The initial stage of invasion by apicomplexan parasites involves the exocytosis of the micronemes-containing molecules that contribute to host cell attachment and penetration. MIC4 was previously described as a protein secreted by Toxoplasma gondii tachyzoites upon stimulation of micronemes exocytosis. We have microsequenced the mature protein, purified after discharge from micronemes and cloned the corresponding gene. The deduced amino acid sequence of MIC4 predicts a 61-kDa protein that contains 6 conserved apple domains. Apple domains are composed of six spacially conserved cysteine residues which form disulfide bridges and are also present in micronemal proteins from two closely related apicomplexan parasites, Sarcocystis muris and Eimeria species, and several mammalian serum proteins, including kallikrein. Here we show that MIC4 localizes in the micronemes of all the invasive forms of T. gondii, tachyzoites, bradyzoites, sporozoites, and merozoites. The protein is proteolytically processed both at the N and the C terminus only upon release from the organelle. MIC4 binds efficiently to host cells, and the adhesive motif maps in the most C-terminal apple domain.

Toxoplasma gondii is a ubiquitous protozoan pathogen infecting human and animals. Like other members of the phylum Apicomplexa, this parasite possesses an elaborate apical apparatus dedicated to host cell invasion. The successive exocytosis of secretory compartments, including rhoptries and micronemes, plays a key role in the invasion process. Micronemal proteins are apparently used for host cell recognition, binding, and motility, whereas the content of the rhoptries likely contributes to the formation of a functional parasitophorous vacuole. T. gondii is remarkable for its ability to invade almost any nucleated cell within its mammalian hosts. This broad host cell specificity suggests that adhesion involves the recognition of ubiquitous surface-exposed host molecules or, alternatively, the presence of various parasite attachment molecules able to recognize multiple host cell receptors. Micronemal proteins identified in several Apicomplexa share common structural features (1) and, in select cases, can even sustain functional complementation across species (2). For example, a family of adhesive proteins containing thrombospondin (thrombospondin-like) and integrin A domains have been described in the micronemes of Plasmodium, Eimeria, Toxoplasma, and Cryptosporidium (3, 4). This family is named from the original member described in Plasmodium as thrombospondin-related anonymous protein or TRAP (1). In T. gondii, three major micronemal proteins have been characterized so far. 1) MIC1 contains only two degenerate thrombospondin-1-like domains (6), MIC2 is the homologue of TRAP (5), and MIC3 contains epidermal growth factor-like domains and forms dimers (7). MIC4 was previously identified as a component of the micronemes (8). In this study, we present the complete characterization of MIC4, a novel type of micronemal adhesin in T. gondii for which homologues exist in two other Apicomplexa; MIC5 in Eimeria tenella (9) and the lectin (SML) in Sarcocystis muris (10).

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—Chemical reagents were obtained from Sigma unless otherwise specified. Mouse hybridomas were obtained by immunization with excretory-secretory antigen (ESA)1 as previously described (11), and mAbs were screened by immunofluorescence assay (IFA) and Western blotting. Polyclonal rabbit serum reacting to Toxoplasma actin (anti-ACT1) was described previously (12). The mAb Tg17–43, which reacts to the dense granule protein GRA1, was provided by Dr. Marie-France Cesbron-Delauw (Lille, France). The monoclonal anti-MIC2 (T34A1) was provided by Dr. Jean-François Dubremetz, and the hydriobome BB2, producing mAbs against Ty1 tag, was provided by Dr. Keith Gull (University of Manchester, Manchester, UK).

Host Cells and Parasite Cultures—Tachyzoites of the RH strain T. gondii were propagated in human foreskin fibroblast (HFF) monolayers grown in Dulbecco’s modified Eagle’s medium containing 3.7 g/liter sodium bicarbonate, 10 mM HEPES, 1 mM t-glutamine, 10% fetal calf serum, and 10 μg/ml gentamicin (referred to as D-10). Parasites were harvested from freshly lysed cultures in Hanks’ balanced salt solution containing 10 mM HEPES and 0.1 mM EGTA (HHE) as previously described (8). Bradyzoites were obtained from mouse brains chronically infected with the ME49 strain of T. gondii (kindly provided by Dr. Steve Parmley, Pulu Alto Medical Research Foundation). Oocysts, including

1 The abbreviations used are: ESA, excretory-secretory antigen; mAb, monoclonal antibody; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; BSA, bovine serum albumin; CBF, cell-bound fraction; PAGE, polyacrylamide gel electrophoresis; MIC4, micronemal protein 4; HXGPR, hypoxanthine-xanthine-guanine-phosphoribosyltransferase; HFF, human foreskin fibroblast; IFA, immunofluorescence assay; MPP1, micronemal protein protease 1; MESNA, 2-mercaptoethanesulfonic acid; TLCK, 1-chloro-3-tosylamido-7-amino-2-heptanone or N-p-tosyl-l-lysine chloromethyl ketone.
nonsporulated, partially sporulated, and fully sporulated preparations, were obtained from cats infected with the VEG strain of T. gondii (kindly provided by Dr. Michael White, Montana State University). A clonal isolate of the RH hsp60-1 strain of T. gondii was used as the recipient strain for all the transfection experiments. DNA Cloning of PCR Products and DNA Sequencing and Analysis—The cosmId library used the SuperCos vector modified with SAGl/ble Toxoplasma selection cassette inserted into its HindIII site. The library was prepared from a Sau3AI partial digest of RH genomic DNA ligated into the BamHI cloning site (kindly provided by D. Howe).

Inserts from TgEST phage clones were amplified using T3 and T7 primers and cloned into pCR2.1 (Invitrogen). DNA sequencing was conducted by cycle-sequencing using ABI Prism Big Dye terminator cycle sequencing reaction kits (ABI, Foster City, CA) and resolved on ABI 377 DNA sequencers. Sequence analysis was conducted with the Genetics Computer Group programs (13), programs available through the National Center for Biotechnology, and programs at the Expasy site.

Construction of Expression Plasmids—The vector pEXMICA1 was constructed by cloning a PCR product corresponding to the N-term region of MIC4 and encompassing the first apple domain (zib-3) and 5′-ggtaatagttggatccacacagctcttt-gc-3′, respectively. In parallel, the same A1 fragment was cloned into a pET vector, and expression protein could also be produced in native soluble form in Escherichia coli BL21.

The expression vector for T. gondii pTMIC4mycHXGPRT was obtained by cloning a PCR product corresponding to the complete coding sequence of MIC4 between the EcoRI and PacI sites of the pThyeHXGPRT vector previously described (14). The primers for PCR were 5′-cggaattccctttttc-gaggtccgaatgcacacatcg-3′ and 5′-tggtgtaggtggatccagatcttt-3′, respectively. An additional epitope tag was introduced at the N terminus of MIC4 to generate pThyMIC4mycHXGPRT. The 11 amino acid Ty-1 tag coding sequence (LEVHTNQDPLD) was inserted as double-stranded oligonucleotides into the unique SstI site at amino acid 47 of MIC4: 5′-ggtttgaacagctctggactgcacgtc3′- and 5′-ttgggggcctgccgacctgcc-3′. An additional expression vector pTMY1MIC4 was constructed by cloning a PCR product corresponding to the complete coding sequence of MIC4 between the EcoRI and PacI sites of the pThyeHXGPRT vector previously described (14). The primers for PCR were 5′-cggaattccctttttc-gaggtccgaatgcacacatcg-3′ and 5′-ggtaatagttggatccacacagctcttt-gc-3′, respectively. In parallel, the same A1 fragment was cloned into a pET vector, and expression protein could also be produced in native soluble form in Escherichia coli BL21.

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Preparation of Micronemal Proteins—For large scale preparation of excretory-secretory antigens (ESA), ~5 × 10^6 tachyzoites were resuspended in 1 ml of HHE and stimulated to discharge micromeres by the addition of ethanol to a final concentration of 1.0% and warming to 37 °C for 30 min (8). Cells were collected by centrifugation at 2000 × g, and the supernatant was kept for binding experiments. To purify the contents of micromeres, ~5 × 10^6 tachyzoites were harvested in HHE as above and subjected to sonication and cell fractionation as described previously (8). Briefly, parasites were resuspended in cold HHE at ~10^7/ml and sonicated while on ice (3 × 30-s pulses at setting 35 on a Branson Sonifier) and transferred to a beaker containing a Microtubeforcelive (Brown Scientific, Rochester, NY) and allowed to sediment. After sonication, large cellular debris was removed by centrifugation at 2000 × g, 10 min, 4 °C, and the supernatant was further clarified by spinning at 8000 × g for 20 min, 4 °C. The micromeres were recovered from the supernatant by centrifugation at 30,000 × g for 30 min at 4 °C. The 30,000 × g pellet was resuspended in PBS, pH 6.0, containing a mixture of protease inhibitors (1 μg/ml E64, 10 μg/ml (4-amidinophenyl)ethyl N-benzoyl carbamyl(methyl)phenylsulfonyl fluoride (BPNF), 1 μg/ml leupeptin) and subjected to three rapid freeze/thaw cycles. The suspension was then sonicated 3 × 15 s using the maximum setting for the microprobe sonicator (550 Sonic Dismembrator, Fisher). The suspension was centrifuged at 100,000 × g for 1 h to remove unbroken micromeres and membranes, and the supernatant containing soluble micronemal proteins was kept for cell binding experiments.

Cell Binding Assays—Confident monoclonals of HFF cells grown in 6-well plates were rinsed in PBS and blocked for 30 min at 12% with 1% BSA in PBS containing 10 mm CaCl_2 and 0.5% MgCl_2 (CM-PBS). Excess BSA was removed by rinsing in CM-PBS, and micronemal proteins (20 μg/ml) were added in a volume of 1 ml of CM-PBS and incubated at 37 °C for 1 h. A;500-cell unbound fraction (referred to as supernatant) was removed, and the monolayers were rinsed four times in cold CM-PBS (referred to as W1, W2, W3, and W4). The cell-bound fraction (CBF) was collected by lysing the monolayer in 1 ml of radiolabeled nucleotide solution [50 μl tritiated EDTA (5 μl Triton X-100, 0.5% sodium deoxycholate, 0.2% SDS, 100 μl NaCl, 5 μl EDTA)] Fractions were counted for 1 min after spin-down in a Beckman LS-100 γ-counter. Dilution standards of the micronemal protein preparations were loaded in parallel to simulate the contents of 1, 5, and 10% of the total input material. ESA treated with 2-mercaptoethanol (MESNA, Sigma) was preincubated at 37 °C for 30 min in the presence of 50 μl MESNA and then diluted 50 times in CM-PBS for the binding assay.

RESULTS

Characterization of Cellular and Secreted Forms of MIC4 and N-terminal Microsequencing of the Secreted Form—To characterize secretory proteins of Toxoplasma, we generated mAbs to the ESA fraction released by extracellular tachyzoites. When the mAb 5B1 was used to probe Western blots, it recognized a sharp band that migrated at 72 kDa in tachyzoite cell

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lysates and 70 kDa in ESA resolved under reducing conditions. Both bands migrated more rapidly and diffusely in the absence of reduction, suggesting the presence of internal disulfide bonds (Fig. 1A). To identify the gene corresponding to the protein recognized by mAb 5B1, we isolated ESA on a large scale and resolved the proteins by SDS-PAGE. Parallel strips were transferred to nitrocellulose for Western blotting to identify the band recognized by mAb 5B1 (Fig. 1B) and to polyvinylidene difluoride membranes for microsequencing. N-terminal sequencing of the band corresponding to the 72-kDa form yielded a partially degenerate sequence of 12 residues (X(G/S)-E(P/N)(D/A)(K/P)LDLA(P/L)V).

Identification and Sequencing of the MIC4 Gene—Comparison of the complete coding sequence against the nonredundant GenBank database using BLAST revealed that the gene was homologous to several micronemal antigens previously described from *S. muris* (18, 19) and to the recently reported *E. tenella* micronemal protein EtMIC5 (9). These proteins share the feature of containing conserved, cysteine-rich domains known as apple motifs, which were detected using Prosite (ExPasy). Such a domain contains six half-cystine residues at highly conserved positions that form a structure resembling an apple (20, 21). The consensus for the internal four cysteine residues of this sequence is C-X₃-C-X₅-C-X₁₁-C. The six apple domains of MIC4 are arranged as follows: A1 (amino acids 67–139), A2 (amino acids 140–230), A3 (amino acids 231–303), A4 (amino acids 304–417), A5 (amino acids 418–490), and A6 (amino acids 491–580) (for alignment of the six apple domains, see Ref. 9). In the case of human plasma prekallikrein, it has been shown that three highly conserved disulfide bonds are linking the first and sixth, second and fifth, and third and fourth apple domains.

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fourth half-cystine residues in each domain (14). Since these cysteine residues are conserved in MIC4, it is likely that disulfide bond formation in MIC4 is similar to prekallikrein. The sequence analysis of MIC4 predicts a signal peptide cleavage site between residues Ala25 and His26 (ExPasy). Although we have not been able to verify the N-terminal sequence of the 72-kDa form of the protein found in cells, it likely corresponds to the mature N terminus generated within the secretory pathway, with removal of an additional 32 residues occurring at the time of secretion into the medium.

Features of MIC4 Gene—The 1743-base pair open reading frame of MIC4 contains no introns. The putative transcription start site of MIC4 was determined by sequencing several clones obtained by 5′ rapid amplification of cDNA ends PCR. A sequence analysis of the promoter region revealed the lack of TATA box and no element resembling an initiator element (Inr) (22). However, a consensus sequence (heptamer motif) found multiple times in the 5′-flanking sequences of several genes (23) is present in the promoter region of MIC4 (Fig. 1C). Two heptamer motifs are positioned at −716 (AGAGACG) and −496 (TGAGACG) from the transcription start site. These elements have been previously mapped and shown to be critical for transcription of the family of GRA genes (23) and are also included in the 27-base pair repeat element of SAG1 gene (24). A single in-frame ATG lies 58 residues upstream of the N-terminal sequence that was obtained from the purified protein. This ATG probably serves as the translational initiation codon based on the facts that 1) it is the first in-frame ATG, 2) the six nucleotides preceding the ATG (CACAAG) are consistent with the consensus sequence for translational initiation in T. gondii (GCNAAA) (25), and 3) the ATG immediately precedes a sequence predicted to encode a hydrophobic signal peptide.

Subcellular Localization and Pattern of Expression of MIC4—We previously reported that the antigen recognized by mAb 5B1 is secreted from Toxoplasma in a manner consistent with it originating from micronemes (8, 26). To confirm that the gene described here corresponds to a micronemal protein, we produced a bacterial recombinant GST-A1 fusion of the N-terminal 186 amino acids of MIC4 encompassing the first apple domain (A1) and raised polyclonal antibodies against the purified protein. The rabbit antisera obtained were tested on immunoblots loaded with the recombinant nonfusion A1 and GST-A1 fusion expressed in E. coli, T. gondii tachyzoites, and Vero cell lysates (Fig. 2A). The sera recognized specifically a 72-kDa protein in tachyzoites. Neither anti-GST antibodies nor the preimmune rabbit sera reacted with T. gondii proteins on Western blots (data not shown).

To determine the pattern of expression of MIC4 in the different life stages of the parasite, cell lysates of tachyzoites, bradyzoites, and oocysts were resolved by SDS-PAGE and probed by Western blotting (Fig. 2B). MIC4 was not detected in unsporulated or partially sporulated oocysts but was not detectable in nonsporulated (NS) or partially sporulated (PS) oocysts. Blotting in parallel with rabbit anti-Toxoplasma actin provided loading control. B, communofluorescence. Rabbit polyclonal anti-MIC4 and mAb anti-MIC2. C, immuno-localization by electron microscopy of MIC4 using the rabbit anti-serum. MIC4 was found in micronemes (arrows) clustered at the apical end of the tachyzoite (a), bradyzoite (b), and merozoite (c). Sections were incubated with rabbit anti-MIC4 followed by goat anti-rabbit IgG conjugated to 10-nm gold. DG, dense granule; R, rhoptry; CW, cell wall; C, conoid; PG, polysaccharide granules.

MIC4 colocalized perfectly with the other micronemal protein MIC2 (27) (Fig. 2B). IFA performed on extracellular parasites only stained parasites permeabilized with Triton X-100 before incubation with the first antibody (data not shown). This observation suggests that MIC4 is predominantly localized in the micronemes and absent from the cell surface. Ultrastructural examination confirmed that the polyclonal antibodies recognized a protein located within the micronemes of tachyzoites (Fig. 2C, a) and bradyzoites (Fig. 2C, b). The few micronemes in the merozoite of mature schizonts were also labeled (Fig. 2C, c). In keeping with the nomenclature of previously established Toxoplasma proteins (27), we named this antigen MIC4.

MIC4 Is Proteolytically Cleaved Only after Release by the Micronemes—The T. gondii micronemal proteins characterized so far are subjected to extensive proteolytic remodeling during their transport and/or secretion (27). Comparison of the amino acid sequence deduced from the MIC4 gene with the information obtained from N-terminal sequencing of the mature pro-
tein is indicative of proteolytic cleavage. To determine whether MIC4 is proteolytically cleaved during its transport to the micronemes, we generated recombinant parasites expressing MIC4 tagged at both ends with epitopes. The construct pTMIC4mycHXGPRT produced MIC4 with epitope tags at the N terminus and/or the C terminus. An additional Ty-1 epitope tag was introduced 10 amino acids upstream of the cleavage site mapped previously by the N-terminal sequencing of secreted form to generate pTty1MIC4mycHXGPRT. Both constructs were stably integrated into T. gondii tachyzoites, and expression of MIC4myc or Ty-1MIC4myc was examined by Western blot and by IFA. The mAbs anti-Myc and anti-Ty-1 recognized the 72-kDa form of MIC4 on Western blot (Fig. 3A) and gave a typical microneme staining on IFA (Fig. 3B). These results demonstrate that the form of MIC4 stored in the micronemes is not proteolytically cleaved beside the cotranslational removal of the signal peptide and, as for MIC2, the processing on MIC4 occurred uniquely post-exocytosis.

MIC4 Is Processed at Both Ends after Release by the Micronemes, and MPP2 Is Likely to Be Responsible for the C-terminal Cleavage—Immunoblot analysis of tachyzoite lysates and ESA material using mAb 5B1 and the rabbit polyclonal antiserum revealed the existence of an additional proteolytic processing at the C terminus that generates the two products of ~50 and 15 kDa (Fig. 3C, lanes 1 and 2, upper and lower panels). The 72-kDa precursor form of MIC4 is present in the micronemes, whereas the processed forms are uniquely detectable in ESA. The polyclonal antibodies were raised against the apple domains A1 and A2 and recognized the 50-kDa form only, whereas the 15-kDa product was detected exclusively by the mAb 5B1. Together, these results document that processing occurs at the surface of the parasite only after release by the micronemes and allowed the mapping of the epitope recognized by the mAb 5B1 within the A6 at the C terminus of MIC4. Recently, distinct protease activities for MIC2 have been described using a variety of protease inhibitors (28). Upon release from the micronemes, MIC2 is proteolytically modified at multiple sites by two distinct enzymes, microneme protein protease 1 (MPP1) and microneme protein protease 2 (MPP2), which probably operate on the parasite surface (28). A subset of serine and cysteine protease inhibitors was shown to block MPP2 activity. Similarly, examination of MIC4 in ESAs from parasites pretreated with various protease inhibitors revealed that the processing of MIC4 into 50- and 15-kDa species was blocked by chymostatin, N-acetyl-l-leucinyl-l-leucinyl-l-norleucinal, and N-acetyl-l-leucinyl-l-leucinyl-methioninol but not any of the other protease inhibitors tested (Fig. 3C). The profile of sensitivity to protease inhibitors strongly suggests that MPP2 processes both MIC2 and MIC4.

MIC4 Binds to Host Cells—Several previous reports indicate that micronemal proteins bind to host cells and may participate in parasite/cell attachment (6, 7, 28, 29). To determine whether MIC4 binds to host cells, we incubated HFF monolayers with derived from tachyzoites treated with a solvent control (Me2SO, lane 1) or parasites expressing HFF-infected-transformed parasites expressing MIC4myc or Ty-MIC4Myec using rabbit anti-MIC4, anti-Ty-1, or anti-myc antibodies. Both epitopes are present on the 72-kDa form of MIC4. B, IFA analysis of HFF-infected-transformed parasites expressing MIC4myc or Ty-MIC4Myec using rabbit anti-MIC4 and colocalization with anti-Myc and anti Ty-1 antibodies. The N- and C-terminal-tagged MIC4s accumulate in the micronemes. C, effect of protease inhibitors on proteolytic processing of MIC4. Western blots of tachyzoite lysate (lane 1) or ESA derived from tachyzoites treated with a solvent control (Me2SO, lane 2) or protease inhibitors (lane 3, pepstatin; lane 4, EDTA; lane 5, 1,10-phenanthroline; lane 6, MMP-1; lane 7, 2,3-dichloroisocoumarin; lane 8, 4-(2-aminoethyl)benzenesulfonyl fluorde hydrochloride; lane 9, 4-amidinophenyl)methanesulfonyl fluoride (APMSF); lane 10, chymostatin; lane 11, TLCK; lane 12, leupeptin; lane 13, E64; lane 14, N-acetyl-l-leucinyl-l-leucinyl-l-norleucinal; lane 15, N-acetyl-l-leucinyl-l-leucinyl-methioninol; lane 16, EGTA). The 50-kDa cleavage product (closed arrowhead) was detected with rabbit anti-MIC4 (upper panel), and the 15-kDa cleavage product (open arrowhead) was recognized by mAb 5B1 (lower panel). Chymostatin, N-acetyl-l-leucinyl-l-leucinyl-l-norleucinal, and N-acetyl-l-leucinyl-l-leucinyl-methioninol blocked production of the 50- and 15-kDa cleavage products, whereas little or no effect was observed with the other protease inhibitors tested.

FIG. 3. MIC4 is proteolytically processed at the N and C terminus after release from the micronemes. A, Western blot analysis of wild type (wt) RH and parasites expressing MIC4myc or Ty-MIC4Myec using rabbit anti-MIC4, anti-Ty-1, or anti-myc antibodies. Both epitopes are present on the 72-kDa form of MIC4. B, IFA analysis of HFF-infected-transformed parasites expressing MIC4myc or Ty-MIC4Myec using rabbit anti-MIC4 and colocalization with anti-Myc and anti Ty-1 antibodies. The N- and C-terminal-tagged MIC4s accumulate in the micronemes. C, effect of protease inhibitors on proteolytic processing of MIC4. Western blots of tachyzoite lysate (lane 1) or ESA derived from tachyzoites treated with a solvent control (Me2SO, lane 2) or protease inhibitors (lane 3, pepstatin; lane 4, EDTA; lane 5, 1,10-phenanthroline; lane 6, MMP-1; lane 7, 2,3-dichloroisocoumarin; lane 8, 4-(2-aminoethyl)benzenesulfonyl fluorde hydrochloride; lane 9, 4-amidinophenyl)methanesulfonyl fluoride (APMSF); lane 10, chymostatin; lane 11, TLCK; lane 12, leupeptin; lane 13, E64; lane 14, N-acetyl-l-leucinyl-l-leucinyl-l-norleucinal; lane 15, N-acetyl-l-leucinyl-l-leucinyl-methioninol; lane 16, EGTA). The 50-kDa cleavage product (closed arrowhead) was detected with rabbit anti-MIC4 (upper panel), and the 15-kDa cleavage product (open arrowhead) was recognized by mAb 5B1 (lower panel). Chymostatin, N-acetyl-l-leucinyl-l-leucinyl-l-norleucinal, and N-acetyl-l-leucinyl-l-leucinyl-methioninol blocked production of the 50- and 15-kDa cleavage products, whereas little or no effect was observed with the other protease inhibitors tested.
indicate that MIC4 binds substantially to the surface of human fibroblasts. As control, GRA1, which is abundantly present in the ESA, does not bind detectably to host cells. The processed forms of MIC4 were examined separately for their ability to bind to host cells using the mAb 5B1 after resolution on 15% SDS-PAGE or the rabbit antiserum (Fig. 4A, right panel). Interestingly, the 15-kDa form but not the 50-kDa form of MIC4 bound to host cells. The mAb 5B1 recognizing the 15-kDa form of MIC4 bound to host cells, and the adhesive motif is restricted to the C terminus encompassing the A6 domain.

To determine the host cell binding activity of the processed forms of MIC4, the CBF was analyzed on a 15% SDS-PAGE using the rabbit anti-MIC4 or the mAb-5B1. The 70- and 15-kDa forms bound to host cells, whereas no binding was detected with 50-kDa form. B, quantification of the binding of MIC4 to host cells. Approximately 11% of MIC4 in the microneme preparation and 26% of MIC4 in ESA was bound to the host cell (*). In contrast, GRA1 was not detected in the CBF. Values are plotted as relative intensity as determined by phosphorimage analysis and compared with loading standards for 1, 5, and 10% of the starting material.

C, ESA prepared from wild type RH and TyMIC4Myc were analyzed in the cell binding assay. The ESA from RH was preincubated for 30 min at 37 °C in absence or presence of 50 mM strong reducing agent MESNA. The treated ESA was then diluted 50 times in CM-PBS before incubation with host cells. D, ESAs prepared from RH and parasites expressing MIC4His, TyMIC4Myc, or two independent clones of MIC4Δ12 were tested and compared in host cell binding assays. The wild type and mutated forms of MIC4 are indicated by an arrow. The rabbit serum anti-MIC4 used in this experiment showed a cross-reaction with host cells, indicated by an asterisk. This signal was also detectable in the sample of HFF cells, which was not incubated in presence of ESA.
product inhibits about 50% of cell binding by the 70-kDa form.² From these results, we concluded that the 15-kDa C-terminal product, which corresponds approximately to the A6 domain, carries the adhesive properties of MIC4.

As mentioned above, the apple structure is maintained by the formation of three disulfide bridges. To test whether these disulfide bridges are necessary for MIC4 binding to host cells we pretreated ESA with the strong reducing agent MESNA. This treated completely abolished MIC4 binding to host cells, providing additional evidence that the adhesive properties of MIC4 are dependent on the presence of intact cystine residues (Fig. 4C). MESNA is not acting on a host cell receptor since preincubation of the ESA with MESNA at room temperature or the addition of the reducing agent to host cells during the binding assay did not impair MIC4 adhesiveness (data not shown). The generation of recombinant parasites expressing a truncated form of MIC4 confirmed these observations. We compared the binding activity of MIC4 with Ty-1MIC4Myc and MIC4his carrying a stretch of eight histidine residues at the C terminus, and a deletion mutant of MIC4 lacking the last 12 amino acids (MIC4C12). MIC4his and MIC4C12 were expressed in a clone of mic4ko mutant parasites lacking MIC4 gene that had been deleted by double homologous recombination.³ Western blot and IFA analysis of the transformed parasites confirmed that MIC4 mutants were of the expected size and appropriately targeted to the micronemes (data not shown). ESAs prepared from these parasites were tested in host cell binding assays. As for the endogenous MIC4, the TyMIC4myc and MIC4hiss proteins bound substantially to host cells. In contrast, ESA corresponding to MIC4C12 failed to show MIC4 adhesiveness, possibly by compromising the proper folding of the domain A6.

**DISCUSSION**

Micronemal proteins are thought to be critical ligands determining host cell specificity at the time of invasion. Recent studies provide strong evidence that the transmembrane micronemal proteins of the TRAP family contribute not only to attachment but also to gliding motility and, thus, actively participate in the invasion process (2, 31). To ensure delivery of ligands at the right time and optimal place, micronemes exocytose adhesins and other factors in a regulated fashion onto the parasite surface during an early phase of invasion (8).

**FIG. 5.** Binding of MIC4 in presence of carbohydrates as competitors. ESA from RH was tested in the presence of increasing concentrations of galactose, N-acetylgalactosamine, or N-acetylgalcosamine.

**FIG. 6.** Schematic representation of the structural and functional domains of MIC4. The sites of proteolytic cleavages on MIC4 are indicated by arrows. The processed forms of MIC4 are depicted, and their corresponding adhesiveness to host cells is indicated with a plus (+) or minus (−). ER, reticulum endoplasmic; VT, valine threonine; SS, serine serine; rAb, rabbit antibodies.

**FIG. 7.** Schematic representation of the structural and functional domains of MIC4. The sites of proteolytic cleavages on MIC4 are indicated by arrows. The processed forms of MIC4 are depicted, and their corresponding adhesiveness to host cells is indicated with a plus (+) or minus (−). ER, reticulum endoplasmic; VT, valine threonine; SS, serine serine; rAb, rabbit antibodies.

apical secretion is sensitive to the kinase inhibitor staurosporine and can be stimulated by calcium ionophore or ethanol treatment (8, 26). These characteristics were used to explore the content of micronemes and to develop a strategy for the identification of novel micronemal proteins and cloning of their corresponding gene (32).

We present here the identification and characterization of a novel micronemal protein identified by this approach. The gene corresponding to MIC4 revealed the existence of a distinct type of adhesive motif called an “apple domain.” MIC4 contains six apple domains and shows a high degree of homology with a small major micronemal protein of *S. muris* containing two apple domains (18, 19) and the much larger micronemal protein from *E. tenella* EtMIC5 (9) with 11 apple domains. The *S. muris* protein is proteolytically processed and released at the apical tip of invading merozoites (33). This protein, called SML, was shown to form noncovalent homodimers and to recognize N-acetylgalactosamine as the dominant sugar (10). Apple do-
mains have been described previously on plasma proteins such as factor XI and prekallikrein (20, 34) and is composed of six half-cystine residues at highly conserved positions. Several studies show that apple domains are implicated in specific interactions between factors of the blood coagulation cascade (35, 36). A single apple domain can exhibit a very specific affinity, as illustrated by the interaction of the third domain A3 of activated factor XI with factor IX (34).

MIC4 has a calculated molecular mass of 61 kDa, and the deduced amino acid sequence from the gene predicts the presence of a signal peptide and six apple domains. We showed that the protein is localized to the micronemes of all infective stages of the parasite. One surprising incidental finding was that the polyclonal anti-MIC4 stained a sub-population of dense granules (wall-forming bodies, type 1) in the macrogametocyte and the outer veil of the early oocyst in the cat intestine. However, it was not possible to identify the molecule recognized as MIC4 or a closely related MIC4-like protein (37).

MIC4 is synthesized and stored in the parasites as a full-length 72-kDa form. Upon discharge from the micronemes, MIC4 is rapidly cleaved at the N terminus to produce a 70-kDa form and less efficiently at the C terminus. The C-terminal cleavage of the 70-kDa species into 50- and 15-kDa products causes a gap in size, as the processed forms do not add up to form 70 kDa. We can not exclude additional cleavage events, but the most likely explanation is inaccuracies in the size estimates or a change in conformation that effects migration. The C-terminal cleavage probably results from the protease activity of MPP2, which mediates the N-terminal processing of MIC2 at the surface of the parasite (28). In the case of MIC2, processing at the C terminus by another protease (MPP1) released the protein from the surface of the parasites and alters drastically the adhesive properties of MIC2. MIC4 binds efficiently to host cells, and the analysis of the diverse processed forms revealed that the adhesive properties of the molecule are confined within the apple domain at the C terminus. In contrast to MIC2, cleavage of MIC4 does not appear to influence the binding properties of MIC4. The 72-kDa precursor as well as 70 and 15 kDa processed forms of MIC4 bind to host cells, and therefore, the biological significance of MIC4 processing is not clear yet. The fact that the 50-kDa form of MIC4 failed to bind to host cells suggested that the adhesive properties of MIC4 are confined strictly to the last 15-kDa form of the protein, which corresponds to the domain A6. A deletion of 12 amino acids at the C terminus of MIC4 confirmed the importance of this region of the molecule for binding. In addition, a pretreatment of ESA with the reducing agent MESNA at 37 °C (but not at room temperature) abrogates completely MIC4 binding, suggesting that an intact apple structure hold by disulfide bridges is prerequisite for adhesion.

In T. gondii, several studies point to a crucial role of sugar-binding proteins in host cell recognition. The glycoprotein, BSA-glucosamidase, competitively blocks infection of human fibroblasts by tachyzoites and depends on the presence of the major surface antigen SAG1 (38). 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 rhotypes in a pattern similar to that observed with the lectins (39). More recent studies suggest that host recognition by T. gondii is mediated by parasite lectins (40) and that sulfated proteoglycans are one determinant used for substrate and cell recognition by MIC2 (30). In competition experiments, MIC4 binding to host cells in the presence of increasing concentrations of carbohydrates showed a biphasic effect. Host cell binding was enhanced at lower concentrations of competitors, but minimal binding was observed at higher doses. The relatively high doses imposed to induce competition suggest that the specificity of the lectin has not yet been identified, and possibly multivalent or more complex carbohydrate structures are involved. A previous study reported the identification of 45, 65, and 71 kDa lectins in T. gondii tachyzoites (40). The association of the 65- and 71-kDa proteins with host cells was abolished in presence of fucoidan, and a biphasic effect was reported in the competition experiments, similar to our observations. MIC4 binding to host cells in the presence of increasing amounts of fucoidan showed no competition, which likely rules out that MIC4 corresponds to the 71-kDa protein described by Ortega-Barrio and Boothroyd (40). Alternatively, like the apple domain studied in the coagulation factors, a yet unknown very specific protein-protein interaction might be responsible for binding.

Since MIC4 lacks a transmembrane or lipid anchor, it likely contributes to parasite adhesion by acting as a bridge between a receptor on the parasite and a receptor on the host cell. Indeed, a recent study has revealed that MIC4 forms a complex with two other micronemal proteins, MIC1 and MIC6 (30). MIC6 is a transmembrane protein that functions as a cargo receptor and ensures proper sorting of MIC1 and MIC4 to the micronemes. MIC6 also likely retains these soluble adhesins at the surface of the parasite during invasion. In this complex, MIC1 is directly and stably associated to MIC4, since the two proteins coimmunoprecipitate even in absence of MIC6. The region of MIC4 interacting with MIC1 is currently being investigated. MIC1, like MIC4, has previously been shown to bind to host cells (6) and does it also in the absence of MIC4 in mutant mic4ko. Therefore MIC1 association with MIC4 represents an interfering parameter in our competition studies that might explain the high doses of galactose necessary to abolish completely host cell binding. For further studies, the nature of the interaction between MIC4 and host cells will have to be examined in the absence of MIC1.

As also observed for other types of micronemal proteins, structural homologues of MIC4 exist in other Apicomplexa (1). The major micronemal antigen of S. muris contains two domains, whereas EtMIC5 exhibits 11 apple domains (GenBank™ accession number AJ245536). A search through the current status of the genome sequencing project of Plasmodium falciparum and the ESTs available for Plasmodium vivax and Plasmodium berghei failed to reveal the presence of a homologue in these members of Apicomplexa. Intriguingly S. muris, T. gondii, and Eimeria infect their hosts via the digestive tract. In contrast, Plasmodium species that are transmitted by an insect vector enter their mammalian host directly by injection into the blood. The existence of several distinct types of adhesins in T. gondii, including MIC1, MIC2, MIC3, and now MIC4, illustrates the diversity of strategies used by the parasite to establish interactions with the host. This diversity confers either a functional redundancy or might accommodate the broad range of host cell type specificity. It will be interesting to examine the possible role of MIC4 in the context of tissue specificity and to determine the nature of the receptor on host cells.

Acknowledgments—We are grateful to James Ajioka for the screening of the cosmid library. We thank Steve Parmley, Michael White, Keith Gull, and Marie-France Cesbron-Delauw for the generous donation of materials used here and Amy Crawford and Maren Lingnau for excellent technical assistance.

REFERENCES
1. Tomley, F. M., and Soldati, D. (2001) Parasitol. Today, in press
2. Kappe, S., Bruderer, T., Guant, S., Fujioka, H., Nussenzweig, V., and Menard, R. (1999) J. Cell Biol. 147, 937–944

*U. Jäkle, and D. Soldati unpublished information.*
