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Invited Review

Emerging roles for protein S-palmitoylation in *Toxoplasma* biology

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ABSTRACT

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 recognised as instrumental for the regulation of protein function by modulating protein interactions, stability, folding, trafficking and signalling. 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-His-Cys (DHHC) 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.

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).

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.

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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 phylum 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 (Nadolski 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 thioether bond (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 (PATs) (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 MGxC, 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.