Protein O- and C-Glycosylation pathways in Toxoplasma gondii and Plasmodium falciparum

Giulia Bandini1,*, Andreia Albuquerque-Wendt2,*, Jan Hegermann3, John Samuelson1 and Françoise H. Routier2

1Department of Molecular and Cell Biology, Boston University, Goldman School of Dental Medicine, 72 East Concord Street, Boston, MA 02118, USA; 2Department of Clinical Biochemistry OE4340, Hannover Medical School, Carl-Neuburg-Strasse 1, 30625 Hannover, Germany and 3Hannover Medical School, Electron Microscopy Facility OE8840, Carl-Neuburg-Strasse 1, 30625 Hannover, Germany

Abstract

Apicomplexan parasites are amongst the most prevalent and morbidity-causing pathogens worldwide. They are responsible for severe diseases in humans and livestock and are thus of great public health and economic importance. Until the sequencing of apicomplexan genomes at the beginning of this century, the occurrence of N- and O-glycoproteins in these parasites was much debated. The synthesis of rudimentary and divergent N-glycans due to lineage-specific gene loss is now well established and has been recently reviewed. Here, we will focus on recent studies that clarified classical O-glycosylation pathways and described new nucleocytosolic glycosylations in Toxoplasma gondii, the causative agents of toxoplasmosis. We will also review the glycosylation of proteins containing thrombospondin type 1 repeats by O-fucosylation and C-mannosylation, newly discovered in Toxoplasma and the malaria parasite Plasmodium falciparum. The functional significance of these post-translational modifications has only started to emerge, but the evidence points towards roles for these glycoprotein glycosylation pathways in tissue cyst wall rigidity and persistence in the host, oxygen sensing, and stability of proteins involved in host invasion.

Introduction

Infections by unicellular protozoan parasites are a major worldwide health concern. It is estimated that parasitic diseases cause more than 1 million deaths every year and billions endure the morbidity of infection. Infections caused by intracellular parasites of the phylum Apicomplexa are the most prevalent, severely compromise human health, and impact animal food production. The most medically important Apicomplexa are the Plasmodium species, the causative agents of malaria, and in particular Plasmodium falciparum, which is responsible for most malaria-related deaths (WHO, 2018). Increased prevention and control measures have led to a marked reduction in malaria mortality rate, but this disease still claims half a million lives every year. Because of their lower mortality burden, other parasitic diseases rarely make headlines. However, toxoplasmosis and cryptosporidiosis also pose important health problems. In immunocompetent hosts, toxoplasmosis is characterized by mild-flu symptoms, whereas newborns and immunocompromised patients may suffer from severe ocular infections or encephalitis. Primary infection with Toxoplasma gondii is also associated with fetal malformations or death of the foetus. Additionally, Cryptosporidium parvum has been recently identified as one of the leading causes of diarrhoeal disease in children below 2 years of age in developing countries (Koloff et al., 2013). Toxoplasma, Cryptosporidium and several other Apicomplexa (e.g. Theileria, Babesia, and Eimeria) also affect livestock and are associated with important economic losses. A lack of effective anti-parasitic vaccines combined with an increase in drug resistance, rapid geographical expansion of vectors, extensive human migration and global transportation of merchandise make parasitic diseases among the most important public health challenges (Nyame et al., 2004). New therapies are needed and a comprehensive understanding of the molecular basis of host–pathogens interactions, together with basic parasite biology, will be crucial for the design of highly specific and efficient anti-parasitic drugs.

Glycans and glycan-binding proteins are known to play a role of paramount importance in host–pathogen interactions. Adhesion of parasites or other microbes to host cells involves interactions between glycan-binding proteins, also called lectins or adhesins, and glycan receptors. These interactions are a prerequisite for infection and often define the tropism of the pathogen. Furthermore, parasite glycans are often antigentic and may trigger both the innate and adaptive immune responses of the host. In addition, glycosylation of intracellular proteins can play roles in signalling and affect parasite proliferation and virulence. Studying the major roles of glycans in promoting parasitic infections and evading host immune responses may lead to the development of novel therapeutic agents, the identification of vaccine candidates and the development of novel diagnostic tools (Guha-Niyogi et al., 2001; Mendonca-Previto et al., 2005; Rodrigues et al., 2015; Goddard-Borger and Boddey, 2018).
Until the mid-1990s only scarce and often controversial information existed regarding glycosylation in apicomplexan parasites (Schwarz and Tomavo, 1993). In this pre-genomic era, glycosylphosphatidylinositols (GPIs) were shown to be synthesized by *T. gondii* and *P. falciparum*. As in other protozoan parasites, these glycans are abundantly present as proteins anchors or as free glycolipids and are essential for parasite survival (reviewed in Debierre-Grockiego and Schwarz, 2010). The availability of whole genome sequences enabled predicting biosynthetic pathways of glycans in various apicomplexa and revealed divergence between species of this phylum (Macedo et al., 2010; Cova et al., 2015; Samuelson and Robbins, 2015). The presence of a few Alg (Asparagine-linked glycosylation) genes in *Plasmodium falciparum* genome challenged the belief that *Plasmodium* does not express any N-glycosylated proteins. Indeed this parasite was shown to synthesize rudimentary N-glycans containing 1 or 2 N-acetylgalactosamine (GlcNAc) residues, in agreement with the set of genes present in this organism (Buskhi et al., 2010). In contrast, *Toxoplasma* and other coccidian parasites maintained a larger N-glycan machinery and are able to synthesize a N-glycan precursor with 2 GlcNAc, 5 mannose (Man) and up to 3 glucose (Glc) residues (reviewed in Samuelson and Robbins, 2015).

In this review, we focus on other types of glycosylation recently described. *Plasmodium falciparum* is thought to be devoid of mucin-type O-glycans since the necessary genes and donor substrate UDP-N-acetylgalactosamidase (UDP-GalNAc) are lacking in this parasite (Templeton et al., 2004; Cova et al., 2015; Lopez-Gutierrez et al., 2017). Similarly, no homologue of the O-N-acetylgalactosaminyltransferase (OGT), responsible for dynamic glycosylation of nuclear and cytoplasmic proteins, is present in the genome of the malaria parasite (Banerjee et al., 2009). In striking contrast, *Toxoplasma* adds O-linked GalNAc to mucin-like proteins, modifies nuclear proteins using an O-fucosyltransferase (OFT) similar to OGT, and glycosylates the cytosolic protein Skp1. In the second part of this review, we address O-fucosylation and C-mannosylation of thrombospondin type 1 repeats (TSRs), newly described in key adhesins of both *Plasmodium* and *Toxoplasma*.

**Mucin-type O-Glycans**

Transfer of N-acetylgalactosamine (GalNAc) to the hydroxyl group of specific serine (Ser) or threonine (Thr) residues initiates O-GalNAc glycosylation, a common post-translational modification of secreted or membrane-associated proteins in eukaryotes. This type of glycosylation is also referred to as mucin-type O-glycosylation, since mucins carry hundreds of heterogeneous O-GalNAc glycans in specific domains composed of Ser-, Thr-, and proline (Pro)-rich tandem repeats. This high heterogeneous O-glycosylation, since mucins carry hundreds of mucin-like domains by pp-GalNAcTs, effectively stained the glycosylated wall of *Toxoplasma* cysts that contain bradyzoites (slow-growing forms) (Sethi et al., 1977). DBA and other lectins that recognize non-reducing terminal GalNAc residues, such as *Vicia villosa* lectin (VVL), were used for affinity purification of *T. gondii* glycoproteins. Co-purified with mass spectrometry (MS), this lectin-capture approach identified candidate glycoproteins, including components of the tissue cyst wall, secreted proteins from the secretory organelles (rhoptries, micronemes and dense granules), and proteins from the parasitophorous vacuole and the inner membrane complex (Luo et al., 2011; Wang et al., 2016) (Fig. 1B). Additionally, transfer of GalNAc to the hydroxyl of Ser/Thr in mucin-like domains by pp-GalNAcTs generates GalNAc Ser/Thr, which is immunogenic in the host. Antibodies directed against this Tn antigen recognized at least 6 proteins ranging from 20 to 60 kDa (Gas-Pascual et al., 2019). DBA staining of the cyst wall is principally due to O-glycosylation of the mucin-like glycoprotein CST1, since deletion of CST1, pp-GalNAcT2, or pp-GalNAcT3 led to a loss of staining by this lectin (Tomita et al., 2013, 2017). CST1 confers structural rigidity to the cyst wall, and the O-GalNAc modification is required for this function (Tomita et al., 2017). Like CST1, SRS13 (surface antigen-1 related sequence 13) contains a Thr-rich mucin-like domain that is heavily O-glycosylated by pp-GalNAcT2 and T3 (Fig. 1C). This protein is upregulated in bradyzoites but is dispensable for cell wall formation (Tomita et al., 2018). In contrast, *T. gondii* proteophosphoglycan 1 (TgPPG1), a Ser/Pro rich-protein that shows similarities to the proteophosphoglycans of *Leishmania* parasites, enhances cell wall formation. Based on its retention in the stacking gel of SDS-PAGE, TgPPG1 is likely highly glycosylated (Craver et al., 2010; Tomita et al., 2013). The prominent role of O-GalNAc glycosylation in the encysted form of *Toxoplasma* is highlighted by deletion of the genes encoding pp-GalNAcT2 and T3 or the nucleotide sugar transporter 1 (TgNST1, TGGT1_267380), which imports UDP-GalNAc and UDP-N-acetylglucosamine (UDP-GlcNAc) into the endoplasmic reticulum/Golgi apparatus for biosynthesis of glycans. Deficiency of pp-GalNAcT2 or T3 leads to fragile brain cysts, likely due to the absence of CST1 glycosylation, while a lower cyst load in the brain was observed in TgNST1-deficient parasites. In contrast, deletion of these genes did not significantly impact the tachyzoite stage of the parasite (Caffaro et al., 2013; Tomita et al., 2017).

**O-GalNAcT1, T2 and T3 are constitutively expressed in both tachyzoites and bradyzoites, whereas pp-GalNAcT4 and T5 are expressed in the cat enteropathtelial stages, and T5 is additionally found in oocysts (Tomita et al., 2017). These enzymes have a type II topology with a short N-terminal cytoplasmic tail, a single transmembrane domain, a stem region and a conserved catalytic domain (Fig. 1A). With the exception of pp-GalNAcT4, they also have a C-terminal, ricin-like lectin domain. The catalytic domain adopts a glycosyltransferase A-fold with a DxH motif involved in divalent ion binding (Stwora-Wojczyk et al., 2004b; Tomita et al., 2017). As in mammals, the enzymes seem to act in a hierarchical manner. The enzyme pp-GalNAcT2 is the priming glycosyltransferase required for initial glycosylation of the mucin-like domain (Tomita et al., 2017). O-glycosylation of neighbouring acceptor sites is then carried out by the follow-on pp-GalNAcTs, pp-GalNAcT1 and T3, leading to a densely glycosylated mucin-like domain (Fig. 1A). In vitro, both pp-GalNAcT1 and T3 are only able to use pre-glycosylated acceptor peptides but not unglycosylated ones (Wojczyk et al., 2003; Stwora-Wojczyk et al., 2004b). Active pp-GalNAcTs have also been described in *Cryptosporidium* (Bhat et al., 2013; Haserick et al., 2017; DeCicco RePass et al., 2018), while the genome of *Plasmodium* lacks genes encoding these enzymes. As early as the late 1970s, it was observed that *Dolichos Biflorus* agglutinin (DBA), a lectin with specificity for GalNAcSer,3GalNAc, recognized a group of proteins ranging from 20 to 60 kDa (Perminov et al., 1973). DBA and other lectins that recognize non-reducing terminal GalNAc residues, such as *Vicia villosa* lectin (VVL), were used for affinity purification of *T. gondii* glycoproteins. Co-purified with mass spectrometry (MS), this lectin-capture approach identified candidate glycoproteins, including components of the tissue cyst wall, secreted proteins from the secretory organelles (rhoptries, micronemes and dense granules), and proteins from the parasitophorous vacuole and the inner membrane complex (Luo et al., 2011; Wang et al., 2016) (Fig. 1B). Additionally, transfer of GalNAc to the hydroxyl of Ser/Thr in mucin-like domains by pp-GalNAcTs generates GalNAc Ser/Thr, which is immunogenic in the host. Antibodies directed against this Tn antigen recognized at least 6 proteins ranging from 20 to 60 kDa (Gas-Pascual et al., 2019). DBA staining of the cyst wall is principally due to O-glycosylation of the mucin-like glycoprotein CST1, since deletion of CST1, pp-GalNAcT2, or pp-GalNAcT3 led to a loss of staining by this lectin (Tomita et al., 2013, 2017). CST1 confers structural rigidity to the cyst wall, and the O-GalNAc modification is required for this function (Tomita et al., 2017). Like CST1, SRS13 (surface antigen-1 related sequence 13) contains a Thr-rich mucin-like domain that is heavily O-glycosylated by pp-GalNAcT2 and T3 (Fig. 1C). This protein is upregulated in bradyzoites but is dispensable for cell wall formation (Tomita et al., 2018). In contrast, *T. gondii* proteophosphoglycan 1 (TgPPG1), a Ser/Pro rich-protein that shows similarities to the proteophosphoglycans of *Leishmania* parasites, enhances cell wall formation. Based on its retention in the stacking gel of SDS-PAGE, TgPPG1 is likely highly glycosylated (Craver et al., 2010; Tomita et al., 2013). The prominent role of O-GalNAc glycosylation in the encysted form of *Toxoplasma* is highlighted by deletion of the genes encoding pp-GalNAcT2 and T3 or the nucleotide sugar transporter 1 (TgNST1, TGGT1_267380), which imports UDP-GalNAc and UDP-N-acetylglucosamine (UDP-GlcNAc) into the endoplasmic reticulum/Golgi apparatus for biosynthesis of glycans. Deficiency of pp-GalNAcT2 or T3 leads to fragile brain cysts, likely due to the absence of CST1 glycoylation, while a lower cyst load in the brain was observed in TgNST1-deficient parasites. In contrast, deletion of these genes did not significantly impact the tachyzoite stage of the parasite (Caffaro et al., 2013; Tomita et al., 2017).
Recently, a study of total O-glycans released by β-elimination from Toxoplasma tachyzoites suggested that this parasite stage expresses only one major mucin-type O-glycan containing two N-acetylhexosamines (Gas-Pascual et al., 2019). The epimerase generating UDP-GalNAc from UDP-GlcNAc (GalE) is necessary for the formation of this O-linked disaccharide. This result indicates the presence of at least one GalNAc residue at the reducing end, which is consistent with the expression of pp-GalNAcTs in this parasite stage. Based on previous studies with lectins (Wang et al., 2016; Tomita et al., 2017), the authors suggest the structure GalNAc-GalNAc and possibly GalNAcα1,3GalNAcα1-Ser/Thr, which is preferentially recognized by DBA (Gas-Pascual et al., 2019). The glycosyltransferase that extends the O-linked GalNAc has not yet been identified (Fig. 1A).

**Nucleocytosolic glycosylation in T. gondii**

Studies performed in the last 30 years have underlined the fact that glycosylation of proteins in the nucleus and cytosol is not an exception, but a conserved feature in most eukaryotes. O-GlcNAcylation, the modification of nucleocytoplasmic proteins with a single GlcNAc residue, was first described in mammals in the early 80s. It was followed a decade later by the initial identification of the Skp1 glycosylation pathway in Dictyostelium...
**The Skp1 glycosylation pathway**

In *T. gondii*, the proline 154 of Skp1 (S-phase kinase-associated protein 1), an adapter of Skp1/Cullin1/F-box protein (SCF)-class E3 ubiquitin ligases, is hydroxylated and further modified by a pentasaccharide (Rahman et al., 2016, 2017; West and Hart, 2017; Gas-Pascual et al., 2019) (Fig. 2A). This glycosylation pathway is involved in the response of *Dictyostelium* to changes in environmental oxygen (West et al., 2010). Detection and monitoring of oxygen levels is a function required by all cells and, in *Dictyostelium* and *Arabidopsis thaliana* (Bandini et al., 2016; Zentella et al., 2017), oxygen sensors (Xu et al., 2012a). While the hydroxylation mechanism is conserved between animals and protists, the protein targets differ. In animals, P4Hs modify the transcriptional co-factor, hypoxia-inducible factor-α (HIFα). At normal oxygen levels, hydroxylation of HIFα leads to its poly-ubiquitination and subsequent proteosomal degradation, blocking the transcription of hypoxia-specific genes (Xu et al., 2012b). In protists such as *Toxoplasma* and *Dictyostelium* a single proline on Skp1 is hydroxylated and then further modified by a pentasaccharide. This modification does not lead to Skp1 degradation, but instead likely influences poly-ubiquitination and targeting to the proteasome of many other proteins (Xu et al., 2012a). In *T. gondii*, gene disruption of either the proline hydroxylase (*phyA*) or the four glycosyltransferases required for the pentasaccharide synthesis, although tolerated, leads to defects in parasite replication in the host. As might be expected, the strongest phenotype is observed in mutants lacking glycosylation, while milder growth defects are observed in mutants with reduced glycosylation (Xu et al., 2012a; Rahman et al., 2016, 2017).

*Toxoplasma* *pentadecasaccharide was defined as Galα1,3Glcα1,3Fucα1,2Galβ1,3GlcNAcα1- (Gal, Galactose; Glc, Glucose; Fuc, Fucose) based on glycopeptidase MS/MS data, NMR studies, and homology to the *Dictyostelium* biosynthetic pathway (Rahman et al., 2016, 2017; West and Hart, 2017). After hydroxylation of proline 154 by TgPhyA (TGGT1_232960) (Xu et al., 2012b), the first glycosyltransferase, TgGnt1 (TGGT1_315885), transfers GlcNAc in an α linkage (Rahman et al., 2016), resulting in an alkali-resistant glycosylation (Fig. 2A). Gnt1 belongs to the Carbohydrate-Active Enzymes (CAZy) glycosyltransferase (GT) 60 family (or GT60), a group that includes also *Trypanosoma cruzi* UDP-GlcNAc polysaccharide α-N-acetylgalcosaminyltransferase (Heise et al., 2009; Lombard et al., 2014). Incubation of DdSkp1 with UDP-[3H]-GlcNAc and cytosolic extracts of wild type *T. gondii*, but not of Δgnt1 parasites, resulted in the transfer of [3H]-GlcNAc to DdSkp1, consistent with the identification of TgGnt1 as the UDP-GlcNAc:HyPro PgtA polypeptide α-N-acetylgalcosaminyltransferase. This activity is dependent on the presence of PhyA, indicating the specificity of Gnt1 for the hydroxylated proline as acceptor (West et al., 2006; Rahman et al., 2016).

Transfer of the second and third sugars is catalysed by TgPgtA (TGGT1_260650), a bifunctional β1,3-galactosyltransferase (β1,3-GalT)/α1,2-fucosyltransferase (α1,2-FucT) (Fig. 2A). PgtA is organized in two separate domains belonging to the glycosyltransferase family GT74 and GT2, each responsible for one specific glycosyltransferase activity. The GT74 family was founded with *D. discoideum* PgtA and contains several eukaryotic and bacterial α1,2-FucTs (West et al., 2010). The GT2 domain of PgtA mediates the transfer of β1,3Gal (Rahman et al., 2016) and belongs to a very large family of functionally diverse inverting GTs. TgPgtA presents a GT74-GT2 tandem organization, where the domains are swapped in *Dictyostelium* PgtA (Van Der Wel et al., 2002). The same assay setup described above for TgGnt1 was used to demonstrate both FucT and GalT activities from *T. gondii* cytosolic extracts. Transfer of Fuc required the presence of UDP-Gal in the reaction, but not vice versa, indicating that Gal and Fuc are transferred sequentially to GlcNAcα-O-Skp1 (Rahman et al., 2016).

*Dictyostelium* *discoideum* and *T. gondii* Skp1 glycosylation differ in the nature of the two distal sugars. In *Dictyostelium*, a single glycosyltransferase DdNagTA transfers two α1,3Gal residues (Ercan et al., 2006; Schafer et al., 2014; Sheikh et al., 2017), while the core trisaccharide of *T. gondii* Skp1 is sequentially modified with an α1,3Glc and an α1,3Gal added by TgGlt1 and TgGat1, respectively.
even though many catalytic residues are conserved (Zentella et al., 2017). Animals and fungi OGTs classify as SE-liked and are involved in O-GlcNAc transfer just as A. thaliana SEC (Hartweck et al., 2002). In contrast, A Spy has recently been shown to be an O-fucosyltransferase (Zentella et al., 2017) and not an OGT. Consistent with this report, knock-out of Spy in T. gondii resulted in the loss of AAL binding, strongly suggesting the nucleocytoplasmic O-fucosyltransferase activity of TgSpy. Spy-deficient parasites were viable but displayed a mild defect in replication in host cells in vitro (Gas-Pascual et al., 2019). Structured illumination microscopy (SIM) showed that AAL binds in a punctate pattern to the nuclear periphery of T. gondii tachyzoites, suggesting that the O-fucosylated proteins are forming assemblies (Fig. 2B). Furthermore a Ser-rich domain fused to YFP is O-fucosylated when expressed in tachyzoites and localizes to the nuclear periphery (Bandini et al., 2016). Co-labelling with a Phenylalanine-Glycine (FG)-repeat nucleoporin suggests that these assemblies are found in close proximity to the nuclear pore complex and four out of the six T. gondii FG-repeat nucleoporins are found in the AAL-enriched fraction (Bandini et al., 2016; Courjol et al., 2017). This pattern was also observed in bradyzoites and sporozoites, but not in oocysts, suggesting that nuclear O-fucosylation might be regulated during the parasite life cycle (Fig. 4).

Nuclear staining with AAL was observed not only in Hammondia hammondi and Neospora caninum, the two species most closely related to T. gondii, but also Cryptosporidium parvum sporozoites (Bandini et al., 2016), which are all predicted to encode a SPINDLY orthologue. Interestingly, glycosylation extracts of T. gondii or C. parvum as well as the recombinant C. parvum SPY enzyme have been shown to have OGT activity in vitro (Banerjee et al., 2009; Perez-Cervera et al., 2011). Recent work has also reported the presence of O-GlcNAcylated proteins both in P. falciparum and T. gondii, using enrichment of proteins with terminal GlcNAc and identification by MS/MS (Kupferschmid et al., 2017; Aquino-Gil et al., 2018). Unfortunately, the peptide fragmentation technique used in these studies did not allow observation of glycopeptides. Future work will likely address the substrate specificity of SPY protein and its potential implication in O-GlcNAcylation. Work published in A. thaliana so far describes only one protein acceptor for SPY, the master regulator DELLA, which is also a substrate for SEC (Zentella et al., 2016, 2017). However, no subcellular localization studies have been performed in this system. Further studies will define if O-fucosylation directs proteins at the nuclear periphery and if this role is conserved in other eukaryotic lineages.

Glycosylation of thrombospondin type 1 repeats

In 2015, Cova and colleagues pointed out the conservation of enzymes for GDP-mannose (GDP-Man) and GDP-Fuc biosynthesis in the genome of different apicomplexan parasites (Cova et al., 2015). These nucleotides sugars, required for glycosylation reactions, were also shown to be present in Plasmodium falciparum blood stages by LC-MS/MS (Sanz et al., 2013). A substantial pool of GDP-Man was expected since Apicomplexa synthesize abundant GPI-anchors, but the presence of GDP-Fuc was more surprising considering that no fucose-containing glycoconjugates had been identified. The existence of fucosylated proteins in the nucleus and the Skp1 pathway mentioned above would explain the requirement for GDP-Fuc in T. gondii and a few other Apicomplexa, but not in Plasmodium spp. However, several Apicomplexa adhesins have thrombospondin type 1 repeats (TSRs), and homologs of the enzymes required for O-fucosylation and C-mannosylation of TSRs in higher eukaryotes can be
identified in this phylum (Buettner et al., 2013; Sanz et al., 2013; Cova et al., 2015).

TSRs are ancient protein modules that emerged before the separation of nematodes and chordates (Hutter et al., 2000). These domains contain approximately 60 amino acids and are typically organized as an elongated three stranded β-sheet, with three conserved disulfide bridges and stacked tryptophan and arginine residues that stabilize the TSR structure (Tan et al., 2002). Present in several proteins in vertebrates, these adhesive domains play roles in immunity, adhesion, neuronal development and signalling (Adams and Tucker, 2000; Shcherbakova et al., 2017). In Apicomplexa, the extracellular domains of several adhesins, including Plasmodium thrombospondin-relative anonymous protein (TRAP, PF3D7_1335900), circumsporozoite protein (CSP, PF3D7_0304600), and T. gondii micronemal proteins 2 (MIC2, TGME49_201780) contain at least one TSR domain (Tucker, 2004; Carruthers and Tomley, 2008) (Fig. 3A). These adhesins interact with receptors present at the host cell surface.

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**Fig. 3.** O-fucosylation and C-mannosylation on TSR repeats. (A) Schematic representation of the two glycosylation pathways in T. gondii. DPY19 transfers Man from dolichol-phosphate-mannose to tryptophan (W) residues on TSRs. POFUT2, a soluble protein in most eukaryotes, modifies Ser/Thr with the CX2−3S/TCX2G motif with Fuc, which can be further elongated by addition of Glc by B3GLCT. This glycosylation requires the GDP-Fuc transporter NST2 and a UDP-Glc transporter. (C) In P. falciparum, POFUT2 and Dpy19 are known to modify TSRS with O-Fuc and C-Man, as detailed for Toxoplasma. The identities of the B3GLCT and the Hex transferred on Fuc have not yet been ascertained. NST2 is the predicted GDP-Fuc transporter.
and are linked via their cytoplasmic tail to the glideosome, a molecular machine necessary for parasite motility and host cell invasion (Frenal et al., 2017). Plasmodium spp. express several TRAP variants that mediate motility, invasion, and egress at different stages of the parasite life cycle, both in the mammalian host and in the mosquito (Sultan et al., 1997; Dessens et al., 1999; Wengelnik et al., 1999; Combe et al., 2009; Steinbuechel and Matuschewski, 2009; Bargieri et al., 2016). In T. gondii, MIC2 plays a comparable role in tachyzoites (Huynh and Carruthers, 2006; Gras et al., 2017). In addition, several Apicomplexa TSR-containing proteins are uncharacterized, and their functions remain unknown.

**O-fucosylation**

Besides database mining, the presence of α-O-fucosylation in Apicomplexa was strongly suggested by MS/MS analyses of TRAP and CSP from *P. falciparum* sporozoites, which demonstrated modification of the TSRs by an O-linked hexose-deoxyhexose (Hex-dHex) (Swearingen et al., 2016) (Fig. 3A). This disaccharide was assumed to be Glcα1,3Fuc-α-O-Ser/Thr as described in mammalian TSRS (Kozma et al., 2006; Luo et al., 2006; Vasudevan and Haltiwanger, 2014). A clear homolog of the protein O-fucosyltransferase 2 (POFUT2) could indeed be identified in the genome of apicomplexan parasites (Cova et al., 2015). POFUT2 catalyses α-linked fucosylation of Ser or Thr residues in the consensus sequence CX_{2-3}s/TCX_{2-3}G, where C1 and C2 are the first two conserved cysteines in the TRS (Luo et al., 2006). The ability of recombinant *Plasmodium vivax* POFUT2 (PVX_098900) to use GDP-Fuc as donor substrate and to modify the CX_{2-3}s/TCX_{2-3}G motif of TRAP and CSP was later demonstrated using an *in vitro* glycosylation transfer assay (Lopaticki et al., 2017). In *T. gondii*, MIC2 is intensely stained with an antibody recognizing the Glcβ1,3Fuc epitope, and four TSRS of MIC2 that contain an O-fucosylation motif (TSR1, 3, 4 and 5) are modified by a Hex-dHex disaccharide by MS/MS analysis of glycopeptides (Fig. 3A). This modification was abolished by deletion of the gene encoding POFUT2 (TGGT1_267730) thereby proving the function of this glycosyltransferase in *T. gondii* (Fig. 3B) (Bandini et al., 2019; Gas-Pascual et al., 2019; Khurana et al., 2019).

Deletion of the gene encoding POFUT2 in *P. falciparum* (PF3D7_0909200) and, in at least one report, in *T. gondii* indicates that, in apicomplexa as in mammals, O-fucosylation plays a role in the stabilization and trafficking of TSR-containing proteins (Lopaticki et al., 2017; Bandini et al., 2019). As in mammals, the loss of α-O-fucosylation affected proteins differently. In *Plasmodium* sporozoites, the cellular level of TRAP was significantly decreased, whereas CSP was not affected (Lopaticki et al., 2017). Consistent with the importance of TRAP in adhesion and motility, hepatocyte invasion by sporozoites was impaired leading to a reduced liver parasite load. Loss of O-fucosylation also impacted on infection of mosquito midgut epithelial cells by ookinetes (Lopaticki et al., 2017). This phenotype could be related to a destabilization of the circumsporozoite and TRAP-related protein (CTRP, PF3D7_0315200), since this protein is involved in the motility of oocystes and contains O-fucosylation motifs (Dessens et al., 1999; Lopaticki et al., 2017). However, several other proteins contain the CX_{2-3}s/TCX_{2-3}G motif and might contribute to the phenotype observed in POFUT2-deficient parasites. In blood stage parasites, POFUT2 is expressed but dispensable for growth (Sanz et al., 2013; Lopaticki et al., 2017). Similarly, no prominent effects were observed in the blood stages of *P. falciparum* and *P. berghei* mutants lacking enzymes involved in GDP-Fuc biosynthesis (Gomes et al., 2015; Sanz et al., 2016). To date, no O-fucosylated protein has been identified in blood stages. The TSR-containing TRAP variants MTRAP (merozoite TRAP), SPATR (sporozoite protein with an altered thrombospondin repeat) and PTRAMP (*Plasmodium* thrombospondin-related apical merozoite protein) are devoid of the canonical POFUT2 consensus motif, suggesting that they are not O-fucosylated (Lopaticki et al., 2017).

As in *Plasmodium*, deletion of *T. gondii* POFUT2 affects the trafficking and cellular levels of the major parasite adhesion MIC2. Using *in vitro* assays, the decreased adhesion and impaired ability to invade displayed by POFUT2-deficient parasites were comparable to those observed in a mutant completely devoid of MIC2 (Gras et al., 2017; Bandini et al., 2019). However, the defects in both egress and parasite replication were much less pronounced in the O-fucosylation mutant than in the parasites lacking MIC2. Add back of *pofut2* rescued the attachment/invasion phenotype of *Δpofut2* parasites and restored cellular MIC2 levels, ruling out off-target mutations being the cause of the phenotype in the knockout. An identical phenotype was obtained by deletion of the nucleotide sugar transporter 2 (NST2, TGGT1_267730) establishing the specificity of this transporter for GDP-Fuc (Bandini et al., 2019) (Fig. 3B). *Toxoplasma gondii pofut2* was

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**Fig. 4.** O- and C-glycosylation pathways in *Toxoplasma gondii* life cycle. *T. gondii* replicates asexually in the intermediate host with tachyzoites as the fast replicative form and bradyzoites in tissue cysts characterizing the chronic stage of infection. In felids, its definite host, *T. gondii* goes through a sexual cycle that concludes with the shedding of unsporulated oocysts that then sporulate in the environment. As shown in the schematic, all the glycosylation pathways reviewed here have been shown to be present in tachyzoites. Transfer of O-GalNAc to mucin-like domains is an important post-translational modification in tissue cyst wall proteins and pp-GalNAcT5 is expressed in oocysts. Nuclear O-fucosylation has been shown to be present also in bradyzoites and sporozoites, but is absent from oocysts.
knocked out in two additional studies (Gas-Pascual et al., 2019; Khurana et al., 2019). Gas-Pascual et al., reported a defect in parasite replication upon its disruption, but did not address invasion or attachment as it was beyond the scope of the screen performed in this study. Finally, in Khurana et al., knock-out of pofut2 had no effect on cellular levels of MIC2, parasite proliferation, or its ability to attach and invade host cells. Discrepant phenotypes between the two reported pofut2 knockouts likely results from the different methods used for generating these cell lines and/or for assaying phenotype (Bandini et al., 2019; Khurana et al., 2019).

In mammals, O-linked fucose on TSRs is typically extended by a β1,3-linked glucose transferred by the glucosyltransferase B3GLCT, which is also involved in protein quality control (Kozma et al., 2006; Luo et al., 2006). A recent study indicates that a small percentage of TRAP and CSP from P. falciparum salivary glands sporozoites is modified with a Hex-dHex whereas only a dHex is found on these proteins in P. vivax and P. yoelii sporozoites (Swearingen et al., 2019). This glucosyltransferase belongs to the large GT31 family, which contains enzymes with various functions (Lombard et al., 2014). In P. falciparum, the parasite-infected erythrocyte surface protein 1 (PIESP1, PF3D7_0310400) shares 31% protein identity with the human B3GLCT and has been proposed as candidate B3GLCT (Swearingen et al., 2016). Surprisingly, this protein was localized at the surface of infected red blood cells (Florens et al., 2004), whereas B3GLCT would be expected to localize in the endoplasmic reticulum (ER) with POFUT2 (Lopaticki et al., 2017). Moreover, by saturation mutagenesis, the PF3D7_0310400 gene is predicted to be essential in asexual blood stages whereas pofut2 is dispensable (Zhang et al., 2018). These data suggest that PIESP1 is not responsible for glucosylation of O-fucose. In T. gondii, however, Gas-Pascual and colleagues recently reported loss of an O-linked Hex-dHex disaccharide upon knock-out of the putative b3glct (TGGT1_239752) indicating that this enzyme is likely responsible for elongating O-Fuc (Fig. 3B). A defect in the lytic cycle characterised by small lytic plaques was also reported in this mutant (Gas-Pascual et al., 2019). While binding of MIC2 by a Glcβ1,3Fuc-specific antibody suggests that at least in T. gondii the hexose modifying O-Fuc is indeed Glc (Bandini et al., 2019), further investigations are necessary to confirm this result and the identity of the hexose in other Apicomplexa.

Nucleotide sugars, required for the biosynthesis of glycans, are mostly synthesized in the cytosol and actively transported into the ER or Golgi by the action of nucleotide sugar/nucleoside monophosphate antiporters (NSTs). The functional identification of TgNST2, the GDP-fucose transporter in T. gondii, has been mentioned above and P. falciparum genome also encodes a putative GDP-sugar transporter, PF3D7_0212000 (Fig. 3C). Similarly, B3GLCT activity requires transport of the UDP-glucose donor into the ER. Bioinformatics searches, using either yeast or human NSTs as templates, identified T. gondii NST3 (TGGT1_254580) and PfUGT (PF3D7_1113300) as putative UDP-Glc/UDP-Gal transporters because of their close homology with the yeast HUT1 transporter (Banerjee et al., 2008; Caffaro et al., 2013). In Plasmodium erythrocyte stages, PfUGT has been localized to the ER and identified as a multidrug resistance gene (Lim et al., 2016). Using a CRISPR screen, the phenotype score associated with the TgNST3 encoding gene (−4.9) was significantly lower than the scores associated with pofut2 (−0.34) and b3glct (−1.36) suggesting that deletion of nst3 would have much more severe effects than the loss of O-fucosylation (Sidik et al., 2014). Similarly, piggyBac transposon insertion mutagenesis suggests that the gene encoding the putative UDP-Glc/UDP-Gal transporter PfUGT is not mutable in asexual blood stages whereas, as mentioned above, O-fucosylation does not seem to play important roles in these parasite stages (Lopaticki et al., 2017; Zhang et al., 2018). These results are difficult to conciliate and suggest that TgNST3 and PfUGT have a different or broader substrate specificity. In metazoa, the function of the HUT1 transporter is also still unclear. Recently, the human orthologue called solute carrier 35B1 (SLC35B1), has been proposed to act as ATP/ADP antiporter (Klein et al., 2018).

C-mannosylation

C-mannosylation is a less known protein modification, mediated by enzymes of the DPY19 family, which, in metazoa, transfer...
an α-mannose residue from dolichol-phosphate-mannose to a tryptophan (Trp) residue located in a WXnW or WXnC motif (Buettner et al., 2013; Niwa et al., 2016; Scherbakova et al., 2017). As in the case of α-O-fucosylation, analysis of Apicomplexa genomes strongly suggested the existence of this modification, and MS/MS analyses confirmed C-hexosylation of tryptophan residues in the TSRS of Plasmodium TRAP and Toxoplasma MIC2, in sporozoites and tachyzoites, respectively (Figs 3A, 4 and 5) (Swearingen et al., 2016, 2019; Bandini et al., 2019; Khurana et al., 2019). In metazoa, this glycosylation is typically found on TSRS carrying a WXnWXnC1 sequence (which directly precedes the O-fucosylation site), as well as type I cytokine receptors characterized by a WSXWS signature (Holsteenge et al., 1999; Julenius, 2007). However, the latter is not found in Apicomplexa. In mammals, two different enzymes (DPY19L1 and DPY19L3) are required for C-mannosylation of both Trp residues in the sequence WXnWXnCnWtetra, whereas Apicomplexa genomes contain a single DPY19 homolog (Buettner et al., 2013; Scherbakova et al., 2017). An in vitro assay using recombinant T. gondii (TGME49_080400) and P. falciparum DPY19 (PF3D7_0806200) indicated that the apicomplexan enzymes are unable to act on a WXnW peptide as their mammalian counterparts but modified WXnWXnC motif, suggesting that they are tailored for TSR modification. Detailed MS/MS analyses of MIC2 further indicated that both tryptophans of the WXnWXnC sequence can be modified (Fig. 3A). Finally, in vitro assays also showed that both T. gondii and P. falciparum DPY19 use dolichol-phosphate-mannose as donor substrate, proving their mannosyltransferase activity (Hoppe et al., 2018).

In mammals, C-mannosylation is suggested to play an important role in stabilization and/or folding of some but not all proteins (Munte et al., 2008; Wang et al., 2009; Buettner et al., 2013; Sasazawa et al., 2015; Siupka et al., 2015; Fujiwara et al., 2016; Niwa et al., 2016; Okamoto et al., 2017; Scherbakova et al., 2017). Using a CRISPR/Cas9 genetic screen or a piggyBac transposon insertional mutagenesis, DPY19 was predicted to confer fitness to T. gondii tachyzoites or P. falciparum asexual stages, respectively (Sidik et al., 2014; Zhang et al., 2018). Further studies are required to define the function of this type of protein glycosylation in apicomplexa.

Concluding remarks
The adaptation of Apicomplexa to parasitic lifestyle was accompanied by a reductive genome evolution involving lineage-specific gene loss (Templeton et al., 2004). This has led to divergent protein glycosylation in the various Apicomplexa genera. Biosynthesis of glycosylphosphatidylinositol is conserved throughout the eukaryotes and these are particularly abundant at the surface of Apicomplexa. In contrast, biosynthetic pathways involved in surface protein N- and O-glycosylation have been considerably reduced or even eliminated. Due to secondary loss of alg genes, Plasmodium expresses rudimentary N-glycans composed of only one or two N-acetylglucosamine residues, whereas Toxoplasma N-glycans are more elaborated (Bushkin et al., 2010; Samuelson and Robbins, 2015).

The synthesis of mucin-type O-glycosylation represents another important divergence between Plasmodium and Toxoplasma. Recent genetic and biochemical work clearly established the existence of mucin-type glycosylation in Toxoplasma and its importance for the rigidity of the cyst wall and parasite persistence. These O-GalNAc glycans likely consist of a disaccharide and are thus less complex than in other eukaryotes. Conversely, Plasmodium apparently lacks the enzymes involved in this protein post-translational modification.

Additionally, two distinct cytoplasmic and nuclear glycosylation pathways were recently described in Toxoplasma. The ubiquitin ligase adaptor Skp1 was shown to be hydroxylated and modified by a pentasaccharide; a post-translation modification required for optimal oxygen-dependent development. Lastly, more than 60 Toxoplasma nuclear proteins were shown to be substituted by one or more fucose residues. This modification carried out by the SPY enzyme is reminiscent of protein O-GlcNAcylation and might have a role in localizing proteins at the nuclear periphery. Within the Apicomplexa, these two pathways seem restricted to a few species closely related to Toxoplasma.

In contrast, both Toxoplasma and Plasmodium possess conserved C-mannosylation and O-fucosylation pathways for modification of TSRs. These repeats are present in key surface adhesins and are possibly acquired by horizontal transfer from an animal source. As in animals, C-mannosylation and O-fucosylation of TSR occur in the early secretory pathway and seem to stabilize these repeats.

The presence of simple O- and C-glycans in Apicomplexans proteins has now been clearly established (Figs 4 and 5). Future work will likely clarify the nature of the proteins carrying these modifications and explore the importance of glycosylation for the parasite biology and pathogenicity.

Author ORCIDs. Giulia Bandini, 0000-0002-8885-3643; Françoise H. Routier, 0000-0002-7163-0590

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