Unusual \(N\)-glycan Structures Required for Trafficking \textit{Toxoplasma gondii} GAP50 to the Inner Membrane Complex Regulate Host Cell Entry Through Parasite Motility* \(\S\)

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\textit{Toxoplasma gondii} motility, which is essential for host cell entry, migration through host tissues, and invasion, is a unique form of actin-dependent gliding. It is powered by a motor complex mainly composed of myosin heavy chain A, myosin light chain 1, gliding associated proteins GAP45, and GAP50, the only integral membrane anchor so far described. In the present study, we have combined glycomic and proteomic approaches to demonstrate that all three potential \(N\)-glycosylated sites of GAP50 are occupied by unusual \(N\)-glycan structures that are rarely found on mature mammalian glycoproteins. Using site-directed mutagenesis, we show that \(N\)-glycosylation is a prerequisite for GAP50 transport from the endoplasmic reticulum to the Golgi apparatus and for its subsequent delivery into the inner complex membrane. Assembly of key partners into the gliding complex, and parasite motility are severely impaired in the unglycosylated GAP50 mutants. Furthermore, comparative affinity purification using \(N\)-glycosylated and unglycosylated GAP50 as bait identified three novel hypothetical proteins including the recently described gliding associated protein GAP40, and we demonstrate that \(N\)-glycans are required for efficient binding to gliding partners. Collectively, these results provide the first detailed analyses of \textit{T. gondii} \(N\)-glycosylation functions that are vital for parasite motility and host cell entry. \textit{Molecular & Cellular Proteomics} 10: 10.1074/mcp.M111.008953, 1–17, 2011.

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1 The abbreviations used are: ER, endoplasmic reticulum; DMEM, Dulbecco’s modified Eagle’s medium; GAP, gliding-associated protein; HFF, human foreskin fibroblast; HA, hemagglutinin; IP, immunoprecipitation; IMC, inner membrane complex; MyoA, myosin A; MLC, myosin light chain; DHB, 2,5-dihydroxybenzoic acid (DHB, Sigma); PFA, paraformaldehyde.
Crucial Roles of Toxoplasma N-glycans

essential for gliding motility in apicomplexan parasites is composed of a tetrameric complex encompassing MyoA, a myosin tail interacting protein also called myosin light chain 1 (MLC1) (5–7), and the two glycososome-associated proteins GAP45 and GAP50, which are anchored to the outer IMC membrane (8, 9). GAP50 is firmly immobilized in the IMC and considered to act as a fixed anchor for the motor complex (10). To power the forward movement of apicomplexan parasites, myosin pulls the actin filaments and their attached adhesins rearward. This requires the GAP-myosin complex to be anchored to the IMC and the underlying cytoskeleton (9–11), in addition to energy and diverse regulatory events. Most importantly, the gliding machinery (including MyoA, MLC1, GAP45, and GAP50) appears to be highly and exclusively conserved across Apicomplexa species (12). Therefore, the elucidation of molecular mechanisms controlling gliding motility and, ultimately the invasion of and egress from host cells could be valuable for the development of new therapeutic strategies against these major human and animal pathogens. A recent study focusing on TgMLC1 demonstrated that the mechanical activity of Class XIV myosins (TgMyoA) can be modulated by a small-molecule inhibitor of myosin motor activity, which interferes with the post-translational modifications of its associated light chains (13). The regulation of gliding motility can also be controlled at additional stages, including actin polymerization (14) and calcium-mediated secretion of adhesins (15). The final assembly of the gliding motor is controlled by phosphorylation of pre-assembled TgGAP45-MyoA-MLC1 complex (16). In addition, the gliding associating protein GAP45 is modified by acylation, whereas GAP50 is an integral membrane protein of the IMC. Both GAP45 and GAP50 were proposed to anchor MyoA to the IMC (9). The precomplex MyoA-MLC1-GAP45 is anchored to the IMC through association with GAP50 and GAP45, whose acylation by both myristoylation and palmitoylation appears as potentially important translational modifications (17). T. gondii is also capable of mobilizing its main source of energy, which was initially suspected by Gaskins et al. (18). This novel location of glycolytic enzymes, which remain pellicle-associated until parasites have completed host cell invasion, probably optimizes ATP delivery required for gliding motility.

Despite the importance of N-glycosylation of proteins, a relatively common post-translational modification of eukaryotic proteins translocated into the endoplasmic reticulum, several studies have suggested that this is a rare event in apicomplexan parasites (19, 20). Indeed, the presence of N-glycosylation for the gliding associated protein TgGAP50, which was initially suspected by Gaskins et al. (9), has only recently been demonstrated by us and others (21, 22). Beyond a very limited number of N-glycoproteins discovered in the parasite, the precise functions of N-glycans are still unknown in T. gondii. Here, we present evidence that the usual N-glycan structures on the TgGAP50 protein are crucial for its trafficking through ER/Golgi apparatus to a final loading into the IMC, and for its subsequent interactions with other key gliding partners. Our study provides insight into how N-glycosylation regulates the trafficking of GAP50 and its association to other members of the actin-myosin motor complex and also demonstrates that N-glycosylation is important for parasite motility and host cell entry.

**EXPERIMENTAL PROCEDURES**

**Culture of Parasites**—Human foreskin fibroblasts (HFFs) were maintained in Dulbecco’s modified Eagle’s medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS, Invitrogen), 2 mM l-glutamine, (Invitrogen) and 10 000 U/ml Penicillin-Streptomycin (Invitrogen). Tachyzoites from T. gondii RH strain were grown in monolayers of HFF cells until they lysed the host cells spontaneously. Freed tachyzoites were harvested and purified using glass wool columns and 3-mm pore filters followed by centrifugation at 2200 rpm for 15 min.

**Cloning of TgGAP50 into T. gondii Expressing Vector and Site-Directed Mutagenesis**—The gene encoding TgGAP50 protein was obtained in http://www.toxodb.org and the following forward and reverse primers, 5'-cccgctgcagATGGCAGGCGCCCCCAGTC-3' and 5'-cccgctgcagTTTATGTAGCGGAGAGACCGTTC-3', were designed to amplify the complete open reading frame of TgGAP50 using cDNA from tachyzoites of RH strain and Accuprim TaqDNA Polymerase High Fidelity (Invitrogen). The PCR product was purified and cloned in pMAH14 plasmid using PstI restriction site and nucleotide sequence accuracy was checked by DNA sequencing. The pMAH14 vector allows fusion at the C-terminus with the HAFLAG tag and expression of TgGAP50-HAFLAG was under control of the GRA1 promoter (23). The selectable marker, bleomycin, was amplified from pTub5Bleo plasmid by PCR, sequenced and subcloned via KpnI restriction sites into pTgGAP50-HAFLAG, resulting in a pMAH14-TgGAP50-HAFLAG vector containing the bleomycin-selectable marker (under the control of tubulin promoter) for the cloning of transgenic stable parasites (23). The replacement of individual and all three potential N-glycosylation sites (Asn101, 136 and 228) of TgGAP50 to Ala was performed by PCR using QuickChange Site-Directed Mutagenesis Kit (Strategene). The plasmid pMAH14TgGAP50-HAFLAG encoding the wild-type GAP50 protein was used as template with the following primer pairs, which allow mutation of Asn residues (underlined): 5'-GGCGGAGTTTCCACCGCTGGCGCACCAGGGTGG-CAATCC-3', 5'-GGATTGCCACCTGGTGTCGGCGGAGCTTGAAAC-TCCG-3' for the first potential N-glycosylation site (mutant was designated pMAH14TgGAP50 N_101-A-HAFLAG); 5'-GGAGTAGCT-GGAGTAGACCGCGTACACCTGCTTGGAGGCTTG-3', 5'-CAGGGCCCTCA-GAGGGTGACCTCTACCTCCAGATGAC-3' for the second potential N-glycosylation site (pMAH14TgGAP50 N_136-A-HAFLAG); 5'-TCCCCTCCTTCCGTTTCTCCTGCGGCGTTGACAGTCG-3', 5'-CACCCGGCGACTCGTCACCCGGACGACGAGCATGAC-3' for the third potential N-glycosylation site (pMAH14TgGAP50 N_228-A-HAFLAG).

**Cloning of TgGAP50 into T. gondii**
5'-ccggccccggtTTGGAGTTGGCCGGAGACG-3' and 5'-ccgggac-tctCGAAGACTTGGTAAGCAGTG-3'.

**Transient and Stable T. gondii Transfections and Immunofluorescence Assay**—Transient transfection was performed using 100 μg of circular plasmids corresponding to wild type and mutated TgGAP50 into 10^7 tachyzoites of the RH strain. After transfection, the parasites were loaded onto confluent monolayers of HFF and 24 h later the intracellular tachyzoites were fixed and processed for immunofluorescence assay. Stable T. gondii transformants were obtained by two selections of transfected tachyzoites in DMEM supplemented with 10 mM Heps and 50 μg/ml pheleomycin (InvivoGen) for 4 h at 37 °C or 5 μg/ml pheleomycin for 10 h at 37 °C and the resistant parasites were cloned by limiting dilution. Intracellular transfected tachyzoites were fixed for 30 min in 4% paraformaldehyde in phosphate-buffered saline (PBS), permeabilized and saturated in PBS containing 0.2% Triton X-100 and 0.1 M glycine at room temperature for 15 min. Samples were blocked with 5% FCS in the same buffer and mouse monoclonal anti-HA.11 antibodies (Covance), diluted at 1:1000, were added to parasites in the same buffer for one hour at 37 °C. Rabbit secondary antibody coupled to Alexa-488 (Molecular Probes) was used at the same dilution. Fluorescence was visualized with a ZEISS Axiosoph microscope whereas confocal imaging was performed with a LSM710 microscope (Zeiss) and a Plan Apochromat objective (Plan-Apochromat 63x/1.40 Oil DIC M27, Zeiss). The associated software (Zen 2008) enabled the adjustment of acquisition parameters. Overlay images were assembled with Image J (National Institutes of Health).

**Gene Knockin for Epitope-tagged Protein Expression in T. gondii**—The plasmid for the gene knockin of the hypothetical proteins was made with the pLIC-HA-DHFR plasmid. A DNA fragment of 2165 bp upstream of the stop codon from the TGM49_049850 genomic sequence was cloned in the PacI restriction site of the pLIC-HA-DHFR plasmid with the ligation independent cloning techniques, using the following primers: 5'-TACTTCATCATCTAACATTAAATGTCGAG-3' and 5'-TCCTCCATCTCATAAATTGGC-3'. For the cloning of the 4386-bp upstream of the stop codon from the TGM49_020950 genomic sequence, the following primers were used: 5'-TACTTCATCATCTAACATTAAATGTCGAG-3' and 5'-TCCTCCATCTCATAAATTGGC-3'. For the cloning of the 2700-bp upstream of the stop codon from the TGM49_058060 genomic sequence, the primers were used: 5'-TACTTCATCATCTAACATTAAATGTCGAG-3' and 5'-TCCTCCATCTCATAAATTGGC-3'.

**Protein Identification and N-glycopeptide Analysis by Mass Spectrometry**—Reduction, alkylation and tryptic in-gel digestion were performed as previously described (22). For N-glycopeptide analysis, the band corresponding to TgGAP50-HAFLAG was also treated overnight with 1.25 μg of chymotrypsin (Roche, Mannheim, Germany) at room temperature. Both digests were analyzed using LC-MS/MS analysis performed with a Q-TOF LC-MS/MS (Bruker Daltonics) mass spectrometer. The complete system was fully controlled by Analyst 1.5.3 software (Bruker Daltonics). For tandem MS experiments, the system was operated in the data-dependent mode with 3 tandem MS (MS/MS) scans.

For N-glycopeptide analysis, oxonium ions for glycopeptide detection were generated using alternate collision energy in the collision cell (10/28 eV). Glycopeptides were highlighted by searching MS data for the presence of the 204.09 m/z HexNac and the 366.14 m/z Hex(HexNac) and confirmed by CID from liquid chromatography (LC)-MS/MS data. The glycopeptide sequences were validated by manual assignment of peptide fragment ions as previously described (24). Mass data collected during nano-MS/LS/MS analysis were processed, converted into .mgf files using Data Analysis 4.0 software (Bruker Daltonics). MS and MS/MS data were analyzed using the Mascot 2.2.0.1 algorithm (Matrix Science, London, UK) and OMSSA (Open Mass Spectrum Search Algorithm) (25). For peptide identification, an in-house generated protein database composed of protein sequences of Toxoplasma gondii downloaded from NCBInr (February 17, 2010) concatenated with reversed copies of all sequences (total 51258 entries) was used. Searches were performed with a mass tolerance of 5 ppm in MS mode and 0.02 Da in MS/MS mode. Enzyme specified was trypsin and one missed cleavage per peptide was allowed. Variable modifications were taken into account such as carbamidomethylation of cysteine residues and oxidation of methionine residues. Neither protein molecular weight nor isoelectric point constraints were applied. The Mascot and OMSSA results were loaded into the Scaffold software (Proteome Software, Portland, OR). To minimize false positive identifications, results were subjected to very stringent filtering criteria as follows: 1) for the identification of proteins with two peptides or more, a Mascot ion score must be higher than 20 and had to be minimum 10 below the 95% Mascot significance threshold (“Identity score”) or an OMSSA E-value above -log(e3) was required; 2) in the case of single peptide hits, the Mascot score of the unique peptide must be greater than 25 and had a minimal “difference score” of 5 than the “Identity score” and the OMSSA E-value must be greater than 6.
The enzymatic reaction was terminated by addition of 1 mM EGTA. The Coomassie-stained bands were excised from the gel, destained and the protein was then reduced and alkylated with iodoacetamide as previously described (28). After PNGase F digestion, N-linked glycans were eluted from the gel pieces and desalted on minicolumns with 10 mg of nonporous graphitized carbon followed by mass spectrometry. MALDI-TOF MS experiments were carried out on Voyager Elite DE-STR Pro instrument (PerSeptive Biosystem, Framingham, MA) equipped with a pulsed nitrogen laser (337 nm) and a gridless delayed extraction ion source. The spectrometer was operated in positive reflectron mode by delayed extraction with an accelerating voltage of 20 kV and a pulse delay time of 200 nsec and a grid voltage of 66%. All spectra shown represent accumulated spectra obtained by 500 laser shots. Sample was prepared by mixing 1 μl aliquot (5 to 10 picomoles) with 1 μl of matrix solution, on the MALDI sample plate. The matrix solution was prepared by saturating methanol-water (1:1) with DHB (10 mg/ml). The on-target α-mannosidase digestion was performed on desalted N-linked glycans dissolved with water at 5 to 10 picomoles/μl and incubated with Jack bean α-mannosidase (EC 3.2.1.24, Sigma Chemicals). One μl of the sample was spotted on the MALDI sample plate and 1 μl of 10 mM ammonium acetate (pH 4.5), and 35 μM of α-mannosidase were added. The MALDI plate was then placed in a crystallization beaker containing water, at 37 °C for 6 h. The enzymatic reaction was terminated by addition of 1 μl of a matrix solution (DHB) and the sample was analyzed by MALDI-TOF-MS. Ribonuclease B (Sigma Chemicals, St. Louis, MO) was used as control N-linked glycoprotein.

Biochemical Analyses of Glycoform Heterogeneity Using LC-MS—Five microliters of chymotrypsin GAP50 digest were analyzed by nanoLC-MS using the nanoACQUITY Ultra-Performance-LC system (UPLC, Waters, Milford, MA) coupled to a SYNAPT High Definition Mass Spectrometry quadrupole time-of-flight tandem mass spectrometer (Waters, Milford, MA) equipped with a nano-electrospray source. Extraction of the current ions corresponding to the different glycoforms, identified by preliminary MS/MS experiments, was performed (m/z 1150.0, 1231.0, 1245.5, 1326.5, 1407.5, 1261.0, 1342.1, 1423.1, ± 0.2 Da) and for each glycosylation site, MS spectra of glycopeptides elution zone were combined.

In Vitro Gliding Assay, Host Cell Invasion and Proliferation Assay—The wild-type parasites, ectopic GAP50-HAFLAG expressers and completely deglycosylated GAP50-HAFLAG parasites, treated or untreated with 5–10 μg/ml of tunicamycin, were released 30 h post-infection and filtered in DMEM containing 10% FCS, 2 mM L-glutamine and 10 000 U/ml Penicillin-Streptomycin. after centrifugation at 1000 rpm for 10 min, the parasite pellet was resuspended in the same medium and the parasites were allowed to move for 15 min at 37 °C on slides previously coated overnight with 50% FCS prepared in PBS. Slides were immediately fixed with 4% PFA and proessed for immunofluorescence assays using antibodies specific to the major surface antigen SAG1 in PBS. Trails of gliding parasites were visualized with a Zeiss Axioshot microscope. These treated or untreated wild type, ectopic expresser and completely deglycosylated GAP50 expressing parasites were also resuspended with DMEM medium containing 10% FCS, 2 mM L-glutamine and 10 000 U/ml Penicillin-Streptomycin, loaded onto confluent monolayer HFF cells and grown for 24 h at 37 °C. The intracellular parasites were fixed and stained with monoclonal antibody specific to SAG1 for immunofluorescence as above and intracellular tachyzoites were counted under Zeiss Axioshot microscope.

**RESULTS**

**Ectopic Expression of the Integral Membrane Anchor GAP50 of the T. gondii Gliding Motor**—We and others have previously shown that GAP50, the membrane anchor for the gliding motor complex in T. gondii, contains potential N-linked oligosaccharides (9, 21, and 22). We have previously investigated the N-glycosylation status of T. gondii by exploring the presence of N-glycosylated peptides isolated from trypsin-digested total detergent extract proteins followed by extensive glycomic and proteomic analyses. This led to the identification of one N-glycopeptide of GAP50, which carries immature oligomannosidic glycans (22). To gain more insight into protein N-glycosylation functions in T. gondii, we decided to determine the fine structures of N-glycan chains of all three potential N-glycosylation sites: Asn residues at position 101, 136, and 228. Toward this goal, we engineered stable T. gondii transgenic parasites, ectopically expressing HAFLAG-tagged GAP50 protein, using both endogenous GAP50 and heterologous dense granule GRA1 gene promoters. We chose the heterologous GRA1 promoter because the weak expression driven by GAP50 promoter yields an insufficient amount of protein for further classical glycomics (gas chromatography coupled to mass spectrometry) and proteomic analyses. To ascertain if, like the wild-type GAP50 protein, the ectopically expressed GAP50-HAFLAG protein (under GRA1 promoter control) localizes in the IMC, we first investigated the localization of GAP50-HAFLAG protein using specific anti-HA antibodies and indirect immunofluorescence visualized by confocal microscopy. This confirmed that the ectopically expressed GAP50-HAFLAG protein (green) was strongly detected in the inner membrane complex, which was stained with monoclonal antibodies specific to IMC1 (red), a major component of the subpellicular network that tightly opposes the cytoplasmic face of the IMC (29) (supplemental Fig. S1, panel A). The GAP50-HAFLAG protein was shown to be in association with the newly formed IMC membranes of daughter parasites (supplemental Fig. S1, panel A). Proteomic analyses using a large scale affinity purification of the HAFLAG-tagged GAP50 and its binding partners under a low salt stringency (see details under “Experimental Procedures”) showed after separation by SDS-PAGE and Coomassie blue staining (supplemental Fig. S1, panel B), all major known gliding partners, such as myosin A (MyoA), myosin-light chain (MLC1), glideosome associated proteins GAP45 and GAP50 (supplemental Table S1). Under these low salt stringency conditions, other proteins identified by proteomics were enolase, fructose-1, 6-bisphosphatase, glyceraldehyde-3-phosphate dehydrogenase, hexokinase, lactate dehydrogenase, phosphofructokinase and numerous hypothetical proteins (supplemental Table S1 and supplemental Fig. S1, panel B). Although, the pull down of the glycolytic enzymes is also consistent with their dual role in both glycolysis and specific relocation from the cytoplasm to IMC before host cell egress.
Table I
Comparison of proteins that bind to N-glycosylated and unglycosylated GAP50-HAFLAG using nanoLC-MS/MS. The affinity purification was performed under high stringency conditions and protein identity was determined using a protein database of T. gondii downloaded from NCBI/ncr databases. The corresponding identity in T. gondii databases (http://www/toxodb.org) and the different genome sequences of T. gondii strains RH, GT1, VEG, and ME49 used for protein identification are shown. We identified three novel putative GAP50 binding proteins including GAP40, which has been independently reported and validated as a novel glideosome component that interacts with GAP45 (34).

| Protein name and function | Protein accession numbers | Protein molecular weight (Da) | N-glycosylated GAP50 | Unglycosylated GAP50 | Number of potential N-glycosylation site | Number of transmembrane domains |
|---------------------------|---------------------------|------------------------------|---------------------|---------------------|----------------------------------------|---------------------------------|
| **Glideosome and regulatory proteins** | | | | | | |
| Actin | gi|1|7|0|3|1|6|0 | 4189 | 3 | 12% | 1 | |
| Actin depolymerizing factor, putative | gi|2|2|1|4|8|1|1|5|1 | 12904 | 2 | 21% | 1 | |
| Gliding-associated protein 45, GAP45 | gi|3|0|8|4|0|6|7 | 27316 | 2 | 13% | 1 | |
| Membrane anchor for myosin XIV precursor, GAP50 | gi|4|6|9|4|8|0|6 | 46590 | 18 | 47% | 11 | 36% | 3 | 1 |
| Myosin A, putative | gi|2|2|1|4|8|3|4|1 | 93319 | 33 | 56% | 11 | 15% | 1 | |
| Myosin light chain TgMLC1 | gi|1|6|5|8|0|1 | 24107 | 8 | 65% | 2 | 11% | 1 | |
| **Glideosome multiprotein complex** | | | | | | |
| Actin | gi|1|7|0|3|1|6|0 | 4189 | 7 | 24% | 8 | 27% | 1 | |
| Gliding-associated protein 45, GAP45 | gi|3|0|8|4|0|6|7 | 27316 | 3 | 20% | 1 | |
| Membrane anchor for myosin XIV precursor, GAP50 | gi|4|6|9|4|8|0|6 | 46590 | 11 | 37% | 8 | 26% | 3 | 1 |
| Myosin A, putative | gi|2|2|1|4|8|3|4|1 | 93319 | 51 | 73% | 15 | 22% | 1 | |
| Myosin light chain TgMLC1 | gi|1|6|5|8|0|1 | 24107 | 4 | 25% | 1 | |
| **Unknown functions** | | | | | | |
| Conserved hypothetical protein | TgME49 020950 | gi|2|2|1|4|8|0|6 | 47891 | 4 | 11% | 5 | 12% | 2 | 1 |
| Conserved hypothetical protein | TgME49 058060 | gi|2|2|1|4|8|7|1 | 23334 | 2 | 10% | 1 | |
| Hypothetical protein, conserved | TgME49 049850 | gi|2|2|1|4|8|4 | 43063 | 2 | 8% | 1 | |

*a* Toxoplasma gondii.
*b* Toxoplasma gondii RH.
*c* Toxoplasma gondii GT1.
*d* Toxoplasma gondii VEG.
*e* Toxoplasma gondii ME49.

The Integral Membrane Anchor GAP50 of T. gondii Gliding Motor Contains Unusual N-glycan Structures—The GAP50 protein was excised and purified from SDS-PAGE described in supplemental Fig. S1, panel B, and the nature of its N-glycans was determined after reduction, carboxamidomethylation, digestion with PNGase F, purification on a non-porous graphitized carbon column followed by MALDI-TOF-MS analyses. The predominant molecular ion corresponding to Hex3HexNAc2 + Na+ (m/z 1419) and two additional weaker ions, Hex2HexNAc2 + Na+ (m/z 1581), and Hex2HexNAc2 + Na+ (m/z 1743) were identified (Fig. 1A). To discriminate the oligomannosylated type N-glycans from the glucose-containing truncated glycans, the glycans were submitted to on-plate α-mannosidase digestion followed by MALDI-TOF-MS analysis (Fig. 1B). After this treatment, two new molecular ions were observed at m/z 771 (Hex2HexNAc2) and 933 (Hex3HexNAc2). The intensity of ion at m/z 1419 (Hex2HexNAc2) was reduced, and this strongly suggests the existence of an oligomannosylated type N-glycans with six mannose residues. However, a significant α-mannose resistant signal was also present (see enlarged insert in Fig. 1B). These data suggest that other isomeric structures, which probably correspond to terminal glucose-containing N-glycans that are unaffected by α-mannosidase, are also present.
on GAP50. We also checked whether α-mannosidase digestion under our experimental conditions is optimal on a positive control using on-plate α-mannosidase digestion applied to free N-glycans of bovine ribonuclease B that contains well-characterized oligomannosylated N-glycans composed of Hex$_6$HexNAc$_2$, Hex$_7$HexNAc$_2$, Hex$_8$HexNAc$_2$, and Hex$_9$HexNAc$_2$. Similarly, two major molecular ions were also observed at m/z 771 (Hex$_6$HexNAc$_2$) and 933 (Hex$_7$HexNAc$_2$) and a minor one at m/z 609 (Hex$_6$HexNAc$_2$) (Fig. 1C). The ions at m/z 1257 (Hex$_7$HexNAc$_2$), 1419 (Hex$_8$HexNAc$_2$), 1581 (Hex$_9$HexNAc$_2$), 1743 (Hex$_9$HexNAc$_2$), and 1905 (Hex$_9$HexNAc$_2$) disappeared after the α-mannosidase treatment, which demonstrates that these oligomannosylated N-glycans of ribonuclease B were efficiently and similarly digested by α-mannosidase as for GAP50. We therefore conclude that biochemical properties of N-glycans at m/z 1581 (Hex$_7$HexNAc$_2$) and 1743 (Hex$_9$HexNAc$_2$) shown to be unaffected by α-mannosidase, are consistent with the assignment of terminal glucose-containing N-oligomannosidic chains to these ions (Fig. 1D). In agreement with this statement, no shift in electrophoretic migration was observed after SDS-PAGE and Western blotting when GAP50 was incubated with endo H, an exoglycosidase known to specifically cleave off oligomannosylated glycans from N-glycoproteins, confirming the occupancy of GAP50 N-glycans by terminal glucose or other sugars (21). In contrast, we demonstrated that GAP50 was sensitive to PNGase F, resulting in a mobility shift (Fig. 5C). Thus, we propose that N-glycan structures composed of oligomannosylated type N-glycans (Hex$_6$HexNAc$_2$) and glucose-containing truncated glycans (Hex$_6$–8 HexNAc$_2$) are both linked to Toxoplasma GAP50 protein even though we cannot rule out the presence of additional minor N-glycans with seven mannose (m/z 1581) or eight mannose residues (m/z 1743) (Fig. 1D).

### Positioning N-glycan Structures on Asn-X-Ser/Thr Sequons of T. gondii GAP50 and Glycoform Heterogeneity

To determine which N-glycan structure is precisely carried by each individual N-glycosylation site in the GAP50 glycoprotein, the in-gel purified protein was separately subjected to trypsin and chymotrypsin digestions. Both digests were first analyzed by LC-MS under conditions which generate glycan-specific fragment ions (m/z 204.09 from HexNAc$^-$ and m/z 366.14 from HexHexNAc$^-$) indicating the location of the glycopeptides in the chromatogram. Based on the glycopeptide fragmentation pattern, high-level confidence assignment for each glycosylated site was identified by LC-MS/MS (Fig. 2, panels A–C).
In the MS/MS spectrum of the doubly protonated parent ion at m/z 1150.48 (Fig. 2A), the most intense fragment ion at m/z 1123.55 (1+) corresponds to the chymotryptic peptide $^{227}\text{SNVTSRAW}^{234}$ with one HexNAc. This interpretation is based on the previous observation indicating that often the MS/MS spectrum of doubly charged ion of a glycopeptide is dominated by a fragment ion corresponding to the peptide carrying a single HexNAc (30). This allows the identification of the N-glycosylation at Asn228 because only one chymotryptic peptide is expected at mass 919.46 Da. The corresponding spectrum displayed a series of single protonated ion fragments higher than molecular ion at m/z 1150.48 and a series of lower double protonated ion fragments, most of them corresponding to successive sugar neutral losses. The other fragment ions are oxonium ions which correspond to internal glycan fragmentations. Taken together, these data demonstrate the presence of Hex$_g$ HexNAC$_g$ structure at position Asn228 of GAP50. In addition, MS/MS spectra of molecular ions m/z 1329.03 (2+) and m/z 1414.31 (3+) displayed intense ion fragments respectively at m/z 1156.54 (1+) and m/z 1532.26 (2+), which represent the two other N-glycosylated sites of the tryptic peptides $^{136}\text{NYTSER}^{143}$ and $^{77}\text{VAANEHISFIASPGSNFLGGVSSLNDTR}^{104}$ carrying one HexNAc on Asn136 and Asn101, respectively (Figs. 2B and 2C). These MS/MS spectra also revealed the structures of the N-linked glycans, Hex$_g$ HexNAC$_g$ at position Asn136 and...
Hex6HexNAc2 at position Asn101, respectively. It should be noted that this MS-based approach led to the identification of 8 distinct glycopeptides assigned to the three glycosylation sites as follows: Hex5–7 HexNAc2 (3 N-glycan chains) at position Asn101, Hex6–8 HexNAc2 (three N-glycan chains) at position Asn136 and Hex6–7 HexNAc2 (two N-glycan chains) at position Asn228. We showed that there was also heterogeneity of oligosaccharides at each three N-glycosylation sites. A better picture of the relative abundance of the different glycoforms present on each of the three N-glycosylated sites was given by an LC-MS experiment (Figs. 2D–2G). The N-glycan Hex6 (HexNAc)2 is the most predominant sugar on Asn228 and Asn101 whereas Hex5 (HexNAc)2 and Hex7 (HexNAc)2 are present in equal amount on the N-glycosylation site Asn136. Together, these data provide insights into the structures, length, heterogeneity and topology of N-glycans present on each Asn-X-Ser/Thr sequon of T. gondii GAP50.

N-glycosylation is Required for Proper Targeting of GAP50 into the Inner Membrane Complex of T. gondii—Previous studies have reported that N-glycosylation inhibition using tunicamycin can affect parasite gliding (21, 22). We speculated that the effect of tunicamycin on N-glycans of GAP50 may occur either by direct inhibition of N-glycosylation syn-
thesis or through indirect and nonspecific effect, as tunicamycin-treatment of mammalian cells can also result in global decrease of cellular protein synthesis (31). In the light of these two possibilities, we first decided to determine whether unglycosylated GAP50 can be used in vivo for proper targeting into IMC and gliding motor assembly using reverse genetic approaches instead of biochemical assays with N-glycosylation inhibitors. To address this issue, T. gondii GAP50 mutants lacking one, two or three of the normally occupied N-glycosylation sites were constructed by site directed mutagenesis (replacement of Asn of the sequon to Ala) and the corresponding expression vectors, under the control of homologous GAP50 promoter, were transfected into the parasites. The localization of mutated GAP50 proteins tagged with HAFLAG was analyzed by indirect immunofluorescence and confocal microscopy. A schematic depicting the position and number of sites mutated in GAP50 is shown on the left corner of Fig. 3. Under the control of GAP50 promoter, the transgenic wild-type GAP50-HAFLAG protein and GAP45 partially colocalized in the IMC, as expected (Fig. 3A). It should be noticed that the signal of the ectopic GAP50-HAFLAG (green), which is transiently expressed after transfection appeared only in few areas of the inner membrane complex that are estimated to cover ~70% (histogram A, yellow) of areas occupied by the endogenous GAP45 signal that is used as a marker delineating the whole IMC surface (red, Fig. 3A). These data also confirm the previous studies that indicate that the endogenous GAP50 has lower turnover rates (10). Consistent with these results, the stably expressing GAP50-HAFLAG protein showed fluorescence signal that occupied the whole IMC surface (supplemental Fig. S1, panel A). The mutation of the first Asn at the position 101 in GAP50, however, revealed a confocal fluorescence signal restricted to the parasite cytoplasm with a significant decrease of co-localization (about 25%) with GAP45 (Fig. 3B, histogram B). It is worth noting that despite the presence of the two other sequons that were demonstrated by glycomic approaches and enzymatic digestions to be readily occupied by N-glycans (Figs. 2 and 5B), a single replacement of the first N-glycosylation site Asn101 by Ala prevents an efficient targeting of GAP50 into the IMC. Moreover, the single mutation of the second N-glycosylation site Asn136 behaves similar to the first Asn101 mutant, with the mutated GAP50 protein detected mostly in the parasite cytoplasm (Fig. 3C). About 25% of co-localization with GAP45 (Fig. 3C, histogram C) was observed despite the presence of the two other sites Asn101 and Asn228 occupied by N-glycans (Fig. 5B). In constrast, the mutation of the third N-glycosylation site Asn228 reveals no changes in the classical IMC localization, about 75% of this mutated GAP50 colocalized with GAP45 in the IMC (Fig. 3D, histogram D), similarly to the wild-type GAP50 (Fig. 3A, histogram A). The mutants lacking all three N-glycosylated sites show behavior similar to the single first or second Asn-x-Ser/Thr sequon with a stronger fluorescence signal surrounding the nucleus and more intra-cytoplasmic vesicles (Fig. 3E). In this case, about 25–30% of deglycosylated GAP50 colocalized with GAP45 (Fig. 3E, histogram E). Taken together, these results strongly suggest that mutation of either first, second or all three N-glycosylation sites may cause the deglycosylated GAP50 protein to remain in the perinuclear compartment defining T. gondii endoplasmic reticulum and Golgi apparatus.

The Deglycosylation of GAP50 Impaired Its Intracellular Trafficking in T. gondii—We next focused on the intracellular trafficking of deglycosylated GAP50 to know if the different mutated GAP50 proteins reside in specific sub-cellular compartments of T. gondii. Therefore, we transfected the expression vectors corresponding to wild type and each deglycosylated GAP50 protein into the transgenic parasite line, which stably expressed a marker of the endoplasmic reticulum, named HDEL, tagged to red fluorescent protein (RFP) (32). The confocal images demonstrate that mutants at the first (Fig. 3G), second (Fig. 3H), and all three (Fig. 3J) N-glycosylated sites displayed fluorescence signals (green) that colocalized with HDEL-RFP (red), suggesting that significant levels of deglycosylated GAP50 (about 70% of colocalization, histograms G, H and J) are retained in the parasite ER.
contrast, the wild-type GAP50 protein (Fig. 3F) and the third N-glycan mutant (Fig. 3I) were mostly found in the IMC, thereby only weakly detectable in the ER (histograms F and I). In addition, when GAP50 protein mutants at the first, second, and all three N-glycosylated sites were transfected in the transgenic parasites, which stably expressed in T. gondii Golgi apparatus, the RFP-tagged Golgi marker GRASP (33, red signal), the fluorescence signal of these deglycosylated GAP50 (green) significantly colocalized (about 75% of colocalization, histograms L, M and O) with GRASP protein (Figs. 3L, 3M, and 3O, respectively). In the case of the wild type and third N-glycan GAP50 mutant, again the deglycosylated and wild-type proteins, which are correctly transported to the IMC cannot be detected in the Golgi apparatus (Fig. 3K and 3N). In this case, only 15–25% of colocalization was observed (histograms K and N). In addition, we also created three independent double mutants GAP50N101_N136AA (Fig. 4C and 4D), GAP50N136_N228AA (Fig. 4E and 4F) and GAP50N101_N228AA (Fig. 4G and 4H), which should lead to simultaneous deglycosylation of GAP50 protein at two Asn sites. Consistently, these double mutations prevent the transport and insertion of their respective double mutated GAP50 protein into the IMC (Fig. 4), and these double deglycosylated transgenic GAP50 retained in the ER and Golgi apparatus (supplemental Fig. S2), similar to the single deglycosylated GAP50 (Fig. 3). Taken together, our data strongly suggest that the two first N-glycosylation sites are more critical for proper trafficking of GAP50 than the third N-glycosylated site.

N-glycosylation is Critical for Association of GAP50 with Other Components of T. gondii Glideosome—To investigate whether the lack of N-glycans on GAP50 might have a direct consequence in the formation of multi-complex proteins assembled as glideosome components, stable transgenic parasites expressing GAP50 mutants and wild-type proteins tagged to HAFLAG were generated (Fig. 5A). We confirmed that in the stable transgenic parasite lines, which were cloned, the deglycosylated GAP50N_101-A, GAP50N_136-A, GAP50N_228-A) collapse into one major and faster mobility band that co-migrates with the triple deglycosylated mutant (GAP50N101_136_228-AAA), itself insensitive to PNGase F, as expected (right panel, lane 5).
Deglycosylation of GAP50 prevents its association with other components of T. gondii glideosome. (A–D) Parasites expressing wild-type GAP50-HAFLAG (GAP50 WT) or mutants lacking one potential N-glycosylation site (GAP50N_101-A, GAP50N_136-A, GAP50N_228-A) or all three N-glycosylation sites (GAP50N_101_136_228-AAA) were subjected to immunoprecipitation (IP) with anti-FLAG beads. Under non-reducing electrophoretic conditions, these Western blots (revealed with polyclonal antibodies specific to MyoA and MLC1) showed that the mutations of the first two N-glycan sites reduced or completely abolished MyoA (A) and MLC1 (B) pull-down. C, Cross-linking parasites expressing wild-type GAP50-HAFLAG (GAP50 WT) or mutants lacking one potential N-glycosylation site (GAP50N_101-A, GAP50N_136-A, GAP50N_228-A) or all three N-glycosylation sites (GAP50_N101_136_228-AAA) with DSP followed by IP and Western blots under reduced electrophoretic conditions demonstrated that the partial deglycosylated GAP50 in GAP50N_101-A, GAP50N_136-A and in the complete unglycosylated GAP50N_101_136_228-AAA transgenics prevent interactions with key glideosome partners in vivo. Note the lack of N-glycan in the third site Asn (GAP50N_228-A) has no consequence on partner binding, instead this partial deglycosylated GAP50 behaves similar to wild-type GAP50. D, Western blots of parasites expressing wild-type GAP50-HAFLAG (GAP50 WT) or mutants lacking one potential N-glycosylation site (GAP50N_101-A, GAP50N_136-A, GAP50N_228-A) or all three N-glycosylation sites (GAP50N101_136_228-AAA) after IP with anti-FLAG beads and staining with polyclonal antibodies specific to GAP45. Note the presence of GAP45 assembled with other glideosome components as large macromolecular complexes under nonreduced electrophoretic conditions (black arrows). Note the presence of a band of 55 kDa in the immunoprecipitated materials derived from the wild type and deglycosylated mutants tested with the anti-GAP45 antibodies. This band, which could represent a cross-reacting protein, was not further investigated.

Deglycosylation of GAP50 proceeds efficiently at some extent on each single mutant even though we noticed faster migrating bands in the first two mutants (Fig. 5B and 5C, lanes GAP50N_101-A and GAP50N_136-A) that perhaps more likely suggested that mutation of the first or second glycosylation site may also affect glycosylation of the other sites. Consistent with this notion, the fastest migrating species in the first mutant comigrates with the fully deglycosylated protein (Fig. 5B and 5C, lanes GAP50_N101-A).

Next, we examined the potential of deglycosylated GAP50 proteins to interact with glideosome partners. The stably transgenic expressing wild type and deglycosylated mutants were lysed with nonionic detergent (Triton X-100) and immunoprecipitated using anti-FLAG beads. Fig. 6 shows that the mutations of the first (GAP50N_101-A), second (GAP50N_136-A) or all three N-glycosylation sites (GAP50N101_136_228-AAA) dramatically reduced the ability to co-immunoprecipitate the key gliding partner, TgMyoA (Fig. 6A). A significant reduction in the level of Myosin A-associated light chain 1 (MLC1, another actin-myosin motor) pulled down by the three deglycosylated GAP50 proteins was also noticed (Fig. 6B). To ensure that the wild type and mutated HAFLAG-tagged GAP50 proteins, used to co-immunoprecipitate partners, would reflect the association of glideosome.
some components in vivo, living transgenic parasites were cross-linked with the reduction sensitive, cell-permeable cross-linker dithiobis succinimidyl propionate (DSP), which has a bond length of 7.7 Ångström. After cross-linking, parasites were lysed with Triton X-100 followed by immunoprecipitation using anti-FLAG column. The binding protein complexes were reduced by dithiothreitol before SDS-PAGE and Western blots confirmed that the mutations of the first, second or all three N-glycosylation sites prevent the ability of these mutated proteins to co-immunoprecipitate GAP45, one of its important partners involved in glideosome formation (Fig. 6C). As expected, the mutation of the third N-glycosylation site, which has no critical effect on trafficking and IMC localization, readily immunoprecipitated GAP45 in a manner similar to the wild-type GAP50 (Fig. 6C, lanes GAP50WT and GAP50N_228-A). Taken together, these data are in good agreement with the two-step assembly of the glideosome: first, the transmembrane type I GAP50 protein is inserted in the nascent IMC during daughter parasite formation and, second, GAP45, TgMyoA and TgMLC are preformed cytoplasmic complexes, which associate to GAP50, already present in the IMC (9). Therefore, we interpreted that the residual binding of MyoA, MLC and GAP45 to deglycosylated GAP50 is likely because of the detergent lysis of transgenic parasites that disrupts the ER/Golgi compartment where deglycosylated GAP50 was stuck, thereby bringing GAP50 to the preformed cytoplasmic GAP45-MyoA-MLC complexes to close vicinity for binding. Thus, the single GAP50 mutant at the first and second N-glycosylation site and the triple GAP50 mutant protein, which result in deglycosylation of GAP50 proteins, preventing proper trafficking to IMC, also implies the absence of association with the other glideosome partners.

**Glideosome Complexes are Disrupted by the Absence of N-glycans on GAP50**—We discovered that, in contrast to previous studies (9), a mild detergent extraction (1% Triton X-100) followed by SDS-PAGE analysis under unreduced conditions (without β-mercaptoethanol or dithiothreitol) in SDS-containing buffers was insufficient to completely dissociate all of the glideosome proteins, even after sample boiling at 100 °C, suggesting that the glideosome proteins can form a large stable and lower migrating complexes of about 125 kDa (Fig. 6D, lanes WT and GAP50N_228-A, black arrows). When this lower migrating protein complex (>120 kDa) was excised after SDS-PAGE (supplemental Fig. S1, panel B) and subjected to in-gel trypsin digestion followed by proteomic analyses, we identified numerous peptides from all known glideosome components such as GAP50, GAP45, MyoA, MLC, and actin (Table I). The presence of all glideosome components was confirmed by proteomic analyses under lower (supplemental Table S1) and higher salt stringency (Table I), and by Western blots using specific antibodies. These data provide new evidence of the existence of a glideosome multiprotein complex containing GAP50, GAP45, MyoA, MLC1, and actin, which cannot be dissociated under mild detergent, boiling and unreduced electrophoretic conditions. In light of the fact that myosin and actin are known to multimerize, it remains to estimate whether all proteins are in near stoichiometric amounts in this glideosome multiprotein complex. However, the tiny amounts of proteins that can be isolated from the glideosome multiprotein complex did not allow us to perform such quantitative proteomic experiments.

To further investigate the implications of N-glycosylation in partner binding by GAP50, we have compared wild type, single and triple deglycosylated GAP50 proteins in pull down assays using anti-FLAG beads. In contrast to wild-type GAP50 and the GAP50N_228-A mutant, we found that the deglycosylated GAP50N_101-A, GAP50N_136-A and fully deglycolylated GAP50N_101_136_228-AAA mutants were not able to form significant levels of the faster migrating band (Fig. 6D, lanes GAP50N_101-A, GAP50N_136-A and GAP50N_101_136_228-AAA), similar to the absence of MyoA and GAP45 binding (Fig. 6A and 6C).

Among the glideosome proteins identified under high salt stringency in Table I is also GAP40, a polytopic protein of the IMC, which has been recently validated as an additional glideosome component (34). Our comparative proteomic analyses also indicated that GAP40 (hypothetical protein TGME49_049850) is present in both monomeric and large macromolecular complexes pulled down with wild-type GAP50, whereas the triple deglycosylated mutant failed to immunoprecipitate GAP40 (Table I). As for GAP40, two other novel hypothetical proteins (TGME49_020950 and TGME49_058060) were also pulled down when GAP50 is N-glycosylated (Table I). To get more insight into the possible roles of these three novel hypothetical proteins that are pulled down by GAP50-HAFLAG, we generated stable transgenic parasites that ectopically expressed these novel proteins using knockin promoter targeting strategy that allowed the expression of HA tagged version of these genes in their corresponding loci in the parasite genome. We confirmed that the hypothetical protein coded by TGME49_049850, which has been recently described as a novel gliding associated protein GAP40 (34) colocalized with two IMC markers, GAP45 and IMC1 (Fig. 7A). In contrast, the two other hypothetical proteins, which were respectively coded by TGME49_020950 and TGME49_058060, were detected in dense granules and also in the parasitophorous vacuole, suggesting that these two other hypothetical proteins are unrelated components that localized in the dense granules. Therefore, these two later hypothetical proteins were not further investigated. Because, we found that GAP40 protein can be only pulled down by N-glycosylated GAP50, the role of N-glycans in the association between GAP40 and other gliding partners was investigated. When the parasites expressing GAP40-HA were incubated with the N-glycosylation inhibitor tunicamycin, neither GAP45 nor MLC1 protein can be immunoprecipitated by GAP40-HA (Fig. 7B, lanes 4). As a positive control, GAP45 and MLC1 can be specifically immunoprecipitated by
N-glycosylation of GAP50 is Pertinent to Parasite Motility and Host Cell Invasion—We and others have previously reported that tunicamycin, an inhibitor which prevents the addition of N-glycans to the Asn residues of N-glycoproteins, also acts in a peculiar manner in *T. gondii* (21, 22) compared with other eukaryotic cells. Although no obvious effects can be seen during the first round of intracellular development of tachyzoites inside the host cells, the tunicamycin-treated parasites were considerably reduced in motility and host cell invasion when they ruptured from the cell and are engaged in a second infection cycle. We observed that tunicamycin induces important alterations in the biogenesis of *T. gondii* IMC and the parasite motile apparatus are impaired. To determine whether the effect of tunicamycin observed specifically involved N-glycosylation of GAP50, we first wanted to knockout the gene encoding wild-type GAP50 in the transgenic parasites already containing a second mutant copy of the gene, which ectopically expressing either N-glycosylated or unglycosylated GAP50 protein. As previously reported (9), our attempts to knock-out GAP50 gene, despite the presence of the second gene copy, have also been unsuccessful. Therefore, we decided to investigate how the wild type and mutant parasites ectopically expressing wild type and unglycosylated GAP50-HAFLAG protein behave in the presence of tunicamycin. It should be noted that we chose to study a transgenic line where fluorescence and Western blot signals of ectopic GAP50-HAFLAG were roughly comparable to endogenous signals (9). In the transgenic parasites, the sum of GAP50 levels is made up of both ectopically expressed GAP50-HAFLAG plus endogenous GAP50 and, as a consequence, the overall GAP50 protein levels are higher than in wild-type parasites. We then monitored the gliding of drug-treated and untreated parasites using immunofluorescence staining to detect the trails left on slides by motile parasites (Fig. 8). When intracellular parasites expressing GAP50-HAFLAG protein ectopically in addition to wild-type GAP50 protein were incubated with tunicamycin, the freshly released ectopic expressers were able to display linear trail motility that was longer (Fig. 8D) than those of the normal wild type (Fig. 8B) and triple deglycosylated GAP50 mutant (Fig. 8F), also treated by the same concentration of tunicamycin. We estimated that the parasites expressing two copies of N-glycosylated GAP50 gene trailed three times longer than the two other parasite lines (Fig. 8G). In the absence of tunicamycin, these ectopically expressing GAP50 parasites displayed a normal circular gliding (Fig. 8C), a behavior typical of either wild type (Fig. 8A) or fully deglycosylated GAP50-expressing parasites (Fig. 8E). Interestingly, we notice that the circular trail, another characteristic of parasite motility, is completely abolished in all parasite lines in the presence of tunicamycin (Fig. 8, compare left panels A, C, and E to right panels B, D, and F). To establish whether tunicamycin could inhibit the host cell invasion of the three parasite lines, we monitored host cell re-infection after the first cycle of intracellular parasite treatment. Fig. 8H shows that the capability of the ectopic GAP50-expressors to display longer trails or motility in the presence of tunicamycin is consistent with these transgenic parasites being able to invade new host cells more efficiently (53%) than wild-type parasites (26%) and unglycosylated GAP50 mutants (29%). This suggests that tunicamycin does not completely shut down glycosylation under our experimental conditions, and the over-expression of GAP50 probably allows obtaining a larger share of the available N-glycans than the wild-type GAP50. We conclude that N-glycosylation plays key roles in the trafficking of gliding components to the IMC, in the assembly of functional glideosome that is required for parasite motility and host cell invasion.

**DISCUSSION**

In this study, we demonstrate that N-linked glycosylation plays two important roles in *T. gondii* GAP50. First, N-linked glycans are essential for the correct intracellular trafficking of GAP50-HAFLAG.
GAP50 and for its insertion into the IMC, a structure that powers parasite motility, migration through host tissues, host cell invasion and egress. Second, the interactions between components of the gliding machinery can be influenced by the N-glycosylation status of GAP50. Although the third N-glycosylation site is apparently not crucial, both the first or second N-linked sugar for GAP50 trafficking in *T. gondii* has to carry at least one N-linked sugar for GAP50 to be properly trafficked to the IMC. When drafting this paper, we were aware about the discovery of GAP40, a novel glideosome component recently validated by Frénal et al. (34), thereby defining this motor machinery as being composed of at least five proteins (MyoA-MLC1-GAP45-GAP50-GAP40). We independently identified GAP40 during our study as a hypothetical protein (TGME49_049850), which is present in both monomeric protein and is associated with the glideosome multiprotein complex pulled down using higher salt stringency. We showed that removal of N-glycans from GAP50 results in dissociation of this glideosome multicomplex complex and losses of several key glideosome components, including GAP40.

Interestingly, we also found that the two first key N-glycosylation sites for GAP50 trafficking in *T. gondii* are well-conserved in two other related apicomplexan parasites, *Neospora caninum* and *Eimeria tenella*, whereas the third N-glycosylation site, apparently not essential, is absent in *E. tenella* (supplemental Fig. S3). Furthermore, the two first putative N-glycosylation sites are also present as divergent sequons in GAP50 proteins of *Plasmodia* species. Despite, the apparent lack of numerous genes coding glycosyl-transferases involved in the assembly of complex N-glycan structures, biochemical studies recently indicated that *Plasmodium falciparum* also synthesizes N-glycans that are shorter than that of...
mammalian and T. gondii counterparts (35, 36). Interestingly, recent studies have also demonstrated that GAP50 of P. falciparum is located in the endoplasmic reticulum in early stage parasites (37). However, it remains to be determined if the shorter N-glycan structures could also play a direct role for trafficking P. falciparum GAP50 to the inner membrane complex.

Our present study provides for first time insights into how N-glycosylation could regulate correct protein folding and trafficking of N-glycoproteins in a model of apicomplexan parasites. It is tempting to hypothesize that in T. gondii, the correct protein trafficking may be ensured by the unusual N-glycan chains, which are present on the three potential glycosylated sites of GAP50. These N-glycan chains are composed of immature and truncated oligomannose-rich sugars, which in some cases contain abnormally uncleaved terminal glucoses that are rarely present on N-glycoproteins of higher eukaryotes. In addition, our studies also provide evidence on the degree of N-glycan heterogeneity and site occupancy on GAP50. The absence of these N-glycans on the first two sites of GAP50 held the mutated protein in the ER/Golgi. Thus, the deglycosylated GAP50 cannot reach the IMC and participate in glideosome formation.

Previous studies have recently proposed models of how the glideosome is inserted in the IMC and this is schematically depicted in Fig. 9, updated according to the potential of N-glycosylation functions reported herein. This model suggests that GAP50 functions as the protein that anchors the glideosome via its transmembrane domain, with the much larger N-terminal part of the protein being located in the lumen of the IMC, except the last C-terminal six amino acids that is involved in binding to GAP45, the novel GAP40 and the motor MyoA-MLC1-Actin. Even though the simplest interpretation of N-glycans functions on GAP50 protein could be that unglycosylated GAP50 is stuck in the trafficking ER and Golgi pathway, thereby preventing its interactions with other glideosome partners, we also demonstrated that deglycosylated GAP50 cannot physically bind to partners. This supports the notion that glycan-glycan or glycan-protein interactions may be involved in the glideosome formation and parasite’s motility. Furthermore, we show in this study that N-glycosylation status of GAP50 is important for parasite gliding and host cell invasion.

Because the three N-glycans of GAP50 protein are located inside the lumen of the IMC, we hypothesize that these N-glycan chains may impact glideosome assembly and functions at the opposite side of the outer IMC membrane through glycan-glycan and/or glycan-protein interactions (Fig. 9). One of these GAP50 interacting partners that could cross the outer IMC is the novel GAP40, which contains nine spanning transmembrane domains and one potential N-glycosylation site. However, the exact topology of

**Fig. 9. Proposed model of N-glycan functions of T. gondii GAP50 in trafficking and binding key glideosome partners.** According to the currently accepted model, which has been recently revisited and improved by Frénal et al. (34), our data indicate that N-glycosylation may be a key post-translational modification for GAP50 transport through the classical ER-Golgi secretory pathway (left panel) until its final destination, the IMC, where it will associate with other glideosome partners (right panel). The N-glycans on GAP50 are not only essential for proper trafficking but probably also important for direct or indirect interactions with the other glideosome partners GAP45, GAP40, MLC1, MyoA, and Actin.
GAP40, its precise N-glycosylation status and contribution to the glideosome’s functions will await further experimental investigations.

The conservation of the motor complex across apicomplexan parasites is further supported by the recent demonstration that gliding motility of these pathogens to invade host cells can be inhibited in *T. gondii* (38), *Cryptosporidium* (39), *Theileria* (40), and *Babesia* (41). Together with the presence of glideosome in *Plasmodium falciparum* parasite motility, such as the unusual and some functions, such as phosphorylation (16), acylation (17) -glycosylation (21, 22, this study). A detailed understanding of the biological functions of factors that modulate parasite motility, such as the unusual N-glycan structures identified in *T. gondii*, and the conservation of this motor complex with numerous hypothetical proteins exclusively found in apicomplexan parasites highlights potential chemotherapeutic targets that may be applicable for a wide variety of these important human and livestock pathogens.

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[This article contains supplemental Figs S1 to S3 and Table S1.]

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