Two Separate, Conserved Acidic Amino Acid Domains within the *Toxoplasma gondii* MIC2 Cytoplasmic Tail Are Required for Parasite Survival*

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Apicomplexan parasites rely on actin-based motility to drive host cell invasion. Motility and invasion also require thrombospondin-related anonymous protein (TRAP) adhesins, which are secreted apically and translocated to the posterior end of the parasite before they are shed by the activity of a rhomboid protease. TRAP orthologs, including *Toxoplasma gondii* MIC2 (microneme protein 2), possess a short cytoplasmic tail, which is essential for motility. Previous studies have shown that aldolase forms a critical bridge between actin filaments and the cytoplasmic domains of MIC2 and TRAP. The cytoplasmic tails of TRAP family members harbor a conserved penultimate tryptophan, which is essential for aldolase binding, and clustered acidic residues. Herein, we determined the role of the conserved acidic residues by using alanine point mutants to investigate aldolase binding in vitro and to test functionality in the parasite. Our studies revealed two separate acidic residue clusters in the cytoplasmic domain of MIC2 that are essential for parasite survival. One region, located at the extreme C terminus, is required for the direct interaction with aldolase, whereas the second upstream acidic region is not necessary for aldolase binding but is nonetheless essential for parasite survival. Both acidic domains are conserved throughout TRAP orthologs, implicating a central role for these motifs in apicomplexan motility.

*Toxoplasma gondii* is a member of the phylum Apicomplexa, which is unified by a complex cytoskeleton; the presence of an apical conoid structure in most members; and three sets of apical secretory organelles: the micronemes, rhoptries, and dense granules (1–3). Apicomplexans are primarily obligate intracellular parasites, contributing to productive dissemination to new sites of infection (5). The ability to invade is absolutely dependent on the actin-based gliding motility of the parasite (6). Gliding motility is a substrate-dependent mode of locomotion that is a hallmark of apicomplexan organisms (4). The locomotion requires actin filaments (7, 8), adhesive proteins secreted from the micronemes (9), and the action of a class XIV myosin (10) anchored in the inner membrane complex (11).

Invasion by *T. gondii* is a multistep process that is largely independent of the host cell. Initially, secretion of the microneme contents results in deposition of adhesive molecules onto the parasite surface, which enables multivalent contacts between the apical end of the parasite and the host cell to be established (9). Translocation of the secreted microneme proteins toward the parasite posterior pulls the organism within the host cell (12). Productive invasion of the host cell also requires a cleavage event, severing the microneme extracellular adhesive component from the parasite surface by an intramembrane rhomboid protease (13, 14).

One of the proteins first demonstrated as integral for gliding motility and subsequent invasion identified in *Plasmodium* was the thrombospondin-related anonymous protein (TRAP)4 (15). TRAP-deficient mutant sporozoites are unable to glide and therefore can invade neither mosquito salivary glands nor mammalian host hepatocytes (16). TRAP paralogs have been identified in all motile *Plasmodium* life stages (17–19), and orthologs are found in numerous species within the phylum, including *Eimeria* (ETP100) (20), *Cryptosporidium* (TRAP-C1) (21), and *T. gondii* (MIC2) (22).

MIC2 (microneme protein 2) and other TRAP family members are type I transmembrane proteins, the extracellular domains of which contain two motifs: an A-domain and one or more type I thrombospondin repeats (22). Additionally, both proteins possess a short C-terminal cytoplasmic domain or tail. The cytoplasmic tails do not exhibit extensive primary sequence similarity, but contain a concentration of acidic amino acid residues and a penultimate tryptophan (23). Deletion of the TRAP cytoplasmic tail (TRApT) in *Plasmodium berghei* sporozoites results in non-motile parasites, despite the presentation of the ectodomain on the parasite surface (24). Similarly, the cytoplasmic tail of MIC2 (MIC2t) is essential for parasite survival (25). The cytoplasmic tails are functionally

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4 The abbreviations used are: TRAP, thrombospondin-related anonymous protein; TRAPt, thrombospondin-related anonymous protein cytoplasmic tail; MIC2t, MIC2 cytoplasmic tail; GST, glutathione S-transferase; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; FACS, fluorescence-activated cell sorter.

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conserved as shown by Kappe et al. (24), who found that wild-type gliding motility can be restored by replacing the TRAPt with the MIC2t, despite the absence of primary amino acid sequence similarity. The experimental results strongly suggest that the cytoplasmic tail has a conserved function in motility.

Because of the crucial roles of the TRAP family of adhesins and the motor complex during effective gliding motility, it was hypothesized the TRAPt and MIC2t might directly interact with the actin cytoskeleton; however, numerous experimental studies ruled out a role for direct binding (26–28). Instead, the cytoplasmic domains of MIC2 and TRAP were shown to interact with aldolase, thus bridging the interaction between extracellular adhesive domains and the cytoskeleton (23). In addition to its participation in glycolysis, the homotetrameric enzyme is capable of bundling and cross-linking actin filaments (29). Thus, aldolase functions as an intermediary to link the parasite cytoskeleton to the extracellular adhesin, powering motility and subsequent invasion.

A previous study demonstrated that the interaction between the TRAPt and aldolase in vitro requires the penultimate tryptophan and acidic residues at the extreme C terminus of the cytoplasmic tail (30). We sought to more thoroughly examine the role of two clusters of acidic residues in the MIC2t in the aldolase interaction in vitro and in survival in vivo. Our findings define two essential interactions in the MIC2t, which are likely conserved in the phylum.

MATERIALS AND METHODS

Growth of Host Cells and Parasite Strains—T. gondii tachyzoites of the RH (ATCC 50174), RH hxgprt^− (31), and EtM1/TgM2KO (32) strains were used in this study. Parasites were maintained by growth in monolayers of human foreskin fibroblasts propagated in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, 2 mM glutamine, 20 mM HEPES (pH 7.5), and 20 μg/ml gentamycin (complete medium).

Protein Sequence Alignment of TRAP Family Cyttoplasmic Tails—Protein sequence alignment of the TRAP family cytoplasmic tails was carried out using ClustalW (available at www.ebi.ac.uk/clustalw/) with the default parameters. The following protein sequences were obtained from the NCBI Protein Database using the indicated accessions numbers: T. gondii MIC2 (accession number AA63303), Neospora caninum MIC2 (accession number AAF01565), Cryptosporidium parvum TRAP-C1 (accession number AAC48311), Plasmodium falciparum merozoite-specific TRAP homolog (accession number PF10_0281), T. gondii MIC6 (accession number AAD28185), P. falciparum CTRP (circumsporozoite protein/thrombospondin-related anonymous protein-related protein; accession number CAB38978), Eimeria tenella MICT (accession number AAD03350), and P. falciparum TRAP (accession number AAA29778).

Cloning of MIC2t Point Mutants—Mutants were generated in a plasmid encoding the MIC2t from Ser721 to Glu769, fused to the C terminus of glutathione-S-transferase (GST), as described previously (23). Point mutants were generated using PCR with the following strategy. The MIC2t was amplified from cDNA using the forward primer FP-MIC2t (5’-GAACCCGGGAGT-TACCAGTATTTTGAGCTC-3’) and a reverse primer encoding the specified point mutation to alanine. The PCR products were purified on QIAquick PCR purification columns (Qiagen Inc.), digested with SmaI and EcoRl (New England Biolabs, Beverly, MA), and cloned into similarly digested pGEX-3X vector (Amersham Biosciences) to generate translational fusions with the C terminus of GST.

The c-Myc epitope-tagged constructs expressing promotorless wild-type MIC2 and point mutants were generated from a previously described full-length version of the MIC2 gene (37). A 1-kilobase pair DNA fragment containing an internal c-Myc epitope tag at Ser659 was PCR-amplified using a 5’-primer appended with a HindIII restriction site (5’-GGGTAATTACACTCTCATCATCATATC-3’) and a 3’-primer appended with a PacI restriction site (5’-GGGTTAATTACACTCTCATATC-3’). The point mutations were generated by altering the sequence of the reverse primer. The resulting PCR products (lacking the C-terminal 9-amino acid hemagglutinin tag) were purified using QIAquick PCR purification columns, digested with HindIII and PacI (New England Biolabs), and cloned into similarly digested MIC2-TAG-P vector as described previously (25). The resulting plasmids contained a promotorless copy of either the wild-type or mutant MIC2 gene containing an internal c-Myc epitope tag. Plasmids for expression of c-Myc epitope-tagged point mutants under the control of the native promoter were generated by digesting the promotorless constructs with PacI and Rsfl. The resulting DNA fragment was then subcloned into a similarly digested MIC2 HXGPRT expression plasmid as described previously (31).

Quantitative Enzyme-linked Immunosorbent Assay (ELISA) Analysis—Recombinant T. gondii aldolase and the GST-MIC2t fusion proteins were purified from the BL21 strain of Escherichia coli as described previously (23). Following purification, recombinant proteins were dialyzed overnight in 1/2× XB buffer (1/2× XB buffer = 50 mM KCl, 10 mM HEPES, 1 mM MgCl2, and 0.1 mM EDTA (pH 7.5)). Protein concentrations were determined using the BCA assay (Pierce). T. gondii aldolase was used to coat high binding 96-well Greiner Bio-One polystyrene ELISA plates (VWR International, Batavia, IL) with 100 μl of 10 μg/ml ELISA coating buffer (50 μM sodium carbonate/bicarbonate (pH 9.6)) overnight at 4 °C. ELISA plates were washed with phosphate-buffered saline (PBS) containing 0.01% Tween 20 and blocked in 10% bovine serum albumin in PBS containing 0.01% Tween 20 for 1 h. 10-Fold serial dilutions of GST-MIC2t fusion protein (wild-type or point mutants) were made in 1× XB buffer. The dilutions were added in triplicate and incubated for 2 h, with shaking at room temperature. After three washes with PBS containing 0.01% Tween 20, the GST-MIC2t/aldolase interaction was detected using a peroxidase-conjugated goat anti-GST antibody (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA). Horseradish peroxidase was detected with equal volumes of substrate A/B from a BD OptEIA kit (BD Biosciences) and quenched with 2 M H2SO4, and the absorbance was read at 450 nm using an EL800 universal plate reader. The KD values were determined using the nonlinear regression Hill equation in the KaleidaGraph graphing software package (Synergy Software, Reading, PA).
**Adhesin/Aldolase Interaction**

**GST-MIC2t Fusion Protein Pulldown Assays**—GST and GST-MIC2t fusion proteins were purified from the *E. coli* BL21 strain as described previously (23). Purified proteins were diazylated and quantitated as described above. Washed glutathione-Sepharose beads (Amersham Biosciences) were added to 60 μg of GST fusion proteins and allowed to bind in PBS for 2 h at 4 °C. During binding, *T. gondii* strain RH tachyzoites were harvested from host monolayers and resuspended to 4×10⁶ parasites/ml in 1× XB buffer. Parasites were lysed by using sequential freeze-thaws in liquid nitrogen and emersion at 37 °C, followed by three sonication bursts of 15 s at setting 3 in a Fisher Model 550 Sonic Dismembrator equipped with a microtip. The insoluble fraction was removed by centrifugation at 14,000 relative centrifugal force for 11 min. After binding, the Sepharose beads were washed twice with 1× XB buffer and resuspended in buffer. The beads were incubated with either 20 μg of purified recombinant *T. gondii* or *P. falciparum* aldolase or 100 μl of soluble parasite lysate for 4 h at 4 °C. Following incubation, the beads were washed five times with 1 ml of ½× XB buffer and resuspended in 1× sample buffer. Bound proteins were resolved by SDS-PAGE and examined by either Coomassie Blue staining or Western blot analysis.

**Immunoblotting and Antibodies**—Protein samples were resolved by 10% SDS-PAGE, immunooblotted with peroxidase-conjugated secondary IgG antibodies (Jackson ImmunoResearch Laboratories, Inc.), and visualized with SuperSignal West Pico chemiluminescent substrate (Pierce). Rabbit anti-*T. gondii* aldolase polyclonal antibody (WU1614) was raised against a GST-aldolase fusion protein and reacts specifically with *T. gondii* aldolase. Anti-c-Myc monoclonal antibody 9E10 was purchased from Zymed Laboratories Inc. (South San Francisco, CA). Rabbit anti-M2AP (MIC2-associated protein) polyclonal antiserum was described previously (32). Rabbit anti-MIC4 polyclonal antibody was described previously (33). Alexa 488 (green)- and Alexa 594 (red)-conjugated secondary IgG antibodies were purchased from Molecular Probes (Eugene, OR).

**Parasite Transfections**—*T. gondii* strain RH *hxgprt*-tachyzoites were transfected by electroporation as described previously (31). Transfected parasites were then allowed to invade host human foreskin fibroblast cells. Complete tissue culture medium was replaced 6 h post-transfection with complete medium containing 25 μg/ml mycophenolic acid (Sigma) and 50 μg/ml xanthine (Sigma). Parasites were grown for 18 days in the presence of drug selection prior to fluorescence-activated cell sorter (FACS) analysis.

*T. gondii* strain EtM1/TgM2KO tachyzoites were transfected by electroporation as described previously (32). Transgenic parasites were then allowed to infect human foreskin fibroblast cells seeded onto coverslips. Tachyzoites were allowed to grow overnight and then processed for immunofluorescence microscopy.

**Flow Cytometry**—Following 18 days of drug selection, extraacellular tachyzoites were harvested from host cell monolayers and stained with anti-c-Myc monoclonal antibody 9E10 and Alexa 488-conjugated secondary antibodies. The stained parasites were examined by flow cytometry to determine the frequency of c-Myc-positive cells. Tachyzoites (1–5×10⁶ parasites/ml) were fixed in PBS containing 4% formaldehyde for 30 min. Fixed parasites were simultaneously blocked and permeabilized in PBS containing 10% fetal bovine serum and 0.02% saponin (blocking solution) for 1 h. Anti-c-Myc epitope tag monoclonal antibody 9E10 was diluted into blocking solution and used to stain parasites for 1 h. Following incubation, parasites were washed three times with PBS containing 0.02% saponin. Alexa 488-conjugated goat anti-mouse IgG (Molecular Probes) was diluted in blocking solution and used to stain parasites for 1 h. Parasites were then washed six times: four times with PBS containing 0.02% saponin and twice with PBS alone. Parasites were resuspended in PBS, and 1×10⁶ parasites were examined using a FACSCalibur flow cytometer (BD Biosciences). The number of epitope-positive parasites was determined with the CellQuest program (BD Biosciences).

**Immunofluorescence Microscopy**—To localize epitope-tagged MIC2 mutants, transiently transfected parasites were inoculated onto host cells seeded on coverslips, grown overnight, and processed for immunofluorescence. Cells were prepared for immunofluorescence as described previously (25). Primary antibodies were diluted in blocking solution and incubated with the coverslips for 1 h. Following six washes with PBS containing 0.1 mm CaCl₂, coverslips were incubated with either Alexa 488- or Alexa 594-conjugated IgG secondary antibodies diluted in blocking solution for 1 h. Following six washes with PBS containing 0.1 mm CaCl₂, coverslips were rinsed in distilled H₂O and mounted with VECTASHIELD containing 4’,6-diamidino-2-phenylindole (Vector Laboratories, Burlingame, CA). Cells were examined using a Zeiss Axioplan microscope equipped with phase-contrast and epifluorescence optics. Images were obtained using a Zeiss AxioCam cooled CCD camera directed by Zeiss AxioVision software (Version 4.5), including a deconvolution package. The images were deconvolved using the inverse relation and the nearest neighbor algorithms, and the resulting images were processed using Adobe Photoshop CS (Version 8.0). The negative control parasite (EtM1/TgM2KO) images were deconvolved using the nearest-neighbor algorithm and matched input compensation.

**RESULTS**

*T. gondii* MIC2 and other members of the TRAP family containing extracellular adhesive domains are anchored in the plasma membrane by a single transmembrane domain and contain short cytoplasmic tails. The short cytoplasmic tails of *T. gondii* MIC2 and *P. falciparum* TRAP mediate a specific interaction with the glycolytic enzyme aldolase (23). Previous studies have shown that the interaction with aldolase is dependent upon the presence of an absolutely conserved tryptophan residue located 1–5 residues from the C terminus (Fig. 1) (23, 30). In addition to the conserved tryptophan residue, the cytoplasmic tails contain a concentration of acidic amino acid residues (Fig. 1). A closer examination of the MIC2t and other members of this family highlights the presence of two clusters of acidic residues, one proximal to the C terminus and the other more distal. The proximal and distal clusters are separated by 5 residues containing a minimum of three hydrophobic residues. In the case of MIC2, the proximal cluster contains a concentration of aspartic acids, whereas the residues distal to the C terminus
The apparent affinity estimated for GST-MIC2t (wild-type) (Table 1) and correlated directly with the results of the protein pulldown assay. Purified recombinant T. gondii aldolase was evaluated using a purified protein pulldown assay. Purified fusion proteins (wild-type or mutant MIC2t) or GST alone was affinity to glutathione-Sepharose beads and incubated with purified recombinant T. gondii aldolase in XB buffer. GST-MIC2t (wild-type) specifically interacted with purified aldolase, whereas GST-W767A disrupted the association, consistent with a previous report (23). GST-D761A,D762A,D763A, GST-D765A, and GST-E769A lost the ability to interact with aldolase (Fig. 2B). GST-M766A and GST-M768A had no effect on the interaction (Fig. 2B). For GST-M766A, there was a slight increase in binding compared with GST-MIC2t. Interestingly, GST-E751A,E752A,E753A and GST-E755A, the residues of which are separated from the proximal acidic residues by the hydrophobic stretch, did not contribute to the interaction with aldolase (Fig. 2B).

The high degree of conservation between the MIC2t and TRAPt led us to also investigate whether the same acidic residues important for the T. gondii aldolase interaction also participate in an association with P. falciparum aldolase. Utilizing the same MIC2t fusion proteins, the purified protein pulldown assay was repeated, replacing T. gondii aldolase with purified P. falciparum aldolase. The results mirrored those obtained with T. gondii aldolase; however, the interaction appeared weaker, as the amount of aldolase recovered by the wild-type MIC2t was less (Fig. 2C).

To provide a more quantitative measure of binding, a quantitative ELISA was developed to monitor binding between aldolase and the MIC2t. Purified recombinant T. gondii aldolase was used to coat high affinity ELISA plates, and the plates were incubated with MIC2t fusion proteins and probed with anti-GST antibody. Binding affinities were estimated as apparent $K_D$ values (based on first-order binding kinetics estimated from a Hill plot) or as 50% of the maximum binding (Table 1). The apparent affinity estimated for GST-MIC2t (wild-type) was ~4.0 nM. GST-W767A had an estimated binding affinity of 82.5 nM, a >20-fold increase over the wild-type fusion protein (Fig. 3). The apparent binding affinities of aldolase for the MIC2t correlated with the strength of the interaction as determined by the purified protein pulldown assay. The binding affinities were determined for all of the point mutants (Table 1) and correlated directly with the results of the
purified protein pulldown assay. As in the pulldown assay, the acidic residues proximal to the C terminus of the MIC2t were essential for the interaction with aldolase, whereas the acidic residues distal to the C terminus did not contribute to the interaction. W767A and E769A had the most pronounced effect upon the interaction, with affinity values in excess of 80 nM (Table 1). The threshold at which the interaction was lost was ~10 nM, as demonstrated by D765A in the purified protein pulldown assay (Fig. 2 and Table 1). The result suggests a tight interaction, which is easily disrupted if the charge interaction is lost.

Recombinantly produced aldolase was used in both the purified protein pulldown assay and ELISA, so we sought to determine whether the same residues are essential for the interaction with aldolase in parasite cell lysates. Extracellular tachyzoites from strain RH were harvested and lysed in XB buffer. The soluble fraction was then incubated with glutathione-Sepharose beads preloaded with GST-MIC2t. The proteins were resolved by SDS-PAGE, and endogenous T. gondii aldolase was identified by Western blot analysis with antibody raised against recombinant T. gondii aldolase (Fig. 4B). The wild-type MIC2t

### Table 1

| MIC2t               | Purified protein pulldown* | Estimated $K_{D}$b | 50% maximum bindingc | Lysate pulldownc | Promoter trapd |
|---------------------|---------------------------|--------------------|-----------------------|------------------|----------------|
| Wild-type Interaction | 4.00                      | 4.0                | Interaction 100       |                   |                |
| E751A,E752A,E753A Interaction | 0.95                      | 0.9                | Interaction 3.3       |                   |                |
| K754A Interaction | 0.44                      | 0.7                | Interaction 135.0     |                   |                |
| E755A Interaction | 1.60                      | 2.2                | Interaction 4.4       |                   |                |
| D761A,D762A,D763A No interaction | 15.26                     | 28.0               | No interaction 8.5   |                   |                |
| S764A Interaction | 3.49                      | 3.6                | Interaction 72.3      |                   |                |
| D765A No interaction | 13.73                     | 10.0               | No interaction 26.4   |                   |                |
| M766A Interaction | 0.96                      | 1.6                | Interaction 65.0      |                   |                |
| W767A No interaction | 82.50                     | 82.0               | No interaction 3.9    |                   |                |
| M768A Interaction | 1.81                      | 3.3                | Interaction 176       |                   |                |
| E769A No interaction | 91.00                     | 89.0               | No interaction 1.9    |                   |                |

* As determined by Coomassie Blue staining (Fig. 2).

b As determined by quantitative ELISA (Fig. 3).

c As determined by Western blot analysis (Fig. 4).

d As determined by promoter-trapping FACS analysis (Fig. 5).

e Percent of promoterless c-Myc epitope-tagged wild-type MIC2.

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**FIGURE 3.** Determination of the binding interaction between the MIC2t and aldolase by quantitative ELISA analysis. The strength of the MIC2t/aldolase interaction was quantitated by ELISA as described under “Materials and Methods.” A representative assay comparing wild-type (WT) MIC2t, W767A, and GST alone is shown. The W767A mutation shifted the binding interaction by ~20-fold. All of the mutants were tested using this assay, and the results are shown in Table 1.

**FIGURE 4.** Identification of essential residues mediating MIC2 and aldolase binding in soluble parasite lysates. A, shown is the sequence of the MIC2t starting with residue 744. The underlined amino acids were mutated to alanine. B, soluble lysates of T. gondii strain RH were incubated with GST-MIC2t (wild-type), GST-MIC2t point mutants, or GST alone. Proteins bound to the GST fusions were resolved by SDS-PAGE and visualized by Western blot analysis with anti-T. gondii aldolase polyclonal antibody (αALD). Coomassie Blue staining was used to demonstrate equal loading of GST fusion proteins (upper panel). The ability to pull down aldolase from soluble lysates was disrupted with the following GST-MIC2t point mutations: E769A, W767A, D765A, and D761A,D762A,D763A (lower panel). Molecular masses are indicated in kilodaltons.
was able to pull down aldolase from soluble lysates, whereas W767A was not, consistent with previous findings (23). The residues shown to be crucial for the interaction in vitro (D761A, D762A, D763A, D765A, W767A, and E769A) also proved to be essential for the recovery of aldolase from the soluble lysate (Fig. 4B). The same trend continued with the distal acidic residues (E751A, E752A, E753A, and E755A) in that these mutations did not affect the ability of the MIC2t to pull
down aldolase from the soluble lysate (Fig. 4B). A previous study showed that, in addition to aldolase, a variety of additional bands are pulled down from lysates using the wild-type MIC2t (22). This pattern did not change with any of the mutants studied here (data not shown), indicating that the defect in distal acidic residues discussed below is not due to failure to precipitate any of the known binding partners.

The conserved nature of the distal acidic residues in the MIC2t and other TRAP family members led us to consider whether these residues contribute to the function of the cytoplasmic tail in vivo. To test this hypothesis, we utilized a reverse genetic approach to test the functionality of MIC2t point mutants within the parasite. *Toxoplasma* does not maintain plasmids episomally, but instead recombines DNA into the chromosomes, primarily in a non-homologous manner (34). Because MIC2 is an essential gene and because many of the mutations tested are likely to have profound phenotypes, we utilized the promoter-trapping method described previously (25). Following transfection of a plasmid containing a promoterless epitope-tagged copy of the MIC2 gene, the frequency of epitope-positive parasites was monitored by FACS analysis (25). Expression of epitope-tagged wild-type MIC2 results from homologous recombination that traps the endogenous promoter (Fig. 5A). In contrast, transfection with promoterless plasmids encoding mutants such as Δmic2t does not result in a detectable signal in this assay (25). The ability to trap the endogenous MIC2 promoter by gene targeting was used as a quantitative assay to test the functionality of various MIC2t point mutants (Fig. 5A). The expected outcome of this assay is that mutation of nonessential residues will result in recovery of parasites that express c-Myc-tagged MIC2, whereas disruption of essential residues will result in a failure to detect epitope expression above background levels. Plasmids encoding similar mutants under the control of the endogenous MIC2 promoter served as a control for transfection efficiency and stability of the epitope-tagged construct. In this case, homologous or non-homologous integration results in a pseudodiploid strain expressing fully wild-type MIC2 and an epitope-tagged form (Fig. 5A).

Following transfection and drug selection, extracellular tachyzoites were harvested, fixed, stained with anti-c-Myc antibody, and analyzed by FACS analysis. When the promoterless form of the wild-type MIC2 plasmid was used, a small second peak of c-Myc-positive parasites was detected as a shift to the right on the FL1 channel in the FACS scan, demonstrating the ability of this construct to rescue parasite growth (Fig. 5B). W767A failed to yield a c-Myc-positive peak, demonstrating the essentiality of this residue for parasite survival (Fig. 5B). M768A resulted in recovery of a c-Myc-positive population, indicating this residue is not essential for parasite survival (Fig. 5B). Not surprisingly, mutation of the acidic residues located proximal to the C terminus of MIC2 resulted in only background c-Myc staining (Table 1), indicating that they are required for parasite survival. Interestingly, mutations of the acidic residues located distal to the MIC2 C terminus (E751A, E752A, E753A and E755A) did not yield a positive c-Myc population, demonstrating they are also essential for parasite survival (Fig. 5B and Table 1). All of the plasmids produced similar levels of c-Myc-tagged proteins under conditions in which they were driven by the endogenous promoter, and none were observed to exert a dominant-negative effect (Fig. 5B) (data not shown).

To rule out the possibility that the in vivo phenotype seen with the two distal acidic acid residue mutants (E751A, E752A, E753A and E755A) is a result of a defect in trafficking to the micronemes, we investigated their localization by immunofluorescence. MIC2 is an essential gene in *T. gondii*; however, Huynh *et al.* (32) were able to disrupt the MIC2 locus by initially transfecting parasites with a plasmid encoding the homologous protein from *E. tenella* (MIC1). This transgenic strain lacks MIC2, yet is still capable of invading host cells, presumably because *E. tenella* MIC1 complements an essential function (32). The EtM1/TgM2KO parasite was transiently transfected with a plasmid encoding c-Myc epitope-tagged MIC2 under the control of the native MIC2 promoter and stained with anti-c-Myc antibody and rabbit anti-MIC4 polyclonal antibody to determine the localization of the mutant proteins (Fig. 6A). Parasites transfected with c-Myc epitope-tagged wild-type MIC2 exhibited apical staining that overlapped with MIC4, indicating proper microneme localization. The two distal acidic residue MIC2t mutants (E751A, E752A, E753A and E755A) also demonstrated an apical staining pattern that co-localized with MIC4 (Fig. 6A). These results demonstrate that the phenotype seen in the promoter-trapping assay is not a result of improper trafficking to the micronemes by the mutant proteins.

In the EtM1/TgM2KO strain, the majority of M2AP is not correctly targeted to the micronemes, but is secreted into the parasitophorous vacuole (32). We reasoned that the reintroduction of MIC2 into the EtM1/TgM2KO strain should rescue the localization defect of M2AP. To investigate this possibility, transiently transfected parasites were also stained with rabbit anti-M2AP polyclonal antibody. In parasites transfected with c-Myc-tagged wild-type MIC2, the majority of M2AP localized...
to the micronemes (Fig. 6B). Likewise, the two distal acidic residue MIC2t mutants (E751A, E752A, E753A, and E755A) rescued the trafficking defect of M2AP (Fig. 6B). These results provide further evidence that the mutations of the distal acidic residues in the MIC2t do not disrupt targeting to the micronemes.

**DISCUSSION**

Members of the phylum Apicomplexa are obligate intracellular parasites, and their survival is linked to successful host cell invasion. Host invasion requires actin-based motility, which relies on a bridge between extracellular adhesins and the parasite actin cytoskeleton (23). In *T. gondii* and *Plasmodium* spp., this link is mediated through an interaction between the TRAP family cytoplasmic tails and the glycolytic enzyme aldolase (23, 30). In this study, we investigated the contribution of conserved acidic residues in the MIC2t to the aldolase interaction. We have shown that acidic residues located proximal to the MIC2 C terminus are required for the aldolase interaction, whereas a series of distal acidic residues do not participate in this association in vitro. The essentiality of the proximal residues to parasite survival was confirmed by promoter-trapping analysis. Additionally, we found that the conserved distal residues are also required for survival. This in vivo phenotype is not a result of nonproductive targeting of the mutant proteins to the micronemes, suggesting instead the requirement of additional proteins in the establishment of a productive link between the MIC2 adhesin and the parasite actin cytoskeleton. The mechanism of gliding motility is conserved across multiple species within Apicomplexa (4), and a molecular understanding of this system may be beneficial in inhibiting invasion by these pathogenic organisms.

A conserved feature throughout all members of the TRAP family of proteins is the concentration of acidic amino acid residues in their cytoplasmic tails. Coupled with this is evidence demonstrating that the interaction of aldolase with filamentous actin is electrostatic (29). We reasoned that the interaction between the MIC2t and aldolase may be similar to the interaction between aldolase and F-actin, and we sought to elucidate the contribution of these acidic residues to the aldolase interaction. The proximal acidic residues in the MIC2t were shown to be essential for binding to aldolase, and the binding affinity of this interaction was very strong (i.e. ~4 nM) under the conditions tested here.

Previous work determined that the kinetics of *T. gondii* actin polymerization are quite different from those of mammalian actin polymerization, resulting in shorter, less stable filaments (35). Physiologically, MIC2 associates with M2AP at a 1:1

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**FIGURE 6. Mutations of the distal acidic residues in the MIC2t do not alter targeting to the micronemes and rescue the M2AP defect in the EtM1/TgM2KO strain.**

A, EtM1/TgM2KO tachyzoites transiently transfected with c-Myc-tagged wild-type (WT), E751A, E752A, E753A, or E755A MIC2 co-localized with MIC4. Epitope-tagged MIC2 was localized using mouse anti-c-Myc antibody (α-C-myc) and Alexa 488-conjugated goat anti-mouse IgG (green). Endogenous MIC4 was visualized using rabbit anti-MIC4 antibody (α-Tg MIC4) and Alexa 594-conjugated goat anti-rabbit secondary antibodies (red). B, EtM1/TgM2KO tachyzoites transiently transfected with c-Myc epitope-tagged wild-type, E751A, E752A, E753A, or E755A MIC2 co-localized with M2AP in the micronemes. The c-Myc epitope was visualized as described for A. The M2AP protein was localized using rabbit anti-M2AP antibody (α-Tg M2AP) and Alexa 594-conjugated goat anti-rabbit secondary antibodies (red). Images were deconvolved using the inverse relation and the nearest neighbor algorithms (described under “Materials and Methods). A representative Z-stack slice is shown. Scale bar = 5 μm.
ratio, forming a heterohexamer complex (25), whereas aldolase exists as a homotetramer. In addition to its role in glycolysis, the aldolase homotetramer possesses multiple actin filament-binding sites and has been shown to function as an F-actin-cross-linking protein (29). The strong affinity between the tail of MIC2 and aldolase could thus serve to stabilize the *T. gondii* actin filaments to promote motility.

The precise stoichiometry between tetrameric aldolase and the trimeric tails of MIC2 heterohexamers is unknown, but the potential for multiple binding sites on aldolase may result in clustering of adhesions and/or allosteric modification of binding affinities.

Previous work examined the role of the proximal acidic residues in the interaction between the TRAPt and aldolase (30); the results of that study are similar to those obtained here for MIC2. However, the aforementioned study did not examine the role of the distal acidic residues, which are conserved in both the MIC2t and TRAPt. Instead, Buscaglia et al. (30) speculated only on the role that these conserved distal acidic residues may play in the interaction with aldolase. The authors suggested that the presumed unstructured nature of the TRAPt would enable the distal residues to interact with the surface of aldolase in an electrostatic manner by associating with other regions of the enzyme. In a more recent study, Buscaglia et al. (36) examined the contributions of the upstream acidic residues to the TRAPt/aldolase interaction. Their results differ from ours and suggest that these residues contribute to an interaction between *Plasmodium* aldolase and GST-TRAPt fusion proteins *in vitro* (36). However, in the present investigation, mutations of the conserved distal residues of GST-MIC2t (E751A,E752A,E753A and E755A) did not have an effect on binding to aldolase. The lack of a phenotype was not a result of excessively permissive buffer conditions, as an increase in salt concentration led to similar results, although the overall binding of all constructs was lower.5 The discrepancy in the *in vitro* results may reflect differences in the biology between these two parasites or in the relative sensitivity of the assays. Although these distal acidic residues did not participate in the aldolase interaction in *T. gondii in vitro*, they were nonetheless essential *in vivo*. The inability to recover a c-Myc-positive population in the promoter-trapping assay strongly suggests that the MIC2t distal acidic residues contribute an essential role in parasite survival.

To eliminate the possibility that the loss of parasite survival associated with the distal acidic residue mutants was a by-product of defective targeting to the micronemes, we transiently transfected a *mic2*-null *T. gondii* strain and established the intracellular location by immunofluorescence. The results established that the essentiality of the MIC2t distal acidic residues *in vivo* is not due to a targeting defect, further supporting the notion that they are required for binding to other protein(s) that may participate in motility. As highlighted earlier, the proximal and distal acidic residues are separated by a hydrophobic stretch, and in MIC2, the hydrophobic stretch harbors a proline residue. The cyclic nature of a single proline residue structurally alters the conformation of the MIC2t such that the

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5 G. L. Starnes and L. D. Sibley, unpublished data.
distal residues could be positioned away from aldolase. Additionally, molecular modeling suggests that, if the proximal acidic residues interact near the aldolase active site, the distal acidic residues are positioned too far away from the active site for them to participate in an interaction there, enabling association with an additional protein(s).\(^6\)

Kappe et al. (24) initially investigated the role that the TRAPt plays in the motility of the \textit{P. berghei} parasite by constructing a series of TRAP truncations to examine gliding and invasion. The authors described a partial tail deletion (ΔS) that resulted in altered motility in which the parasite demonstrated a “pendulum” type of gliding. This deletion truncates the last 12 residues, removing the tryptophan and proximal acidic residues, but leaving the distal acidic cluster intact. As a result of the altered motility, the mutant parasites could not invade mosquito salivary glands. The nonproductive nature of this motility suggests that the truncated TRAPt may still be weakly associated with aldolase and the actin cytoskeleton. The TRAPΔS deletion partially overlaps the distal acidic residues described in this study. Collectively, the results of Kappe et al. (24) and our findings suggest that aldolase is not the sole constituent in the motility complex and that the presence of additional protein(s) may be required to link TRAP family adhesins to the apicomplexan cytoskeleton.

Our findings demonstrate a critical role for the acidic residues in the proximal region of the MIC2t in binding to aldolase. This interaction is essential \textit{in vitro} and \textit{in vivo}, demonstrating the importance of charge interaction in bridging adhesins to the actin cytoskeleton. Additionally, we have shown that the conserved cluster of distal acidic residues is not essential for the interaction with aldolase, but is nonetheless required for parasite survival. The elucidation of the accessory protein(s) responsible for this second interaction is an important goal in defining the mechanism of motility in \textit{T. gondii}. Within Apicomplexa, gliding motility is a unique method of locomotion, which is conserved across multiple species. Because of the high level of conservation, a detailed mechanistic understanding may offer insights into novel targeted therapies to prevent the invasion of host cells by these obligate intracellular organisms.

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\(^6\) J. Sygusch, personal communication.