N-terminal palmitoylation is required for *Toxoplasma gondii* HSP20 inner membrane complex localization

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Abstract

Toxoplasma gondii is an obligate intracellular parasite and the causative agent of toxoplasmosis. Protein palmitoylation is known to play roles in signal transduction and in enhancing the hydrophobicity of proteins thus contributing to their membrane association. Global inhibition of protein palmitoylation has been shown to affect *T. gondii* physiology and invasion of the host cell. However, the proteins affected by this modification have been understudied. This paper shows that the small heat shock protein 20 from *T. gondii* (TgHSP20) is synthesized as a mature protein in the cytosol and is palmitoylated in three cysteine residues. However, its localization at the inner membrane complex (IMC) is dependent only on N-terminal palmitoylation. Absence or incomplete N-terminal palmitoylation causes TgHSP20 to partially accumulate in a membranous structure. Interestingly, TgHSP20 palmitoylation is not responsible for its interaction with the daughter cells IMCs. Together, our data describe the importance of palmitoylation in protein targeting to the IMC in *T. gondii*.

Keywords

Toxoplasma gondii; small heat shock protein 20; palmitoylation; inner membrane complex; endodyogeny

1. Introduction

Fatty acylation of proteins is a widespread feature of eukaryotic cells. This post-translational modification (PTM) plays a key role in membrane localization of signal-transducing proteins [1, 2] as well as in regulation of mitochondrial metabolism [3, 4]. Fatty acylation includes myristoylation and palmitoylation. Whereas myristoylation refers to the irreversible

attachment of 14-carbon myristate in a co- [5, 6] or post- [7–10] translational manner, palmitoylation is the post-translational attachment of the 16-carbon fatty acid palmitate onto cysteine residues via a labile thioester bond. Palmitate is transferred onto proteins enzymatically [11–13] or in a spontaneous manner [4, 14] with both mechanisms being operational in cells.

Apicomplexan parasites are a large group of obligate intracellular parasites that include *Eimeria spp., Cryptosporidium spp., Neospora spp., Plasmodium spp., Theileria spp. and T. gondii*. Protein palmitoylation has been described to operate in *E. tenella* [15], *P. falciparum* [16–19] and *T. gondii* [15, 20, 21]. There are several proteins known or predicted to be palmitoylated in *T. gondii* [22]. In general, protein palmitoylation is important for proper membrane localization of the modified protein. In this regard, it has been described that mutation of the predicted palmitoylation sites in *T. gondii* inner membrane complex sub-compartment protein 4 (ISP4) and calcium-dependent protein kinase 3 (CDPK3), alters their localization from the pellicle to the cytosol [23–25]. We recently described the functional significance of this modification in *T. gondii*, where inhibition of protein palmitoylation was able to interfere with the parasite's physiology and host-cell invasion [20]. Interestingly, the host-cell invasion process depends on an actin- and myosin-based complex located at the pellicle, between the plasma membrane (PM) and inner membrane complex (IMC)[26]. The same effect on host-cell invasion was observed in *P. falciparum* [27]. In general, these results underscore an important role for palmitoylation of IMC-associated proteins.

T. gondii small heat shock protein 20 (TgHSP20) displays interesting characteristics which could be used to study the role and dynamics of protein palmitoylation. TgHSP20 localizes to the parasite IMC in a set of discontinuous stripes [28]. Recombinant TgHSP20 forms multimers [29], binds phosphatidylinositol 4-phosphate and phosphatidylinositol 4,5 biphosphate phospholipids [28] and has chaperone activity *in vitro* [29]. It has been reported that antibodies against HSP20 reduce host-cell invasion by *B. divergens*, *T. gondii* and *N. caninum* [30, 31]. In *Plasmodium*, deletion of the *hsp20* gene results in altered sporozoite speed and substrate adhesion leading to an impaired natural malaria transmission in the host [32, 33]. Although HSP20 is predicted to be synthesized in the cytosol, it localizes to the IMC in *T. gondii* [28], *P. falciparum* [32] and to the pellicle in *Babesia* [34]. Moreover, in *T. gondii* HSP20 is incorporated to the IMC at intermediate stages of daughter cell formation [28]. Here, we confirmed that all the TgHSP20 cysteine residues are palmitoylated *in vivo* but only N-terminal palmitoylation is necessary for localization at the IMC. Interestingly, palmitoylation was not necessary for the interaction with the IMC of daughter cells during budding, as a non-palmitoylable version of TgHSP20 localized to the IMC along the daughter cell development. Finally, we discuss a possible model of palmitoylation events and sub-cellular localization of *T. gondii* HSP20 to the IMC.

2. Materials and methods

2.1 Antibodies and reagents

Specialized and common reagents were from Sigma, unless specified. [9,10-^{3}H]-palmitic acid and [^{35}S]-methionine/[^{35}S]-cysteine were from PerkinElmer Life and Analytical Sciences. ECL Plus was from GE Biosciences. Alexa-conjugated secondary antibodies were from Molecular Probes. Tissue culture reagents were from Invitrogen. The serum anti-Ty was kindly provided by Dr Dubremetz (Université de Montpellier, France), anti-IMC1 antibody was generously provided by Dr Ward (University of Vermont, USA) and anti-SAG1 antibody was kindly provided by Dr. Marina Clemente (Universidad de San Martin, Argentina).

2.2 *Toxoplasma* and host-cell cultures

T. gondii tachyzoites of the RH Δ*hxgprt* strain [35] were used throughout the study. Parasites were maintained by serial passage on confluent monolayers of human foreskin fibroblasts (HFFs) in Dulbecco's Modified Eagle Medium (DMEM; Gibco BRL) supplemented with 10% v/v bovine serum albumin (BSA), 100 i.u. (international units)/ml penicillin and 100 μg/ml streptomycin (Gibco BRL).

2.3 Metabolic labeling with [^{3}H]-palmitic acid and immunoprecipitation

Freshly lysed (10^9) tachyzoites were purified using a 3 μm polycarbonate filter and incubated for 4 h with 100 μCi of [9,10-^{3}H]-palmitic acid previously conjugated to BSA fatty acid free (1:1, mol: mol ratio). Then, the cells were washed twice with PBS and finally resuspended in 2 ml of immunoprecipitation buffer (IP buffer; 20 mM Tris-HCl, 150 mM NaCl, 1% TX-100 and Complete protease inhibitor cocktail-Roche, pH 7.4). After incubation for 2 h with rotation at 4°C, the lysate was centrifuged at 10,000 *x g* for 10 min at 4°C, the pellet was discarded and 5 μl of the appropriate antiserum was added to the supernatant (rabbit anti-HSP20, rabbit preimmune or mouse anti-Ty, mouse preimmune). Protein A/G Plus sepharose (Santa Cruz Biotechnology) was then added (50 μl of slurry previously equilibrated in IP buffer) and incubated 16–18 h with rotation at 4°C. The immunocomplexes were washed three times with IP buffer and finally resuspended in SDS-PAGE loading buffer without any reducing agent. Each sample was divided in three: one gel was transferred to a PVDF membrane and western blotted for the proteins (loading control). The other two gels were subjected to hydroxylamine treatment and its control.

2.4 Western blot analysis

A SDS-PAGE gel was used as a loading control of the immunoprecipitation. The gel was electrophoresed at 100 V for 1 h onto a PVDF membrane and blocked in 5% w/v skim milk in PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM KH_2PO_4, pH 7.4) for 1 h. Then rabbit anti-HSP20 (1/1,000) or mouse anti-Ty (1/500) serum was added and incubated for another hour followed by washes in PBS, PBS-T (PBS with 0.1% v/v Tween-20) and PBS. Secondary antibodies conjugated to HRP were added (1/10,000), incubated for 1 h followed by washes in PBS, PBS-T and PBS. Finally proteins were immunodetected by ECL Plus (GE healthcare).

2.5 Hydroxylamine treatment

Gels were treated with either 1M Tris-HCl pH 7.0 (control) or with 1M NH_2OH-NaOH pH 7.0 for 48 h, changing for fresh solutions every 12 h. Then the gels were stained with Coomassie Brillant Blue, destained and incubated for 30 min in ddH_2O. Finally the gels were incubated for 30 min in 1M sodium salicylate pH 6.5, dried for 2 h at 80°C using a gel dryer machine and exposed to autoradiographic film typically for a month.

2.6 *In silico* analyses

Analysis of HSP20 amino acid sequence in Apicomplexa was performed using *Toxoplasma gondii* (TgHSP20; NCBI accession number AAT66039), *Neospora caninum* (NcHSP20; NCBI accession number CD537778), *Eimeria tenella* (EtHSP20; NCBI accession number AI758032), *Plasmodium falciparum* (PfHSP20; NCBI accession number CAD51208), *Plasmodium berghei* (PbHSP20; NCBI accession number AAL07529), *Plasmodium vivax* (PvHSP20; NCBI accession number XP_001615006), *Plasmodium yoelii yoelii* (PyyHsp20; NCBI accession number EAA16935), *Babesia bigemina* (BbiHS20; NCBI accession number AAK11630), *Babesia bovis* (BbiHSP20; NCBI accession number AAK11624) and *Theileria parva* (TpHSP20; NCBI accession number XP_763804). BioEdit software version 4.0 (Ibis Biosciences) was used for multiple alignment and sequence analysis.

2.7 Treatment with 2-bromopalmitate

A monolayer of HFF cells was infected with *T. gondii* in a 24-well dish. After 10 min on ice, the parasites were allowed to invade for 1 h at 37°C. Then, the cells were washed twice with PBS and the media was changed with a mix of 1% v/v of 100 μM BSA fatty acid free and 2-bromopalmitate respectively (final concentrations). The infected cells were incubated for 16–18 h at 37°C then were subjected to indirect immunofluorescence studies.

2.8 Indirect immunofluorescence studies

All the steps were carried out at room temperature. Media was discarded and the cells were washed twice with PBS. Cells were fixed with formaldehyde 4% v/v in PBS for 30 min followed by a 1 min wash in PBS. Then the cells were permeabilized with 0.3% v/v Triton X-100 in PBS for 20 min and blocked with 3% w/v BSA in PBS for 30 min. After this, appropriate primary antibodies were added diluted in 3% w/v BSA in PBS for 60 min followed by extensive washes. Anti-species secondary antibodies were added and incubated for another 60 min followed by extensive washes. Finally the samples were mounted with Fluoromont (Abcam). The primary antibodies used were: mouse monoclonal anti-Ty (1/200), rabbit anti-IMC1 (1/1,000), mouse anti-IMC1 (1/2,000), rabbit anti-HSP20 (1/1,000). Parasites were imaged using a Delta Vision deconvolution microscope (Applied Precision).

2.9 Generation of TgHSP20 mutants

To generate the TgHSP20-FL-Ty mutant version, the TgHSP20 ORF was amplified as previously described [28]. To change cysteine in position 3 to serine in TgHSP20, we used the following forward primer: 5′-AGATCTATGAGTTCCTGTGGCGGTAC -3′ (altered nucleotide underlined), to change cysteine 4 to serine: 5′-AGATCTATGAGTTGCTCTGGCGGTAC -3′ and to change cysteines 3 and 4 to serines: 5′-AGATCTATGAGTTCCTCTGGCGGTAC-3′. In order to replace cysteine in position 160 to serine we used the QuikChange® II XL site-directed mutagenesis kit (Agilent Technologies), using as a forward primer 5′-GTTGGAGGTTAAAATCTCCTCCATTCAAACC-3′ and the reverse primer 5′-AGGAGATTTTAACCTCCAACAATCCATTTG-3′. The plasmid ptubHSP20$_{FL}$-Ty was used as template to generate ptubHSP20$_{C160S}$-Ty. The plasmid ptubHSP20$_{C3,4}$s-Ty was used as template to generate TgHSP20$_{C3,4,160S}$-Ty. Mutations were verified by sequencing and 50 μg of the wild type and each mutagenized vector was transfected into RHΔ*hpt* parasites. The transfected populations were selected under xanthine and mycophenolic acid pressure and cloned by limiting dilution, as previously described [36].

2.10 Transmission electron microscopy analysis

Infected monolayers were rinsed with 2% glutaraldehyde in 0.1 M Na-phosphate buffer pH 7.4. Intracellular parasites were then pelleted for 2 min at full speed and floated in fresh fixative for 90 min. After two washes in phosphate buffer, cells were fixed using 1% osmium tetroxide in phosphate buffer during 1 h at RT. After washing in ddH2O twice, the sample was dehydrated in ethanol and embedded in epon resin containing propylene oxide. Thin sections were obtained with a Leica ultramicrotome and collected in formvar-coated single hole grids.

3. Results

3.1 HSP20 is palmitoylated in *T. gondii* and the predicted palmitoylation sites are conserved across the phylum

TgHSP20 localizes to the IMC (Figure S1A; [28]). However, its amino acid sequence neither contains a signal peptide (http://www.cbs.dtu.dk/services/SignalP), nor a predicted hydrophobic region or a transmembrane domain (http://www.vivo.colostate.edu/molkit/hydropathy). In addition, there is no evidence for maturation (Figure S1B) that could explain its IMC localization. Hence, we decided to study whether post-translational modifications could account for its localization. *In silico* analysis shows that TgHSP20 could be subjected to several post-translational modifications (PTM) including phosphorylation, glycosylation with O-linked N-acetylglucosamine and palmitoylation (Figure S1C). Of all these PTM, palmitoylation is the only modification that could facilitate TgHSP20 localization to the IMC. *T. gondii* HSP20 contains three cysteine residues at positions 3, 4 and 160 (C3, C4 and C160) all of which are predicted to be palmitoylated (Figure S1C).

In order to experimentally confirm whether TgHSP20 is palmitoylated, we performed metabolic labeling of tachyzoites with [^{3}H]-palmitic acid followed by immunoprecipitation. Figure 1A shows that this protein is palmitoylated *"in vivo"*. Furthermore, TgHSP20 radiolabel was removed by neutral hydroxylamine treatment, confirming that the palmitic acid is bound to TgHSP20 through a labile thioester bond. It is important to highlight that the three cysteine residues found in TgHSP20 predicted to be palmitoylated, are conserved in Apicomplexa (Figure 1B). These results could suggest that palmitoylation may be important for TgHSP20 localization and/or function in Apicomplexan parasites.

3.2 Palmitoylation of HSP20 is required for proper localization to the IMC

Since palmitoylation has been shown to localize soluble proteins to membranes, we tested whether protein palmitoylation was responsible for TgHSP20 localization to the IMC. In order to do this, intracellular tachyzoites were incubated with the palmitoylation inhibitor 2-bromopalmitate (2-BP; [37]). Figure 1C shows that TgHSP20 changes localization from the IMC to the cytosol and formed an unidentified structure after 2-BP treatment (white arrowhead), suggesting that TgHSP20 is anchored to the IMC by palmitoylation.

3.3 TgHSP20 is palmitoylated in all its cysteine residues

In order to determine which residue/s is/are palmitoylated, different additional copies of HSP20 in which each putative palmitoylated cysteine residue (C3, C4 and C160) were changed to serines as indicated in the scheme depicted in Figure 2A. These mutated genes were cloned into a *Toxoplasma* expression vector containing a Ty-tag and expressed in tachyzoites. These tachyzoites were metabolically labeled with [^{3}H]-palmitic acid followed by immunoprecipitation with an anti-Ty antibody. Western blot analyses of the immunoprecipitated proteins are shown in Figure 2B. The endogenous TgHSP20 was visualized as a single band in Western blots using anti-TgHSP20 antibody (Figures 1A and S2). However, some of the Ty-constructs appeared as a doublet when using the anti-Ty antibody (Figure 2B and S2) in which the lower band co-migrates with the endogenous TgHSP20 (Figure S2). Mass spectrometry analysis of these bands confirmed that both bands corresponded to TgHSP20 (data not shown). These two bands may correspond to different post-translational modifications or structural differences of the protein. Nevertheless, more studies are required to elucidate the nature of the observed doublet of the TgHSP20-Ty constructs. The expression of all constructs produced proteins with some level of palmitoylation, with the exception of TgHSP20$_{C3,4,160S}$-Ty (Figure 2B, lower panel). This result shows that TgHSP20 is palmitoylated on all of its cysteine residues. Furthermore,

neutral hydroxylamine treatment removed the radiolabel from all the constructs, demonstrating that the lipid attachment was due to a labile thioester bond (Figure 2B).

3.4 N-terminal palmitoylation determines TgHSP20 localization

In order to establish the role of palmitoylation of each cysteine residue on the localization of TgHSP20, we analyzed the sub-cellular localization of the different Ty-tagged TgHSP20s. Figure 2C shows that the $TgHSP20_{FL}$-Ty construct localized to the IMC, as previously reported for the endogenous TgHSP20 [28]. Interestingly, stable lines expressing $TgHSP20_{C3S}$-Ty and $TgHSP20_{C4S}$-Ty mutants also showed a faint IMC localization and a strong signal in an unidentified structure (Figure 2C, arrowheads). This structure was also observed when tachyzoites were treated with 2-BP (Figure 1C, arrowhead). Of note, this unidentified structure did not react against IMC3 or SAG1 by indirect immunofluorescence (data not shown). $TgHSP20_{C3,4S}$-Ty localized to the cytosol and displayed a very intense signal in the unidentified structure (indicated with an arrowhead). The non-palmitoylable form of TgHSP20 ($TgHSP20_{C3,4,160S}$-Ty) displayed a cytosolic localization without the presence of the unidentified structure. Localization of $TgHSP20_{C160S}$-Ty is similar to that of full length TgHSP20 (Figure 2C).

In order to study the nature of the TgHSP20-positive unidentified structure observed in some mutant lines, a transmission electron microscopy analysis was performed with tachyzoites stably expressing $TgHSP20_{C3,4S}$-Ty. The image suggests the presence of some accumulation of membranes within the parasite (Figure 3; [38]).

3.5 Palmitoylation is not required for localization of TgHSP20 in the IMC of daughter cells

Apicomplexan parasites divide by internal budding into a variable number of daughter cells. *T. gondii* divides by the simplest mode in the phylum, endodyogeny, and assembles two daughter cells every round of replication. Prior to cytokinesis, daughter cells are dotted with a fully formed pellicle, which includes the IMC [39]. In order to determine how palmitoylation modulates TgHSP20 targeting to the IMC of daughter cells, intracellular replicating parasites were treated with 2-BP and analyzed by indirect immunofluorescence microscopy with anti-HSP20 antibodies. Interestingly, palmitoylation seems not to play a role on TgHSP20 attachment to the IMC of daughter cells (Figure 4A). To confirm this result, stable lines expressing different palmitoylable versions of TgHSP20 were analyzed for their sub-cellular localization in replicating parasites. All the mutant lines lysed the infected monolayer at a similar time than the parental cell-line, not showing any evident phenotype associated to parasite growth (data not shown). All the Ty-tagged TgHSP20s localized to the IMC of daughter cells regardless of the cysteine that was modified (Figure 4B). This indicates that the initial attachment of TgHSP20 to the IMC of daughter cells is a process independent of palmitoylation. Furthermore, the stable line expressing a non-palmitoylable form of TgHSP20 ($TgHSP20_{C3,4,160S}$-Ty) localized to the IMC of daughter cells but to the cytosol of mature parasites. As such, we decided to investigate at which point of the endodyogeny process $TgHSP20_{C3,4,160S}$-Ty changed its localization. Figure 5 shows that $TgHSP20_{C3,4,160S}$-Ty localizes to the IMC of daughter cells until late endodyogeny and moves to the cytosol only when parasites are mature. As such, palmitoylation seems to be required to retain TgHSP20 at the mother IMC in intracellular parasites.

4. Discussion

Our data shows that TgHSP20 is synthesized as a mature protein in the cytosol and that its initial localization to the parasite IMC is independent of palmitoylation, but this PTM of TgHSP20 is required to maintain such localization in the mature parasite. Interestingly, these results indicate that a differential regulation of IMC protein targeting may occur in

daughter and mature parasites. Although palmitoylation of *T. gondii* proteins has been predicted for numerous proteins, mainly for proteins localized to the IMC, there are little experimental evidences of such modification. One interesting finding of this investigation is that TgHSP20 is palmitoylated at the three cysteines present in its amino acid sequence. Up to date, the Apicomplexa phylum presents two IMC proteins where their palmitoylation status determines their localization to the pellicle [19, 22, 40]. The glideosome association protein GAP 45 localizes at the space between the plasma membrane and the IMC and is part of the motor complex of this parasite. However, this protein does not localize to the IMC of daughter cells and it seems to be associated to the plasma membrane from where it interacts with the IMC of mature parasites [41]. The *T. gondii* enzyme hypoxanthine-xanthine-guanine phosphoribosyltransferase (HXGPRT) presents two isoforms [40]. Whereas the HXGPRT–I localizes to the cytosol, the isoform-II presents an extension at the N-terminal region, which is palmitoylated and that determines its IMC localization [40]. Other interesting examples of *T. gondii* proteins that localize to the IMC are the IMC Sub-compartment Proteins (ISP1/2/3/4) [42] and the calcium-dependent protein kinase 3 (TgCDPK3) [24, 25]. ISPs 1–3 and TgCDPK3 present predicted myristoylation and palmitoylation sites at the N-terminal region [24, 25, 42], whereas ISP4 presents two predicted palmitoylation sites [23]. Mutation of the predicted N-terminal acylation sites delocalizes the proteins from the pellicle to the cytosol [23–25, 42]. However, the palmitoylation status of all these proteins remains to be demonstrated biochemically. In addition, *T. gondii* presents several IMC-associated proteins predicted to be palmitoylated such as IMC 1,4,6,11,12,13,14,15, MCL1 and PKG1 [15, 22, 43]. Taken together, it seems that palmitoylation of at least a subset of IMC-associated proteins could be important to retain these proteins in this compartment, being TgHSP20 an example of them.

Analysis of the dynamics of the incorporation of TgHSP20 into the IMC shows some interesting traits. We found that HSP20 was targeted to the IMC at an intermediate stage of budding. Once at the IMC, TgHSP20 is retained in this sub-compartment until the late steps of parasite replication, apparently without requirement for palmitoylation as observed for the non-palmitoylable form of TgHSP20 (TgHSP20$_{C3,4,160S}$-Ty). We expected to observe contact between TgHSP20 and the IMC because all palmitoyl acyl-tranferases (PATs) are transmembrane proteins [44]. In fact, data mining of the *T. gondii* database (www.toxodb.org) reveals that putative TgPATs contain a signal peptide and several transmembrane domains [22]. However, in mature tachyzoites, after cytokinesis, the presence of TgHSP20 at the IMC requires at least cyteine 3 or 4 to be palmitoylated. Similarly, in higher eukaryotes a well characterized example of protein palmitoylation-dependent sorting is the H- and N-Ras. After translation, these proteins are farnesylated allowing them to associate with ER and Golgi membranes [45]. This single lipidation step produces a transient and weak attachment to the membrane, in this case with the ER. Subsequent palmitoylation steps generate the trapping necessary to keep the stable membrane association, particularly at the PM and Golgi. Palmitoylation and de-palmitoylation of Ras regulates diffusional exchange between the cytosol and endo-membranes [46, 47]. In a similar manner, our data suggest that TgHSP20 palmitoylation at cysteines 3 and 4 traps the chaperone at the IMC.

The fact that TgHSP20 is targeted to the IMC without being palmitoylated indicates that it could have an interacting molecule. Although these parasites express the endogenous TgHSP20 and the mutants versions could tag along with its localization, similar results were obtained when TgHSP20$_{C3,4,160S}$-Ty was expressed in a TgHSP20-knock out parasite (Figure S3). Up to now, we were unable to detect any protein partner either as interactor and/or substrate [28] but one of them could be the specific PAT that palmitoylates TgHSP20, a speculation that needs to be further explored. Another possibility is that TgHSP20 binds IMC lipids as it has been shown for the recombinant TgHSP20 [28]. This

intrinsic property could account for the initial binding of TgHSP20 to the IMC of nascent cells, whereas palmitoylation, as mentioned above, is required to maintain the IMC localization in mature intracellular parasites. In this case, a change in membrane composition or fluidity at the final steps of parasite replication [48, 49] could account for the generation of an unstable environment for non- palmitoylated TgHSP20. In fact, the mature IMC present a different topology along the parasite with palmitoylated proteins locating at three different regions [42]. This suggests that once TgHSP20 is at the IMC, palmitoylation could confer properties to stably interact or bind with specific regions along this sub-compartment. Actually, TgHSP20 presents a distribution in short stripes from the basal to the polar region of the parasite. It is interesting to note that *hsp20* expression at the mRNA level is clearly associated to cell cycle, peaking at the mitosis phase (Figure S4), as described by other IMC proteins and suggesting that it occurs because they accompany the building of the new daughter cell [50]. This is in agreement with the possibility that TgHSP20 mature together with the IMC during parasite replication. Further studies would be necessary to address this point.

After treatment of tachyzoites with 2-BP, TgHSP20 showed a localization similar to that observed for the TgHSP20$_{C3,4S}$-Ty, demonstrating that palmitoylation on cysteine 160 does not play a role in TgHSP20 trapping at the IMC. Considering that cysteine 160 is conserved, the role of its palmitoylation remains to be elucidated. On the other hand, lack of palmitoylation at cysteine 160 (TgHSP20$_{C160S}$-Ty) did not produce the TgHSP20-positive unidentified structure, suggesting that this structure depends on palmitoylation at this residue. We speculate that this structure may contain misfolded products of TgHSP20. Interestingly, this structure seems to contain membrane structures that are reminiscent of the membranous myelin fibers observed when the apicoplast biogenesis was disturbed [38]. The significance of this finding needs to be further studied. The fact that TgHSP20$_{C3,4,160S}$-Ty did not produce any unusual structure supports our hypothesis of the idea of a previous requirement of TgHSP20 association to the IMC to generate such structure. Even though the role of cysteine 160 is unclear, palmitoyaltion at this site may stabilize the anomalous attachment of TgHSP20 to the IMC. Of note, these unidentified structures are not naturally observed in the parasite.

In conclusion, we observed that the *T. gondii* IMC protein HSP20 is palmitoylated at three sites and that these palmitoylated sites have different roles at least in the targeting the chaperone to the IMC. We also observed that TgHSP20 is translated in the cytosol and does not require rapid palmitoylation to bind to the nascent IMC. However, a fine modulation of protein localization can occur at later steps of parasite replication, including palmitoylation as one of such process.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Highlights

- TgHSP20 is synthesized is palmitoylated in three cysteine residues

- N-terminal palmitoylation determines its IMC localization

- Incomplete palmitoylation causes TgHSP20 to partially accumulate in a membranous structure

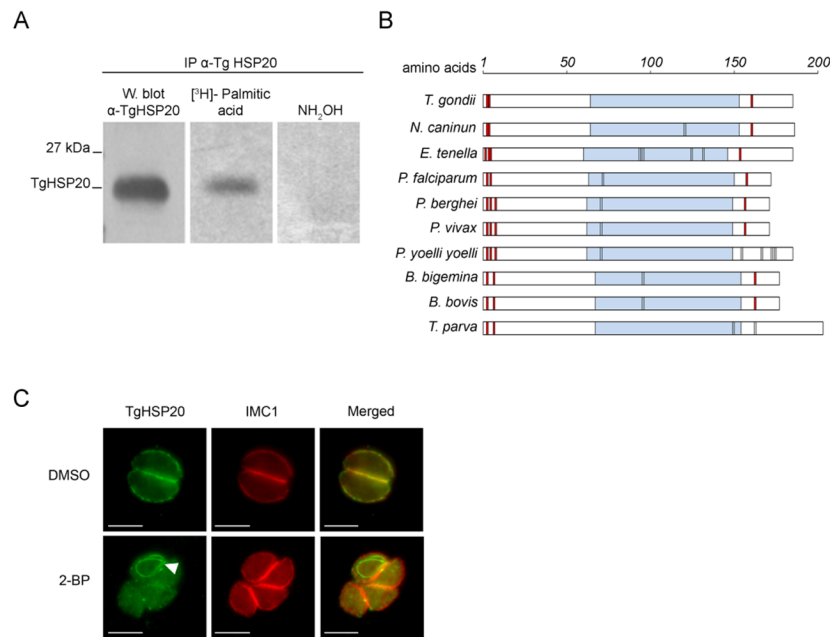- TgHSP20 palmitoylation is not responsible for its interaction with daughter cells IMCs

Figure 1. TgHSP20 is palmitoylated in vivo and this modification is responsible for its IMC localization

A) TgHSP20 is palmitoylated *in vivo*. Metabolic labeling of wild type *T. gondii* parasites using [³H]-palmitic acid followed by specific immunoprecipitation with anti-TgHSP20 antibody. After the immunoprecipitation, the sample was divided in three and run on SDS-PAGE: one sample was used for western blotting as a loading control. The two remaining samples were used to detect the radiolabeled HSP20 as indicated in Materials and Methods. B) Schematic representation of Apicomplexan HSP20 amino acid sequences. The analysis includes the amino acid sequences of HSP20 from *Toxoplasma gondii* (TgHSP20; NCBI accession number AAT66039), *Neospora caninum* (NcHSP20; NCBI accession number CD537778), *Eimeria tenella* (EtHSP20; NCBI accession number AI758032), *Plasmodium falciparum* (PfHSP20; NCBI accession number CAD51208), *Plasmodium berghei* (PbHSP20; NCBI accession number AAL07529), *Plasmodium vivax* (PvHSP20; NCBI accession number XP_001615006), *Plasmodium yoelii yoelii* (PyyHSP20; NCBI accession number EAA16935), *Babesia bigemina* (BbiHSP20; NCBI accession number AAK11630), *Babesia bovis* (BbiHSP20; NCBI accession number AAK11624) and *Theileria parva* (TpHSP20; NCBI accession number XP_763804). Light-blue boxes indicate α-crystallin domain. Red boxes represent cysteine residues predicted to be palmitoylated. Black lines depict cysteine residues. C) TgHSP20 depends on palmitoylation for IMC localization. Intracellular parasites were treated for 16–18 h with either 100 µM 2-bromopalmitate (2-BP) or DMSO and then TgHSP20 localization was analyzed by indirect immunofluorescence. The localization of the protein IMC1 was unaffected by the treatment. An unknown structure stained with TgHSP20 antibodies is shown in cells treated with 2-BP (arrowhead). Scale bars = 5 µm.
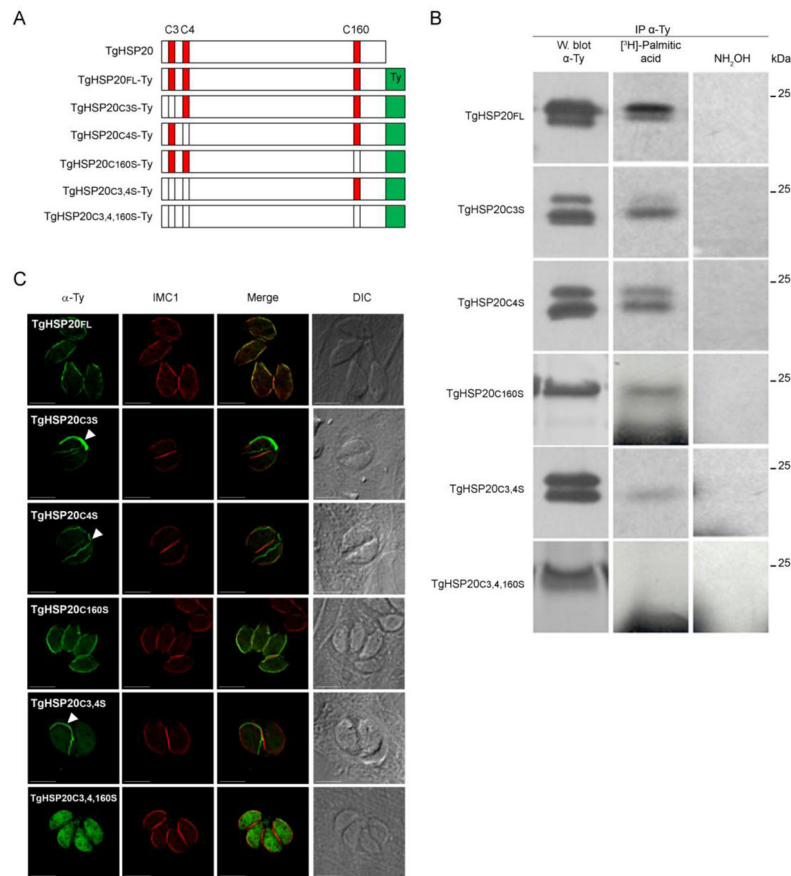
Figure 2. TgHSP20 is palmitoylated in all its cysteine residues but only N-terminal palmitoylation determines TgHSP20 localization to the IMC
A) Schematic representation of all Ty-tagged constructs generated and used in this study. The red boxes indicate the putative palmitoylated cysteine residues. B) *T. gondii* HSP20 is palmitoylated on all of its cysteine residues. Stable cell-lines over-expressing the different Ty-tagged constructs were metabolically labeled with [^{3}H]-palmitic acid followed by anti-Ty immunoprecipitation. Then, the samples were divided in three and run on SDS-PAGE: one sample was used for Western blot analysis as a loading control. The two remaining samples were used to detect if the radiolabel was incorporated to the protein. C) Palmitoylation of cysteines 3 and 4 is important to confer IMC localization. Intracellular tachyzoites overexpressing the different Ty-tagged constructs were fixed and the localization of the constructs was determined by indirect immunofluorecence assays. Differential interference contrast (DIC) microscopy is shown on the right. White arrowheads indicate a TgHSP20-postive unidentified structure. Scale bars= 5 μm..
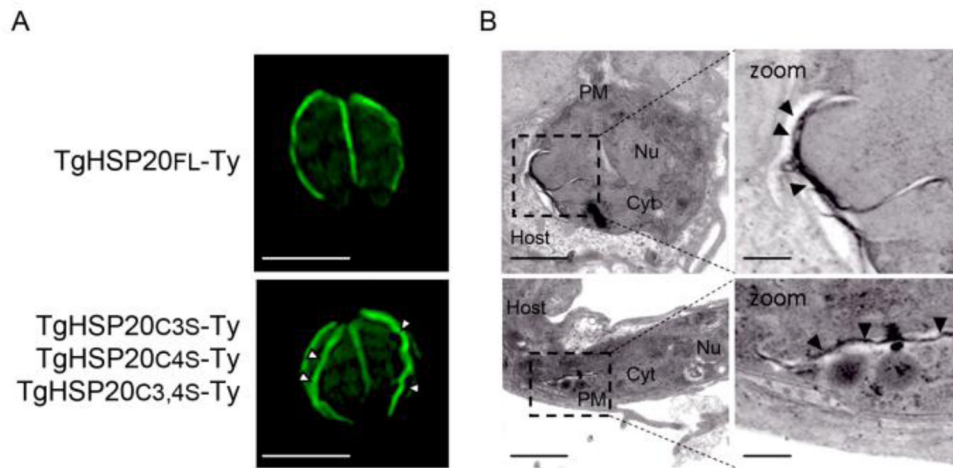
Figure 3. Transmission electron microscopy of the TgHSP20$_{C3,4S}$-Ty construct suggests the presence membrane accumulation

Lack or partial palmitoylation at the N-terminal of TgHSP20 forms an unidentified structure. A) Immunofluorescence assay showing the sub-cellular localization of full-length TgHSP20-Ty (TgHSP20$_{FL}$-Ty; upper panel) or parasites over-expressing different N-terminal palmitoylable TgHSP20 constructs (same result either with TgHSP20$_{C3S}$-Ty, TgHSP20$_{C4S}$-Ty or TgHSP20$_{C3,4S}$-Ty; lower panel). Scale bars= 5 μm. B) Transmission electron microscopy of the parasites observed in panel A. The white arrowheads show the atypical localization of TgHSP20 with indirect immunofluorescence and black arrowheads in the electron microscopy analysis. PM: plasma membrane; Nu: nucleus; Cyt: cytoplasm. Scale bars= 1 μm and 200 nm for the zoomed images.
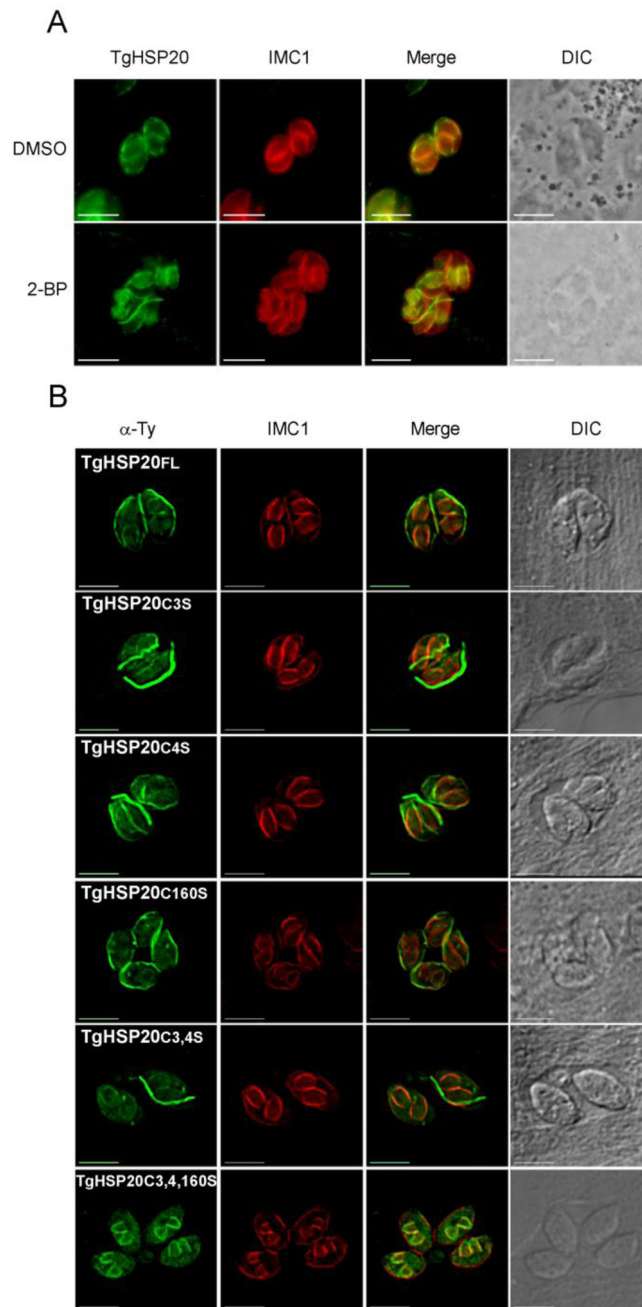
Figure 4. Palmitoylation is not required for TgHSP20 attachment to the IMC of daughter cells
A) TgHSP20 localization at daughter' IMC is not affected by 2-BP treatment. Replicating parasites were treated with 2-BP (100 µM) or DMSO for 16–18 h, then fixed and TgHSP20 localization was assessed by indirect immunofluorescence microscopy. Differential interference contrast (DIC) images are shown in the right panels. Scale bars= 5 µm. B) IMC localization at the daughter cell is maintained regardless of the TgHSP20 palmitoylation status. Intracellular replicating parasites overexpressing different Ty-tagged constructs were fixed and TgHSP20 localization was assessed by indirect immunofluorescence microscopy. DIC pictures are in the right panels. The anti-IMC1 antibody labels the IMC from both the mother and daughter cells. Scale bars= 5 µm.
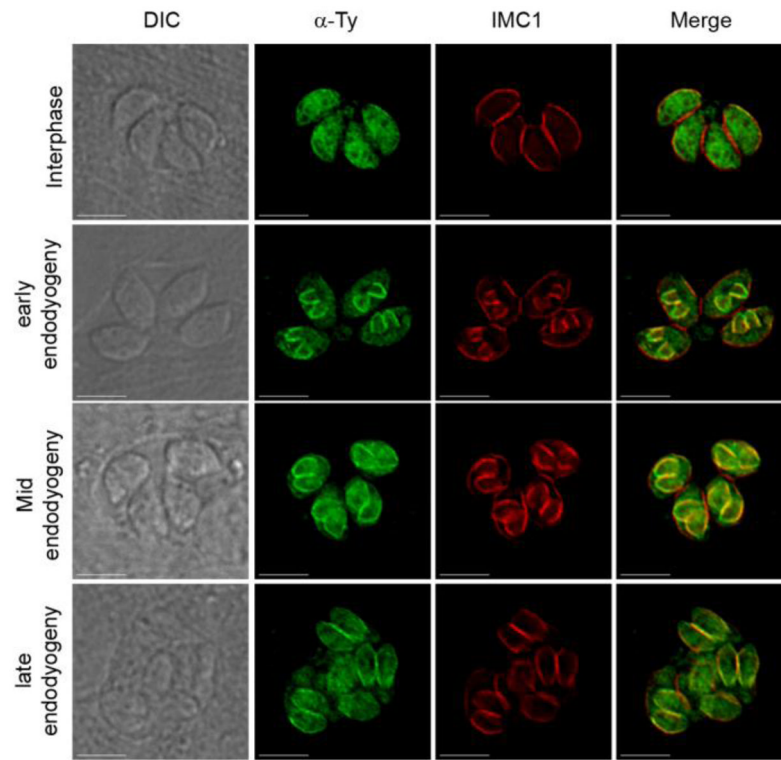
Figure 5. TgHSP20 interacts with IMC throughout endodyogeny in a palmitoylation-independent manner
The non-palmitoylable TgHSP20$_{C3,4,160S}$-Ty construct is able to interact with the daughter IMC until the late steps of endodyogeny. Samples of replicating parasites overexpressing the TgHSP20$_{C3,4,160S}$-Ty construct were obtained at different stages of the endodyogeny process and the Ty localization was assessed by indirect immunofluorescence assays. DIC pictures are in the right panels. Scale bars: 5 µm.