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Abstract
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Invited Review

Emerging roles for protein S-palmitoylation in Toxoplasma biology

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Post-translational modifications are refined, rapidly responsive and powerful ways to modulate protein function. Among post-translational modifications, acylation is now emerging as a widespread modification exploited by eukaryotes, bacteria and viruses to control biological processes. Protein palmitoylation involves the attachment of palmitic acid, also known as hexadecanoic acid, to cysteine residues of integral and peripheral membrane proteins and increases their affinity for membranes. Importantly, similar to phosphorylation, palmitoylation is reversible and is becoming recognized as instrumental for the regulation of protein function by modulating protein interactions, stability, folding, trafficking and signaling. Palmitoylation appears to play a central role in the biology of the Apicomplexa, regulating critical processes such as host cell invasion which is vital for parasite survival and dissemination. The recent identification of over 400 palmitoylated proteins in Plasmodium falciparum erythrocytic stages illustrates the broad spread and impact of this modification on parasite biology. The main enzymes responsible for protein palmitoylation are multi-membrane protein S-acyl transferases harbouring a catalytic Asp-His-Cys-CHHC motif. A global functional analysis of the repertoire of protein S-acyl transferases in Toxoplasma gondii and Plasmodium berghei has recently been performed. The essential nature of some of these enzymes illustrates the key roles played by this post-translational modification in the corresponding substrates implicated in fundamental processes such as parasite motility and organelle biogenesis. Toward a better understanding of the depalmitoylation event, a protein with palmitoyl protein thioesterase activity has been identified in T. gondii. TgPPT1/TgASH1 is the main target of specific acyl protein thioesterase inhibitors but is dispensable for parasite survival, suggesting the implication of other genes in depalmitoylation. Palmitoylation/depalmitoylation cycles are now emerging as potential novel regulatory networks and T. gondii represents a superb model organism in which to explore their significance.

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1. Introduction

Members of the phylum Apicomplexa are obligate intracellular protozoan parasites. Many of them are pathogens for humans and/or animals and cause severe diseases. The best example is probably Plasmodium, which is transmitted via infected mosquitoes and is responsible for malaria, causing approximately one million deaths each year (WHO, 2012). Toxoplasma gondii, the causative agent of toxoplasmosis, is one of the most successful parasites and is mainly transmitted via the ingestion of contaminated food, leading to chronic infection in one-third of the world’s population. This infection is mostly asymptomatic and the response raised by the immune system pushes the parasite to differentiate into a slowly dividing, encysted form that persists for the life span of its host (Innes, 2010). Two other parasites of the phylum, Eimeria and Theileria spp., can cause economic losses affecting poultry and cattle, respectively (Bishop et al., 2004; Tewari and Maharana, 2011).

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A unique attribute of this phylum is that the parasites use their substrate-dependent gliding motility to actively enter their target cells, a step necessary for their survival. Following intracellular expansion, they also utilise motility to egress from infected cells and invade neighbouring cells. The glideosome is the molecular motor that powers parasite motility (Opitz and Soldati, 2002). It has been extensively studied and several components of this machinery are highly modified by post-translational modifications (PTMs); notably phosphorylation and palmitoylation (Jacot and Soldati-Favre, 2012). Very little is known about lipid modification in apicomplexans, however palmitoylation appears to be widely used by these parasites. The functional importance of palmitoylation for the lytic cycle of T. gondii has recently been highlighted by a series of striking examples of palmitoylated substrates critically involved in diverse processes such as motility, invasion (Gaskins et al., 2004; Frenal et al., 2010), calcium signalling (Garrison et al., 2012; Lourido et al., 2012; McCoy et al., 2012), organelle positioning (Beck et al., 2013; Mueller et al., 2013) and parasite division (Beck et al., 2010). Importantly, these cases implicate proteins that are broadly conserved across the phylum Apicomplexa.
This review provides an update of the recent studies on palmitoylated proteins, illustrating how instrumental this PTM is in revealing unexpected elements of the biology of apicomplexans. Moreover, it describes current knowledge concerning the palmitoylation/depalmitoylation machinery across the phyllum and in *T. gondii* in particular.

2. Acylation impacts on the fate of proteins

Proteins are often modified by PTMs that influence their function, impacting on their conformation, stability, trafficking and partner interactions. The best-studied PTM and probably the most notorious is phosphorylation, however acylation, also known as fatty acylation or lipidation, is emerging as a widespread PTM (Nadolinski and Linder, 2007). In particular, palmitoylation might have been underestimated for decades due to its labile nature that can be disrupted by reducing conditions (Levental et al., 2010) and due to the low sensitivity of radioactive palmitate labelling that necessitated weeks to months of exposure of autoradiograms. However, the recent development of new technologies to capture and identify palmitoylated proteins (Hannoush and Sun, 2010) has led to the realisation that this PTM is widely used not only by eukaryotes, but also by viruses and bacteria that are able to subvert the host palmitoylation machinery (Blanc et al., 2013).

Acylation comprises three main types of modifications that are known to influence the membrane interaction properties of their substrates due to their hydrophobic nature. Myristoylation and prenylation are irreversible and lead to a loose association with membranes. Protein myristoylation consists of the co-translational addition of a 14-carbon saturated fatty acid (myristate) to a N-terminal glycine (Boutin, 1997; Farazi et al., 2001), whereas prenylation post-translationally links a 15- (farnesyl) or 20-carbon (geranylgeranyl) isoprenoid to a C-terminal cysteine through a thioester post-translationally (Zhang and Casey, 1996; Amaya et al., 2011) (Fig. 1A and B). In contrast, S-palmitoylation is a labile thioester linkage arising from the post-translational addition of a 16-carbon saturated fatty acid (palmitate) to a cysteine residue that can be within either soluble or integral membrane proteins (Linder and Deschenes, 2007). Soluble proteins are profoundly modified by saturated lipids (myristate and palmitate) that promote their insertion into microdomains or lipid rafts (Levental et al., 2010; Yang et al., 2010) (Fig. 1C). In contrast, the role of palmitoylation for transmembrane domain (TMD)-containing proteins has been more enigmatic. Nevertheless recent studies have highlighted the key contribution of palmitoylation in changing the conformation of the TMD itself, impacting protein interactions directly (Blaskovic et al., 2013) (Fig. 1D).

Palmitoylation has aroused considerable interest due to its enzymatically reversible nature, its predicted impact on the localisation and stability of its substrates and their interaction with other proteins (Linder and Deschenes, 2007; Baekkeskov and Kanaani, 2009). The best-studied examples are probably the two small GTPase isoforms H- and N-Ras, members of the proto-oncogene family that shuttle between the Golgi apparatus and the plasma membrane (PM) to regulate activity and signalling (Ahearn et al., 2012). Concomitantly, the enzymes responsible for palmitoylation and depalmitoylation have been associated with important human diseases and cancers (Greaves and Chamberlain, 2011; Resh, 2012).

In the kinetic trapping model (Peitzsch and McLaughlin, 1993; Shahinian and Silvius, 1995), palmitoylation acts in concert with two other types of acylation to ensure a stable interaction within membrane leaflets (Fig. 1B). First, myristoylation or prenylation allows the protein to interact loosely with the membrane, whereas subsequent palmitoylation enhances and tethers this interaction. The initial transient interaction can be stabilised by proximal positively charged amino acid motifs and is in fact necessary for the subsequent palmitoylation step. Indeed, it maintains the substrate in proximity to the membranes, where the multi-pass TMD-containing enzymes responsible for transferring palmitate are embedded in the lipid bilayer. These enzymes are known as protein S-acyl transferases (PATSs) (Mitchell et al., 2006). Palmitoylation is not only found in combination with other types of acylation; some proteins can also be uniquely modified by palmitoylation, however the molecular basis that mediates the initial contact with the membrane remains to be identified. Given that palmitoylation is reversible, a depalmitoylation reaction can occur and is catalysed by a class of enzymes known as acyl protein thioesterases (APTs) or palmitoyl protein thioesterases (PPTs) (Camp and Hofmann, 1993; Zeidman et al., 2009). It is conceivable that palmitoylation/depalmitoylation cycles constitute an efficient way to modulate the functions of proteins and biological processes.

3. Global analysis of palmitoylated proteins in apicomplexans

3.1. In silico prediction of the palmitoylated proteome

To date, no consensus motif other than the presence of an accessible cysteine has been identified within substrates as a signature for palmitoylation. It is therefore particularly difficult to make in silico predictions of palmitoylated cysteine residues within a protein, even with the number of experimentally identified sites rising (Ren et al., 2008). The reliability of the prediction is considerably increased when myristoylation or prenylation sites are predicted as well, because these are often coupled to palmitoylation. For example, a cysteine residue upstream of the prenylated motif can become palmitoylated. Palmitoylation is frequently associated with myristoylation of an N-terminal glycine residue and it has been experimentally determined that the modified cysteines are, in this case, preferentially found within the 20 amino acids following the modified glycine (Navarro-Lerida et al., 2002). We performed a search for such dual N-terminal acylation across the genome of *T. gondii* with the motif MGxG, where x can be zero to 18 residues of any kind. The search produced 112 hits after the elimination of the proteins that had a signal peptide or contained one or more TMD (Supplementary Table S1). Among these 112 proteins, some kinases (CDPK3, CDPK4, PKG), gliding-associated proteins (GAP45, GAP70 and GAP80), inner membrane complex (IMC) sub-compartment proteins (ISP1, 2 and 3) and the rhoptry-associated protein with armadillo repeats only protein (ARO) were found. These proteins are probably truly acylated, since most of them become cytosolic after mutation of the predicted modified residues (Beck et al., 2010, 2013; Garrison et al., 2012; Lourido et al., 2012; McCoy et al., 2012; Mueller et al., 2013). Many of these proteins are broadly conserved across the phylum Apicomplexa and for some of them, palmitoylation is essential for their function. In support of this, treatment of *T. gondii* and *Plasmodium falciparum* with 2-bromopalmitate (2-BP), an inhibitor of palmitoylation, is deleterious to parasite survival (Alonso et al., 2012; Jones et al., 2012), although these findings should be interpreted with caution, given that 2-BP is considered to be poorly specific and highly toxic (Davda et al., 2013).

Despite the existence of an algorithm for the prediction of palmitoylation sites in proteins (CSS-Palm 3.0) (Ren et al., 2008) that has recently been improved thanks to experimentally verified palmitoylation sites manually collected from the scientific literature, confidence in the identification of palmitoylated proteins based on in silico analysis remains low. Therefore, the methods of choice for investigating palmitoylation in a given organism or cell type rely on global biochemical approaches that result in identification of the whole palmitoylated proteome or palmitome.
3.2. Experimental palmitome

In the procyclic form of the protozoan parasite Trypanosoma brucei, an experimental palmitome analysis was performed by an Acyl-Biotin Exchange (ABE) method (Emmer et al., 2011) (Fig. 2A). This study revealed that the main part of this proteome is constituted by uniquely palmitoylated proteins; 50% being integral proteins and 33% being hydrophilic proteins, while dually acylated proteins represent approximately 17%. Although no palmitome has been reported in T. gondii to date, the recently established palmitome in the schizont stage of P. falciparum is highly informative. This study combined, for the first time, two methods: ABE and Metabolic Labelling and Click Chemistry (MLCC) (Fig. 2), to isolate palmitoylated proteins prior to their identification by mass spectrometry (Jones et al., 2012). The ABE method consists of blocking all free cysteine thiols prior to the cleavage of the cysteine-palmitoyl thioester linkages and labelling of the newly exposed thiols with biotin for affinity purification. A number of false positives can occur due to incomplete blockage of free cysteine residues and incomplete cleavage of the thioester linkage. To increase the level of confidence, parasites were metabolically labelled with the non-radioactive palmitate analogue, 17-octadecyloic acid (17-ODYA), which exhibits an alkyne group for subsequent click chemistry with biotin-azide (Martin and Cravatt, 2009). This recent technology allows the isolation of proteins which are able to incorporate the palmitate analogue during the time of metabolic labelling and to include more of the dually acylated proteins that are relatively resistant to cleavage in the ABE method (Ladygina et al., 2011). More than 400 putative P. falciparum palmitoylated proteins were identified in the highly enriched fraction (Jones et al., 2012). This is significantly more than what has been reported to date for the palmitome of Saccharomyces cerevisiae (50 proteins) (Roth et al., 2006), T. brucei (124 proteins) (Emmer et al., 2011) and mammalian cells (between 125 and 260 proteins) (Kang et al., 2008; Martin and Cravatt, 2009; Yount et al., 2010). These findings led to the assumption that P. falciparum makes substantial use of palmitoylation, wider than previously thought, with implications for many diverse biological processes including signalling, development, invasion as well as cytoadherence and drug resistance (Jones et al., 2012). Indeed, a large diversity of substrates has been identified including both soluble and TMD proteins, in addition to the known palmitoylated proteins such as the gliding-associated protein PfGAP45 (Rees-Channer et al., 2006) or PfCalpain, a cysteine protease required for cell cycle progression that shuttles between the endoplasmic reticulum (ER) and the nucleus (Russo et al., 2009a,b). Several additional proteins already suspected to be palmitoylated were also found, such as the calcium-dependent protein kinase PCDPK1, the myosin A tail-associated protein PfMTIP and the alveolin-containing proteins PfALV4 and PfALV5.
4. Palmitoylated proteins in T. gondii

4.1. IMC-localised palmitoylated proteins are important for division

Members of the Apicomplexa belong to the group Alveolata which also includes the dinoflagellates and ciliates. These highly diverse protozoans share a unique structural feature: a three-layered pellicle composed of the PM and the closely apposed dual layer, named the IMC or alveoli (Gould et al., 2011). Most of the palmitoylated proteins described to date are found anchored to the membrane of the IMC or the PM (Table 1). In Toxoplasma, the IMC is formed from patches of flattened sacs joined together by sutures, except for the apical cap, where only one truncated cone-shaped plate is observed, and the posterior end where the IMC is interrupted (Dubremetz and Torpier, 1978). This unique organelle plays a structural role, contributing to the shape and robustness of the parasite, and acts as a scaffold for the growing daughter cells (Striepen et al., 2007). The IMC also serves as a platform for the

![Diagram]

Fig. 2. Two methods used to establish the palmitome of Plasmodium falciparum schizonts. Schematic representation of the acyl-biotin exchange (ABE) steps (A) and of the in vivo metabolic labelling with the palmitate analogue 17-octadecynoic acid (17-ODYA) that harbours an alkyne group for a subsequent click chemistry step (B). TCEP, Tris (2-carboxyethyl)phosphine; NEM, N-ethylmaleimide; HPDP-biotin, N-[(6-biotinamido)hexyl]-3’-(2’-pyridylthio)-propionamide. Adapted from Yang et al. (2010).
anchoring of the molecular machinery, the glideosome, which powers parasite motility necessary for invasion of, and egress from, infected cells (Opitz and Soldati, 2002). Although the IMC is a central organelle, little is known about its biogenesis, organisation and regarding the trafficking of proteins to this unique compartment.

A family of conserved proteins in apicomplexans has been localised to different sub-compartments of the IMC and these are termed IMC sub-compartment proteins (ISPs) (Beck et al., 2010; Fung et al., 2012). TgISP1, 2 and 3 are myristoylated and palmitoylated at their N-terminus, whereas TgISP4 contains two pairs of predicted palmitoylated cysteine residues but no myristoylation site (Fung et al., 2012). TgISP1 is restricted to the apical cap of the parasite, whereas TgISP3 is found in the IMC but is excluded from the cap. Notably, TgISP2 and TgISP4 are localised to a central zone of the IMC that starts under the apical cap and stops around the end of the microtubules, one-third of the distance above the posterior pole (Beck et al., 2010). TgISP1 appears to play a gate-keeper role for the apical sub-compartment, since its deletion allows the relocation of the three other ISPs to the cap (Beck et al., 2010; Fung et al., 2012). The importance of acylation in targeting the TgISPs to the IMC is clearly established, since mutation of the predicted palmitoylation sites leads to their cytosolic localisation. However, it remains unclear what is necessary for targeting to the IMC sub-domains. Indeed, whereas the expression of the N-terminal parts of TgISP1 (first 29 residues) and TgISP3 (first 36 residues) containing the myristoylated and palmitoylated sites are sufficient to target GFP to the expected sub-compartments, the first 40 residues of ISP2 confer no specificity, and target the fusion to the entire IMC (Beck et al., 2010). A residue outside the N-terminal part is therefore likely to be critical to restrict TgISP2 to the central portion of the IMC. In addition, TgISP4 localises to the same sub-compartment but lacks the two acylation sites, suggesting the participation of another targeting determinant or an interacting partner. TgISP2 appears to control cell division and daughter cell formation, since its deletion often leads to the budding of more than two parasites (Beck et al., 2010), whereas the other TgISPs might be functionally redundant.

The cytoplasmic face of the IMC is intimately associated with the basket of subpellicular microtubules that run along the apical two-thirds of the parasite length (Morrissette et al., 1997) through a network of intermediate-like filaments formed by alveolin-containing proteins (named ALVs or IMCs) that are conserved in apicomplexans (Mann and Beckers, 2001; Gould et al., 2008; Anderson-White et al., 2011). The IMC family is composed of 14 members in T. gondii and none of them has a predicted TMD, but 10 have putative palmitoylation sites that probably anchor the proteins to the cytoplasmic face of the IMC (Anderson-White et al., 2011). Consistent with these predictions, PFALV4 and PFALV5, corresponding to TgIMC4 and TgIMC15, respectively, were identified in the palmitome of P. falciparum (Jones et al., 2012). In T. gondii, the IMCs belong to several groups, each having a different timing of expression during daughter cell development; some IMCs are detectable only in the mature parasites, whereas one (TgIMC15) appears as early as the appearance of the two centro-somes. TgIMC11 is restricted to the apical cap, whereas a group of four IMCs are targeted to the basal complex (Anderson-White et al., 2011). For two IMCs that do not contain predicted palmitoylation sites, it is clear that their alveolin repeats are the main but indirect targeting determinants, possibly interacting with a palmitoylated IMC protein (Anderson-White et al., 2011). The targeting of these IMC proteins is tightly regulated in time and space and palmitoylation might play a central role in this orchestration.

Finally, the small heat shock protein HSP20, which is strictly conserved in apicomplexans, has been localised to the outer leaflet of the IMC facing the PM, in mature parasites and in stripes that follow the microtubules but extend further, suggesting that it could be associated with other structural elements in growing daughter cells (de Miguel et al., 2008) (Table 1). Three palmitoylated cysteine residues have been identified by radioactive palmitate labelling, however only the N-terminal pair of cysteine residues were found to be important for targeting, based on site-directed mutagenesis. Unexpectedly, acylation is not required for the interaction of HSP20 with the IMC of the developing daughter cells but is necessary to maintain the protein at the IMC of the mature parasites (De Napoli et al., 2013). The HSP20 orthologue in Plasmodium berghei has been reported to regulate sporozoite motility (Montagna et al., 2012).

4.2. A dually acylated protein is essential for apical positioning of the rhoptry organelles

The apicomplexan invading zoites are highly polarised cells that possess an apical complex that includes two types of specialised, regulated secretory organelles, the micronemes and rhoptries, whose contents are sequentially discharged during invasion (Car ruthers and Boothroyd, 2007).

Table 1
Summary of the putative or confirmed palmitoylated substrates described in this review for Toxoplasma and Plasmodium.

<table>
<thead>
<tr>
<th>Name</th>
<th>Localisation</th>
<th>Method of identification</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>TgISP1, 2, 3</td>
<td>IMC</td>
<td>Site-directed mutagenesis, N-terminal part fused to GFP</td>
<td>Beck et al. (2010)</td>
</tr>
<tr>
<td>TgISP4</td>
<td>IMC</td>
<td>Site-directed mutagenesis</td>
<td>Fung et al. (2012)</td>
</tr>
<tr>
<td>TgHSP20</td>
<td>IMC</td>
<td>Site-directed mutagenesis, [3H] palmitate labelling</td>
<td>De Napoli et al. (2013)</td>
</tr>
<tr>
<td>TgIMC1, 4, 6, 9, 10, 11, 12, 13, 14, 15</td>
<td>IMC</td>
<td>Site-directed mutagenesis, [3H] palmitate labelling</td>
<td>Anderson-White et al. (2011)</td>
</tr>
<tr>
<td>TgMLC1</td>
<td>Pellicle</td>
<td>Site-directed mutagenesis</td>
<td>Frenal et al. (2010)</td>
</tr>
<tr>
<td>TgGAP45</td>
<td>PM</td>
<td>Site-directed mutagenesis</td>
<td>Garrison et al. (2012)</td>
</tr>
<tr>
<td>TgCDPK3</td>
<td>PM</td>
<td>Site-directed mutagenesis</td>
<td>McCoy et al. (2012)</td>
</tr>
<tr>
<td>TgMLC2</td>
<td>PM</td>
<td>Site-directed mutagenesis</td>
<td>Lourido et al. (2012)</td>
</tr>
<tr>
<td>PIGAP45</td>
<td>PM</td>
<td>[3H] palmitate labelling</td>
<td>Unpublished data</td>
</tr>
<tr>
<td>PIMTIP</td>
<td>Pellicle</td>
<td>P.f. schizont palmitome</td>
<td>Rees-Channer et al. (2006)</td>
</tr>
<tr>
<td>PVARO</td>
<td>Rho try</td>
<td>Site-directed mutagenesis, N-terminal part fused to GFP, ABE</td>
<td>Cabrer a et al. (2012)</td>
</tr>
<tr>
<td>PFA LV4, 5</td>
<td>IMC</td>
<td>P.f. schizont palmitome</td>
<td>Jones et al. (2012)</td>
</tr>
<tr>
<td>PFA Calpain</td>
<td>ER/nucleolus</td>
<td>Site-directed mutagenesis, [3H] palmitate labelling</td>
<td>Russo et al. (2009a)</td>
</tr>
</tbody>
</table>

ABE, acyl-biotin exchange; ER, endoplasmic reticulum; IMC, inner membrane complex; P.f., Plasmodium falciparum; PM, plasma membrane.
ARO is a conserved protein across the phylum that localises at the surface of the rhoptries (Hu et al., 2010; Cabrera et al., 2012). PFARO and TgARO decorate the cytosolic face of the rhoptry membrane through N-terminal myristoylation and palmitoylation (Table 1). The first 20 amino terminal residues of ARO are sufficient to target GFP to the rhoptries, and both the organellar targeting and membrane association critically depend on the acylation sites, together with the two downstream conserved residues, R9 and K14 (Cabrera et al., 2012). Surprisingly, substitution of the arginine with a negatively charged glutamate residue is sufficient to abolish the membrane association of the GFP fusion, whereas exchange with a positively charged lysine residue targets the chimera to the PM. Additionally, whereas mutation of the lysine residue K14 to a positively charged arginine does not affect rhoptry localisation, its substitution with a negatively charged glutamate results in association of the fusion protein with the IMC membrane (Frenal et al., 2012). This meticulous dissection of the N-terminal portion of the dually acylated ARO suggests that the selective targeting signal that discriminates between IMC, PM and rhoptries is subtle and might rely on key critical residues that need to be recognised by specific PATs.

Inducible knockout of TgARO has been generated and led to a defect in apical positioning of the fully mature rhoptry organelles, which are instead found dispersed throughout the cytosol. Depletion of TgARO did not interfere with the apical localisation or secretion of micronemes and the mutant parasites were able to egress from the infected cells and to glide. However, discharge of the rhoptry content did not occur and the parasites failed to invade host cells (Beck et al., 2013; Mueller et al., 2013). Acylation of ARO is critical for the anchoring of the complex that ARO forms with its identified interacting partners, myosin F, ARO interacting partner (AIP) and adenylate cyclase beta (ACβ). These partners potentially assist ARO in bringing the rhoptries to the apical pole of the parasite (Mueller et al., 2013).

4.3. Acylation of GAP45 is crucial for cohesion of the pellicle during motility

Toxoplasma gondii motility relies on the glideosome (Opitz and Soldati, 2002), an actomyosin system that is highly conserved across the phylum and is composed of six proteins. The myosin heavy chain A (MyoA) is the molecular motor and interacts with the myosin light chain MLC1 (or myosin A-associated protein MTIP in Plasmodium spp.) (Herm-Gotz et al., 2002) and the coccidian-specific essential light chain 1 (ELC1) (Nebl et al., 2011). Both MLC1 (MTIP) and ELC1 are predicted to be palmitoylated and PM-TIP was detected in the P. falciparum palmitome (Jones et al., 2012). Two integral membrane proteins, the gliding-associated proteins GAP40 and GAP50 (Gaskins et al., 2004; Frenal et al., 2010), anchor the complex in the outer membrane of the IMC, ensuring a firm attachment to the underlying cytoskeleton. In this context, the glideosome has been reported to interact with a family of glideosome-associated proteins with multiple membrane spans, termed GAPMs, which are integral membrane proteins of the IMC (Bullen et al., 2009). Two proteins of this family have also been identified in the palmitome (Jones et al., 2012). Finally, GAP45 is an acylated protein that recruits the motor complex to the IMC via its C-terminal domain (Frenal et al., 2010). PGAP45 has been experimentally shown to be myristoylated and palmitoylated at its N-terminus (Rees-Channer et al., 2006) and these two types of acylation are necessary to anchor TgGAP45 to the PM (Frenal et al., 2010). Although the mutation of these N-terminal acylation sites did not impair the recruitment of the motor complex to the IMC by TgGAP45, parasites expressing such a mutant failed to substitute for TgGAP45 function in motility, invasion and egress. This uncovered a critical role for TgGAP45 in pellicle integrity that participates in the maintenance of cohesion between IMC and PM during the tension imposed by invasion (Frenal et al., 2010). Palmitoylation presumably also occurs at the C-terminal region of PGAP45, which might act as an anchor to the IMC (Jones et al., 2012). Toxoplasma gondii possesses two additional proteins related to TgGAP45, TgGAP70 and TgGAP80 that are tailored to the apical and posterior pole of T. gondii and recruit MyoA and MyoC, respectively (Frenal et al., 2010; Frenal, unpublished data).

4.4. Calcium signalling

Members of the calcium-dependent protein kinases (CDPKs) are important players in the signalling cascade leading to infection (Billker et al., 2009). Two of these, TgCDPK3 and TgCDPK4, are predicted to be myristoylated and palmitoylated and TgCDPK3 has been linked to egress, notably via the identification of several mutations in the corresponding locus that cause resistance to calcium ionophore-induced egress (Garrison et al., 2012). TgCDPK3 is localised to the PM and the first 15 N-terminal amino acids are sufficient for this targeting (Garrison et al., 2012; Lourido et al., 2012; McCoy et al., 2012). Mutations in the acylation sites hampered complementation of a knockout strain (Garrison et al., 2012; McCoy et al., 2012), thus confirming that lipid anchoring to the membrane is essential for the function of TgCDPK3 in parasite egress from infected cells.

5. Enzymes implicated in protein palmitoylation

Non-enzymatic palmitoylation of protein can in principle take place, however a family of enzymes responsible for catalysing S-palmitoylation has initially been identified in yeast and subsequently in mammals (Bartels et al., 1999; Roth et al., 2002; Fukata et al., 2004; Huang et al., 2004). The PATs are only found in eukaryotic cells and exhibit a catalytic Asp-His-His-Cys motif, present within a cysteine-rich domain (CRD), and are therefore described as DHHCs (Fig. 3A). In contrast to other acyl transferases that are soluble, the DHHCs are multi-membrane-spanning domain-containing proteins with the catalytic motif facing the cytosol (Mitchell et al., 2006). Site-directed mutagenesis established a direct involvement of the DHHC motif in palmitate transfer, since substitution of the cysteine residue was enough to abolish both auto-palmitoylation of the PAT and palmitoylation of substrates (Roth et al., 2002; Smotrys et al., 2005). It was recently clearly demonstrated that the auto-palmitoylated PAT serves as a covalent intermediate between the donor palmitoyl-CoA and the acceptor in a two-step process (Jennings and Linder, 2012). Virtually nothing is known about the mode of recognition between these enzymes and their substrates, but specificity appears to be determined by regulatory domains outside of the DHHC-CRD (Huang et al., 2009). In addition, other domains involved in protein–protein interactions such as ankyrin repeats, PDZ or SRC Homology 3 (SH3) domains are also found in several DHHCs and might participate in substrate recognition.

The DHHCs are found in variable numbers among protozoans and mammals. For example, the repertoire of DHHCs in S. cerevisiae (Mitchell et al., 2006) includes seven enzymes, whereas T. brucei possesses 12 (Emmer et al., 2011) and Arabidopsis thaliana has 23 (Batistic, 2012), the same number as found in humans (Putlina et al., 1999). The P. falciparum genome encodes 12 DHHC-containing proteins, whereas T. gondii possesses the largest family in the Apicomplexa with 18 genes, 16 of which are expressed in the tachyzoite stage (Frenal et al., 2013). These large repertoires in apicomplexans again argue for an important role for palmitoylation. Recently, a global analysis of the subcellular localisation, and essentiality of DHHC-containing proteins, has been performed.
Fig. 3. The DHHC motif-containing family of protein S-acyl transferases (PATs) in apicomplexans. (A) Alignment of the DHHC-cysteine rich domain (CRD) of Toxoplasma gondii PATs as well as the DPG and TTxE conserved motifs (with x being any residue), whose function is still unknown, performed with multalin (Corpet, 1988). The residues in red are conserved in at least 16 of the 18 sequences (>90%) and the residues in blue are conserved in 12–16 of the 18 sequences (70–90%). Gene IDs are from ToxoDB version 8.2 (Aurrecoechea et al., 2007). (B) Phylogenetic tree of apicomplexan DHHC-containing proteins based on maximum likelihood (ML). Nodes supported by bootstrap values <95 were considered insignificant and have been collapsed to highlight the significant clusters (coloured boxes). Protein accession numbers are given according to the EuPathDB website (Aurrecoechea et al., 2007). Sequence alignment used to compute the phylogenetic tree was previously published (Frenal et al., 2013). Each colour refers to a specific organism: red, T. gondii; orange, Neospora caninum; light blue, Plasmodium falciparum; dark blue, Plasmodium berghei; purple, Cryptosporidium parvum; black, Eimeria tenella; magenta, Theileria parva; and green, Babesia bovis.
simultaneously in *T. gondii* and *P. berghei* (Frenal et al., 2013). As in humans and yeast (Ohno et al., 2006), these DHHCs are targeted to distinct endomembrane compartments, with several of them found in the Golgi apparatus (Table 2). In addition, some enzymes are found at the PM and in the apicomplexan-specific organelles including the IMC and the rhoptries. Nothing is yet known about the determinants that address these polytopic enzymes to their specific destinations. Interestingly, five DHHCs appear to be essential for *T. gondii* survival and include the apicomplexan-specific IMC and rhoptry enzymes (Table 2). Despite the existence of eight Golgi-localised DHHCs, two of them probably exhibit unique features, as the corresponding genes could not be disrupted (Frenal et al., 2013). This is the first organism for which five PATs appear to individually fulfil some critical functions whereas all of the yeast or *T. brucei* PATs are apparently dispensable or functionally redundant (Roth et al., 2006; Emmer et al., 2011).

DHHC7 is the PAT that localises to the rhoptries of *T. gondii*, *P. falciparum* and *P. berghei* (Frenal et al., 2013). A critical role in invasion was established based on two distinct strategies leading to down-regulation of TgDHHC7 expression that resulted in the mis-positioning and defective secretion of the rhoptries (Beck et al., 2013; Frenal et al., 2013). DiCre-dependent excision of the TgARO gene (Frenal et al., 2013). The defects in rhoptry discharge and organelle positioning and defective secretion of the rhoptries (Beck et al., 2013; Frenal et al., 2013). These findings strongly suggest that TgARO is the main substrate of TgDHHC7 and hence constitutes the first essential substrate/PAT pair identified in *T. gondii*. To date, no other substrate for TgDHHC7 has been identified.

Numerous palmitoylated proteins have been found to be associated with the IMC and two PATs are localised to this compartment in *T. gondii* (Frenal et al., 2013). Whereas TgDHHC2 is present in all sub-compartments of the IMC, TgDHHC14 appears to be excluded from the apical cap. TgDHHC14 is predictably responsible for the palmitoylation of proteins located in the corresponding sub-compartments of the IMC and might act as a trafficking determinant. Both IMC-DHHCs are conserved across the phylum (Fig. 3B) and appear to be essential, reflecting their key contribution to the biogenesis of this compartment and their impact on the development and maturation of the daughter cells. In *Plasmodium*, the IMC is not sub-compartmentalised, so it is plausible that the function of the three IMC-DHHCs is redundant.

Finally, two DHHC-containing proteins found in the PM are tentatively responsible (by their localisation) for the palmitoylation of the N-terminal domains of TgGAP45, TgGAP70 and TgCDPK3. In addition, TgMLC2, a myosin light chain that anchors myosin D to the inner leaflet of the PM, is presumably also a substrate for one of the PM-DHHCs, since mutation of two cysteine residues predicted to be palmitoylated led to the cytosolic relocation of the ML22-MyoD complex (Polonais et al., 2011; unpublished data). TgDHHC4 and TgDHHC13 are individually dispensable, suggesting some functional redundancy to ensure palmitoylation of these substrates, reported to be critical for parasite dissemination. Intriguingly, although the two PM-DHHCs might be able to catalyse lipid modification of the same substrates, they are not phylogenetically closely related and only TgDHHC13 is conserved across the phylum (Fig. 3B).

### 6. Enzymes implicated in protein depalmitoylation

S-Palmitoylation is the only reversible post-translational lipid modification that in principle can allow membrane cycling of a substrate. This reversibility offers a flexible, rapid and precise mode of regulation of protein activity, comparable with phosphorylation (Hemsley and Griersson, 2008). Genes encoding four enzymes with the capacity for depalmitoylation have been identified in higher eukaryotic genomes to date, but have been less extensively studied than the DHHC-PATs. All have an active Ser-Asp-His catalytic triad and cleave the labile thioester bond that attaches palmitate to the substrate, however they can be split into two categories. The palmitoyl protein thioesterases, PPT1 and PPT2, are involved in degradation of lipid-modified proteins in lysosomes, with each having distinct substrate specificity due to divergence in the substrate-binding regions (Camp and Hofmann, 1993; Camp et al., 1994; Soyombo and Hofmann, 1997). Loss of either PPT1 or PPT2 function results in the accumulation of lipid-modified material characteristic of lysosomal storage disorders such as infantile neuronal ceroid lipofuscinosis (INCL), and ultimately leads to cell death (Vesa et al., 1995; Gupta et al., 2001, 2003; Mitchison et al., 2004). APT1 and APT2 have been associated with dynamic palmitoylase cycling. The preferred substrates of APT1 were identified as thioacylated proteins (Duncan and Gilman, 1998; Hirano et al., 2009) although APT1 was originally identified as a lysophospholipase (Sugimoto et al., 1996). Further characterisation of human APT1 (hAPT1) has shown that it is responsible for cleaving palmitate from substrates both in vitro and in vivo (Duncan and Gilman, 1998; Tomatis et al., 2010). It is possible that the distinct substrate specificities of APT1 and APT2 are due in part to their differential expression between cell types, although some overlap might exist (Tomatis et al., 2010). The palmitoylation cycle that controls the correct compartmentalisation of H-Ras and N-Ras is the most intensively studied example of an APT1 substrate (Baekkeskov and Kanaani, 2009).

In apicomplexans, evidence for the existence of a functional APT enzyme has recently been deciphered in two studies that led to the characterisation of an active serine hydrolase, phylogenetically related to APT1 and APT2 but restricted only to the coccidian subgroup of the Apicomplexa, which can cleave palmitoylated substrates in vitro (TGME49_228290). TgPPT1 also known as TgASH1). The term APT could not be assigned to this gene in *T. gondii* since this abbreviation has already been used to describe an unrelated gene (Child et al., 2013; Kemp et al., 2013). TgPPT1 is a target of a small molecule inhibitor, chloroacetocoumarin, which exhibits
enhanced microneme secretion, motility and invasion efficiency (Child et al., 2013). Genetic ablation of the TgPPT1 gene alleviated the effect of chloroisoconomarin. In the parallel chemical-genetic study, TgPPT1/TgASH1 was identified as the target of two classes of inhibitors against hAPT1 that show different structures and levels of specificity against serine hydrolases, and disruption of the gene showed no detectable impairment in either microneme secretion, gliding motility or invasion (Kemp et al., 2013).

Together with the lack of a clear APT homologue outside of the coccidian group of the Apicomplexa, the dispensability of TgPPT1 suggests that either depalmitoylation is not critical for these parasites, or that it occurs through the action of another related enzyme. The T. gondii genome possesses over 50 proteins that contain ω-hydrolase domains. Using Hidden Markov Models (HMM) (Gough et al., 2001), 20 of these can be grouped into families that are potentially relevant for APT activity (Kemp et al., 2013). Three candidates exhibit the most significant homology to TgPPT1 and present across the Apicomplexa phylum and were investigated further. Two of these were dispensable (TgASH2 – TGME49_254690 and TgASH3 – TGME49_223510), whereas TgASH4 (TGME49_262490) was refractory to gene disruption and hence constitutes a primary candidate for an enzyme implicated in palmitoylation cycles.

7. Conclusion and perspectives

The large repertoires of PATs in apicomplexans and the prevalence of palmitoylated proteins deduced from the palmitome of asexual-stage P. falciparum parasites strongly support the preponderant role of palmitoylation as a PTM in these parasites. Although the palmitoylation cycle is a conceptually attractive mechanism to rapidly and efficiently fine-tune biological processes, there is limited evidence for its exploitation in apicomplexans. To date, the only clear example of a parasite protein shown to go through cycles of palmitoylation and depalmitoylation with an impact on its targeting and function is the calpain in P. falciparum. This protein shuttles between the ER and the nucleolus of the parasite in a palmitoylation-dependent manner (Russo et al., 2009a). Importantly, the palmitome analysis combined with 2-bromo-palmitate treatment also suggests that a series of parasite proteins might be subject to palmitoylation/dDepalmitoylation cycles and if this enzyme constitutes a possible target for intervention.

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Appendix A. Supplementary data

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References


