Identification of the membrane receptor of a class XIV myosin in *Toxoplasma gondii*

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Acipicomplexan parasites exhibit a unique form of substrate-dependent motility, gliding motility, which is essential during their invasion of host cells and during their spread between host cells. This process is dependent on actin filaments and myosin that are both located between the plasma membrane and two underlying membranes of the inner membrane complex. We have identified a protein complex in the acipicomplexan parasite *Toxoplasma gondii* that contains the class XIV myosin required for gliding motility, TgMyoA, its associated light chain, TgMLC1, and two novel proteins, TgGAP45 and TgGAP50. We have localized this complex to the inner membrane complex of *Toxoplasma*, where it is anchored in the membrane by TgGAP50, an integral membrane glycoprotein. Assembly of the protein complex is spatially controlled and occurs in two stages. These results provide the first molecular description of an integral membrane protein as a specific receptor for a myosin motor, and further our understanding of the motile apparatus underlying gliding motility in acipicomplexan parasites.

Introduction

The human and animal pathogen *Toxoplasma gondii* is an obligate intracellular parasite of the phylum Apicomplexa, which also includes *Plasmodium*, the causative agent of malaria, as well as *Eimeria* and *Cryptosporidium*, causative agents of enteritis. These protozoan parasites have to be motile in order to escape their host cell at the end of infection and invade new host cells, yet they lack the structures normally associated with cell motility, such as cilia, flagella, pseudopodia, or lamellipodia. Instead, these organisms move by a unique process called gliding motility, a substrate-dependent process characterized by circular and forward twisting movements (Hakansson et al., 1999). The process of gliding motility is still poorly defined, and only some of the key players have been identified to date. An actin–myosin-based motility system has been implicated in movement and host cell invasion of *Toxoplasma, Cryptosporidium*, and *Plasmodium* using F-actin destabilizing agents and inhibitors of myosin function (Miller et al., 1979; Dobrowolski and Sibley, 1996; Dobrowolski et al., 1997; Forney et al., 1998; Pinder et al., 1998; Kappe et al., 1999; Wetzel et al., 2003). In *Toxoplasma*, gene disruption experiments indicate that its myosin-A isoform (TgMyoA; Meissner et al., 2002), a class XIV myosin, and its associated light chain TgMLC1 (Herm-Gotz et al., 2002) are critical for the gliding motility of the parasite. For that reason this complex has been referred to as the glideosome (Opitz and Soldati, 2002).

The cell wall or pellicle of acipicomplexan parasites consists of the plasma membrane and the closely associated, flattened cisternae of the inner membrane complex. Both actin and the myosin-A homologues have been localized to the space between the plasma membrane and the inner membrane complex of *Toxoplasma* and *Plasmodium* (Dobrowolski et al., 1997; Pinder et al., 1998). In *Toxoplasma*, actin filaments appear to be associated with the plasma membrane of the parasite through an interaction with the cytoplasmic tail of the transmembrane adhesin MIC2, which in turn may be mediated by the glycolytic enzyme aldolase (Jewett and Sibley, 2003). TgMyoA was originally also believed to be associated with the plasma membrane (Dobrowolski et al., 1997). However, more recent evidence in *Plasmodium yoelii* suggests that its myosin-A is associated with the inner membrane complex, as judged by the localization of MTIP, a myosin light chain-like protein that interacts with the myosin-A tail (Bergman et al., 2003).

The manner in which the acipicomplexan myosins associate with membranes is not known. A dibasic motif in the carboxy...
terminus of TgMyoA has been found to be essential for localization to the pellicle and has been proposed to mediate association with membranes (Hettmann et al., 2000). As TgMyoA is easily extracted using high pH, it appears to be a peripheral membrane protein (Hettmann et al., 2000). However, it is not known whether its membrane association

Figure 1. GAP45 is associated with the inner membrane complex of T. gondii. (A) Distribution of TgGAP45 was compared with that of the plasma membrane protein SAG1 in control cells and in parasites treated with C. septicum α-toxin. In untreated cells, the plasma membrane protein SAG1 is evenly distributed over the parasite circumference, whereas TgGAP45 distribution reveals an anterior gap (arrows) consistent with localization to the inner membrane complex. In toxin-treated parasites, the plasma membrane is markedly distended, whereas the distribution of TgGAP45 is not affected, confirming its association with the inner membrane complex. Bars, 4 μm. (B) Diagram illustrates the basic elements of the Toxoplasma pellicle: the plasma membrane, the two membranes of the inner membrane complex, the membrane skeleton, and the subpellicular microtubules. Isolated pellicle preparations were incubated with control antiserum or TgGAP45 antiserum, followed by goat anti–rabbit secondary antibodies conjugated to 10-nm gold. The arrowhead indicates the plasma membrane, the arrows the gold particles, and the asterisks the subpellicular microtubules. Bars, 150 nm. (C) Multiple alignment of GAP45 sequences from different apicomplexan parasites. Amino acid residues identical in at least three of the four sequences are highlighted in black; similar residues in gray. The amino-terminal N-myristoylation motif in the GAP45 sequences is indicated with an asterisk. TgGAP45: T. gondii GAP45, GenBank/EBML/DDBJ accession no. AAP41369; NcGAP45: N. caninum GAP45 sequence, assembled from EST sequences with accession no. NcEST3c79 and NcEST3d11b08; PfGAP45: P. falciparum GAP45, accession no. AAN36304; PyGAP45: P. yoelii GAP45, accession no. EAA23022; CpGAP45: C. parvum GAP45, accession no. CAD98387.
is mediated by an integral membrane protein or through a direct interaction with phospholipid head groups, in a manner analogous to that proposed for myosin IC and IB (Dobberstein and Pollard, 1992; Reizes et al., 1994). In the case of two class V myosins, Myo2p in Saccharomyces cerevisiae and myosin-Va in melanocytes, specific receptors have been identified on the membranes of cargo organelles, the yeast vacuole and melanosome, respectively. In the melanocyte, myosin-Va is linked by melanophilin to Rab27a that is, in turn, associated with the melanosome membrane through two geranylgeranyl moieties at its carboxy terminus (Wu et al., 2002a,b). In S. cerevisiae, Myo2p is linked by Vac17p to Vac8p that is associated with the vacuole membrane through an N-myristoyl and multiple palmitoyl groups at its amino terminus (Ishikawa et al., 2003).

Here, we show that the two known components of the Toxoplasma glideosome, TgMyoA and TgMLC1, are associated with two novel proteins, TgGAP45 and TgGAP50. Although the function of TgGAP45 is not clear at this time, TgGAP50 is an integral membrane glycoprotein that anchors the glideosome in the inner membrane complex of T. gondii, and thus performs a critical function in parasite motility.

Results
Identification of TgGAP45
As several factors required for Toxoplasma motility are known to be associated with the parasite pellicle, we set out to identify additional structural and regulatory factors required for parasite motility by a general characterization of the isolated pellicle. Isolated pellicle preparations were subjected to sequential detergent extraction with Triton X-100 (TX100) and sodium deoxycholate (DOC) to preferentially solubilize proteins in the plasma membrane and inner membrane complex, respectively (Mann and Beckers, 2001). Antisera were produced to the TX100- and DOC-soluble fractions, and these were used to screen expression libraries of Toxoplasma cDNA. Several of the reactive clones contained novel sequences and were further characterized by the isolation of full-length cDNAs and the production of monospecific antisera.

The antisera to one of the clones detected with antisera to the DOC-soluble fraction labels the periphery of parasites in permeabilized (Fig. 1 A, GAP45) but not intact parasites (unpublished data), suggesting it is associated with a cytoplasmic aspect of the Toxoplasma pellicle. However, the staining pattern of this protein, TgGAP45, does not overlap precisely with that of the plasma membrane marker SAG1. Specifically, discontinuities are observed at the anterior end of the parasite, suggesting the protein is associated with the inner membrane complex rather than the plasma membrane. To confirm this, immunofluorescence was performed on parasites treated with Clostridium septicum α-toxin, which causes their plasma membrane to swell away from the inner membrane complex (Wichroski et al., 2002). After toxin treatment, the staining patterns for TgGAP45 and SAG1 are clearly distinct (Fig. 1 A), also suggesting that the protein is present in the inner membrane complex of the parasite rather than the plasma membrane.

To determine the exact localization of TgGAP45 in the parasite, pellicle preparations were incubated with the mono-

*Figure 2. GAP45 is associated with myosin-A, myosin light chain-1, and a novel 50-kD protein. (A) The TgGAP45 antiserum recognizes a single 45-kD protein on immunoblots. A Toxoplasma lysate was fractionated by SDS-PAGE and immunoblot analysis using either preimmune serum or a TgGAP45 antiserum. (B) Immunoprecipitation analysis with the TgGAP45 antiserum. Intracellular parasites were metabolically labeled with 35S-labeled methionine and cysteine, lysed in a buffer containing either SDS or TX100, and subjected to immunoprecipitation with the TgGAP45 antiserum. Only one protein, GAP45, is immunoprecipitated from denaturing extracts prepared with SDS, whereas three additional proteins are immunoprecipitated from TX100 extracts. (C) Metabolically labeled parasites or HFF cells were lysed in TX100-containing buffer and subjected to immunoprecipitation with the TgGAP45 antiserum. The immune complex was subjected to SDS-PAGE and transferred to nitrocellulose. Different molecular mass ranges of the blot were excised and incubated with monospecific antisera to TgMyoA, TgGAP45, and TgMLC1; each recognize their respective proteins in the immunoprecipitated complex. Asterisks indicate the IgG heavy and light chains that cross react with the secondary antibody used.
A), larger than the predicted molecular mass of 27.3 kD. As recombinant fusion proteins containing the putative coiled-coil domain (but not the remainder of the protein) display a similar anomalous migration behavior during SDS-PAGE, we believe this to be due to an elongated structure or the high content of charged residues (unpublished data).

TgGAP45 is associated with Toxoplasma myosin-A, myosin light chain-1, and a novel protein TgGAP50

As the predicted amino acid sequence did not reveal any obvious functional homology to known proteins, we analyzed this protein by immunoprecipitation from metabolically labeled parasites. When cells are lysed and denatured in an SDS-containing buffer, only a single 45-kD protein is detected in immunoprecipitates (Fig. 2 B). However, when cells are lysed in a TX100-containing buffer, three additional proteins are immunoprecipitated with apparent molecular masses of 93, 50, and 32 kD (Fig. 2 B). When this immunoprecipitate is further analyzed by immunoblotting with an antiserum to TgGAP50, only the 45-kD subunit reacted (Fig. 2 C; unpublished data), suggesting the other proteins are tightly associated, unrelated proteins.

Considering the fact that TgGAP45 is found in the Toxoplasma pellicle and that the associated 93- and 32-kD proteins are similar in size to the pellicle-associated myosin-A (TgMyoA) and its associated light chain (TgMLC1), we analyzed the immunoprecipitates with antisera to both proteins. As is shown in Fig. 2 C, the 93- and 32-kD proteins do indeed react with antisera to TgMyoA and TgMLC1, respectively. All TgMyoA and TgMLC1 in the parasite is apparently associated with TgGAP45, as judged by immunoblot analysis of parasite extracts after immunodepletion of TgGAP45 (unpublished data). However, the 50-kD protein does not react with any of the antisera used, suggesting it represents a novel protein, and is henceforth referred to as TgGAP50.

As the complex of TgMyoA and TgMLC1 has been demonstrated to participate in the gliding motility of Toxoplasma and other apicomplexan parasites, it has been named the glideosome (Opitz and Soldati, 2002). Based on their association with this complex and their apparent molecular mass, the two novel proteins will therefore be referred to as glid-
In order to assess the role of TgGAP50 in the glideosome complex, parasites were metabolically labeled with [35S]-methionine/cysteine and incubated in the absence or presence of PNGase-F. Bands corresponding to the fully glycosylated and deglycosylated TgGAP50 are indicated.

The glideosome complex was immunoprecipitated from metabolically labeled parasites and incubated in either the absence or presence of PNGase-F. Bands corresponding to the fully glycosylated and deglycosylated TgGAP50 are indicated.

TgGAP50 is an integral membrane glycoprotein of the inner membrane complex

TgGAP50 was purified by preparative two-dimensional gel electrophoresis, and tryptic digests were analyzed by mass spectrometry. The amino acid sequences of four tryptic fragments were obtained and used to identify a candidate gene in the database of Toxoplasma genomic DNA. The complete ORF was subsequently identified by RT-PCR analysis and isolation of a full-length cDNA clone. The TgGAP50 ORF predicts a 431-residue protein with a predicted molecular mass of 46.6 kD. Analysis of the predicted amino acid sequence reveals the presence of putative transmembrane domains at the amino terminus (residues 25–45) and the extreme carboxy terminus (residues 402–426), suggesting that TgGAP50 is an integral membrane protein (Fig. 3).

Database searches reveal the presence of orthologues in three other apicomplexan parasites: P. falciparum, P. yoelii, and Eimeria tenella (Fig. 3). These proteins are highly homologous (41–58% identity) along their entire sequences, except for the amino-terminal signal peptide, which show little or no similarity. Therefore, it is likely that the TgGAP50 orthologues perform similar functions in the motility of all apicomplexan parasites. Interestingly, TgGAP50 demonstrates a 22–26% identity to various purple acid phosphatases of plants and animals, although the amino acid residues critical for enzymatic activity of these phosphatases were mostly not conserved in the sequence of Toxoplasma TgGAP50 or its apicomplexan orthologues (Fig. 3).

The amino-terminal transmembrane domain of TgGAP50 appears to act as a cleavable signal peptide, as direct amino acid sequencing of purified protein reveals that the amino terminus of the mature protein corresponds to residue 51 in the predicted sequence. As the predicted molecular mass of the mature protein was smaller than that observed in SDS-PAGE and as the TgGAP50 sequence is predicted to have three N-linked glycosylation sites (Fig. 3), we subjected immunoprecipitates to digestion with the endoglycosidase PNGase-F. As can be seen in Fig. 4, this results in a substantial decrease in molecular mass of TgGAP50 to a value close to the one predicted for the mature protein. This result demonstrates that TgGAP50 is indeed N-glycosylated in Toxoplasma. The decrease in molecular mass is consistent with the removal of about three N-linked glycans, suggesting that all three predicted glycosylation sites are indeed used.

When a fusion protein of TgGAP50 and YFP is expressed in T. gondii, it is efficiently targeted to the inner membrane complex of mature parasites and growing daughter parasites (Fig. 5), and is not present in the parasite plasma membrane as judged by the following observations. In nonreplicating parasites, TgGAP50-YFP colocalizes with the cell surface marker SAG1 except at the extreme apical end of the organism, a region that lacks the inner membrane complex (Fig. 5 A, arrows). Treatment of these parasites with C. septicum α-toxin, which induces selective swelling of the plasma membrane without affecting inner membrane complex morphology (Wichroski et al., 2002), results in clear separation of the plasma membrane marker SAG1 and TgGAP50-YFP (Fig. 5 A). Together, these observations indicate that TgGAP50 is an integral membrane protein of the inner membrane complex rather than the plasma membrane. This conclusion is supported by the observation that in replicating parasites, TgGAP50-YFP colocalizes with the inner membrane complex–associated protein TgIMC1 (Mann and Beckers, 2001) in the mother cell and enclosed daughter parasites (Fig. 5 B).

Parasites expressing TgGAP50-YFP in a stable fashion were metabolically labeled and analyzed by immunoprecipitation with antisera to TgGAP45 and to GFP, which cross reacts with YFP. When these parasites were lysed and denatured in SDS, the GFP antiserum immunoprecipitates only a single protein of ~82 kD, consistent with the predicted molecular mass of the TgGAP50-YFP fusion protein (Fig. 6 A). However, when parasites were lysed in TX100-containing buffers, the GFP antiserum immunoprecipitated the TgGAP50-YFP protein together with TgMyoA, TgMLC1, and TgGAP45, but no untagged TgGAP50 (Fig. 6 A). In this immunoprecipitate, TgGAP50-YFP was clearly present in excess of the other subunits, suggesting that a substantial fraction of this protein is not present in glideosomes. TgGAP45 antiserum immunoprecipitates TgMyoA, TgGAP50, TgGAP45, and TgMLC1 from these parasites, along with the TgGAP50-YFP fusion protein (Fig. 6 A).

Combined, these data indicate that mature TgGAP50 is an integral membrane protein of the Toxoplasma inner membrane complex containing a 351-residue lumenal do-

Figure 4. Toxoplasma GAP50 is modified by N-linked glycosylation. The glideosome complex was immunoprecipitated from metabolically labeled parasites and was incubated in the absence or presence of PNGase-F. Bands corresponding to the fully glycosylated and deglycosylated TgGAP50 are indicated.
main with N-linked glycans at residues 101, 136, and 228, a single transmembrane domain, and a six-residue cytoplasmic domain at the carboxy terminus. TgGAP50 is part of the glideosome complex along with TgGAP45, TgMyoA, and TgMLC1. As TgGAP50 is the transmembrane protein in this complex, it is likely to act as the membrane anchor for the glideosome, and thus to play a pivotal role in parasite motility. The presence of highly conserved orthologues in other apicomplexan parasites suggests the function of TgGAP50 is conserved throughout this phylum.

The glideosome is assembled in two stages

Immunofluorescence analysis of Toxoplasma expressing the TgGAP50-YFP fusion protein reveals a distinct difference in the localization of the different components of the glideosome. TgGAP50 is found in the inner membrane complex of both mature parasites and immature daughters, as judged by its colocalization with the marker TgIMC1 (Fig. 5). In contrast, TgMyoA, TgMLC1, and TgGAP45 are only found associated with the inner membrane complex of mature parasites and are entirely absent from immature daughters (Fig. 5). This observation suggests that the glideosome may be assembled in multiple stages during cell division in T. gondii.

To test this hypothesis, we used pulse-chase analysis and immunoprecipitation with TgGAP45 antiserum to determine if there were any changes in glideosome composition over time. As can be seen in Fig. 7 A, a complex containing TgMyoA, TgMLC1, and TgGAP45 can be isolated after a 15-min pulse labeling, but TgGAP50 is absent. In contrast, after a 4-h chase, all glideosome subunits, including TgGAP50, are present in the complex. Together with the data in Fig. 5, these observations demonstrate that the glideosome complex is assembled in two stages. During or shortly after their synthesis, the three glideosome subunits synthesized on cytoplasmic ribosomes, TgMyoA, TgMLC1, and TgGAP45, associate with each other into a complex, the proto-glideosome. TgGAP50, on the other hand, is cotranslationally inserted into the parasite ER and is transported to the inner membrane complex where, in mature parasites, it associates with the TgMyoA/TgGAP45/TgMLC1 proto-glideosome to form the functional, membrane-associated glideosome.

GAP50 acts as the membrane anchor for the glideosome

If TgGAP50 acts as the membrane anchor of the glideosome complex, one would predict that association of the glideosome with membranes would be dependent on the presence of TgGAP50 in the complex. To test this hypothesis, we separated homogenates of pulse-labeled and chased parasites into soluble and particulate fractions and immunoprecipitated from each the proto-glideosome and glideosome complex using TgGAP45 antiserum. As can be seen in Fig. 7 B, the proto-glideosome in pulse-labeled cells is found almost entirely in the soluble fraction. The mature glideosome in chased cells, in contrast, is largely present in the particulate fraction. Together, the data in Fig. 7 clearly demonstrate that the glideosome complex is assembled in distinct stages. The TgMyoA, TgMLC1, and TgGAP45 subunits first assemble into a soluble proto-glideosome in the cytoplasm, followed by its association with the membrane anchor.
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TgGAP50 and formation of the glideosome proper on the inner membrane complex.

Because the association of the proto-glideosome with TgGAP50 most likely occurs through the latter’s short carboxy-terminal cytoplasmic domain, we generated a mutant TgGAP50-YFP fusion protein, TgGAP50^H9004(427-431)YFP, in which this entire domain was deleted. Although expression of the full-length TgGAP50-YFP is not deleterious to the parasite and stable transfectants can be obtained with ease, we were unable to obtain stably transfected parasites expressing TgGAP50^H9004(427-431)YFP. In fact, at 48 h after transfection 20–40% of parasites expressed normal TgGAP50-YFP, but no parasites expressing the mutant protein were observed at that time. This observation indicates that expression of TgGAP50^H9004(427-431)YFP exerts a dominant lethal effect on Toxoplasma.

To determine whether this was due to an effect on assembly of the glideosome, Toxoplasma transiently expressing this construct were metabolically labeled 24 h after transfection and subjected to immunoprecipitation with antisera to TgGAP45 or GFP. Unlike full-length TgGAP50-YFP, TgGAP50^H9004(427-431)YFP does not associate with the other glideosome subunits (Fig. 6 B), suggesting that the carboxy-terminal cytoplasmic domain is indeed essential for that process.

Discussion

The gliding movement of T. gondii and other apicomplexan parasites appears to be mediated by the interaction of a myosin motor with plasma membrane–associated actin filaments (Jewett and Sibley, 2003). Here, we report on the discovery of two novel proteins, TgGAP45 and TgGAP50, found associated with the major myosin heavy and light chains of Toxoplasma, TgMyoA and TgMLC1. The term glideosome has been used previously to describe the complex of TgMyoA and TgMLC1 (Opitz and Soldati, 2002); we propose here to update its definition to also include TgGAP45 and TgGAP50. Preliminary analysis of the subunit stoichiometry in this complex suggests it may be composed of one copy of each protein. TgGAP45 is associated with the outer face of the inner membrane complex in mature parasites. TgGAP50, on the other hand, is an integral membrane glycoprotein found in the inner membrane complex of both mature and immature parasites. This protein also is the first membrane protein identified in that structure in apicomplexan parasites.

The role of TgGAP45 is not clear at this time, although the failure to obtain viable parasites with a disrupted TgGAP45 gene using various strategies (Kim et al., 1993;
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Pulse-chase analysis and subcellular fraction revealed that members of the glideosome complex (see below). Proteins probably reflects its role in the interaction with other short carboxy-terminal cytoplasmic domains of the GAP50 of the inner membrane complex. The near identity of the prising and may indicate that this domain interacts in a spe-
mains of apicomplexan GAP50 proteins and the purple acid phosphatase activity in preparations of recombinant phosphatases. This notion is supported by our inability to de-
phatases, a family of secreted enzymes found in animals and plants (Oddie et al., 2000; Schenk et al., 2000). However, the amino acid residues critical to phosphatase activity
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The assembly of the glideosome in two stages may serve several purposes for Toxoplasma. Most likely, it limits assembly of the fully active glideosome to the location where it is needed, the outer face of the inner membrane complex of the mature parasite. However, it is also possible that the proto-glideosome association with the cytoplasmic tail of TgGAP50 is reversible, and thus offers a mechanism for control of parasite motility.

In our working model of Toxoplasma motility, glideosomes on the inner membrane complex interact with actin filaments that are associated with the adhesin MIC2 in the parasite plasma membrane. This arrangement would allow the glideosome to move with respect to the MIC2 anchored on a host cell or other substrate. However, in order for this to result in parasite motility it is critical that the glideosome is not only attached to the inner membrane complex, but also that it is immobilized within the plane of the membrane. This could be accomplished most easily by direct or indirect interaction of the glideosome with stable elements of the Toxoplasma cytoskeleton. Such structures have not been described on the side of the inner membrane complex that faces the plasma membrane. However, on the cytoplasmic side a dense fibrillar network forms a membrane skeleton along the length of the parasite (Mann and Beckers, 2001). In addition, 22 microtubules are present on that side, although these extend only partly along the length of the parasite (Nichols and Chiappino, 1987). As the inner membrane complex consists of two membranes, association of the glideosome with either cytoskeletal element would require
the presence of structures that span both membranes. Freeze fracture analysis of the inner membrane complex has in fact revealed candidates that could fulfill this function in the form of large numbers of intramembranous particles present in both membranes and distributed in a manner suggesting they are associated with both the membrane skeleton and the microtubules (Morrissette et al., 1997). Experiments are in progress addressing, in general, the immobilization of the glideosome and specifically its association with the intramembranous particles. The possibility that TgGAP50 may, in fact, interact directly or indirectly with the cytoskeleton is suggested by the observation that expression of a mutant TgGAP50 lacking its cytoplasmic domain is lethal for Toxoplasma. As expression of this protein does not disrupt assembly of the glideosome on endogenous TgGAP50, its lethality may be explained by a dominant disruption of the glideosome–cytoskeleton interaction.

The identification in T. gondii of TgGAP50 as the receptor for a complex of TgMyoA, TgMLC1, and TgGAP45 in the inner membrane complex provides the first evidence that the class XIV myosins in apicomplexan parasites associate with membranes through interaction with a transmembrane protein. Further analyses are needed to determine how the different subunits interact with each other and how complex assembly is controlled in the parasite. Moreover, identification of the manner in which TgGAP50, and therefore the glideosome as a whole, is immobilized in the plane of the inner membrane complex is of critical importance in understanding the mechanism of gliding motility in Toxoplasma and the other apicomplexan parasites.

Materials and methods

Culture of Toxoplasma and isolation of pellicle proteins

The RH (HXGPRT A strain of Toxoplasma was maintained as described previously (Mann et al., 2002). A pellicle fraction was prepared from 4 × 107 parasites by sonication and differential centrifugation as described previously (Mann and Beckers, 2001). This fraction was extracted in 1% TX100 in PBS and 1 mM PMSF for 10 min on ice. TX100-insoluble material was collected by centrifugation at 10,000 rpm for 10 min, washed once in 1% TX100 in PBS, and extracted for 10 min on ice in 1% DOC in PBS and PMSF. DOC-soluble material was separated from insoluble material by centrifugation as above. The TX100- and DOC-soluble fractions were used to immunize two mice by subcutaneous injection (Cocalico Biologicals, Inc.).

Library screening with anti-pellicle antisera

A T. gondii cDNA library in AZAPII (AIDS Research and Reference Program, McInnes Biosciences, Rockville, MD) was screened using antisera to pellicle proteins with the ProteinSpot Immunoscreening System (Promega). Positive clones were initially grouped based on restriction enzyme digestion patterns, and a single representative of each group was sequenced (Keck Biotechnology Resource Laboratory, Yale University, New Haven, CT).

Preparation of monospecific antisera to recombinant TgGAP54

For production of monospecific antisera to TgGAP45, a BamHI and XhoI fragment containing the carboxy-terminal 72 amino acids of the predicted ORF was inserted in-frame behind GST in pGEX2 (Amersham Biosciences). GST-GAP45 fusion protein was expressed in Escherichia coli JM109 and purified on glutathione-agarose (Sigma-Aldrich). The fusion protein was used to immunize mice and rabbits (Cocalico Biologicals, Inc.).

Metabolic labeling of T. gondii

Parasites were allowed to invade a monolayer of human foreskin fibroblast (HFF) cells. After 14–16 h, cells were incubated in methionine/cysteine-free medium (Mediatech) for 1 h before addition of 0.1 mCi [35S]-labeled methionine/cysteine (Amersham Biosciences) per ml medium. Parasites were harvested on ice after a 20–24-h incubation at 37°C.

To perform pulse-chase experiments, HFF cells grown in multiple flasks were infected and starved in methionine/cysteine-free medium as described above. To each flask, 0.25 mCi [35S]-labeled methionine/cysteine was added per ml of label medium. After 15 min at 37°C, one flask (pulse) was placed on ice. Unlabeled methionine and cysteine were added to the other flasks to final concentrations of 1 and 0.2 mM, respectively, and these were incubated at 37°C for the time indicated.

Immunoprecipitation of the glideosome

Parasites (5–10 × 10^7/ml) were lysed with 1% TX100 or 1% SDS in the presence of protease inhibitors (P8340; Sigma-Aldrich). For TX100 lysis, cells were resuspended in IP buffer (1% TX100, 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, and 5 mM EDTA). For SDS lysis, cells were resuspended in 100 μl 1% SDS in water plus protease inhibitors and heated for 5 min at 95°C, followed by ninefold dilution in IP buffer. SDS- or TX100-lysed cells were incubated on ice for 10 min, followed by centrifugation at 14,000 rpm for 10 min at 4°C. The supernatant was incubated at 4°C for 1 h with anti-GAP45 or anti-GFP antisera. Protein A-Sepharose (Zymed Laboratories) was added and the incubation was continued for 30 min at 4°C. Immune complexes were washed three times in IP buffer and separated by SDS-PAGE. Gels containing radiolabeled immune complexes were subjected to fluorography using Enhance (NEN Life Science Products), dried, and exposed to film.

Purification of immunoprecipitates was performed as suggested by the manufacturer (CalBiochem). The protein A beads were resuspended after the last wash in 25 μl 50 mM sodium phosphate, pH 7.5, 50 mM 2-mercaptoethanol, and 0.1% SDS, and were heated for 5 min at 95°C. After cooling and addition of TX100 to a final concentration of 1%, 5 μl PNGase-F was added and the mixture was incubated for 4 h at 37°C followed by SDS-PAGE and fluorography as described above.

Subcellular fractionation

Toxoplasma-infected HFF cells were pulse labeled with [35S]-labeled methionine/cysteine as described above and either placed on ice or chased for an additional 4 h at 37°C. Cells and parasites were collected as described above and frozen in liquid nitrogen. After thawing on ice, cell pellets were resuspended in 500 μl TBS with 5 mM EDTA and protease inhibitors, and were subjected to five 10-s bursts in a cell disrupter (Misonix). Half of each homogenate was placed on ice and the remainder was centrifuged for 30 min at 150,000 g in a rotor (SW25; Beckman Coulter). TX100 was added to the total homogenate sample and the soluble fraction to a final concentration of 1%. The particulate fraction was resuspended in 250 μl IP buffer. After addition of protease inhibitors and a 10-min incubation on ice, all fractions were clarified by centrifugation for 10 min at 14,000 g and analyzed by immunoprecipitation with TgGAP45 antisera as described above.

Mass spectroscopy, protein sequencing, and cloning of TgGAP50

The glideosome complex was isolated from 107 parasites using anti-GAP45 antibodies covalently attached to CnBr-activated Sepharose-4B (Amersham Biosciences). Bound proteins were eluted using 7 M urea, 2 M thiocyanate, and 0.1% SDS, and were separated by two-dimensional electrophoresis using a pH 4–7 IPG strip (Bio-Rad Laboratories) in the first dimension and SDS-PAGE in the second dimension. Proteins of interest were detected by staining with Coomassie brilliant blue, excised, and subjected to in-gel digestion using trypsin. Tryptic peptides were analyzed by lipid chromatography coupled with tandem mass spectroscopy at the Mass Spectrometry Shared Facility in the UAB Comprehensive Cancer Center (University of Alabama at Birmingham, Birmingham, Alabama).

For direct sequencing of the TgGAP50 amino terminus, the glideosome proteins were transferred, after two-dimensional electrophoresis, to PVDF membrane (Bio-Rad Laboratories). The TgGAP50 spot was excised after staining with Coomassie brilliant blue and subjected to direct amino acid sequencing at the Protein Chemistry Facility of the UAB Comprehensive Cancer Center.

Toxoplasma genomic DNA and mRNA sequences in ToxoDB encoding TgGAP50 were identified using the peptide sequences obtained by mass spectroscopy. The complete predicted ORF and stop codon of TgGAP50 was amplified from total Toxoplasma RNA by RT-PCR using Pu Turbo DNA polymerase (Stratagene) and the primers 5’-gggacccgtactagcggcgc-3’ and 5’-ggcctaggatatctagtgacg-3’. This PCR product was cloned into the TOPO® TA vector (Invitrogen) and sequenced in its entirety. Several cDNA clones
encoding the TgGAP50 ORF and flanking regions were isolated from a library of *Toxoplasma* cDNA in AZAPI (AIDS Research and Reference Agent Program) and sequenced in their entirety.

**Plasmid construction and expression in *Toxoplasma***

to generate the TgGAP50-YFP construct, the ORF was amplified using the primers 5'-GGTTCAGATGCGCGACCAGAGAG-3' and 5'-ggctatgtgctggtgttg-3'. The PCR product was digested with BglII and AvrII and inserted between the BglII and AvrII sites in pGEM-1-MC1-YFP/sagCAT (Hu et al., 2002). To generate the TgGAP50Δ(427-431)YFP construct, the ORF minus the six carboxy terminal residues was amplified using the same forward primer as above and the reverse primer 5'-gccctag-gACCGTCGACTGCGACCAGAGAG-3', digested with BglII and AvrII, and inserted between the BglII and AvrII sites in pGEM-1-MC1-YFP/sagCAT.

TgGAP50-YFP and TgGAP50Δ(427-431)YFP constructs were transfected into *Toxoplasma* by electroporation, and stable transfectants expressing TgGAP50-YFP were obtained by selection with chloramphenicol (Mann et al., 2002) and cloning by limiting dilution.

The HA-TgMyoA construct was amplified using primers 5'-cgggatc-cATGCGCCGACCAGATCACGT-3' and 5'-GTCTAGACCACCCGGCTGACAAAGTCG-3'. The resulting PCR product was digested with BamHI and cloned between the BamHI and filled-in NotI sites of pEXP-NermHA (Mann et al., 2002). The HA-TgMyoA construct was transfected into *Toxoplasma* expressing TgGAP50-YFP as described above, and parasites were analyzed by immunofluorescence 24 h after transfection.

**Immunofluorescence and immuno-EM**

Untreated extracellular parasites, parasites treated with *C. septicum* α-toxin, or infected cells were fixed and permeabilized for 5 min in cold (−20°C) methanol or for 15 min in 3% PFA and 0.25% glutaraldehyde in PBS, and for 5 min in 1% TX100 in PBS. Epifluorescence microscopy was performed as described previously (Mann et al., 2002) using a microscope (model BX60; Olympus), and images were collected with a SPOT camera (Diagnostic Instruments) and processed in Adobe Photoshop.

For the α-toxin experiments, parasites were treated with 20 nM toxin for 4 h and processed as described previously (Wicherski et al., 2002), except that fixed and permeabilized parasites were incubated first with primary antibody diluted at 1:2,000, followed by incubation with a 1:400 dilution of Alexa Fluor®-conjugated secondary antibody (Molecular Probes, Inc.).

For preembedding immuno-EM, isolated pellicles were incubated with primary antibody (anti-TgGAP5 at 1:500) diluted in 3% BSA in PBS for 3 h on ice. The sample was washed in PBS by sedimentation (10,000 rpm for 10 min) and resuspension. Incubation with 10-nm gold-conjugated goat anti–mouse IgG secondary antibody (anti-TgGAP5 at 1:500) diluted in 3% BSA in PBS for 3 h on ice. The sample was washed in PBS by sedimentation (10,000 rpm for 10 min) and resuspension. Incubation with 10-nm gold–conjugated goat anti–mouse IgG secondary antibody (Sigma-Aldrich) diluted 1:25 in 3% BSA in PBS was performed overnight at RT. The sample was washed in PBS as described above and fixed in 1% glutaraldehyde in PBS for 20 min. After another PBS wash, the sample was fixed in 1% osmium tetroxide in PBS for 20 min, washed in PBS, dehydrated in increasing concentrations of ethanol, embedded in Spurr’s resin (Electron Microscopy Sciences), and polymerized overnight at 70°C. Thin sections were prepared, stained with uranyl acetate and lead citrate, and observed with an electron microscope (model H7100; Hitachi).

**SDS-PAGE and immunoblotting**

Protein preparations were separated by SDS-PAGE on 12% polyacrylamide mini gels (Bio-Rad Laboratories). To obtain optimal resolution between TgGAP45 and TgGAP50, electrophoresis was continued for 15 min at 150 V after the bromophenol blue front had run off the gel. Transfer to nitrocellulose and immuno blot analysis was performed as described previously (Mann and Beckers, 2001).

**Analysis of TgGAP45 and TgGAP50 sequences**

DNA and predicted protein sequences were analyzed using BLAST and FASTA. Multiple sequence alignments were performed using ClustalW. Putative transmembrane domains were identified using TMHMM (http:// www.cbs.dtu.dk/services/TMHMM-2.0/) and HMMTOP (http://www.enzim. hu/hmmtop/). Potential N-linked glycosylation sites were identified using NetNGlyc (http://www.cbs.dtu.dk/services/NetNGlyc/). Preliminary genomic and cDNA sequence data were accessed via http://ToxoDB.org. Sequence data for the *P. falciparum* genome were accessed via http://plasmodb.org/bioz. We are grateful to Ms. Lindsay Peck for her expert technical assistance. We especially thank Dr. Dominique Soldati (Imperial College London, London, UK) for helpful discussions and for providing us with antisera to the carboxy-terminal tail of TgMyoA and TgMLC1 and a plasmid clone of TgMyoA. We thank Dr. M. Heintzelman (Dartmouth Medical College, Hanover, NH) for antiserum to TgMyoA and Dr. Rodney Tweten (University of Oklahoma Health Sciences Center, Oklahoma City, OK) for *C. septicum* α-toxin. We thank Leigh Millic for her excellent assistance with EM.

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