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A novel palmitoyl acyl transferase controls surface protein palmitoylation and cytotoxicity in *Giardia lamblia*

María C. Touz,[†] John T. Conrad and Theodore E. Nash*
From the Laboratory of Parasitic Diseases, NIAID, NIH, Bethesda, MD 20892, USA.

Summary

The intestinal protozoan parasite *Giardia lamblia* undergoes surface antigenic variation whereby one of a family of structurally related variant-specific surface proteins (VSPs) is replaced in a regulated process by another antigenically distinct VSP. All VSPs are type I membrane proteins that have a conserved hydrophobic sequence terminated by the invariant hydrophilic amino acids, CRGKA. Using transfected *Giardia* constitutively expressing HA-tagged VSPH7 and incubated with radioactive [^{3}H]palmitate, we demonstrate that the palmitate is attached to the Cys in the conserved CRGKA tail. Surface location of mutant VSPs lacking either the CRGKA tail or its Cys is identical to that of wild-type VSPH7 but non-palmitoylated mutants fail to undergo complement-independent antibody specific cytotoxicity. In addition, membrane localization of non-palmitoylated mutant VSPH7 changes from a pattern similar to *rafts* to non-*rafts*. Palmitoyl transferases (PAT), responsible for protein palmitoylation in other organisms, often possess a cysteine-rich domain containing a conserved DHHC motif (DHHC-CRD). An open reading frame corresponding to a putative 50 kDa *Giardia* PAT (gPAT) containing a DHHC-CRD motif was found in the *Giardia* genome database. Expression of epitope-tagged gPAT using a tetracycline inducible vector localized gPAT to the plasma membrane, a pattern similar to that of VSPs. Transfection with gPAT antisense producing vectors inhibits gPAT expression and palmitoylation of VSPs *in vitro* confirming the function of gPAT. These results show that VSPs are palmitoylated at the cysteine within the conserved tail by gPAT and indicate an essential function of palmitoylation in control of VSP-mediated signalling and processing.

Accepted 30 August, 2005. *For correspondence. E-mail tnash@niaid.nih.gov; Tel. (+1) 301 496 6920; Fax (+1) 301 402 2689. [†]Present address: Instituto Mercedes y Martín Ferreyra, Friuli 2434, CP 5000, Córdoba, Argentina.

Introduction

Variant-specific surface proteins (VSPs) are a family of related proteins that coat the entire surface of *Giardia* trophozoites including the flagella. *Giardia* undergoes antigenic variation by changing the constitutively expressed VSP for another similar but antigenically different one. VSPs are proteins with frequent CXXC motifs and are transported through the ER by their signal peptides (McCaffery and Gillin, 1994). To date, all known VSPs are membrane proteins that contain similar 25-amino acid transmembrane domain and a highly conserved CRGKA cytoplasmic tail (Mowatt *et al*., 1994). This conserved C-terminus may function as a membrane-anchoring domain (Papanastasiou *et al*., 1996) or as a sorting motif to the plasma membrane (Marti *et al*., 2003a). However, because neither deletions nor mutations of CRGKA altered its surface localization (Touz *et al*., 2003), its function remains unknown.

Many VSPs are palmitoylated (Papanastasiou *et al*., 1997a; Hiltpold *et al*., 2000). In other organisms, the most common type of palmitoylation is S-palmitoylation, which consists of the addition of palmitate (C16:0) to cysteine residues by way of thioester linkages. Similar to VSPs, almost all proteins that undergo this modification are membrane associated (Linder and Deschenes, 2003). No well-defined consensus sequence has been identified for palmitoylation other than the requirement for the presence of a cysteine residue that is usually located close to the transmembrane/cytoplasmic tail (Smotrys and Linder, 2004). Analysis of the conserved VSPs C-terminus revealed two cysteine residues that could serve as palmitoylation sites (Fig. 1).

Only three *Saccharomyces cerevisiae* proteins that promote palmitoylation have been identified including effector of Ras function (Erf2), ankyrin-repeat-containing protein (Akr1) and the SNARE protein Ykt6 (Lobo *et al*., 2002; Roth *et al*., 2002; Veit, 2004). Erf2 and Ykt6 have a common Asp-His-His-Cys-cysteine-rich domain (DHHC-CRD) motif that likely confers palmitoyl transferase (PAT) activity. The biological function of palmitoylation is incompletely understood but appears to be important in protein trafficking, cell signalling and localization to lipid *rafts* that are subdomains of the plasma membrane rich in cholesterol and sphingolipids.

Very little is known about palmitoylation in *Giardia*. Prior studies suggested that palmitoylation occurs within the C-

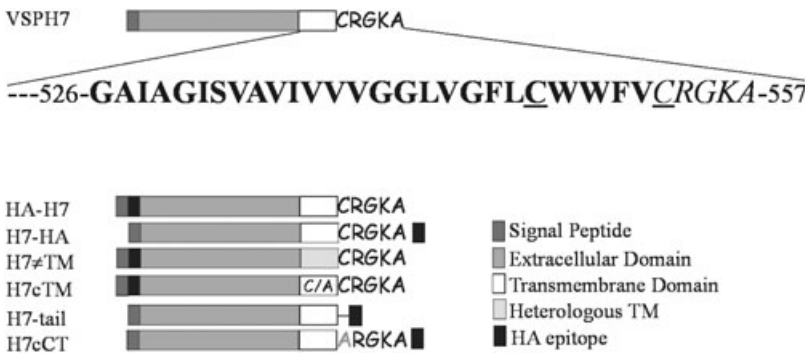

Fig. 1. Schematic representation of VSPH7 and transgenic VSPH7 proteins. VSPH7 ORF contains a signal peptide, an extracellular domain, a transmembrane domain (TM) and a cytoplasmic tail. The enlargement shows the sequence of conserved C-terminus of VSPH7; in bold represents the transmembrane domain TM; in italic represents the cytoplasmic tail and the putative sites of palmitoylation are underlined. HA-H7 has the VSPH7 gene plus the HA epitope sequence at the N-terminus and H7-HA has the HA epitope sequence at the C-terminus. H7≠TM stands for VSPH7 with the TM of the Encystation-specific cysteine protease TM (Touz *et al*., 2003). H7cTM denotes a point mutation of the Cys for Ala inside VSPH7 TM. H7-tail is a tail-deleted VSPH7 and H7cCT is VSPH7 containing a point mutation of the Cys residue of its cytoplasmic tail. Each vector possesses a puromycin acetyl transferase gene for selection. All genes are expressed under the control of the tubulin promoter.

terminus but neither the precise location(s) nor function(s) are known (Papanastasiou *et al*., 1997b). In the present study we confirm that VSPs are palmitoylated, determine the site of palmitoylation, characterize the enzyme responsible for this activity, and determine the biological consequences of this modification.

Results

VSPH7 localization on the plasma membrane

VSPH7 is an 85 kDa variant-specific surface protein of *Giardia* clone GS/M-H7 that possesses a single transmembrane domain and a conserved CRGKA cytoplasmic tail and covers the entire cell surface, including the flagella (Nash and Mowatt, 1992). VSPH7 is not genetically codified nor expressed in *Giardia* clone WB/1267, allowing detection of the extracellular portion of VSPH7 at the surface of transfected WB trophozoites using VSPH7-specific mAb G10/4 (Nash *et al*., 1995; Elmendorf *et al*., 2001). To track the fate of the carboxyl and amino termini of H7, an HA epitope was added to the N-terminus (HA-H7) or C-terminus (H7-HA) and transfected into WB/1267 trophozoites (Fig. 1). Transgenic expressed proteins were detected with anti-VSPH7 G10-4 mAb or anti-HA mAb. Immunofluorescence assays showed that both HA-H7 or H7-HA were localized to the plasma membrane similar to native VSPH7 (Fig. 2A) (Nash and Mowatt, 1992; Touz *et al*., 2003) and that the addition of the HA epitope did not change its surface localization in agreement with our previous report (Touz *et al*., 2003). Immunoblot experiments using G10-4 mAb, which recognizes the extracellular region of VSPH7, showed an ≈85 kDa band (corresponding to the whole protein) and 55 kDa band (corresponding to the N-terminus) in GS/H7 trophozoites expressing native H7 or WB/1267 trophozoites transfected with HA-H7 or H7-HA (Fig. 2B, left panel). On the other hand, immunoblotting using anti-HA mAb detected the 85 kDa band (whole protein) and a 55 kDa band (N-terminus) for HA-H7 and an 85 kDa (whole protein) and a 40 kDa band (C-terminus) for H7-HA (Fig. 2B, right panel). In this assay, the native VSPH7 was not detected by anti-HA mAb. Thus, the presence of the 85 and 55 kDa bands recognized by G10-4 mAb (corresponding to the N-terminus) and a 40 kDa lower band (corresponding to the C-terminus) demonstrates that VSPH7 is cleaved at the C-terminus as was suggested for other VSPs (Papanastasiou *et al*., 1996). The higher intensity of the 55 kDa band in HA-H7 and H7-HA using G10-4 mAb indicates that this process may be exaggerated in transfected cells. There may also be different degrees of recognition by these two mAbs in immunoblotting; still, a higher and a lower band can be seen (Fig. 2B).

In order to define the influence of VSP palmitoylation in VSPH7 localization, a series of modifications were introduced into the C-terminal domain of VSPH7: replacement of the VSPH7's TM domain (H7≠TM) for the TM of the Encystation-specific Cysteine Protease TM (Touz *et al*., 2003), deletion of VSPH7 cytoplasmic tail (H7-tail), and point mutations of the putative palmitoylation sites at the TM (H7cTM) and cytoplasmic tail (H7cCT) (Fig. 1). When these transgenic proteins were assayed by immunofluorescence, all were localized at the plasma membrane showing that the TM/cytoplasmic tail domains or palmitoylation does not influence VSPH7 subcellular localization to the plasma membrane (Fig. 2C). In addition, immunofluorescence assays using unfixed-non-permeabilized cells and G10-4 mAb showed surface localization of native VSPH7 and all transgenic VSPH7

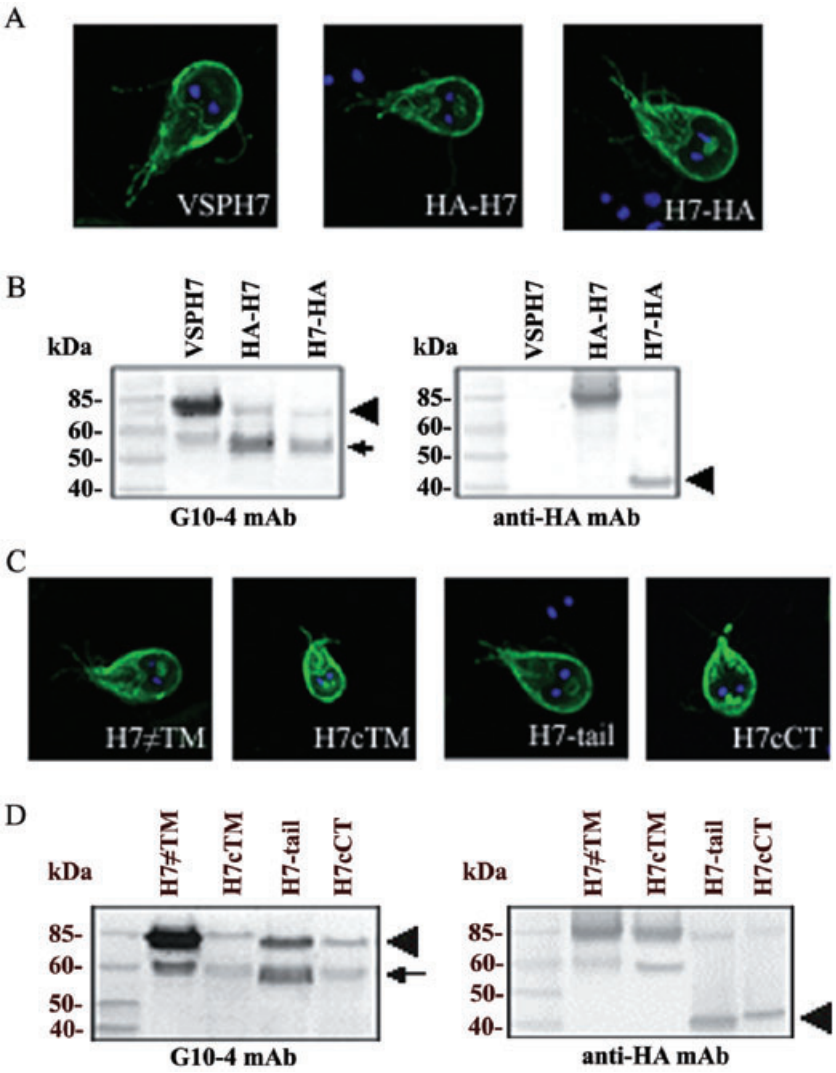

Fig. 2. VSPH7 and its modified versions localize to the plasma membrane in *Giardia* trophozoites.
A. Immunofluorescence using G10-4 mAb that specifically recognizes the extracellular domain of VSPH7 shows the surface localization of VSPH7, HA-H7 and H7-HA by confocal microscopy.
B. Immunoblotting using G10-4 mAb (left panel) demonstrates a double band for VSPH7, HA-H7 and H7-HA corresponding to the entire protein (arrowhead) and the N-terminal portion (arrow). Using anti-HA mAb in immunoblot assay (right panel), the cleaved C-terminal domain (arrowhead) can be detected in *Giardia* expressing the H7-HA transgenic protein.
C. The transgenic proteins H7≠TM, H7cTM, H7-tail and H7cCT are also detected on the surface of transfected *Giardia* trophozoites by using G10-4 mAb in immunofluorescence assays and confocal microscopy.
D. Immunoblotting using G10-4 mAb (left panel), show the whole protein band (arrowhead) and the N-terminal portion of the proteins (arrow). Using anti-HA mAb the 85 kDa band (whole protein) and the 55 kDa band (N-terminus) are detected for H7≠TM and H7cCT while the 85 kDa and the 40 kDa band (C-terminus, arrowhead) are detected for H7-tail and H7cCT (Fig. 2D, right panel).

confirming the correct expression and folding of transgenic VSPs at the surface of the parasite (data not shown). Immunoblotting using G10-4 mAb, showed a dominant high molecular weight band for H7≠TM, H7cTM, H7-tail and H7cCT, as was expected (Fig. 2D, left panel). Anti-HA mAb that recognizes the N-terminus of H7≠TM and H7cTM and the C-terminus of H7-tail and H7cCT detected the 85 kDa band (whole protein) and a 55 kDa band (N-terminus) and the 85 kDa (whole protein) and the 40 kDa band (C-terminus) respectively (Figs 1 and 2D, right panel).

VSPH7 palmitoylation site

To determine the specific palmitic acid attachment site (Fig. 1), [9,10–^{3}H]palmitic acid uptake was determined in GS/H7 cells (VSPH7) as well as in various VSPH7 mutants (HA-H7, H7≠TM, H7cTM, H7-HA, H7-tail, or H7cCT) transfected into WB/1267. Immunoprecipitation (IPP) of VSPH7, HA-H7, H7≠TM, H7cTM, H7-HA using G10-4 mAb followed by fluorography, detected a strongly labelled band. In contrast labelling was not found in the H7-tail and H7cCT samples (Fig. 3A, G10-4). Immunoblotting, using biotin-labelled G10-4 mAb, showed that even though protein expression after IPP varied among samples they are clearly comparable (Fig. 3A, G10-4). In addition, a similar result was seen when anti-HA mAb was used for IPP showing that HA-H7, H7≠TM, H7cTM and H7-HA are palmitoylated but H7-tail and H7cCT are not. The 40 kDa palmitoylated band of H7-HA corresponding to the C-terminus of the protein was also observed. Native VSPH7 and VSP1267 were not immunoprecipitated because they do not carry the HA epitope. Immunoblotting using alkaline phosphatase-labelled anti-HA mAb showed the protein expression for each sample (Fig. 3A, anti-HA). Non-transfected WB/1267 served as a negative control. It was reported that palmitoylation is influenced by the sequence surrounding the cysteine to

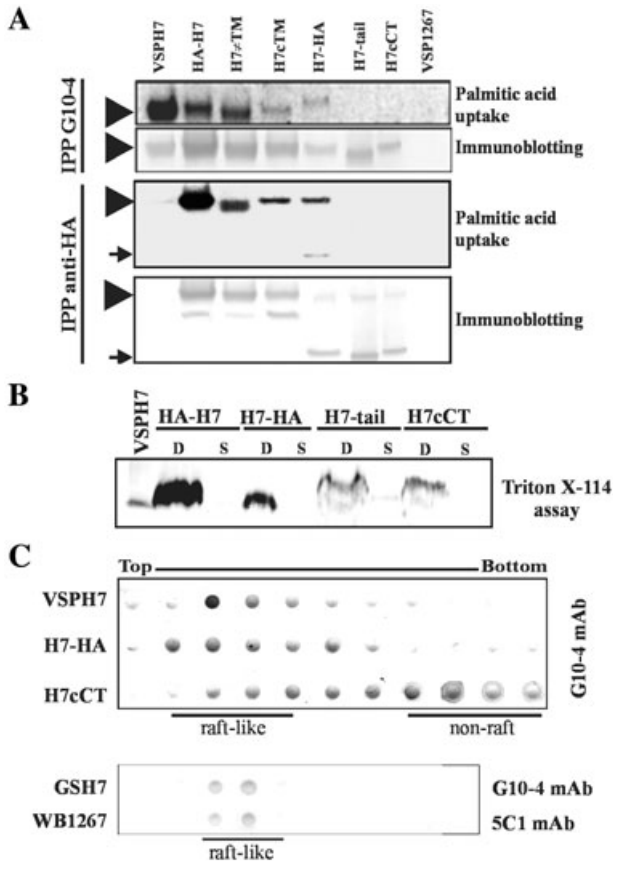

Fig. 3. The cytoplasmic tail of VSPH7 is palmitoylated and associated with *raft*-like domains.
A. Palmitate uptake assay using G10-4 mAb for IPP (G10-4, upper panel) shows the labelled high molecular weight band (85 kDa) for the native VSPH7 and the transgenic proteins HA-H7, H7≠TM, H7cTM and H7-HA (arrowhead) that is absent for H7-tail, H7cCT, as well as the negative control VSP1267. Using anti-HA mAb for IPP, the palmitoylated upper band (85 kDa) is seen for HA-H7, H7≠TM, H7cTM and H7-HA (anti-HA, upper panel, arrowhead) and an additional 40 kDa band corresponding to the C-terminus of H7-HA (arrow). No palmitoylation is observed for H7-tail and H7cCT, same as was observed with G10-4 IPP. Immunoblotting shows the protein expression after IPP (G10-4 and anti-HA, bottom panel). VSPH7 and VSP1267 were used as negative controls.
B. Phase separation assay results using Triton X-114 show that the palmitoylated VSPH7, H7-HA, the non-palmitoylated H7-tail, and H7cCT partition in the membrane fraction. D, detergent phase; S, soluble phase.
C. Solubilization in Triton X-100, sucrose gradient separation, and dot-blot assays show that the native VSPH7 and the control H7-HA are found in *rafts*-like domains while the non-palmitoylated H7cCT is mainly found in not-*raft* fractions (top panel). VSP1267 is also found in *raft*-like domains similar to VSPH7 (bottom panel).

be palmitoylated (Ponimaskin and Schmidt, 1998; Bijlmakers and Marsh, 2003) and that may explain the decrease in palmitoylation of the H7≠TM, H7cTM and H7-HA mutants. Nevertheless, the lack of palmitoylation of H7-tail and H7cCT mutants strongly suggest that the specific site of palmitoylation of VSPH7 is the cysteine located within its cytoplasmic tail.

VSPH7 in raft-like domains

Next, we examined possible changes in VSPH7 membrane association in palmitoylated and non-palmitoylated VSPH7 by phase separation using Triton X-114. Transgenic VSPH7 without the tail or the palmitoylated cysteine partitioned to the detergent phase similar to native protein or the control, verifying that loss of palmitoylation does not affect membrane association (Fig. 3B). The fact that in other systems membrane protein modification with palmitate impacts the lateral distribution of proteins on the plasma membrane by targeting them to lipid *rafts* prompted us to investigate if this were the case for VSPs. To determine whether native VSPH7, control H7-HA, and non-palmitoylated H7cCT proteins were present in lipid *rafts*, Triton X-100 lysates of GS/H7 and trophozoites transfected with related H7 mutants and controls were analysed by sucrose density gradient centrifugation. Fractions were analysed for the presence of VSPH7 by immunoblotting using mAb G10-4. VSPH7, the control H7-HA, and the mutants H7≠TM and H7cTM (not shown), partitioned into the Triton X-100-insoluble, low buoyant density lipid *raft* fractions of the gradient (*raft*-like fractions) while H7cCT, which lacks the palmitoylation modification, shifted mostly to non-*raft* fractions (Fig. 3C, top panel) similar to the non-palmitoylated form of RGS16 (regulator of G-protein 16) (Hiol *et al*., 2003). With the same approach using WB trophozoites highly expressing VSP1267, we also demonstrate that VSP1267 behaves as VSPH7 partitioning to *raft*-like fractions (Fig. 3C, bottom panel). This result suggests that palmitoylation was important in targeting VSPs to lipid *rafts*. Additional experiments where more protein was analysed showed a very low presence of VSPH7 and VSP1267 in non-*raft* domains (data not shown), suggesting that, even though VSPs seems to be mainly palmitoylated, the cycle between palmitoylation/depalmitoylation occurs also in *Giardia*.

Cytotoxicity assay using VSPH7 variants

Antibodies to the extracellular portion of specific VSPs caused detachment and aggregation of trophozoites, and exhibited complement-independent cytotoxic effect towards the parasite (Aggarwal *et al*., 1989; Stager *et al*., 1997). One of the possible effects of lack of palmitoylation is loss of antibody-mediated cytotoxicity. Transductants selected by fluorescence-activated cell sorter (FACS) expressing 100% of mutated H7, non-mutated tagged-H7, or native VSPH7, were exposed to anti-GS antisera, anti-H7 mAb, or a control mAb and the number of trophozoites surviving or killed determined. WB clone 100% expressing VSP1267 was used as negative control. Immediately after addition of G10-4 mAb (specific for VSPH7), mouse polyclonal antiserum (anti-GS/VSP pAb, raised against GS/H7

Table 1. Cytotoxicity assay at 24 h post addition of specific anti-VSP antibodies.

Cells 24 h	Without Ab	G10-4 mAb	Anti-GS/VSPs pAb	8G8 mAb	Progeny
WB1267	–	–	–	–	VSPH7-negative
GS/H7	–	Immobilization Aggregation Detachment Killing	Immobilization Aggregation Detachment Killing	–	VSPH7-negative
H7-HA	–	Immobilization Aggregation Detachment Killing	Immobilization Aggregation Detachment Killing	–	VSPH7-negative
H7-tail	–	–	–	–	VSPH7-positive
H7cCT	–	–	–	–	VSPH7-positive

Cells expressing the palmitoylated VSPH7 and H7-HA show a significant cytotoxic effect after addition of the anti-VSPH7 mAb G10-4 or the anti-GS/VSPs polyclonal antibodies. Trophozoites expressing the non-palmitoylated H7-tail and H7cCT, as well as VSP/1267 (negative control) are not affected by antibody addition. Analysis of surviving trophozoites shows that the progenies of GS/H7 and H7-HA are VSPH7-negative while for H7-tail and H7cCT are VSPH7 positive. 8G8 is a non-VSP-specific mAb. The dash (–) means no effects. Assays were performed in triplicate and scored by persons unaware of the contents of the wells.

Giardia clone), or anti-CWP2 8G8 mAb (Lujan *et al*., 1995), no effect was observed compared with trophozoites not exposed to antibody (not shown). However, at 3 h, low cell immobilization and aggregation was observed for GS/H7 and H7-HA when G10-4 mAb or anti-VSP pAb was added (not shown). After 24 h, immobilization, aggregation, detachment and complement-independent cytotoxicity was observed only for GS/H7 and H7-HA in the presence of G10-4 mAb and anti-VSP pAb (Table 1). The strong cytotoxic effect of G10-4 mAb and anti-VSP pAb on *in vitro* cultivated *Giardia* trophozoites is illustrated in Fig. 4. The number of adherent viable cells, compared with cells without addition of antibodies, showed that both GS/H7 and H7-HA had a cell survival = 5% after 24 h. Similar effect was observed when transgenic cells expressing H7≠TM or H7cTM were used (data not shown). Conversely, recovery of WB/1267, H7-tail, or H7cCT viable cells after 24 h showed no cytotoxicity compared with controls (Fig. 4). In addition, progeny analysis revealed that surviving GS/H7 and H7-HA trophozoites were VSPH7 negative while the surviving for H7-tail and H7cCT trophozoites were 100% VSPH7 positive (Table 1). Negative controls (WB/1267 cells, no addition of antibody, or addition of an 8G8 non-specific mAb) failed to show immobilization, aggregation, detachment, or killing as expected. These findings strongly suggest that VSP palmitoylation and *raft* localization are involved in signalling and cell survival in *Giardia*.

Identification of Giardia *palmitoyl transferase (gPAT)*

Palmitoyl transferase or protein acyltransferase (PAT) participates in the enzymatic addition of palmitate to cysteine residues. We used two well described PATs, Erf2/Erf4 and Akr1 (Lobo *et al*., 2002; Roth *et al*., 2002), to perform BLAST searches for homologous *Giardia* genes. Sequence analysis identified a protein (*Giardia* PAT – gPAT; GenBank™ Accession number EAA36893) that possesses 28%, 37% and 35% identities with PAT Erf2 and the DHHC proteins Y0L003c (yeast) and DHHC-PAT (*Cryptosporidium*) respectively (Fig. 5A). gPAT shares several characteristics with the PATs such as numerous transmembrane domains (gPAT has five TMs), the DHHC-CRD (Asp-His-His-Cys-cysteine-rich domain), hypothesized to be a PAT domain located between the second and the third TM, and its localization at the plasma membrane (Fig. 5A–C) (Lobo *et al*., 2002; Roth *et al*., 2002). In addition, using the same search strategy, we found two other *Giardia* protein sequences that are similar to gPAT and Erf2, that

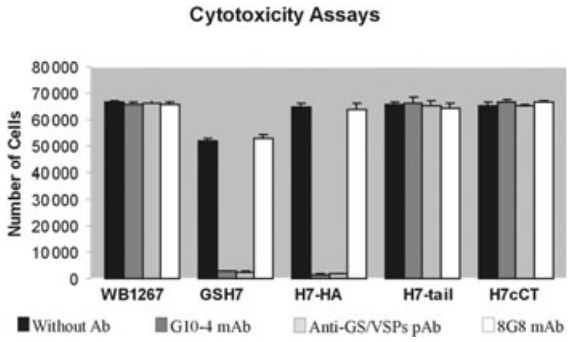

Fig. 4. Cytotoxicity assays of *Giardia* trophozoites at 24 h post addition of anti-VSPH7 specific antibodies. *Giardia* trophozoites were cultured without antibiotic selection with mouse polyclonal antiserum or mAbs (1:20). The effects of antibody (immobilization, aggregation, detachment and killing) were scored at 24 h by estimating the number of adherent viable parasites. GS/H7 and H7-HA cells show a survival = 5% after 24 h after addition of the VSPH7-specific G10-4 mAb or anti-VSPs pAb. Conversely, H7-tail and H7cCT cells are not affected by addition of these antibodies. Note that cells without treatment, WB/1267 cells (negative control), and the use of the non-VSP specific mAb 8G8 do not exhibit any cytotoxicity in all samples tested. The data represent the total number of surviving parasites. Results are the means ± SE of three independent experiments.

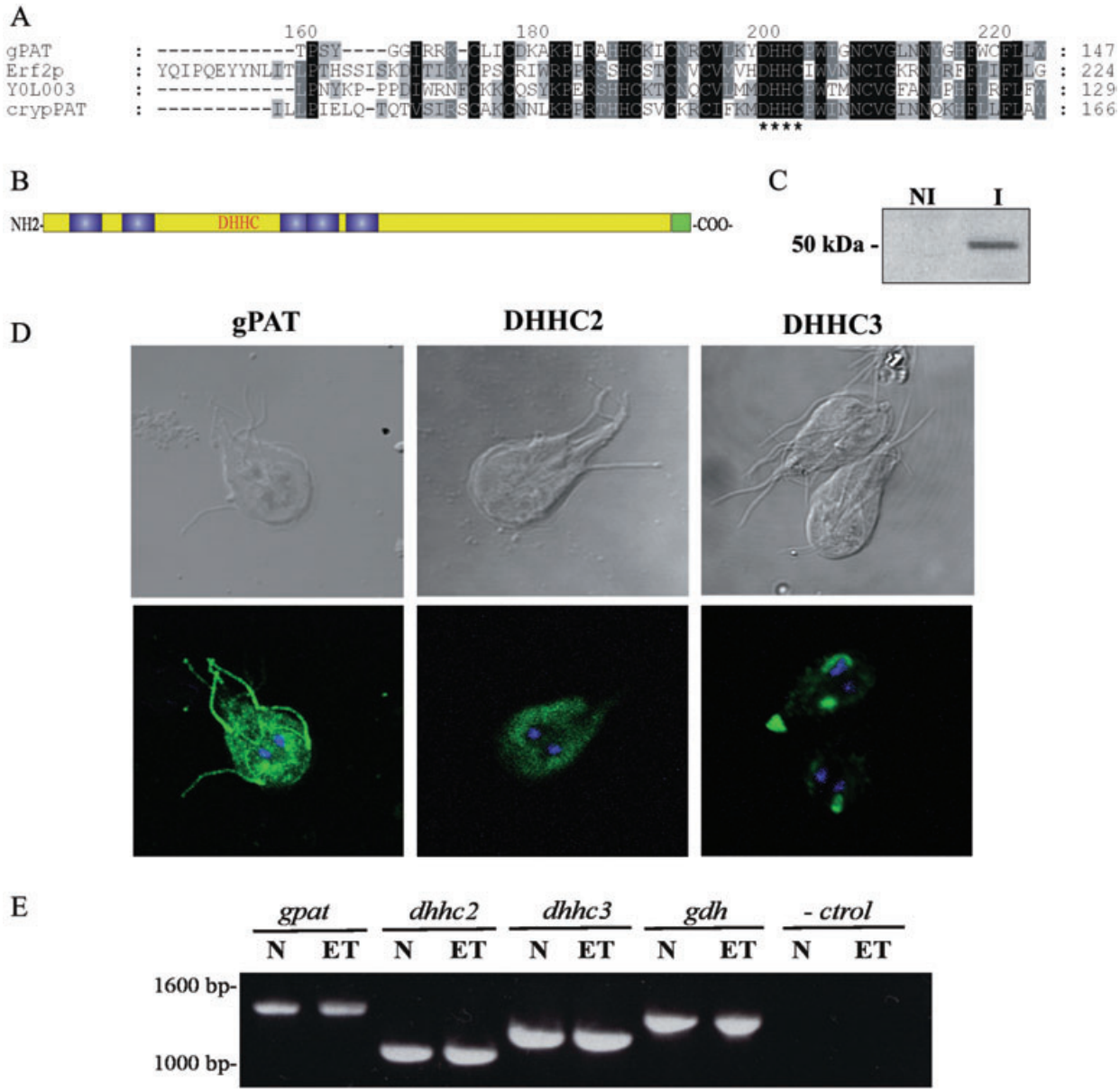

Fig. 5. gPAT is a DHHC protein located at the plasma membrane.
A. Alignment of gPAT with other described PATs shows homology in the sequence adjacent to the DHHC motif.
B. Schematic representation of the transgenic gPAT containing five transmembrane domains (blue) and the V5-tag added at the C-terminus (green).
C. Immunoblotting detects a 50 kDa band in cells tetracycline-induced (I) to express gPAT by using anti-V5 mAb. No band is observed in non-induced cells (NI).
D. Immunofluorescence assay (bottom panels) shows that gPAT is localized to the trophozoite plasma membrane (using anti-V5) while the DHHC2 and DHHC3 *Giardia* proteins are localized around the nuclei and in vesicles by confocal microscopy (using anti-HA mAbs).
E. Semi-quantitative RT-PCR experiment shows that the transcripts of gPAT, DHHC2 and DHHC3 are normally expressed during the parasite growth and differentiation. N, non-encysting trophozoites; ET, encysting trophozoites; *gdh*, glutamate dehydrogenase.

we call DHHC2 (GenBank™ Accession number EAA38877.1) and DHHC3 (GenBank™ Accession number EAA37657.1). Immunofluorescence analysis of epitope-tagged proteins expressed in WB trophozoites showed that gPAT is localized to the plasma membrane of *Giardia* trophozoites, while DHHC2 and DHHC3 are located close to the nuclei and in vesicles (Fig. 5D). Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR) showed that gPAT, DHHC2 and DHHC3 mRNA are expressed equally during *Giardia* growth and 24 h encystation (Fig. 5E). Even though these three *Giardia* proteins are similar, the subcellular localization

of gPAT at the plasma membrane together with its PAT activity (see below) suggested it is involved in VSPs palmitoylation.

gPAT activity

To determine whether gPAT is responsible for VSP palmitoylation, gPAT expression was depressed by production of antisense transcripts to mRNA of gPAT using a tetracycline inducible vector (Fig. 6A). After tetracycline induction (I), the steady-state levels of gPAT, DHHC2 and DHHC3 mRNA were determined by semi-quantitative RT-PCR. Only gPAT showed a decrease in mRNA expression; in contrast *gpat* antisense production did not affect the expression of DHHC2 or DHHC3 (Fig. 6B).

PAT activities have been characterized in different cell lines, however, attempts to isolate PAT using standard protein purification techniques have been unsuccessful due to the extreme instability of their activities (Linder and Deschenes, 2003). To examine whether gPAT is directly involved in the palmitoylation of VSPs, we performed an *in vitro* palmitoylation assay using recombinant VSPH7 and VSP1267 (rVSPH7 and rVSP1267) and *Giardia* homogenate. Incubation of rVSPH7 and rVSP1267 with homogenate of trophozoites from non-expressing *gpat* antisense (NI) in the presence of [9,10–^{3}H]palmitic acid led to the incorporation of the label into the recombinant VSPs (Fig. 6C, top panel – NI). On the other hand, when the homogenate containing *gpat* antisense was added (I), rVSPH7 and rVSP1267 showed approximately a 37% and 55% reduced uptakes of radioactive palmitate respectively (Fig. 6C, top panel – I). Similarly, a 30% reduction on palmitate uptake was observed for the native VSP9B10 after gPAT antisense production in *in vivo* studies (Fig. 6C, top panel). As expected, incorporation of [9,10–^{3}H]palmitic acid was prevented by heating the homogenate (Φ) prior to adding the substrates (Fig. 6C, top panel – Φ). Immunoblotting using anti-V5 mAb, showed that rVSPH7 and rVSP1267 loaded and recovered from the IPP were equal for NI, Φ and I samples (Fig. 6C, bottom panel). In addition, knock-down assays involving DHHC2 and DHHC3 genes showed no alteration on VSP9B10 palmitoylation *in vivo* (Fig. S1). These results demonstrate that reduction of gPAT expression also decreases palmitoylation of VSPs suggesting that gPAT participates in VSP palmitoylation.

Discussion

Giardia lamblia trophozoites express on their surface one protein of a family of surface antigens or VSPs. These proteins are associated with the survival of the parasite in the natural environment (Nash, 1997; Adam, 2001), however, the unique characteristic of these molecules suggest a more complex role beyond the evasion of the immune response in the vertebrate small intestine. In this study, we analysed the function of the C-terminal conserved sequence of VSPs. Previous reports suggested that the

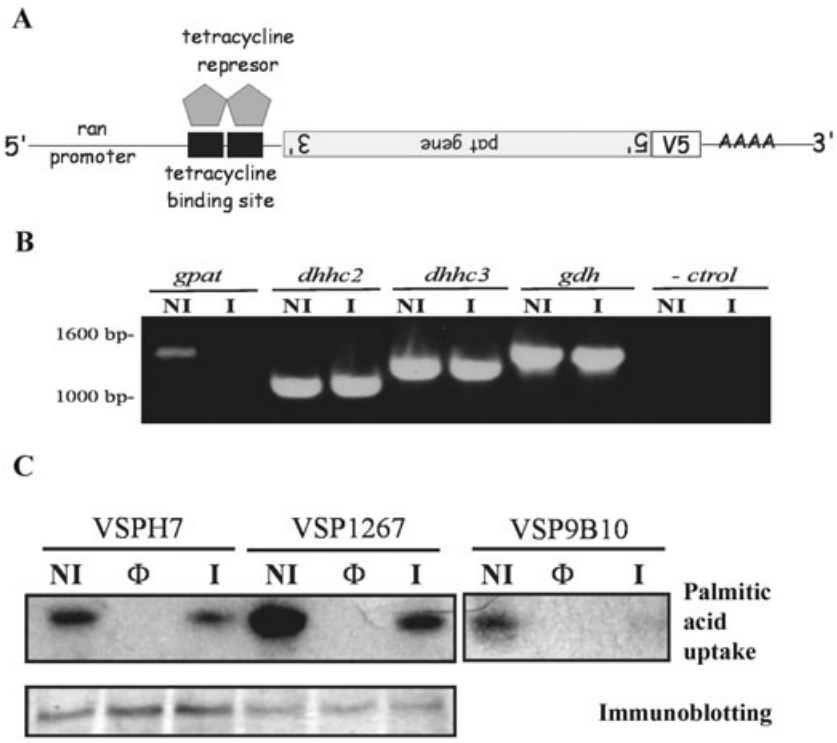

Fig. 6. gPAT possesses palmitoyl transferase activity.
A. Schematic representation of the *gpat* antisense plasmid containing the *Giardia ran* promoter controlled by the tetracycline repressor.
B. Semi-quantitative RT-PCR experiment shows that after induction of *gpat* antisense the transcript of gPAT is absent or highly reduced. On the contrary, DHHC2 and DHHC3 mRNAs are not affected by *gpat* antisense. NI, non-induced by tetracycline; I, induced by tetracycline; *gdh*, glutamate dehydrogenase.
C. Palmitic acid uptake assay (top panel) shows that gPAT palmitoyl transferase activity is greatly reduced after *gpat* antisense expression when the transgenic VSPH7, VSP1267 and the native VSP9B10 were assayed. The film was exposed for 1 week. NI, non-tetracycline induced homogenate; I, tetracycline induced homogenate; Φ, boiled homogenate. Immunoblotting assay using anti-V5 mAb (bottom panel) shows the VSPH7 and VSP1267 protein expression.

conserved transmembrane domain (TM) of all VSPs as well the invariant cytoplasmic tail CRGKA (CT) are important for the attachment and sorting of these proteins to the plasma membrane respectively (Marti *et al*., 2003a; Papanastasiou *et al*., 1997b). Here, we show that alteration of the TM or CT does not modify VSPH7 subcellular localization and postulate that the nature of the sequence of these domains is not involved in membrane anchoring. In fact, using a tetracycline-inducible vector we were able to produce low expression of VSPH7 using constructs containing the entire VSPH7's open reading frame (ORF) (iH7-HA) or without the CT (iH7-tail). Because mutated VSPH7 still localized to the surface of these transfected trophozoites, these results do not support the suggestion by others that high over-expression of VSPH7 account for their surface localization (Marti *et al*., 2003b). Instead, these results confirm our findings that neither the sequence of the TM nor the CT play a significant role in VSP surface localization (Fig. S2). Our results disagree with those of Marti *et al.* (Marti *et al*., 2003a) who showed that deletion of the VSPH7's CT led to retention of this protein in the ER suggesting an essential role of the CT sequence in sorting of VSPs. The differences reported by both groups may be due to the use of a chimera molecule by Marti *et al*. This unnatural molecule differs considerably from the native VSPH7 and may result in its misfolding and retention in the ER. In addition, the promoter driving expression of this chimera was the CWP1 promoter, that is regulated and transcriptionally active only during encystation (Mowatt *et al*., 1995), the stage of the life cycle of the organisms where VSPs transcription is not well characterized.

Our studies showed that VSPH7 is specifically palmitoylated on the cysteine residue located in the invariant VSPs five-amino acid cytoplasmic tail, CRGKA. Although the addition of palmitate to VSPH7 was demonstrated earlier, the precise location was not defined (Hiltpold *et al*., 2000). Palmitoylation targets proteins to lipid *raft*, subdomains of the plasma membrane that are enriched in sphingolipids and cholesterol (Edidin, 2003). Targeting of proteins to *raft* via palmitoylation relies on the ability of the palmitate to fit in an ordered lipid environment rather than a function of hydrophobicity. Using a standard assay to identify *raft* domains, native VSPH7 and VSP1267 as well as transfected non-mutated epitope-tagged VSPH7 associate with *raft*-like membrane fractions. In contrast, mutated non-palmitoylated VSPH7 shifted away to more dense fractions. These results suggest that the role of palmitoylation in the VSPs is linked to the segregation of these proteins to specific domains of the *Giardia* plasma membrane. In *Giardia*, the presence of subdomains of the plasma membrane (including *rafts*) has not been reported. Nevertheless, an essential function of the availability of cholesterol in induction of the encystation process has been extensively studied (Lujan *et al*., 1996a,b) suggesting an alteration of the surface membrane and protein distribution during this process (Touz *et al*., 2002a; A. R. Zurita and H. D. Lujan, pers. comm.). In this regard, a more complete study of plasma membrane composition to define the presence of plasma membrane subdomains should be carried out in this parasite.

In peripheral membrane proteins, palmitoylation can promote membrane interactions. In integral membrane proteins, however, the function of palmitoylation is not clear. Localization of signal proteins in *rafts* appears to be important for their function (Kabouridis *et al*., 1997; Lin *et al*., 1999). It was suggested that signal transduction could be regulated by sequestering signalling protein into different plasma membrane domain until they are brought together by an activating signal (Zhang *et al*., 1998). It is possible that the association of VSPs with *raft*-like domains permits signalling to occur. When we performed antibody cytotoxicity assays, we found that after addition of VSP-specific mAb, only cells transfected with the entire VSPH7 exhibited aggregation and cytotoxicity whereas trophozoites expressing VSPH7 without the CT or the palmitoylation-specific site survived. Moreover, we observed that in the case of VSPH7 (transgenic or wild-type) the surviving progeny no longer expressed surface VSPH7 while surviving H7-tail and H7cCT trophozoites still expressed the surface VSPH7 transgenic protein. These results have important biological implications. They suggest that complement-independent antibody-mediated cytotoxicity is due to specific changes or signalling mediated by the palmitoylated CRGKA cytoplasmic tail.

One obvious consequence of antibody-mediated cytotoxicity permitted by palmitoylation is the quick elimination of populations of parasites expressing VSPs or epitopes recognized by the host and the replacement by organisms expressing unrecognized VSPs. This may allow the maintenance of increased numbers of trophozoites and subsequently production and shedding of enlarged number of cysts. This would permit better chance of infection of other hosts.

Similar to other lipid modifications, protein palmitoylation is thought to be an enzymatic reaction mediated by palmitoyl transferase or acyltransferase (PAT). Here, we describe for the first time a DHHC protein in *Giardia*, gPAT, and demonstrate its PAT activity *in vitro* and *in vivo*. Only three proteins that promote palmitoylation have been described in *S. cerevisiae*: the effector of Ras function (Erf2), the ankyrin-repeat-containing protein (Akr1), and the SNARE protein Ykt6 (Lobo *et al*., 2002; Roth *et al*., 2002; Dietrich *et al*., 2004). Erf2 and Ykt6, similar to gPAT, contain a conserved Asp-His-His-Cys cysteine-rich domain (DHHC-CRD) motif and behave as integral membrane proteins. The common DHHC sequence is critical for the activity of the enzymes (Wedegaertner and

Bourne, 1994; Lobo *et al*., 2002). Many proteins containing the DHHC-CRD motif are found throughout the eukaryotic kingdom. In *Giardia*, only three fulfil the characteristics of an acyltransferase: gPAT, DHHC2 and DHHC3 (Reiner *et al.,* 2003; this work). Of the three, only gPAT was found at the surface membrane and therefore closely associated with VSPs. Moreover, lower expression of gPAT, but not DHHC2 and DHHC3, paralleled the decrease in palmitoylation of recombinant VSPs *in vitro* and native VSP9B10 *in vivo*, suggesting that this enzyme is responsible for the C-terminus VSPs palmitoylation *in vivo.*

Similar to *S. cerevisiae* (Zhang *et al*., 1998; Linder and Deschenes, 2003), in which none of the three DHHC-CRD PATs appear essential for survival, almost complete reduction of gPAT activity did not show any effect on parasite growth or survival *in vitro*. Nevertheless, addition of inhibitors of palmitoylation hydroxylamine or bromopalmitate to growing trophozoites in culture, induced cell death indicating that palmitoylation itself is critical for cell survival (M. C. Touz and T. E. Nash, unpubl. results). Palmitoylation is clearly important in *Giardia* and may be essential. Because *Giardia* derives from the most primitive branch of the eukaryotic lineage of decent, it offers unique ways to study the evolution of fundamental cellular processes. The demonstration that a novel PAT is involved in VSPs palmitoylation and that this process controls cell survival represents a novel and special form of protein modification and might open new ways to study and further understand signalling regulation in higher eukaryotes.

Experimental procedures

Giardia lamblia cultivation and transfection

Trophozoites of the isolate WB, clone 1267 (Nash *et al*., 1988) or GS, clone H7 (Nash and Mowatt, 1992) were cultured as described (Keister, 1983). Encystation of trophozoite monolayers was accomplished by the method described by Boucher and Gillin (Boucher and Gillin, 1990). Trophozoites were transfected with the constructs by electroporation and selected by puromycin as previously described (Yee and Nash, 1995; Singer *et al*., 1998; Elmendorf *et al*., 2001).

Expression of VSPH7 and VSPH7 variants in WB/1267 trophozoites

To constitutively express VSPH7, the plasmid pTubH7pac was used (Touz *et al*., 2003). The plasmids pTubHAH7pac (L. Kulakova and T. E. Nash, unpubl. results) or pTubH7HApac (Touz *et al*., 2003) were employed to express VSPH7 tagged with the influenza haemagglutinin (HA) epitope at the N-terminus or C-terminus (Fig. 1). For H7≠TM (VSPH7 with a different transmembrane domain -TM-), the plasmid pTubHAH7pac was modified by exchange of the TM of VSPH7 for the TM of the Encystation-specific Cysteine Protease TM (Touz *et al*., 2002b) as was described before (Touz *et al*., 2003). The plasmid ΔH7 (Touz *et al*., 2003) was used to express H7-tail (VSPH7 without the cytoplasmic tail). A site-directed mutagenesis kit (QuikChange, Stratagene) was employed to construct point mutations of VSPH7. In H7cTM, the cysteine in the TM of VSPH7 was replaced with alanine and in H7cCT, the cysteine residing within VSPH7 cytoplasmic tail was replaced with alanine as described (Touz *et al*., 2003) (Fig. 1). Deletion and mutations were confirmed by sequencing using dye terminator cycle sequencing (Beckman Coulter).

Expression of gPAT and DHHCs proteins in WB/1267 trophozoites

The tetracycline-inducible vector pINDG (Singer *et al*., 1998; Sun and Tai, 2000; Elmendorf *et al*. pers. comm.) was modified by introduction of the V5 epitope (GKPIPNPLLGLDST) at the C-terminus of the luciferase gene by plasmid restriction and ligation using the SphI and the PstI sites (creating the pINDG-V5 vector). The *gpat* gene replaced the luciferase gene by restriction and ligation using the BamHI and the SphI sites. The *dhhc2* and *dhh3* genes were cloned into pTubApaH7pac (Touz *et al*., 2003) by restriction and ligation using the NcoI and the EcoRV or HpaI sites. After transfection and clone selection, protein expression was induced by the addition of 10 μg ml^{-1} of tetracycline to the growth medium. All sequences were confirmed by sequencing using dye terminator cycle sequencing (Beckman Coulter).

Immunofluorescence assays

For indirect immunofluorescence assays of fixed cells, trophozoites cultured in growth medium were harvested and processed as described (Lujan *et al*., 1995; Zheng and Gao, 1999). Briefly, cells were washed two times with PBSm (100 ml PBS $1 \times + 1$ ml complete medium, pH 7.4) and allowed to attach to the slides for 30 min at 37°C. After fixation with 4% formaldehyde in PBS for 40 min at room temperature the cells were washed with PBS and blocked with 10% normal goat serum in 0.1% Triton-X100 in PBS for 30 min at 37°C. Cells were then incubated with the specific mAb (1:100) in PBS containing 3% normal goat serum and 0.1% Triton-X100 for 1 h at 37°C, followed by incubation with FITC-conjugated goat anti-mouse (1:200) secondary antibody at 37°C for 1 h. Preparations were finally washed with PBS and mounted in Vectashield mounting medium with DAPI (Vector Laboratories, CA). Primary VSPH7 specific G10/4 mAb (Nash *et al*., 1995) were used to detect VSPH7 and VSPH7 variants constructs or anti-HA mAb (Sigma, St. Louis, MO) for VSPH7 variants constructs and DHHCs. Anti-V5 mAb (Sigma, St. Louis, MO) was used for detection of gPAT. The specimens were viewed with a Leica TCS-NT/SP confocal microscope. Controls included omission of primary antibody and assays using non-transfected cells.

Immunoblot analysis

Western blotting was performed as previously reported (Lujan *et al*., 1995). Briefly, 10 μg of total protein/lane from

transfected non-encysting trophozoites were suspended in 30 μl of Laemmli sample buffer (Bio-Rad, Hercules, CA) and electrophoresed into a 4–12% Tris-glycine polyacrylamide gel. The proteins were transblotted onto nitrocellulose membranes (Invitrogen, Carlsbad, CA) and probed with anti-HA mAb (1:1000 dilution) (Sigma, St. Louis, MO) or VSPH7 specific G10-4 mAb (Nash *et al*., 1995) (1:500 dilution). To detect gPAT, cells were suspended in 30 μl of Laemmli sample buffer with 2-mercaptoethanol and boiled for 5 min before the electrophoresis. For gPAT detection, anti-V5 mAb (Sigma, St. Louis, MO) was used at a 1:1000 dilution.

Palmitate incorporation

The assay followed the procedure described by Papanastasiou *et al*. (Papanastasiou *et al*., 1997b). Briefly, trophozoites transfected with H7 constructs were washed and suspended in RPMI containing 1 mCi of [9,10–^{3}H]palmitic acid for 2 h at 37°C. After centrifugation, the pellets were lysed in 1 ml of lysis buffer for 1 h at 4°C. The samples were then centrifuged 5 min at 2500 r.p.m., the supernatants mixed with 2 μl of G10-4 mAb, and the IPP protocol followed as described below. The samples were loaded onto SDS–PAGE and the gels fixed with 7.5% acetic acid, 20% methanol for 15 min, soaked in Enlighning (DuPont NEN) for 30 min, dried, and analysed by fluorography after a month.

Immunoprecipitation

Immunoprecipitation was performed as described before (Lujan *et al*., 1995) using anti-HA mAb (Sigma, St. Louis, MO) or VSPH7 specific G10-4 mAb (Nash *et al*., 1995). Briefly, *Giardia* trophozoites were disrupted in lysis buffer (50 mM Tris, pH 8.0, 120 mM NaCl, 5 mM EDTA, 1% Triton X-100 and protease inhibitors) for 30 min on ice and centrifuged at 13 000 *g* for 5 min at 4°C. The cell lysate was then precleared by using protein A/G-Sepharose beads (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 min at 4°C, and subsequently subjected to IPP using 2 μl of specific mAb or an unrelated mAb (control). After incubation overnight at 4°C, protein A/G-Sepharose was added, and the incubation continued for 4 h. The immunoprecipitates were washed three times in lysis buffer before immunoblot analysis.

Phase separation of integral membrane proteins in Triton X-114 solution

Giardia trophozoites were suspended in 10 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 1.0% Triton X-114 at 0°C and subjected to detergent and aqueous phase partitioning as described previously (Bordier, 1981).

Biochemical isolation of detergent-insoluble membranes

Lipid *rafts* were isolated as previously described (Hiltbold *et al*., 2003). Briefly, trophozoites transfected with the H7 constructs were washed with MNE buffer (25 mM MES pH 6.5, 150 mM NaCl, 2 mM EDTA) and lysed in 0.4 ml of 1% Triton X-100/MNE/protease inhibitors on ice for 1 h. The lysates were brought to 1 ml with 90% sucrose/MNE, and overlaid with 2 ml of a 30% sucrose/MNE solution and 1 ml of a 5% sucrose/MNE solution. The samples were subjected to ultracentrifugation at 100 000 *g* for 16 h at 4°C. Nine or 11 fractions were collected from the top of the tube and solubilized in 1% octylglucoside for 1 h at 4°C. The fractions were solubilized in SDS sample buffer without boiling before SDS–PAGE analysis. VSPH7 variants were detected by dot-blot analysis using specific mAb.

Cytotoxicity assay

Giardia trophozoites expressing native VSPH7 or transgenic VSPH7 were grown in complete medium, washed with PBS, and subjected to live-immunofluorescence assay using a non-lethal concentration (1:5000) of VSPH7-specific G10-4 mAb. Trophozoites expressing the same amount and intensity of VSPH7 on the surface were selected and harvested after three sequential selection runs using a FACS. A WB/1267 clone expressing 100% VSP1267 was used as a negative control. The cells were collected and analysed using CELLQuest software and a FACScalibur flow cytometer (Becton Dickinson, San Jose, CA). These clones were grown until confluence before a second IFA analysis using G10-4 mAb to test for 100% expression of VSPH7. Cytotoxicity assays were performed in triplicate in 96-well microtitre plates. Briefly, each well contained 40 000 *Giardia* trophozoites in 200 μl of complete medium and 2 μl of mouse polyclonal antiserum or mAbs (1:20). The effects of antibody (immobilization, aggregation, detachment and killing) were scored by an observer unaware of the contents immediately after addition of the reagents (0 h), and at 3 h and 24 h. Plates were maintained anaerobically without antibiotic selection. Antibodies were heated at 56°C for 40 min to eliminate complement-mediated cytotoxicity. Cytotoxicity was determined in triplicate after 24 h by estimating the number of adherent viable parasites. Briefly, the medium was decanted and the attached trophozoites washed twice with prewarmed complete medium. The plates were then chilled on ice for 15 min and the trophozoites collected and counted using a Coulter Z1 Particle Counter (Coulter, Miami, FL, USA). Progeny were analysed by growing the trophozoites for 24 h without puromycin selection and labelling of viable trophozoites after addition of goat anti-mouse FITC-conjugated antibody (Cappel).

Knock-down of gPAT by antisense strategy

The *gpat* ORF was amplified, restricted with BamHI and SphI enzymes, and ligated to the pINDG in the opposite direction resulting in the antisense vector that was used for inhibition of gPAT expression. Trophozoites transfected with this plasmid were grown in complete medium and the antisense induced (or not, for control) with 10 μg ml^{-1} of tetracycline.

Semi-quantitative RT-PCR

Total RNA from 2×10^6 trophozoites was isolated using RNA STAT-60 reagent (Tel-Test, Friendswood, TX) followed by digestion with 50 U of DNAse (Roche Diagnostics, Indianap-

olis, IN) for 15 min at 25°C and final purification using the SV Total RNA Isolation System (Promega, Madison, WI). For reverse transcription and PCR amplification, the One-step RT-PCR kit (Qiagen, Valencia, CA) was employed and total RNA was diluted serially with final amounts of RNA ranging from 0.2 ng to 20 ng per reaction in a final reaction volume of 50 μl. The reverse transcription reaction was performed at 50°C for 30 min following by inactivation of the reverse transcriptase at 95°C for 15 min. For PCR, 30 cycles (30 s at 94°C, 30 s at 50°C and 1 min at 72°C) were used ending with a final extension of 10 min at 72°C. To determine the level of sense wild-type mRNA of *gpat*, *dhhc2* and *dhhc3* cDNA, gPATf/gPAT3′UTR, DHHC2f/DHHC2r and DHHC3f/DHHC3r set of primers were used respectively. DNA-contamination control (– control) was performed by adding gPATf/gPAT3′UTR primers at the PCR step of the RT-PCR reaction. To control for amounts of RNA loaded into wells, expression of the constitutive glutamate dehydrogenase enzyme (GDH) was determined with RT-PCR using primers GDHf/GDHr. Aliquots (5 μl) of the RT-PCR reaction were size-separated on 1.2% agarose gel in TAE (E-Gel, Invitrogen Corporation, Carlsbad, CA) prestained with ethidium bromide. Amplification products were directly quantified by densitometric scanning of the fluorescence intensity under UV light using EagleSight® software for image acquisition, documentation and analysis (Stratagene, La Jolla, CA).

gPAT activity assay

Palmitoyl transferase activity of gPAT was assayed by measuring the incorporation of tritiated palmitate. Homogenates of non-induced (NI) or induced (I) trophozoites to generate gPAT antisense were produced by sonication in PBS buffer at 4°C. A portion of NI trophozoites homogenate was boiled for 15 min (Φ) and used as a negative control of gPAT activity. The assay was performed by adding 20 μg rVSPH7 or rVSP1267 produced *in vitro* using Expressway™ Plus System, following the manufacture's instructions (Invitrogen Life Technologies, Caribad, CA) to 100 μl of NI, Φ and I trophozoites homogenate and incubated 15 min at 37°C in presence of 1 mCi of [9,10–^{3}H]palmitic acid. After heat inactivation at 100°C for 15 min, the samples were subjected to IPP using anti-V5 mAb for rVSPH7 and rVSP1267, or anti-9B10 mAb for VSP9B10, and analysed by SDS–PAGE and fluorography as described above. Activity was quantified by densitometric scanning using EagleSight® software for image acquisition, documentation and analysis (Stratagene, La Jolla, CA).

Oligonucleotide primers used (5′-3′ orientation)

For VSPH7 point mutations:

H7cTMf:GTCGGAGGCCTCGTCGGCTTCCTCGCCTGGT GGTTCGTCTGCCGCGGGAAG
H7cTMr:CTTCCCGCGGCAGACGAACCACCAGGCGAGG AAGCCGACGAGGCCTCCGAC
H7cCTf:GGCTTCCTCTGCTGGTGGTTCGTCGCCCGCGG GAAGGCGGATATCTATCCA
H7cCTr:TGGATAGATATCCGCCTTCCCGCGGGCGACGAA CCACCAGCAGAGGAAGCC

For gPAT and DHHCs cloning:

gPATf:GTT**GGATCC**ATGGTCAGTTGTGTCGACAAGATCT TC (**BamHI**)
gPATr:GTAT**GCATGC**ATTTTCCAGACCCTGGACAATTCTA TC (**SphI**)
DHHC2f:GTT**CCATGG**GCAAGGAGGGCCCCAATAGG (**NcoI**)
DHHC2r:GAT**GATATC**ATTCATCTCTTCACCCGATAAATC (**EcoRV**)
DHHC3f:GTT**CCATGG**AAGTCCCCGATGGAAATAGT (**NcoI**)
DHHC3r:GAT**GTTAAC**TTCGTGATCCGTTGATTGCATCGG (**HpaI**)

For gPAT antisense:

gPATasF:GTT**GCATGC**ATGGTCAGTTGTGTCGACAAGA TCTTC (**SphI**)
PATasR:GAT**GGATCC**ATTTTCCAGACCCTGGACAATTCT ATC (**BamHI**)
gPAT3′UTR:TCTAACGAGCAGATGAAACGATGGTCA;
GDHf:CACCATGCCTGCCAGACGATCGAGGAG
GDHr: TCACACGCAGCCCTGCTCGATCATCAT

Restriction sites are denoted in bold cases.

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Supplementary material

The following supplementary material is available for this article online:

Fig. S1. Palmitoyl transferase activity *in vivo*. VSP9B10 in wild-type or transgenic trophozoites was immunoprecipitated after ^{3}Hpalmitate labelling and counted in a scintillation counter. Only transgenic trophozoites expressing gPAT antisense show a decrease in VSP9B10 palmitate binding when comparing with the wild type, inactivated (heat), DHHC2 and DHHC3 antisense cells. Values represent the mean ± SE of three independent experiments.

Fig. S2. Normal surface location of low-expressed VSPH7 transgenic proteins. Confocal microscopy of immunofluorescence assay using G10-4 mAb shows that both iH7-HA and iH7-tail are located on the plasma membrane when its expression is induced by 5 $\mu g\ ml^{-1}$ and 10 $\mu g\ ml^{-1}$ of tetracycline.

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