A Conserved Molecular Motor Drives Cell Invasion and Gliding Motility across Malaria Life Cycle Stages and Other Apicomplexan Parasites*\textsuperscript{(S)}

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Apicomplexan parasites constitute one of the most significant groups of pathogens infecting humans and animals. The liver stage sporozoites of \textit{Plasmodium} spp. and tachyzoites of \textit{Toxoplasma gondii}, the causative agents of malaria and toxoplasmosis, respectively, use a unique mode of locomotion termed gliding motility to invade host cells and cross cell substrates. This amoeboid-like movement uses a parasite adhesin from the thrombospondin-related anonymous protein (TRAP) family and a set of proteins linking the extracellular adhesin, via an actin-myosin motor, to the inner membrane complex. The \textit{Plasmodium} blood stage merozoite, however, does not exhibit gliding motility. Here we show that homologues of the key proteins that make up the motor complex, including the recently identified glideosome-associated proteins 45 and 50 (GAP40 and GAP50), are present in \textit{P. falciparum} merozoites and appear to function in erythrocyte invasion. Furthermore, we identify a merozoite TRAP homologue, termed MTRAP, a micronemal protein that shares key features with TRAP, including a thrombospondin repeat domain, a putative rhomboid-protease cleavage site, and a cytoplasmic tail that, \textit{in vitro}, binds the actin-binding protein aldolase. Analysis of other parasite genomes shows that the components of this motor complex are conserved across diverse Apicomplexan genera. Conservation of the motor complex suggests that a common molecular mechanism underlies all Apicomplexan motility, which, given its unique properties, highlights a number of novel targets for drug intervention to treat major diseases of humans and livestock.

Parasites from the phylum Apicomplexa represent some of the most significant human and agricultural pathogens. Their ranks include \textit{Theileria parva} and \textit{Theileria annulata}, parasites that give rise to lymphoproliferative diseases of cattle, the opportunistic pathogens \textit{Toxoplasma gondii} and \textit{Cryptosporidium parvum} that can cause life-threatening, prolonged infection in immunocompromised patients, and the most lethal of the group, the genus \textit{Plasmodium}, in particular \textit{Plasmodium falciparum}, the cause of millions of human deaths and as many as 500 million infections annually (1).

Apicomplexa are a monophyletic group of obligate intracellular parasites that invade a wide range of host cells but lack the classical means of motility such as a flagellum or cilia. Instead, they move by a unique form of actin-based locomotion called gliding motility (for recent reviews, see Refs. 2–4). Efficient motility and invasion requires the release of proteins from secretory organelles located at the apical prominence, the defining structure of the phylum. These organelles, the micronemes, rhoptries, and dense granules contain many of the key proteins needed for directional attachment, cell invasion, and establishment of the parasitophorous vacuole (PV)\textsuperscript{(5)} within the host cell (5). Much of our understanding of gliding motility comes from studies with the liver stage parasite from \textit{Plasmodium spp.}, the sporozoite, or the morphologically similar tachyzoite of \textit{Toxoplasma}. Micronemal adhesive proteins are released onto the zoite (motile parasite form) surface prior to invasion and are transported to the posterior end of the cell, being cleaved and released on their way, thus creating a dynamic treadmill (2–4). Ultimately, the extracellular adhesin is linked internally to an anchor that is attached to a series of membrane structures, underlying the outer plasma membrane of the parasite, called the inner membrane complex (IMC) (6, 7). Between the plasma membrane and IMC lies an internal actin-myosin motor that drives cell motility (2–4). In addition to driving cell motility in sporozoites and tachyzoites, the same molecular components also appear to be used for host cell invasion (8–11). The thrombospondin-related anonymous protein (TRAP) (12) from \textit{Plasmodium} is the essential adhesin needed for sporozoite motility and for liver cell invasion (9) and defines a family of related proteins conserved across Apicomplexan genera and parasite life cycle stages (e.g. TgMIC2 in \textit{Toxoplasma} (reviewed in Ref. 11) and PICTRP in \textit{Plasmodium} ookinetes (13)). TRAP is a type I integral membrane protein that binds to sulfated glycosaminoglycans on liver cells (14). Key to its adhesive function is the presence of a thrombospondin repeat (TSR) domain (14), a well characterized protein domain that is present on surface proteins across diverse organisms and is implicated in several biological functions such as cell adhesion and cell motility (15). Two essential \textit{Plasmodium} proteins that contain a TSR domain, the aforementioned TRAP and liver stage circumsporozoite protein (CSP) (16), are both...
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included in ongoing vaccine trials, suggesting that other TSR proteins may also provide novel avenues for anti-malaria therapeutics.

The cytoplasmic tails of the Plasmodium TRAP and TgMIC2 in Toxoplasma have both been shown to interact with the filamentous (F)-actin binding protein aldolase (17, 18). This would then constitute the bridge between the extracellular adhesin and F-actin (17, 18). F-actin interacts dynamically with the class XIV myosin A (MyoA) (6) and in Toxoplasma is anchored to the IMC via the myosin light chain (MLC) (19) and the membrane-anchored glideosome-associated protein TgGAP50 (20) and its associated TgGAP45 (20). A homologue of MLC has been described in both P. yoelii and P. falciparum (7), referred to as the MyoA tail-interacting protein (MTIP). The chain of interaction of these components (TRAP-aldolase-actin-MyoA-MLC/MTIP-GAP45-GAP50) constitutes what we refer to as the motor complex.

Unlike sporozoites and tachyzoites, merozoites, the blood stage malaria parasites, do not demonstrate gliding motility, and the molecular mechanisms that underlie their invasion of the erythrocyte are still largely unknown. Evidence for the involvement of an actin-myosin motor system in merozoites comes from the inhibition of invasion when cultures are pretreated with drugs that target actin (cytochalasin B (21)) or myosin (butane-2,3-dione monoxide (6)). This suggests that, despite the absence of gliding motility, the merozoite may utilize actin polymerization, myosin, and by inference a homologous molecular motor for efficient erythrocyte invasion.

Here we show that the components of the motor complex characterized in sporozoites and tachyzoites are expressed in developing merozoites, localize consistently with their inferred function, and form complexes that support their having a role in erythrocyte invasion. We identify a putative essential merozoite-specific TRAP homologue (MTRAP) that is localized to the micronemes, released and processed during invasion, and interacts in vitro with aldolase, key features suggesting that it is the blood stage invasion adhesin that links the cell surface to the motor complex. A search for homologues of each of these motor proteins in the recently published *P. falciparum* (22) and *C. parvum* (23) genomes as well as partial genome sequences for *T. annulata* (22) and *Eimeria* (24) suggests that it is the blood stage invasion adhesin that links the cell surface to the motor complex. A search for homologues of each of these motor proteins in the recently published *T. annulata* (22) and *C. parvum* (23) genomes as well as partial genome sequences for *T. annulata* (22) and *Eimeria* (24) confirms that the motor complex is universally present and likely to form the foundation for all of the different modes of cell motility and invasion seen across the phyllum.

**EXPERIMENTAL PROCEDURES**

**Identification of *P. falciparum* TRAP Paralogues and Motor Protein Orthologues**—The TSR sequence of TRAP (PlasmoDB ID: PF13_0201 (12)) and CTRP (PF0640w (13)) were used as BLASTP and TBLASTN queries to search the *P. falciparum* genome (available on the World Wide Web at www.PlasmoDB.org) to identify further TRAP paralogues. Alignments were carried out using PRALINE, available on the World Wide Web at www.PlasmoDB.org) to identify orthologues of the *P. falciparum* TRAP-aldolase-actin-MyoA-MLC/MTIP-GAP45-GAP50) constitutes what we refer to as the motor complex.

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immunoblots, saponin-lysed parasite pellets from highly synchronous schizont and ring stage 3D7 parasites (40–48 and 0–8 h, respectively) as well as culture supernatants (post-schizont rupture) were separated in sample buffer on 4–12% SDS-NuPAGE gels (Invitrogen) under reducing conditions and transferred to nitrocellulose membranes (Schleicher & Schuell). Rabbit and mouse antisera were diluted in 0.1% Tween 20-phosphate-buffered saline with 1% (w/v) skim milk. Appropriate secondary antibodies were used, and immunoblots were developed by ECL (Amersham Biosciences). Commercial antibodies against HA (3F10; Roche Applied Science) and GFP (7.1/13.1; Roche Applied Science) were used for tagged proteins.

Parasite Cultures—*P. falciparum* asexual parasites were maintained in human erythrocytes (blood group O+) at a hematocrit of 4% with 10% Albumax™ II (Invitrogen) (25). 3D7 strain parasites were originally obtained from David Walliker at Edinburgh University. Cultures were synchronized as previously described (26).

Vector Construction and Transfection—The Gateway MultiSite™ system (Invitrogen) was used for C-terminal tagging of MTRAP and PfGAP45 with either GFP or a triple hemagglutinin (3*HA) tag. The destination vector pCHDR-3/4 (27) that carries the human dihydrofolate reductase gene, conferring resistance to the antifolate drug WR99210, was recombined with pENTR vectors: pENTR-4/1_pAMA1 containing the *AMA1* (apical membrane antigen 1) promoter (28), pENTR-2/3_FGFmptu2 (27) or pENTR2/3_3*HA vectors that provided the C-terminal GFP or 3*HA tag, and pENTR1/2 vectors for MTRAP and PfGAP45. These last two vectors were generated by cloning PCR products into the pENTR-D/TOPO vector (Invitrogen), using a toposomerase I-based reaction. Genes were amplified with the 5′ primer containing the CACC motif to facilitate directional cloning using the toposomerase enzyme (27). *MTRAP* (Plasmodb accession number PF10_0281) was amplified using the primers 5′-CACCgaaaaaagATGTAGCAGTGAGGCGGAG-3′ and TTCGAGT-CACGATTAGTTTCGATTCATCAATCATT-3′. *PfGAP45* (Plasmodb accession number PF11090w) was amplified using the primers 5′-CACATatagGAGAGAAAATAGAATATTATGTTTG-3′ and 5′-GCTC-AATTAAATGGTTGATCGGATATACT-3′. Resulting PCR products were incubated overnight at room temperature as per the manufacturer’s instructions with pENTR-D/TOPO (Invitrogen) to yield pENTR-1/2_MTRAP and pENTR1/2_PfGAP45.

Final expression vectors were generated by mixing one of each of the four plasmids (pENTR-4/1, pENTR-1/2, pENTR-2/3, and pCHDR-3/4) in the presence of a recombination enzyme mix according to the manufacturer’s instructions (Invitrogen). The three generated plasmids pCHDR-MTRAP-GFP, pCHDR-MTRAP-3*HA, and pCHDR-PfGAP45-GFP contained the gene of interest flanked by the A*MA1 promoter and either GFP or a 3*HA tag in a destination (transfection) clone containing the human dihydrofolate reductase-selectable marker. 3D7 parasites were transfected as described previously (29) with 100 μCi/ml [35S]methionine (PerkinElmer Life Sciences) until multinucleated schizonts were apparent, and proteins were extracted as described previously (33) in 1-mL volumes of 1% T-Net (1% Triton X-100, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA) with Complete (Roche Applied Science) protease inhibitor. Immunoprecipitations were performed using polyclonal rabbit antibodies against PfMTRIP,6 PfGAP45, and PfGAP50 with protein G-Sepharose (Amersham Biosciences). Proteins were separated by SDS-PAGE and visualized either by enhancement with Amplify™ fluorescence reagent (Amersham Biosciences) and autoradiography or by Western blot with mouse antibodies against PfGAP45, PfGAP50, PfMTRIP, and PfMyoA.

Protein Pull-down Assays—The entire cytoplasmic regions of PfTRAP, PfTTRAP, MTRAP, and PfTRAMP containing 45, 45, 25 residues, respectively, were amplified from either *P. berghei* (ANKA strain) or *P. falciparum* (3D7) genomic DNA using the following primers: PfTRAPtailIF (5′-GATCTcggattgagatatTTTAAATGAGGAG-TAGCGC-3′) and PfTRAPtailIR (5′-GATCtcgtggTTAGTTTCTTCTTCTTCATCG-3′) and PfTTRAPtailR (5′-GATCTcggattgagatatTTTAAATGAGGAG-TAGCGC-3′) and PfTTRAPtailR (5′-GATCtcgtggTTAGTTTCTTCTTCTTCATCG-3′) and PfMTRAPtailIF (5′-GATCtcggattgagatatTTTAAATGAGGAG-TAGCGC-3′) and PfMTRAPtailR (5′-GATCtcgtggTTAGTTTCTTCTTCTTCATCG-3′) and PfTRAMPtailIF (5′-GATCtcgtggTTAGTTTCTTCTTCTTCATCG-3′) and PfTRAMPtailR (5′-GATCtcgtggTTAGTTTCTTCTTCTTCATCG-3′). Protein pull-down were constructed using the pFTRAPtailIF primer along with the reverse primers pFTRAPtailW_Air (5′-GATCtcgtggTTAGTTTCTTCTTCTTCTTCTTCATCG-3′) (underlined bases indicate Trp to Ala mutation). PCR products were treated with BamHI/Xhol, purified, and cloned into pGEX 4T-1 (Amersham Biosciences). A His-tagged PfAldo (His-PfAldo) was amplified with PfAldoF and PfAldoR primers (see above) and cloned into pProEX-HTB (Invitrogen) to yield BamHI/Xhol sites. GEX fusions and His-PfAldo were expressed in Escherichia coli and purified by immobilization on glutathione-Sepharose microbeads equipped with an AxioCam MRm camera. The shown single 2-stacks were processed using the AxioVision version 4.2 deconvolution software package. For indirect immunofluorescence, air-dried smears of parasites were fixed for 5 min with 100% methanol at −20 °C, blocked for 30 min in 3% bovine serum albumin (Sigma) in phosphate-buffered saline, and then incubated for 1 h with the relevant antisera: rabbit MTRAP-TSR (1:20), rabbit PGAP45 (1:500), rabbit PGAP50 (1:500), mouse PfMyoA (1:500), rabbit PfAMA1 (30:1,500), mouse GFP monoclonal (Roche Applied Biosciences) (1:50), mouse PIRAP1 (31:1,100), or mouse PfMTIP™ (1:200). Following two 5-min washes in 3% bovine serum albumin/phosphate-buffered saline, slides were incubated for 1 h with appropriate Alexa Fluor 488/594 secondary antibodies (Molecular Probes) and mounted in Vectashield® (Vector Laboratories) with 10 μg/ml DAPI (Roche Applied Science).

For electron microscopy, schizont stage 3D7 parasites were enriched by Plasmagel and fixed with 4% formaldehyde and 0.1% glutaraldehyde on ice for 30 min. Embedding, immunolabeling, and contrast staining were performed as described (32). Ultrathin sections were incubated with affinity-purified rabbit MTRAP-TSR antibody (1:5), IgG-purified rabbit PGAP45 antibody (1:200), or mouse PGAP50 serum (1:200) followed by 10- or 25-nm gold-labeled anti-rabbit or mouse IgG. Cells were viewed on a Philips CM120 transmission electron microscope.

**IMC Immunoprecipitation**—Trophozoites from synchronized parasites were incubated with 300 μCi/ml [35S]methionine (PerkinElmer Life Sciences) until multinucleated schizonts were apparent, and proteins were extracted as described previously (33) in 1-mL volumes of 1% T-Net (1% Triton X-100, 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 5 mM EDTA) with Complete (Roche Applied Science) protease inhibitor. Immunoprecipitations were performed using polyclonal rabbit antibodies against PfMTRIP,6 PfGAP45, and PfGAP50 with protein G-Sepharose (Amersham Biosciences). Proteins were separated by SDS-PAGE and visualized either by enhancement with Amplify™ fluorographic reagent (Amersham Biosciences) and autoradiography or by Western blot with mouse antibodies against PfGAP45, PfGAP50, PfMTRIP, and PfMyoA.

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To determine whether the PfGAP proteins are expressed in the erythrocyte stage parasites, localization to a ring of fluorescence around the developing merozoites within schizonts or around free merozoites (Fig. 1C). This pattern is consistent with localization to the IMC that lines the length of the merozoite under the plasma membrane (6, 7). In the very rare event of capturing an invading merozoite (Fig. 1C, bottom panels), fluorescence appeared to be concentrated at the point of interface between the merozoite and the erythrocyte membrane (white arrows), consistent, although not unequivocally, with localization to the moving junction that migrates along the length of the invading zote (35, 36). Immunoelectron microscopy of invading merozoites, which to our knowledge has not yet been possible with P. falciparum, would enable confirmation of the localization of these proteins to the moving junction.

In P. yoelii sporozoites, MTIP has previously been shown to localize to the IMC (7). Using antibodies that recognize the P. falciparum orthologue, IFAs showed consistent co-localization between PfMTIP and PfGAP45 and, to a limited extent, with PfGAP50 (Fig. 2A). Lack of complete co-localization between the components is not unexpected, since the intact motor complex is predicted to form only transiently at the moving junction (20, 37). Close inspection of the reactivity with anti-PfMTIP (Fig. 2A) suggests that there is a reduction in fluorescence at the extreme tip of the invading merozoite (white arrow on inset), which is consistent with an absence of IMC at the apical pole (20). We also observed partial co-localization of the PfGAP proteins with mouse antiserum raised against a PfMyoA peptide (Fig. 2A), which gives a diffuse appearance around the periphery of merozoites forming a gradient with greatest intensity at the apical region (Fig. 2A), consistent with previous reports (6, 38). Again, lack of complete co-localization is not unexpected, given the transient nature of the motor complex. As predicted, we saw no co-localization by IFA with antibodies against SARA5, a PV protein, whose fluorescence is lost following schizont, and therefore PV, rupture (data not shown).

Transmission electron microscopy of P. falciparum schizont sections showed the typical trilaminar appearance predicted from the plasma membrane and two inner membranes of the IMC (Fig. 2B, white arrows). The parasitophorous vacuolar membrane and plasma membrane are likely to be too close together to differentiate at this late stage. Immunelectron microscopy of these sections with anti-PfGAP45 (25-nm gold particles) localized the protein to an electron-dense band around the periphery of forming merozoites with little to no labeling in the internal cytoplasmic regions (Fig. 2B). This structure has previously been interpreted as the IMC (6, 7). We also attempted double labeling of schizont sections with anti-PfGAP50 (10-nm gold particles); however, these reacted poorly, with few gold particles per merozoite, although when they were found, they consistently located close to the larger gold particles of PfGAP45 (Fig. 2B, white asterisk, and data not shown).

The predicted molecular mass of PfGAP45 is 24 kDa, whereas those against PfGAP45 recognized a doublet at ~37 to ~40 kDa (Fig. 1B). PfGAP50 has a predicted molecular mass of 44.5 kDa, and the immunoblot reactivity was consistent with its predicted size. PfGAP45 has a predicted molecular mass of 24 kDa, but its migration through SDS-PAGE is likely to be affected by its elongated structure or the high content of charged residues as with its T. gondii orthologue (20). The extra band present for PfGAP45 (Fig. 1B) suggests that it undergoes limited processing, consistent with TgGAP45 in T. gondii (20).

FIGURE 1. Characterization of PfGAP50 and PfGAP45 proteins. A, expression through the P. falciparum asexual life cycle of six genes that encode proteins homologous to those known to play key roles in Apicomplexan gliding motility and invasion. Data are from Ref. 34. Blue, down-regulation; yellow, up-regulation. Expression for the first five genes (PfGAP50, PfGAP45, PfActin, PfMTIP, and PfMyoA) peaks ~30 h postinvasion. PfAldolase has a flatter expression profile, consistent with a dual role in PfAldolase PfGAP45 recognize specific protein products. C, rabbit antiserum against recombinant PfGAP50 recognizes a single product at both glycolysis and invasion (17) (Fig. 1C). This profile is consistent with localization to the IMC that lines the length of the merozoite under the plasma membrane (6, 7). In the very rare event of capturing an invading merozoite (Fig. 1C, bottom panels), fluorescence appeared to be concentrated at the point of interface between the merozoite and the erythrocyte membrane (white arrows), consistent, although not unequivocally, with localization to the moving junction that migrates along the length of the invading zote (35, 36). Immunoelectron microscopy of invading merozoites, which to our knowledge has not yet been possible with P. falciparum, would enable confirmation of the localization of these proteins to the moving junction.

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beads (Amersham Biosciences) or on Ni\(^{2+}\)-nitrilotriacetic acid-agarose beads (Qiagen), respectively.

Pull-downs with recombinant TRAP tails (or a GST negative control) and either His-PfAldo or P. falciparum schizont-enriched parasite lysate were carried out essentially as described (17, 18). Schizont-enriched lysate was taken from saponin-lysed cultures ~40 h postinvasion (representing ~10\(^8\) parasites), resuspended in 50 mM KCl, 10 mM HEPES (pH 7.7), 1 mM MgCl\(_2\), 1 mM EDTA, and 0.2% Tween 20 (buffer A) (17, 18). A negative control, using buffer A alone, was included with each pull-down. Bound proteins were loaded in 2 \times \text{ sample buffer and separated by SDS-PAGE. Pull-downs involving His-PfAldo were visualized by Coomassie staining, whereas those with schizont lysate were visualized by Western blot probed with rabbit polyclonal antiserum raised against recombinant PfGAP45 similarly suggests localization to the IMC. A second putative invading merozoite also suggests PfGAP45 is concentrated at the interface (white arrows) with the erythrocyte, consistent with the moving junction. Scale bar, 1 \mu m.

RESULTS

Motor Complex Proteins PfGAP45 and PfGAP50 Are Expressed in Merozoites—Recent studies have identified three proteins that link MyoA to the IMC in Toxoplasma tachyzoites, MLC1 (also called MTIP) (7, 19), TgGAP45, and TgGAP50 (20), all of which have orthologues identified in P. falciparum (7, 20). We analyzed P. falciparum microarray data through the asexual erythrocytic cycle (34) for expression of PfGAP genes, PfMTIP, and three other components of the actin-myosin motor complex (PfActin, PfMyoA, and PfAldolase) and found that each of them has a pattern of expression consistent with involvement in invasion, peaking in transcription ~32–44 h postinvasion (Fig. 1A). PfAldolase has a flatter expression profile, consistent with a dual role in both glycolysis and invasion (17) (Fig. 1A).

To determine whether the PfGAP proteins are expressed in the erythrocytic stage of P. falciparum, we raised antiserum to recombinant PfGAP50 and PfGAP45. When analyzed by immunoblot, mouse and rabbit antisera against PfGAP50 recognized a single product at ~45 kDa, whereas those against PfGAP45 recognized a doublet at ~37 to ~40 kDa (Fig. 1B). PfGAP50 has a predicted molecular mass of 44.5 kDa, and the immunoblot reactivity was consistent with its predicted size. PfGAP45 has a predicted molecular mass of 24 kDa, but its migration through SDS-PAGE is likely to be affected by its elongated structure or the high content of charged residues as with its T. gondii orthologue (20). The extra band present for PfGAP45 (Fig. 1B) suggests that it undergoes limited processing, consistent with TgGAP45 in T. gondii (20).
Conservation of the Apicomplexan Actin-Myosin Invasion Motor

To investigate the trafficking of PfGAP proteins through the asexual cycle, we generated parasites that expressed PfGAP45 with a C-terminal GFP tag. Visualization of these parasites at different life cycle stages consistently showed PfGAP45 localizing to the divisions between forming merozoites, giving a circumferential fluorescence in pre- and post-trypomastigot merozoites (Fig. 2C) in a pattern very similar to that shown for PfMyoA through the erythrocytic cycle (38). Taken together with the IFA and electron microscopy data, this demonstrates that, like their orthologues in Toxoplasma, the PfGAP proteins localize to the IMC in *P. falciparum* merozoites and suggests that the inner portion of the motor complex, MyoA-MTIP-GAP45-GAP50, is conserved between the two genera.

The MyoA-MTIP-GAP Complex Is Conserved in Merozoites—Having shown that the components of the inner portion of the motor complex are present in merozoites, we undertook immunoprecipitations with radiolabeled schizont preparations harvested from late stage cultures (40–48 h postinvasion) using polyclonal rabbit antibodies against PfMTIP. Immunoprecipitations consistently gave rise to protein bands at apparent molecular masses of ~90, ~45, ~40, ~35 and 25 kDa (Fig. 3A). Western blots with specific antibodies to each subunit reacted with products at the same predicted molecular weight, strongly suggesting that these proteins were PfMyoA, PfGAP50, PfGAP45, and PfMTIP, respectively (Fig. 3B). Additional immunoprecipitations using antibodies against PfGAP45 and PfGAP50 similarly pulled down bands reactive with anti-PfMyoA, PfMTIP, and the two PfGAP proteins (Fig. 3B). The precipitation of minimal amounts of PfGAP50 with anti-PfGAP45 is surprising, given that the reciprocal immunoprecipitation successfully isolated all of the components, and suggests that the complex may not be very stable under our current isolation conditions (Fig. 3B). This may also explain why antibodies raised against PfMyoA did not precipitate any of the components (data not shown). Overall, the interaction between these four components of the motor complex in vivo argues strongly for the functional conservation of the inner portion of the motor complex in *P. falciparum* merozoites as originally identified in *T. gondii*.

Identification of a Merozoite-specific TRAP Homologue—Having identified conservation in the inner portion of the motor complex, we sought to identify a merozoite adhesin that might serve as the extracellular link to the internal motor complex. TSR-containing proteins have been shown to be essential for *T. gondii* tachyzoite and both *Plasmodium* sporozoite and ookinete motility and invasion (8–10) and have also been shown to form the extracellular link to the internal motor complex (17, 18). To determine whether there was also a merozoite-specific TRAP/MIC2 (micronemal protein 2) homologue that functions for erythrocyte invasion, we searched the genome sequence of *P. falciparum* with BLASTP and TBLASTN algorithms using the TSR domain of TRAP and CTRP. Eight TSR-containing genes, all with signal peptides, were identified (Fig. 4A). RT-PCR with specific primers for each of the genes suggests that of these, only the circumsporozoite protein generally identified as TRAP/MIC2 (16), a recently described erythrocytic TSR protein PTRAMP (39), and two unidentified genes PF10_0281 and MAL8P1.45 were transcribed in trophozoites and schizonts late in the erythrocytic cycle, consistent with a role in invasion (Fig. 4B). PF10_0281 was transcribed at considerably lower levels compared with PTRAMP, consistent with the idea that PTRAMP performs a specific role in erythrocytic invasion.
greater levels than all the other genes through the life cycle (Fig. 4B). Our identification of eight TRAP-related proteins excluded the recently identified secreted protein with altered thrombospondin repeat, PISPATR (40), that, despite having a modified TSR domain, does not have a definable transmembrane domain or a cytoplasmic tail.

The presence of a cytoplasmic tail with a concentration of acidic residues and a subterminal tryptophan residue is thought to be central to the interaction of TgMIC2 and TRAP with the internal actin-myosin motor via the glycolytic enzyme aldolase (17, 18). Of the eight genes, five have a concentration of acidic residues, of which four have a subterminal Trp residue (PTRAMP has a tyrosine residue) (Fig. 4C); acidic residues are labeled in yellow; and recessed C-terminal aromatic residues (Trp or Tyr) are labeled in red.

By a process of elimination, PF10_0281, which peaks in expression late in the erythrocytic cycle and has a putative rhomboid cleavage site (44); acidic residues are labeled in yellow; and recessed C-terminal aromatic residues (Trp or Tyr) are labeled in red.

In addition to features of the cytoplasmic tail, intramembrane cleavage of TRAP/MIC2 also appears to be essential for TRAP function (41), with cleavage thought to occur via an intramembrane rhomboid protease (42–45). Alignment of the C-terminal regions of the eight TSR-containing genes (with TgMIC2), shows that only four of the eight have a putative rhomboid cleavage site (44–48 h postinvasion) with mouse and rabbit antisera recognized a ~70-kDa band with an additional band running at ~25 kDa (Fig. 5A). Although the larger product is greater than the predicted size of MTRAP (which is 58 kDa), this may be due to the high concentration of negative residues in the region between the TSR and transmembrane domain, which are likely to affect the running of the full-length protein through SDS-PAGE. The specificity of the rabbit antibody was confirmed by expressing an additional MTRAP copy with a triple hemagglutinin (3HA) or GFP tag at the end of the cytoplasmic tail (Fig. 5A).

To investigate whether a functional product of MTRAP is expressed, we raised antiserum to a recombinant GST fusion protein encompassing the MTRAP-CSR domain. Western blot of schizont pellet material (44–48 h postinvasion) with mouse and rabbit antisera recognized a ~70-kDa band with an additional band running at ~25 kDa (Fig. 5A). Although the larger product is greater than the predicted size of MTRAP (which is 58 kDa), this may be due to the high concentration of negative residues in the region between the TSR and transmembrane domain, which are likely to affect the running of the full-length protein through SDS-PAGE. The specificity of the rabbit antibody was confirmed by expressing an additional MTRAP copy with a triple hemagglutinin (3HA) or GFP tag at the end of the cytoplasmic tail (Fig. 5A).

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the rhoptry or in the micronemes (Fig. 5B). To definitively differentiate between these two compartments, we carried out immunoelectron microscopy of schizont sections with the MTRAP-TSR antibody (Fig. 5C). Gold particles (10 nm) consistently localized to the micronemes, easily distinguished from the rhoptries as small vesicular bodies at the apical tip of developing merozoites. This confirms that MTRAP is indeed located in the micronemes (Fig. 5C).

To investigate the function of MTRAP, we attempted to disrupt the endogenous MTRAP gene using standard protocols (48). Despite repeated attempts in different parasite strains, we were unable to knock out the gene (data not shown). This suggests that MTRAP may play an essential role in parasite development, which is consistent with its being of equivalent importance to erythrocyte invasion as Plasmodium TRAP and CTRP are to invasion of liver cells and mosquito midgut cells, respectively (9, 10).

MTRAP Is Released onto the Merozoite Surface and Processed during Invasion—To visualize the trafficking and investigate whether MTRAP is released apically during merozoite development, we expressed a copy of the full-length MTRAP gene with a C-terminal GFP or triple HA tag. Both tagged proteins localize to the apical tip (Fig. 6A; data not shown). In the developing schizont and immediately after merozoite release, MTRAP-GFP localizes to a singular point at the extreme apical tip (Fig. 6, A and B), confirming localization seen with the MTRAP-TSR antibody (Fig. 5B). In some free merozoites, an apical capping of the merozoite is also seen (Fig. 6A). This is clearly the apical tip, since it is anterior to the nucleus and overlaps with a darker region that is thought to be the rhoptries (visible under bright field; see 0 h). This capping pattern of fluorescence has been described previously with another microneme protein AMA1 (49) and suggests that MTRAP may also be released onto the surface of the invading merozoite prior to invasion (30). This suggestion is confirmed by co-localization of MTRAP with AMA1, both of which are apical before schizont rupture (Fig. 6B, top panel) and associated with the surface of the invading merozoite, concentrating at the apical pole (Fig. 6B, middle panel).

Following invasion, both the cytoplasmic tail (Fig. 6, A and B) and TSR domain (data not shown) are detectable by IFA in newly invaded rings. Close inspection of Fig. 6B (bottom panel, inset) shows that there does not appear to be exact co-localization between AMA1 and the GFP-tagged tail. This would be predicted if the tail remains bound to the merozoite plasma membrane, whereas the ectodomain of AMA1 (to which this antibody is raised (30)) would remain in the PV. To further investigate MTRAP processing, we carried out Western blots with highly synchronous 3D7 and 3D7-MTRAP-3 HA cultures using parasite preparations harvested pre- and post-invasion. Immunoblots of wild-type and MTRAP-3 HA schizont preparations (44–48 h post-invasion), parasite supernatants (postschizont rupture), and early ring stage parasites (<8 h) show that the full-length MTRAP, MTRAPfull, is diminished post-invasion with only a small amount of the unprocessed form being carried through to the ring stages (Fig. 6C, band 1). No MTRAPfull was detected in supernatant, consistent with its being membrane-bound at the C terminus (Fig. 6C, band 1). The ~25-kDa processed form that contains the TSR domain, MTRAP25, was detected in supernatant and was also present in the newly formed ring (Fig. 6C, band 2). Release of MTRAP25 into the supernatant is consistent with MTRAP being cleaved in its extracellular domain during invasion. Its presence in very young rings may be due to excess TSR domain remaining in the PV or due to its remaining bound to either an erythrocyte receptor or other merozoite invasion proteins that are carried through into the newly formed ring (as would also appear to apply to AMA1) (Fig. 6B, bottom panel). Immunoblots with 3D7-MTRAP-3 HA parasite material showed three additional bands to MTRAPfull at ~48, ~45, and 10–20 kDa, respectively (Fig. 6C, bands 3–5). The ~48-kDa band may represent the loss of the TSR domain, since it is not recognized by anti-TSR, with further processing leading to the slightly shorter ~45-kDa band. The size of the 10–20-kDa band is consistent with a cleavage event near or within the transmembrane domain of the tagged protein, leaving the cytoplasmic tail. Such a cleavage event is supported by the presence of a putative rhomboid cleavage site in the transmembrane domain of MTRAP (Fig. 4C) and the essential nature that this cleavage has for other TRAP homologues (42, 43, 45). A ~30-kDa band detectable in 3D7-MTRAP-GFP parasite material (Fig. 5A, right panel, lane c) is likely to be the equivalent cytoplasmic tail, factoring in the increased mass given by the GFP tag. Together, this suggests that at least two cleavage events occur in MTRAP proteolysis during invasion (Fig. 6D), leaving a cytoplasmic tail that has a predicted size consistent with its being the product of intramembrane cleavage by a rhomboid protease.

The Cytoplasmic Tail of MTRAP Binds to Recombinant and Native P. falciparum Aldolase—Central to the function of TRAP in sporozoites and TgMIC2 in tachyzoites is the interaction of the cytoplasmic tail with the rest of the motor complex, an interaction that may be mediated by aldolase, a protein known to bind F-actin (17, 18). To investigate whether MTRAP also had the ability to bind aldolase, we expressed the cytoplasmic tails of MTRAP and PfTRAP along with a mutant MTRAP tail (MTRAPW/A, having a Trp to Ala mutation in the fourth residue from the C terminus) fused to GST (Fig. 7A) and investigated their binding to recombinant PfAldo expressed with a His8 tag. GST-MTRAPtail and GST-PfTRAPtail immobilized on GSH-Sepharose beads bound recombinant PfAldo (Fig. 7B). Binding of all tails to PfAldo was not very efficient, with a maximum of 0.1 µg bound/µg of added tail fusion protein (Fig. 7B; data not shown). This may indicate that the interaction in vivo is not very strong, although it may also be the result of poor refolding of our recombinant PfAldo. Binding of GST-
MTRAP<sub>W/A</sub> tail was almost 60% less than that in the wild type (Fig. 7C), a reduction that is consistent with the Trp residue being important for stabilizing the interaction with aldolase (18). As additional controls, immobilized GST was shown to have minimal binding to PfAldo (Fig. 7B), whereas PbTRAP bound to PfAldo to almost the same degree as PfTRAP (Fig. 7B and C). We also expressed the cytoplasmic tail of another erythrocytic TSR protein, PTRAMP (39). This bound PfAldo at a level between that of MTRAP<sub>W/A</sub> and GST, suggesting that PTRAMP, despite having acidic residues and a terminal Tyr residue (that is also aromatic) in its cytoplasmic tail, may not be functionally equivalent to that of TRAP (Fig. 7B and C).

To further characterize the in vitro interaction of the MTRAP tail with aldolase, we carried out pull-down assays using the same GSH-Sepharose-immobilized GST-MTRAP<sub>W/A</sub> tail but this time incubated the recombinant tail with whole parasite lysate taken from schizont-enriched cultures (~40 h postinvasion). Immunoblots of bound parasite proteins probed with rabbit polyclonal antisera raised against recombinant PfAldo recognized a single band at ~40 kDa (Fig. 7D). This corresponds to the predicted molecular mass of <i>P. falciparum</i> aldolase at 40.1 kDa. A similar band was detectable in a pull-down with the GST-PTRAMP<sub>W/A</sub> tail and with the GST-MTRAP<sub>W/A</sub> tail (Fig. 7D). This confirms the affinity of the MTRAP and TRAP tails for native aldolase. Pull-downs using the GST-PTRAMP<sub>W/A</sub> tail or GST did not bring down any detectable aldolase (Fig. 7D). This further argues against any functional equivalence between TRAP and PTRAMP.

The in vitro interaction of the MTRAP C terminus with aldolase supports its role as the functional homolog of TRAP for the merozoite, although the functional significance of the aldolase interaction is still unknown. The similarities between MTRAP and TRAP and the presence of the other components linking the IMC to an extracellular adhesin suggests that the actin-myosin motor complex is conserved between the liver stage sporozoite and the blood stage merozoite of <i>Plasmodium</i> parasites despite their very different modes of motility.

Conservation of the Apicomplexan Actin-Myosin Invasion Motor—Given the apparent conservation in the motor complex that is used to drive both sporozoite motility and invasion and merozoite invasion and the known
Conservation of the Apicomplexan Actin-Myosin Invasion Motor

conservation between *Plasmodium* zoites and *Toxoplasma* tachyzoites in the way they move (8), we sought to determine whether each of the subunits now identified in the merozoite is also seen across other Apicomplexan genera. Searches of the publicly available genomes for *T. annulata* (22), *C. parvum* (23), *E. tenella* (available on the World Wide Web at www.GeneDB.org), and *B. bigemina* (available on the World Wide Web at www.sanger.ac.uk) show that orthologues for all contributing genes (TRAP homologue-aldolase-actin-MyoA-MTIP/MLC-GAP45-GAP50) are present (Table 1, supplemental Fig. S3). The presence of orthologues to all of the key components supports the notion that the same actin-myosin-based molecular motor drives cell motility and invasion across all Apicomplexan parasites.

**DISCUSSION**

General conservation in the molecular mechanisms that underlie gliding motility and cell invasion across Apicomplexan parasites has previously been suggested following heterologous replacement of cell surface adhesins (8, 50) and by the presence of orthologues to different components of the motor complex in three genera of Apicomplexan parasites (7, 19, 20). However, until now, this conservation has not been demonstrated for the relatively nonmotile blood stage merozoite of *Plasmodium* spp. Here we show that the same basic motor complex that drives gliding motility in the amoeboid-like *Plasmodium* sporozoite is also present and likely to drive cell invasion in the merozoite. Furthermore, we show that the generic complex, consisting of TRAP homologue-aldolase-actin-MyoA-MTIP/MLC-GAP45-GAP50, can be identified in each of the available Apicomplexan genomes, representing six highly divergent genera. This suggests that the motor complex is evolutionarily conserved across the phylum Apicomplexa.

Primary support for the role of the conserved motor in *P. falciparum* erythrocyte invasion comes from the expression and localization of each of the key components of the motor complex and their association together. PfGAP45, PfGAP50, and MTIP all localize to the merozoite periphery by IFA (Figs. 1 and 2), which is confirmed by electron microscopy to be the IMC (Fig. 2B) (7). Although localization by microscopy is not definitive, immunoprecipitation of the complex using antibodies against both PfGAP proteins and MTIP confirms that these components do associate in vivo (Fig. 3). Although this may only involve a small fraction of the different subunits, this is likely to be determined by the transient nature of the moving junction in the invading merozoite and not necessarily a demonstration of low affinity between different subunits. Unlike gliding motility in more motile Apicomplexan life cycle stages,

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### Table 1

| Subunit Type   | Sequence Details                                                                 |
|---------------|----------------------------------------------------------------------------------|
| GST-MTRAP     | gYFLRKEK7EKWQETKKEFENFVMFDADLGKDNKAMDEEWFAL                  |
| GST-PTRAP     | gYFLRVPAGAPYPYAGEPEAPFDELTGEEDKLDPEQFRLPEENW                     |
| GST-MTRAP_{WA}| gYFLRKEKEK7EKWQETKKEFENFVMFDADLGKDNKAMDEEWFAL                  |
| GST-PTRAMP    | gYFLRVPAGAPYPYAGEPEAPFDELTGEEDKLDPEQFRLPEENW                     |
| GST-PbTRAP    | gYFLRVPAGAPYPYAGEPEAPFDELTGEEDKLDPEQFRLPEENW                     |

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**FIGURE 7.** The MTRAP cytoplasmic tail interacts with aldolase in vitro. A, schematic of recombinant TRAP homolog tails cloned in frame at the C terminus of GST. The amino acids from the 5’ BamHI cloning site are shown in lowercase type, and the aromatic residue or replacement of Trp for Ala in GST-MTRAP_{WA} is shown in boldface type. B, GST-MTRAPtail interacts with recombinant PfAldo. Equal amounts of each GST fusion tail, immobilized to GSH-Sepharose, were incubated with His-PfAldo and bovine serum albumin. Bound fractions were washed, separated by SDS-PAGE, and visualized by Coomassie staining. GST negative control and *P. berghei* TRAP positive controls are included. C, percentage binding of PfAldo with respect to GST-MTRAPtail. The error bars indicate a 95% confidence interval from three independent experiments. D, GST-MTRAPtail interacts with native PfAldolase. Equal amounts of each GST fusion tail (excluding PbTRAP), immobilized to GSH-Sepharose, were incubated with whole parasite lysate from schizont-enriched cultures 40 h postinvasion. Immunoblots of bound proteins, separated by SDS-PAGE, were probed with polyclonal rabbit antiserum raised against recombinant GST-PfAldo. Schizont lysate represents 1% of the material used in the plus lysate pull-down with the sample indicating pull-down in the presence of buffer A alone (see “Experimental Procedures”).
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Table 1

| Gene      | PfMyoA | TgMyoA | CpMyoA | TaMyoA | EtMyoA | BbMyoA |
|-----------|--------|--------|--------|--------|--------|--------|
| GAP45     | PfGAP45| TgGAP45| PfGAP45|        |        |        |
| MTIP      | CpMTIP| TaMTIP| EtMTIP| BbMTIP|        |        |
| Actin     | PfACT1| TgACT1| CpACT1| TaACT1| EtACT1| BbACT1|
| Aldolase  | PfAldo| TgAldo| PfAldo|        |        |        |
| TRAP      | TRAP  |        |        |        |        |        |

Entries for **P. falciparum** and **T. gondii** are from GenBank. Entries for **C. parvum**, **T. annulata**, and **B. bigemina** are from www.sanger.ac.uk.

For GAP45, sequence conservation in the extreme N and C terminus highlights these regions as potentially critical to function. This includes the Met-Gly N-myristoylation motif in the first two amino acid positions (20). The addition of a C-terminal GFP tag to PIGAP45 was not detrimental to parasite development here, suggesting that the interaction with the other components may be via the N terminus. It is possible that a functional domain may still be accessible in the GFP mutant (since there is a linker between the C terminus and GFP) or that endogenous PIGAP45 is sufficient to sustain function despite the presence of the episomal GFP copy. Further characterization of the motor complex with the PIGAP45-GFP-expressing line will help to resolve whether the tagged form is indeed functional.

There are long stretches of amino acid identity in the N-terminal region of the GAP50 protein (supplemental Fig. S3). The formation of the Toxoplasma motor complex relies critically on a functional GAP50 C-terminal tail (20), which, along with conservation in the six amino acids that make up the C terminus among orthologues from Plasmodium and Eimeria, was used to argue that the complex with GAP45, MTIP, and MyoA is associated with the short cytoplasmic tail of GAP50 (20). Including orthologues from Cryptosporidium, Theileria, and Babesia reduces the degree of sequence homology in the cytoplasmic tail, suggesting that the conservation may have been overestimated (supplemental Fig. S3). We believe that the high degree of conservation in N-terminal regions between the six Apicomplexan species argues in favor of the complex being assembled using the much larger N terminus and not the short C terminus of the GAP50 anchor.

The level of sequence homology between MTIP and TgMLC1 is well characterized (7) and appears to hold across Apicomplexan genera. Binding of MTIP to PfMyoA was shown previously to be dependent on a large portion of the MTIP protein (7) and a dibasic motif in the PfMyoA tail (51). The dibasic motif is maintained across the six genera, as are three highly conserved amino acid stretches within the C-terminal portion of MTIP, which may constitute the sites that are central to the interaction with MyoA or have other functional roles (positions 97–100, 111–119, and 195–202; supplemental Fig. S3). The CpMTIP ortholog has a very large additional C-terminal tail. Further investigation will determine whether this is an error in the gene annotation or if the long cytoplasmic tail has unique properties or functions.

A TRAP-based Motor Complex—Conservation of the molecular mechanism that underlies all Apicomplexan motility is strengthened by the presence of a TRAP homologue in each of the Apicomplexan genera (Table 1). However, the generality of this assertion has not been extended to erythrocyte invasion by Plasmodium merozoites. Here, we have identified a novel, putatively essential, erythrocytic protein that has a number of features that suggest it may be the functional TRAP homolog for merozoites, which we have named MTRAP. With its identification, there are now at least two *P. falciparum* merozoite-specific TRAP homologues (MTRAP, PF10_0281; PTRAMP (39), PFL0870w).

Four main observations support the notion that MTRAP is a probable merozoite-specific functional homolog of TRAP: (a) MTRAP is expressed at high levels in middle to late stage asexual parasites; (b) it is
released onto the merozoite surface prior to invasion; (c) MTRAP is processed during invasion in a manner that is consistent with there being intramembrane cleavage by a rhomboid protease; and (d) it is putatively essential as demonstrated by repeated attempts to knock out the endogenous gene. Further support for the functional homology also comes from the ability of the cytoplasmic tail of MTRAP to bind in vitro both recombinant and native aldolase and the reduction in binding following mutation of the terminal Trp residue (Fig. 7B). However, despite the observed interactions of TRAP, TgMIC2, and now MTRAP with aldolase (17, 18), the functional significance of this interaction is still unknown. Furthermore, we believe that other proteins are likely to be involved and may be critical for stable interaction between the cytoplasmic tail and F-actin. Studies are currently under way to identify other actin-binding proteins that may link with the extracellular adhesin.

We have not been able to demonstrate direct binding of the MTRAP TSR domain to the host cell surface,7 and whereas this may not rule out a direct interaction with an erythrocyte receptor, it is possible that MTRAP binds the erythrocyte indirectly via another parasite protein. Such an association of MTRAP with a secondary binding partner would not be unprecedented, since TgMIC2 associates closely with M2AP in Toxoplasma (52), disruption of which severely impairs host cell entry (53). A potential secondary partner for MTRAP might be one of the erythrocyte binding antigens, such as EBA-175, which does not have a TSR domain and has not been shown to interact with the motor complex. In support of this, it has been proposed that trafficking of EBA proteins to the micronemes requires an unidentified escorter protein (54). One possibility is that a micronemal protein, such as MTRAP, may function as both the escorter and the link between invasion adhesins and the motor complex in merozoites. The ability of the TSR domain to interact with a conserved domain, such as the 3′ Cys-rich domain of EBAs (55), would provide a generic link to a variable extracellular invasion ligand. The conserved domain of Rh proteins represents another potential site of interaction (56). The recent observation of an association between proteins from different cellular compartments at the moving junction in Toxoplasma (35) supports such a complex being involved in invasion. The micronemal protein TgAMA1 (whose P. falciparum orthologue does not bind to the erythrocyte surface despite being essential for invasion (28)) is associated with a complex of rhoptry neck proteins RON2 and RON4 (35). Studies are currently under way to determine whether the MTRAP TSR is involved in a similar tight junction complex with other micronemal or rhoptry proteins.

The extracellular domain of MTRAP does not appear to be under positive selection, as are analogous regions of AMA1 (57) and EBA-175 (58). In comparison with its inferred orthologue in P. reichenowi (supplemental Fig. S2), there is no significant evidence for an excess of non-synonymous polymorphism in PfMTRAP (Fisher’s exact p = 0.56) (59). This may suggest that MTRAP is hidden from the host immune response by associating in a complex or, alternatively, that its release in vivo is later than other micronemal proteins and occurs in the tight junction away from host antibodies. In support of this lack of exposure, invasion assays with polyclonal serum raised against the MTRAP TSR domain did not inhibit invasion.8

The conservation of the motor complex across Apicomplexan parasites is further supported by the recent demonstration that Cryptosporidium uses gliding motility to invade host cells (60) and that invasion or motility can be inhibited in Cryptosporidium (60), Theileria (61), and Babesia (62), following the addition of drugs that inhibit actin or myosin. The presence of the complex in Cryptosporidium is particularly striking, since this genus represents a phylogenetically early branch on the Apicomplexan tree more closely related to gregarines (parasites of invertebrates) than it is to either Plasmodium or Toxoplasma (63). Together, this suggests that a generic motor complex is used across the phylum Apicomplexa and has been adapted to the variety of hosts and tissues targeted and the diversity of cell morphology found among Apicomplexan parasites. The conservation of this complex despite the diversity of Apicomplexan parasites highlights a number of potential targets for chemotherapeutic inhibition that may be applicable in a wide variety of diseases of both humans and livestock.

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