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Toxoplasma gondii micronemal protein MIC1 is a lactose-binding lectin

Elaine V. Lourenço[2,3], Sandra R. Pereira[4,7], Vitor M. Faça[4,7], Arlete A. M. Coelho-Castelo[3], José R. Mineo[6], Maria-Cristina Roque-Barreira[1,2,3], Lewis J. Greene[2,4,5,7], and Ademilson Panunto-Castelo[2,3]

[2]Departamento de Biologia Celular e Molecular e Bioagentes Patogênicos, Faculdade de Medicina de Ribeirão Preto, USP, Av. Bandeirantes 3900, 14049-900, Ribeirão Preto, SP, Brazil,[3]Pós-graduação em Imunologia Básica e Aplicada, Universidade de São Paulo, Ribeirão Preto, SP 14049-900, Brazil, [4]Centro de Química de Proteínas, Universidade de São Paulo, Ribeirão Preto, SP 14049-900, Brazil,[5]Departamento de Ginecologia e Obstetrícia, Faculdade de Medicina de Ribeirão Preto, Universidade de São Paulo, Ribeirão Preto, SP 14049-900, Brazil, [6]Instituto de Ciências Biomédicas, Universidade Federal de Uberlândia, Uberlândia, MG 38400-902, Brazil, and [7]Departamento de Bioquímica, Universidade Federal de São Paulo, São Paulo, SP 04044-020, SP, Brazil.

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Host cell invasion by *Toxoplasma gondii* is a multistep process with one of the first steps being the apical release of micronemal proteins that interact with host receptors. We demonstrate here that micronemal protein 1 (MIC1) is a lactose-binding lectin. MIC1 and MIC4 were recovered in the lactose-eluted (Lac$^+$) fraction on affinity chromatography on immobilized lactose of the soluble antigen fraction from tachyzoites of the virulent RH strain. MIC1 and MIC4 were both identified by N-terminal microsequencing. MIC4 was also identified by sequencing cDNA clones isolated from an expression library following screening with mouse polyclonal anti-60/70 kDa (Lac$^+$ proteins) serum. This antiserum localized the Lac$^+$ proteins on the apical region of *T. gondii* tachyzoites by confocal microscopy. The Lac$^+$ fraction induced hemagglutination (mainly type A human erythrocytes), which was inhibited by β-galactosides (3 mM lactose and 12 mM galactose) but not by up to 100 mM melibiose (α-galactoside), fucose, mannose, or glucose or 0.2 mg/ml heparin. The lectin activity of the Lac$^+$ preparation was attributed to MIC1, because blotted MIC1, but not native MIC4, bound human erythrocyte type A and fetuin. The copurification of MIC1 and MIC4 may have been due to their association, as reported by others. These data suggest that MIC1 may act through its lectin activity during *T. gondii* infection.

Key words: Toxoplasma gondii/microneme/MIC1/MIC4/ lactose-binding lectin.

Introduction

Recent studies of the pathogenesis of parasitic disease have focused on the molecular mechanisms used by organisms to recognize and invade host cells. This is largely mediated by specific parasite molecules and complementary ligands on host cells. The properties of sugar recognition and discrimination by carbohydrate-binding proteins enable these proteins to contribute specifically to cell–parasite interactions (Cummings, 1999).

Host cell–parasite interaction is a complex stepwise process involving multiple receptors and complementary ligands on both cell types. *Toxoplasma gondii* is an obligate intracellular parasite and a highly cosmopolitan protozoan capable of invading and replicating within nucleated cells of warm-blooded animals (Jacobs, 1956). During the invasion process the parasite exhibits gliding motility on the surface of the cell, continually probing it with the conoid, an apical organelle. In response to a still unidentified signal during the probing process, the parasite reorients itself with its apical end attached to the host cell surface. Once the parasite has been oriented, invasion is preceded and accompanied by apical protein release from two secretory organelles of the parasite, that is, micronemes (Achbarou *et al.*, 1991) and rhoptries (Saffer *et al.*, 1992). The secretion of micronemal proteins is the first event to be visualized, and it is followed by parasite attachment to the host cell. This is rapidly followed by the discharge of rhoptries from the apical end, which decrease the viscosity of the target cell membrane, initiate its invagination, and enhance invasion (Bonhomme *et al.*, 1999). There is a great interest in the identification of ligand and receptor in the process of attachment and infection by *T. gondii*. The broad host range of *T. gondii* suggests that one or more host cell receptors are fairly common across different cell types or that multiple potential receptors are present.

In the present study we report lectin activity in a protein obtained from soluble *T. gondii* antigen adsorbed to immobilized lactose and eluted with a lactose solution. This preparation, designated Lac$^+$, presented two major proteins identified as the micronemal proteins MIC1 and MIC4. When the nitrocellulose-transferred Lac$^+$ proteins were incubated with erythrocytes or biotinylated fetuin, MIC1, but not MIC4, bound these ligands. These data demonstrate that MIC1 is a lectin.

Results

The total soluble antigen (STAg) fraction from *T. gondii* was submitted to affinity chromatography using an α-lactose-agarose column. The bound material eluted with lactose (Lac$^+$) corresponded to 0.2% of total protein based on amino acid analysis after acid hydrolysis. The Lac$^+$ fraction agglutinated type A

[1]To whom correspondence should be addressed

human erythrocytes. Its specific hemagglutination activity was 40 UH/µg for type A erythrocytes and 10 UH/µg for type B or O erythrocytes. Of the various sugars tested for their ability to inhibit agglutination of type A erythrocytes by the Lac+ preparation, only lactose and D-galactose were effective. Their minimal inhibitory concentrations were 3 mM and 12 mM, respectively. High concentrations (100 mM) of D-mannose, D-glucose, L-fucose, or melibiose or 0.2 mg/ml of heparin had no effects on agglutination induced by the Lac+ fraction.

The Lac+ fraction was characterized by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) under nonreducing and reducing conditions (Figure 1). Under nonreducing conditions (Figure 1, lane 2), the Lac+ fraction contained one protein of ~60 kDa, a double band at ~70 kDa, and a protein of ~120 kDa. Some less abundant smaller proteins were also observed. Under reducing conditions (Figure 1, lane 3), two major proteins of ~63 and ~78 kDa were observed.

To aid in identifying the 60-kDa and 70-kDa proteins, recovered under nonreducing conditions (Figure 1, lane 2), they were obtained by preparative SDS–PAGE, emulsified in Freund's adjuvant, and injected intraperitoneally (IP) into mice to generate antisera. By Western blot analysis, this mouse polyclonal anti-60/70-kDa serum was shown to be specific because it recognized the three components of the Lac+ preparation including the double band at 70 kDa (Figure 2, lane 1). The antiserum reacted only with the 60- and 70-kDa bands of STAg (Figure 2, lane 2) and was used to isolate clones from the cDNA library of *T. gondii*. Two clones were isolated and partially sequenced and both were identified as MIC4.

Because the Lac+ preparation contained at least two major proteins and only MIC4 was identified in the cDNA library, we determined the N-terminal sequence of each of the bands by automatic Edman degradation (Table I). The 60-kDa band had an N-terminal sequence of SH(S/A)H(A/S)PARPRY, which is identical to residues 26–36 of MIC1. Note that two amino

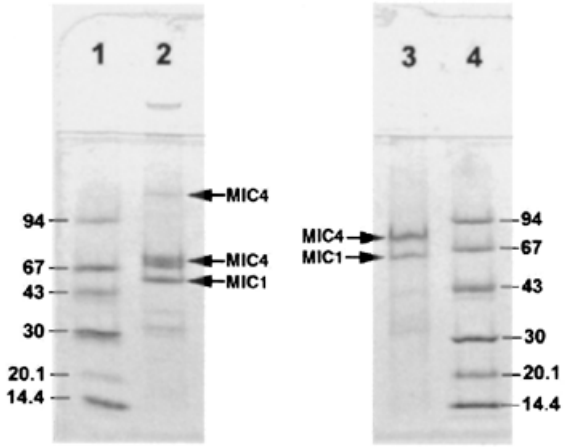

Fig. 1. SDS–PAGE of the Lac+ preparation under nonreducing and reducing conditions. Lanes 2 and 3 contain 2.5 µg of the Lac+ electrophoresed under nonreducing and reducing conditions, respectively. Lanes 1 and 4 contain 0.25 µg protein of the molecular mass standards proteins electrophoresed under nonreducing and reducing conditions, respectively. The molecular masses (kDa) of standard marker proteins are indicated on both sides of the figure. The identification of the micronemal proteins was based on N-terminal sequencing.

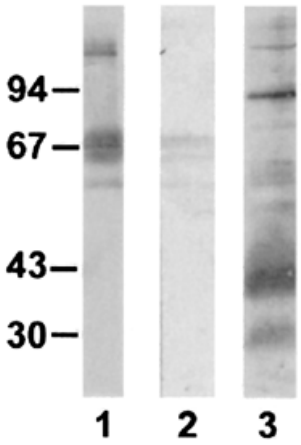

Fig. 2. Specificity of mouse polyclonal anti-60/70-kDa serum for Lac+ proteins. Lac+ fraction (20 µg) (lane 1) or STAg (lanes 2 and 3) were separated by SDS–PAGE under nonreducing conditions and transferred to nitrocellulose membranes. Membranes were incubated with either mouse anti-60/70-kDa serum (lanes 1 and 2) or mouse anti-STAg serum (lane 3). The bands were visualized using a peroxidase-conjugated goat anti-mouse IgG. The molecular masses (kDa) of standard marker proteins are indicated on the left side of the figure.

Table I. Comparison of N-terminal sequence of 60-, 70-, and 120-kDa Lac+ proteins, obtained by Edman degradation, with the MIC1 and MIC4 protein sequences predicted from cDNA sequences

Res. No.	26[a]	27	28	29	30	31	32	33	34	35	36
MIC1	S	H	S	H	S	P	A	R	P	R	Y
60 kDa	S	H	S/A	H	A/S	P	A	R	P	R	Y
Res. No	45	46	47	48	49	50	51	52	53	54	
MIC4	I	T	P	A	G	D	D	V	S	A	
70 kDa	I	T	P	A	G	D	D	V	S	A	
120 kDa	I	T	P	A	G	D	D	V	S	A	

[a]The numbers above the amino acids refer to the putative sequences of MIC1 and MIC4 (accession numbers Z71786 and AF143487, respectively, in GenBank). The amino acids were identified as amino acid phenylthiohydantoins produced by Edman degradation.

acids were obtained at residue position 3 and 5. The 70-kDa and 120-kDa bands both had N-terminal sequences of ITPAGDDVSA, which are identical to MIC4 (Table I). When Lac$^+$ was submitted to reverse-phase high-performance liquid chromatography (RP-HPLC) (Figure 3) the two major peaks were identified as MIC1 and MIC4 on the basis of their N-terminal sequences (data not shown).

The mouse polyclonal anti-60/70-kDa serum was used to label *T. gondii* tachyzoites. Under confocal microscopy, the parasites were labeled exclusively in the apical region (green in Figure 4), opposite to the nucleus (red in Figure 4), which is consistent with the localization of micronemes in Apicomplexan parasites.

To identify which protein was responsible for the lectin activity, the Lac$^+$ fraction was separated by SDS–PAGE and transferred to nitrocellulose membranes, which were incubated with erythrocytes (Figure 5, lane 2) or biotinylated fetuin (Figure 5, lane 3). Both erythrocytes and fetuin bound to the 60-kDa protein MIC1, but not to the 70-kDa protein MIC4 or to the 120-kDa protein presumably related to MIC4. When STAg was analyzed by the same technique, binding of fetuin to the 60-kDa protein, MIC1 was observed (Figure 5, lane 4). An additional weak staining of an 85-kDa protein in STAg was observed, but no staining of 70-kDa (MIC4) or 120-kDa proteins was demonstrable.

To determine if the denaturing conditions under which SDS–PAGE was carried out prevented the reaction of MIC4 with fetuin, we performed PAGE under mild conditions (pH 8.8) in the absence of SDS. The native gel of Lac$^+$ showed two major bands (Figure 6, lane 1). The faster one was sharp and identified by amino acid sequencing as MIC4. The slower running band was diffuse and provided insufficient protein for identification by sequencing. Because both native proteins were recognized by mouse polyclonal anti-60/70-kDa serum in the immunoblotting assay (Figure 6, lane 2), we assume that the diffuse band corresponds to MIC1. The data in Figure 6, lane 3 show that fetuin bound only to the diffuse band, MIC1, but not to MIC4.

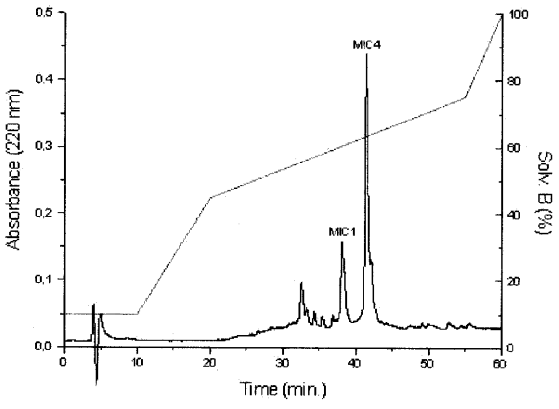

Fig. 3. RP-HPLC chromatographic profile of the Lac$^+$ proteins. Lac$^+$ proteins (30 µg) were applied to a Vydac C4 column (220 × 2.1 mm i.d.). The straight line represents the elution gradient, as described in *Materials and methods*. The peaks were identified by N-terminal sequencing.

Discussion

We have shown here that MIC1 from *T. gondii* is a lectin that recognizes β-galactoside. MIC1 was one of two major components of the lactose-bound fraction (Lac$^+$) obtained from STAg. The other component was MIC4, for which we could not demonstrate lectin activity. These conclusions were based on (1) the inhibition of hemagglutinating activity of Lac$^+$ by lactose and D-galactose, but not glucose, mannose, fucose, melibiose, or heparin; (2) correspondence of amino acid sequence between the 60- and 70-kDa proteins from Lac$^+$ with MIC1 and MIC4, respectively; (3) binding of human erythrocytes and fetuin to blotted MIC1 but not MIC4 even when separated under native conditions. These data strongly suggest that MIC1 is the lectin present in Lac$^+$ responsible for the adsorption to immobilized lactose and for the hemagglutinating activity.

Lectins, because of their property of recognition and their ability to discriminate among sugars, are excellent candidates to mediate interactions of parasites with complementary ligands on host cells. In an Apicomplexa species closely related to *T. gondii*, *Sarcocystis muris*, a microneme protein, was characterized as a lectin with affinity for N-acetylgalactosamine (Klein *et al.*, 1998). Although several investigators have suggested the participation of lectin molecules in the host cell invasion by the *T. gondii*, no lectin from *T. gondii* has been identified. Some clues to the presence of lectin in the *T. gondii* have been obtained, although the precise involvement of glycoconjugates or lectins in infection has remained elusive. Incubation of tachyzoites in the presence of gold-labeled albumin-N-acetyl-D-glucosamine or albumin-galactose, but not in the presence of albumin-mannose, led to labeling of the rhoptries (Carvalho *et al.*, 1991). The interaction between SAG1 and host cell surface can be inhibited by the bovine serum albumin (BSA)–glucosamide, suggesting that glycan receptors might be important in infection of cells (Mineo *et al.*, 1993). Gross *et al.* (1993) showed that fetuin bound specifically to a 15-kDa antigen of tachyzoites, a phenomenon inhibited by sialic acid and the lectin *Sambucus nigra* agglutinin. Monteiro *et al.* (1998) demonstrated that mutant cells that present few or no surface-exposed sialic acid residues were infected to a lower extent by *T. gondii*. Similar results were obtained if sialic acid residues were removed by previous neuraminidase treatment. These investigators observed that the addition of sialic acid residues to surface-exposed glycoconjugates using fetuin as a sialic acid donor and the trans-sialidase of *Trypanosoma cruzi* rendered the cells more easily infected by *T. gondii*.

Lac$^+$ has an electrophoretic profile (two main bands of 70 and 60 kDa) similar to the pattern obtained by Ortega-Barria and Boothroyd (1999), who examined electrophoresed *T. gondii* tachyzoite proteins following transfer to nitrocellulose and their interactions with glutaraldehyde-fixed rabbit erythrocytes. These investigators reported hemagglutination activity in the cytoskeleton, membrane, and total antigen from *T. gondii*. Although this hemagglutination was inhibited by three sulfated carbohydrates (fucoidan, dextran sulfate, and heparin) or by glycoproteins (fetuin and asialofetuin), no inhibition was observed with lactose or D-galactose, the inhibitory sugars for the hemagglutination reaction triggered by Lac$^+$ in the present study. They reported that sulfated carbohydrates, depending on the concentration used, could also facilitate or block parasite

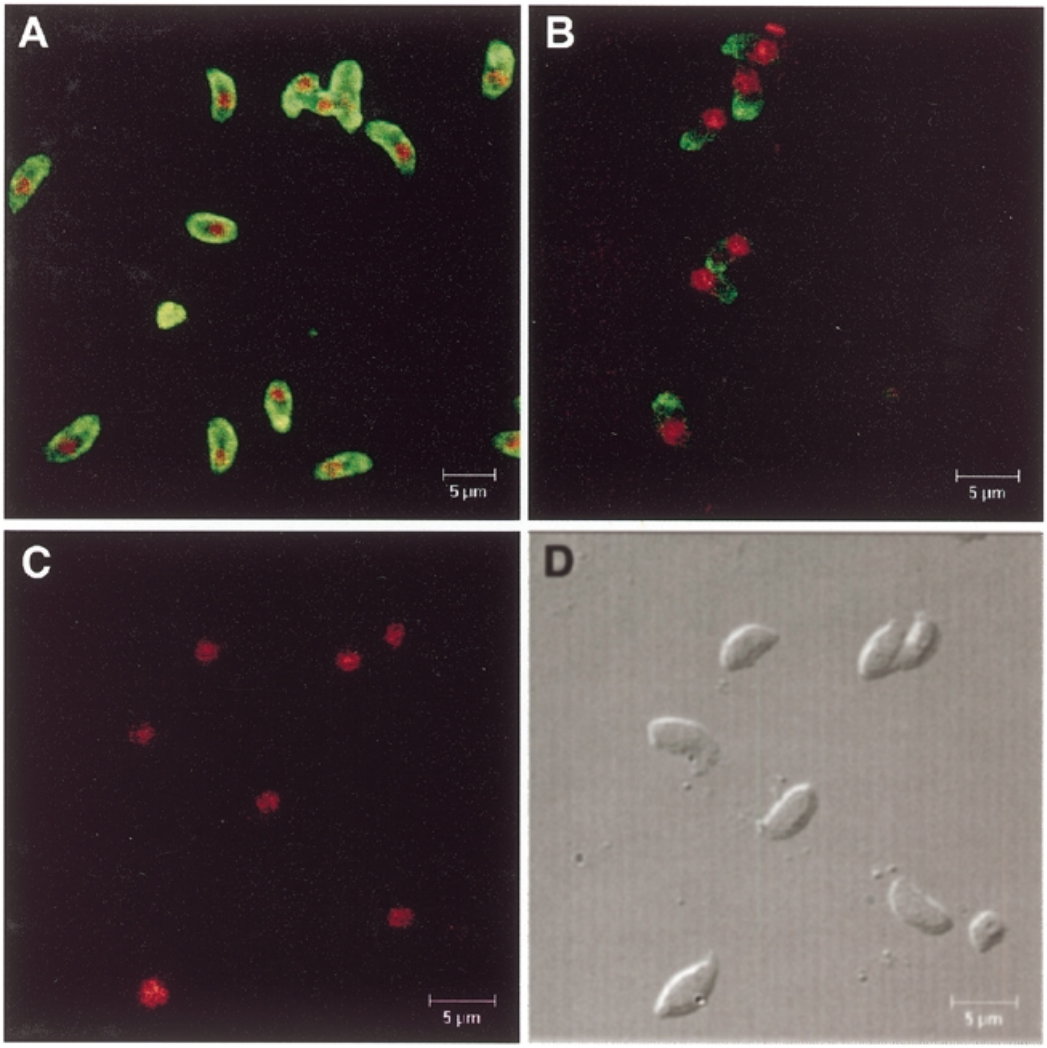

Fig. 4. Labeling of the apical region of *T. gondii* by mouse polyclonal anti-60/70-kDa serum. *T. gondii* tachyzoites were fixed on glass slides and incubated with mouse anti-STAg serum (**A**), mouse anti-60/70-kDa (Lac^+) serum (**B**), or serum from nonimmunized mouse (**C**). Reactions were developed with fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse IgG (green). Panel **D** corresponds to the DIC of panel **C**. Propidium iodide-labeled nuclei are visualized in red.

attachment to host cells. In addition, *T. gondii* shows a decreased ability to bind to cells that are deficient in proteoglycan synthesis. In contrast, we found that heparin did not interfere with Lac^+ lectin activity, even when used at concentrations as high as 0.2 mg/ml. It is known that D-galactose in aqueous equilibrium preferentially occurs as the β-galactopyranoside. Thus, the inhibition of the hemagglutinating activity of Lac^+ by lactose and D-galactose, but not by other carbohydrates, including melibiose, is indicative that the lectin contained in the Lac^+ preparation preferentially recognizes β-galactoside. Interestingly, when the nitrocellulose-transferred proteins were incubated with erythrocytes or biotinylated fetuin, MIC1, but not MIC4, bound ligands even when Lac^+ was electrophoresed under native conditions. This lectin activity of MIC1 was inhibited by 100 mM lactose, but not by 100 mM mannose (data not shown). Although a variety of glycosaminoglycans may function as receptors for *Toxoplasma* in the adhesion to the substratum during gliding and for attachment to host cells during invasion (Carruthers *et al.*, 2000a), glycosaminoglycan-binding proteins from *T. gondii* were not identified. Our results demonstrate that Lac^+ from *T. gondii* has a lectin activity different from that described by Ortega-Barria and Boothroyd (1999) and that probably is not a glycosaminoglycan-binding protein.

Our N-terminal data for MIC4 do not correspond to those reported by Brecht *et al.* (2001). Their amino terminal sequence [X(G/S)E(P/N)(D/A)(K/P)LDLA(P/L)V] starts at residue 14 of the MIC4 sequence obtained in the present study (ITPAGDDVSANVTSSEP). This suggests a difference in the posttranslational processing of the protein. Posttranslational processing of MIC2 has also been reported (Carruthers *et al.*, 2000b).

We propose that MIC1 is a lectin and that MIC4, complexed to this lectin, may copurify with MIC1 on the lactose-agarose

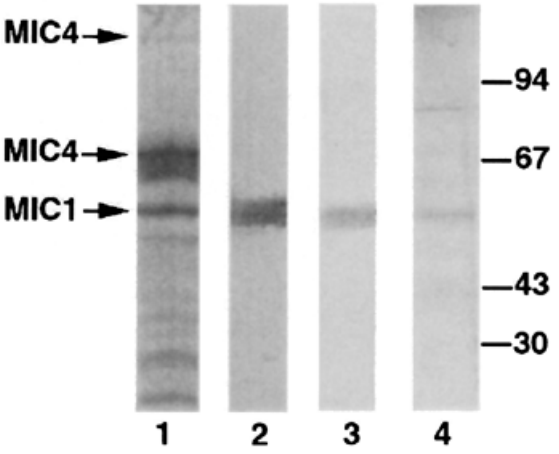

Fig. 5. Binding of denatured proteins of the Lac$^+$ preparation to human erythrocytes and fetuin. The Lac$^+$ preparation (20 µg) (lanes 2 and 3) and STAg (lane 4) were separated by nonreduced SDS–PAGE and transferred to nitrocellulose membranes. Membranes were incubated with erythrocytes (lane 2) or biotinylated fetuin (lanes 3 and 4). The binding of erythrocytes to the transferred proteins was identified visually, whereas the binding of biotinylated fetuin was detected with streptavidin-peroxidase, as revealed by DAB in the presence of hydrogen peroxide. Coomassie blue staining of the electrophoresed Lac+ proteins is shown in lane 1. The molecular masses (kDa) of standard marker proteins are indicated on the right side of the figure.

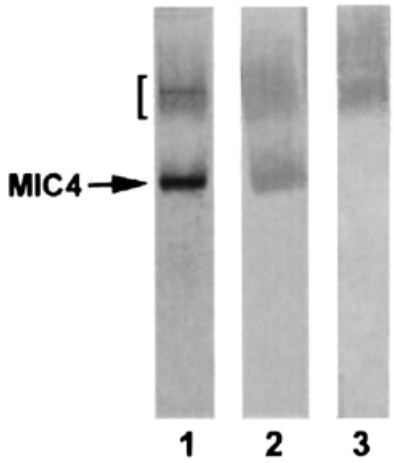

Fig. 6. Binding of native proteins of Lac$^+$ preparation to fetuin. Lac$^+$ (20 µg) was separated by native PAGE and transferred to nitrocellulose membranes. Membranes were incubated with anti-60/70-kDa serum (lane 2) or biotinylated fetuin (lane 3). Binding of biotinylated fetuin was detected with streptavidin-peroxidase, and mouse polyclonal anti-60/70-kDa serum with peroxidase-conjugated goat anti-mouse IgG. Coomassie blue staining of the electrophoresed Lac$^+$ proteins is shown in lane 1. The faster band was blotted and identified as MIC4 by amino acid sequencing. The bracket indicates the diffuse band assumed to contain MIC1.

column. Brecht *et al.* (2001) suggested that MIC4, which lacks a transmembrane or lipid anchor domain, could act as a bridge between an unknown receptor in the parasite and a receptor on the host cell, and that MIC4 forms a complex with MIC1 and MIC6. In this complex MIC1 may be directly and stably associated with MIC4, because the two proteins were coimmunoprecipitated, even in the absence of MIC6. The present demonstration of lectin activity in MIC1, but not MIC4, can partly explain the results obtained by Brecht *et al.* (2001), who observed that increasing concentrations of free galactose had a biphasic effect on MIC4 binding to host cells. Lower concentrations of the carbohydrate enhanced the MIC4 binding to host cell, whereas minimal binding inhibition was observed at higher doses. The role played by MIC1, through its lectin activity, in *T. gondii* infection is not known.

Materials and methods

Parasites

Tachyzoites of the virulent RH strain of *T. gondii* were maintained by intraperitoneal passage in Swiss mice and were collected 48–72 h after infection (Camargo *et al.*, 1978). Parasites obtained from the peritoneal exudate were washed three times with phosphate buffered saline (PBS, 10 mM sodium phosphate containing 0.15 M NaCl, pH 7.2) by centrifugation at 1000 × *g* and resuspended in PBS.

Preparation of STAg

Parasites resuspended in PBS containing 0.8 mM phenylmethylsulfonyl fluoride (PMSF, Sigma Chemical Co., St. Louis, MO) were submitted to sonication (Vibra-cell, Sonics & Materials Inc., Danbury, CT) with three pulses of 10 kHz, 50 W, for 4 min each. The soluble proteins recovered by centrifugation at 3000 × *g*, for 30 min at 4°C, constituted the STAg preparation.

Isolation of lactose-binding proteins

Twenty-milligram portions of STAg were submitted to affinity chromatography on a 5.0 ml α-lactose-agarose column (Sigma) previously equilibrated at 4°C with PBS containing 0.5 M NaCl. After washing with equilibrating buffer, the adsorbed material (Lac$^+$) was eluted with 10 ml of 0.1 M lactose in equilibrating buffer, concentrated, and dialyzed against water in an ultradiafiltration system using YM10 membrane (Amicon® Division, W.R. Grace & Co., Beverly, MA).

Assay for hemagglutinating activity

Human blood samples groups A, B, and O were collected into heparinized tubes. Erythrocytes were separated from platelet-rich plasma and the buffy coat by centrifugation at 150 × *g* for 15 min. They were then pelleted by centrifugation at 350 × *g* for 15 min, washed three times in PBS, and resuspended in the same buffer at a concentration of 2% (v/v). Hemagglutination assays were performed as previously described (Rüdiger, 1993) using 96-conical well microtiter plates (Nalge Nunc International Co., Naperville, IL). Twenty-five microliters of Lac$^+$ protein (40 µg/ml) were serially diluted (twofold) in PBS, and 25 µl of one of the 2% suspensions of human erythrocytes were added to each diluted sample. After 1 h at room temperature, each well was examined for agglutination. Lectin titer is reported as the reciprocal of the highest dilution of Lac$^+$ protein able to cause detectable agglutination of erythrocytes. One hemagglutinating unit (HU) is defined as the minimum lectin concentration required to provide complete agglutination. Specific hemagglutination activity corresponds to HU per

microgram lectin (HU/μg). For inhibition of hemagglutinating activity, 25 μl of a serial dilution of a potential carbohydrate inhibitor (D-glucose, D-mannose, D-galactose, L-fucose, lactose, melibiose, or heparin) was mixed with an equal volume containing 2 HU of Lac^+ preparation, for 1 h at room temperature. At the end of this incubation, 25 μl of a 2% suspension of human erythrocytes were added, and the minimum concentration of carbohydrate able to inhibit hemagglutination was recorded after 1 h at room temperature.

PAGE and electroblotting

SDS–PAGE was performed with either a 10–15% PhastGel gradient using a Phast System (Amersham-Pharmacia Biotech, Uppsala, Sweden) (Figure 1) or a 10% polyacrylamide gel using a Mini V-8.10 Vertical Gel Electrophoresis System (Gibco BRL, Rockville, MD) (Figures 2 and 5). For the PhastGel System, dried samples were suspended in 10 mM Tris–HCl buffer containing 1 mM ethylenediaminetetraacetic acid (EDTA), pH 8.0, with 2.5% (w/v) SDS, and, in some cases, 1% (w/v) dithiothreitol was also added to reduce the samples. After running (250 V, 10 mA), gels were stained with Coomassie blue. Low molecular mass standards from Amersham Pharmacia Biotech were used.

For the 10% gel, samples were suspended in 0.5 M Tris–HCl buffer, pH 8.8, with 12% (v/v) glycerol, 2% (w/v) SDS (Laemmli, 1970), and running at 200 V, 80–120 mA. Gels were stained as described above.

Native PAGE was performed in a 10% polyacrylamide gel without SDS or reducing agent using a Mini V-8.10 Vertical Gel Electrophoresis System (Gibco BRL) (Figure 6). Samples were suspended in 0.5 M Tris–HCl buffer, pH 8.8, with 12% (v/v) glycerol. Running and staining were performed as described above.

In some experiments, after electrophoresis proteins were blotted to nitrocellulose or polyvinylidene difluoride (PVDF) membranes using the Mini V-8.10 Blot System, according to the manufacturer's instructions. The PVDF membrane was stained with Coomassie blue, and the bands were cut out for sequencing by Edman degradation. The nitrocellulose membranes were blocked with 3% (w/v) gelatin in PBS for 2 h at 37°C and immunostained with mouse polyclonal antiserum or used to assay binding of erythrocytes or fetuin to electrophoretically separated proteins.

Lectin-binding assays

Erythrocyte binding. The nitrocellulose membrane containing electrophoresed Lac^+ proteins was incubated for 1 h at room temperature in PBS containing 1% (v/v) Triton X-100, followed by 1 h in PBS containing 1% (w/v) BSA. The membrane was incubated with a 1% suspension of human erythrocytes (A, B, or O) in 1% BSA and the suspension allowed to settle for 1 h at room temperature. After gentle washing, the sheet was fixed for 10 min in 3% buffered formalin and rinsed with PBS.

Glycoprotein binding. The nitrocellulose membrane containing electrophoresed Lac^+ proteins was incubated with biotinylated fetuin (14 μg/ml) for 1 h at room temperature, washed three times with PBS, and incubated with streptavidin-peroxidase (Pierce Chemical Co., Rockford, IL) for 30 min at room temperature. The reaction was visualized with diaminobenzidine (DAB) (Sigma) in the presence of 0.03% hydrogen peroxide (Sigma).

Production of mouse polyclonal antiserum

Bands of 70 and 60 kDa from the Lac^+ preparation obtained by preparative SDS–PAGE under nonreducing conditions were emulsified in Freund's complete adjuvant (Sigma). Mice were injected IP with 10 μg protein. On days 21, 28, and 35 postinoculation, they were reinjected IP with the same amount of antigen emulsified in Freund's incomplete adjuvant (Sigma). After 8 days, the animals were bled, and the titer of specific serum IgG was tested by enzyme-linked immunosorbent assay.

Immunoblotting assay

Nitrocellulose membranes containing electrophoresed and blotted *T. gondii* proteins were incubated with 1:3000 mouse polyclonal anti-STAg serum, 1:3000 mouse polyclonal anti-60/70-kDa serum, or 1:500 nonimmunized mouse serum for 1 h at room temperature, washed three times with PBS, and developed with 1:1500 peroxidase-conjugated goat anti-mouse IgG antibody (Sigma) for 1 h at room temperature. The reactions were visualized with DAB (Sigma) in the presence of 0.03% hydrogen peroxide (Sigma).

Screening of a T. gondii cDNA library

A cDNA library from ME49-PDS *T. gondii* tachyzoites was prepared in λZAP II (Stratagene, La Jolla, CA) and kindly provided by Dr. Ian Manger, Department of Microbiology and Immunology, Stanford University School of Medicine (Stanford, CA). The cDNA library (amplified titer, 4×10^8 pfu/ml) was screened with polyclonal mouse anti-Lac^+ preparation antiserum. Clone protein expression was induced using nitrocellulose filters soaked in 10 mM isopropyl-1-thio-β-D-galactopyranoside. Positive plaques were identified using a 1:4000 dilution mouse anti-Lac^+ preparation antiserum, 1:2000 alkaline phosphatase-conjugated goat anti-mouse IgG antibody (Gibco BRL), and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate (Gibco BRL). Two positive plaques denoted E1 and E7 were submitted to three rounds of purification. The isolated λ phage clones were then converted into pBluescript SK (–) phagemids with the ExAssist helper phage (Stratagene) according to the manufacturer's instructions. Double-stranded sequencing of clones was determined using an automatic DNA sequencer (ALFexpress DNA Analysis System, Amersham Pharmacia Biotech) and a Thermo Sequenase Fluorescent Labeled Primer Cycle Sequencing Kit (Amersham Pharmacia Biotech). Sequence homologies were obtained by using Basic Local Alignment Search Tool (BLAST) (Altschul *et al.*, 1990).

Protein quantification by amino acid analysis

Amino acid analyses were performed using the phenylthiocarmyl derivative method (Bidlingmeyer *et al.*, 1984) after acid hydrolysis with 6 N HCl containing 1% phenol in the vapor phase, at 110°C for 22 h. A mixture containing 2.5 nmol of each amino acid (Standard H, Pierce Chemical Co., Rockford, IL) was derivatized, and 4% (100 pmol) was injected.

Edman degradation

The N-terminal protein sequences of PVDF-blotted proteins were determined with a Procise sequenator (Procise Microsequencer, model 491, Applied Biosystem, Foster City, CA)

using gas-phase chemistry with online identification of phenylthiohydantoin derivatives. The phenylthiohydantoin amino acid standard contained 10 pmol of each derivatized amino acid, and 10 pmol of lactalbumin was used to determine the efficiency of the system.

RP-HPLC

Protein separations were carried out using a Thermo Separation Products system (Riviera Beach, FL) employing two pumps (model constaMetric 3500 and 3200) and a dual wavelength spectromonitor 4200 controlled by the LC-Talk (version 2) software. The proteins retained on the column were eluted with a gradient (10% B for 10 min, 10–50% in 10 min, 50–75% in 35 min, and 75–100% in 5 min) using trifluoroacetic acid (TFA)/acetonitrile (ACN) (solvent A was 0.1% TFA and solvent B was 80% ACN, 20% water, and 0.085% TFA) at 0.2 ml/min flow at room temperature. The column was reequilibrated with 10% B for 20 min. HPLC peaks of the Lac^+ fraction were identified by N-terminal sequencing.

Fluorescence confocal microscopy

T. gondii tachyzoites were adsorbed to glass slides to obtain 10–20 parasites/field at a magnification of 400× and fixed with 1% (v/v) formaldehyde in PBS for 30 min at room temperature. The slides were washed three times with PBS to remove excess formaldehyde and the parasites were permeabilized with 0.01% (w/v) saponin (Amersham Pharmacia Biotech) in PBS. After incubation with 1% (w/v) BSA in PBS for 30 min at room temperature, slides were then incubated with 1:1000 mouse polyclonal anti-STAg serum, 1:1000 mouse polyclonal anti-60/70-kDa serum, or 1:1000 nonimmunized mouse serum for 1 h at room temperature. They were washed three times with PBS and developed with 1:1500 fluorescein-conjugated goat anti-mouse IgG antibody (Sigma) for 1 h at room temperature. After washing twice in PBS, slides were incubated with propidium iodide (0.5 μg/ml; Sigma) and after three washes with PBS, the slides were mounted in glycerol buffered with 50 mM carbonate, pH 9.5. Samples were imaged on a Leica TCN-NT confocal system, using a 100× N.^{a} 1.4 Plan-Apochromatic (DIC) oil-immersion objective.

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Abbreviations

ACN, acetonitrile; BSA, bovine serum albumin; DAB, diaminebenzidine; HU, hemagglutinating unit; IP, intraperitoneal; Lac^+, lactose-binding fraction from STAg; MIC, micronemal protein; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate buffered saline; PMSF, phenylmethylsulfonyl fluoride; PVDF, polyvinylidene difluoride; RP-HPLC, reverse phase-high performance liquid chromatography; SAG1, surface antigen 1; SDS, sodium dodecyl sulfate; STAg, soluble *Toxoplasma gondii* antigen; TFA, trifluoroacetic acid.

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