HHS Public Access

Author manuscript

Microbes Infect. Author manuscript; available in PMC 2017 March 04.

Published in final edited form as:

Microbes Infect. 2016 March ; 18(3): 199–210. doi:10.1016/j.micinf.2015.11.004.

Extracellular *Toxoplasma gondii* tachyzoites metabolize and incorporate unnatural sugars into cellular proteins

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Abstract

Toxoplasma gondii is an obligate intracellular parasite that infects all nucleated cell types in diverse warm-blooded organisms. Many of the surface antigens and effector molecules secreted by the parasite during invasion and intracellular growth are modified by glycans. Glycosylated proteins in the nucleus and cytoplasm have also been reported. Despite their prevalence, the complete inventory and biological significance of glycosylated proteins in *Toxoplasma* remains unknown. In this study, we aimed to globally profile parasite glycoproteins using a bioorthogonal chemical reporter strategy. This strategy involves the metabolic incorporation of unnatural functional groups (i.e., "chemical reporters") into *Toxoplasma* glycans, followed by covalent labeling with visual probes or affinity tags. The two-step approach enables the visualization and identification of newly biosynthesized glycoconjugates in the parasite. Using a buffer that mimics intracellular conditions, extracellular *Toxoplasma* tachyzoites were found to metabolize and incorporate unnatural sugars (equipped with bioorthogonal functional groups) into diverse proteins. Covalent chemistries were used to visualize and retrieve these labeled structures. Subsequent mass spectrometry analysis revealed 89 unique proteins. This survey identified novel proteins as well as previously characterized proteins from lectin affinity analyses.

Keywords

Apicomplexa; glycobiology; "click" chemistry; metabolic labeling; parasite

1. Introduction

Glycosylation is a ubiquitous post-translational modification that is critically important for diverse cellular functions, including protein folding and sorting, receptor interactions and signal transduction [1, 2]. Glycans are built off polypeptide chains by the actions of

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Conflict of interest:
The authors declare no conflict of interest.

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numerous glycosyltransferases (enzymes that transfer activated nucleotide sugars to growing glycan chains or underlying protein/lipid units). Glycan structures can be further modified by glycosidases (enzymes that trim sugars from larger structures) or enzymes that add sulfate or acetyl groups to the monosaccharide units. Several proteins synthesized in the rough ER are modified by N-linked glycans (typically attached to asparagine residues), while others are modified via O-linked glycans (attached to serine, threonine, tyrosine, hydroxylysine, or hydroxyproline residues). Some extracellular proteins also comprise glycosylphosphatidylinositol (GPI) anchors, carbohydrate-containing groups that anchor the proteins into the external leaflet of the plasma membrane [3, 4]. In recent years, numerous intracellular proteins relevant to cell signaling, metabolism, and gene expression have been discovered to harbor β-O-GlcNAc residues [5, 6].

The prevalence and central roles of protein glycosylation are only beginning to be appreciated in apicomplexan pathogens such as *Toxoplasma gondii. T. gondii* is an obligate intracellular parasite, capable of infecting all nucleated cell types in diverse warm-blooded animals and birds [7]. *Toxoplasma* tachyzoites are the rapidly proliferating form of the parasite that cause acute stage infection. This form and other life cycle stages contain characteristic eukaryotic organelles including a nucleus, endoplasmic reticulum (ER), Golgi apparatus, mitochondrion and plastid, as well as specialized membrane-bound secretory organelles termed micronemes, rhoptries and dense granules. Tachyzoites are bounded by a tri-laminar pellicle consisting of a plasma membrane and underlying inner membrane complex (IMC) and the tachyzoite surface is particularly enriched in GPI-linked surface proteins such as TgSAG1 [8]. In order for *Toxoplasma* to survive and replicate, extracellular parasites must actively invade host cells in infected organisms, including humans. Invasion of host cells begins with the initial attachment of GPI-anchored surface antigens on *Toxoplasma* to glycans on the host cell [9]. Secretion from micronemes and rhoptries is required for parasite motility, host cell invasion and establishment of a non-phagosomal membrane-bound parasitophorous vacuole [10, 11]. Within the vacuole, tachyzoites undergo an asexual reproduction by endodyogeny, a process in which two daughter cells are produced inside a mother cell. In the absence of a host cell, the parasite is not able to replicate and will not survive. Trafficking of proteins to the parasite surface and to the microneme, rhoptry and dense granule compartments occurs via post-Golgi sorting [12–14] and is likely to require protein glycosylation.

We have an incomplete understanding of glycan modification in apicomplexan parasites due, in part, to a lack of tools for studying these non-templated biopolymers in their native contexts [15]. Lectins (receptor proteins that bind to glycans) [16, 17] and glycan-specific antibodies [18] are available to survey parasite glycans, but these reagents often bind multiple types of structures and thus provide limited information on the types of glycans present. Additionally, many well-known reagents available for studies of mammalian glycosylation may not recognize unique glycan motifs produced by the parasite. Early reports suggested that GPI anchors are the predominant form of carbohydrate modification of *Toxoplasma* proteins [18–21]. However, there is now ample evidence that *Toxoplasma* proteins can be modified by both N- and O-linked pathways [18, 22–27]. Intracellular *Toxoplasma* proteins decorated with β-O-GlcNAc residues have also been identified [27, 28]. Curiously, the predominant glycans in *Toxoplasma* tachyzoites are unusual

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oligomannosidic ($Man_{5-8}(GlcNAc)_2$) and paucimannosidic ($Man_{3-4}(GlcNAc)_2$) sugars, which are rarely present on mature vertebrate glycoproteins [29]. Significantly, tunicamycin-treated parasites exhibited reduced motility, host cell invasion, and growth, suggesting that parasite glycosylation may represent a significant future drug target [17, 27].

Two reports have identified glycosylated proteins in *Toxoplasma* tachyzoites using lectin affinity chromatography and mass spectrometry (MS). In the first study, a Concanavalin A (ConA) column was used to enrich and identify putative glycoproteins, including cytoskeletal proteins (TgMyoA, TgMyoB/C, TgIMC2, actin, tubulin and TgGAP50), secreted proteins (the microneme proteins TgAMA1 and TgPLP1 and several rhoptry proteins) and likely components of the membrane trafficking machinery (putative sortilin and Sec61 orthologs) [17]. More recently, a set of 132 proteins that are likely modified by glycans was identified by serial lectin affinity chromatography (SLAC) and MS [16]. This glycoproteome included surface antigens, microneme, dense granule and rhoptry components, heat shock proteins, as well as a number of hypothetical proteins. The lectins ConA, wheat germ agglutinin (WGA) and Jacalin were used individually and in serial purification protocols to isolate glycoproteins in this study. While some glycoproteins bound to all three lectins, others were only retained by one or two of the reagents.

As noted above, although lectins can be used to profile some aspects of parasite glycosylation, these reagents do not provide a complete picture of glycan biosynthesis. Complementary information can be captured using the bioorthogonal chemical reporter strategy [30–32]. In this approach, a monosaccharide substrate is modified with a functional group (the reporter) that is chemically inert in biological systems. Upon administration to cells, the modified sugar is processed similarly to its native counterpart and integrated into cellular glycans. Finally, the labeled glycans are reacted with a detectable probe using highly selective, covalent chemistries. Depending on the nature of the probe, this strategy permits both the visualization of glycans and/or their enrichment from complex mixtures for subsequent analyses. In this manuscript, we utilize the bioorthogonal chemical reporter strategy to label and profile proteins in living *Toxoplasma* tachyzoites. This approach identified both known and novel candidate glycoproteins in the parasite and, importantly, demonstrated that tachyzoites can metabolize and incorporate sugar derivatives into cellular structures in the absence of host cell machinery. Subsets of the identified proteins appear to be modified by O-linked structures, suggesting an important role for these conjugates in the parasite life cycle.

2. Materials and methods

2.1. Culture of host cells and parasites

Toxoplasma (RH and Me49 strains, and Me49-derived lines) were grown in human foreskin fibroblast (HFF) host cells [33]. HFF cells were cultured in DMEM media (GIBCO, Invitrogen) supplemented with 10% fetal bovine serum in a humidified incubator (37 °C, 5.0% CO_2).

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2.2. Metabolic labeling of parasites

Confluent monolayers of HFF cells were infected with *Toxoplasma* 24–48 h prior to metabolic labeling. Once the host cells were infected to maximum capacity, they were washed with Endo buffer (44.7 mM K_2SO_4, 106 mM sucrose, 10 mM $MgSO_4$, 20 mM Tris, 5 mM glucose, 3.5% BSA pH 8.2), a buffer that mimics intracellular conditions [34], and then collected by scraping. Parasites were released from host cells by syringe passage (27-gauge needle) and filtered through a 3-μm mesh. The parasites were pelleted at 1500 ×*g* for 20 min, aspirated, and resuspended in 5 mL of Endo buffer. The parasites were added to 60-mm plates that had been pre-coated with Ac_4GlcNAc [35], Ac_4GlcNAz [36], or Ac_4GlcNAlk [37].

2.3. Cell lysate preparation

Labeled parasites were collected by centrifugation (1500 ×*g* for 20 min), washed twice with PBS and resuspended in 75 μL of lysis buffer (1% NP-40, 150 mM NaCl, 50 mM triethanolamine, pH 7.4) containing protease inhibitors (Roche Biosciences). Samples were subjected to five freeze-thaw cycles, pelleted, and the resulting supernatants were collected for analyses. Total protein concentrations were measured using a bicinchoninic acid (BCA) assay (Pierce, ThermoScientific).

2.4. Covalent protein labeling and SDS-PAGE

2.4.a. Cu(I)-catalyzed "click" reactions—*Toxoplasma* lysates (50 μg) were diluted with lysis buffer to a final concentration of 1 μg/μL. To each sample was added rho-alk, rho-az, biotin-alk [38], or biotin-az [39] (100 μM, from a 10 mM stock solution in DMSO), along with a freshly prepared cocktail of "click" chemistry reagents (sodium ascorbate: 1 mM, from a 50 mM stock solution in water; tris[(1-benzyl-1-H-1,2,3-triazol-4-yl)methyl]amine (TBTA): 100 μM, from a 10 mM stock solution in DMSO); $CuSO_4$•$5H_2O$: 1 mM, from a 50 mM stock solution in water). The final reaction volume was 50 μL in all cases. The reaction mixtures were vortexed and incubated at RT for 1 h. To precipitate the labeled proteins, the samples were treated with ice-cold methanol (1 mL) and placed at −80 °C for 2 h. The precipitates were pelleted at 13,000 ×*g* for 10 min (at 4 °C), and the supernatants were discarded. The samples were air-dried for 1 h at RT prior to the addition of 15 μL of resuspension buffer (4% SDS, 150 mM NaCl, 50 mM thiethanolamine pH 7.4) and 15 μL of 2× SDS-PAGE loading buffer (20% glycerol, 0.2% bromophenol blue, 1.4% β-mercaptoethanol). The proteins were denatured at 95°C and then resolved on Tris-glycine SDS-PAGE gels. Labeled proteins were detected with antibodies on immunoblots or in-gel fluorescence was used (below).

2.4.b. The Staudinger ligation—*Toxoplasma* lysates (50 μg) were reacted with phosphine-FLAG-His_6 or phosphine-biotin (250 μM, 500 μM stock solution in PBS) at RT for 12 h [40]. The samples were then treated with 4X SDS-PAGE loading buffer (4% SDS, 40% glycerol, 0.4% bromophenol blue, 2.8% β-mercaptoethanol). The samples were then incubated at 95 °C and the proteins were resolved on Tris-glycine SDS-PAGE gels. In some cases, the labeled proteins were detected with antibodies on immunoblots, whereas in other cases, in-gel fluorescence was used (below).

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2.5. Visualization of labeled proteins

2.5.a. Immunoblots—Proteins separated by SDS-PAGE were electroblotted to nitrocellulose membranes. Blots with biotinylated proteins were blocked using a solution of 7% BSA in PBS containing 0.1% Tween-20 (PBS-T) for 1 h at RT. FLAG-His_6-labeled blots were blocked using a solution of 5% non-fat milk (in PBS-T) for 1 h at RT. The blots were incubated with HRP-α-biotin (Jackson ImmunoResearch, 1:10,000 dilution), IRDye 800 CW streptavidin (Li-Cor, 1:10,000 dilution) or HRP-α-FLAG (Sigma, 1:5,000 dilution) in the appropriate blocking buffer for 1 h at RT, then rinsed with PBS-T (6 × 10 min). Detection of membrane-bound antibodies was accomplished by chemiluminescence (SuperSignal chemiluminescence substrate, Pierce) or near infrared spectroscopy on an Odyssey Infrared Imaging System. Densitometry analyses were performed using ImageJ software. The intensity from each immunoblot lane was divided by the intensity of the corresponding lane from the Ponceau S image. The resulting numbers were normalized to the negative control (background) and plotted.

2.5.b. In-gel fluorescence scanning—Following SDS-PAGE, some gels were incubated with destaining solution (50% methanol/10% acetic acid in water, 10 min), followed by water (10 min). In-gel fluorescent signals were measured on a Typhoon Trio+ scanner. Densitometry analyses were performed using imageJ software. The intensity from each fluorescent lane was divided by the intensity of the corresponding lane from the Coomassie stained gel. The resulting numbers were normalized to the negative control (background) and plotted.

2.6. Immunoprecipitation

Toxoplasma samples were prepared and reacted as previously described. In order to purify TgSAG1, samples were incubated with anti-P30/SAG1 (DG52) [41] at 4 °C, with mixing. The protein-antibody complex was isolated using Pierce Protein A/G Magnetic Beads (Thermo Scientific). Samples were analyzed via immunoblot as above.

2.7. Fluorescence microscopy

Syringe-lysed parasite cultures were purified using a PD-10 desalting column (GE Healthcare) or filter (Millipore, Millex-SV, 5.00 um PVDF membrane) and washed with DMEM media (Corning) supplemented with 3% (vol/vol) heat inactivated FBS (Omega Scientific), penicillin (100 U/mL), and streptomycin (10 μg/mL). The parasites were resuspended in Endo buffer containing Ac_4GlcNAz (250–1000 μM) or Ac_4GlcNAc (250–1000 μM). After 8 h, the parasites were washed with PBS (3 × 0.5 mL) and DMEM (3 × 0.5 mL). The parasites were then centrifuged onto poly-L lysine coated (0.01%, Sigma) glass coverslips (1500 rpm for 7 min) and fixed with 4% paraformaldehyde in PBS for 15 min at RT. After washing with PBS (3 × 0.25 mL), the parasites were permeabilized with 0.1% Triton-X in PBS for 5 min at RT. The parasites were rinsed with PBS (3 × 0.25 mL) and treated with freshly prepared “click” chemistry cocktail containing biotin-alk as described above. The samples were washed with PBS (3 × 0.25 mL), then blocked for 1 h at RT with PBS + 5% BSA (0.5 mL). The parasite samples were then treated with streptavidin-AlexaFluor594 (Jackson Labs; 1:1000 in PBS) for 30 min at RT, then washed with PBS (3 ×

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0.25 mL). In some cases streptavidin-AlexaFluor488 (Jackson Labs; 1:1000 in PBS) was used, and the resulting images were false-colored red for consistency. The cover slips were mounted on glass slides with Vectashield mounting media (Vector Laboratories). All samples were prepared in triplicate. Images were acquired on a Nikon Eclipse Ti inverted microscope with NIS-Elements Microscope imaging Software and analyzed with ImageJ.

In some cases, parasites incubated with unnatural sugars in Endo buffer were used to infect HFF cells. HFF cells were grown on glass coverslips submerged in 0.5 mL DMEM media supplemented with 10% FBS (vol/vol), penicillin (100 U/mL), and streptomycin (100 μg/mL). The cells were infected at a multiplicity of infection (MOI) of 5–15 in duplicate, then processed and imaged as above.

2.8. Deglycosylation experiments

2.8.a. PNGaseF treatment— *Toxoplasma* lysates (40 μg) were diluted with lysis buffer (to total 10 μL), 10% NP-40 (1.3 μL), and reaction buffer (New England Biolabs deglycosylation kit, 1.3 μL). PNGaseF (New England Biolabs 200 U, 0.4 μL) was added to each sample, and the reactions were incubated at 37 °C for 8 h. Proteins were then labeled with biotin-alk and analyzed via immunoblot as above.

2.8.b. Hexosaminidase-f treatment— *Toxoplasma* lysates (50 μg) were diluted with lysis buffer (to total 54 μL), G2 reaction buffer (New England Biolabs, 6 μL). Hexosaminidase-*f* (New England Biolabs 5 U, 1.0 μL) was added to each sample, and the reactions were incubated at 37 °C for 16 h. Proteins were then labeled with biotin-alk and analyzed via immunoblot as above. As a control, 45 μL of 0.1 mg/mL 4-nitrophenyl-*N*-acetyl-β-D-glucosaminide (Sigma) was incubated with 5 μL of G2 reaction buffer and 1 μL of hexosaminidase *f* (active or heat-killed) or 1 μL of water for 2 h at RT. Released 4-nitrophenol was measured via absorbance at 400 nm.

2.8.c. Tunicamycin treatment— HFF cells were cultured in T-175 flasks for 12 d, then treated with 5 μg/mL tunicamycin for 48 h. The cells were then infected with *Toxoplasma* tachyzoites. After 24 h growth in tunicamycin-treated host cells, the parasites were isolated as above. Extracellular parasites were then incubated with unnatural sugars in the presence of tunicamycin for an additional 8 h prior to covalent labeling and immunoblot analysis.

2.8.d. OGA treatment— *Toxoplasma* lysates (40 μg) were diluted with lysis buffer (to total 20 μL) and treated with OGA enzyme as in [42] at 37 °C for 24 h. Proteins were then labeled with biotin-alk and analyzed via immunoblot as above.

2.8.e. β-Elimination— *Toxoplasma* lysates (40 μg) were diluted with lysis buffer (to total 20 μL) and then labeled with biotin-alk. After the addition of 5 μL of a β-elimination reagent mixture (Sigma), the reaction was incubated at 4 °C for 8–24 h. Proteins were then analyzed via immunoblot as above.

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2.9. Enrichment of proteins for MudPIT analysis

Toxoplasma lysate (6 mg total protein in 1 mL of lysis buffer) was treated with 110 μL of 10 mM phosphine-biotin in DMSO (working concentration = 1 mM). The reaction mixture was incubated under an Ar atmosphere for 4 h at 37 °C. Next, 1 mL of ice-cold methanol for each 250 μL of reaction mixture was added, and the samples were incubated at −80 °C for 2 h to precipitate proteins. The precipitates were pelleted at 13,000×*g* (4 °C, 10 min). The supernatants were discarded, and the samples were re-suspended in ice-cold methanol and precipitated at −80 °C two more times. Precipitates were dissolved in 1% SDS in PBS, and incubated with avidin agarose beads at RT overnight. The beads were washed with 2 column volumes of 1% SDS in PBS (pH 7.4), followed by 2 column volumes of 6 M urea in PBS (pH 7.4), then 2 column volumes of 4 M NaCl in PBS (pH 7.4), and 2 column volumes of 100 mM NH_4HCO_3 (pH 7.4). The bound species were eluted by boiling the beads in SDS-PAGE loading buffer at 95 °C for 10 min, and then loaded onto Tris-glycine SDS-PAGE gels. The gels were stained with Coomasssie blue and submitted to the UCI Mass Spectrometry Facility for MudPIT analysis. Following in-gel digestion with porcine trypsin, extracted peptides were separated on a C18 column and analyzed by MSE on a SYNAPT G2 instrument with a triziac source (Waters). Data were analyzed using ProteinLynx Global Server software (PLGS 3.0) with the *Toxoplasma* UniProt database. A spreadsheet of *Toxoplasma* proteins identified by mass spectrometry using the ToxoDB/uniprot database was further organized using BLASTP and NCBI data to categorize hits into subgroups (Chaperones/stress, Cytoskeleton, Enzymes, Membrane Compartments and Trafficking, Secretory Pathway and Other Cellular Processes). A set of uncharacterized protein hits was also noted and annotated with any available information on protein motifs. Similar data were obtained from two replicate experiments (labeled 1 and 2 in Table 1).

3. Results

3.1. Extracellular *Toxoplasma* tachyzoites metabolize and incorporate unnatural sugars

We utilized the bioorthogonal chemical reporter strategy to target glycoconjugates in living *Toxoplasma* tachyzoites. In this technique, peracetylated azido or alkynyl sugars are metabolized by cells and incorporated into glycan structures (Figure 1A). The acetyl groups on each unnatural sugar facilitate cellular uptake, and the free sugars are liberated by esterase activity or hydrolysis. Following installation into proteins or other targets, the unique azido and alkynyl groups can be covalently reacted with probes for visualization or purification [30]. Prior to this report, the chemical reporter strategy had not been used to observe glycosylation or other metabolic processes in *Toxoplasma* tachyzoites. Labeling parasites within a host cell presented a challenge, as the unnatural sugars had to traverse both the host cell and parasite plasma membranes in order to reach the tachyzoite cytoplasm. Indeed, we were unable to robustly detect the unnatural sugar labels in intracellular tachyzoites even at high doses of sugar. This was likely due to the action of host cell esterases and competition with the host cell metabolic machinery, preventing monosaccharide delivery into the tachyzoite cytoplasm. Since *Toxoplasma* tachyzoites are most metabolically active when they are intracellular, we labeled extracellular *Toxoplasma* tachyzoites in established ionic conditions that mimic intracellular cues (Intracellular Endo Buffer) [34].

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Azido and alkynyl variants of *N*-acetylglucosamine (Ac_4GlcNAz and Ac_4GlcNAlk, Figure 1A) have been used to target O-linked and O-GlcNAcylated proteins in mammalian cells and bacteria, and have the potential to target N-linked structures as well [43–46]. When these reagents were administered to extracellular *Toxoplasma* tachyzoites, they labeled similar subsets of proteins, suggesting that the distinct chemical reporters do not influence metabolism of the monosaccharide (Figures 1B, S1–3). Importantly, tachyzoites incorporate Ac_4GlcNAz and Ac_4GlcNAlk in a concentration and time dependent manner (Figures 1B–C, S1–6). Previous studies have demonstrated that the *N*-acyl chain of GlcNAc and related analoggs can be cleaved in cells and subsequently used for protein acetylation [47, 48]. To investigate whether the *N*-acyl units of Ac_4GlcNAz or Ac_4GlcNAlk were removed and appended to *Toxoplasma* proteins, we synthesized *N*-azido and *N*-alkynyl acetyl units and examined their incorporation (Figure 1D, S7). Since the labeling patterns were distinct across multiple experiments, we believe that many proteins targeted with Ac_4GlcNAz or Ac_4GlcNAlk are not simply acetylated. Metabolic incorporation was also visualized using fluorescence microscopy. Ac_4GlcNAz-labeled parasites were reacted with biotinylated probes and subsequently stained with streptavidin-conjugated Alexa488 (Figure 2A). Signal was specific to Ac_4GlcNAz-treated parasites (likely revealing glycoproteins or glycolipids) and appeared most concentrated in the region around the nucleus, perhaps reflecting ER localization. Importantly, Ac_4GlcNAz-treated samples were still able to infect host cells, suggesting that metabolic labeling was tolerated by the parasites (Figures 2B).

3.2 Some proteins appear to be modified by O-linked sugars

To elucidate the type of labeled structures, we first investigated the common carbohydrate-cleaving enzyme *N*-glycosidase F (PNGaseF). PNGaseF treatment did not eliminate Ac_4GlcNAz-dependent signal in our immunoblot analyses, suggesting that the majority of the label is not present in N-linked glycans or that the enzyme may not recognize features of carbohydrate modification in *Toxoplasma* (Figures 3A, S8–9). We also examined the effect of tunicamycin treatment on Ac_4GlcNAz-labeled parasites. Tunicamycin inhibits *N*-linked glycosylation in eukaryotes. However, cells must be treated for 24–72 h with the drug to observe any effect, and extracellular parasites are not viable for that length of time. We chose to treat host cells with tunicamycin prior to infection with tachyzoites. Parasites were allowed to replicate in treated cells for 24 h, and then were released and subjected to further tunicamycin treatment (extracellularly) in the presence of the unnatural sugars. Minimal reduction in Ac_4GlcNAz-dependent signal was observed in this experiment, indicating that most of the labeled proteins are not susceptible to the effects of tunicamycin, or that insufficient drug entered the parasites (Figures 3B, S10).

Ac_4GlcNAz can target a second major class of glycans in eukaryotes: O-linked glycans. Boothroyd and coworkers recently reported a *Toxoplasma* line that is deficient in certain mucin-type O-linked glycans. This strain is null for a nucleotide sugar transporter (NST1) relevant to complex glycan biosynthesis (Figures 3C, S11). When these parasites were treated with Ac_4GlcNAz, though, no reduction in labeling was observed compared to the complemented strain. While it is not possible to rule out the incorporation of azido sugar into complex mucins, our data suggest that the majority of Ac_4GlcNAz likely targets proteins with simpler O-linked structures, β-O-GlcNAc residues, or other post-translational

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modifications. Attempts to verify the fate of Ac_4GlcNAz by treating lystates with *O*-GlcNAcase (OGA), an enzyme capable of removing single β-O-GlcNAc residues found on numerous intracellular proteins, were not successful (data not shown). Additionally, no diminishment in signal was observed with a related recombinant lysosomal hexosaminidase (Figures 3D, S12). Beta-elimination conditions reduced Ac_4GlcNAz-dependent signal in the parasites, consistent with at least some of the label being localized to O-linked structures (Figures 3E, S13).

3.3. Global profiling reveals both predicted and novel candidate glycosylated proteins

We purified a set of proteins that incorporated the Ac_4GlcNAz label using biotinylated reactants and streptavidin beads. The captured and eluted proteins were analyzed via mass spectrometry (MS) and the resulting 89 candidate proteins are listed in Table 1. Importantly, the hits included orthologs of proteins that are known to be O-GlcNAcylated in other species (HSP60, enolase, GAPDH) [49, 50] as well as proteins that were identified in two lectin MS surveys of tachyzoite proteins (myosin A, GAP50) [16, 17]. Some proteins are previously characterized components that are specific to *Toxoplasma* (SAG1, SAG2) [51, 52] while others are novel hypothetical proteins identified in the *Toxoplasma* genome. This dataset included orthologs of proteins that are markers of the ER (protein disulfide isomerase, a reticulon domain containing protein and the SERCA calcium ATPase) as well as components that mediate membrane trafficking (BET1, Sec63 and the dynamin family member Gbp1p). Parasite-specific effectors known to be secreted from the rhoptries, micronemes or dense granules (AMA1, NTPase1, NTPase2, ROP5, and ROP7) were also identified. We anticipate that these secreted effectors and membrane compartment markers are likely modified by complex glycan structures. It should also be noted that our MS survey identified known acetylated proteins and processing enzymes. These data suggest that while Ac_4GlcNAz is capable of targeting glycan biosynthetic pathways, the probe can be diverted to other metabolic pathways and thus report on other post-translational modifications in *Toxoplasma*. Simlar results have been observed in cultured human cells [45, 48, 53].

3.4. SAG1 is modified with the metabolic probe

One of the most well-characterized proteins identified by our survey is surface antigen 1 (SAG1). Immunoprecipitation of SAG1 from Ac_4GlcNAz-labeled cells verified that it was labeled with the unnatural reporter (Figure 4A). However, we were unable to confirm whether the azido group was present in an O-glycosidic linkage (Figure 4B). SAG1 is a GPI-linked protein [54]. Therefore, the unnatural probe could potentially be associated with the GPI modification or may reflect an N-linked (at N178 and/or N241) site or alternative modification. We also attempted to determine the amino acid site of modification. However, we were unable to detect the altered peptide fragment with MS, most likely because the bulk of SAG1 was synthesized prior to incorporation of the unnatural sugar and the signal from the minor, azide-modified fraction was too low.

4. Discussion

The details of protein glycosylation in *Toxoplasma* are less well understood than the process of carbohydrate modification in vertebrate cells, but important features are beginning to

emerge from studies that have used a variety of reagents to demonstrate carbohydrate incorporation into modified proteins. Our understanding of glycan biology in *Toxoplasma* or other apicomplexan parasites is complicated by the need to dissect contributions of the host cell away from parasite-specific processes. This is critically important, as previous results suggest that the specific nature of carbohydrate modification of parasite proteins is influenced by host cell type [55]. However, the *Toxoplasma* genome has between 14 and 18 annotated glycosyltransferases [56] and the results described here demonstrate that parasites can metabolize and incorporate unnatural sugars in the absence of host cells. Previous studies indicate that the predominant glycans in *Toxoplasma* are oligomannosidic ($Man_{5-8}(GlcNAc)_2$) and paucimannosidic ($Man_{3-4}(GlcNAc)_2$) sugars, which are rarely present on mature vertebrate glycoproteins [17]. This is consistent with the observation that the *Toxoplasma* genome lacks annotated glycosidases, suggesting that transferred sugars are not further trimmed.

Unnatural sugars are a promising toolset to begin to elucidate parasite glycosylation machinery. Using these probes, we identified candidate glycoproteins in *Toxoplasma* tachyzoites complementary to other reports using using lectin affinity chromatography and MS. The first study profiled ConA-purified components while the second survey used ConA, WGA and Jacalin for affinity chromatography. There is a significant overlap of our results with both lectin datasets. In several instances, all three surveys identify the same specific proteins (GAP50, Myosin A, TgAMA1, ROP7, β-tubulin and actin). Our identification of an overlapping protein dataset reinforces the evidence that these components are likely modified by glycosylation. Moreover, we also identified proteins that were not revealed in the previous surveys. This is not surprising, as lectins bind to subsets of glycan structures and may not identify all glycosylated proteins in *Toxoplasma*. One limitation of our strategy is that the unnatural sugars were only reproducibly incorporated into proteins when we labeled extracellular parasites. This is likely due to the action of esterases in the host cell cytoplasm which may prevent the sugar probes from accessing the parasite cytoplasm. While we were able to identify a number of secreted and surface proteins, these may be less abundantly synthesized in non-replicating parasites. A second limitation is that unnatural sugars can be diverted to other metabolic pathways in *Toxoplasma*, complicating the assignments of all metabolic end products. Deconvoluting the fates of the unnatural sugars, and the potential crosstalk between metabolic pathways in *Toxoplasma*, will benefit from additional studies with more refined chemical probes. These reagents, in combination with genetic tools, promise to bolster studies of parasite glycosylation and other post-translational modifications, in additon to the roles of glycoconjugates in mediating essential parasitic processes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We are grateful to Melissa Lodoen (UCI), Peter Bradley (UCLA) and John Boothroyd (Stanford) for useful discussions on this work. Research presented in this paper was supported by funds from the UCI School of Physical

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Sciences to JAP. We also thank members of the Morrissette, Lodoen, and Prescher labs for manuscript edits and helpful discussions.

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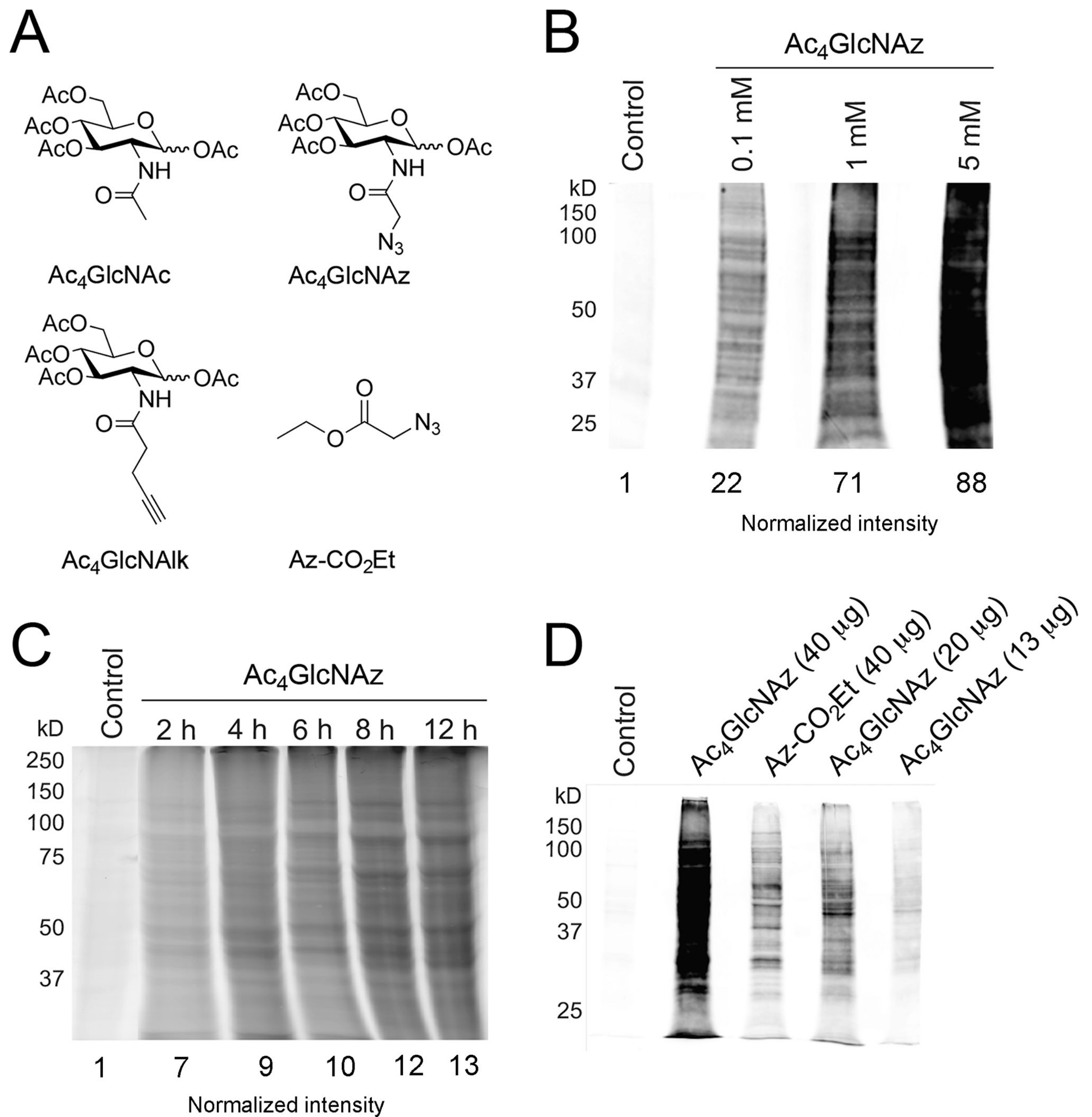

Figure 1. *Toxoplasma* tachyzoites metabolize unnatural sugars

(A) Structures of peracetylated N-acetylglucosamine (Ac_4GlcNAc) and the corresponding alkynyl (Ac_4GlcNAlk) and azido (Ac_4GlcNAz) analogs used in metabolic labeling studies. The acetyl groups facilitate sugar uptake into cells and are removed by the action of non-specific esterases in the cytoplasm. An azide-functionalized acyl chain (Az-CO_2Et) used in various control experiments is also pictured. The azide and alkyne functional groups can be detected using bioorthogonal "click" chemistries. (B) *Toxoplasma* tachyzoites incorporate Ac_4GlcNAz in a dose-dependent manner. Parasites were incubated with unnatural sugar

(0.1–5 mM) or the control sugar $Ac_4GlcNAc$ for 8 hours, lysed, and reacted with a biotin-alk tag via "click" chemistry. The labeled proteins were separated via gel electrophoresis and detected via immunoblot with streptavidin. Equivalent protein loading was confirmed via staining with Ponceau S (Figure S1) and normalized intensities are listed. (C) *Toxoplasma* tachyzoites incorporate $Ac_4GlcNAz$ in a time-dependent manner. Parasites were incubated with $Ac_4GlcNAz$ (1 mM) or the control sugar $Ac_4GlcNAc$ (1 mM) for 2–12 h hours, lysed, and reacted with a rhodamine-alkyne probe. Labeled proteins were separated via gel electrophoresis and analyzed via in-gel fluorescence. Equivalent protein loading was confirmed via staining with Ponceau S (Figure S4) and normalized intensities are listed. (D) $Ac_4GlcNAz$ treatment reveals a unique glycoprotein fingerprint. Parasites were incubated with $Ac_4GlcNAz$ (1 mM), $Ac_4GlcNAc$ (1 h) or a non-sugar probe (Az-CO_2Et) for 8 hours, lysed, and reacted with a biotin-alk tag. Labeled proteins were analyzed via immunoblot as in (C). Equivalent protein loading was confirmed via staining with Ponceau S (Figure S7).

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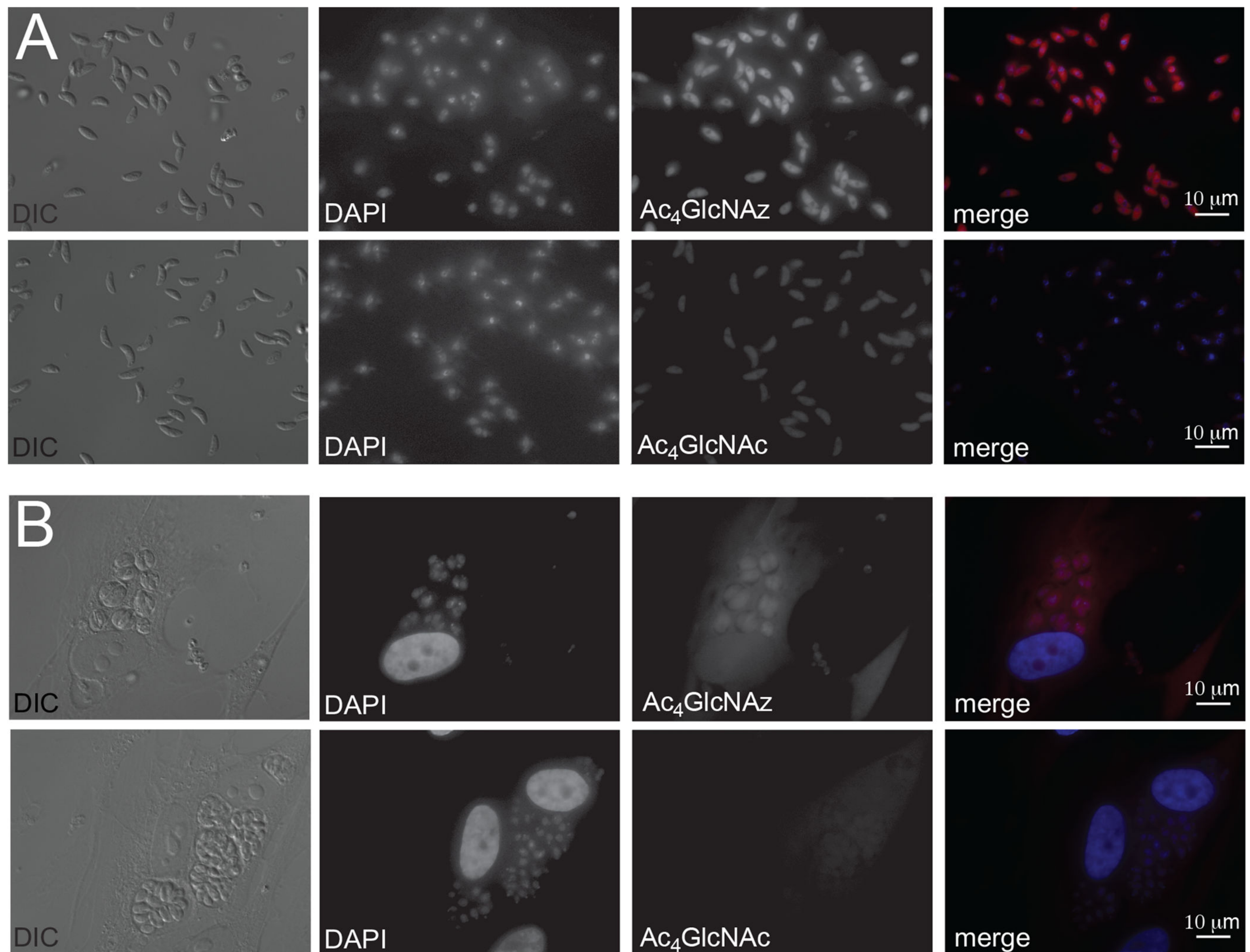

Figure 2. Chemical reporters can be visualized in *Toxoplasma* tachyzoites via fluorescence microscopy

(A) Parasites were incubated with $Ac_4GlcNAz$ (0.5 mM) or $Ac_4GlcNAc$ (0.5 mM) for 8 h, prior to fixation with 4% paraformaldehyde in PBS for 15 min at rt. Subsequent reaction with biotin-alk and incubation with streptavidin-AlexaFluor594 enabled fluorescence detection of modified bioconjugates. Exposure times were established with $Ac_4GlcNAz$ samples and kept constant for the control samples. (B) $Ac_4GlcNAz$-treated parasites remain viable. Parasites were incubated with $Ac_4GlcNAz$ (250 μM) or $Ac_4GlcNAz$ (250 μM) for 8 h and then added to HFF monolayers. After 36 h, the samples were fixed, labeled, and imaged as in (A).

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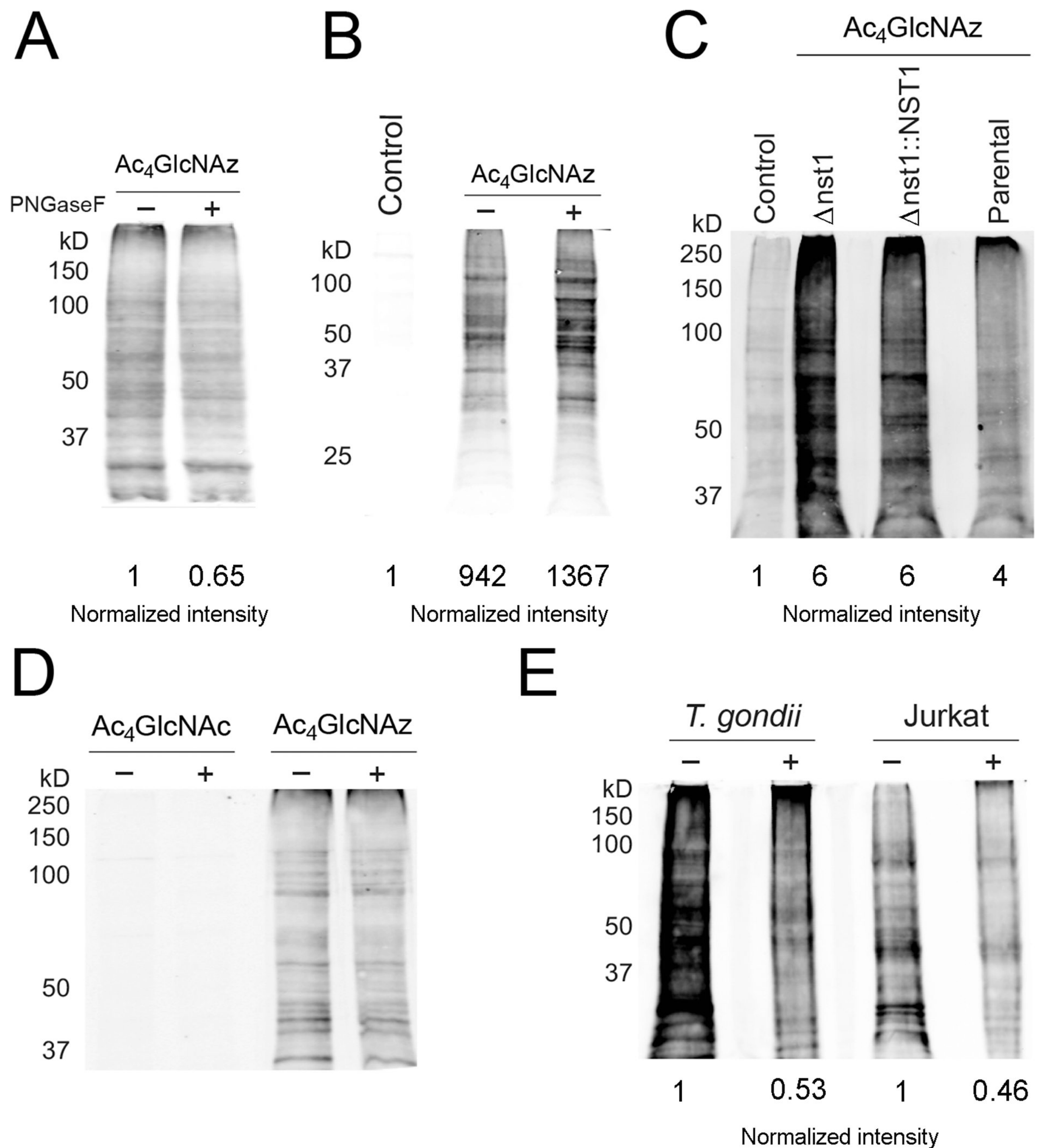

Figure 3. Ac_4GlcNAz appears to target some O-linked glycans in *Toxoplasma* tachyzoites

(A) Parasites were incubated with Ac_4GlcNAz (1 mM) or Ac_4GlcNAc (1 mM), then lysed and treated with PNGase F. All samples were labeled with biotin-alk and analyzed via immunoblot as in Figure 1B. (B) Parasites were grown in tunicamycin-treated HFFs for 24 h, then harvested and incubated in Endo Buffer with Ac_4GlcNAz (1 mM) or Ac_4GlcNAc (1 mM) and additional tunicamycin. After 8 h, the parasites were lysed and protein samples were reacted and analyzed via immunoblot as in Figure 1B. (C) Parasites deficient in a UDP-GlcNAc nucleotide sugar transporter (nst1) were treated with Ac_4GlcNAz (1 mM) or

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Ac_4GlcNAc (1 mM) for 8 h, then lysed and analyzed as in Figure 1B. The complemented strain (nst1::NST1) and parental strain (Me49) were similarly processed and analyzed. (D) Parasites were incubated with Ac_4GlcNAz (1 mM) or Ac_4GlcNAc (1 mM), then lysed and treated with hexosaminidase-f. All samples were labeled with biotin-alk and analyzed via immunoblot as in Figure 1B. (E) Parasites were treated with Ac_4GlcNAz (1 mM) or Ac_4GlcNAc (1 mM) for 8 h, The samples were then lysed, and proteins were subjected to elimination, labeled with biotin-alk and analyzed as in Figure 1B. As a control, Jurkat cells were labeled with Ac_4GlcNAz (0.1 mM) for 48 h, then lysed and subjected to the same conditions as parasite cell lysate.

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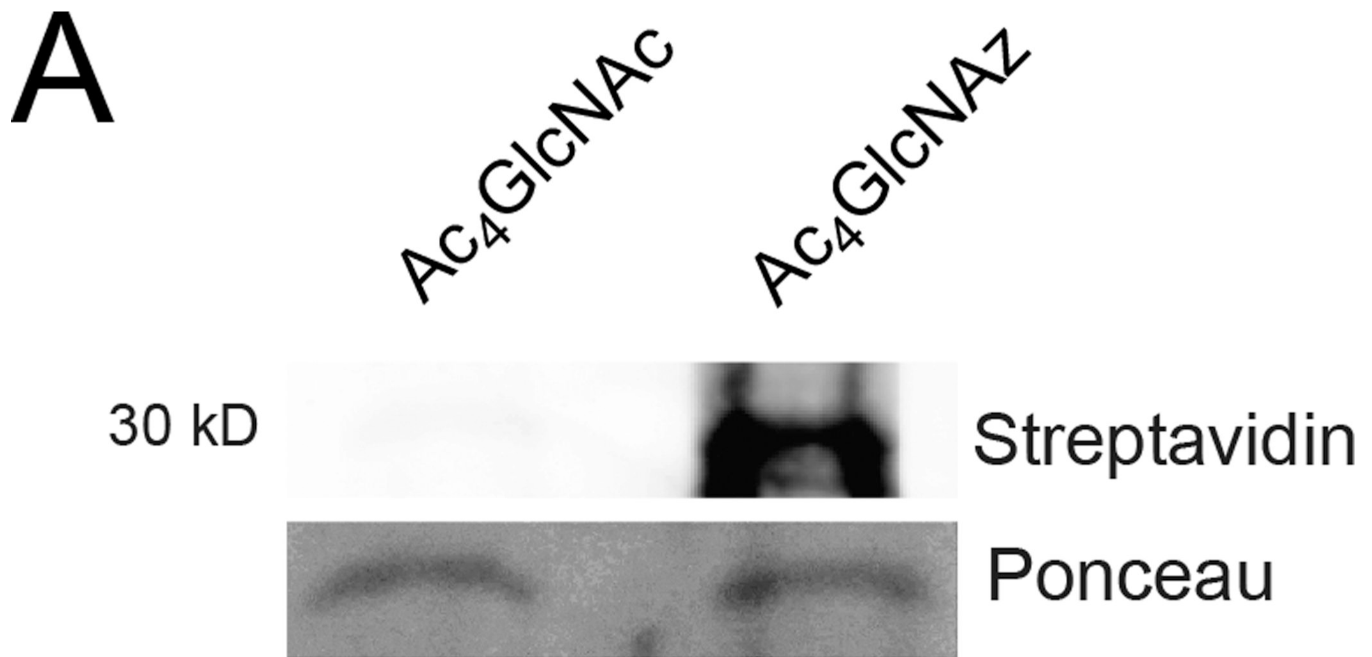

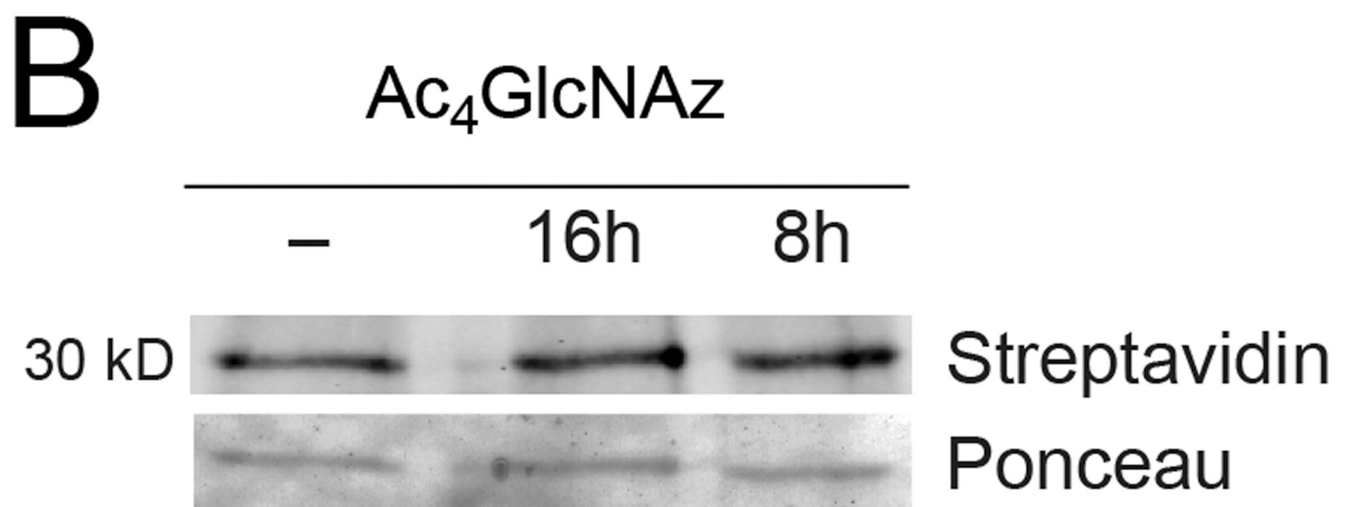

Figure 4. Azido label is detected in TgSAG1

(A) Parasites were labeld with Ac_4GlcNAz (1 mM) or Ac_4GlcNAc (1 mM, control) for 8 h. The samples were lysed and treated with biotin-alk as above, then incubated with anti-gp30/SAG1 (DG52) antibody overnight at 4°C. The protein-antibody complex was isolated using Pierce Protein A/G Magnetic Beads and analyzed via immunoblot. (B) Parasites were labeled with Ac_4GlcNAz (1 mM) or Ac_4GlcNAc (1 mM, control) for 8 h. The samples were then lysed, and proteins were subjected to beta-elimination, labeled with biotin-alk as above, then incubated with anti-gp30/SAG1 (DG52) antibody overnight at 4° C. The protein-

antibody complex was isolated using Pierce Protein A/G Magnetic Beads and analyzed via immunoblot.

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Table 1

Glycosylated proteins identified by mass spectroscopy

Protein Name	Uniprot Number	Predicted Function	MS Expt	Other Evidence	Lectin MS Surveys
Chaperones/stress (11)					
CDC48 (ATPase, proteasome)	B9PFU8	chaperone	2		
Heat shock protein 90	Q2Y2Q8	stress response	1,2	yes	yes
Heat shock protein 90	F0VBM9	stress response	2	yes	
Heat shock protein 90	F0VEH8	stress response	2	yes	
Heat shock protein 70	Q9U540	stress response	1,2	yes	yes
Heat shock protein 70	Q9UAE9	stress response	1	yes	
Heat shock protein 70	O76274	stress response	2	yes	
Heat shock protein 70	B6KHU4	stress response	2	yes	
Heat shock protein 60	F0VQU9	stress response	1	yes	yes
Heat shock protein 20	B9PGE9	stress response	2		
TCP-1 cpn60 family chaperonin	B6KFE8	chaperone	2		
Cytoskeleton (8)					
Actin	P53476	cytoskeleton	1,2		yes
α-tubulin1	B9PJD4	cytoskeleton	2	yes	
β-tubulin1	F0V8J8	cytoskeleton	1,2		
GAP50 (Acid phosphatase)	Q6PQ42	myosin complex	1,2	yes	yes
IMC3	Q6GYB1	cytoskeleton	2		
Myosin A	B9PW84	myosin complex	1,2	yes	yes
Myosin light chain 1	Q95UJ7	myosin complex	2		
Myosin D	MYOD	myosin complex	2		
Enzymes (32)					
Acid phosphatase	B9PQM6	phosphatase	1	yes	
Aconitate hydratase (aconitase)	B9PMS3	TCA	2		
ADP-ATP carrier (translocase)	Q9BJ36	mitochondria	1,2		

Protein Name	Uniprot Number	Predicted Function	MS Expt	Other Evidence	Lectin MS Surveys
Asparaginyl tRNA synthetase	B6KCZ4	tRNA synthetase	2		
ATP dependent DNA helicase II	B9PQX8	helicase	2		yes
ATP synthase α-subunit	B9PUY4	ATP synthesis	1,2	yes	
ATP synthase β-subunit	Q309Z7	ATP synthesis	1,2	yes	
Ca^{2+}-dependent protein kinase 1	Q3HNM6	Ca^{2+} signaling	2	yes	
Citrate synthase	B6KCK9	mitochondria	2		
Cytochrome P450	B6K9N1	mitochondria	2		
Cytosol aminopeptidase	B9PTG8	protein turnover	2	yes	
Enolase	B9PH46	glycolysis	2	yes	
Fructose 1,6-bisphosphatase	Q8MY84	gluconeogenesis	1		
Fumarase	B9Q1R2	aa metabolism	2		
Isocitrate dehydrogenase 2	B9PW21	ox decarboxylation	2		
Long chain fatty acid CoA ligase	B9PJN2	FA breakdown	2	yes	
Long chain fatty acid CoA ligase	B9PJM7	FA breakdown	2	yes	yes
Malate quinone oxidoreductase	B6KKB7	pyruvate metabol	2		
Branch ch α-keto acid dehydrogenase	Q1KSF2	mitochondria	2		
Mitochondrial processing peptidase					
α-subunit	B9PUJ6	mitochondria	1,2		
β-subunit	B9PW21	mitochondria	2		
Peroxiredoxin 3	Q86GL5	antioxidant	2		
Phosphate carrier protein (TMD)	B9PRN1	mitochondria	2		
Prolyl endopeptidase	B9QH13	endopeptidase	2	yes	
Phosphofructokinase	B9Q857	glycolysis	2	yes	
Pyridine nucleotide diS oxidoreductase	B9PXF3	oxidoreductase	2		
Pyruvate carboxylase	B9PSZ5	mitochondrial	2	yes	
Pyruvate kinase	B6KVA2	glycolysis	2	yes	
Succinate CoA synthetase α-subunit	B9PTH6	mitochondrial	1,2		
Succinate CoA synthetase β-subunit	Q1KSE5	mitochondrial	1,2		
Succinate dehydrogenase	B9PZU5	mitochondrial	2		
Tryptophanyl tRNA synthetase	B6KKA3	tRNA synthetase	2		

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Protein Name	Uniprot Number	Predicted Function	MS Expt	Other Evidence	Lectin MS Surveys
Other Cellular Processes (13)					
Histone H2A	F0VGI1	histone	1	yes	
AP2 transcription factor (AP2X-9)	B9PZQ1	transcription factor	1	yes	
Calmodulin	B9PZ33	Ca^{2+} signaling	2		
Elongation Factor-1α	B6KN45	transcription	2	yes	yes
Elongation Factor-2	F0VEU2	transcription	2	yes	
Elongation factor Tu	B6KC06	transcription	2	yes	
Nucleosome assembly protein	B6KAS9	nucleosomes	1		
Prohibitin	B9PP22	transcription	2	yes	
Prohibitin	B9PGD2	transcription	2	yes	
Ribosomal S3Ae family	F0VIB8	translation	2		
Ribosomal: 60s ribosomal protein	F0VBQ9	translation	1		yes
Thioredoxin	B9PM19	redox signaling	2		
Ubiquitin	F0VPK9	ubiquitination	1,2		yes
Membrane Compartments & Trafficking (10)					
α-importin (nuclear transport)	B9QIQ9	transport	2		
Gbp1p protein	B9PLQ7	dynamin superfam	2		
Protein disulfide isomerase	Q9BLM8	ER, diS bonds	1,2	yes	yes
Rab23 (nuclear transport)	B9PSV9	transport	2		
Ranbp1 domain containing protein	F0V739	transport	2		
Reticulon domain containing protein	B9PMP2	ER curvature	2		
SERCA Calcium ATPase	Q5IH90	Ca^{2+} pump	2	yes	
Signal recognition particle	B9Q211	rough ER	2		
Vacuolar ATP synthase β-subunit	B9PQR4	H^{+} pump	2	yes	
Vacuolar ATP synthase subunit E	F0VAR6	H^{+} pump	2	yes	
Secretory Pathway (11)					
Apical membrane antigen (AMA1)	B9Q2L9	secreted, MN	2		yes

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Protein Name	Uniprot Number	Predicted Function	MS Expt	Other Evidence	Lectin MS Surveys
Mitochondrial association factor	B9Q3S3	secreted, DG	2		yes
Nucleoside triphosphatase 1 (DG)	Q27893	secreted, DG	2		
Nucleoside triphosphatase 2 (DG)	Q27895	secreted, DG	1		
Rhoptry protein ROP5B (rhoptries)	F2YGS5	secreted, rhoptries	2		yes
Rhoptry protein ROP5C (rhoptries)	F2YGS4	secreted, rhoptries	1,2		yes
Rhoptry protein ROP7 (rhoptries)	B6KR07	secreted, rhoptries	2		yes
Rhoptry protein ROP13 (rhoptries)	B9PK73	secreted, rhoptries	2		yes
Rhoptry protein ROP44 (rhoptries)	B6KPH7	secreted, rhoptries	1		
Surface antigen-1 (SAG1, P30)	C7E5U4	secreted, surface	1,2	yes	yes
Surface antigen-2 (SAG2)	Q27004	secreted, surface	2		yes
Uncharacterized (4)					
M protein repeat containing protein	B9PQT9	No predicted TMD	2	N/A	
Uncharacterized protein	B6K8Z6	*Borrelia* motif, TMD	1,2	N/A	
Uncharacterized protein	B9Q2P2	No predicted TMD	2	N/A	
Uncharacterized protein	F0VB85	No predicted TMD	2	N/A	

"Other evidence" of glycosylation summarized in [46].