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Characterization of *T. gondii* tissue cyst lectin reactivity and the identification of novel glycoproteins

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Introduction

The success of *Toxoplasma gondii* as a pathogen is contributed to by the extensive host range of the asexually replicating forms (1,2). As such any warm blooded animal can serve as a reservoir for the parasite providing a means of transmission through the act of carnivory due to the ingestion of tissue cysts (2,3). Tissue cysts are typically maintained for the life of the host and the factors contributing to their reactivation and the potential for symptomatic disease in the context of immune suppression remain elusive (2). Understanding mechanisms of *Toxoplasma* tissue cyst biogenesis and maintenance are crucial to defining their biology and pathogenic potential in the context of immune suppression.

The study of glycosylation pathways has been fairly limited in *Toxoplasma gondii*. Much of what we know about the glycans present in the parasite comes from studies that defined the spatial binding patterns of several lectins (4-8) as well as more recent biochemical studies (9-11). Insights into the potential for glycan diversity can be gleaned from the presence or absence of specific enzymatic activities in the parasite genome. Such studies indicate the potential for both N-linked (9,12) and O-linked modifications (13-15) are encoded in the genome. Notably, the *in silico* data suggests that *T. gondii* (and other Apicomplexa) possess a truncated pathway for N-glycosylation (9,12), raising questions regarding the complexity of N-glycans modifying target proteins.

Glycosyltransferases (GT) tend to possess narrow specificities with regard to the specific monosaccharide they add, the nature of the linkage and the target terminal sugar, amino acid, or lipid they modify. This narrow specificity combined with the observed diversity of glycan structures necessitates a broad range of enzymatic activities. Not surprisingly, there are 92 distinct families of GTs with additional sequences that cannot be readily fit into any specific family (http://www.cazy.org/GlycosylTransferases.html). At this time the diversity of GT's encoded in the *T. gondii* genome is not known. We further reasoned that the presence of lectin reactivity associated with the parasite or vacuole/tissue cyst, in the absence of a potential GT to catalyze the reaction in the parasite genome, would suggest the involvement of a host activity.

Recent work suggest that transient fusion events occur between the tachyzoite parasitophorous vacuole membrane (PVM) and the associated host endoplasmic reticulum (ER) (16). In addition data from the Coppens lab (personal communication) suggests that host Golgi derived vesicles are intercepted by the *T. gondii* vacuole. Such dynamic interactions between the PVM and the 2 primary host organelles involved in glycosylation (17) present a potential mechanism for the delivery of host cell activities to the vacuole. We hypothesize that potential fusion at the PVM of cellular organelles in the tachyzoite vacuole and during cystogenesis contributes to the glycosylation of the developing cyst wall.

Lectin reactivity of *T. gondii* tissue cysts

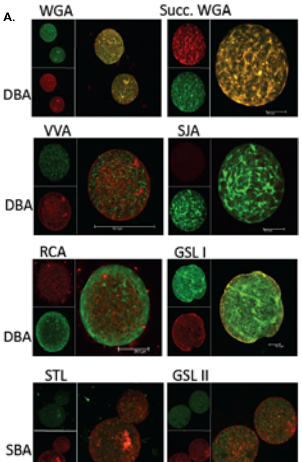
We selected a group of lectins to stain both tachyzoites and tissue cysts based on 2 criteria. The first was the revalidation of prior published studies in the organism and within the vacuole/tissue cyst. The second criterion was the use of the lectins, which were used to select for host cell lines resistant to their cytotoxic effects in the classic studies by the Stanley laboratory (reviewed in (18)). We reason that these well defined lines can be exploited to establish whether or not host activities are involved in the glycosylation of the targets within the parasite vacuole or cyst.

Lectin	Sugar Specificity	Brain Tissue Cyst	Brain Cyst Wall	Induced Cyst (tissue culture)	Tachyzoite Label
Dolichos (DBA)	Term α-GalNAc	Wall and Matrix	++++	Cyst wall	Weak Rhoptry
Soybean (SBA)	Term α-GalNAc	Wall and Matrix	++++	Cyst wall	Rhoptry
Viccia villosa (VVA)	Term α-GalNAc	Wall and Matrix	++++	Cyst wall and ER	Rhoptry
Peanut (PNA)	Unblocked Galactosyl-β1,3 GalNAc	No staining	No staining	Puncta in parasite Weak cyst wall	No staining
Jacalin lectin (JAC)	Sialic acid blocked OR unblocked Galactosyl-β1,3 GalNAc	Wall and Matrix	+++	ER Weak cyst wall label	No staining
Concanavalin A (ConA)	Mannose	Cyst Matrix	No staining	ER	ER
Datura Stramonium (DSA)	Tri-Tetra-antennary Complex N-Glycans (chitobiose)	Cyst Matrix	No staining	No staining	No staining
Lens culinaris (LCA)	Bi-Tri antennary Complex N Glycans	Cyst matrix	No staining	No staining	Weak ER
Ulex europaeus (UEA)	α1,2-Fucose	Wall and Matrix	+	No staining	ER and Weak Apical
Griffonia I-I (GSL I)	α-GalNac,α-Gal	Wall and Matrix	++	Not determined	Not determined
Griffonia L-II (GSL II)	α or β GlcNAc	Bradyzoite Puncta	no staining	Weak ER	Weak Apical
Wheat Germ Ag (WGA)	GlcNAc3-sialic acid	Wall and Matrix	+++	Weak cyst wall	No staining
Succinylated WGA (SuccWGA)	GlcNAc3 not sialic acid	Wall and Matrix	+++	No staining	No staining

Table 1. Outlines the staining patterns of warious lectins in brain cysts, induced cysts, and tachyzoites. The green highlighted rows are sialic acid recognizing lectins. The yellow highllighted rows recognize N-glycosylated sugars.

Lectin reactivity in brain cysts

Type II ME49 cysts were purified from chronically infected mouse brains on a Percoll gradient. The tissue cysts were washed and tittered and deposited on a glass slide using a Cytospin centrifuge. The slides were fixed in cold methanol (-20ºC), blocked with Carbo-free blocking buffer (Vector Labs), and stained with either FITC or rhodamine conjugated lectins. All lectins were purchased from vector labs. The lectin reactivity patterns were examined and revealed staining for the cyst wall (DBA, WGA, UEA, GSL I), cyst matrix (DBA, WGA, ConA, LCA, UEA), and bradyzoites (GSL II, VVL).



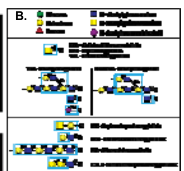


Figure 1. A Immunofluorescent images of brain derived tissue cysts. Tissue cysts were stained with either DBA or SBA to outline the cyst wall and a second lectin to determine the staining pattern **B** Schematic of lectin reactivity illustrating the sugars the lecitns recognize.

The diversity of lectin reactivity suggests a more complex repertoire of glycans than has been appreciated. Correlating lectin reactivity to the activities encoded in the genome will allow the establishment of parasite or host involvement.

N-Glycosylation pathway in *T. gondii*

The machinery of N-linked glycosylation in eukaryotes has been extensively characterized (19,20) (Figure 2). While the pathway is intact in yeast, it is much reduced in Apicomplexa (9,11,12). The extent of attrition in this pathway has become apparent from both bioinformatic (summarized in Table 1) and biochemical analyses (10,11,21). It is notable that the requirements for the diversity in glycan rich structures may dictate the extent of completeness in the pathway. What is also notable is that NONE of the Apicomplexan genomes appear to have homologues for ALG3, ALG9 and ALG12 (9,11,12). Thus the genetic capacity for N-linked glycosylation appears linked to the requirements for glycosylation in the life cycles for the parasite.

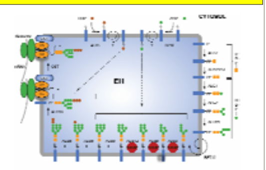
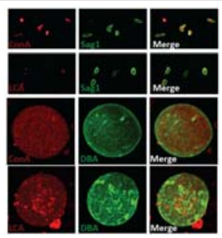


Fig. 2. Enzymatic Machinery of N-Glycosylation. The process of N-glycosylation begins with the addition of GlcNAc to the Dolichol (Dol) lipid precursor. The addition of mannose residues in the cytoplasm is catalyzed by ALG1, ALG2 and ALG11. A flippase activity transfers the lipid linked glycan into the lumen of the ER where the sequential addition of mannose is catalyzed by ALG3, ALG9 and ALG12. The ALG 6, ALG8 and ALG10 activities add glucose completing the glycan for en bloc transfer to the polypeptide by the oligosaccharide transferase (OST). Further modification of the sugar occurs in the Golgi.

The *T. gondii* genome lacks ALG3, ALG9 and ALG12 and is believed to be deficient in the synthesis of complex N-Glycans

Detection of complex N-glycosylation in cysts



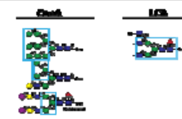


Figure 3: N-glycosylation in tachyzoites and brain derived tissue cysts. Tachyzoites label robustly with Concanavalin A (ConA) but weakly with *Lens culinaris* (LCA) which detects complex N-glycans. SAG1 labels the parasite surface. In contrast robust LCA staining is seen with LCA in tissue cysts. Note that ConA/LCA staining is excluded from the cyst wall seen with Dolichos DBA(green).

Complex N-glycans are found in the matrix of mature tissue cysts but are not extensive in tachyzoites.

Purification of glycosylated proteins

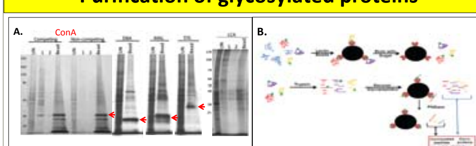


Figure 4A.
Lectin affinity pulldowns reveal a diversity of glyco-proteins in *T. gondii* tachyzoites.

ConA competing sugar : 200mM α-methyl-mannoside,
ConA non-competing sugar : 200mM α-acetyl galactosamine
LCA competing sugar : 200mM α-methyl-mannoside
Arrowhead- Lectin band.

Figure 4B: Proteomic scheme for the identification of glycoproteins (example shown is for N-glycosylated proteins): Extracts of tachyzoites and tissue cysts will be incubated with Lectin conjugated magnetic beads. N-glycosylated proteins (green circle) captures by the lectin will be eluted with the appropriate competing sugar. The eluate will be prepped by TSGE and trypsinized prior to MuDPIT identification of targets. In order to identify the specific glycan-modified peptides within the targets lectin bead affinity will be used. The bead captured glycopeptides will be treated with PNGase to liberate the peptides which following TSGE will be identified by MuDPIT.

T. gondii cysts are sialyated

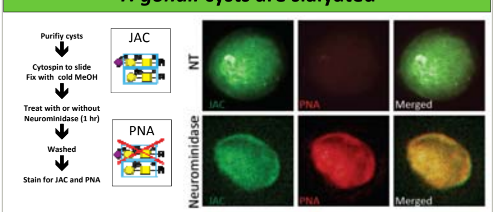


Figure 5. Detection and confirmation of sialylation on the tissue cyst surface. Tissue cysts label robustly with Jaclin (JAC) lectin but not Peanut Agglutanin (PNA) even though both recognize the same glycan (galactosyl-1,3 GalNac (T-antigen)). **While JAC binds T antigen in both the presence or absence of a terminal sialic acid, this modification blocks PNA binding**. We treated brain derived tissue cysts with Neuraminidase as described. **Neuraminidase results in the removal of the terminal sialic acid now permitting PNA binding.**

The detection of terminal sialic acid on the tissue cyst suggests that *T. gondii* must possess sialyltransferases (SiaT) (Fig. 6A). Alternatively the parasite may hijack host cell activities. SiaT's are defined by the presence of a distinct motif, the Sialyl-motif, that defines the substrate (cmp-Neuraminic (sialic) acid) (22,23) (Figure 6B) which allows for the bioinformatic identification of potential homologs in the *T. gondii* genome. A failure to identify a potential homolog would suggest that host SiaT's catalyze the modification of Galactosyl-1,3 GalNAc in the tissue cyst.

Figure 6A The sialyltransferase reaction. And classes of mammalian SiaT's. **B.** The Sialyl-motif of ST3-GAIII

Bioinformatic Search for Sialyltransferases in the Toxoplasma genome

Text search for Toxoplasma and Sialyltransferase in PubMED, ToxoDB, EupathDB produced NO hits.

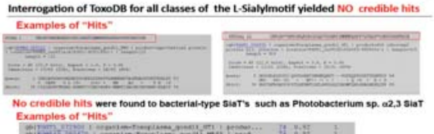
Text Search for "Sialyltransferase " in NCBI yielded 305 hits for whole genome sequences though none in Toxoplasma or Apicomplexa. 75 hits were obtained in the human genome. Analysis of all 42 NCBI databases yielded 28526 genome survey sequence records (GSS) with none in Toxoplasma or EupathDB.

Interrogation of ToxoDB for all classes of the L-Sialylmotif yielded NO credible hits

Examples of "Hits"

No credible hits were found to bacterial-type SiaT's such as Photobacterium sp. α2,3 SiaT

Examples of "Hits"



Conclusion: The Toxoplasma genome does NOT encode any Sialyltransferases

Does the host cell play a role in cyst formation?

The contribution of PVM-organelle interaction in tissue cyst formation has not been investigated. The classic studies of Ferguson (24-27) and others (28) investigated the organization of the tissue cyst wall in brain derived cysts by EM. Could the fate of PVM-associated ER be its incorporation into the tissue cyst wall? More relevant could cystogenesis be promoted by the delivery of host GT's to the nascent cyst wall? Our observation of extensive vacuolation and fusion at the PVM (Figure 7) of Compound 1 (29) treated parasites suggests dynamic interactions at the parasite host interface accompany tissue cyst formation. The host ER/Golgi and the parasitophorous vacuole represent distinct membrane bound structures within the infected cell. Recent work from the Sher and Coppens laboratories demonstrate the localization of host ER proteins (16) and Golgi lipids (Coppens, personal communication) in the vacuolar space although the mechanisms by which they are delivered remain elusive. Such a mechanism, accelerated during cyst formation (Figure 7) could provide a mechanism for the delivery of host GT's to the parasite.

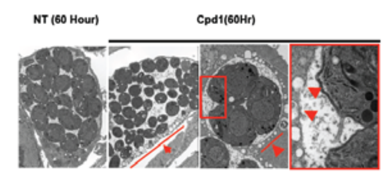
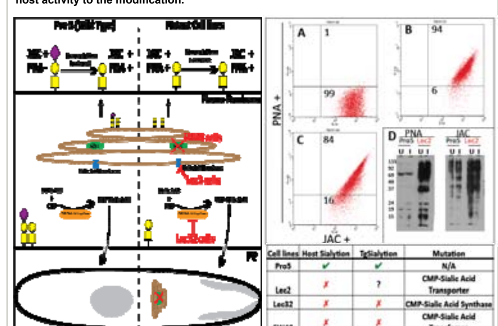


Figure 7. Vacuolation and potential fusion of host vesicles at the PVM accompany induced cyst formation. *T. gondii* (ME49) vacuole at 60 hours post infection of HFF cell (NT). Note the organization of the tubule-vesicular network within the vacuolar space. Tissue cyst formation induced by treatment with compound 1 for 60 hours results in the development of granular matrix and the appearance of vesicular structures lining the PVM (red line and arrow panel2,3). Higher magnification of the boxed region in panel 3 reveals potential fusion events at the PVM with vesicles of unknown provenance (4th panel, arrowheads).

Use of mutant host cell lines

The use of lectins to investigate the activities involved in glycosylation has a long history. The cytotoxic properties of lectins were exploited by the Stanley group to isolate CHO cell mutants based on their resistance to exogenously added lectins (a comprehensive review is found in (18)). This panel of mutants has been instrumental in the identification of diverse activities contributing broadly to each step of the glycosylation machinery. Mapping the genetic basis for resistance has resulted in the identification of enzymes involved the charging of nucleotide sugars, their transport to the sites of glycan synthesis as well as GT's themselves. The mutant lines are classified as "Lec#" lines based on their phenotypes. We can also target specific host genes using RNAi knockdowns.

In our initial screen for lectin reactivity (Table 1) we focused on lectins that were used by the Stanley group to isolate the "Lec" mutants (18). Several of these exhibited reactivity in *T. gondii* tachyzoites and/or tissue cysts (Table 1). We reasoned that if lectin reactivity is in fact observed, and the gene(s) required for the specific modification is absent in the parasite genome (based on the analysis of the GT repertoire) then we would have ready access to a deficient cell line to rapidly test the contribution of the specific host activity to the modification.



Cell lines	Host Sialytion	TgSialytion	Mutation
Pro5	✔	✔	N/A
Lec2	✗	?	CMP-Sialic Acid Transporter
Lec32	✗	✗	CMP-Sialic Acid Synthase
SW48	✗	✗	CMP-Sialic Acid Transferase

Figure 8. Use of host cell mutants with defects in sialylation. Left panel: Schematic representation of the phenotypes of Wild type (Pro5-) and mutant cells. The **Lec2 mutant** is defective in the Golgi CMP-sialic acid transporter. **Lec32 cells** cannot make the substrate CMP-sialic acid in the host cytoplasm and **SW48** cells lack all SIaT's**. Top right: A.** Flow cytometry of untreated **Pro5-** cells following labeling with PNA and JAC. **Pro5- cells are JAC+PNA-**. **B**. The failure to sialylate proteins renders **LEC2 cells JAC+PNA+ C**: Wild type **Pro5- cells treated with neuraminidase become JAC+ PNA+** proving that the PNA target is blocked by sialylation. **D.** Lectin blots using PNA and JAC validate the FACS data. **Bottom Right**: Expected host and *T. gondii* sialylation phenotypes in wild type and mutant host cells.

Establishing the host role in the sialyaltion of *T. gondii* proteins. Our results suggest that the *T. gondii* cyst has terminally sialylated proteins (Figure 5, Table 1) despite the parasite lacking known sialyltrasferases (Figure 6). The Lec2.6A CHO cell line exhibits minimal reactivity with sialic acid directed lectins in both intact cells and lectin blots relative to the control cells (30). The inability of Lec2.6A cells to sialylate its glycans stems from a defect in the CMP-sialic acid Golgi transporter which results in the resident SiaT's lacking their substrate (30). CMP-sialic acid is however made in the host cytoplasm which would make it accessible the PV lumen due to the pore activity of the PVM (31) (Figure 8) . SW48 cells (available from ATCC) are reported to lack all SiaT activities (32,33) (Figure 8). Infection of these cells can be used to discriminate whether the potential sialylation of parasite proteins seen in Lec2.6A cells is due to host or parasite encoded activities. If the former is true, we expect to see no sialylated proteins. In contrast if parasite encoded activities are responsible then we would expect to find sialylated proteins in the infected SW48 cells. The contribution of host CMP-sialic acid to the sialylation of parasite proteins will be confirmed using the LEC32 line which is deficient in CMP-sialic acid synthase (18) (Figure 8).

Summary

1. **The diversity of lectin reactivity indicates the glycome of the parasite is more complex than previously thought.**
2. **Selectivity of the lectins can be exploited to identify *T. gondii* glycoproteins.**
3. **Lectin reactivity for complex N-glycans and sialic acid is observed despite the *T. gondii* genome lacking key genes.**
4. **Tissue cyst glycosylation may in part be mediated by the host cell.**
5. **Host glycosylation mutants can directly address the host contribution.**

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