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ORIGINAL ARTICLE

Direct evidence of *O*-GlcNAcylation in the apicomplexan *Toxoplasma gondii*: a biochemical and bioinformatic study

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Abstract *Toxoplasma gondii* and *Plasmodium falciparum* are apicomplexan parasites responsible for serious diseases in humans. Many studies have focused on the post-translational modifications (PTMs) found in the two protists including phosphorylation, acetylation or SUMOylation but only a few of these are concerned with the nuclear and cytosolic-specific glycosylation *O*-GlcNAcylation. *O*-GlcNAcylation is a highly dynamic PTM—regulated by the ON and OFF enzymes: *O*-GlcNAc transferase and *O*-GlcNAcase—that can compete with phosphorylation but its function remains unclear. In this work, we directly prove the *O*-GlcNAcylation in *T. gondii* using antibodies specifically directed against the modification and we strongly suggest its occurrence in *P. falciparum*. We found that the inducible 70 kDa-Heat Shock Protein is *O*-GlcNAcylated, or associated with an O-GlcNAc-partner, in *T. gondii*. Using anti-OGT antibodies we were able to detect the expression of the glycosyltransferase in *T. gondii* cultured both in human foreskin fibroblast and in Vero cells and report its putative sequence. For the first time the presence of *O*-GlcNAcylation is unequivocally shown in *T. gondii* and suspected in *P. falciparum*. Since the *O*-GlcNAcylation is implicated in many biological fundamental processes this study opens a new research track in the knowledge of apicomplexans' life cycle and pathogenic potential.

Keywords *Toxoplasma gondii* · *Plasmodium falciparum* · *O*-GlcNAc · *O*-GlcNAcylation · *O*-GlcNAc transferase · OGT

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Introduction

The obligate intracellular parasites *Toxoplasma gondii* and *Plasmodium falciparum* are responsible for two major infectious diseases, toxoplasmosis and malaria, respectively. These two ubiquitous protozoans have been intensively studied during the last hundred years. *T. gondii* was identified in 1908 in the North African rodent gundi (*Ctenodactylus gundi*) by Nicolle and Manceaux (Nicolle and Manceaux, 1908). This parasite infects warm-blooded animals including humans and in the majority of the cases the infection has no serious consequences for the immunocompetent patients. Nevertheless a reactivation of the latent parasite in immunocompromised individuals may cause encephalitis, an acute inflammation of the brain, and it is also noteworthy that cerebral toxoplasmosis is a major

cause of morbidity and mortality among AIDS patients. It has been calculated that the seroprevalence for toxoplasmosis for the pregnant women was near 45% in France (Berger et al. 2009) and it is estimated that a third of humans worldwide are infected. *Plasmodium* was observed for the first time by Alphonse Laveran (who was awarded the Nobel Prize for Medicine in 1907) in red blood cells of individuals infected with the parasite. Malaria, transmitted by the female anopheles mosquito, is a mild illness when it is caused by *P. vivax*, *P. malariae* or *P. ovale* but it can be severe when it is caused by *P. falciparum*. Each year *P. falciparum* is responsible for 300–500 million documented cases worldwide and causes the death of 2–3 millions, mainly children. Numerous post-translational modifications (PTMs) occurring in *T. gondii* and *P. falciparum* have been described (for reviews see Weiss et al. 2009 and Chung et al. 2009). Of these PTMs we find as regards *T. gondii N*-glycosylation (Fauquenoy et al. 2008; Luk et al. 2008), glypiation (modification with Glycosyl-Phosphatidyl Inositol (GPI) Striepen et al. 1997; Debierre-Grockiego et al. 2009), SUMOylation (modification with a Small Ubiquitin-like MOdifier Braun et al. 2009), perhaps ubiquitination and neddylation (Frickel et al. 2007), acetylation and glutamylation of α-tubulin (Plessmann et al. 2004), and methylation of α- and β-tubulin (Xiao et al. 2010). Concerning *Plasmodium*, phosphorylation (Ward et al. 2004; Schneider and Mercereau-Puijalon 2005; Dorin-Semblat et al. 2007), prenylation (Chakrabarti et al. 2002), glypiation (Gerold et al. 1994; Wichmann et al. 2007), acetylation (Cui et al. 2007; Andrews et al. 2009), methylation (Cui et al. 2008), SUMOylation, ubiquitination (Horrocks and Newbold 2000), palmitoylation, and protein cleavage (for review see Chung et al. 2009) have been reported. Of all these PTMs, the existence of *O*-GlcNAcylation in *T. gondii* and *P. falciparum* remained controversial. The *O*-GlcNAcylation occurs within the cytosolic and the nuclear compartments of eukaryotes (for reviews see Hart et al. 2007; Butkinaree et al. 2010) and it has recently been shown that mitochondrial proteins could also be *O*-GlcNAcylated (Hu et al. 2009). Virtually it is assumed that *O*-GlcNAcylation is widely expressed in all the living organisms from viruses to humans through bacteria and nematodes with the exception of yeast. Until recently there was a doubt concerning the existence of this PTM in apicomplexans. The first study, reporting the putative existence of *O*-GlcNAcylation in apicomplexans, was done in the early '90s (Dieckmann-Schuppert et al. 1993). It was shown that erythrocytes infected with *P. falciparum* contained *O*-glycans with terminal GlcNAc and further analyses suggested that these structures could be simply *O*-GlcNAc. In the same report, it was attested that *P. falciparum* possessed its own *O*-GlcNAc transferase (OGT) using the synthetic peptide Pro-Tyr-Thr-Val-Val. Later Hoessli et al. (2003) demonstrated that MSP1 (Merozoite Surface Protein-1) that is expressed and GPI-anchored at the cell surface of *P. falciparum* bore *O*-GlcNAc moieties. But owing to its extracellular localization we cannot assume that this glycosylation is related to the nuclear and cytoplasmic-specific *O*-GlcNAcylation.

Recently the OGTs expressed by *Cryptosporidium parvum*—that is phylogenetically close to *Plasmodium* and *Toxoplasma*—and by the minimalist protist *Giardia lamblia* have been characterized (Banerjee et al. 2009). This study strongly supports the hypothesis that *O*-GlcNAcylation occurs in lower eukaryotes and that the two protists make their *O*-GlcNAc moieties using their own OGTs. Nevertheless, none of these three studies showed direct evidence of *O*-GlcNAcylation in these parasites.

In the present report we used two distinct anti-*O*-GlcNAc directed antibodies, namely, RL2 and CTD110.6, to show that *T. gondii* unambiguously expresses the nuclear and cytoplasmic modification and to suggest its occurrence in *P. falciparum*. Among the plethora of *O*-GlcNAcylated proteins observed, Hsp70 was identified either as being itself modified or associated with an *O*-GlcNAcylated partner. We also found that *T. gondii* expresses its own OGT and using sequences' alignment we found that the parasite's enzyme shares a high identity with SPindlY.

Materials and methods

Culture and purification of the parasites

T. gondii grown in African green monkey kidney cells (Vero cells, ATCC CCL-81), were cultured in DMEM (Dulbecco's Modified Eagle Medium, Gibco BRL), and supplemented with 10% fetal calf serum (FCS, Gibco), 2 mM glutamine, 100 units/mL penicillin, and 0.1 mg/mL streptomycin. Parasites (5×10^7) were added to confluent monolayer of cells (175 cm^2), harvested after being cultivated for 72 h, and liberated from their host cells using a Mixer Mill homogenizer (Retsch). The suspension was run through a 20 mL glass-wool column to remove cellular debris. The purity of the tachyzoite suspension was monitored microscopically. Cell lines and parasites were routinely tested for *Mycoplasma* contamination. To control the efficiency of parasite purification, *T. gondii* cells liberated from their host cells were mixed with homogenized Vero cells, and the mixture was purified using glass-wool columns as described previously (Grimwood et al. 1979).

The *P. falciparum* strain FCBR was obtained from B. Enders, Behring Co. (Marburg, Germany) and was maintained as previously described (Schmidt et al. 1998). Development and multiplication of plasmodial cultures were monitored by microscopic evaluation of

Giemsa-stained smears. Parasite cultures were routinely checked for *Mycoplasma* contamination. Parasite multiplication was assessed as described previously (Dieckmann-Schuppert et al. 1992). Trophozoites were isolated from rings and non-infected erythrocytes by magnetic separation using SuperMACS and D columns (Miltenyi Biotec GmbH, Bergisch Gladbach, Germany).

SDS-PAGE and Western blotting

All the cells and the parasites used in this study were lysed in the following homogenization buffer (HB): 10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0,1% (w/v) SDS, proteases inhibitors, pH 7.4. After centrifugation at 20,000*g* for 10 min, the supernatant was mixed with Laemmli buffer and boiled for 10 min. The proteins were run on 8 or 10% SDS-PAGE in reducing conditions. The gels were either stained with brilliant blue or electroblotted onto a nitrocellulose sheet. The transfer efficiency was checked using the Ponceau red staining. The blots were saturated with 5% (w/v) non-fat milk in TBS (Tris-buffered saline)-Tween (15 mM Tris, 140 mM NaCl, 0.05% (v/v) Tween) or with 3% (w/v) bovine serum albumin in TBS for 45 min. The primary antibodies were incubated overnight at 4°C. The mouse monoclonal anti-*O*-GlcNAc (RL2, Ozyme), mouse monoclonal anti-alpha-tubulin (Santa-Cruz Biotechnologies), and rabbit polyclonal anti-actin (Sigma) were respectively used at dilutions of 1:1,000; 1:1,000 and 1:10,000. The mouse monoclonal (IgM) anti-*O*-GlcNAc CTD110.6 (generously provided by Prof. G.W. Hart) was used at a dilution of 1:4,000. The rabbit polyclonal anti-OGT antibodies AL25 and AL35 (gifts from Prof. G.W. Hart) were both used at a dilution of 1:10,000 and the rabbit polyclonal anti-Hsp70 (Stressgen) was used at a dilution of 1:30,000. The specificity of the RL2 and of the CTD110.6 antibodies was checked by co-incubation with 1 M of free GlcNAc. Then the membranes were washed three times for 10 min in TBS-Tween and incubated with either an anti-mouse (IgG or IgM) horseradish peroxidase-labeled secondary antibody or an anti-rabbit IgG horseradish peroxidase-labeled secondary antibody (GE Healthcare) at a dilution of 1:10,000. Finally, three washes of 10 min each were performed with TBS-Tween and the detection was carried out with enhanced chemiluminescence using Hyperfilms (GE Healthcare).

Measurement of the OGT activity

The *T. gondii* OGT activity was assayed by using the procedure described by Banerjee et al. 2009 with the following slight differences. The activity was measured in triplicate on the nucleocytoplasmic *T. gondii* (or HepG2 as a positive control) proteins using 0.4 µCi of UDP-[^{3}H] GlcNAc. After labeling, the proteins were precipitated with trichloro-acetic acid (TCA) at a final concentration of 20% (*m*/*v*), and then recovered on glass microfiber filters (GF/A, Whatman), and intensively washed with 0.1% (*m*/*v*) TCA under vacuum. The precipitates were rinsed with absolute ethanol and then counted on the filters in a liquid scintillation counter (Beckman).

Immunoprecipitation

HeLa cells or *T. gondii* were lysed in 1 mL of HB. After centrifugation at 20,000 *g*, supernatants were incubated overnight at 4°C with 10 µL of anti-*O*-GlcNAc (RL2). Then 50 µL of proteins G was added to the samples that were newly placed for 1 h at 4°C. Four washes were realized in the following order: HB, HB supplemented with 0.5 M NaCl, HB/TNE (10 mM Tris/HCl, 150 mM NaCl, 1 mM EDTA, pH 7.4) (v/v) and finally TNE alone. Immunoprecipitates were resuspended in Laemmli buffer and boiled. Immunoprecipitation experiments were controlled by replacing the anti-*O*-GlcNAc specific-directed antibodies by non-immune antibodies.

BLAST and sequences' alignment

The sequence of the catalytic domain II of the human OGT (AAH38180.1; GI:23315618) was used for performing the BLAST analyses. The databases used were ApiDB (http://apidb.org) and ToxoDB (http://toxodb.org/toxo/). MultAlin (http://multalin.toulouse.inra.fr/multalin/multalin.html) and ClustalW2 (http://www.ebi.ac.uk/Tools/clustalw2/index.html) were used for the realization of the sequences' alignments.

Results

The crude extracts of *T. gondii* and *P. falciparum* were resolved by SDS-PAGE and the proteins were stained brilliant blue. It was observed that the parasites display distinct protein profiles when compared to Vero/HFF and erythrocytes, respectively (Fig. 1a), suggesting that there was cross-contamination between the different organisms. This absence of contamination is unequivocally attested by the anti-α-tubulin staining (Fig. 1b); α-tubulin is expressed in Vero cells, HFF, and *T. gondii* but the difference of molecular weight observed between the two cell types and the parasite asserts that there was no contamination during the preparation of *T. gondii*. With regard to the preparation of *P. falciparum* a high expression of α-tubulin is observed for the trophozoites, whereas no α-tubulin is observed for the erythrocytes.

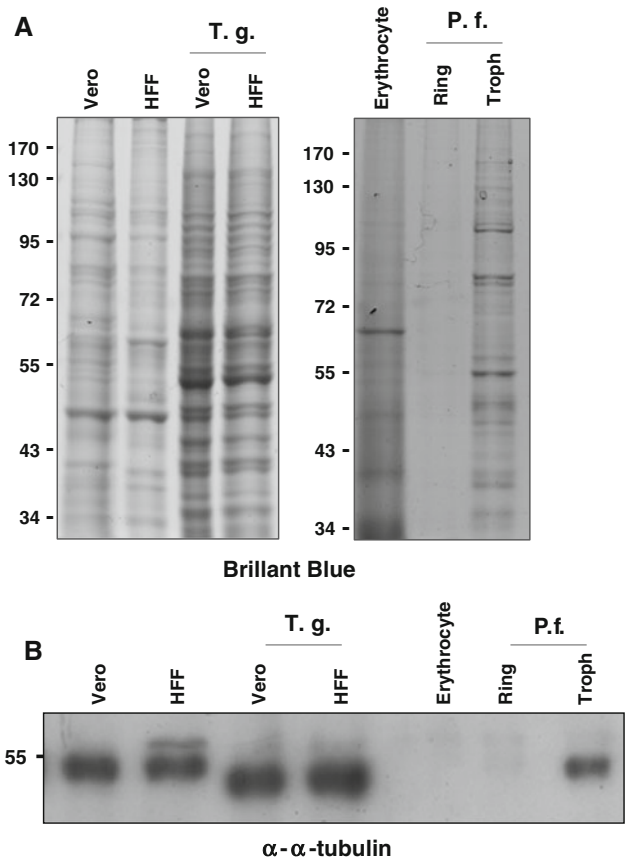

Fig. 1 *T. gondii*, *P. falciparum* and their host cells display distinct protein profiles. The Vero cells, HFF, erythrocytes, and protozoan cells were lysed in the homogenization buffer and run using SDS-PAGE. The extracts were either stained with brilliant blue (**a**) or electroblotted onto nitrocellulose and analyzed for their alpha-tubulin content (**b**). *HFF*, human foreskin fibroblast; *T.g.*, *Toxoplasma gondii*; *P.f.*, *Plasmodium falciparum*

T. gondii and *P. falciparum* express both a myriad of *O*-GlcNAcylated proteins

In order to prove that *O*-GlcNAcylation occurs in *T. gondii* and *P. falciparum* we analyzed the parasites' proteins using two anti-*O*-GlcNAc specific antibodies (RL2, Fig. 2a and CTD110.6, Fig. 2b) that recognize a wide range of *O*-GlcNAcylated proteins. The RL2 antibody was originally designed for recognizing a group of eight proteins located to the nuclear pore complex (Snow et al. 1987) and further analyses indicated that the antibody was directed against the *O*-GlcNAc moieties of these proteins (Holt et al. 1987). The CTD110.6 antibody (mouse IgM) was designed against the consensus heptapeptide YSPTSPS (Comer et al. 2001; Teo et al. 2010) that is repeated in a high number of copies in the Carboxyl-Terminal Domain of the RNA polymerase II. Using the RL2 we can see that the proteins expressed by *T. gondii* and *P. falciparum* are heavily *O*-GlcNAcylated (Fig. 2a). The *T. gondii* and the Vero cells' *O*-GlcNAc profiles are rather different since *O*-GlcNAcylated proteins having an apparent molecular weight more than 130 kDa are found for *T. gondii* but are undetectable for the Vero cells. The band having a 34 kDa apparent molecular weight in the erythrocyte lane (asterisk) and which displays a high reactivity with the anti-*O*-GlcNAc antibody is not found in the *P. falciparum* corresponding lane. In the same idea the bands having an apparent molecular weight close to 38–41 kDa in the Vero cells' lane (asterisks) are not found in the *T. gondii* lane ruling out the concern of a contamination during the preparation of the parasites. The specificity of the RL2 was asserted by incubating the antibody with free N-acetylglucosamine. As for RL2, the staining with the CTD110.6 reveals that the *O*-GlcNAcylation profiles are rather different when the *T. gondii* and the *P. falciparum* extracts are compared to their host cells and that many proteins bear the *O*-GlcNAc residue (Fig. 2b). The specificity of the CTD110.6 was also checked by incubating the antibody with free *N*-acetylglucosamine (data not shown). It is noteworthy that *O*-GlcNAcylated proteins were also detected when *T. gondii* was cultured in HFF (data not shown).

Since one of the *O*-GlcNAcylated proteins detected in *T. gondii* with the RL2 and the CTD110.6 displays a molecular mass close to the 72 kDa mass marker we sought that it may be the 70 kDa-Heat Shock Protein (Hsp70), a well-recognized *O*-GlcNAcylated protein (Guinez et al. 2006). For that purpose the *O*-GlcNAcylated proteins expressed in *T. gondii* and in HeLa (positive control) cells were immunoprecipitated either with the RL2 antibody or with non-immune IgG (negative control). The resultant immunoprecipitates were blotted with an anti-Hsp70 antibody (Fig. 2c). We can observe that in *T. gondii*, Hsp70 is *O*-GlcNAc-modified or at least tightly associated with an *O*-GlcNAcylated partner.

The nuclear and cytoplasmic *O*-GlcNAc transferase is found in *T. gondii*

The next step of our study was to prove OGT in the parasites. To tentatively detect the enzyme responsible for the attachment of the sugar moiety we have tested a panel of anti-OGT antibodies. Using the AL35 and the AL25 antibodies against the proteins expressed by *T. gondii* we were able to detect a band migrating close to the OGT expressed by the Vero cells and the HFF (Fig. 3a). In contrast, we did not find any band that might correspond to OGT with the *P. falciparum* extracts. According to this result, we cannot rule out that the proteins detected with the anti-*O*-GlcNAc antibodies with the *P. falciparum* extracts are the previously described GPI-anchored *O*-GlcNAcylated proteins (Hoessli et al. 2003).

We have assayed the endogenous OGT activity found in *T. gondii* and compared it to the HepG2 cells since this

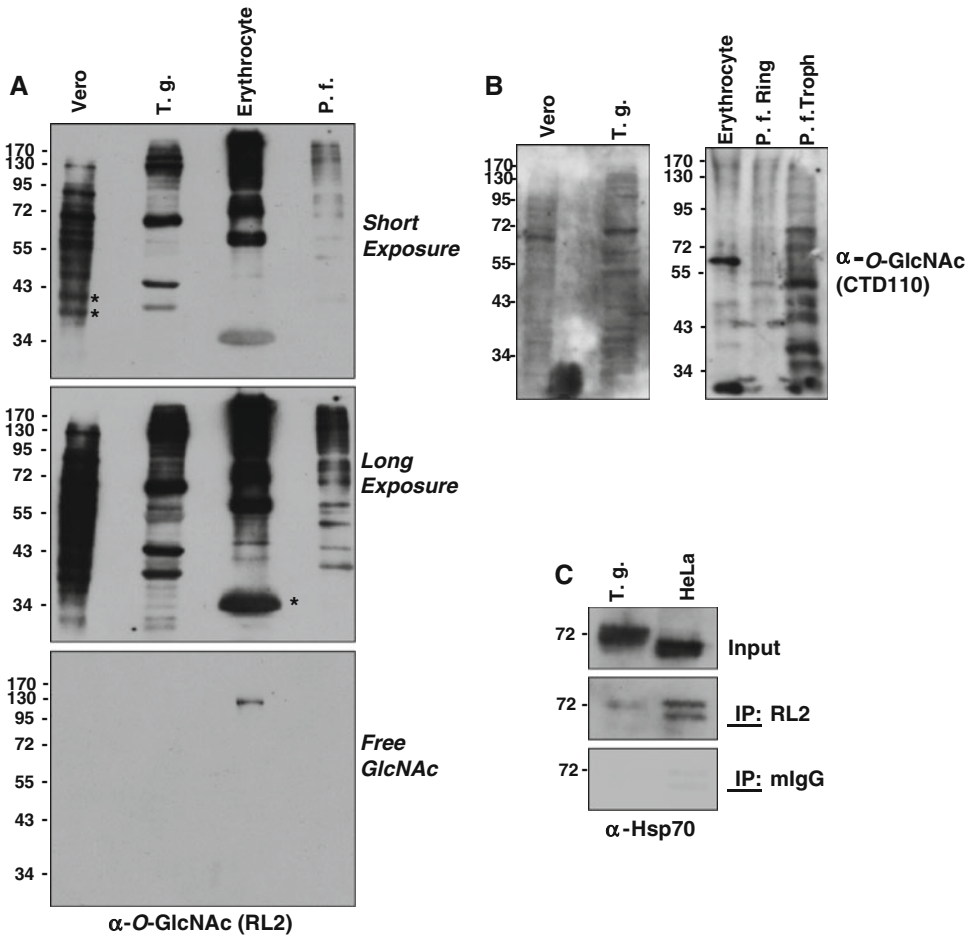

Fig. 2 *T. gondii* and *P. falciparum* express a myriad of *O*-GlcNAcylated proteins. After host cells and parasites lysis, the proteins were run on SDS-PAGE, electroblotted onto nitrocellulose sheets and analyzed for their *O*-GlcNAc content either with the RL2 antibody (**a**) or the CTD110.6 antibody (**b**). The *asterisks* indicate *O*-GlcNAcylated proteins expressed in Vero cells or in the erythrocytes and that are not found in the parasites taking away any problem of contamination. The specificity of the RL2 was checked by incubating the antibody with free *N*-acetylglucosamine (GlcNAc). HeLa cells and *T. gondii* extracts were incubated with the RL2 antibody in order to immunopurify *O*-GlcNAcylated proteins (**c**). After running on SDS-PAGE the immunoprecipitated proteins were analyzed by Western blot according to their Hsp70 content. A negative control was realized by replacing the anti-*O*-GlcNAc antibody by non-immune mouse IgG. *T.g.*, *Toxoplasma gondii*; *P.f.*, *Plasmodium falciparum*; *OGT*, *O*-GlcNAc transferase

human hepatocarcinoma cell line is known to possess appreciable levels of *O*-GlcNAcylation (Fig. 3b). For that purpose we followed the procedure described by Banerjee et al. (2009) who measured the OGT activities for *Giardia* and *Cryptosporidium* nucleocytosolic extracts. We detected a significant *T. gondii* OGT activity that is sevenfold higher than the background. Nevertheless this activity is threefold lower than that of the HepG2 cells.

Using the amino acids' sequence of the catalytic domain II (CDII; Lazarus et al. 2005) of the human OGT we have studied the *Apicomplexan Genome Resource* (ApiDB) by using the "Basic Local Alignment Search Tool" (BLAST). We found the sequence *TGGT1_112580* as the putative *T. gondii* OGT (Fig. 4). This sequence was aligned with SPY (SPindlY, from *Arabidopsis thaliana*), with the predicted sequence corresponding to the *Cryptosporidium parvum* (cgd1_1300 SPindlY-like TPR repeats; 1032 amino acids) and published by Banerjee et al. (2009), with the *Cryptosporidium muris* predicted sequence (*CMU_036020* TPR_repeat containing protein; 1070 amino acids) found in our Blast researches and with some OGT sequences found in other organisms (Fig. 4). The maximal identity/homology found for these eight sequences set around the two last thirds of the proteins that is in the catalytic domains. These alignments revealed that most of the amino acids essential for the binding properties and for the catalytic activity (Lazarus et al. 2005; Martinez-Fleites et al. 2008; Clarke et al. 2008; Martinez-Fleites et al. 2010) of the glycosyltransferase are present in the two herein described sequences, i.e., *T. gondii* and *C. muris* OGTs (Fig. 4). The mobile loop found in the catalytic pocket between the βN4 and the αN4 structures is found for

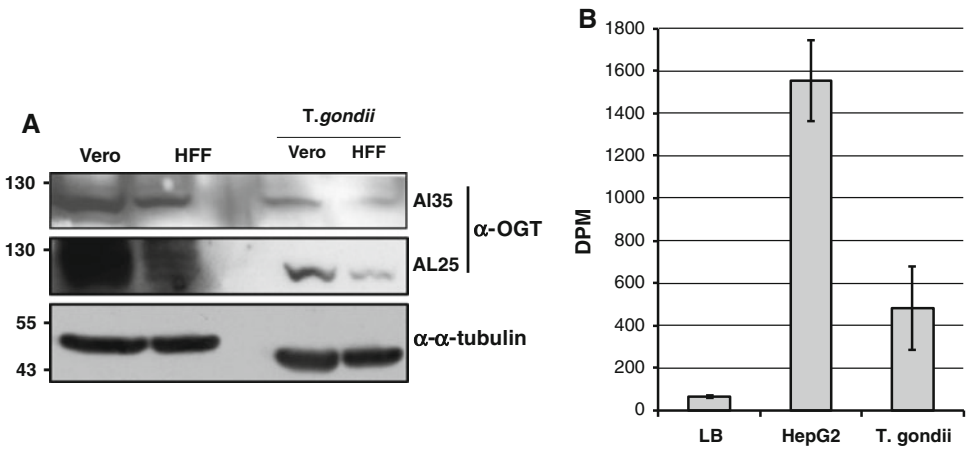

Fig. 3 The OGT is expressed in *T. gondii*. The protein extracts form Vero cells, HFF and from *T. gondii* cultured in both host cells were run on SDS-PAGE, electroblotted and analyzed according to their OGT content either with the AL35 or the AL25 antibodies (**a**). The relative activities of *T. gondii* and HepG2 OGTs were measured using a labeling with UDP-[^{3}H] GlcNAc as described under the 'Materials and methods' section (**b**). *HFF*, human foreskin fibroblast; *T.g.*, *Toxoplasma gondii*; *LB*, labeling buffer (negative control); *DPM*, disintegrations per minute

T. gondii and *C. muris* except that the basic residue (*Hs*OGT Arg647; *Tg*OGT Arg701) is changed to a cysteine in *C. muris* OGT (Cys781). The glycine and the threonine residues located within this motif are conserved along with the eight species tested (*Hs*OGT Gly641/Thr643). The residues implicated in the binding of the beta-phosphate of UDP-GlcNAc are strictly conserved among the species (*Hs*OGT Lys852/Thr931: *Tg*OGT Lys793/Thr876; *Cm*OGT Lys871/Thr955). The alpha-phosphate between the two residues defined as necessary for bridging the threonine is also strictly conserved (*Hs*OGT Thr932: *Tg*OGT Thr877; *Cm*OGT Thr956), whereas the second residue is divergent (*Hs*OGT Gln848; *Tg*OGT Asn790; *Cm*OGT Arg869). The residue stabilizing the ribose part of the nucleotide-sugar is homologous for the species since we find either an aspartic acid or a glutamic acid residue (*Hs*OGT Asp935; *Tg*OGT Glu880; *Cm*OGT Glu959). In our alignments we also found the histidine 558, which is conserved among all the species (*Tg*OGT His623; *Cm*OGT His668) and which mutation inactivates the enzyme. Finally in the *T. gondii* C-terminal OGT sequence we find the Lys937/Arg938 dipeptide sequence (HsOGT Lys981/Lys982) necessary for the binding of OGT to the phosphoinositides (Yang et al. 2008) and named PPO for PIP-binding activity of OGT.

Discussion

O-GlcNAcylation is widely expressed in many organisms including viruses, bacteria, and *C. elegans*. In eukaryotes, this modification preferentially occurs within the cytosolic, the nuclear (Hart et al. 2007) and the mitochondrial (Hu et al. 2009) compartments. *O*-GlcNAcylation differs from typical *N*- and *O*-glycosylations by many features, one of the most surprising being its high dynamism. The functions played by *O*-GlcNAcylation are multiple and include the regulation of the cell cycle, the intracellular trafficking of proteins, their turnover, and many others (for recent review see Butkinaree et al. 2010). To date no study has clearly reported the presence of *O*-GlcNAcylation in yeast and in apicomplexans. In the present work we definitively prove that *T. gondii* possess a large variety of *O*-GlcNAcylated proteins and in a second part we report the potential sequence of *T. gondii* OGT. We have also arguments for suggesting that *P. falciparum* *O*-GlcNAcylates its intracellular proteins, but since we failed to find a putative OGT sequence, we cannot certainty assert that what we detected with this parasite is indeed the classical nucleocytoplasmic *O*-GlcNAcylation. We also attempted to prove the OGA in *T. gondii* and *P. falciparum* by Western blot and by *in silico* analyses but the two approaches proved unfruitful. This lack of finding is in accordance with the previously published data by Banerjee et al. (2009) who did not find homologues of the OGA for their parasites.

OGT is a glycosyltransferase (EC. 2.4.1.94) assigned to the GT41 family in the CAZY (Carbohydrate-Active enZYme) database (Cantarel et al. 2009). It belongs to the Glycogen Phosphorylase/GlycosylTransferase Family (GPGTF), which includes the glycogen phosphorylase, the UDP-GlcNAc 2-epimerase and the glycosyl transferase MurG (Wrabl and Grishin 2001; Lazarus et al. 2005). The human *OGT* gene resides on the Xq13.1 locus (Nolte and Müller 2002), a region associated with neurological disorders, and its deletion results in cell lethality (Shafi et al. 2000). OGT has been identified in many kinds of organism

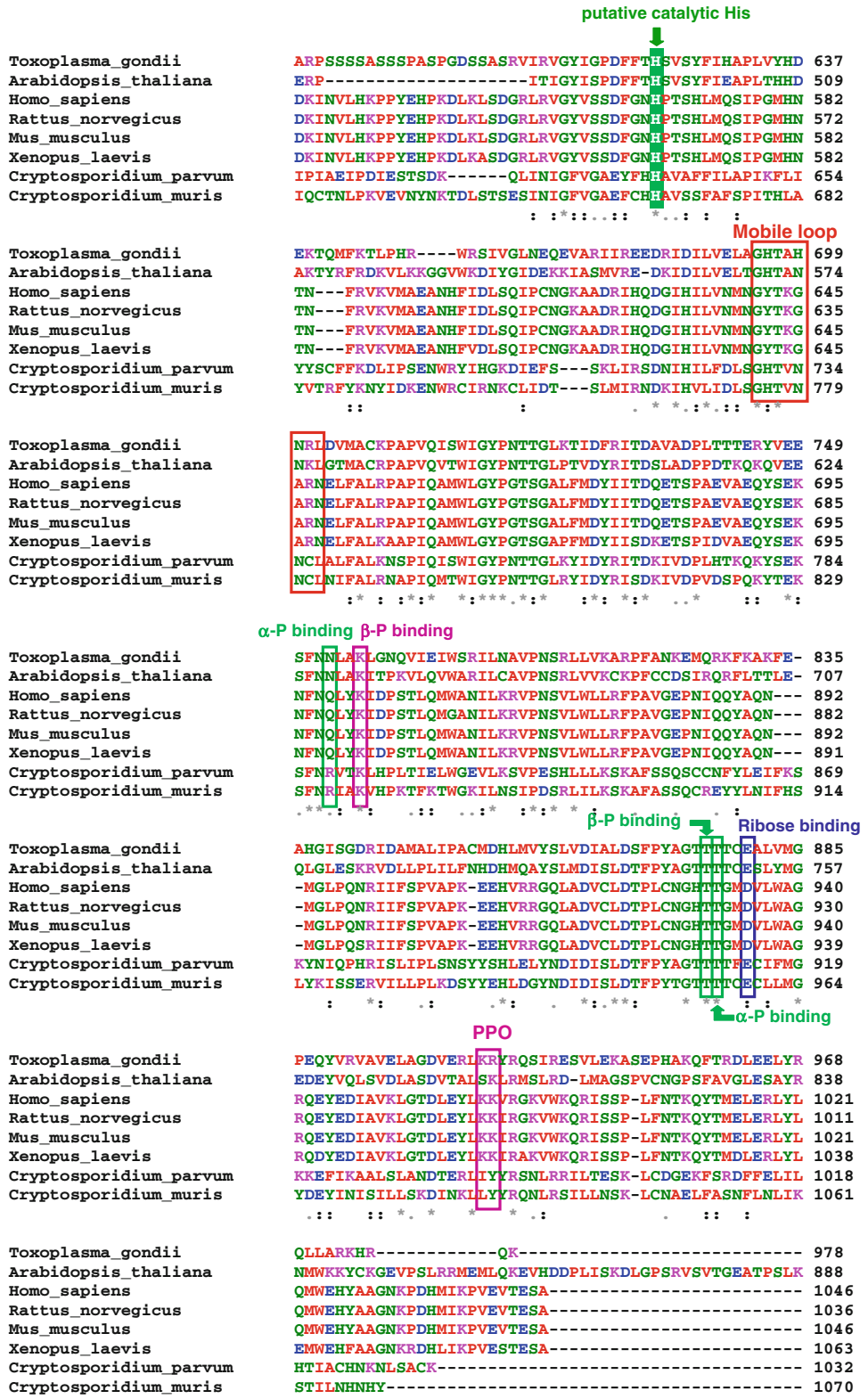

Fig. 4 The *T. gondii* putative OGT sequence shares similar features with other species. The putative OGT sequence (*TGGT1_112580*) found for *T. gondii* was aligned with other known OGT sequences and with the putative *Cryptosporidium muris* OGT sequence (*CMU_036020*). The putative catalytic histidine, the mobile loop located within the catalytic pocket and described by Martinez-Fleites et al. (2008), the binding sites for the UDP-GlcNAc ribose, alpha-phosphates and beta-phosphates, and PPO (PIP-binding activity of OGT) are *highlighted*

(Wrabl and Grishin 2001). For example, the plant *Arabidopsis* expressed two OGTs, namely, SPY for SPindlY and SEC for SECret agent (Hartweck et al. 2002) and the bacteria *Listeria monocytogenes* expresses the OGT GmaR that controls the flagellar motility (Shen et al. 2006). In humans, there are three known OGT isoforms differing in their N-terminal region (for review see Lazarus et al. 2009): the nuclear and cytoplasmic OGT (ncOGT) that

contains 11.5 TPRs (TetraTricopeptide Repeats; amino acids 1–473 in the human OGT sequence); the mitochondrial OGT (mOGT) which contains 9.5 TPR, and a mitochondrial targeting sequence and the short OGT isoform (sOGT) found within the nucleus and the cytoplasm and which only contains 2.5 TPRs. All these isoforms share a common sequence in the C-terminal region. In this part of the enzyme two catalytic domains (CD) are found (amino acids 504–1046 in the human OGT), the CDI and the CDII (Lazarus et al. 2005). In our study we used the sequence of the second catalytic domain for finding putative apicomplexans OGTs. We found several sequences between which are *TGGT1_112580* for *T. gondii* OGT and *CMU_036020* for *Cryptosporidium muris* OGT. The alignment and the analysis of these sequences with other previously described OGTs show that the most conserved part of the enzyme is located in the second half, the region where are located the two catalytic domains (Fig. 4). In our alignments the *T. gondii* OGT shares a maximal identity with the plant (*Arabidopsis thaliana*) OGT homologue SPindlY: 40% for the full-lengths OGTs and 50% for the catalytic domain of the enzymes. As detailed in the 'Results' section we found most of the amino acids essential for the binding properties that are for the ribose and the α/β- phosphates binding. We also found the histidine 558 necessary for the catalytic activity of OGT. It seems that this crucial residue is at the catalytic basis of the enzyme since it may serve for the deprotonation of the Ser/Thr hydroxyl group of the acceptor (Lazarus et al. 2009; Martinez-Fleites et al. 2010). At last we found the KR dipeptide instead of the KK dipeptide described by Yang et al. (2008) and crucial for the PIP-binding (PPO domain). In conclusion the putative *T. gondii* OGT sequence possesses the main crucial amino acids residues necessary for its biological properties. This finding that *T. gondii*, along with perhaps other protozoan parasites, expresses its own OGT opens up new therapeutic perspectives. Indeed it has been established that OGT is necessary for stem cells' viability (Shafi et al. 2000), and numerous works have shown that its knock-down using si/shRNA interferes with many fundamental biological functions. The few differences found for the UDP-GlcNAc binding properties of *T. gondii* and human OGTs suggest that specific pharmacological inhibitors could be designed and synthesized so as to specifically block the parasite OGT. The generation of new molecules targeting the *Toxoplasma* metabolism may be an alternative to the resistance developed by the parasite toward the current drug therapies. In this view it has been recently observed that the use of the small-molecule inhibitor tachypleginA blocked the motility of *T. gondii* by inducing an unidentified PTM of TgMLC1, a protein partner of the myosin motor protein TgMyoA (Heaslip et al. 2010). This finding is very interesting since it may be envisaged that this inhibitor, or a derivative one, can be used for stopping the *T. gondii* infectivity through targeting an enzyme involved in protein modification. In addition to be dependent upon the motor proteins, the invasion process is also tightly orchestrated by the microtubule network. Xiao et al. (2010) recently showed that *T. gondii* α-tubulins and β-tubulins were highly modified. The different PTMs occurring on microtubules are undoubtedly fundamental actors of the invasion process and blocking their metabolism by the use of inhibitors should interfere with the infection pathway.

The next step in these studies will be to identify which proteins are *O*-GlcNAcylated in *T. gondii*. This identification will be helpful for the understanding of the function played by the modification in the parasites life cycles and in the infection processes.

Finally this study is another proof that *O*-GlcNAcylation is ubiquitous and it shows that this PTM is fundamental even for the simplest organisms.

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Conflict of interest The authors declare that they have no conflict of interest.

References

Andrews KT, Tran TN, Wheatley NC, Fairlie DP (2009) Targeting histone deacetylase inhibitors for anti-malarial therapy. Curr Top Med Chem 9:292–308

Banerjee S, Robbins PW, Samuelson J (2009) Molecular characterization of nucleocytosolic *O*-GlcNAc transferases of *Giardia lamblia* and *Cryptosporidium parvum*. Glycobiology 19:331–336

Berger F, Goulet V, Le Strat Y, Desenclos JC (2009) Toxoplasmosis among pregnant women in France: risk factors and change of prevalence between 1995 and 2003. Rev Epidemiol Sante Publique 57:241–248

Braun L, Cannella D, Pinheiro AM, Kieffer S, Belrhali H, Garin J, Hakimi MA (2009) The small ubiquitin-like modifier (SUMO)-conjugating system of *Toxoplasma gondii*. Int J Parasitol 39:81–90

Butkinaree C, Park K, Hart GW (2010) O-linked beta-*N*-acetylglucosamine (O-GlcNAc): extensive crosstalk with phosphorylation to regulate signaling and transcription in response to nutrients and stress. Biochim Biophys Acta 1800:96–106

Cantarel BL, Coutinho PM, Rancurel C, Bernard T, Lombard V, Henrissat B (2009) The Carbohydrate-Active EnZymes database (CAZy): an expert resource for Glycogenomics. Nucleic Acids Res 37:D233–D238

Chakrabarti D, Da Silva T, Barger J, Paquette S, Patel H, Patterson S, Allen CM (2002) Protein farnesyltransferase and protein prenylation in *Plasmodium falciparum*. J Biol Chem 277:42066–42073

Chung DW, Ponts N, Cervantes S, Le Roch KG (2009) Post-translational modifications in *Plasmodium*: more than you think!. Mol Biochem Parasitol 168:123–134

Clarke AJ, Hurtado-Guerrero R, Pathak S, Schüttelkopf AW, Borodkin V, Shepherd SM, Ibrahim AF, van Aalten DM (2008) Structural insights into mechanism and specificity of *O*-GlcNAc transferase. EMBO J 27:2780–2788

Comer FI, Vosseller K, Wells L, Accavitti MA, Hart GW (2001) Characterization of a mouse monoclonal antibody specific for *O*-linked *N*-acetylglucosamine. Anal Biochem 293:169–177

Cui L, Miao J, Furuya T, Li X, Su XZ, Cui L (2007) PfGCN5-mediated histone H3 acetylation plays a key role in gene expression in *Plasmodium falciparum*. Eukaryot Cell 6:1219–1227

Cui L, Fan Q, Cui L, Miao J (2008) Histone lysine methyltransferases and demethylases in *Plasmodium falciparum*. Int J Parasitol 38:1083–1097

Debierre-Grockiego F, Molitor N, Schwarz RT, Lüder CG (2009) *Toxoplasma gondii* glycosylphosphatidylinositols up-regulate major histocompatibility complex (MHC) molecule expression on primary murine macrophages. Innate Immun 15:25–32

Dieckmann-Schuppert A, Bender S, Odenthal-Schnittler M, Bause E, Schwarz RT (1992) Apparent lack of *N*-glycosylation in the asexual intraerythrocytic stage of *Plasmodium falciparum*. Eur J Biochem 205:815–825

Dieckmann-Schuppert A, Bause E, Schwarz RT (1993) Studies on *O*-glycans of *Plasmodium-falciparum*-infected human erythrocytes. Evidence for *O*-GlcNAc and *O*-GlcNAc-transferase in malaria parasites. Eur J Biochem 216:779–788

Dorin-Semblat D, Quashie N, Halbert J, Sicard A, Doerig C, Peat E, Ranford-Cartwright L, Doerig C (2007) Functional characterization of both MAP kinases of the human malaria parasite *Plasmodium falciparum* by reverse genetics. Mol Microbiol 65:1170–1180

Fauquenoy S, Morelle W, Hovasse A, Bednarczyk A, Slomianny C, Schaeffer C, Van Dorsselaer A, Tomavo S (2008) Proteomics and glycomics analyses of *N*-glycosylated structures involved in *Toxoplasma gondii*–host cell interactions. Mol Cell Proteomics 7:891–910

Frickel EM, Quesada V, Muething L, Gubbels MJ, Spooner E, Ploegh H, Artavanis-Tsakonas K (2007) Apicomplexan UCHL3 retains dual specificity for ubiquitin and Nedd8 throughout evolution. Cell Microbiol 9:1601–1610

Gerold P, Dieckmann-Schuppert A, Schwarz RT (1994) Glycosylphosphatidylinositols synthesized by asexual erythrocytic stages of the malarial parasite, *Plasmodium falciparum*. Candidates for plasmodial glycosylphosphatidylinositol membrane anchor precursors and pathogenicity factors. J Biol Chem 269:2597–2606

Grimwood BG, Hechemy K, Stevens RW (1979) *Toxoplasma gondii*: purification of trophozoites propagated in cell culture. Exp Parasitol 48:282–286

Guinez C, Losfeld ME, Cacan R, Michalski JC, Lefebvre T (2006) Modulation of HSP70 GlcNAc-directed lectin activity by glucose availability and utilization. Glycobiology 16:22–28

Hart GW, Housley MP, Slawson C (2007) Cycling of *O*-linked beta-*N*-acetylglucosamine on nucleocytoplasmic proteins. Nature 446:1017–1022

Hartweck LM, Scott CL, Olszewski NE (2002) Two *O*-linked *N*-acetylglucosamine transferase genes of *Arabidopsis thaliana* L. Heynh have overlapping functions necessary for gamete and seed development. Genetics 161:1279–1291

Heaslip AT, Leung JM, Carey KL, Catti F, Warshaw DM, Westwood NJ, Ballif BA, Ward GE (2010) A small-molecule inhibitor of *T. gondii* motility induces the posttranslational modification of myosin light chain-1 and inhibits myosin motor activity. PLoS Pathog 6:e1000720

Hoessli DC, Poincelet M, Gupta R, Ilangumaran S, Nasir-ud-Din (2003) *Plasmodium falciparum* merozoite surface protein 1. Eur J Biochem 270:366–375

Holt GD, Snow CM, Senior A, Haltiwanger RS, Gerace L, Hart GW (1987) Nuclear pore complex glycoproteins contain cytoplasmically disposed *O*-linked *N*-acetylglucosamine. J Cell Biol 104:1157–1164

Horrocks P, Newbold CI (2000) Intraerythrocytic polyubiquitin expression in *Plasmodium falciparum* is subjected to developmental and heat-shock control. Mol Biochem Parasitol 105:115–125

Hu Y, Suarez J, Fricovsky E, Wang H, Scott BT, Trauger SA, Han W, Hu Y, Oyeleye MO, Dillmann WH (2009) Increased enzymatic *O*-GlcNAcylation of mitochondrial proteins impairs mitochondrial function in cardiac myocytes exposed to high glucose. J Biol Chem 284:547–555

Lazarus BD, Roos MD, Hanover JA (2005) Mutational analysis of the catalytic domain of *O*-linked *N*-acetylglucosaminyl transferase. J Biol Chem 280:35537–35544

Lazarus BD, Love DC, Hanover JA (2009) *O*-GlcNAc cycling: implications for neurodegenerative disorders. Int J Biochem Cell Biol 41:2134–2146

Luk FC, Johnson TM, Beckers CJ (2008) N-linked glycosylation of proteins in the protozoan parasite *Toxoplasma gondii*. Mol Biochem Parasitol 157:169–178

Martinez-Fleites C, Macauley MS, He Y, Shen DL, Vocadlo DJ, Davies GJ (2008) Structure of an *O*-GlcNAc transferase homolog provides insight into intracellular glycosylation. Nat Struct Mol Biol 15:764–765

Martinez-Fleites C, He Y, Davies GJ (2010) Structural analyses of enzymes involved in the *O*-GlcNAc modification. Biochim Biophys Acta 1800:122–133

Nicolle C, Manceaux L (1908) Sur une infection à corps de Leshman (ou organismes voisons) du gondi. CR Acad Sci 147:763

Nolte D, Müller U (2002) Human *O*-GlcNAc transferase (OGT): genomic structure, analysis of splice variants, fine mapping in Xq13.1. Mamm Genome 13:62–64

Plessmann U, Reiter-Owona I, Lechtreck KF (2004) Posttranslational modifications of alpha-tubulin of *Toxoplasma gondii*. Parasitol Res 94:386–389

Schmidt A, Schwarz RT, Gerold P (1998) *Plasmodium falciparum*: asexual erythrocytic stages synthesize two structurally distinct free and protein-bound glycosylphosphatidylinositols in a maturation-dependent manner. Exp Parasitol 88:95–102

Schneider AG, Mercereau-Puijalon O (2005) A new Apicomplexa-specific protein kinase family: multiple members in *Plasmodium falciparum*, all with an export signature. BMC Genomics 6:30

Shafi R, Iyer SP, Ellies LG, O'Donnell N, Marek KW, Chui D, Hart GW, Marth JD (2000) The *O*-GlcNAc transferase gene resides on the X chromosome and is essential for embryonic stem cell viability and mouse ontogeny. Proc Natl Acad Sci USA 97:5735–5739

Shen A, Kamp HD, Gründling A, Higgins DE (2006) A bifunctional *O*-GlcNAc transferase governs flagellar motility through anti-repression. Genes Dev 20:283–295

Snow CM, Senior A, Gerace L (1987) Monoclonal antibodies identify a group of nuclear pore complex glycoproteins. J Cell Biol 104:1143–1156

Striepen B, Zinecker CF, Damm JB, Melgers PA, Gerwig GJ, Koolen M, Vliegenthart JF, Dubremetz JF, Schwarz RT (1997) Molecular structure of the "low molecular weight antigen" of *Toxoplasma gondii*: a glucose alpha 1–4 *N*-acetylgalactosamine makes free glycosyl-phosphatidylinositols highly immunogenic. J Mol Biol 266:797–813

Teo CF, Ingale S, Wolfert MA, Elsayed GA, Nöt LG, Chatham JC, Wells L, Boons GJ (2010) Glycopeptide-specific monoclonal antibodies suggest new roles for *O*-GlcNAc. Nat Chem Biol 6:338–343

Ward P, Equinet L, Packer J, Doerig C (2004) Protein kinases of the human malaria parasite *Plasmodium falciparum*: the kinome of a divergent eukaryote. BMC Genomics 5:79

Weiss LM, Fiser A, Angeletti RH, Kim K (2009) *Toxoplasma gondii* proteomics. Expert Rev Proteomics 6:303–313

Wichmann D, Schwarz RT, Ruppert V, Ehrhardt S, Cramer JP, Burchard GD, Maisch B, Debierre-Grockiego F (2007) *Plasmodium falciparum* glycosylphosphatidylinositol induces limited apoptosis in liver and spleen mouse tissue. Apoptosis 12:1037–1041

Wrabl JO, Grishin NV (2001) Homology between *O*-linked GlcNAc transferases and proteins of the glycogen phosphorylase superfamily. J Mol Biol 314:365–374

Xiao H, El Bissati K, Verdier-Pinard P, Burd B, Zhang H, Kim K, Fiser A, Angeletti RH, Weiss LM (2010) Post-translational modifications to *Toxoplasma gondii* alpha- and beta-tubulins include novel C-terminal methylation. Proteome Res 9:359–372

Yang X, Ongusaha PP, Miles PD, Havstad JC, Zhang F, So WV, Kudlow JE, Michell RH, Olefsky JM, Field SJ, Evans RM (2008) Phosphoinositide signalling links *O*-GlcNAc transferase to insulin resistance. Nature 451:964–969