Proteomic Analysis of Cleavage Events Reveals a Dynamic Two-step Mechanism for Proteolysis of a Key Parasite Adhesive Complex*

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The transmembrane micronemal protein MIC2 and its partner M2AP comprise an adhesive complex that is required for rapid invasion of host cells by the obligate intracellular parasite *Toxoplasma gondii*. Recent studies have shown that the MIC2/M2AP complex undergoes extensive proteolytic processing on the parasite surface during invasion, including primary processing of M2AP by unknown proteases and proteolytic shedding of the complex by an anonymous protease called MPP1. While it was shown that MPP1-mediated cleavage is necessary for efficient invasion, it remained unclear whether the adhesive complex was liberated by juxtamembrane or intramembrane proteolysis. Here, using a three-phase strategy of assigning cleavage sites based on intact matrix-assisted laser desorption/ionization mass followed by confirmation by enzymatic digestion and inhibitor profiling, we demonstrate that M2AP is processed by two parasite-derived proteases called MPP2 and MPP3. We also define the substrate repertoire of MPP2 by two-dimensional differential gel electrophoresis using fluorescent tags. Finally, we use complementary mass spectrometric techniques to unequivocally show that MIC2 is shed by intramembrane cleavage within its anchoring domain. Based on the properties of this cleavage site, we conclude that the sheddase, MPP1, is likely a multipass membrane protease of the Rhomboid family. Our data support a novel two-step proteolysis model that includes primary processing of the MIC2/M2AP complex followed by secondary cleavage to shed the complex from the parasite surface during the final steps of invasion. Molecular & Cellular Proteomics 3:565–576, 2004.

The protozoan parasite *Toxoplasma gondii* is an intracellular pathogen classified in the phylum Apicomplexa. Infection with *T. gondii* causes severe illness (toxoplasmosis) when the pathogen is contracted congenitally or when it is reactivated in immune-suppressed individuals (1). Despite the usually effective antibiotics available for toxoplasmosis, undesired side effects of these drugs complicate current treatments. Recent studies have revealed that proteolytic processing plays crucial roles in parasite survival (reviewed in Ref. 2), and inhibition of proteases by selective inhibitors has been shown to block *Toxoplasma* invasion and intracellular development (3, 4). Accordingly, new insight into the proteolytic events in *T. gondii* has the potential to lead to the development of novel therapeutic strategies. In addition, *T. gondii* has recently gained attention as a model organism for apicomplexan parasites because it shares common apical structures and a similar mechanism of active invasion with relatives such as the malaria parasite *Plasmodium*, but is genetically easier to manipulate (5).

One of the first events in *T. gondii* invasion, coincident with apical attachment of the parasite to the host cell, is the release of numerous "soluble" and transmembrane (TM)-anchored proteins from secretory vesicles called micronemes (6, 7). These micronemal proteins (MICs) contain a variety of conserved adhesive domains, and several have been demonstrated to bind to receptors on mammalian cells (8). MIC2 (9) is a micronemal TM protein consisting of six thrombospondin-1-The abbreviations used are: TM, transmembrane; MICs, micronemal proteins; M2AP, MIC2-associated protein; mM2AP, mature M2AP; MPP1, Microneme protein protease 1; MPP2, microneme protein protease 2; MPP3, microneme protein protease 3; amu, atomic mass units; ESA, excretory/secretory antigen; DMEM, Dulbecco’s modified Eagle’s medium; 2DE, two-dimensional gel electrophoresis; MS, mass spectrometry; MALDI, matrix-assisted laser desorption/ionization; CID, collision-induced dissociation; Cy3, 1-(5-carboxypentyl)-11032N-propylindocarbocyanine halide; Cy5, 1-(5-carboxypentyl)-11032N/hydroxy-succinimidyl ester; TFA, trifluoroacetic acid; IPG, immobilized pH gradient; HPLC, high-pressure liquid chromatography; PMSF, phenylmethanesulfonyl fluoride; TOF, time-of-flight; QTOF, quadrupole TOF; RIP, regulated intramembrane proteolysis; JP, juxtamembrane proteolysis; MSP, merozoite surface protein.

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like adhesive repeats (M-domain), an integrin-like adhesive sequence (A-domain), and a short cytoplasmic tail indirectly connected to the actin-myosin system beneath the plasma membrane of the parasite (10). Within 15 min of synthesis, MIC2 is assembled into a complex with another micronemal protein, M2AP (11). M2AP is composed of a short pro-domain, a β-sheet domain, and a C-terminal coil domain. The complex remains associated during transit through the Golgi complex and storage in the micronemes. Upon parasite attachment to a host cell, the MIC2/M2AP complex is rapidly secreted onto the apical surface of the parasite. As *T. gondii* uses its actin-myosin system to actively penetrate into a target cell (12, 13), the MIC2/M2AP complex is translocated over the parasite body to its posterior end (14), where the ectodomain of MIC2 (A-domain and M-domain) is released through C-terminal proteolysis of MIC2, with M2AP still bound in the complex (15). The observation that discharge and translocation of MIC2/M2AP is tightly correlated with invasion has strongly implied an important role for the complex during invasion (11, 14). This is further supported by recent evidence showing that blocking C-terminal proteolysis of MIC2 can impair host cell entry (16). Together with the observation that M2AP-null mutant parasites are markedly defective in host-cell attachment and invasion (17), the collective evidence strongly suggests that MIC2/M2AP is a key protein complex that is required for efficient invasion. Despite this progress, however, the precise molecular details of how MIC2/M2AP facilitates invasion remain poorly understood.

MIC2 and M2AP undergo extensive proteolytic processing during maturation and invasion (11, 15). Indeed, proteolytic processing is a common post-translational modification for *Toxoplasma* MICs including, for example, AMA1 (18), MIC3 (19), and MIC6 (20). Processing occurs either within the parasite secretory pathway during protein maturation or on the parasite surface during invasion. In the case of M2AP, the pro-domain of M2AP is removed in the Golgi or nascent micronemes. When M2AP is discharged onto the surface of the parasite, it is further truncated into four smaller polypeptides (11). However, the precise cleavage sites and the proteases responsible for the processing remain unknown. Extensive processing of MIC2 also occurs on the parasite surface where this adhesin is multiply cleaved at its N terminus by a parasite-derived protease MPP2 (15). In addition, another parasite-derived protease MPP1 processes MIC2 at an undefined site in its C terminus, releasing the ectodomain into the medium. Recently, the Soldati group (20) demonstrated that a MIC2 hybrid protein containing multiple mutations within its TM anchor was resistant to C-terminal cleavage and shedding. This genetic evidence suggested that C-terminal cleavage might occur within the membrane. However, another recent genetic study by the Sibley group (16) demonstrated that a lysine-containing motif outside the TM segment is necessary for C-terminal processing. Because this C-terminal proteolysis is required for invasion, it is important to address this apparent discrepancy with compelling structural evidence. Also, because MPP1 has not been molecularly cloned or characterized, analysis of its cleavage site specificity could reveal important clues to its enzymatic properties.

Herein we unequivocally demonstrate that MIC2 is liberated from the parasite surface by intramembrane cleavage, based on precise identification of the cleavage site using complementary mass spectrometric approaches. Furthermore, we used proteomic analysis of cleavage sites and protease inhibitor profiling to show that M2AP is multiply processed by MPP2 and at least one additional novel parasite-derived protease we term MPP3. Based on these new findings, we propose a detailed two-step model for the dynamic processing of MIC2/M2AP, which includes primary and secondary processing steps that culminate in shedding of the adhesive complex from the parasite surface.

**EXPERIMENTAL PROCEDURES**

**Materials**—Porcine trypsin (sequencing grade) was obtained from Promega (Madison, WI), and endoproteinase Asp-N (excision grade) was obtained either from Calbiochem (La Jolla, CA) or Roche (Indianapolis, IN). 18O water was purchased from Isotec (CK Gas Products Ltd., Hampshire, United Kingdom). Mass spectrometry (MS) calibration mixture II (peptide mass standard kit) was obtained from Applied Biosystems (Framingham, MA), and calibration protein (aldolase) was obtained from Sigma ProteomassTM kit (Sigma, St. Louis, MO). Trifluoroacetic acid (TFA) was obtained from Pierce (Rockford, IL). Electrophoresis reagents and molecular marker proteins were obtained from BioRad (Hercules, CA). Fluorescent dyes (1-(5-carboxypropyl)-1′-propylindocarbocyanine halide (Cy3) and 1-(5-carboxypentyl)-1′-methylindodi-carboxyanine halide N-hydroxysuccinimidyl ester (Cy5)) and immobilized pH gradient (IPG) strips were obtained from Amersham Biosciences (Piscataway, NJ). Protease inhibitors were obtained from either Sigma or Roche. The matrix-assisted laser desorption/ionization (MALDI) matrix, α-cyano-4-hydroxycinnamic acid, and sinapinic acid were purchased from Sigma. All the organic solvents are high-pressure liquid chromatography (HPLC) grade. All other reagents and chemicals were obtained from either Fisher or Sigma and were of the highest purity available.

**Parasite Culture and Large-scale Excretory/Secretory Antigen (ESA) Preparation**—Parasite culture and ESA preparation was performed according to the protocol described previously (15). Briefly, *T. gondii* strain 2F was propagated in human foreskin fibroblast cells. Freshly egressed tachyzoites, a rapidly dividing parasite stage responsible for acute infection, were harvested by passage twice through a 20-gauge needle followed by filtration through a 3-μm pore size membrane to remove host cell debris. Parasites were washed twice by centrifugation in invasion medium prior to use in experiments. Large-scale preparation of ESA proteins was performed by incubating ~4 × 10^13 2F strain tachyzoites in 15 ml of Dulbecco’s modified Eagle’s medium (DMEM) containing 2 mM glutamine, 10 mM HEPES, and 1% (v/v) ethanol at 37 °C for 20 min followed by cooling on ice 5 min. Parasites were removed by centrifugation (1000 × g, 10 min, 4 °C), and the supernatant was concentrated to ~400 μl using C-20 concentrators according to the manufacturer’s instructions (Milipore, Bedford, MA).

**Secretion Experiment and Protease Inhibitor Assay**—Tachyzoites (4 × 10^7/ml) in modified invasion medium (DMEM + 10 mM HEPES) were incubated for 3 min at 37 °C with 1% (v/v) ethanol to stimulate microneme secretion. Tachyzoites were removed by centrifugation (twice at 1000 × g, 10 min, 4 °C), and supernatants were mixed with...
SDS-PAGE sample buffer containing 2.0% (v/v) 2-mercaptoethanol for SDS-PAGE and Western blotting (see below). Before being as- sayed for secretion as described above, tachyzoites were preincu- bated for 10 min at room temperature with 1% DMSO (solvent con- trol); 1 μM cytochalasin D (CytD); 5 μM pepstatin; 10 mM EDTA (sodium salt); 10 mM EGTA (sodium salt); 5 mM 1,10-phenanthroline; 50 μg ml−1 matrix metalloprotease-1 inhibitor; 10 μg 3,4-dichlorois- coumarin; 100 μM N-[1-chloro-3-[4-tosylamido]-7-amino-2-heptanone HCl; 100 μg 4-(2-aminoethyl)benzene-sulfonfylfluoride hydrochloride; 1 mg phenylmethylsulfonyl fluoride (PMSF); 40 μg ml−1 leupeptin; 10 μg E-64; and 40 μg ml−1 calpain inhibitor I (N-acetyl-Leu-Leu- norleucinal (ALLN)) or 40 μg ml−1 calpain inhibitor II (N-acetyl-Leu- Leu-methioninal (ALLM)).

** SDS-PAGE and Western Blotting—**Proteins were resolved on 12.5% or 10% reducing SDS-polyacrylamide gels. For Western blotting, PAGE gels were semidry transferred to nitrocellulose mem- branes. Blotting was performed as described previously (9) using RmrM2AP (whole serum, 1:5000).

**Two-dimensional Gel Electrophoresis (2DE) and Two-dimensional Differential Gel Electrophoresis (2D-DIGE)—**ESA proteins were sepa- rated in the first dimension using the Ettan IPGphor isoelectric focusing system (Amersham Biosciences). Then 120 μg of ESA protein was mixed with rehydration buffer containing 8 M urea, 2% 3-[3-cholami- dopropyl]dimethylammonionio]-1-propanesulfonic acid (CHAPS), 0.5% carrier ampholytes buffer, and 65 mM dithiothreitol (DTT). Rehydration and isoelectric focusing were performed according to the manufac- turer’s instructions using pH 4–7 IPG strips (13 cm for 2DE, 18 cm for 2D-DIGE). Following isoelectric focusing, proteins were reduced and alkylated by successive 15-min treatments with equilibration buffer containing 2% dithiothreitol followed by 2.5% iodoacetic acid. Then, proteins were resolved in the second dimension on a 12.5% SDS- PAGE gel. Resolved proteins were then either transferred directly to PVDF membrane for N-terminal sequencing or stained with colloidal Coomassie Blue stain (21) for MS analysis. 2D-DIGE experiments (22) were carried out as follows: 50 μg of lyophilized ALLN-treated and solvent (DMSO) control-treated ESA were each dissolved in 10 μl of labeling buffer (30 mM Tris, 8 M urea, 2% CHAPS, pH 8.5). Then 400 pmol each of Cy3 and Cy5 was added to the ALLN and control samples, respectively, and samples were incubated on ice for 30 min in the dark. After labeling, 1 μl of 10 mM lysine was added to stop the reaction. Cy3- and Cy5-labeled samples were mixed together and resolved by 2DE as described above. Fluorescence scanning was performed using a Typhoon 9200 variable mode imager (Molecular Dynamics, Sunnyvale, CA).

**N-terminal Sequencing—**Proteins on a 2DE gel were transferred to Immobilon™ PdIQ membranes (Millipore) and stained with Coomassie Brilliant Blue R-250. Bands were excised and subjected to Edman degradation on a Perkin-Elmer Biosystems Model 477A Gas-Phase Protein Sequencer (Wellesley, MA).

**Sequence Analysis—**Protein sequence alignments, pI, and molec- ular mass predictions were performed using the ExPASy PeptideMass (ca. 5 kDa) program, which used the web-based programs, ExPASy PeptideMass (ca. 5 kDa) program, which used the ExPASy PeptideMass database (http://www.expasy.org/tools/findpept.html). A threshold mass deviation of 50 ppm was used to search against the Toxoplasma NCB (National Center for Biotechnology Information) database. For in-gel digestion with 18O labeling, digests were performed in 50 mM NH4HCO3 solution and subjected to MS analysis. For MALDI-MS analysis of intact protein, the mass error of aldolase was less than 10 amu at 50% height. For assessing experimental deviation, three spectra were acquired for each sample deposited on the plate.

MALDI-MS Analysis of Digested Peptides—Protein spots from PAGE gels were excised, in-gel digested with endoproteinases (12.5 ng μl−1 trypsin or 10 ng μl−1 Asp-N) in 50 mM NH4HCO3, and solvent-extracted as described previously (24). Peptides excised from one-dimensional (1D) gels were reduced with DTT (50 mM DTT in 100 mM NH4HCO3, 50 °C, 1 h) and alkylated with iodoacetamide (275 mM iodoacetamide in 100 mM NH4HCO3 in the dark at room temperature 45 min) prior to digestion. After extraction, peptide mixtures were dried down and redissolved in 2 μl of 50% acetonitrile and 0.3% TFA, mixed with saturated α-cyano-4-hydroxy cinnamic acid (matrix) solu- tion, and deposited on the MALDI-MS target plate according to a general two-layer method (25). The MS spectra were acquired in reflection mode with delayed extraction and were calibrated using calibration peptide mixture II. Peptide mass fingerprints (PMF; a list of highly accurate peptide monoisotopic masses) were used to search against the Toxoplasma NCBI database via the MS-Fit algorithm (prospector.ucsf.edu) or searching the FindPept tool (ca.expasy.org/ tools/findpept.html). A threshold mass deviation of 50 ppm was used to judge true positive matches in all cases unless otherwise specified.

**Electrospray Ionization (ESI) Tandem Mass Spectrometry—**Peptide sequencing was performed by liquid chromatography tandem MS analysis of peptide fragments from collision-induced dissociation (CID). Peptides were fractionated by reverse-phase HPLC in a 0–60% methanol gradient with 0.5% formic acid over 30 min at ~200 nl min−1 on a 75 μm × 100 mm C18 PepMap column using an UltiMate HPLC system, equipped with a FAMOS autosampler (LC Packings, San Francisco, CA). Eluting peptides were sprayed through a 10-μm picotip (New Objectives, Woburn, MA) directly into a QSTAR Pulsar quadrupole orthogonal TOF tandem mass spectrom- eter (Applied Biosystems/MXD Sciex, Foster City, CA), equipped with a Protana XYZ manipulator source (MDS Protana AS, Odense, Den- mark). Using the software of Analyst QS (Applied Biosystems), spray, declustering, and focusing potentials were set at 1500, 60, and 220 V, respectively. Peptides were selected for fragmentation in information-dependent acquisition experiments monitoring charge states 2–4 of the two most intense ions. Parent ions were surveyed for 0.5 s, and selected ions were fragmented by CID at 25, 30, and 35 V collision energies. Fragmentation spectra were collected in enhance-all mode over 1.5-s durations. Specific ions of interest were placed in the included list for fragmentation. Errors on fragmentation ions were less than 0.02 amu. The acquired MS data were analyzed with the pro-
RESULTS

N-terminal Sequencing Reveals C-terminal Proteolysis of M2AP—Proteolytic processing is a common feature for all MICs (8). We previously reported (11) that in addition to the 40-kDa mature M2AP (mM2AP; i.e. prodomain removed), a series of four smaller M2AP polypeptides appeared in the ESA fraction. This suggested that mM2AP undergoes proteolytic processing on the surface of the parasite. 2DE of a large-scale ESA preparation confirmed the presence of the four processed products (termed M2AP-1, M2AP-2, M2AP-3, and M2AP-4), which shifted to the alkali end of the first dimension (Fig. 1, B and C). Based on this shift, we hypothesized that M2AP-1–4 were proteolytic products derived from successive proteolysis of the highly acidic C-terminal domain. Confirming this, microsequencing demonstrated that M2AP-1–4 each possess the same N terminus (TFLEL . . . ) as mM2AP (11).

Fig. 1. MALDI-MS analysis of intact masses of mM2AP and its processing products (M2AP1–4). A, schematic depiction of M2AP showing the propeptide (Pro) cleavage site (left arrow), the N-terminal sequence (TFLEL . . . ) of the β domain, and approximate positions of processing sites that correspond to M2AP1–4. B, coomassie-stained 2DE gel of secretory ESA proteins. Spots corresponding to mM2AP and M2AP1–4 are shown within the boxed area. Approximate boundaries of the pH 4.0–7.0 gradient are indicated along the top of the gel and positions of molecular mass markers (in kDa) are shown to the left. C, enlarged region of interest indicating the N-terminal sequences obtained from each spot by Edman degradation. D, spots were excised, extracted, and analyzed directly by MALDI-TOF. The average masses of mM2AP and M2AP1–4 are indicated in the respective spectra (indicated peaks correspond to singly charged molecular ions [M+H]+).
Assignment of C-terminal Cleavage Sites of M2AP Based on MALDI Intact Mass Analysis—To determine the precise C-terminal cleavage sites of M2AP processing, we first assigned the cleavage sites on the basis of the intact masses of mM2AP and M2AP1–4. Protein spots corresponding to mM2AP and M2AP1–4 were excised, solvent-extracted, and analyzed by MALDI-TOF. Calibrating with a standard protein (aldolase, 39212.28 average mass) and acquiring MS spectra under parallel acquisition conditions, the masses of mM2AP, M2AP-1, M2AP-2, M2AP-3, and M2AP-4 were measured as 30,438.35, 27,319.87, 23,621.91, 22,057.39, and 21,762.38 Da, respectively (Fig. 1). Because relative mass differences are more accurate than absolute masses, we calculated the sizes of the truncated fragments of M2AP1–4 by subtracting their masses from that of mM2AP. Based on this, we assigned C-terminal cleavage sites for each of the M2AP isoforms (see Fig. 2 and Table I).

Mass Spectrometric Peptide Fingerprints of M2AP1–4 Validate the C-terminal Cleavage Sites—To confirm the M2AP C-terminal cleavage sites, we used specific endoproteases for in-gel digests of 2DE gel-excised M2AP1–4 spots before acquiring PMFs of the proteolytic digests by MALDI-MS. Tryptic digests of M2AP2–4 in MS spectra revealed peptides with masses that, in every case, closely matched predicted peptide masses corresponding to the cleavage sites defined by intact mass analysis. For example, M2AP-2 showed a peak at m/z 4048.677 corresponding to the peptide 1TPLELVEVPDCSNVQVSNMQMVKKLQVAGSDNGVLLBPFYTVQK (theoretical m/z 4048.879) (Fig. 3B). Similarly, for M2AP-3 a prominent peak at m/z 2481.044, corresponding to the peptide 2AIPQPLTESVLPLPSKQHSGQEMSPHSGQPAEFLPPSPGLSPEEN (theoretical m/z 2481.153), was observed (Fig. 3C). Also, for M2AP-4, a peak at m/z 2140.978 closely matched the predicted m/z 2140.987, for the peptide 1TPLELVEVPDCSNVQVSNMQMVKKLQVAGSDNGVLLBPFYTVQK (theoretical m/z 2140.908). These peaks are exactly 1 Da more accurate than the theoretical values.

Assignment of C-terminal Cleavage Sites of M2AP—To confirm the M2AP C-terminal cleavage sites, we used specific endoproteases for in-gel digests of 2DE gel-excised M2AP1–4 spots before acquiring PMFs of the proteolytic digests by MALDI-MS. Tryptic digests of M2AP2–4 in MS spectra revealed peptides with masses that, in every case, closely matched predicted peptide masses corresponding to the cleavage sites defined by intact mass analysis. For example, M2AP-2 showed a peak at m/z 4048.677 corresponding to the peptide 1TPLELVEVPDCSNVQVSNMQMVKKLQVAGSDNGVLLBPFYTVQK (theoretical m/z 4048.879) (Fig. 3B). Similarly, for M2AP-3 a prominent peak at m/z 2481.044, corresponding to the peptide 2AIPQPLTESVLPLPSKQHSGQEMSPHSGQPAEFLPPSPGLSPEEN (theoretical m/z 2481.153), was observed (Fig. 3C). Also, for M2AP-4, a peak at m/z 2140.978 closely matched the predicted m/z 2140.987, for the peptide 1TPLELVEVPDCSNVQVSNMQMVKKLQVAGSDNGVLLBPFYTVQK (theoretical m/z 2140.908). These peaks are exactly 1 Da more accurate than the theoretical values.

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Mass Spectrometric Peptide Fingerprints of M2AP1–4 Validate the C-terminal Cleavage Sites—To confirm the M2AP C-terminal cleavage sites, we used specific endoproteases for in-gel digests of 2DE gel-excised M2AP1–4 spots before acquiring PMFs of the proteolytic digests by MALDI-MS. Tryptic digests of M2AP2–4 in MS spectra revealed peptides with masses that, in every case, closely matched predicted peptide masses corresponding to the cleavage sites defined by intact mass analysis. For example, M2AP-2 showed a peak at m/z 4048.677 corresponding to the peptide 1TPLELVEVPDCSNVQVSNMQMVKKLQVAGSDNGVLLBPFYTVQK (theoretical m/z 4048.879) (Fig. 3B). Similarly, for M2AP-3 a prominent peak at m/z 2481.044, corresponding to the peptide 2AIPQPLTESVLPLPSKQHSGQEMSPHSGQPAEFLPPSPGLSPEEN (theoretical m/z 2481.153), was observed (Fig. 3C). Also, for M2AP-4, a peak at m/z 2140.978 closely matched the predicted m/z 2140.987, for the peptide 1TPLELVEVPDCSNVQVSNMQMVKKLQVAGSDNGVLLBPFYTVQK (theoretical m/z 2140.908). These peaks are exactly 1 Da more accurate than the theoretical values.

### Table I

| Species   | Measured intact mass | Mass difference from mM2AP | Mass deviation of cleavage site |
|-----------|----------------------|----------------------------|---------------------------------|
| mM2AP     | 30,438.35 ± 14.56    | 0                          | 0                               |
| M2AP-1    | 27,319.87 ± 13.06    | 3,118.48 ± 26.98           | 9.34 (E256/E257)                |
| M2AP-2    | 23,621.91 ± 21.79    | 6,816.44 ± 36.26           | 31.48 (E222/S223)               |
| M2AP-3    | 22,057.39 ± 11.76    | 8,380.96 ± 24.71           | 27.34 (A207/E208)               |
| M2AP-4    | 21,762.38 ± 31.62    | 8,675.96 ± 43.23           | 18.04 (S203/A204)               |

* a Intact mass (± S.D., triplicate measurements) measured by MALDI-TOF MS as described in “Experimental Procedures.”
* b Mass (± S.D., triplicate measurements) difference determined by subtracting the measured intact masses of M2AP1–4 from the measured mass of mM2AP.
* c Mass deviation from the calculated value based on the cleavage site shown in parentheses.

the N-terminal side of aspartic acid residues. As shown in Fig. 3A, we observed two interesting peaks at m/z 595.279 and 611.238 in the Asp-N digest. These peaks are exactly 1 Da larger than the theoretical masses, m/z 594.279 and 610.238, of the peptide 252DNSMQ286 without and with oxidization of M255, respectively. According to previous studies (27, 28), the 1-Da difference is likely due to deamination of N253. The observation of a parallel addition of 1 Da in its methionine-oxidized derivative strongly supports this interpretation. Collectively, the above data demonstrate that M2AP is processed at highly discrete cleavage sites at Q256/E257, L222/S223, A207/E208, and S203/A204, with the last site corresponding to almost complete removal of the C-terminal coiled domain. M2AP Is Processed by Two Distinct Proteolytic Activities—To examine the protease(s) responsible for the surface proteolysis of the M2AP coiled domain, we performed protease inhibitor profiling experiments. As shown in Fig. 4A, proteolytic processing of M2AP was affected by a subset of protease inhibitors. These included chymostatin and PMSF, which showed partial inhibition, and the calpain inhibitors ALLN and ALLM, which strongly blocked processing. These inhibitors blocked the formation of the three smallest fragments of M2AP (M2AP2–4), without impairing the two largest fragments (mM2AP and M2AP-1). It should be noted that because the C-terminal coiled domain is highly antigenic, the smaller, more heavily processed species are less well recognized by the antiserum. Therefore, inhibition of M2AP2–4 formation by ALLN and ALLM is more pronounced than indicated by Western blotting. ALLN and ALLM are tripeptide aldehydes previously shown to block removal of an N-terminal extension from MIC2 by MPP2 (15). The following additional evidence further supports that MPP2 is responsible for generating M2AP2–4. First, similar to the observed process-
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Fig. 3. MALDI-TOF analysis of M2AP1–4 enzymatic digests. A, Asp-N PMF spectrum of M2AP-1. Ions indicated with arrows correspond to deaminated peptides derived from C-terminal cleavage of M2AP-1. a, Asp-N autolysis product; m, matrix ions. B, tryptic PMF spectrum of M2AP-2 showing an m/z 4048.677 peak corresponding to C-terminal cleavage at M2AP-2. C, tryptic PMF spectrum of M2AP-3 revealing an m/z 2481.044 peak derived from C-terminal cleavage at M2AP-3. D, tryptic PMF spectrum of M2AP-4. The m/z 2140.908 peak corresponds to C-terminal cleavage at M2AP-4. Ions marked with an asterisk indicate peptides derived from M2AP1–4.

Fig. 4. Effects of protease inhibitors on proteolytic processing of M2AP. A, ESA samples were prepared from tachyzoites treated with the protease inhibitors indicated at the top. Blots were probed with RurM2AP for detection of M2AP. DMSO, dimethylsulfoxide (solvent control); 1,10-Phen, 1,10-phenanthroline; MMP-1, matrix metalloprotease-1 inhibitor; DCI, dichloroisocoumarin; AEBSF, 4-(2-aminoethyl)benzenesulfonylfluoride hydrochloride; PMSF, phenylmethanesulfonyl fluoride; ALLN, N-acetyl-leu-leu-norleucinal; ALLM, N-acetyl-leu-leu-methioninal; CytD, cytochalasin D; CytD Post, CytD control (CytD was added to the sample just prior to gel-loading to control for a direct effect on migration). B, 2D-DIGE analysis ESA proteins collected from parasites treated with DMSO or ALLN were labeled with Cy3 (green) or Cy5 (red), respectively, and separated by 2DE (pH 4–7 first dimension and 12.5% SDS-PAGE second dimension). Proteins that were diminished by ALLN treatment are green. Marked proteins were excised and identified by PMF.

...ing of M2AP, trimming of the MIC2 N terminus occurs in multiple steps resulting in removal of a loosely structured region adjacent to a globular domain. Second, M2AP processing is dramatically enhanced by parasite treatment with CytD (Fig. 4A), which was previously shown to promote processing by MPP2 (15). Finally, based on processing of MIC2 at V50, G66, and S53, MPP2 prefers to cleave at a small amino acid in the P1 position, which is consistent with the cleavage specificity for M2AP2–4 (L222, A207, and S203, respectively). Although formation of M2AP2–4 appeared to be somewhat sensitive to EGTA, because levels of mM2AP and M2AP-1 were also lower, at least some of this effect is due to inhibition...
of microneme secretion, which is calcium dependent.

In contrast to M2AP2–4, formation of M2AP-1 was largely resistant to ALLN and ALLM and instead appeared to accumulate in the presence of these inhibitors. These results suggest that M2AP-1 is generated by a distinct parasite protease we term MPP3. This conclusion is further supported by the observation that the cleavage site specificity (NSMQ/EVEN) of MPP3 is different from that of MPP2. It remains to be determined whether primary cleavage by MPP3 is necessary for subsequent cleavage by MPP2.

In addition to the current findings for M2AP, MPP2 has been previously reported to cleave MIC2 (15) and MIC4 (29). However, it is not known whether MPP2 is exclusively dedicated to these substrates or if it is responsible for processing additional parasite proteins. To globally examine the MPP2 substrate repertoire, we compared ESA proteins from control and ALLN-treated parasites by 2D-DIGE. Control ESA proteins were covalently labeled with Cy3 (pseudocolored green) while ALLN ESA proteins were labeled with Cy5 (pseudocolored red), and the samples were mixed and separated on a single 2D gel. Protein spots that are diminished or absent in the ALLN sample are green, whereas proteins that increase in abundance are red. Spots that do not change significantly are yellow or shades thereof. As expected (Fig. 4B), ALLN treatment inhibited the formation of M2AP2–4, MIC2XQ (product of MPP2’s removal of the N-terminal extension), and the MIC4 cleavage products including the ~50-kDa N-terminal product and the “15”–kDa C-terminal product (29). However, ALLN also disrupted processing of SUB1, a micronemal subtilase of unknown function, by blocking the formation of several isoforms in the 70-kDa cluster (SUB170) and the 40-kDa product (SUB140). Although this implies that MPP2 is responsible for the processing of SUB1, we cannot rule out the possibility that ALLN inhibits other proteases in addition to MPP2. Verification of the complete MPP2 substrate repertoire will require its molecular cloning and the identification of additional selective inhibitors.

MIC2 Is Shed from the Parasite Surface by Intramembrane Cleavage—As mentioned above, recent studies have implied that MIC2 is released from the parasite surface by cleavage within the membrane (20) or outside the membrane (16). To resolve this issue, we purified the secreted form of MIC2 within the membrane (20) or outside the membrane (16). To determine whether primary cleavage by MPP3 is necessary for subsequent cleavage by MPP2.

To unambiguously verify the identity of the C-terminal peptide, we analyzed the Asp-N digests of the shed form of MIC2 with an ESI quadrupole–TOF (QTOF) tandem mass spectrometer. As expected, a triply charged ion ([M+3H]3+) with m/z 916.7 whose singly charged ion ([M+H]+) corresponds to the MALDI ion m/z 2748.262 was observed in the ESI-MS spectra. Subsequently, this peptide was selected and fragmented by CID. In the acquired fragmentation spectrum (Fig. 5C), the major fragmentation peaks were assigned to “y” and “b” series ions representing truncation from the C and N terminus of the peptide, respectively (fragmentation ions are labeled according to Biemann’s nomenclature (31)). Based on the assigned “y” series ions (y1, y2, y3, y4, y5, y7, y8, and y9; although the y6 ion was missing, the corresponding b6 ion was observed), the partial sequence . . . GSGIAGAIA704 was specified, which precisely terminates at the cleavage site identified above. Similarly, the N-terminal sequence 678DENETPTN . . . was largely specified by the “b” series ions in the spectrum (b2, b3, b4, b5, b7, b8, b9. the b1, and b6 ions were missing, but the corresponding y27 and y32 ions were observed). Similar to MALDI-TOF analysis, ESI-QTOF also failed to detect a peptide corresponding to juxtamembrane cleavage at the conserved lysine motif that is necessary for processing. Based on the above MS evidence, we conclude that MIC2
is shed from the parasite surface by intramembrane proteolytic cleavage at a site near the N-terminal side of its TM anchor. As shown in Fig. 6, alignment of the TM sequences of MIC2 and related proteins reveals that the cleavage site is strikingly well conserved, strongly suggesting that intramembrane cleavage is a general mechanism for shedding adhesive proteins from the surface of apicomplexan parasites.

**DISCUSSION**

Based on cleavage site specificity and inhibitor profiling data, primary processing of MIC2 and M2AP is mediated by two surface proteases, MPP2 and MPP3. While the detailed properties of these proteases await their molecular cloning and characterization, the available data suggest several clues to their features. First, MPP2 is probably a chymotrypsin-like serine protease or calpain-like cysteine protease based on partial inhibition by chymostatin and PMSF and near complete inhibition by ALLN and ALLM. Because these inhibitors may not be absolutely specific for either class of protease, screening of additional inhibitors will be required to better define the classification of MPP2. Second, we predict that MPP2 resides on the apical surface of the parasite. This is based on the observation that MPP2-mediated processing is dramatically enhanced by treatment with CytD, which dis-
related proteins showing the uniformly conserved amino acids (blue) within the TM anchor. Lysine residues implicated in C-terminal cleavage are indicated in red. The underlined sequence represents the C-terminal peptide derived from N-terminal cleavage by Asp-N and C-terminal cleavage by MPP1. The TM anchor region is highlighted in gray.

rupts the actin-dependent redistribution of the MIC2/M2AP complex toward the posterior end of the parasite, effectively trapping the enzyme and substrate together. Finally, MPP2 appears to have a limited substrate repertoire consisting primarily of MIC2, M2AP, MIC4, and SUB1, based on DIGE analysis of the parasite secretory products. While the processing of these substrates appears to be incomplete when assayed using extracellular parasites, it should be noted that in this situation the substrates are probably quickly released from the parasite surface. In contrast, the substrates appear to be stabilized on the parasite surface during invasion (7, 14), presumably leading to more efficient processing.

C-terminal processing of MIC2 by MPP1 is probably a crucial step in invasion based on the finding that parasites expressing a noncleavable copy of MIC2 were significantly impaired in cell entry (16). However, as mentioned above, conflicting evidence regarding the precise cleavage site was reported in two recent studies. Based on biochemical analysis of MIC6 and genetic analysis of MIC2 and MIC12, Soldati and Freeman (20) proposed that MIC2 is cleaved within its TM anchor by regulated intramembrane proteolysis (RIP), a recently identified mechanism of liberating membrane proteins. On the other hand, data reported by Sibley and coworkers (16) suggested that MIC2 is cleaved by juxtamembrane proteolysis (JP) at a dibasic motif consisting of K692/K693, i.e., several residues outside the TM anchor. In the present article, we resolve this issue by unequivocally demonstrating that MIC2 is cleaved within its TM anchor at A704/G705. While we cannot completely rule out the possibility of a separate JP cleavage, especially because it has been reported (32, 33) that JP often precedes RIP, we prefer the model that cleavage occurs exclusively by RIP for the following reasons. First, when ALLN or CytD treatment is used to eliminate MIC2 size heterogeneity due to N-terminal processing, MIC2 migrates as a well-defined band, suggesting it is generated by a single cleavage event (15). Second, as exemplified by its similarity to the Rhomboid substrate Spitz (Fig. 6), the MIC2 TM cleavage site closely resembles the recognition sequence for a Rhomboid-like protease (34), which is one of several enzymes capable of mediating RIP. Third, several genes encoding Rhomboid-like proteases are evident in the Toxoplasma genome database (D. Soldati, personal communication). Fourth, a recent study demonstrated that Drosophila Rhomboid-1 is capable of cleaving a hybrid protein containing the TM region of MIC2 (34). Finally, Rhomboid proteases typically do not require primary processing by JP (32). If indeed MIC2 processing occurs exclusively by RIP, it is possible that the membrane-proximal dibasic motif necessary for MIC2 C-terminal processing is either a distal recognition site for MPP1 (a putative Rhomboid-like protease) or is necessary to maintain the structural integrity of MIC2 for proper intramembrane cleavage.

RIP is generally carried out by multipass membrane proteases and occurs in a wide variety of organisms ranging from bacteria to humans (33). Recently, RIP has gained much attention as a recognized mechanism for intercellular and intracellular signaling (32). It was revealed that RIP mediates important signal transduction events associated with sterol regulation (e.g., SREBP cleaved by S2P (35)), cell fate selection (e.g., surface receptor Notch cleaved by γ-secretase (36)), and developmental growth regulation (e.g., Rhomboid cleavage of the Drosophila epidermal growth factor-like membrane proteins Spitz, Keren, and Gurken (37)). Also, the recently identified mammalian signal peptide peptidase is a multipass membrane aspartyl protease responsible for secondary processing of secretory signal peptides (38). In addition, RIP of amyloid precursor protein to produce the amyloid β peptide plays a key role in Alzheimer’s disease (39). Despite these established instances, the mechanism of RIP is only beginning to be unveiled, largely because the lipid bilayer presents a challenging experimental environment. Nonetheless, it was recently shown that RIP proteases probably use catalytic mechanisms similar to known proteases that require water to hydrolyze the peptide (32). Given this and considering that TM segments generally adopt a difficult-to-cleave helical conformation, the TM domain of the substrate protein must be at least partially unwound and exposed to the aqueous environment before or during the cleavage to allow water access to the cleavage site buried in the membrane. Accordingly, Urban and Freeman (34) recently suggested that Rhomboid substrates are susceptible to RIP cleavage because their TM domain contains potential helix-breaking residues (e.g., G and A), which tend to form a partially disordered conformation. This presumably contributes to the specificity of Rhomboid-like RIP proteases. Our defined RIP cleavage site at A704/G705 in the outer portion of the MIC2 membrane segment appears to be consistent with this mechanism for specificity.

Based on the analysis of MIC2/M2AP proteolysis presented herein, we propose a two-step processing model for MIC2/M2AP during invasion (Fig. 7). Once the parasite’s apical end attaches to a host cell, the MIC2/M2AP complex is mobilized from the micronemes onto the parasite surface where it un-
Processing of a Toxoplasma Adhesive Complex

Toxoplasma gondii (T. gondii) is an intracellular parasite that causes toxoplasmosis in humans and other animals. The parasite is known for its ability to invade erythrocytes and other cell types. The invasion process involves several critical steps, including interaction with host receptors, secretion of adhesive proteins, and proteolytic processing.

**Fig. 7. A two-step model for proteolytic processing of the MIC2/M2AP complex.** The MIC2/M2AP complex is assembled in the parasite endoplasmic reticulum (ER) before undergoing proteolytic maturation to remove a propeptide from M2AP as it transits through the Golgi apparatus en route to the micronemes (M). MIC2 is comprised of an N-terminal extension (N), which according to the known crystal structures of integrin A-domains probably connects with the base of the MIC2 A-domain (yellow sphere). The M-domain (blue) of MIC2 likely adopts an extended configuration based on its six subdomains that are similar to thrombospondin type-1 repeats. The TM anchor (red) connects the extracellular domains with the C-terminal cytosolic domain. M2AP is comprised of a globular β-domain (green) and an extended coiled domain (purple). The MIC2/M2AP complex is mobilized from the micronemes to the parasite anterior surface where it engages host receptors during parasite attachment. As the complex is actively translocated by the parasite actin-myosin motility machinery (motor complex) toward the posterior end, MIC2 and M2AP encounter two parasite-derived proteases, MPP2 and MPP3, which remove MIC2’s N-terminal extension and M2AP’s coiled domain. This primary processing might facilitate interaction with host receptors or clustering of adhesive proteins on the parasite surface. Upon further translocation, the complex undergoes secondary processing by a third parasite protease, MPP1, which is depicted as a multisplice integral membrane protein (yellow cylinders). RIP cleavage results in shedding of the complex and presumably aids in extracting the C-terminal domain from the membrane to facilitate degradation.

**Primary Processing**

- **1° Processing**
  - De novo incorporation of MIC2/M2AP into the membrane.
  - Cleavage within the TM domain of MIC2. If MPP1 is confined to the posterior end of the parasite, this step would be spatially regulated by movement of the substrate into the vicinity of the protease. This type of spatial regulation is commonly associated with RIP (35, 37). However, because the MIC2/M2AP complex is efficiently cleaved by MPP1 in the presence of CytD, at least some MPP1 is likely present on the apical surface. Definitive identification, molecular cloning, and analysis of the MPP1 should help clarify the regulatory mechanism governing the release of adhesive complexes.

**Secondary Processing**

- **2° Processing**
  - RIP cleavage of MIC2 by MPP1 likely fulfills at least four important roles. First, it severs the molecular connection between the parasite and its target host cell, thereby facilitating sealing of the parasitophorous vacuole membrane prior to the completion of cell invasion. Patch clamp studies have shown that this is the slowest, and therefore rate-limiting, step in invasion (40). Second, MPP1 cleavage appears to reduce the affinity of the MIC2/M2AP complex for its receptors (15), thereby preventing retention of the complex on the host plasma membrane after invasion. This probably serves as a mechanism to minimize detection by host immune surveillance. Third, in the absence of host cells, MPP1 cleavage prevents MIC2/M2AP complex from accumulating on the parasite posterior surface. Presumably this is detrimental for invasion because the parasite would then attach to the host cell via its posterior end, thereby precluding correct apical invasion (16). Finally, as suggested by others (33), RIP may facilitate extraction of the C-terminal domain from the membrane for rapid degradation. Indeed, this C-terminal domain appears to be highly unstable because it is undetectable even after maximally stimulating microneme secretion (15). This property likely serves to avoid the C-domain acting as a sink for components of the actin-myosin machinery, which would result in a dominant-negative effect on motility. Because at least MIC2, MIC6, and MIC12 (20) undergo intramembrane cleavage, RIP is likely a general mechanism used by T. gondii to shed adhesive proteins during invasion.

Interestingly, proteins on the surface of the malaria merozoite are also subjected to proteolytic processing and shedding during invasion (41). Merozoite surface protein-1 (MSP-1) is initially present as a high-molecular-mass protein expressed on the surface of malaria merozoites and noncovalently complexed with other parasite proteins (41). The 200-kDa MSP-1 precursor is processed into four smaller fragments coincident with merozoite release from an infected cell. During erythrocyte invasion, one of these primary processing products (MSP-1<sub>142</sub>) is further subjected to a secondary processing, thereby releasing the fragmented MSP-1 ectodomain and its associated partners into the surrounding medium. Only the 19-kDa processed product, MSP-1<sub>19</sub>, remains on the merozoite surface. The *Plasmodium falciparum* ortholog of AMA1 (PFAMA1) is also shed as a result of juxtanemembrane cleavage following its translocation from micronemes onto the merozoite surface (26). Similar to MPP1, the protease responsible for shedding of both these malaria proteins is a membrane-bound enzyme (26, 41). MSP-1 secondary processing has been demonstrated to be essential for successful erythrocyte invasion, and accordingly MSP-1<sub>19</sub> is...
emerging as a promising target for drug or vaccine development designed to block invasion (42). However, despite the striking similarities between processing of these malaria proteins and MIC2, several important differences exist in their features. First, MSP-1 circumferentially decorates the free merozoite surface, whereas MIC2 is a transient surface protein that is initially deployed only to the parasite apical surface. Second, in contrast to MSP-1, which is “shaved” off the merozoite surface as the moving junction slides over the parasite, MIC2 is excluded by the moving junction, resulting in it being swept toward the posterior end of the parasite during penetration. While some shedding of MIC2 may occur throughout invasion, it appears that much of the surface MIC2 is released from the posterior end of the parasite just prior to completion of invasion (14). Third, and most importantly, shedding of both malaria proteins occurs at JP cleavage sites, whereas secondary processing of MIC2 by MPP1 occurs within the TM anchor by RIP.

In summary, the present study suggests that T. gondii uses a dynamic and complex system for processing of MIC proteins during invasion, culminating in the intramembrane cleavage of adhesive complexes from the parasite surface. Given that these proteolytic events are likely crucial for Toxoplasma invasion, these findings shed new light on the invasive mechanism of T. gondii and provide new insight regarding the properties of parasite proteases that could be attractive therapeutic targets. In addition, these studies provide a novel example of sequential processing associated with RIP-mediated liberation of membrane proteins.

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