasitized erythrocytes upon the addition of PPMP at ring stage (0 h; lane 3). These results imply that PPMP impairs the stage progression from ring to trophozoite as well.

Discussion

In this study, we identified a plasmodial gene (named *PfNSM*) encoding a neutral SMase. As far as we know, this is the first report in the molecular cloning of a protozoan SMase. When GST-PfNSMase, a fusion protein of the *PfNSM* product with GST, was expressed in *E. coli*, the cells produced SMase activity liberating phosphocholine from SM. Enzyme properties of GST-PfNSMase are consistent in various respects with those of SMase detected in *P. falciparum*-infected erythrocytes and isolated parasites. They require Mg^{2+} and anionic phospholipids for SMase activity in vitro, and are efficiently inhibited by scyphostatin (27; Fig. 4). The optimum pH of the activity is around pH 7.5, and the apparent K_m for SM is 50–100 μM. Further characterization of GST-PfNSMase expressed in *E. coli* has revealed that the plasmodial SMase is capable of hydrolyzing LCPLs such as lysoPtdCho and lysoPAF in vitro (Fig. 3). The membrane fraction from *P. falciparum* parasites also retained this LCPL-PLC activity (data not shown). Henceforth, we refer to the *PfNSM* product as a plasmodial SM/LCPL-PLC.

LCPL-PLC activity of the plasmodial SM/LCPL-PLC was hardly detected in the presence of 0.1% Triton X-100, although the same enzyme source showed a high activity of SMase in the presence of the detergent. It is currently unknown as to why PLC activities to different substrates by the same enzyme are differently affected by detergents. Because both activities of SMase and LCPL-PLC that are associated with the protein encoded in the *PfNSM* are similarly inhibited by scyphostatin (Fig. 4), it is unlikely that this enzyme has two catalytic sites and each of them mediates one of two reactions. Efficient hydrolysis of lysoPtdCho and lysoPAF under detergent-free condition was also observed in a mammalian SMase (nSMase-1; reference 19). Not only the plasmodial SM/LCPL-PLC but also bacterial SMases and a mammalian SMase (nSMase-1) are capable of hydrolyzing lysoPtdCho (19, 41; Fig. 3 A). However, the ratios of lysoPtdCho-PLC activity determined under detergent-free conditions to SMase activity determined under the mixed micelle conditions are 0.5 or more for the plasmodial SM/LCPL-PLC and mammalian nSMase-1, but

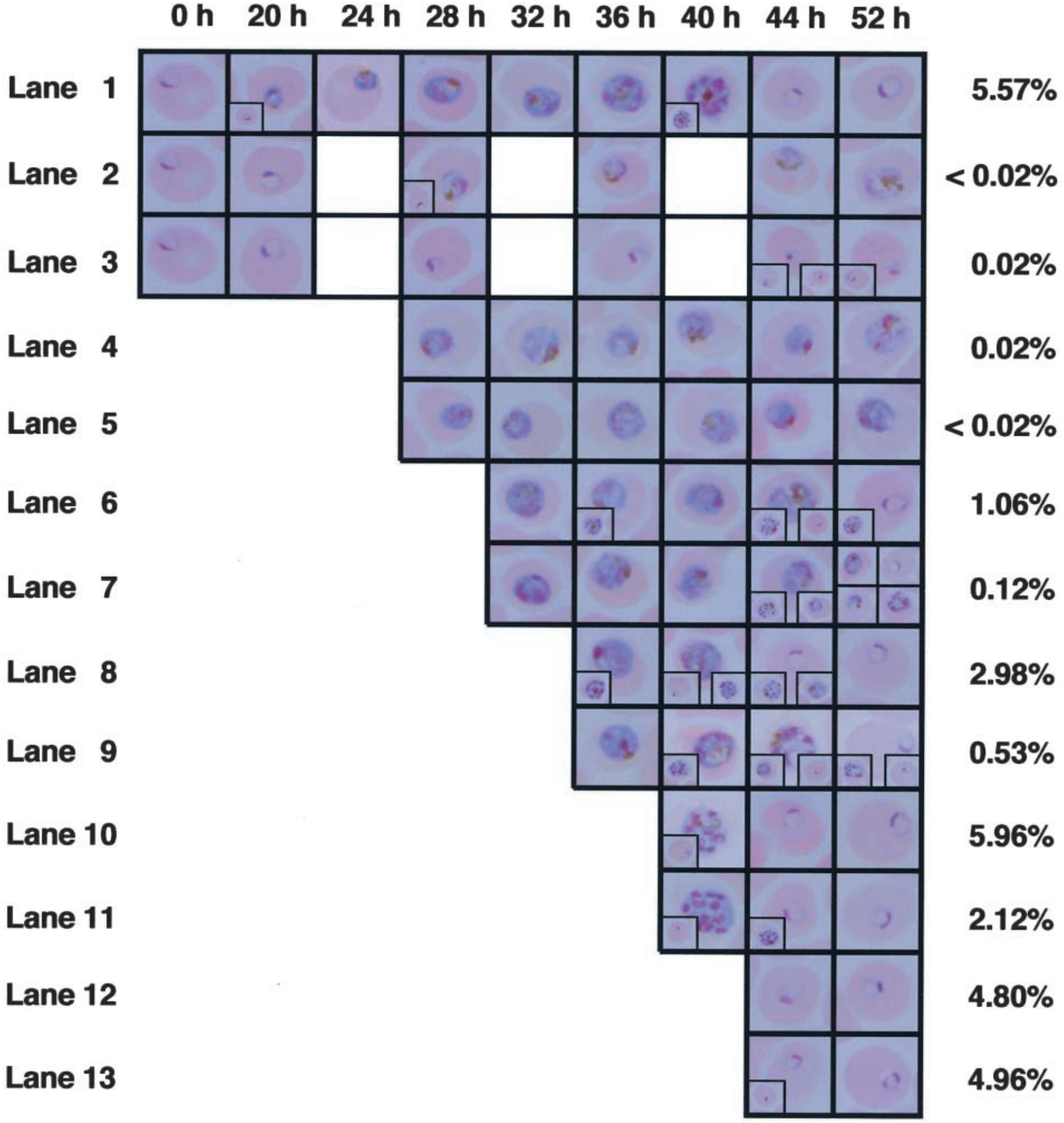


Figure 6. Effect of scyphostatin and PPMP on the intraerythrocytic development of *Plasmodium falciparum*. Parasite cells were treated with 1 μM scyphostatin or 5 μM PPMP at different stages. Lane 1, control medium containing 0.1% DMSO; lanes 2, 4, 6, 8, 10, and 12, medium containing 1 μM scyphostatin; lanes 3, 5, 7, 9, 11 and 13, medium containing 5 μM PPMP. The parasite culture was transferred from the control medium into the medium containing inhibitor in place of DMSO at 24 h (lanes 4 and 5), 28 h (lanes 6 and 7), 32 h (lanes 8 and 9), 36 h (lanes 10 and 11), and 40 h (lanes 12 and 13). Each panel shows the major morphology of the intraerythrocytic parasite in each treatment at the indicated time. Inset shows the other morphology that when observed, comprises >50% of the major one. However, in some occasions, no major morphology was observed. The percentage indicated at the left is the parasitemia of newly formed rings at 52 h. Mean values from duplicate slides are shown, results are reproducible based on two independent experiments.

∼0.01 for bacterial SMases (this study, and references 19 and 41).

The ID_{50} values of scyphostatin and PPMP for the parasite growth in a serum-free medium determined by the [^{3}H]hypoxanthine incorporation assay are 1.2 and 3.5 μM, respectively, whereas by microscopic assay those of scyphostatin and PPMP are 0.2 and 0.6 μM, respectively (Fig. 5 B). In the microscopic analysis, parasitized erythrocyte exhibiting an abnormal trophozoite-like morphology and a tiny ring- or early trophozoite-like morphology observed in the culture treated with scyphostatin (at 52 h in lane 2, Fig. 6) and PPMP (at 52 h in lane 3, Fig. 6), respectively, were evident at 0.1–1 μM scyphostatin and 0.3–3 μM PPMP. These morphologies, however, could not be observed at 96 h with concentrations >0.1 μM scyphostatin and >2 μM PPMP in a similar assay condition (data not shown). The parasites exhibiting such abnormal morphologies might be committed to cell death, but still retain metabolic activity with respect to hypoxanthine incorporation into nucleic acid, thereby giving differences in ID_{50} values depending on assay methods (Fig. 5 B).

Transcription of *PfNSM* is initiated from the young trophozoite stage and becomes most active in segmented schizonts (Fig. 2 C), and scyphostatin impairs specifically the maturation of trophozoite into schizont during the intraerythrocytic development of *P. falciparum* (Fig. 6). This good correlation of the time for the *PfNSM* transcription and the developmental stage on which scyphostatin exerts its effect leads us to suggest that the inhibition of the intraerythrocytic development of the parasite results from the inhibition of the plasmodial SM/LCPL-PLC activity. Lauer et al. (7) indicated that PPMP inhibits SM synthase activity associated with *P. falciparum* cells, and also inhibits proliferation of the parasite in vitro. PPMP does not inhibit plasmodial SM/LCPL-PLC activity, while scyphostatin does not inhibit SM synthase activity, indicating that the target enzymes of the two inhibitors differ (27). The stage progression influenced by scyphostatin overlaps with that of PPMP (progression from trophozoite to schizont), although the stage influenced by scyphostatin is narrower. Parasites treated with both inhibitors at the trophozoite stage show similar morphological changes (Fig. 6). We assume that a certain functional linkage to maintain the normal intraerythrocytic development at least in the maturation of trophozoite into schizont of *P. falciparum* might exist between SM synthase, the target of PPMP, and SM/LCPL-PLC, the target of scyphostatin. The knowledge obtained in this study together with previous studies (7, 27) would emphasize the importance of the enzymes involved in SM metabolism as rational targets for malaria chemotherapy, although the development of specific inhibitors to plasmodial enzymes is needed in this direction. Interestingly, the ID_{50} value of scyphostatin to bovine brain SMase is ∼5-fold higher than that of plasmodial SMase (Fig. 4), indicating the possibility that derivation of scyphostatin leads to more selective or specific inhibitors to plasmodial SM/LCPL-PLC.

Although physiological and pathological roles of the SM/LCPL-PLC in *P. falciparum* cells remain unclear, there are several possible roles. First, as we have been proposing (reference 27, and above), the plasmodial SM/LCPL-PLC might degrade host-derived SM to supply the parasite with ceramide, which would modulate the cell cycle progression of intraerythrocytic parasites and/or would be used for resynthesis of SM within parasitized erythrocytes. In this regard, we have proposed the possibility that the host-cell SM is accessible to the plasmodial SM/LCPL-PLC through the tubovesicular membrane structure (27). This proposal is supported by the recent finding demonstrated by Lauer et al. (42) that degradation of externally supplied sphingomyelin analogs in parasitized-erythrocytes is inhibited when cells were treated with PPMP which impaired the formation of the tubovesicular membrane structure. Note that the stage-specific transcription of *PfNSM* gene correlates well with the C_6-NBD-SM hydrolyzing activity levels (this study, and reference 42). Second, the plasmodial SM/LCPL-PLC might degrade host-derived lysoPtdCho to supply the parasites with phosphocholine and/or monoacylglycerol for their efficient intraerythrocytic growth since lysoPtdCho is abundant in the human plasma, especially in forms associated with lipoproteins (43, 44). Third, the plasmodial SM/LCPL-PLC might be implicated to detoxify LCPLs. It has been recently shown that human lipoproteins treated with bee venom phospholipase A_2 are highly toxic to the in vitro culture of *P. falciparum* (45). As the most abundant type of phospholipids associated with lipoproteins is PtdCho (43, 44), treatment of lipoproteins with phospholipase A_2 is predicted to enhance greatly the level of lysoPtdCho. If so, the plasmodial SM/LCPL-PLC could be involved in the detoxification of potentially harmful lysoPtdCho. Finally, a previous study showed that the level of plasma lysoPAF in patients with severe malaria is reduced to ∼10% from the normal level, and suggested that the reduction of the plasma lysoPAF level was due to the increased production of PAF in malaria patients (46). Our finding in this study raises an alternative, but not mutually exclusive, explanation that an enhanced degradation of lysoPAF through plasmodial SM/LCPLs-PLC results to a decrease in the plasma lysoPAF level.

Success in the identification of the gene for *P. falciparum* SM/LCPL-PLC and production of the plasmodial SM/LCPL-PLC in *E. coli* as a recombinant protein could facilitate elucidation of the physiological functions of this enzyme and create invaluable tools that can be exploited to localize this enzyme within parasitized erythrocytes, to study for the regulation of gene expression, and to perform transfection experiments.

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