Structural Basis of *Toxoplasma gondii* MIC2-associated Protein Interaction with MIC2*

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**Background:** M2AP and the adhesin MIC2 form a complex involved in *T. gondii* gliding motility and invasion.

**Results:** M2AP adopts a galectin-like fold featuring a hydrophobic ligand binding site required for association with MIC2.

**Conclusion:** M2AP utilizes a modified galectin fold to bind a key adhesive protein.

**Significance:** The galectin fold is adaptable for binding carbohydrate and protein substrates.

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*Toxoplasma gondii* parasites must actively invade host cells to propagate. Secretory microneme proteins have been shown to be important for both gliding motility and active invasion. MIC2-M2AP is a protein complex that is essential for productive motility and rapid invasion by binding to host cell surface receptors. To investigate the architecture of the MIC2 and M2AP complex, we identified the minimal domains sufficient for interaction and solved the NMR solution structure of the globular domain of M2AP. We found that M2AP adopts a modified galectin fold similar to the C-terminal domain of another microneme protein, MIC1. NMR and immunoprecipitation analyses implicated hydrophobic residues on one face of the M2AP galectin fold in binding to the membrane proximal sixth thrombospondin type I repeat domain of MIC2. Our findings provide a second example of a galectin fold adapted for microneme protein-protein interactions and suggest a conserved strategy for the assembly and folding of diverse protein complexes.

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*Toxoplasma gondii* is a widespread intracellular pathogen infecting approximately one-third of the global human population. Although healthy individuals may experience mild flu-like symptoms during acute infection, immunocompromised persons are prone to more severe consequences, such as blindness or encephalitis due to reactivation of latent infection (1). As a member of the phylum Apicomplexa, *T. gondii* parasites must actively invade host cells to propagate. Gliding motility, a process where the parasite slides along the surface of the host cell, is essential for invasion. One protein complex that fulfills this requirement is MIC2 and its partner protein M2AP. The importance of this complex in motility and invasion was established through genetic deletion or tetracycline-regulated knockdown of either protein, resulting in reduced parasite invasion and virulence in mouse infection (6–8). MIC2 is the transmembrane (TM) member of the complex, with an extracellular region composed of an integrin-like I-domain followed by five thrombospondin-like repeats (TSRs) and a degenerate sixth TSR near the TM domain. The C-terminal cytosolic tail is thought to interact with the actin-myosin motility system, but the molecular basis of this connection remains to be determined (9, 10).

M2AP is a soluble protein with an N-terminal propeptide, a central β-domain, and a predicted C-terminal coil domain (11). M2AP does not appear to play a direct role in the binding of host cells. Rather, its expression is necessary for the proper trafficking and secretion of MIC2 (12). The propeptide of M2AP is required for correct trafficking of the MIC2-M2AP complex (12). Interestingly, M2AP mutants that are resistant to propeptide cleavage show normal trafficking of MIC2, implying that such mutants retain an ability to form a complex with MIC2 that is sufficiently strong to support trafficking to the micronemes. However, these mutants are defective in secretion of MIC2, and the complex appears to disassociate upon isola-
tion. These findings suggest that failure to remove the propeptide results in deficient formation of a tight complex but one that is nonetheless sufficient for trafficking within the parasite (12).

Crystal structures of the MIC2 I-domain (13) and the I-domain with the first TSR (14) suggested that the adhesive function of the I-domain is regulated by conformational changes, potentially induced by tension created during gliding motility. Low resolution data from small angle x-ray scattering implied that MIC2-M2AP adopts a dimeric structure with MIC2 appearing as an elongated rod and M2AP appearing as a “foot” at the C-terminal end of the M-domain (14). The study also showed that TSR6 is likely the major determinator for the interaction with M2AP; however, direct evidence for this was not provided, and the precise interface of the complex and the structure of M2AP were not addressed.

In this study we sought to decipher the domains and specific interfaces of MIC2 and M2AP that are necessary for the association of this protein complex. Using a combination of genetic, biochemical, and structural studies, we confirm that the membrane-proximal TSR6 of MIC2 principally interacts with M2AP. Our findings also reveal marked structural similarities between M2AP and MIC1, suggesting a conserved strategy for assembling MIC complexes.

**EXPERIMENTAL PROCEDURES**

**Parasite Culture**—Parasites were grown in human foreskin fibroblast cells and harvested as described previously (7).

**Expression Constructs**—Constructs for transfection into *T. gondii* parasites were based on the M2AP complement vector, pM2AP (6), containing the 5’ and 3’ sequences flanking M2AP. The M2AP cassette was replaced with MIC2 full-length and deletion sequences in the NsiI and PacI restriction sites. Deletion mutations were generated by fusion PCR using a Myc-tagged MIC2 template (pHLEM_TgMIC2, provided by L. D. Sibley, Washington University, St. Louis, MO), containing a SHuffle DE3 strain (New England Biolabs). For *Escherichia coli* expression, the MIC2 cDNA pHLEM_TgMIC2 was ligated into the NotI and KpnI restriction sites of the pBud-M2AP complement vector with the same primers as those used for M2AP. The M2AP cassette was replaced with MIC2 full-length constructs under the EF-1α promoter, pM2AP (6), containing the 5’-domain mutations were generated using the QuikChange XL mutagenesis kit (Stratagene). Primers used in the cloning experiments are listed in Table 1.

**Toxoplasma M2AP-MIC2 Interaction**

Transfection, Immunoprecipitation, Immunoblotting, and Immunofluorescence—MIC2 or M2AP constructs in pM2AP were transfected into MIC2-null parasites, EtM1/TgM2KO/ EtM2, or Δm2ap parasites, respectively (15). Egressed parasites were isolated 48 h post-transfection and lysed with radioimmunoprecipitation assay buffer (50 mM Tris, pH 7.5, 1% Triton X-100, 5% sodium deoxycholate, 0.2% SDS, 100 mM NaCl, 5 mM EDTA, pH 8.0). After a 20-min incubation for lysis, lysates were centrifuged at 13,000 × g for 10 min at 4 °C. The supernatant was removed and incubated with 1 μl of rabbit anti-M2AP antibody overnight at 4 °C with nutation followed by incubation with protein G-Sepharose beads for 1.5 h. Beads were washed 4× with radioimmunoprecipitation assay buffer and boiled in 1× SDS-PAGE sample buffer.

CHO K1 cells were maintained in Ham’s F-12, 10% FBS, 50 μg/ml penicillin/streptomycin at 37 °C and 5% CO₂. Cells were plated into 6-well plates at a density of 2.5 × 10⁴/ml for 20 h and transfected with 1 μg of plasmid DNA using FuGENE 6 transfection reagent (Roche Applied Science) at a 1:3 ratio of FuGENE:DNA. At 48 h post-transfection, cells were washed with phosphate buffered saline (PBS; 2.6 mM KCl, 1.47 mM KH₂PO₄, 137.9 mM NaCl, 8.09 mM Na₂HPO₄) and scraped into 1 ml of PBS. 100 μl was pelleted and resuspended in 1 ml of 1× SDS-PAGE sample buffer for cell lysates, and 900 μl was pelleted and resuspended in radioimmunoprecipitation assay buffer for immunoprecipitation (IP) as described above for parasite lysates. IPs were performed as described above.

Cell lysates and IPs were run on 10% SDS-PAGE gels and blotted by semi-dry transfer (Bio-Rad) to polyvinylidene fluoride (PVDF) membrane. Membranes were blocked with 5% nonfat milk, probed with mouse anti-myc 9E10 monoclonal antibody (Developmental Studies Hybridoma Bank) in 1.25% nonfat milk, PBS, 0.1% Tween 20 (PBS-T), washed in PBS-T, and incubated with goat anti-mouse HRP in 1.25% milk, PBS-T. Blots were incubated with enhanced chemiluminescent (ECL) substrate (Super Signal West Pico, Pierce) and exposed to film.

Immunofluorescence staining was performed as described (6) using rabbit anti-M2AP (11), mouse mAb 6D10 anti-MIC2 (16), or affinity-purified rabbit anti-VP1 (17).

**Quantification of Western Blots**—Cell lysates and IPs were prepared and performed as per ECL detection with the following modifications. SDS-PAGE gels were semidy transferred onto Immobilon-FL PVDF (Millipore) and blocked with 1.25% fish gelatin (Sigma). Membranes were probed with mouse anti-myc antibody at 1:5000 in PBS-T, washed 4× in PBS-T, and incubated with goat anti-mouse fluorophore-800 at 1:20,000 (Licor Biosciences). After washes in PBS-T, membranes were imaged on a LiCor Odyssey imager and quantified using LiCor Software.

**Protein Expression for NMR Spectroscopy**—Truncated wild type and mutant M2AP (residues 47–228), MIC2-TSR12 (residues 271–402), and MIC2-TSR56 (residues 532–648) were generated by PCR and then subcloned into pET-32 Xa/LIC plasmid (Novagen). The protein was then expressed in SHuffle® *Escherichia coli* strain (New England Biolabs). For [15N,13C] labeling of wild type M2AP, minimal media containing 0.07% [15N]NH₄Cl and 0.2% [13C₆]glucose was used; otherwise Luria-Bertani media was used. For protein production, cultures were grown in minimal media.
Toxoplasma M2AP-MIC2 Interaction

TABLE 1
Primer names and sequences

| Primer name                     | Primer sequence |
|--------------------------------|-----------------|
| pM2AP complement vector        |                 |
| IMC (WT MIC2)                  |_MIC2.3.Nsil.F   |
| M2AP                          |_MIC2.2307.Pac1.R|
| MIC2-ΔI                       |_MIC2.76.808.F  |
| MIC2-ΔM                       |_MIC2.808.76.R  |
| MIC2-ΔTSR1–2                  |_MIC2.808.1207.F|
| MIC2-ΔTSR1–4                  |_MIC2.1207.808.R|
| MIC2-ΔTSR3–6                  |_MIC2.1184.myc679.F|
| MIC2-ΔTSR5–6                  |_MIC2.1565.myc679.F|
| pBud-CE4 vector               |                 |
| IMC (WT MIC2)                  |_MIC2.1.Notl.Koz.F|
| M2AP                          |_MIC2.2310.KpnI.R|
| TSR5–6                        |_MIC2.1567.1579.F|
| MIC2                            |_MIC2.1780.915.F|
| M2AP-F123A                    |_M2AP.F123A.F    |
| M2AP-L125A.R                  |_M2AP.L125A.R    |
| M2AP-W134A,R                  |_M2AP.W134A.R    |
| M2AP-V136A,R                  |_M2AP.V136A.R    |
| M2AP-K76A                      |_M2AP.K76A.F     |
| M2AP-K80A                      |_M2AP.K80A.F     |
| M2AP-K100A                     |_M2AP.K100A.F    |
| M2AP-K76A-K80A                 |_M2AP.K76A-K80A.F|

The frequencies window tolerances for assigning NOEs were ±0.05 ppm for direct proton dimensions and ±0.05 ppm for indirect proton dimensions and ±0.5 ppm for nitrogen dimensions and ±1.1 ppm for carbon dimensions. The 100 lowest energy structures had no NOE violations >0.5 Å and no dihedral angle violations >5°. For NMR interaction mapping of TSR fragments with M2AP, 1H,15N HSQC spectra of 15N-labeled M2AP were recorded at ~100 μM M2AP concentration in the absence and presence of at least 6-fold molar excess of TSR1–2 or TSR5–6.

RESULTS

The TSR3–6 Region of MIC2 Is Important for Binding

M2AP—MIC2 and M2AP form a stable complex soon after synthesis in the endoplasmic reticulum, and they remain together indefinitely thereafter. To elucidate the domains responsible for this tight association, we generated serial deletion constructs containing a Myc epitope tag near the MIC2 TM anchor for an initial assessment by transient expression in the parasite (Fig. 1A). Domain truncation constructs included deletion of the two major extracellular domains, the integrin-like I-domain (MIC2-ΔI), and the domain containing the TSRs (MIC2-ΔM). To examine this interaction in the endogenous system and a MIC2-null background, constructs were transiently transfected into EtM1/TgM2KO/EtM2 parasites (15). These parasites express the Eimeria tenella orthologs of MIC2 and M2AP termed EtMIC1 and EtMIC2, respectively. Our previous work demonstrated that expressing the Eimeria func-

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were grown at 37 °C before induction with 1 mM isopropyl-β-1-thiogalactopyranoside at 18 °C for 12 h. The protein was purified using nickel-nitritoltriacetic acid-agarose (Promega). After incubation with Factor Xa protease, the tag was removed by cleavage using nickel-nitritoltriacetic acid-agarose (Promega). After purification, the protein was purified by size exclusion chromatography using a Superdex 75 column (GE Healthcare) and re-applying the mixture to nickel-nitritoltriacetic acid-agarose.

Heteronuclear single quantum coherence (HSQC) spectra were collected at 298 K on Bruker DRX500 and DRX800 spectrometers equipped with z-shielded gradient triple resonance cryoprobes.

The side-chain assignments of the protein were completed using HCCCH two-dimensional total correlation spectroscopy (TOCSY) and (H)CC(CC)NH TOCSY. The distance restraint from TALOS (20) were also incorporated in the calculation.

ARIA (19) was used for completion of the NOE assignment and structure calculation. Dihedral angle restraints derived from TALOS (20) were also incorporated in the calculation. The frequency window tolerances for assigning NOEs were ±0.05 ppm for direct proton dimensions and ±0.05 ppm for indirect proton dimensions and ±0.5 ppm for nitrogen dimensions and ±1.1 ppm for carbon dimensions. The 100 lowest energy structures had no NOE violations >0.5 Å and no dihedral angle violations >5°. For NMR interaction mapping of TSR fragments with M2AP, 1H,15N HSQC spectra of 15N-labeled M2AP were recorded at ~100 μM M2AP concentration in the absence and presence of at least 6-fold molar excess of TSR1–2 or TSR5–6.

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tional complex permitted deletion of MIC2, thus creating a null background for expression of MIC2 domain deletion mutants. A MIC2 direct knock-out was recently reported (8), but this strain was not available when we initiated the current study. Due to the low signal from transient transfection, expression of the domain deletion constructs was assessed by immunoprecipitating MIC2 with anti-MIC2 and detection with anti-Myc. The MIC2 interaction with M2AP was tested by IP using anti-M2AP and detection with anti-Myc. M2AP efficiently co-immunoprecipitated with full-length MIC2 (IMC) and MIC2-H9004 even though the expression of MIC2-H9004 was below the limit of detection (Fig. 1A). M2AP showed less efficient association with the MIC2-H9004M, implicating the M-domain in binding M2AP. With respect to M2AP domains involved in the interaction, the propeptide of M2AP is cleaved during trafficking, and the coiled tail domain is serially processed after secretion, yet the MIC2-M2AP complex remains associated. This suggests the predominant interaction is between the M domain of MIC2 and the central β-domain of M2AP. Accordingly, we focused on serial deletions of the M domain to assess interactions with M2AP.

The M domain is composed of five conserved TSRs together with a single degenerate TSR in the sixth position (16). Constructs were made with block deletions of 2 or 4 adjacent TSRs: the first two, first four, last four, and last two (Fig. 1B). Transient expression of these constructs in EtM1/TgM2KO/EtM2 parasites showed that the interaction with constructs lacking the first two or four TSRs was similar to that observed with full-length MIC2 (Fig. 1B), which implicates TSRs 5 and 6 in binding to M2AP.

**MIC2 TSR6 Is Sufficient for Interaction with M2AP**—The IP results of the MIC2 mutants indicated that the two TSRs (5 and 6) closest to the TM domain were important for interaction with M2AP. To determine whether TSR5 and/or TSR6 is suffi-


Cys-61–Cys-68 and Cys-103–Cys-110 staple together two antiparallel β-strands (Fig. 2B). Two disulfide bonds, Cys-61–Cys-77 and Cys-110–Cys-120, staple together two antiparallel β-hairpins, namely 5'-face of the sandwich. This region from two molecules of MIC1 interacts with a single MIC6 via its second and third EGF domains. A search using program DALI (22) confirmed M2AP as most similar in structure to the galectin-like domain of MIC1. M2AP superimposes with a root mean standard deviation of 3.0 Å over 106 equivalent backbone Cα atoms of the MIC1 galactin-like domain (Z-score 6.5) (Fig. 2C). Like MIC1, M2AP does not possess a canonical carbohydrate binding site but displays features more akin to a protein–protein interaction surface in its place. A prominent hydrophobic patch is delineated by Phe-123, Leu-125, Trp-134, and Val-136 (Fig. 2D), which also coincides with the MIC6 binding surface on MIC1 (Fig. 2C).

**Mapping of TSR56 Interaction Sites on M2AP—**Our NMR assignments and solution structure provide the opportunity to interpret NMR titration experiments and identify the interacting surfaces between M2AP and MIC2 TSR5–6. Despite successful efforts to produce MIC2 TSR pairs using recombinant expression systems, fragments were poorly folded as determined from dispersion in NMR spectra (data not shown). Nonetheless, chemical shift deviations were observed in M2AP spectra in the presence of TSR5–6. The most significant chemical shift changes (Fig. 2E) localized to the β2-β11-β4-β5-β6-β7 face of the β-sandwich in titration (Fig. 2F, left). However, nearly identical chemical shift perturbations on the β2-β11-β4-β5-β6-β7 face were also seen in titrations with TSR1–2 (data not shown), suggesting that this negatively charged surface displays a significant nonspecific interaction with partially folded TSRs.

**Several Residues on the β2-β11-β4-β5-β6-β7 Face of M2AP Are Not Required for Interaction with MIC2 TSR6—**Although chemical shifts on the β2-β11-β4-β5-β6-β7 face were not unique to interaction with TSR5–6, we nonetheless wanted to test their potential role in binding. Because MIC2 TSR6 is sufficient for interaction with M2AP and its isoelectric point is low (5.2), to test the potential contributions we focused on mutating positively charged residues on the β2-β11-β4-β5-β6-β7 face of M2AP. Two lysine residues showing chemical shift changes (Lys-80 and Lys-100) along with one residue (Lys-76) that did not show a shift but is in the vicinity of shifted residues were mutated to alanine and expressed in CHO cells for interaction with TSR6. A portion of the transfected CHO cells was used for lysate, and the remainder was used for IP. The myc tag was used for normalization within each lane to control for the differences in expression levels of the various M2AP mutants. The interaction of TSR6 with M2AP was quantified by infrared fluorescence imaging. The results show that binding of the single point mutants (K76A, K80A, K100A) or a double mutant (K76A/K80A) to TSR6 is indistinguishable from binding of WT ΔproM2AP (Fig. 3A and B). Although these findings suggest that positively charged residues on the β2-β11-β4-β5-β6-β7 face of M2AP are not required for interaction with TSR6, the possibility that they play a supplementary role in the interaction cannot be excluded.

**M2AP Hydrophobic Patch Residues Are Necessary for Interaction with MIC2 TSR6—**We noted small but discernable chemical shift changes on the β1-β12-β3-β8-β9-β10 face proximal to the hydrophobic patch noted above (Fig. 2F, right panel). Because TSR6 is sufficient for formation of the complex, it likely forms a high affinity protein–protein interaction with M2AP involving burial of an exposed hydrophobic surface. Furthermore, the resemblance between the hydrophobic patch of M2AP and the EGF binding interface of MIC1 lead to the

### Table 2

| Structure calculation statistics for M2AP |
|----------------------------------------|
| **NMR distance and dihedral constraints** |
| **Distance constraints** |
| Total NOE | 2542 |
| Intraresidue | 910 |
| Interresidue | 1632 |
| Sequential (| |) | 535 |
| Medium range (| |) | 143 |
| Long range (| |) | 47 |
| Total dihedral angle restraints | 907 |
| φ | 94 |
| ψ | 94 |
| Structural statistics |
| Violations (mean and S.D.) |
| Distance constraints (Å) | 0.040 ± 0.007 |
| Dihedral angle constraints (°) | 1.54 ± 0.43 |
| Maximum dihedral angle violation (°) | 0.89 |
| Maximum distance constraint violation (Å) | 0.23 |
| Deviations from idealized geometry |
| Bond length (Å) | 0.005 ± 0.000 |
| Bond angle (°) | 0.59 ± 0.003 |
| Improper s (°) | 2.15 ± 0.145 |
| Average pairwise rmsd"(Å) |
| Heavy | 0.579 |
| Backbone | 0.253 |
notion that this may represent a binding site for the degenerate TSR6.

To further investigate the role of M2AP hydrophobic patch residues in the interaction of M2AP with MIC2-TSR6, we created alanine substitutions of the M2AP at four positions in the hydrophobic patch (Phe-123, Leu-125, Trp-134, and Val-136) in constructs expressing MIC2 TSR6 or TSR5–6. Expression of the TSR6 and the M2AP mutants in CHO cells was relatively similar (Fig. 4A, Lysate), whereas the level of interaction was significantly reduced in mutants F123A, L125A, and W134A, indicating a role for these residues in binding TSR6 (Fig. 4A, IP). In contrast, the V136A mutation had no effect on the interaction. Quantification of multiple experiments revealed a statistically significant reduction in the interaction of F123A, L125A, and W134A mutants with MIC2 TSR6 (Fig. 4B). Similar results were obtained with constructs expressing M2AP mutants and similar (Fig. 4A, Lysate), whereas the level of interaction was significantly reduced in mutants F123A, L125A, and W134A, indicating a role for these residues in binding TSR6 (Fig. 4A, IP). In contrast, the V136A mutation had no effect on the interaction. Quantification of multiple experiments revealed a statistically significant reduction in the interaction of F123A, L125A, and W134A mutants with MIC2 TSR6 (Fig. 4B). Similar results were obtained with constructs expressing M2AP mutants and
Toxoplasma M2AP-MIC2 Interaction

FIGURE 3. Positively charged residues on the β2-β11-β4-β5-β6-β7 face of M2AP are not required for interaction with MIC2 TSR6. A, expression of M2AP mutants and TSR6 in CHO cells (lysate lanes) and immunoprecipitation with RbαM2AP followed by detection of M2AP mutants and TSR6 with MscMyc (IP lanes). B, quantification of expression versus interaction of M2AP mutants with TSR6. Interaction:expression ratios for mutant M2APs were normalized to that of WT M2AP set to 100%. Data are compiled from three independent experiments. Error bars represent S.D. n.s., not significant.

TSR5–6 (Fig. 4C), except that the W134A mutation had less of an effect on binding TSR6 than on TSR5–6 (Fig. 4D). Expression of the M2AP mutant proteins was similar to that of wild type M2AP, implying that lack of interaction was not due to gross misfolding of M2AP. To investigate this further we assessed the structural integrity of recombinant M2AP mutants by 1H NMR spectroscopy, which showed that all four mutants are well folded and exhibit similar upfield aliphatic peak patterns to wild type M2AP (Fig. 4E). Altogether our findings suggest that Phe-123 and Leu-125 are principal residues for association with TSR6 and that Trp-134 plays a contributing role.

Necessity of M2AP Hydrophobic Patch Residues for Stable Interaction with MIC2 Is Recapitulated in Transgenic Parasites—To validate the above findings under more physiologic conditions, we stably expressed the M2AP hydrophobic patch mutants in Δm2ap parasites (6) and assessed their localization and interaction with MIC2. Whereas F123A, L125A, and V136A showed prominent localization to the micronemes similar to WT M2AP, W134A showed extensive staining near the parasite nucleus in a pattern consistent with the parasite endoplasmic reticulum. Nonetheless, limited association of W134A with MIC2 was noted, which is consistent with this mutant being sufficiently functional to guide MIC2 to the apical micronemes more effectively than parasites completely lacking M2AP (compare W134A with Δm2ap in Fig. 5A). Δm2ap parasites show partial retention of MIC2 in a subapical compartment marked with vacuolar pyrophosphatase 1 (VP1) (12), which is not seen in W134A parasites (Fig. 5B).

To assess the impact of M2AP hydrophobic patch mutations on association with MIC2, we prepared parasite lysates and used a portion to determine M2AP expression levels and the remainder for immunoprecipitation reactions. Quantification was performed as described for the interactions in CHO cells. Consistent with the results from CHO cells, F123A, L125A, and W134A mutants showed significantly less association with MIC2 than WT M2AP, an M2AP complement, or the V136A mutant (Fig. 5C and D). The greatest effect was observed with the W134A mutant, where interaction with MIC2 was nearly completely abolished.

M2AP is processed from a proform to a mature form during trafficking to the micronemes (12, 23). Immunoblots detecting M2AP mutants in transfected parasites showed that the W134A mutant is almost exclusively in the proform (Fig. 5C, bottom panel), possibly reflecting the population that is retained in the parasite endoplasmic reticulum since maturation is known to occur in a post-Golgi compartment(s) (12). Interestingly, the propeptide in the L125A is processed efficiently, indicating its exposure to post-Golgi maturation, which is dependent upon association with MIC2 (6), and yet interaction with MIC2 was significantly reduced. This, coupled with the correct trafficking of MIC2 in all of the mutants, likely reflects weak residual association with MIC2. Weak association is maintained by high local concentrations within the exocytic system of the parasite but is subsequently lost upon extraction for immunoprecipitation.

DISCUSSION

MIC2-M2AP is an abundant microneme complex that adopts a stable conformation soon after synthesis and remains together perpetually even after proteolytic shedding from the parasite surface. To determine the specific domains and residues necessary for this tight interaction, domain deletions of MIC2 and site mutations of M2AP were generated. Expression of full-length MIC2, ΔI-domain, and ΔM-domain constructs in the parasite suggested that TSRs in the M-domain are principally responsible for interaction with M2AP. These findings were confirmed by subsequent deletion constructs showing that the membrane proximal TSR6 is necessary and sufficient for binding M2AP. Nonetheless, a modest association was still seen between the ΔM-domain mutant and M2AP, implying a residual interaction with the I-domain. The extent that these supplementary interactions influence the fully assembled MIC2-M2AP complex remains to be determined by structural studies of the entire complex.

Alignments of the TSRs from T. gondii MIC2 and EtMIC1 revealed conserved features that may have implications for the structure and function of their respective protein complexes (16). The first 5 TSR domains are consistent with the canonical TSRs from thrombospondin-1, but the sixth TSR is less well conserved particularly in the CSVTCG signature motif. Divergence from the canonical sequence and other sequences in this TSR could indicate specialization of this domain for interaction with M2AP. Additionally, the membrane proximity of TSR6 likely has functional implications for placing M2AP near the membrane. M2AP, and more specifically amino acids in its pro-
peptide, are required for correct trafficking of the MIC2-M2AP complex to the micronemes (12, 23). The M2AP propeptide was proposed to interact with a hypothetical cargo receptor that guides the complex from the endosomal-like organelles to the micronemes. Membrane proximity could place M2AP in an optimal position to interact with such a receptor. Although a sortilin-like cargo receptor termed TgSORTLR was recently shown to bind several MICs and is crucial for microneme bio-

**FIGURE 4.** Several residues in the hydrophobic patch of mature M2AP are required for interaction with TSR6 and TSR5–6 in CHO cells. A and C, expression of M2AP mutants and TSR5–6 or TSR6 (Lysate lanes) in CHO cells and IP with RbuM2AP. B and D, quantification of expression versus interaction of M2AP mutants with TSR6 or TSR5–6. Interaction:expression ratios for mutant M2APs were normalized to that of WT M2AP set to 100%. Data were compiled from at least three independent experiments. *** p value < 0.001; ** p value < 0.006. Error bars represent S.D. n.s., not significant. E, upfield methyl region from the 1H NMR spectra for the four M2AP mutants overlaid with that of wild type M2AP. The WT and W134A major peaks are truncated.
genesis, it remains unclear whether TgSORTLR is responsible for M2AP-dependent trafficking of the MIC2-M2AP complex to the micronemes (24).

The mode of recognition previously observed for the galectin-like domain of MIC1 suggested a more generalized means of constructing functional assemblies (2, 3). The EGF domain augments one edge of the galectin β-sheet as well as clamping onto the neighboring exposed sheet surface. A TSR domain comprises an irregular, antiparallel β-sheet with a rippled first strand. The core is localized on one face of the sheet and is stabilized by layered arrangement of tryptophan and arginine residues. It is conceivable that in tandem TSR domains the pair could interact with the galectin fold of M2AP in an analogous fashion to the two β-hairpins of EGF domains seen in the MIC1-MIC6 interaction. The relative location of the exposed hydrophobic patch on the galectin β-sheet face is conserved between MIC1 and M2AP (Fig. 2, C and D), and our mutagenesis of this region confirms its crucial role in binding MIC2 in CHO cells and in the parasite. Two residues in this region also show modest chemical perturbations in the NMR titration, namely Val-122 and Phe-123. These comparatively small chemical shift changes may reflect averaging at the M2AP-TSR6 interface as our recombinant TSRs have been shown to be only partially folded. Nonetheless our combined mutagenesis and NMR data provide the first direct evidence for an interaction between the hydrophobic patch in M2AP and TSR6 in MIC2 being the major determinants for complex formation. The ability of TSR6 to stably associate with M2AP when the two proteins are expressed together likely reflects the need for chaperone-mediated folding and facilitation of complex assembly in vivo. Our data provide evidence to support a recent small angle x-ray scattering model that suggests the proximal TSR domain (i.e. TSR6) is the likely interaction point of M2AP and that this assists in the extension of MIC2 away from the parasite surface for optimal presentation of the I-domain and M-domain to host receptors (14).

FIGURE 5. Expression of M2AP hydrophobic patch mutants in Δm2ap parasites confirms their role in tight binding to MIC2. A, immunofluorescence staining of M2AP and MIC2 to assess trafficking to the micronemes. Overnight replicated RH wildtype or Δm2ap parasites were fixed and stained with rabbit anti-M2AP and mouse mAb 6D10 anti-MIC2. Arrows indicate retention in a post-Golgi compartment. Bar, 5 μm. B, immunofluorescence staining of VP1 and MIC2 to assess retention in a post-Golgi compartment(s). Parasites were stained as for A except for using affinity purified rabbit anti-VP1. Arrows indicate retention in a post-Golgi compartment. C, expression of M2AP mutants and TSR6 in Δm2ap parasites (Lysate lanes) and immunoprecipitation with RbM2AP (IP lanes). MW, molecular weight. D, quantification of expression versus interaction of M2AP mutants with TSR6. Interaction:expression ratios for mutant M2APs were normalized to that of WT M2AP set to 100%. Data are compiled from three independent experiments. Error bars represent S.D. n.s., not significant.
Tandem stretches of closely spaced, cysteine-rich domains (e.g. EGF and TSR) are often susceptible to misfolding. Apicomplexan parasites have evolved specialized partner proteins, and likely chaperones, to assist in their folding and stability during protein transport. Although unrelated at the amino acid sequence level, we reveal a second example of a galectin-like fold that functions in guiding proper formation of a microneme complex. Rather than a canonical carbohydrate binding surface, a surface-exposed hydrophobic patch shared by the galectin domains of both MIC1 and M2AP presents a platform for protein interaction and hierarchical assembly of multicomponent protein complexes.

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