Protein palmitoylation in protozoan parasites

Maria Martha Corvi[1], Luc Gerard Berthiaume[2], Maximiliano Gabriel De Napoli[1]

[1]Laboratorio de Parasitologia Molecular, Instituto Tecnologico de Chascomus (IIB-INTECH), CONICET-Universidad Nacional de General San Martin, Camino de Circunvalacion Laguna Km. 6 CC 164 (B7130IWA), Chascomus, Provincia de Buenos Aires, Argentina, [2]Department of Cell Biology, School of Molecular and Systems Medicine, Faculty of Medicine and Dentistry, University of Alberta, Edmonton, Alberta T6G 2H7, Canada

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1. ABSTRACT

Palmitoylation plays an important role in the regulation of the localization and function of the modified protein. Although many aspects of protein palmitoylation have been identified in mammalian and yeast cells, little information is available of this modification in protozoan parasites. Protein palmitoylation has been described for a few set of proteins in *E.tenella*, *P. falciparum*, *T. gondii*, *G. lamblia* and *T. cruzi*. Interestingly, in all these parasites palmitoylated proteins appears to be involved in vital processes such as invasion and motility. In addition, most of these parasites contain in their genomes genes that encode for putative palmitoyl-acyl transferases, the enzymes catalyzing the palmitoylation reaction. Although protein palmitoylation could be playing key roles in invasion and motility in a variety of parasites, little is known about this important reversible modification of proteins that typically plays a role in membrane tethering. As such, this review will focus on the main features of protein palmitoylation as well as provide an overview of the state of knowledge of this modification in protozoan parasites.

2. INTRODUCTION

Protozoan parasites are a diverse group of eukaryotic cells which have evolved as specialized cells, gaining new cellular compartments and presenting complex protein trafficking pathways (reviewed in (1)). Almost all secreted and transmembrane domain containing proteins follow the classical endoplasmic reticulum-Golgi apparatus-organelle/secretion. However, there are some proteins that are synthesized in the cytoplasm and targeted directly to membrane structures. In addition, some proteins that follow the secretory pathway are localized in membrane microdomains such as lipid rafts. There are several manners in which a protein without transmembrane regions could associate to lipid bilayers and one of them is protein lipidation.

Protein lipidation, the covalent modification of proteins by lipids, not only provides the modified protein enough hydrophobicity to allow association with cellular membranes but also modulates protein-protein interactions, stabilizes protein structure and regulates protein activity as well. Protein lipidation is divided in four main categories:

prenylation, glypiation, cholesteroylation and fatty acylation. Prenylation refers to the covalent modification of proteins by the isoprenoids farnesyl (C15:0) and geranylgeranyl (C20:0). These lipids are attached post-translationally to one or more cysteine residues at or near the C-terminus of the protein via a stable thioether bond. Protein prenylation has been studied in detail in protozoan parasites and several inhibitors of the farnesyl transferase are being tested for the treatment of malaria and African trypanosomiasis (reviewed in (2, 3)). Glypiation is the covalent modification of proteins at their C-terminus by glycosylphosphatidylinositol (GPI) structures. GPI-anchored proteins are abundant in protozoan parasites and also represent the most important carbohydrate modification of cell-surface proteins in these parasites (4). Cholesteroylation refers to the covalent modification of proteins by cholesterol (5) and up to date there are no reports of this modification in protozoan parasites. Finally, protein fatty acylation describes the covalent modification of proteins by long chain fatty acids and is divided into two categories: myristoylation and palmitoylation (6). N-myristoylation involves the co- or post-translational attachment of myristate (C14:0) to an N-terminal glycine residue of a protein via a stable amide linkage (7, 8). Myristoylation alone is not sufficient for stable membrane interaction. A second feature such as a cluster of positively charged amino acids or a palmitoylation signal is required to provide stable membrane binding and membrane or sub-membrane domain targeting information (6, 7, 9, 10). Myristoylated proteins have been reported in many protozoan parasites and include cGMP dependent protein kinase, a flagellar calcium-binding protein and the glideosome associated protein GAP45 (11-16). Finally, protein palmitoylation typically refers to the covalent attachment of palmitate (C16:0) to cysteine residues via a thioester linkage (17). Palmitoylation has also been shown to occur on N-terminal amino acid of proteins via an amide bond (e.g. Hedgehog and G alpha s (18, 19)). Palmitoylation plays a significant role in subcellular trafficking of proteins between membrane compartments, as well as in modulating protein-protein interactions. In contrast to prenylation and myristoylation, palmitoylation is the only lipid modification that can be dynamically regulated, in part due to the nature of the thioester bond. This is highlighted by the fact that the lifetime of the palmitoyl moiety on a protein is shorter than the lifetime of the palmitoylated protein itself. This reversible modification allows the cell to actively regulate the location and function of specific proteins. A detailed description of the main characteristics of protein palmitoylation is reviewed in the following section.

Because protozoan parasites incorporate lipids from their host into their proteins, including and predominantly palmitate (20, 21), the contributions of palmitoylation to the regulation of the localization and function of proteins in the life cycle of protozoan parasites will be reviewed.

3. PROTEIN PALMITOYLATION

3.1. General aspects

S-acylation is the post-translational attachment of fatty acids to cysteine residues of proteins usually through a thioester bond (17). Since palmitic acid (C16:0), is the most common fatty acid to be linked to proteins in this manner, S-acylation is often referred to as palmitoylation. However, other fatty acids can be attached in this manner -both saturated and unsaturated fatty acids, these include myristic, oleic, arachidonic and stearic acids (22-24). The trafficking functions of protein palmitoylation have been thoroughly reviewed by Henis *et al.* (25) and Iwanaga *et al.* (26). For protein palmitoylation to occur, palmitate must be activated in the form of its coenzyme A derivative, palmitoyl-CoA (27, 28). Palmitoylation is a reversible modification (29) that has been shown to be required for the membrane attachment and function of several proteins involved in signal transduction processes. The target of this modification can be both cytosolic and transmembrane proteins. A significant aspect of protein palmitoylation is that the labile thioester linkage permits repeated cycles of palmitoylation and depalmitoylation of a protein (30-32) and, as such, can act as a molecular switch for proteins.

3.2. Functions

Protein palmitoylation has been shown to be important for membrane anchoring, targeting of proteins to lipid rafts, trafficking proteins from the early secretory pathway to the plasma membrane, regulating protein activity and modulating the gene transcription of mitochondrial proteins.

Palmitoylation mainly functions in protein tethering to membranes and sub-membrane domains enriched in sphingolipids, cholesterol and some specific membrane proteins (33, 34). These domains are commonly known as lipid rafts. These rafts are small and dynamic membrane domains that can cluster or segregate membrane components. Whereas proteins modified with GPI anchors are processed through the secretory pathway and found on the cell surface, palmitoylated proteins are typically localized on the cytoplasmic leaflet of the plasma membrane and endomembranes (35). Several palmitoylated proteins have been found in the mitochondria (36) or found to be secreted (e.g. Apolipoprotein B (37, 38), Hedgehog (39, 40), Wnt (41-43)). Several studies showed that blocking protein palmitoylation through site-directed mutagenesis (44-46) or its prevention by the use of the palmitoylation inhibitor 2-bromopalmitate (47) abrogates the localization of numerous proteins to lipid rafts. Interestingly, cells treated with polyunsaturated fatty acids (PUFA) show a reduction in the association of proteins with lipid rafts (24). These results highlight the importance of palmitoylation in raft targeting and cell signaling.

Palmitoylation is also involved in trafficking lipid-modified signal transducers. A classical example of the role of palmitoylation in trafficking signals transducers are the Ras GTPases proteins (recently reviewed in (25)). All Ras proteins are synthesized on soluble ribosomes and farnesylated in the cytoplasm by farnesyltransferase. Ras proteins are further processed by proteolytic cleavage and carboxymethylation at the surface of the ER en route towards the plasma membrane (48, 49). It is at this point that Ras isoforms diverge in their trafficking. As a result, they show a differential localization under steady state

conditions. While K-Ras is targeted to the plasma membrane via the microtubule network, palmitoylated N- and H-Ras do so via the secretory pathway. Non-palmitoylated H-Ras accumulates early in the secretory pathway suggesting that palmitoylation is a signal to exit from the ER. Newly synthesized heterotrimeric G proteins appear to use a similar strategy relying on palmitoylation to exit the secretory pathway en route to the plasma membrane (50, 51). Protein activity can also be modified by palmitoylation, although this regulation is not well understood. G-protein-mediated signaling is regulated by palmitate at multiple levels (recently reviewed in (52)). Palmitoylation not only regulates the localization of heterotrimeric G proteins at the plasma membrane but also regulates the transducing signal. Palmitoylation also modifies the interactions of G-protein alpha subunits and regulators of G-protein signaling (RGS) proteins. RGS proteins constitute a large family that modulate G-protein-coupled receptors (GPCRs) signaling by accelerating GTP hydrolysis on active G alpha subunits (53), thereby reducing the amplitude and duration of signaling.

In 2010 a new role of protein palmitoylation as a regulator of transcription was described. It has been demonstrated that palmitoylation of the mitochondrial enzyme 3-hydroxy-3-methylglutaryl-CoA synthase (HMGCS2) promotes PPAR alpha interaction. This results in transcriptional activation at the PPAR alpha response element in the Hmgcs2 gene with a ensuing a feed forward regulation cycle (54). Interestingly, eight more mitochondrial proteins harboring nuclear receptor interacting motif were found to be palmitoylated, suggesting that a novel and perhaps general role for palmitoylation as a modulator of transcription of mitochondrial genes may exist (54).

3.3. Depalmitoylation

The half-life of the palmitate moieties on proteins is significantly shorter than that of the acylated protein itself, indicating that during its lifetime a protein can go through more than one cycle of depalmitoylation and repalmitoylation. The dynamic of palmitoylation was measured in pulse-chase experiments which showed the half-life of palmitate ranging from 20-180 min, far less than the half-life of the protein for which it was measured (recently reviewed in (55)). Depalmitoylation can occur either in response to signals or spontaneously and occurs everywhere in the cell (56). In this manner, a signaling cascade could be regulated. In fact an agonist induced increase in palmitate turnover has been observed for several proteins such as the beta 2-adrenergic receptor, G alpha s, endothelial nitric oxide synthase, H-Ras and PSD-95 (30, 57-59). Palmitoyl-protein thioesterase I (PPT1) and PPT2 are lysosomal hydrolases involved in the cleavage of acyl-cysteine linkages during the process of protein degradation (60, 61). A second acyl protein thioesterase (APT1) that depalmitoylates G alpha subunits and Ras have been cloned (62). APT1 is a 29 kDa cytosolic thioesterase that depalmitoylates proteins by catalyzing the cleavage of the thioester bond between the fatty acyl group and the protein. APT1 was originally purified as a lysophospholipase (63), although the enzyme prefers palmitoylated protein

substrates to lipid substrates (62). ATP1 has a higher affinity and catalytic efficiency towards palmitoylated protein substrates and is widely conserved from yeast to humans (62). In addition, RGS4, H-Ras, and eNOS are substrates *in vitro* for mammalian APT1 (62, 64). It has recently been reported that APT1 is palmitoylated which could facilitate the interaction of this cytosolic protein with membrane-associated palmitoylated susbtrates (65). APT2 is a mammalian protein that presents a 60 % homology to APT1. However, no depalmitoylation activity has been detected for APT2 yet.

3.4 Palmitoyl-acyl transferases

Contrary to what was observed in mitochondria where palmitoylation occurs in a spontaneous fashion (36, 66, 67), protein palmitoylation is mainly mediated through two different families of proteins: DHHC-containing proteins and membrane bound-O-acyltransferase (MBOAT) proteins.

3.4.1 zDHHC-containing proteins

It is now well accepted that cytosolic proteins are palmitoylated by a family of proteins containing a zinc molecule and an "asp-his-his-cys" motif inserted in a cysteine rich domain (zDHHC-CRD) (68). The human genome contains at least 23 members of the zDHHC PAT family. In general terms, the gene product of the members of this family are predicted to have an even number of transmembrane domains and seem to be expressed on ER, Golgi and the PM (69). Although the exact mechanism of protein palmitoylation has not been determined yet, it has been established that mutation of the cysteine residue of the DHHC motif on PATs abolishes substrate palmitoylation (70-73). A recent report provided new insights on the critical involvement of another cysteine residue outside the DHHC consensus sequence in catalysis for the yeast PATs Swf1 and Pfa3 function *in vivo* (74).

3.4.2 MBOAT proteins

Secreted proteins and peptides are substrates of members of the MBOAT (membrane bound-O-acyltransferase) family (75). Similar to what occurs with zDHHC members, this group of proteins present 8-12 membrane-spanning domains and contain a conserved histidine or leucine residue in one of the transmembrane domains that is thought to be involved in its activity. MBOATs that palmitoylate proteins such as Hedgehog (39), Spitz (76), Wnts (77) and ghrelin (78) have been described. A recent review on palmitoylation of secreted proteins can be obtained from Chang and Magee (79).

4. PROTEIN PALMITOYLATION IN PROTOZOAN PARASITES

Protein palmitoylation could be detected in different protozan parasites (see section below). In addition to these observations, protozoan parasites also present putative DHHC-PATs. In fact, by data mining of different genome databases (www.eupathdb.org.) we could identify that all studied parasites showed several putative PATs (Table 1). The search was performed by pfam information using the DHHC motif as target. An overall analysis

Protein palmitoylation in protozoan parasites

Table 1. Prediction of DHHC-containing gene products in protozoan parasites

Organism	PAT gene ID	DHHC zinc finger domain	TM regions	Other domains
Entamoeba histolytica [1]	EHI_148370	Yes	4	Plexin repeat
	EHI_049860	Yes	4	--
	EHI_198560	Yes	5	--
	EHI_073590	Yes	4	--
	EHI_004220	Yes	4	--
	EHI_134770	Yes	2	--
	EHI_008430	Yes	4	--
	EHI_169960	Yes	4	--
	EHI_027690	Yes	4	--
	EHI_054500	Yes	2	--
	EHI_025320	Yes	4	--
	EHI_016420	Yes	4	Plexin repeat
	EHI_119280	Yes	6	--
	EHI_081800	Yes	5	--
	EHI_007270	Yes	4	--
	EHI_170440	Yes	4	--
	EHI_047770	Yes	4	--
	EHI_052120	Yes	4	Plexin repeat
	EHI_180820	Yes	0	Protein tyrosine kinase family, catalytic domain
	EHI_048840	Yes	3	---
	EHI_037820	Yes	4	Plexin repeat
Cryptosporidium hominis [2]	Chro.70184	Yes	4	--
	Chro.70359	Yes	4	--
	Chro.60520	Yes	4	--
	Chro.40233	Yes	4	--
	Chro.80543	Yes	4	--
	Chro.10160	Yes	4	--
	Chro.10101	Yes	4	Ankyrin repeats
	Chro.50258	Yes	4	--
	Chro.20236	Yes	4	--
Giardia lamblia [3]	GL50803_2116	Yes	3	--
	GL50803_6733	Yes	4	--
	GL50803_1908	Yes	4	--
	GL50803_96562	Yes	6	--
	GL50803_16928	Yes	4	--
	GL50803_9529	Yes	4	--
	GL50803_8619	Yes	4	--
	GL50803_8711	Yes	4	--
Encephalitozoon intestinalis [4]	Ein04_0200	Yes	4	--
	Ein08_1410	Yes	4	--
	Ein08_1470	Yes	4	--
	Ein09_0220	Yes	4	Plexin repeat
	Ein10_0670	Yes	3	Ankyrin repeats
Plasmodium falciparum [5]	PFB0140w	Yes	5	--
	PFB0725c	Yes	4	--
	PFC0160w	Yes	5	Ankyrin repeats
	PFE1415w	Yes	4	--
	PFF0485c	Yes	4	--
	MAL7P1.68	Yes	4	--
	PFI1580c	Yes	4	--
	PF11_0167	Yes	4	Plexin repeat
	PF11_0217	Yes	4	--
	MAL13P1.117	Yes	4	--
	MAL13P1.126	Yes	8	Ankyrin repeats and BAR domain
Toxoplasma gondii [6]	TGME49_052200	Yes	3	--
	TGME49_066940	Yes	4	--
	TGME49_013550	Yes	2	--
	TGME49_046650	Yes	2	--
	TGME49_078850	Yes	4	--
	TGME49_024290	Yes	2	--
	TGME49_049380	Yes	4	--
	TGME49_084170	Yes	3	--
	TGME49_093730	Yes	2	Ankyrin repeats
	TGME49_029160	Yes	1	--
	TGME49_024310	Yes	2	--
	TGME49_069150	Yes	4	--
	TGME49_093220	Yes	2	--
	TGME49_017870	Yes	4	--
	TGME49_050870	Yes	4	--
	TGME49_055650	Yes	6	--
	TGME49_072320	Yes	1	--
Trichomonas vaginalis [7]	TVAG_453850	Yes	2	--
	TVAG_423280	Yes	2	--

TVAG_201890	Yes	4	--
TVAG_202150	Yes	4	--
TVAG_490930	Yes	2	--
TVAG_164710	Yes	4	--
TVAG_221770	Yes	3	--
TVAG_417920	Yes	4	--
TVAG_349350	Yes	4	--
TVAG_039820	Yes	2	--
TVAG_483450	Yes	4	--
TVAG_203860	Yes	4	--
TVAG_096710	Yes	4	--
TVAG_233440	Yes	4	--
TVAG_445720	Yes	4	--
TVAG_313610	Yes	3	--

[1]Amoeba database version 1.1 (http://amoebadb.org/amoeba/) [2]Crypto database version 4.3 (http://cryptodb.org/crypto/) [3]Giardia database version 2.2 (http://giardiadb.org/giardia/) [4]Microsporidia database version 1.1 (http://microsporidiadb.org/micro/) [5]Plasmo database version 6.5 (http://plasmodb.org/plasmo/) [6]Toxoplasma database version 6.1 (http://toxodb.org/toxo/) [7]Trichomonas database version 1.2 (http://trichdb.org/trich/)

showed that every DHHC-containing protein detected in the databases contains several transmembrane domains as is expected for PATs (reviewed in (68)). Apart from the DHHC motif inserted in a cysteine rich domain, several of the identified putative transferases presented other features such as ankyrin and plexin repeats, which could be responsible for either substrate specificity or interaction with a possible regulator. In fact, ankyrin repeats are also observed in human (DHHC13 and DHHC17) and yeast (Akr1 and Akr2) PATs. Interestingly, the number of putative PATs ranged from 5 to 21, being *Entamoeba histolytica* the protozoan with the higher number of putative PATs. These findings support the idea that protein palmitoylation may be playing a key role in the cell biology of these protozoan and warrants further characterization of not only the palmitoylated proteins but also the palmitoyltransferases themselves.

4.1. Protein palmitoylation, cell biology and invasion in Apicomplexan parasites

Apicomplexan parasites cause a wide variety of diseases in animals. This group of obligate intracellular parasites includes *Eimeria spp.*, *Cryptosporidium spp.*, *Neospora spp.*, *Plasmodium spp.*, *Theileria spp.* and *Toxoplasma spp*. Apicomplexan parasites have a complex life cycle and pass through asexual and sexual stages of development. In contrast to bacterial pathogens, these apicomplexan parasites are eukaryotes and share many metabolic pathways with their animal hosts. Since the most experimentally accessible apicomplexan parasite is *T. gondii*, it has been used as a model of this phylum (Figure 1).

T. gondii presents a compartmental organization similar to other eukaryotes and a group of secretory organelles located at the apical tip of the parasite, which are involved in attachment and invasion (reviewed in (80)). Apart from the nucleus and a single perinuclear, polarized endoplasmic reticulum (ER), *T. gondii* presents a single Golgi stack composed of 3-5 cisternae located adjacent to the only ER exit site (81). All members of the Apicomplexa phylum do not possess peroxisomes (82, 83), but they do contain two plastid organelles: a single tubular mitochondrion and a four membrane apicoplast (84, 85). Besides, acidocalsisomes –organelles responsible of calcium storage-are also found in this phylum (86).

Micronemes and rhoptries are organelles that are localized at the apical tip and their content are discharged at the time of invasion. Although the dense granules (DG) are distributed throughout the cell body, their contents are also secreted during the invasion mechanism (87). Trafficking of secreted proteins throughout this intramembrane network has been described in detail elsewhere (1). Another important organelle, the inner membrane complex (IMC), together with the plasma membrane (PM) forms the pellicle, a triple lipid bilayer typical of apicomplexan parasites. The IMC is a continuous patchwork of flattened vesicles located below the PM and is thought to be originated either from the ER or Golgi (88, 89).The IMC is of critical importance for the parasite's cellular processes such as replication, gliding motility and invasion. Apicomplexan parasites share common means of a substrate-dependent locomotion system termed "gliding motility" that is actively used by the parasite for tissue migration and host-cell invasion (80). The driving motor system is localized at the space between the PM and the IMC with a complex formed by a class of myosin (MyoA), a myosin light chain (MLC) and a gliding-associated protein 45 (GAP45). This complex is anchored to the IMC by the integral protein GAP50, which is also part of the complex. F-actin and aldolase connect this complex with proteins of the parasite's PM (80). *T. gondii* actively penetrates its host-cell. This invasion process occurs in several steps, which include the initial and apical attachments, the moving junction formation step (the site where the parasite enters the host-cell and forms the parasitophorous vacuole), the rhoptry discharge and the invasion, closure and separation steps. A different set of proteins is required in each step (80). Of importance we will mention AMA1 that forms a complex with the rhoptry-neck proteins RON2, RON4 and RON5 to form the moving junction, which is the site of the parasite's entry into the host cell (80). As mentioned before, lipid rafts are extremely important membrane microdomains where palmitoylated proteins are found and function as platforms for intracellular signaling cascades. Lipid rafts in apicomplexan parasites are very poorly studied and given the high content of palmitoylated proteins in lipid rafts, they will be discussed further herein.

4.1.1. *Eimeria spp.*

Until now only one report on protein palmitoylation in *E. tenella* has been published (11). In this report, Donald and Liberator describe that the coccidian
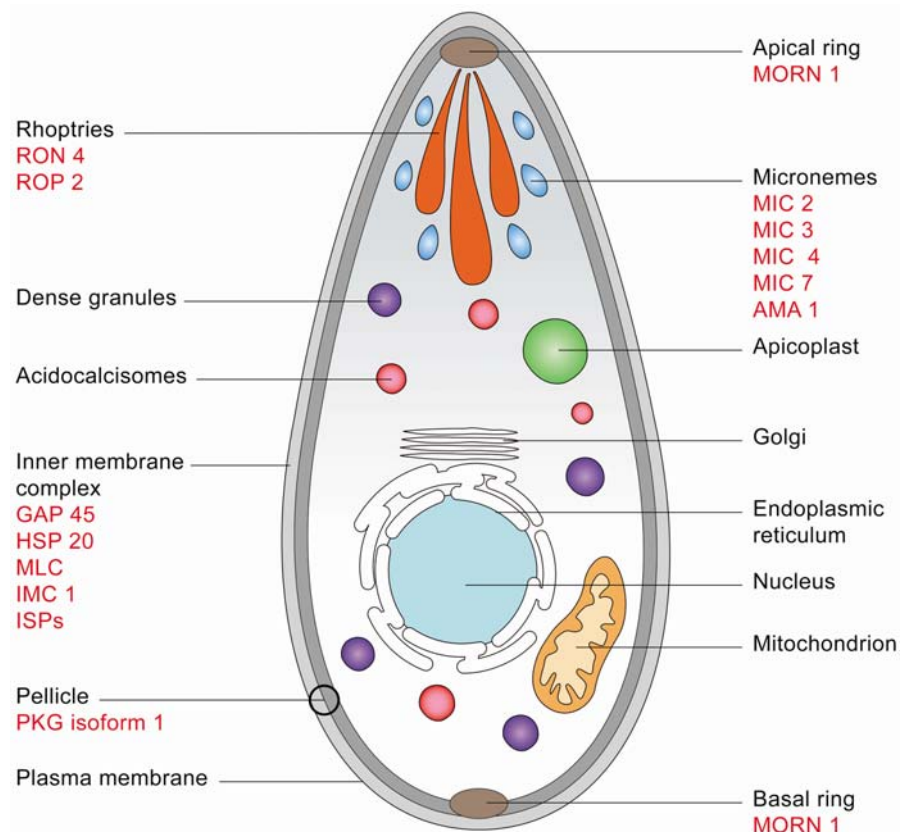
Figure 1. Schematic representation of *T. gondii* tachyzoite morphology. Palmitoylated proteins are shown in red and below the organelle where they are localized.

enzyme cGMP-dependent protein kinase (PKG) gene presents two isoforms as a result of an internal translational initiation methionine codon. Whereas isoform-I is mainly associated to the pellicle, isoform-II remains cytosolic. In addition, deletion mapping analysis demonstrated that the first 50 amino acids at the N-terminus are sufficient to confer membrane association (11). Furthermore, this strong membrane association seems to be dependent on N-terminal glycine-2 myristoylation and cysteine-4 palmitoylation since mutation of these residues significantly reduced the exclusive membrane association. In addition, metabolic labeling with tritiated myristate and palmitate of wild type and mutant PKG constructs confirmed the relationship between fatty acylation and subcellular localization of this enzyme. Although N-myristoylation was important for PKG localization; it does not seem to play any role in the regulation of its activity (11). It will eventually be interesting to assess whether fatty acylation targets the protein to the IMC or the plasma membrane.

It is important to highlight that the presence of flotillin-1 containing lipid rafts on *E. tenella* sporozoites were recently described (90, 91). An antibody against human flotillin-1 labeled the apical region of the parasite, which mediates host cell invasion. Interestingly, treatment of sporozoites with this antibody was sufficient to block parasite invasion indicating that this protein is required for the internalization into the host cells (90). Incubation of parasites with the anti-coccidian drug monesin, a lipophilic ion-selective ionophore which causes neutralization of acidic intracellular organelles, disrupted flotillin-1 raft association. The presence of flotillin-1 predominantly at the apical tip suggests that flotillin-1 is involved in the invasion machinery of *E. tenella* (91). As such, the authors speculate that upon interaction with host cell surface, lipid rafts could function as platforms triggering an intracellular signaling cascade that leads to the later events of the invasion mechanism.

4.1.2. *Plasmodium spp.*

Protein palmitoylation in *P. falciparum* seems to be critical for proper protein localization. On one hand, *P. falciparum* calcium-dependent protein kinase 1 (PfCDPK1) which was demonstrated to be essential for the parasite's survival (13), was observed to be myristoylated, palmitoylated and also contained a poly-basic cluster at its N-terminus. The presence of this triple membrane attachment motif is apparently required for its targeting to the parasitophorous vacuole. This triple motif is required and sufficient for the export of the kinase to the vacuolar space where it can exert its function (92). However, PfCDPK1 is not the only protein reported to be acylated in *P. falciparum*. The only and atypical calpain expressed in

Table 2. Prediction of *T. gondii* palmitoylated proteins involved in invasion

Name	Accession number [1]	Position of palmitoylated cysteine residues [2]
AMA1	TGME49_055260	16, 476
RON4	TGME49_029010	20
IMC1	TGME49_031640	5, 9, 12, 13, 607, 609
Tubulin alpha chain	TGME49_116400	315
MIC2	TGME49_001780	2
MIC3	TGME49_119560	107, 108
MIC4	TGME49_008030	12
MIC7	TGME49_061780	10
ROP2	TGME49_015780	4, 12
MLC	TGME49_057680	8, 11
GAP45	AAP41369 [3]	5
HSP 20	AAT66039[3]	3, 4, 160
MORN 1	AAZ99885[3]	4

[1] Accession number in toxoDB v 6.1 (http://www.toxodb.org/toxo/home.jsp) [2] Prediction using CSS-Palm 2.0 (http://csspalm.biocuckoo.org/) with a high threshold cut-off [3] NCBI accession number (http://www.ncbi.nlm.nih.gov)

P. falciparum which is essential for cell cycle progression (93), has also been reported to be myristoylated at glycine 2 and dually palmitoylated at cysteines 3 and 22. Calpain localization seems to be regulated by fatty acylation and this modification may be critical for its function in cell cycle progression (15). A third protein reported to be dually acylated *in vivo* in *P. falciparum* was the 45 kDa gliding-associated protein (GAP45). Initial N-myristoylation was proposed to play a role in directing the motor-complex in which GAP45 takes part to the IMC where is inserted. In a second step, palmitoylation at an undetermined cysteine residue at the targeted membrane is thought to provide a stronger affinity interaction (14).

It is interesting to emphasize that the *P. falciparum* genome codes for thirteen different DHHC containing proteins (94). However up to date the presence of only one possible PAT has been reported (94). This protein was shown to localize at the Golgi apparatus and contains five ankyrin repeats at its N-terminus and a DHHC motif before the third transmembrane domain. Due to the timing of expression, it is thought that this DHHC-containing protein could have a role in protein sorting to apical organelles during the development of the asexual stage of the parasite (94). As such, it could be extremely important during the invasion process.

An important aspect of *Plasmodium* infection is that appears to rely strongly on proteins resident in erythrocyte lipid rafts (95). It has been observed that disruption of lipid rafts without affecting membrane cholesterol, significantly reduces parasite invasion and assigns a vital role to lipid rafts in invasion of erythrocytes by *P. falciparum* (96).

4.1.3. *Theileria spp.*

So far, there are not reports on palmitoylated proteins, DHHC-containing proteins or lipid rafts in *Theileria parva* itself, but it has been reported that this parasite utilizes lipid rafts present in the bovine B and T cells to infect them. After infection, these cells acquire a phenotype that is compatible to leukemic cells because of their proliferative capacity *in vitro* (97), invasive tumor-like behavior in athymic, irradiated mice (98, 99) and the capability of forming colonies in soft agar. Apparently, this altered phenotype is caused by the synergistic effect of activated myristoylated and palmitoylated Hck - a member

of the Src family of protein tyrosine kinases, and, PI-3K. It is thought that this effect is due to both kinases localizing in lipid rafts and as such, triggering an intracellular cascade of events that lead to the permanent activation of the transcriptional factor AP-1, in a parasite-dependent manner (100). The authors speculate that lipid rafts in the infected cells are "hot spots" for the regulation of the kinases involved in the host cell transformed phenotype (100).

4.1.4. *Toxoplasma spp.*

Protein palmitoylation has been reported for one of the two isoforms of the enzyme cGMP-dependent protein kinase (PKG) in *Toxoplasma gondii*. Pulse labeling experiments indicate that this enzyme does not goes through proteolytic processing. Similar to what was observed in *E. tenella*, N-terminal myristoylation and palmitoylation are responsible for isoform-I localization to the parasite's pellicle (11). However, the non-fatty acylated isoform-II remains in the cytosol. We speculate that protein palmitoylation may play a key role in the invasion process in *T.gondii* since many proteins involved in this process are predicted to contain this post-translational modification and include AMA1, RON4, ROP2, MIC2, MIC3, MIC4, MIC7 and MLC (Table 2, Figure 1). Similar to what was observed in *P. falciparum* (14), TgGAP45 contains an N-terminal myristoylation and palmitoylation signal (101). Other proteins related to the IMC and/or the invasion processes that are predicted to be palmitoylated (102) include IMC1 and TgHSP20. TgHSP20 is a chaperone localized to the IMC and its function is unknown yet. Our preliminary results indicate that this chaperone is palmitoylated (MMC and MGDN unpublished data). Another interesting protein predicted to be palmitoylated isTgMORN1. TgMORN1 is a protein associated to the cytoskeleton and localized to ring structures at the apical and posterior end of the IMC and to the centrocone, a specialized nuclear structure that organizes the mitotic spindle (103). This protein is recruited to the basal complex early in the replication that plays a key role in maintaining the parasite's integrity during cytokinesis (104). How TgMORN1 interacts with the IMC is still unknown but they show a tight interaction (103). TgMORN1 does not contain a transmembrane domain or signal peptide on it. Palmitoylation could indeed anchor MORN1 in the membrane, but MORN domains by themselves can also associate directly with membrane.

Table 3. DHHC-containing proteins in *T. gondii*

Name	Accession number toxoDB 6.1 [1]		
	TGGT1	TGVEG	TGME49
TgDHHC-1	TGGT1_074120	TGVEG_014050	TGME49_093730
TgDHHC-2	TGGT1_001120	TGVEG_055920	TGME49_052200
TgDHHC-3	TGGT1_076010	TGVEG_085950	TGME49_066940
TgDHHC-4	TGGT1_036010	TGVEG_032390	TGME49_084170
TgDHHC-5	TGGT1_077170	TGVEG_078600	TGME49_055650
TgDHHC-6	TGGT1_118670	TGVEG_026340	TGME49_029160
TgDHHC-7	TGGT1_111510	TGVEG ---	TGME49_072320
TgDHHC-8	TGGT1_107340	TGVEG_016250	TGME49_069150
TgDHHC-9	TGGT1_078810	TGVEG_065070	TGME49_024310
TgDHHC-10	TGGT1_078790	TGVEG_065050	TGME49_024290
TgDHHC-11	TGGT1_028150	TGVEG_006540	TGME49_017870
TgDHHC-12	TGGT1_101050	TGVEG_002490	TGME49_049380
TgDHHC-13	TGGT1_102430	TGVEG_001220	TGME49_050870
TgDHHC-14	TGGT1_103510	TGVEG_029470	TGME49_078850
TgDHHC-15	TGGT1_039070	TGVEG_034770	TGME49_013550
TgDHHC-16	TGGT1_026450	TGVEG_005340	TGME49_046650
TgDHHC-17	TGGT1_073570	TGVEG_014680	TGME49_093220

[1] Accession number of the three strains in toxoDB v 6.1 (www.toxodb.org)

Recently, N-terminal myristoylation and palmitoylation have been reported to be responsible for membrane targeting of ISP1-3 (IMC-sub-compartment proteins) to the IMC (105). Similar to what observed for MORN1, these proteins lack a predicted signal peptide or even a transmembrane domain. In this report, Beck *et al.*, demonstrate that mutation of glycine in position 2 or mutation of both cysteine residues in position 7 and 8 relocalize the proteins to the cytosol. Figure 1 illustrates *T. gondii*'s morphology and the proteins that are reported or predicted to be palmitoylated.

T. gondii's genome has 17 DHHC-containing proteins (Table 3) that only share high homology with the human counterparts in the cysteine-rich domain where the DHHC motif is inserted. Lipid rafts were also detected in *T. gondii*, where an isoform of 14-3-3 protein was localized. The presence of a detergent resistant membrane- specific isoform of 14-3-3 protein was proposed to be associated with lipid raft-dependent signal transduction (106).

All these observations suggest that proteins that are reported to be palmitoylated (PKG isoform 1, HSP 20 and GAP 45, the later at least in Plasmodium) are synthesized in the cytosol and then are incorporated to the pellicle (Figure 1). It will be of interest to determine whether protein palmitoylation is responsible for this targeting by itself or in conjunction with other undiscovered signal. At the same time, the palmitoylation predictor shows that many proteins that are secreted from different organelles could be palmitoylated as well (Figure 1). Finally we will mention that it would be of high importance to determine whether protein palmitoylation plays a role in the invasion process of the parasite to its host cell using chemical inhibitors or si/shRNA methodology.

4.2. *Giardia lamblia*

Giardia lamblia is a protozoan that parasitizes the small intestine of vertebrates. It is a cause of intestinal infection and diarrhea and infects millions of people worldwide. The first report on protein palmitoylation in *G. lamblia* appeared in 2000. Indeed, Hiltpold *et al.*, (2000) demonstrated that several variant surface proteins (VSPs) are palmitoylated as well as glycosylated. These proteins are type I transmembrane proteins and this human pathogenic parasite undergoes antigenic variation by changing the constitutively expressed VSP for another similar but antigenically different one. The main importance of this work was to show for the first time the presence of protein palmitoylation in this parasite. However, the report unfortunately did not demonstrate the function of such modifications nor was the acceptor amino acid residue identified (107). In 2005, Touz *et al.*, showed that the palmitic acid was incorporated in the hydrophilic invariant C-terminal region of the VSP-H7. VSP-H7 palmitoylation regulated its rafts-like localization and non-palmitoylated mutants failed to undergo complement-independent antibody specific toxicity. Furthermore, this paper reported for the first time the presence of a functional *G. lamblia* PAT that was able to palmitoylate VSP *in vitro* (108). In 2009, a report on palmitoylation of an alpha 19-giardin demonstrated that this protein is dually acylated. Both myristoylation and palmitoylation were shown to be required for its unique ventral flagellar localization (109).

4.3. Trypanosomatides

Trypanosome cruzi calcium-binding protein (FCaBP) was shown to be both myristoylated and palmitoylated at its N-terminus (12). These lipid modifications were necessary and sufficient for the protein targeting to the flagella of this parasite. Interestingly, this dual fatty acylation do not only targets FCaBP to the flagella, but also to lipid rafts that are part of the flagellar membrane (16). The protein mislocalizes if these rafts are disrupted. The requirement of dual acylation for flagellar localization was also recently described for a family of small acylated proteins in *Leishmania* (110).

5. CONCLUDING REMARKS

Protein palmitoylation in protozoan parasites, although it is still an unexplored field, is rising in significance as shown by the increasing number of recent

reports on this topic. In fact, all the work already published in *Eimeria spp.*, *Plasmodium spp.* and *Toxoplasma spp.* on protein palmitoylation suggests that this modification is of importance in conferring proper membrane localization to the proteins that are modified. In addition, lipid rafts found in the pathogens are suggested to serve as platforms where the intracellular signaling may take place during the invasion process. As such, roles played by protein palmitoylation in protozoan parasites do not seem to be much different than those observed in other eukaryotic cells (e.g. human or yeast). Whether protein palmitoylation also affects protein function in parasites is still unknown. In addition, all protozoan parasites contain in their genome sequences that encode possible PATs indicating that they have the machinery necessary to carry out this modification. More interesting is the observation that vital processes such as invasion, survival and motility are altered by protein palmitoylation. This suggests that PATs could represent *bona fide* antiparasitic drug targets. With development of new chemical tools that allow the rapid assessment of protein fatty acylation (36, 111-114) we are convinced that in the next years more palmitoylated proteins will be identified, including perhaps the entire palmitoylated protein complements or palmitoylomes of one of these parasites. In addition, these new tools will likely provide further insights on the parasite's fatty acyl transferases in the upcoming years. Unraveling the palmitoylomes of these parasites is certain to reveal key features on the importance of palmitoylation in the biology of protozoan parasites.

6. ACKNOWLEDGEMENTS

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Abbreviations: GPI: glycosilphosphatidylinositol, PPT: palmitoyl-protein thioesterase, APT: acyl protein thioesterase, IMC: inner membrane complex, Myo A: myosin A, MLC: myosin light chain, RON: rhoptry neck protein.

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Send correspondence to: Maria Corvi, Laboratorio de Parasitologia Molecular Instituto Tecnologico de Chascomus (IIB-INTECH) CONICET-Universidad Nacional de General San Martin, Camino de Circunvalacion Laguna Km 6 CC 164 (B7130IWA) Chascomus Provincia de Buenos Aires Argentina, Tel: 54-2241-424045 (ext 103), Fax: 54-2241-424048, E-mail: mcorvi@intech.gov.ar