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Initial Characterization of CST1, a *Toxoplasma gondii* Cyst Wall Glycoprotein

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***Toxoplasma gondii* is an important protozoan pathogen of humans that can cause encephalitis in immunocompromised individuals such as those with AIDS. This encephalitis is due to reactivation of latent infection in *T. gondii*-seropositive patients. Latent organisms survive within tissue cysts, which are specialized parasitophorous vacuoles containing bradyzoites. The cyst wall of this structure is produced by modification of the parasitophorous vacuole by the parasite and is important in cyst survival. The components of the cyst wall have been poorly characterized. By using immunofluorescence and immunoelectron microscopy, we have identified a monoclonal antibody (MAb 93.18) that reacts with the cyst wall. This antibody recognizes a 116-kDa glycoprotein, which we have termed CST1, containing sugar residues that bind *Dolichos biflorans* lectin (DBA). CST1 is distinct from *T. gondii* antigen labeled with succinyl *Triticum vulgare* lectin (S-WGA) and represents the major DBA-binding component in *T. gondii*. The carbohydrate components of the tissue cyst, such as CST1, are probably important in both providing stability and facilitating persistence in its host. As is seen in the carbohydrate capsules of fungi, glycoproteins in the *T. gondii* cyst wall may protect cysts from the immune response of the host. Further characterization of the formation of the cyst wall and its components should lead to insights into the mechanism of tissue cyst persistence and may suggest novel therapeutic approaches to eliminate tissue cysts of this organism.**

Toxoplasma gondii is a ubiquitous Apicomplexan parasite of humans and other animals and birds. It is an important opportunistic pathogen of immunocompromised hosts and is a major opportunistic pathogen of the AIDS epidemic (28, 29, 35). Although overwhelming disseminated toxoplasmosis has been reported, the predilection of this parasite for the central nervous system, causing necrotizing encephalitis, constitutes its major threat to patients with human immunodeficiency virus infection (AIDS) (28, 29).

The factors affecting the transition of tachyzoites to the latent bradyzoite stage remain to be defined. Although these stages are well defined morphologically, little is known about how interconversion from one to the other stage occurs or what signal(s) mediates this transformation (43). The identification of early and late bradyzoite differentiation markers is an important avenue of investigation. Several studies using transmission electron microscopy or bradyzoite-specific monoclonal antibodies (MAbs) have demonstrated the development of cyst-like structures in vitro (2, 3, 13, 20, 26, 34, 40, 45). Feeding experiments in cats have demonstrated that tissue culture-derived cysts are biologically identically to cysts obtained from animal tissues (11).

In the brain, *T. gondii* tissue cysts develop intracellularly within both neurons and astrocytes, persisting for months to perhaps years. Tissue cysts can be 50 to 100 μm in diameter and are separated from the host cell cytoplasm by a thick cyst wall (12). The cyst wall is thought to be important in maintaining the integrity of the cyst in host cells for long periods. The cyst wall consists of a highly invaginated outer membrane underlaid with a dense osmiophilic matrix containing membranous vesicles. It is 200 to 850 nm thick. An association of the host cell intermediate filaments with parasitophorous vacuoles, the membrane-bound compartment in which the tachyzoite stage replicates, has been described in Vero cells, and a similar association of glial fibrillary acidic protein is seen with the bradyzoite (cyst) vacuole (17–19). There is no evidence of structural integration of host cell intermediate filaments in the cyst wall; instead, glial filaments encase the cysts in the host cell during cyst development in host cells in vitro (19). This filament wrapping of tissue cysts may play a role in bradyzoite differentiation and/or cyst stabilization in the host cell cytoplasm.

Study of the *T. gondii* cyst wall, as well as clarification of the host cell contribution to this structure, is now possible due to the development of the reagents that recognize bradyzoites in vitro (13, 43). These reagents have demonstrated that modification of the parasitophorous vacuole into a developing cyst wall is an early event in differentiation. As early as 1 day postinfection in vitro, bradyzoite-specific cyst wall antigens, such as that recognized by MAb 73.18 (L. M. Weiss, D. LaPlace, H. B. Tanowitz, and M. Wittner, Letter, J. Infect. Dis. **66:**213–215, 1992), are already expressed and localized to the cyst wall. In this paper we present data on the localization and carbohydrate modifications of CST1, the 116-kDa cyst wall antigen recognized by MAb 73.18. The carbohydrate modifications of CST1 are probably important in providing stability to the tissue cyst and in facilitating cyst persistence in the host.

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In addition, as is seen in the carbohydrate capsules of fungi, this glycoprotein may be important in protecting tissue cysts from the immune response of the host.

MATERIALS AND METHODS

Parasite strains, tissue culture, and parasite purification. *T. gondii* type II strains ME49 and R5 (an atovaquone-resistant PDS mutant) (41) and a type I strain, RH, were utilized for these studies (21). R5 was used for these studies because it readily differentiates to bradyzoites under stress conditions (e.g., pH change or sodium nitroprusside treatment [43]). Parasites were maintained by serial passage in confluent monolayers of human foreskin fibroblast cells (ATCC CCD-27SK) grown in Dulbecco's modified Eagle's medium (pH 7.1) (Gibco-BRL, Gaithersburg, Md.) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin solution (Gibco-BRL). To induce the expression of bradyzoite-specific proteins, the culture medium was replaced with pH 8.1 medium 2 h after inoculation, and the medium was changed daily to keep the pH constant (45). Parasites were harvested at 5 to 6 days postinfection and released from the parasitophorous vacuoles by sequential passage of host cells through 23-, 25-, and 26-gauge needles. This material was passaged through a 3.0-μm-pore-size Nuclepore filter and centrifuged at 400 × *g* for 10 min. Parasites were resuspended in phosphate-buffered saline (PBS) and centrifuged at 400 × *g* for 10 min, and the pelleted parasites were stored at −70°C for protein analysis and purification.

In vivo cyst formation. BALB/c^{dm2} mice, which have a deletion of the *Ld* gene at the *HLA-2L* locus, were infected intraperitoneally with 20 cysts of *T. gondii* ME49 (7). Brains were removed from the mice at 4 to 8 weeks postinoculation, and ME49 cysts were isolated from murine brain tissue using isopycnic centrifugation as previously described (10; Weiss et al., Letter). Yields averaged 3,000 cysts per mouse with each cyst containing 500 to 2,000 bradyzoites.

Primary astrocyte culture. Murine astrocytes from C 57BL/6 × SV129 mice were cultivated from the brains of neonatal (<24-h-old) mice as previously described (16, 19). Murine pups were sacrificed, the brain was removed from the cranium, the forebrain was dissected, and the meninges were removed. The tissue was minced and incubated in 0.25% trypsin for 5 min at 37°C. After 5 min, the trypsin was inactivated with a solution containing DNase and soybean trypsin inhibitors and the tissue was further disrupted by trituration in a 20-ml pipette. The dissociated cells were filtered through a 74-μm-pore-size Nitex mesh, centrifuged at 200 × *g*, suspended in growth medium at a concentration of 10^6 cells/ml, and plated onto poly-L-lysine-coated dishes. Astrocytes were maintained in endotoxin-free minimal essential medium (Gibco-BRL) supplemented with 20% fetal bovine serum (Gibco-BRL), 5% glucose, and 100 U of penicillin and streptomycin per ml (Gibco-BRL). The growth medium was changed every 3 days. After 7 days in vitro, a confluent layer of 10^4 astrocytes/cm^2 was reached. By this method, the cells were found to be >95% astrocytes, as judged by positive staining for glial fibrillary acidic protein. Cultures contained <5% microglia, as identified by staining with the lectin BS1-B4 (Sigma, St. Louis, Mo.). Astrocytes were dissociated in trypsin-EDTA, replated onto poly-L-lysine-coated coverslips or 24-well plates at 10^4 cell/cm, and cultured for 7 to 10 days after replating. These astrocytes were then infected with *T. gondii* ME49 as described below.

In vitro cyst formation of *T. gondii* in murine astrocytes. *T. gondii* ME49 was used to inoculate murine astrocytes. To induce bradyzoite and cyst formation in vitro, the method previously described by Weiss et al. (45) was used with the following modifications. Briefly, murine astrocytes were infected with 10^4 ME49 *T. gondii*, and by 3 days postinoculation, 10 to 25% of the infected cells contained cysts. In some cultures, sodium nitroprusside (50 μM) was added to the culture medium to enhance bradyzoite differentiation and cyst formation.

Bradyzoite-specific antibodies. Monoclonal hybridomas were prepared as previously described (Weiss et al., Letter) from mice immunized intraperitoneally with 5,000 solubilized (sonicated) ME49 cysts, purified from BALB/c $H2^{dm2}$ mouse brains, that were emulsified with complete Freund's adjuvant. One of the bradyzoite-specific MAbs identified, MAb 73.18 (immunoglobulin G3 [IgG3]), reacted with the cyst wall of cysts purified from mouse brain (46). Another antibody, MAb 74.1.8 (IgG2b), has been demonstrated to recognize the cytoplasmic antigen BAG1/hsp30 (also known as BAG5) (1, 36). In addition, rabbit antisera to the recombinant bradyzoite-specific antigens MAG1, a matrix antigen (37), and BAG1 (33, 49) were utilized.

Indirect-immunofluorescence staining. Cultures were washed with Hanks balanced salt solution, fixed in 4% paraformaldehyde for 10 min, and washed three times with PBS. The slides were then permeabilized and blocked for 30 min in a solution containing 10% FBS, 10% lamb serum, and 0.1% Triton X-100. For immunofluorescent staining, cells were incubated in MAb 73.18 (diluted 1:50) for 1 h and then washed three times with PBS. They were then incubated with the secondary antibody rhodamine-conjugated anti-mouse IgG (no. 605-140; Boehringer Mannheim), washed three times with PBS, overlaid with 2.5% DABCO (1,4-diazabicyclo-[2,2,2] octane)–PBS, and viewed with a Nikon epifluorescence microscope. Some cultures were double labeled with MAb 73.18 and 1:100 biotinylated *Dolichos biflorus* lectin (DBA) (no. L6533; Sigma) and then incubated with streptavidin-Texas red (no. S-6370; Molecular Probes) and fluorescein-conjugated anti-mouse IgG. After being washed three times with PBS, the slides were overlaid with 2.5% DABCO–PBS and then viewed with a Nikon epifluorescence microscope. The lectin and all antibodies were diluted in PBS containing 10% FBS.

Transmission electron microscopy. Cysts isolated from mouse brain were fixed overnight at 4°C in 2.5% glutaraldehyde buffered in 0.1 M sodium cacodylate (pH 7.2). Following fixation, the cells were rinsed in 0.1 M sodium cacodylate buffer, postfixed in 1% OsO_4, dehydrated in a graded ethanol series, placed in propylene oxide, and embedded in Epon. Thin sections were placed on copper grids, stained with 4% uranyl acetate and 0.1% lead citrate, and then examined with a Philips JEOL 1200 transmission electron microscope operated at 80 kV.

Immunoelectron microscopy. Cysts isolated from murine brains were fixed in 0.5% glutaraldehyde–2% paraformaldehyde in 0.1 M cacodylate buffer for 1 h at 4°C, rinsed in cacodylate buffer, dehydrated through a graded ethanol series, embedded in LR White, and polymerized for 48 h at 60°C. The tissue blocks were sectioned, placed on nickel grids, and coated with Formvar and carbon. The grids were incubated in blocking buffer (1% bovine serum albumin and 1% Tween 20 in PBS) for 30 min and then incubated in MAb 73.18 (1:20 dilution) for 2 h and washed five times in blocking buffer. They were then incubated for 1 h in anti-mouse IgG conjugated to 20-nm gold particles. Some grids were incubated for 2 h in DBA conjugated to 10-nm gold particles (no. L4643 [Sigma], 1:20 dilution). All antibodies and the lectin were diluted in blocking buffer. Following the incubation with secondary antibody or lectin conjugated to gold, the grids were successively washed in blocking buffer, PBS–1% Tween, and PBS. They were then postfixed in 1% glutaraldehyde for 10 min and washed in PBS and then in water. They were stained with 4% uranyl acetate, counterstained with 0.1% lead citrate, and then examined on a JEOL 1200 transmission electron microscope operated at 80 kV.

Analysis by 2D SDS-PAGE. Purified *T. gondii* was resuspended in PBS to a concentration of 2×10^9 to 3×10^9 *T. gondii* cells/ml, freeze-thawed four times, mixed with an equal volume of first-dimension sample buffer (9.5 M urea, 2% Triton X-100, 5% β-mercaptoethanol, 1.6% Bio-Lyte 5/7 ampholye, 0.4% Bio-Lyte 3/10 ampholyte [Bio-Rad, Hercules, Calif.]), and incubated at room temperature for 15 min. Following incubation, the mixture was centrifuged at 14,000 × *g* for 10 min and the supernatant was used as crude extract for analysis.

Proteins were separated according to their isoelectric points in the first-dimension tube by using a Bio-Rad mini-Protean II two-dimensional (2D) system employing the method of O'Farrell et al. First-dimension capillary tube gels (4% acrylamide, 9.2 M urea, 20% Triton X-100, 1.6% BioLyte 5/7, 0.4% BioLyte 3/10, 0.01% ammonium persulfate, 0.1% *N,N,N′,N′*-tetramethylethylerediamine TEMED) were cast 1 day prior to electrophoresis. The tubes were prerun using the following protocol: 200 V for 10 min, 300 V for 15 min, and then 400 V for 15 min. After this prerun, 25 to 50 μl of prepared extract (≈4×10^8 parasites) was loaded into the capillary gel and the tubes were run at 500 V for 10 min followed by 750 V for 3.5 h. The tubes were then placed on the top of a 10% polyacrylamide slab gel and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V for 1.5 to 2 h in the second dimension by method of Laemmli as previously described (48). After electrophoresis, the gels were stained using Coomassie blue or transferred to a nitrocellulose membrane at 100 V for 1.5 h using the method of Towbin as previously described (48).

Immunoblotting and lectin binding. Nitrocellulose protein blots were washed thoroughly with PBS and blocked for 1 to 2 h in 5% nonfat dried milk in PBS. Immunoblotting was performed by room temperature incubation of these blots for 1 h with 1:200 MAb 73.18 in PBS containing 0.1% Tween 20 (PBS-T). The blots were then washed with PBS, incubated with a goat anti-mouse IgG-IgM-alkaline phosphatase conjugate (Tropix, Bedford, Mass.) at 1:5,000 for 1 h at room temperature, and washed with PBS-T. Antibody binding was visualized using nitroblue tetrazolium–5-bromo-4-chloro-3-indolylphosphate (NBT-BCIP) or by chemiluminescence using [disodium 3-(4-methoxyspiro{1,2-dioxetane-3,2′-(5′-chloro)tricyclo[3.3.1.$1^{3,7}$] decan}-4-yl)phenyl phosphate (CSPD; Tropix, Bedford, Mass.). Lectin binding was performed in an analogous fashion. After incubation for 1 h with 5% nonfat dried milk, the blots were incubated with either 20 μg of DBA-alkaline phosphatase conjugate per ml or 10 μg of succinyl *Triticum vulgare* lectin (S-WGA)-horseradish peroxidase conjugate (EY Laboratories, San Mateo, Calif.) per ml for 1 h at room temperature. The blots were then washed in PBS-T, and lectin binding was identified by incubation with

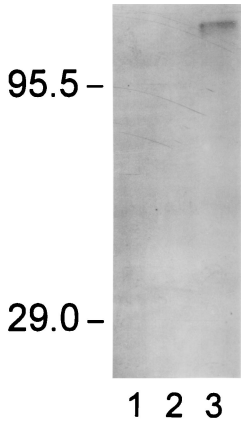

FIG. 1. Immunoblot using MAb 73.18. Lanes: 1, Human fibroblast cells; 2, pH 7.1-grown tachyzoites of *T. gondii* RH; 3, pH 8.1-treated *T. gondii* R5 purified from human fibroblast cells. Equal amounts of protein were loaded in lanes 1 to 3.

NBT-BCIP or CSPD for DBA-alkaline phosphatase and 4-chloro-1-naphthol for S-WGA–horseradish peroxidase.

Protein and carbohydrate analysis. *T. gondii* R5 strain extracts grown at pH 8.1 (10^7 parasites per lane) were separated by SDS-PAGE and transferred onto nitrocellulose membranes. The blots were treated either with 25 mM sodium metaperiodate in 50 mM sodium acetate buffer (pH 4.3) for 1 h in the dark or with 1 mg of proteinase K per ml in 50 mM Tris-HCl buffer (pH 7.5) for 1 h at room temperature. Following this treatment, the blots were washed thoroughly and blocked with 5% nonfat dried milk in PBS prior to antibody incubation.

Purification of CST1 by lectin chromatography. An extract of *T. gondii* R5 strain grown at pH 8.1 was prepared by four freeze-thaw cycles in PBS containing 4% 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate (CHAPS). The detergent (CHAPS) was then removed by affinity matrix chromatography using an Extracti-Gel D-CHAPS column (Pierce, Rockford, Ill.). The extract was then loaded on a DBA-Sepharose column (EY Laboratories), and the column was extensively washed with LAC buffer (10 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.02% NaN_3) to remove nonbinding proteins. The bound material was eluted using *N*-acetyl-D-galactosamine at 60°C, and the eluate was concentrated using an Ultrafree-15 filter (Millipore, Waterford, Mass.) for further analysis.

RESULTS

MAb 73.18 recognizes a bradyzoite-specific 116-kDa glycoprotein. MAb 73.18 reacted with a 116-kDa antigen by immunoblot analysis both in cysts of *T. gondii* ME49 isolated from mouse brain (data not shown) and in *T. gondii* R5 cysts developing in vitro (Fig. 1, lane 3). There was no reactivity of MAb 73.18 with either human fibroblasts (lane 1) or *T. gondii* RH tachyzoites (lane 2). Tachyzoites from either ME49 or R5 at pH 7.1 also had no reactivity with MAb 73.18 (data not shown). A similar-size protein has been demonstrated by radioiodination of intact cysts followed by SDS-PAGE (38, 39). Immunoreactivity of MAb 73.18 to R5 lysate was destroyed by incubation of blots with either 25 mM sodium metaperiodate (pH 4.3 acetate buffer) or proteinase K (1 mg/ml) (data not shown). Thus, MAb 73.18 appears to be directed at a carbohydrate epitope (consistent with its IgG3 subclass) on a glycoprotein found in bradyzoites and cysts of *T. gondii*. We have termed this 116-kDa reactive antigen CST1.

Localization of MAb 73.18 reactivity to the cyst wall. CST1 is located in the cyst wall (Fig. 2), i.e., the limiting membrane, of the bradyzoite parasitophorous vacuole both in vitro (Fig. 2B) and in vivo (Fig. 2C). In vitro bradyzoites that ruptured out of the parasitophorous vacuole had some cytoplasmic reactivity with MAb 73.18; however, this was of much lower intensity than that seen in the cyst wall. No reactivity was seen in tachyzoites that ruptured out of the tachyzoite parasitophorous vacuole or tachyzoites within this vacuole. During formation of the bradyzoite parasitophorous vacuole, CST1 reactivity (MAb 73.18) was evident on day 1 after exposure to stress conditions (pH 8.1 or SNP) that induce bradyzoite formation. Its expression was as rapid as that of BAG1. Parasite vacuoles positive for CST1 also express the bradyzoite-specific markers BAG1 and MAG1 (49).

Localization of CST1 by electron microscopy. *T. gondii* ME49 cysts were isolated from mouse brain and fixed for transmission and immunoelectron microscopy. Transmission electron microscopy of the cyst wall demonstrated a cyst wall, as characterized by an invaginated outer membrane and an underlying dense osmiophilic matrix (Fig. 3A). Immunoelectron microscopy of cysts stained with MAb 73.18 demonstrated labeling of the cyst wall matrix (20-nm gold [Fig. 3B]). A small amount of label was also seen in the cytoplasm of bradyzoites (data not shown), but this did not localize to any specific organelle. Immunoelectron microscopy of cysts stained with DBA also demonstrated labeling of the cyst wall matrix (10-nm gold, [Fig. 3C]).

Colocalization of lectin binding and CST1 by microscopy. Murine astrocytes were infected with *T. gondii* ME49 and in-

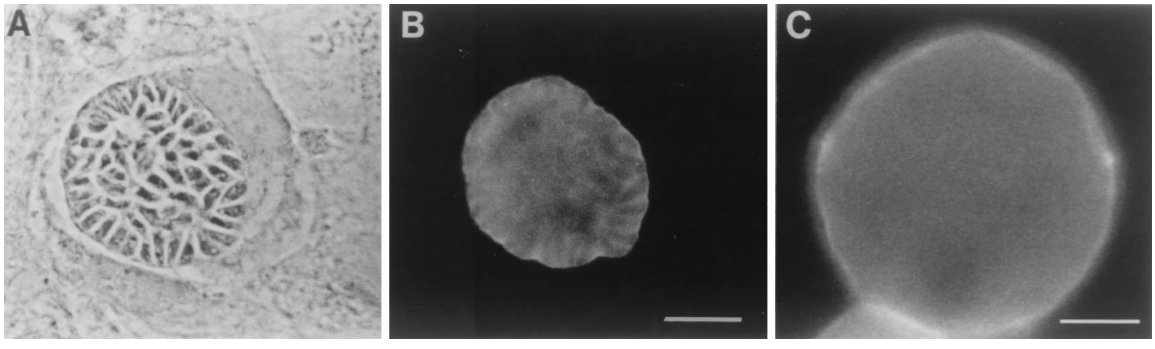

FIG. 2. Reactivity of MAb 73.18 with *T. gondii* cysts. (A) Phase-contrast microscopy of cyst from in vitro astrocytes infected with *T. gondii* ME49. (B) Immunofluorescence microscopy of the cyst in panel A stained with MAb 73.18. Bar, 10 μm. (C) Immunofluorescence microscopy of a cyst, isolated at 8 weeks from the brain of a mouse infected with *T. gondii* ME49, stained with MAb 73.18. Bar, 15 μm.

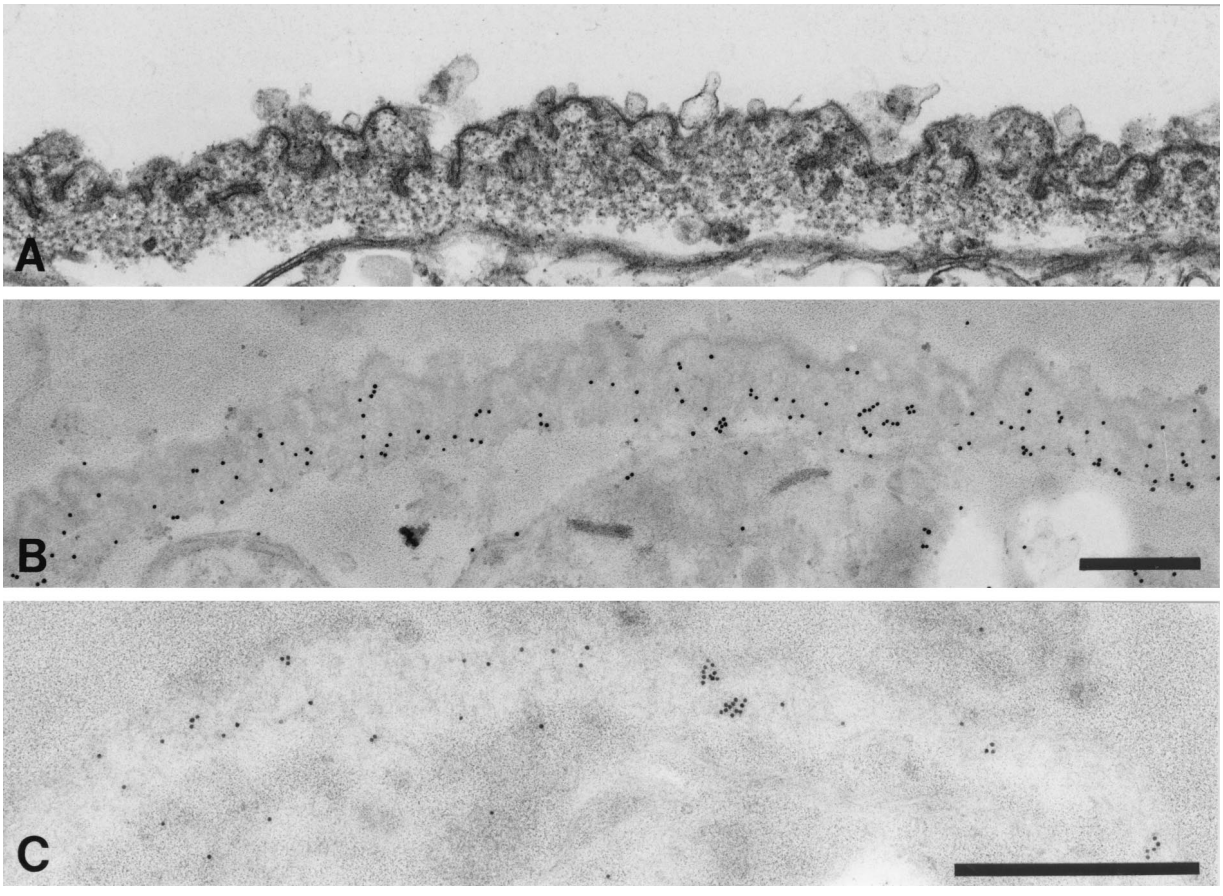

FIG. 3. Electron microscopy of the cyst wall of *T. gondii* cysts isolated from mouse brain. (A) Transmission electron microscopy of the cyst wall. (B) Immunoelectron microscopy of cysts stained with MAb 73.18, showing labeling of the cyst wall matrix (20-nm gold particles). (C) Immunoelectron microscopy of cysts stained with DBA, showing labeling of the cyst wall matrix (10-nm gold particles). Bars, 1 μm.

cubated at pH 8.1 for 3 days to induce bradyzoite formation in vitro. In vitro-derived cysts reacted with MAb 73.18 (Fig. 4A) and with DBA (Fig. 4B). An overlay of the vacuoles stained with both DBA and MAb 73.18 demonstrates a yellow color (Fig. 4C), indicating colocalization of MAb 73.18 and DBA staining.

Analysis of CST1 by 2D SDS-PAGE and lectin binding. Since the cyst wall is a critical structure in the stability and formation of intact tissue cysts of *T. gondii*, we sought to purify the CST1 glycoprotein for further analysis. Freeze-thawed extracts of *T. gondii* R5 grown for 5 days at pH 8.1 were analyzed using 2D SDS-PAGE (Fig. 5). A large number of proteins were evident when a 10% discontinuous polyacrylamide gel was used under reducing conditions (Fig. 5A). After the proteins were transferred to nitrocellulose, an immunoblot was performed using MAb 73.18 (Fig. 5B) and/or a lectin overlay with DBA (20 μg/ml) (Fig. 5C). A single major band was obtained at pI 5.7 and 116 kDa that reacted with both MAb 73.18 (Fig.

FIG. 4. Immunofluorescence of the cyst wall and lectin staining of *T. gondii* cysts formed in vitro in murine astrocytes. (A) Cysts stained with MAb 73.18 (FITC). (B) DBA staining (Texas red-streptavidin). (C) Overlay of MAb 73.18 and DBA staining; note the yellow color, indicating colocalization of the cyst wall and lectin staining.

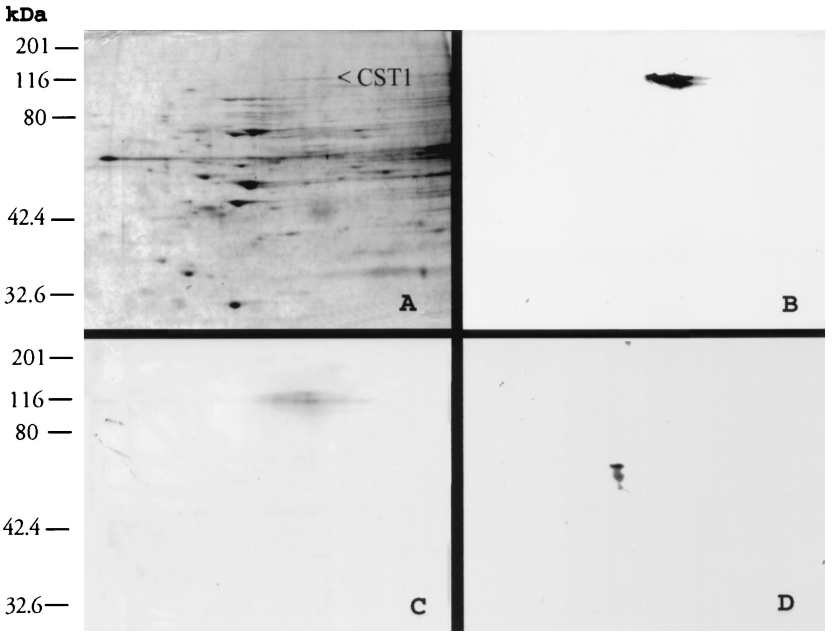

FIG. 5. 2 D electrophoresis of *T. gondii* R5 grown in human fibroblasts at pH 8.1. (A) Silver-stained 2D gel. The band indicated by < at pI 5.7 and 116 kDa corresponds to CST1, as defined by reactivity to MAb 73.18. (B) Immunoblot of a 2D gel with MAb 73.18, demonstrating that this antibody reacts with a 116-kDa band at pI 5.7. (C) Lectin overlay of a 2D gel using DBA. The identified band is at pI 5.7 and 116 kDa. This band can be superimposed on the immunostained band identified by MAb 73.18. (D) Lectin overlay using S-WGA. This demonstrates that S-WGA localizes to a protein distinct from that identified by DBA and MAb 73.18.

5B) and DBA (Fig. 5C). When the lectin S-WGA was used, the 116-kDa protein was not labeled, but instead a different antigen of about 48 kDa was labeled (Fig. 5D). Both DBA and S-WGA have been reported to label the cyst wall of *T. gondii* (6). This suggests that the carbohydrate modifications recognized by DBA and S-WGA are present on different proteins that make up the cyst wall.

Lectin affinity chromatography was used to further purify CST1. A pH 8.1-grown *T. gondii* R5 extract was loaded on a DBA-Sepharose column, and the column was extensively washed with LAC buffer to remove nonbinding proteins. No MAb 73.18-reactive material was found in the eluate. The bound material was eluted using *N*-acetyl-D-galactosamine at 60°C. On 2D electrophoresis, the eluted material was CST1, as defined by MAb 73.18 reactivity (Fig. 6) at 116 kDa, and the corresponding protein band was visible at 116 kDa using SYPRO orange.

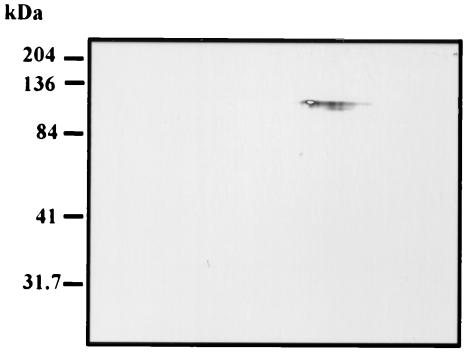

FIG. 6. Immunoblot of DBA chromatography-purified protein. Immunoblot of a 2D gel using the DBA affinity chromatography-purified material (see Materials and Methods) with MAb 73.18, demonstrating that this antibody reacts with a 116-kDa band at pI 5.7, consistent with purification of CST1.

DISCUSSION

The formation of the cyst wall and parasitophorous vacuole matrix is an early event that accompanies bradyzoite differentiation (4, 5, 14, 43). The vacuole, i.e., tissue cyst, in which bradyzoites develop can contain several thousand bradyzoites. The tissue cyst is entirely within a host cell, and the limiting membrane of this host cell can be seen by electron microscopy. The tissue cyst wall is a modification of the limiting membrane of the parasitophorous vacuole and is elastic, argyrophilic, and periodic acid-Schiff reactive (12, 24). The cyst wall and matrix probably protect bradyzoites from harsh environmental conditions and also provide a physical barrier to host immune defenses.

Both DBA and S-WGA have been used to demonstrate bradyzoite (cyst) formation in cell culture and are useful alternatives or adjuncts to immunofluorescence with bradyzoite-specific MAbs (6, 22, 43, 46). The cyst wall binds both DBA and S-WGA. This binding can be inhibited by competition with the sugar haptens *N*-acetylgalactosamine (GalNAc) for DBA and *N*-acetylglucosamine (GlcNAc) for S-WGA (6). Treatment with chitinase disrupts the cyst wall and eliminates S-WGA binding, consistent with the presence of chitin in this structure (6). Binding of DBA to the cyst wall of cysts of the related coccidian parasite *Neospora caninum* developing in vitro is also seen (46).

As demonstrated in this study, the 116-kDa antigen identi-

fied by MAb 73.18 (CST1) binds to DBA. A similar-size antigen, i.e., 116 kDa, is recognized by the sera of animals with chronic infection (38, 39, 51) and by rat MAb CC2 (15). By 2D electrophoresis, CST1 accounts for all of the DBA-binding activity in the cyst wall. CST1 does not bind S-WGA, and thus there appears to be a second cyst wall component that is responsible for the binding of S-WGA to *T. gondii* cysts. With the exception of glycosylphosphatidylinositol anchors, *T. gondii* proteins do not usually have carbohydrate modifications, and in fact little to no lectin binding is seen to the surface of either bradyzoites or tachyzoites (13). The carbohydrate modifications of CST1 are probably important in providing stability to the cyst and its persistence in the host. In addition, as is seen in the carbohydrate capsules of fungi (9), cyst wall glycoproteins may be important in protecting the tissue cysts from the immune response of the host either by being poorly immunogenic or by masking other epitopes.

In extracellular protozoa, such as *Giardia lamblia* and *Entamoeba histolytica*, trophozoites undergo fundamental biologic changes to survive outside the intestine of their host by differentiating into environmentally resistant cyst stages that contain carbohydrates in their cyst walls (23, 32). In *G. lamblia*, this is triggered by lipid starvation and other stresses, and formation of the cyst wall is associated with induction of hsp78/BiP, a heat shock protein (30, 31). In *T. gondii*, bradyzoite differentiation is also triggered by environmental stress (44, 45, 47) and is associated with heat shock protein expression. It is interesting that in the *T. gondii* EST database (http://www.cbil.upenn.edu/ParaDBs/Toxoplasma/index.html), 12 of the 13 clones with identity to hsp78/BiP expression are found in the ME49 bradyzoite in vivo EST library and only one is from the RH tachyzoite library. hsp 78/BiP may act as a chaperone for glycoproteins involved in the formation of the cyst wall during bradyzoite differentiation. In *G. lamblia*, the cyst wall is carbohydrate rich and contains GalNAc. Cyst formation is associated with the expression of UDP-*N*-acetylglucosamine pyrophosphorylase (8). Similarly, in yeast the unfolded-protein response is associated with induction of glycosylation enzymes (42). It is possible that tissue cyst formation in *T. gondii* is also associated with the induction of enzymes for the carbohydrate modification of proteins. Gene homologs associated with glycosylation, e.g., UDP-*N*-acetylglucosamine-1-phosphate transferase (GenBank no. N59933), are present in the *T. gondii* EST database. These pathways may offer new therapeutic targets for treatment of the bradyzoite stage of *T. gondii*.

Assembly of the cyst wall in *T. gondii*, as in *G. lamblia*, yeast, or bacteria, is likely to involve the expression and assembly of a series of genes and proteins in a sequential manner (27). This process is most probably dependent on both the timing of expression and the spatial localization of proteins during the assembly process. To date, only two genes have been identified that correspond to proteins identified in either the cyst wall or parasitophorous vacuole matrix of *T. gondii*. MAG1 (GenBank no. U09029) is a 65-kDa protein expressed in the matrix of the parasitophorous vacuole between bradyzoites and is also seen at the edge of the cyst wall by immunoelectron microscopy (37). The dense-granule protein GRA5 (GenBank no. L06091) (25) is found in both tachyzoites and bradyzoites within dense granules and the parasitophorous vacuole matrix. In tachyzoites, faint staining of the parasitophorous membrane is seen using antibody to GRA5; however, in bradyzoites, there is strong staining of the cyst wall, i.e., the limiting parasitophorous membrane (24). Like BAG1, both MAG1 and GRA5 appear early in bradyzoite vacuole formation, with antibody staining being evident by 1 day following stress induction (24). Other dense-granule antigens (GRA1 and GRA2) have been found in the matrix of both tachyzoite and bradyzoite vacuoles. While no other cyst wall-reactive MAbs have been reported, several MAbs have been identified that recognize bradyzoite-specific molecules localized to the parasitophorous vacuole matrix of developing cysts. These include a 29-kDa matrix antigen identified by MAb E7B2 (50) and a 19-kDa matrix antigen recognized by MAb1.23.29 (L. M. Weiss, unpublished data). The corresponding genes have not been identified.

The present paper describes the initial characterization of CST1, a glycosylated protein component of the cyst wall. Further studies on this protein, including identification of the corresponding gene, as well as investigation of the nature of the carbohydrate modifications of the cyst wall, are in progress. The cyst wall is the defining structure of the *T. gondii* tissue cyst and is most probably responsible for the persistence of bradyzoites in tissue. This modification of the limiting membrane of the parasitophorous vacuole may limit the communication of this intracellular parasite with its host cells. Additionally, the cyst wall probably limits antigen presentation to the host, contributing to the persistence of this intracellular parasite. Studies on the structure and function of the cyst wall may suggest novel therapeutic strategies for the elimination or prevention of latency during *T. gondii* infection.

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