The Dual Origin of *Toxoplasma gondii N*-Glycans[†]

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ABSTRACT: N-Linked glycosylation is the most frequent modification of secreted proteins in eukaryotic cells that plays a crucial role in protein folding and trafficking. Mature *N*-glycans are sequentially processed in the endoplasmic reticulum and Golgi apparatus through a pathway highly conserved in most eukaryotic organisms. Here, we demonstrate that the obligate intracellular protozoan parasite *Toxoplasma gondii* independently transfers endogenous truncated as well as host-derived *N*-glycans onto its own proteins. Therefore, we propose that the apicomplexan parasite scavenges N-glycosylation intermediates from the host cells to compensate for the rapid evolution of its biosynthetic pathway, which is primarily devoted to modification of proteins with glycosylphosphatidylinositols rather than *N*-glycans.

In eukaryotes, asparagine-linked glycosylation (ALG) is initiated in the endoplasmic reticulum (ER) membrane by the synthesis of lipid-linked oligosaccharides, the dolichol-pyrophosphate-oligosaccharide (DolPPOS)[1] (Figure 1A) (*1*−*5*). The precursor oligosaccharide $Glc_3Man_9GlcNAc_2$-PP-Dol is assembled in a stepwise manner by the sequential actions of specific glycosyltransferases (ALG1−ALG14) and glycosidases. The five first mannose residues are transferred from GDP-Man to the chitobiose core, leading to the original structure $Man_5GlcNAc_2$. After the oligosaccharide is flipped to the luminal side of the ER (*6, 7*), four other mannose residues are attached, provided by Dol-P-Man and three glucose residues from Dol-P-Glc. The complete oligosaccharide moiety ($Glc_3Man_9GlcNAc_2$) is then transferred to selected asparagines of the nascent proteins on the consensus motif Asn-X-Ser/Thr (Figure 1A). This central reaction is catalyzed by the hetero-oligomeric protein oligosaccharyltransferase (OST) complex (*8*), which contains a catalytic subunit, STT3 (*9, 10*).

Whereas many reports are available concerning N-glycosylation in free-living organisms, information regarding obligate intracellular parasites is still fragmentary. The data concerning ALG genes in several eukaryotic organisms suggest that database mining may allow the prediction of DolPPOS structures synthesized by any organism (*11*). This bioinformatic approach has been verified in several unicellular organisms such as *Trypanosoma cruzii*, *Tetrahymena thermophilia*, and *Leishmania major*. Interestingly, the capacity of classical O-linked and N-linked glycosylations appears to be greatly reduced in some species exhibiting an obligate intracellular lifestyle; for example, the apicomplexan parasite *Plasmodium falciparum* contains low levels of N-glycosylation capability (*12*−*14*), and two microsporidian species, *Encephalitozoon cuniculi* and *Antonospora locustae*, seem to have lost the ability to synthesize N-linked glycans through the secondary losses of several key enzymes of the N-glycosylation pathway (*15, 16*). Similar to *P. falciparum*, glycosylphosphatidylinositol (GPI) anchors represent the major carbohydrate modification of proteins in *T. gondii* (*17, 18, 14*). *T. gondii* is a coccidian parasite found worldwide that infects a wide range of warm-blooded vertebrates and has emerged as an important opportunistic pathogen for immunocompromised persons. For example, co-infection with HIV often leads to fatal encephalitis (*19*−*21*). *T. gondii* shares common features with other apicomplexan parasites and represents a promising model for the study of the biosynthesis and role of glycans in the apicomplexa. The first direct biochemical evidence of N-glycosylation of the proliferative-stage, tachyzoite, glycoprotein gp23 provided impetus for the study of N-glycosylation in *T. gondii* (*22*). Furthermore, it was recently reported that N-glycosylation is essential for successful infection (*23, 24*). It is noteworthy that the lack of solid structural data represents a major obstacle to deciphering glycoconjugate metabolism using bioinformatic tools. *T. gondii* clearly expresses a functional STT3p (*25*); however, genome analysis indicates that orthologs of ALG3, ALG9, and ALG12 are absent. These correspond to the set of luminal mannosyltransferases that use Dol-P-Man as the sugar donor [Table S1 of the Supporting Information (*11, 23*); http://www.toxodb.org/toxo/home.jsp], indicating a profound modification in the *N*-glycan biosynthetic pathway of the parasite. Thus, on the basis of the presence of a specific subset of orthologous ALG genes, *T.*

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[1] Abbreviations: GPI, glycosylphosphatidylinositol; DolPPOS, dolichol-pyrophosphate-oligosaccharide; ER, endoplasmic reticulum; OST, oligosaccharyltransferase; Man, mannose; Glc, glucose; GlcNAc, *N*-acetylglucosamine; MS, mass spectrometry.

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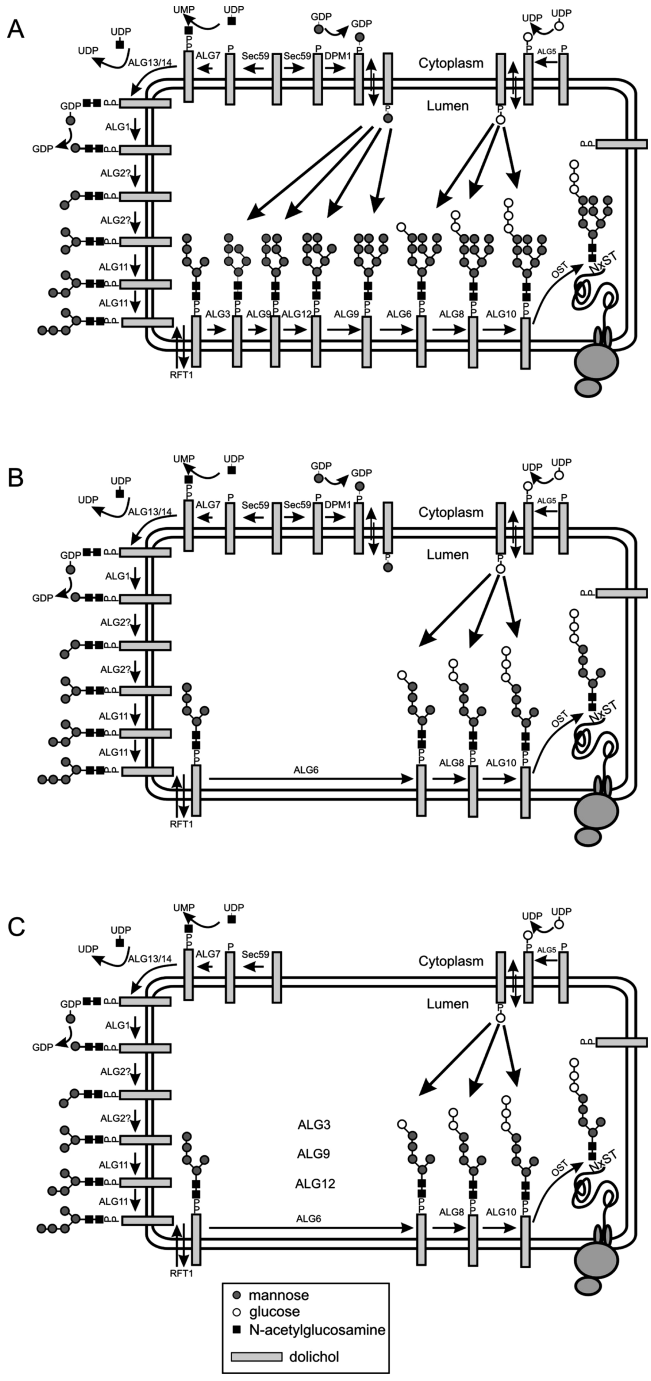

FIGURE 1: Inventory of ALG glycosyltransferase genes and predicted dolichol-linked *N*-glycans in (A) classical mammalian cells, (B) *Toxoplasma gondii*, and (C) the CHO B3F7 mutant cell line.

gondii possesses the enzymatic equipment for the biosynthesis of truncated $Glc_3Man_5GlcNAc_2$-PP-Dol, but not complete $Glc_3Man_9GlcNAc_2$-PP-Dol oligosaccharide precursors (Figure 1B). Luk and colleagues recently suggested that *N*-glycans in *T. gondii* differ from classical oligomannosyl-type structures and could correspond to $Glc_3Man_5GlcNAc_2$ (*23*). However, until now, the exact nature of oligosaccharides transferred onto nascent parasite proteins has been unknown.

MATERIALS AND METHODS

Cell and Parasite Cultures. T. gondii grown in African green monkey kidney cells (Vero cells, ATCC CCL-81), human foreskin fibroblasts (HFF, ATCC CRL-1635), CHO Pro-5, and CHO B3F7 were cultured in DMEM (Gibco BRL), supplemented with 10% 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 grown in CHO B3F7 liberated from their host cells were mixed with homogenized Vero cells, and the mixture was purified using glass-wool columns as described previously.

Strains and Media. The *Saccharomyces cerevisiae* and *Escherichia coli* strains used in this work were *YPH* 499 (Mat a; *ura* 3-52; *lys* 2-801*amber*; *ade* 2-101*ochre*; *trp* 1-63; *his* 3-200; *leu* 2-1) (Stratagene) and *E. coli* strain *XL1-blue* (Stratagene), which was used for subcloning and other standard recombinant DNA procedures. *S. cerevisiae* strains were grown in YPAD medium [1% (w/v) Bacto yeast extract, 2% (w/v) Bactopeptone, 2% (w/v) dextrose, and 4 mg/L adenine] or SD medium (0.17% Bacto yeast nitrogen base, 0.5% ammonium sulfate, and 2% dextrose) containing the nutritional supplements necessary to complement strain auxotrophs or allow selection of transformants. *YPH499-HIS-GAL-ALG7* was maintained on SGR medium (4% galactose, 2% raffinose, 0.17% Bacto yeast nitrogen base, and 0.5% ammonium sulfate) in which dextrose is replaced with galactose and raffinose as a source of carbohydrates. *E. coli* strains were grown in LB medium.

Extraction of Glycoproteins and Preparation of N-Glycans. Collected *T. gondii* (10^9 parasites) or cells were homogenized by ultrasonic treatment at 4 °C. Lipids were extracted by sequential extraction with 20 volumes of a chloroform/methanol mixture (2:1, v/v) and then with a chloroform/methanol/water (40:20:3) solution. Delipidated homogenates were suspended in a solution of 6 M guanidinium chloride and 5 mM ethylenediaminetetraacetic acid (EDTA) in 0.1 M Tris-HCl (pH 8) and agitated for 4 h at 4 °C. Dithiothreitol was then added to a final concentration of 20 mM and incubated for 5 h at 37 °C, followed by the addition of iodoacetamide to a final concentration of 50 mM and further incubation overnight in the dark at room temperature. The reduced and alkylated sample was dialyzed against water at 4 °C for 3 days and lyophilized. The recovered protein samples were then sequentially digested with TPSK-treated trypsin overnight at 37 °C, in 50 mM ammonium bicarbonate buffer (pH 8.4), and with chymotrypsin. Crude peptides and glycopeptides were loaded onto a C18 Sep-Pak cartridge. Glucan polymers and other hydrophilic contaminants were washed off with 5% aqueous acetic acid, and the bound peptides and glycopeptides were eluted with a step gradient of 20, 40, and 60% 1-propanol in water. Eluted fractions were pooled, dried down, and then incubated with *N*-glycosidase F (Roche, Basel, Switzerland) overnight at 37 °C in 50 mM ammonium bicarbonate buffer (pH 8.4). Released *N*-glycans were separated from peptides and glycopeptides using the same C18 Sep-Pak procedure. Pooled propan-1-ol fractions were then digested with *N*-glycosidase

A from almond (0.5 milliunits, Calbiochem) in 50 mM ammonium acetate buffer (pH 5) at 37 °C overnight. The *N*-glycans that were released were likewise separated from the peptides by the same C18 Sep-Pak procedure.

Chemical Derivatization. For MALDI-MS analyses, the glycan samples were permethylated using the NaOH/dimethyl sulfoxide slurry method (*26*). The permethyl derivatives were then extracted in chloroform and repeatedly washed with water.

Coupling Oligosaccharides with 2-Aminopyridine. After hydrolysis, oligosaccharides were reductively aminated with 2-aminopyridine at the reducing end (*27*). Then, to remove excess reagent and purify 2-PA oligosaccharides, two methanol solutions (75 and 85%) were successively added to the reaction mixture and dried under a nitrogen stream. After addition of 1 mL of water, the pH was adjusted to 10 by adding NH_4OH (25%). The aqueous phase was washed 10 times with chloroform to eliminate excess 2-AP. The aqueous phase was then transferred into a clean tube and pH-neutralized with glacial acetic acid before lyophilization. Finally, derivatized oligosaccharides were purified by solid phase extraction (SPE) onto a C18 Sep-Pack column.

Mannosidase Treatment of 2-Aminopyridinylated N-Glycans. 2-PA-coupled PNGase F-released oligosaccharides were digested with α-mannosidase from *Aspergillus saitoi* (GKX5009 Glyko). Each sample was treated with α(1,2)-mannosidase in 100 mM sodium acetate and 2 mM Zn^{2+} (pH 5.0) and incubated overnight at 37 °C. The mixture was finally applied on a C18 Sep-Pak column to purify 2-aminopyridinylated oligosaccharides.

MS Analyses of Glycans. For MALDI time-of-flight (MALDI-TOF) MS glycan profiling, native compounds in water were mixed 1:1 with a 2,5-dihydroxybenzoic acid (DHB) matrix (10 mg/mL in a 50:50 MeOH/water mixture), spotted on the target plate, and dried under vacuum. Data acquisition was performed manually on a Voyager instrument (Applied Biosystems) operated in the reflectron mode. Laser shots were accumulated until a satisfactory signal-to-noise ratio was achieved when combined and smoothed.

Extraction of Lipid-Linked Oligosaccharides. Extractions were performed according to ref *28*. Briefly, cell pellets were extracted with 2 mL of a chloroform/methanol/H_2O solution (3:2:1). After centrifugation, the upper phase and proteins were extracted twice with 1.5 mL of theoretical lower phase (70 mL of MeOH, 5 mL of 4 mM $MgCl_2$, and 430 mL of chloroform). Then proteins were washed four times with 3 mL of theoretical upper phase (240 mL of MeOH, 225 mL of H_2O, 9.4 mL of 100 mM $MgCl_2$, and 15 mL of CH_3Cl_3). After pellets had been partially dried, Dol-PP-OS were extracted with 1.5 mL of a CH_3Cl_3/MeOH/H_2O (10:10:3, v/v) mixture. Dried Dol-PP-OS were released by mild acid treatment (0.1 M HCl in tetrahydrofuran at 50 °C for 2 h). Released oligosaccharides were dried under a stream of nitrogen and purified on a carbograph column (Alltech carbograph SPE column).

RESULTS AND DISCUSSION

The Complementation by Tg-alg7 in the ALG7-Deficient S. cerevisiae Conditional Mutant Strain Suggests the Presence of a Functional Set of ALG Enzymes in T. gondii. The alg7 gene encodes GlcNAc-1-P transferase that initiates biosynthesis of *N*-glycan precursors. Therefore, as an initial step toward an evaluation of genetic regulation of the *T. gondii* N-glycosylation, the alg7 gene homologue from *T. gondii* was identified. Initially, a 338 bp fragment of putative *T. gondii* alg7 was identified by a BLAST search of the EST database (http://www.toxodb.org/toxo/home.jsp) using the sequences of known orthologs from other organisms. This was subsequently used to screen a *T. gondii* Lambda ZAP cDNA library, and a clone containing a 1503 bp open reading frame predicted to encode a 54 kDa type III transmembrane protein was isolated (EMBL Data Bank accession entry AJ436993). The deduced protein sequence is 36.3 and 43% identical to amino acid sequences of the *S. cerevisiae* and *Homo sapiens* homologue proteins, respectively. Furthermore, the putative *T. gondii* alg7 gene was able to complement a conditional lethal yeast mutant (Figure S1 of the Supporting Information), strongly indicating that it encodes a functional GlcNAc-1 transferase. Additionally, in an attempt to inactivate the alg7 locus, we modified the disruption construct TUB5/CAT (*28*) that contains a chloramphenicol acetyltransferase (CAT) expression cassette. The regions flanking the CAT cassette in the 5′ and 3′ positions were replaced with 1.2 and 2.8 kb DNA fragments, respectively, derived from the untranslated 5′ and 3′ sequences of the ALG7 locus. After selection, chloramphenicol-resistant clones were further analyzed by PCR. Every PCR-tested clone was false-positive (data not shown). We thus assumed that disrupting ALG7 may be lethal for the parasite, suggesting that N-glycosylation is essential for *T. gondii*'s life cycle. The fact that *T. gondii* possesses genes encoding at least two key enzymes responsible for the initiation of DolPPOS precursor biosynthesis (ALG7) and the transfer of the final products to nascent proteins (STT3) (*25*) strongly suggests that the parasite exhibits a functional, although possibly truncated, N-glycosylation pathway.

Analysis of DolPPOS from T. gondii Contradicts in Silico Prediction. In a first approach, to evaluate the independent ability of *T. gondii* to synthesize *N*-glycans, we performed experiments on isolated *T. gondii*. In that case, our attempts to analyze DolPPOS by MS were negative, due to very small quantity of isolated material. We also observed that [^{3}H]mannose and methionine incorporation were virtually absent compared with that in normal cells. We then chose to analyze intracellular parasites in parallel to their host cells. The presence of the *de novo* glycosylation pathway in the parasite was confirmed after specific extraction of DolPPOS (*29*), hydrolysis of the oligosaccharide moiety, and profiling by mass spectrometry (MS). We observed differences between the DolPPOS profiles of the parasite and those of its host cells. Whereas the profile of DolPPOS oligosaccharide moieties from control African green monkey kidney epithelial (Vero) cells is characterized by prominent $Man_8GlcNAc_2$, $Man_9GlcNAc_2$, and $Glc_{1-3}Man_9GlcNAc_2$ (Figure 2A and Table 1), *T. gondii* tachyzoïtes exhibit a simpler pattern containing only $Hex_8GlcNAc_2$ and $Hex_9GlcNAc_2$ (Figure 2B and Table 1). Surprisingly, the presence of precursors with more than eight hexoses does not correlate with the gene repertoire of the parasite, whose genome is likely to lack a number of genes required for the biosynthesis of *N*-glycan (Figure 1B).

The N-Glycosylation Profile of T. gondii Is Host Cell-Dependent. To reconcile our apparently contradictory ob-

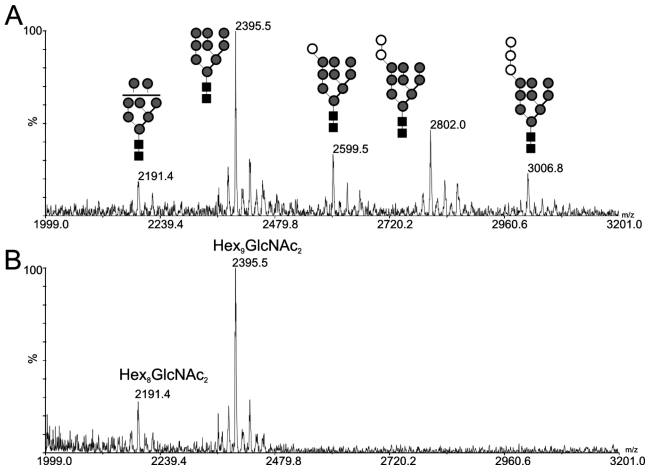

FIGURE 2: MALDI-TOF MS comparison of DolPPOS acid-hydrolyzed released oligosaccharides from (A) Vero cells and (B) *T. gondii* grown in Vero cells.

Table 1: DolPPOS Acid-Hydrolyzed Released Oligosaccharides from Vero Cells and *T. gondii* Grown in Vero Cells and a Summary of Permethylated Compounds Observed by MS in Vero Cells and *T. gondii* Grown in Vero Cells

signal (*m/z*)	composition	Vero	*T. gondii*
1783	$Hex_6GlcNAc_2$	√	
1987	$Hex_7GlcNAc_2$	√	
2191	$Hex_8GlcNAc_2$	√	√
2395	$Hex_9GlcNAc_2$	√	√
2599	$Hex_{10}GlcNAc_2$	√	
2802	$Hex_{11}GlcNAc_2$	√	
3006	$Hex_{12}GlcNAc_2$	√	

servations, we defined the nature of the final *N*-glycan biosynthetic products using a glycomic approach, releasing glycans from the total pool of proteins from *T. gondii* grown in different host cells and analyzing their distribution using mass spectrometry. Control Vero and HFF cells exhibit major oligomannosyl-type structures as well as minor hybrid and complex-type *N*-glycans (Figure 3A, Figure S2 of the Supporting Information, and Table 2). We did not identify sialylated *N*-glycans, although their presence was suggested

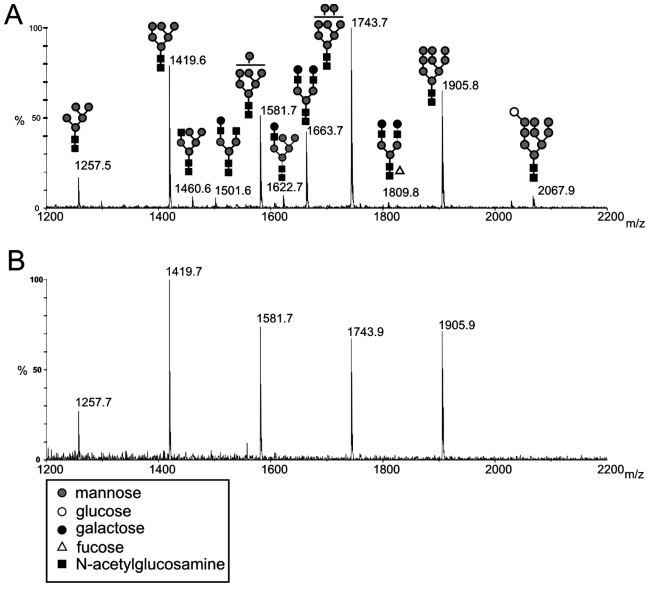

FIGURE 3: MALDI-TOF MS comparison of native PNGase F-released oligosaccharides from (A) Vero cells and (B) *T. gondii* grown in Vero cells.

Table 2: Summary of Native and 2-AP Derivative Compounds Observed by MS in Vero Cells and *T. gondii* Grown in Vero Cells

native (*m/z*)	2-PA (*m/z*)	composition	Vero	*T. gondii*
933	1011	$Man_3GlcNAc_2$, Na^+	√	√
1079	1157	$(Fuc)Man_3GlcNAc_2$, Na^+	√	
1095	1173	$Hex_1Man_3GlcNAc_2$, Na^+	√	√
1257	1335	$Hex_2Man_3GlcNAc_2$, Na^+	√	√
1419	1497	$Hex_3Man_3GlcNAc_2$, Na^+	√	√
1460	1538	$GlcNAcMan_5GlcNAc_2$, Na^+	√	
1501	1579	$GlcNAc_2Man_3GlcNAc_2$, Na^+	√	
1581	1659	$Hex_4Man_3GlcNAc_2$, Na^+	√	√
1622	1700	$GalGlcNAcMan_5GlcNAc_2$, Na^+	√	
1663	1741	$Gal_2GlcNAc_2Man_3GlcNAc_2$, Na^+	√	
1743	1821	$Hex_5Man_3GlcNAc_2$, Na^+	√	√
1809	1887	$FucGal_2GlcNAc_2Man_3GlcNAc_2$, Na^+	√	
1905	1983	$Hex_6Man_3GlcNAc_2$, Na^+	√	√
2012	2090	$FucGal_2GlcNAc_3Man_3GlcNAc_2$, Na^+	√	
2028	2106	$Gal_3GlcNAc_3Man_3GlcNAc_2$, Na^+	√	
2067	2145	$Hex_7Man_3GlcNAc_2$, Na^+	√	√
2174	2252	$FucGal_3GlcNAc_3Man_3GlcNAc_2$, Na^+	√	

by GC analysis, but evidenced several sialylated glycosphingolipids (data not shown). It has been recently established that micronemal proteins from *T. gondii* bind specifically sialylated oligosaccharides (*30*). We assume that those gangliosides likely correspond to membrane compounds recognized by *T. gondii* during invasion. This comes as no surprise since gangliosides are particularly abundant in mammalian cell membranes and play a key role in a variety of essential cellular processes (*31*).

In contrast to host cells, tachyzoïtes grown in Vero cells were found to lack complex-type *N*-glycans (Figure 3B and Table 2). Similar results were obtained with *T. gondii* cultivated in HFF cells (Figure S2B of the Supporting Information). Indeed, MS profiling exclusively revealed oligomannosylated type *N*-glycans ranging from $Man_3HexNAc_2$ to $Man_9HexNAc_2$ that are typically observed in mammalian cells. The presence of high-mannose-type glycans (more than six mannose residues) on parasitic proteins is again in contradiction with bioinformatic predictions, leaving in question the origin of these compounds.

To address this discrepancy, we cultivated tachyzoïtes in a CHO mutant cell line deficient in Dol-P-Man synthase, B3F7 (*31*). These cells synthesize $Glc_{1-3}Man_5GlcNAc_2$-PP-Dol, instead of the classical $Glc_{1-3}Man_9GlcNAc_2$-PP-Dol lipidic precursors, and transfer truncated $Glc_3Man_5GlcNAc_2$ onto their newly synthesized proteins [Figure 1C (*32*)]. Accordingly, the N-glycosylation profile of the host B3F7 cell line is dominated by truncated $Man_3GlcNAc_2$ to $Man_5GlcNAc_2$ as well as complex-type *N*-glycans that are likely to result from the trimming and processing of $Glc_3Man_5GlcNAc_2$ (Figure S3A of the Supporting Information and Figure 4C) (*33*). Then, a set of signals with *m/z* values corresponding to $Hex_{6-8}HexNAc_2$ were tentatively attributed to glucosylated $Glc_{1-3}Man_5GlcNAc_2$ based on genomic data. Both *T. gondii* and B3F7 cells are expected to synthesize similar truncated glycans as a result of the deficiency in different activities. Indeed, B3F7 cells are deficient in Dol-P-Man synthase, whereas *T. gondii* lacks Dol-P-Man using luminal mannosyltransferases. If the synthesis of $Man_9GlcNAc_2$ observed in *T. gondii* grown in Vero and HFF cells was the result of the complementation of parasite-deficient ALG3, ALG9, and ALG12 enzymatic activities by the host cell, we would then also find this glycan in *T. gondii* grown in B3F7 cells. However, *N*-glycan

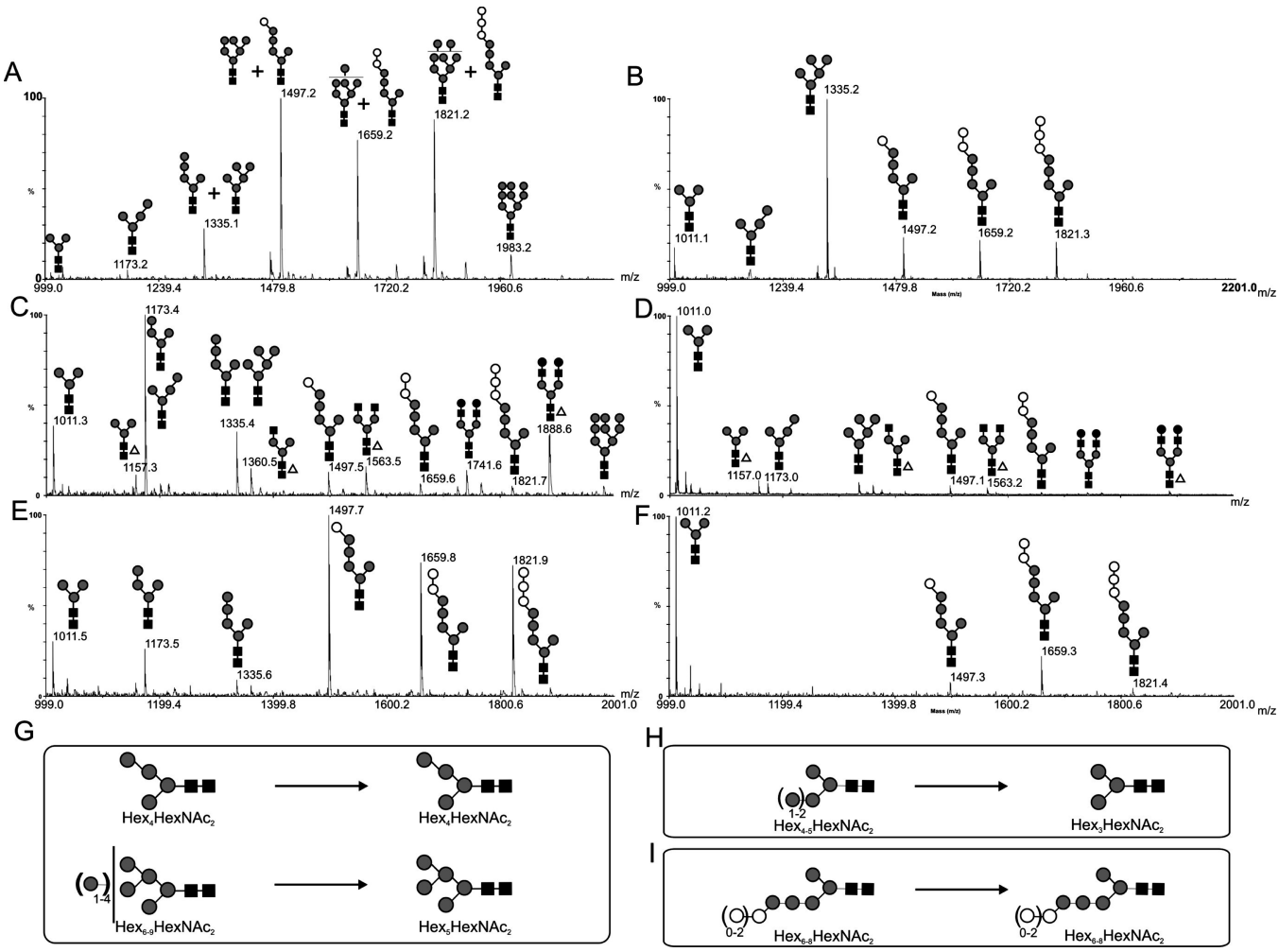

FIGURE 4: MS profiles of oligosaccharides released from proteins of (A and B) *T. gondii* grown in Vero cells, (C and D) B3F7 cells, (E and F) *T. gondii* grown in B3F7 cells (A, C, and E) before and (B, D, and F) after *A. saitoi* exomannosidase digestion. Susceptibility to *A. saitoi* exomannosidase digestion of (G) branched Golgi-derived oligomannosyl types, (H) ER-derived *N*-glycans, and (I) ER-derived linear truncated *N*-glycans. Oligosaccharides were analyzed by MALDI-TOF MS after derivatization as 2PA oligosaccharides.

profiling demonstrates that *T. gondii* grown in B3F7 mutant cells synthesize oligosaccharides varying from $Hex_3GlcNAc_2$ to $Hex_8GlcNAc_2$, but not $Hex_9GlcNAc_2$ (Figure 4E and Figure S3B of the Supporting Information). Furthermore, we never observed any complex-type *N*-glycans in the parasite, regardless of the host cell in which it had been grown. The specific absence of $Hex_9GlcNAc_2$ signals in *T. gondii* grown in B3F7 cells compared with other host cells established that parasite glycosylation capacities were somehow dependent on the host. However, the parasite does not use its host enzymatic repertoire to complement its own incomplete repertoire. On the basis of the known mammalian glycosyltransferase repertoire, the $Hex_{5-8}HexNAc_2$ signals observed in Vero and HFF cells are classical branched mature *N*-glycans derived from the trimming of $Glc_3Man_9GlcNAc_2$, while in B3F7 cells, they correspond to truncated $Glc_{0-3}Man_5GlcNAc_2$. It is noteworthy that the mutant (B3F7) is not completely deficient in DolPMan synthase activity as minute, but measurable, amounts of $Man_9GlcNAc_2$ were also detected.

T. gondii Grown in B3F7 Cells Exclusively Synthesize Glucose-Containing Biantennary N-Glycans. Finally, to assess the relative contributions of the parasite and host cells to *T. gondii* N-glycosylation, we looked for the precise origin of the protozoan *N*-glycans in various host cells. The exquisite specificity of *A. saitoi* exomannosidase toward the α(1-2) linkage permits differentiation of the Golgi degradation products (Figure 4G) from the linear ER-related compounds (Figure 4H) and the glucose-containing truncated glycans (Figure 4I). The efficiency of this enzymatic treatment was evaluated by MS using a standard mixture of high-mannose-type chains ranging in size from $Man_6GlcNAc_2$ to $Man_9GlcNAc_2$. This demonstrated that glycans ranging from $Man_7GlcNAc_2$ to $Man_9HexNAc_2$ were almost quantitatively degraded to branched $Man_5HexNAc_2$ and residual $Man_6GlcNAc_2$ (Figure S4 of the Supporting Information). On the other hand, $Man_4GlcNAc_2$ and $Man_5GlcNAc_2$ from tachyzoïtes grown in B3F7 cells were completely degraded to $Man_3GlcNAc_2$ by enzymatic treatment (Figure 4E,F). This indicated that these two oligosaccharides are ER-derived compounds. Furthermore, resistance of $Hex_6GlcNAc_2$, $Hex_7GlcNAc_2$, and $Hex_8GlcNAc_2$ to mannosidase activity, as well as the absence of a branched $Man_5GlcNAc_2$ degradation product, establishes that these compounds are $Glc_{1-3}Man_5GlcNAc_2$ truncated glycans. *N*-Glycan samples were digested twice to rule out the possibility of partial degradation. Then, identical results were obtained from several independent parasite productions. Altogether, these

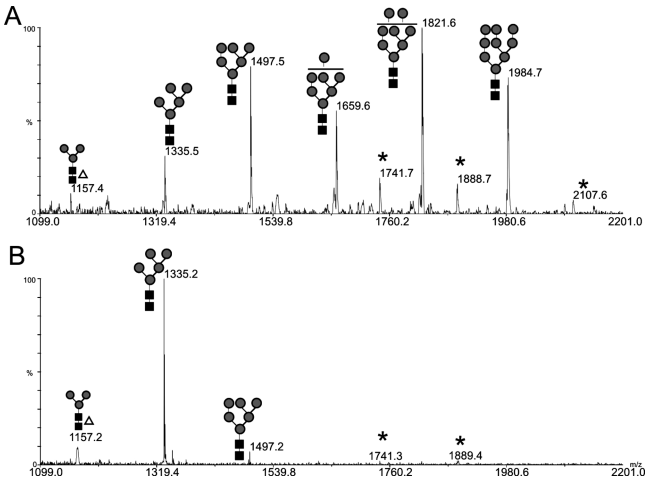

FIGURE 5: Susceptibility of Vero cell-derived *N*-glycans to *A. saitoi* exomannosidase digestion. MS profiles of 2-AP-derivatized oligmannosyl-type *N*-glycans (A) before and (B) after mannosidase digestion. Asterisks denote complex-type *N*-glycans.

data strongly suggest that *N*-glycans from *T. gondii* grown in B3F7 cells correspond to $Glc_{1-3}Man_5GlcNAc_2$ truncated glycans.

A similar strategy confirmed that truncated *N*-glycans represent also the major type of glycosylation in B3F7 cells (Figure 4C,D). It also established the presence of small amounts of branched glycans, not observed in *T. gondii*. These are certainly the result of a residual Dol-P-Man synthase activity in B3F7 cells, as suggested by the presence of a $Man_9GlcNAc_2$ signal. Taken together, these data establish that *T. gondii* transfers truncated *N*-glycans onto newly synthesized proteins. However, considering that both parasite and B3F7 mutant cells potentially synthesize identical truncated *N*-glycans, we cannot determine at this stage if parasite truncated *N*-glycans are genuinely synthesized by the parasite or scavenged from the host cells.

T. gondii Synthesizes Truncated N-Glycans and Scavenges Oligomannosyl N-Glycans from Host Cells. To establish the origin of parasitic *N*-glycans, we re-evaluated the structure of *T. gondii N*-glycans grown in Vero cells by using mannosidase. As expected, mannosidase treatment of Vero cell-derived *N*-glycans eliminated $Man_{7-9}GlcNAc_2$, confirming that all mature *N*-glycans are indeed Golgi-modified products (Figure 5). As observed with the standard *N*-glycans (Figure S4 of the Supporting Information), their complete degradation generated a prominent branched $Man_5GlcNAc_2$ and a minor $Man_6GlcNAc_2$. In contrast, $Hex_{7-9}GlcNAc_2$ isolated from *T. gondii* grown in Vero cells was partially resistant to digestion by mannosidase, indicating the presence of truncated $Glc_{1-3}Man_5GlcNAc_2$ glycans (Figure 4A,B). However, the concomitant marked increase in the level of branched $Man_5GlcNAc_2$ and the disappearance of $Hex_9GlcNAc_2$ clearly indicate the presence of branched $Man_{6-9}GlcNAc_2$, as well. To confirm that the $Hex_9GlcNAc_2$ signal originated from *T. gondii* rather than from a host cell contaminant, we mixed parasites released from B3F7 cells with homogenized Vero cells and analyzed the resulting *N*-glycan profile. This analysis revealed the absence of $Hex_9GlcNAc_2$, thereby indicating that any contamination from host cells is below the level of detection (Figure S5 of the Supporting Information).

CONCLUSION

Analyses of *N*-glycans extracted from tachyzoïtes grown in different host cells clearly demonstrated that the parasite is able to synthesize and transfer truncated glucosylated biantennary *N*-glycans to parasite proteins (*23, 25*), in a host-independent manner. These results are in agreement with *in silico* prediction of the *T. gondii* glycosyltransferase pathway (*11, 23*).

However, when grown in Vero and HFF cells, *T. gondii* also transfers classical *N*-glycans identical to those of its host cells, as indicated by the presence of Golgi-derived oligomannosylated types. These glycans are not synthesized by the parasite through functional complementation of the incomplete *T. gondii* glycosyltransferase pathway by host cell enzymes, since parasites grown in B3F7 cells (which still maintain this activity) do not synthesize mature *N*-glycans. Therefore, we hypothesize that *T. gondii* scavenges DolPPOS from its host cell in a nonspecific manner and subsequently transfers its oligosaccharidic moieties onto its own newly synthesized proteins, as evidenced by the presence of functional STT3 (*25*). Once transferred, the destiny of these glycans remains unclear; however, they seem to undergo very limited processing in contrast to mammalian cells. Indeed, although B3F7 mutant cells extensively process the $Glc_{1-3}Man_5GlcNAc_2$ precursor glycans toward $Man_4GlcNAc_2$ and complex types, proteins from *T. gondii* grown in these cells mainly harbor $Glc_{1-3}Man_5GlcNAc_2$. This is in good agreement with the apparent lack of most proteins involved in the quality control of newly synthesized proteins in *T. gondii* (*34*).

To the best of our knowledge, this represents the first documented example of the scavenging of glycan intermediates from the host cell by an intracellular parasite. In a similar way, *T. gondii* has been shown to mobilize selected host lipids to fulfill its high metabolic requirements during proliferation (*35*−*37*). These exchanges could be facilitated by the close association of the parasitophorous vacuole membrane with the host endoplasmic reticulum, where the early steps of *N*-glycan biosynthesis take place. From an evolutionary perspective, it is noteworthy that the absence of Dol-P-Man-dependent mannosyltransferase activities in the N-glycosylation pathway is counterbalanced by the synthesis of very large quantities of GPI-type glycosylation (*38*). Considering the postulated common evolutionary origin of ALG enzymes and PIG enzymes involved in the biosynthesis of phosphatidylinositol glycans (*39*), one may postulate that Dol-P-Man-dependent mannosyltransferases could have rapidly evolved for the exclusive synthesis of GPI in *T. gondii*. The presence of such enzymes in apicomplexan protozoa is in agreement with this hypothesis (*40*). The opportunity for the parasite to use *N*-glycan precursors synthesized by the host could then have lifted the selective pressure to maintain an intact reticular N-glycosylation pathway, acting as a powerful drive for the evolution of glycosylation machinery in intracellular obligatory parasites.

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SUPPORTING INFORMATION AVAILABLE

Five figures (Figure S1−S5) and their corresponding legends and one table (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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