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JACOT, Damien, et al.

Abstract

Members of the phylum Apicomplexa possess a highly conserved molecular motor complex anchored in the parasite pellicle and associated with gliding motility, invasion and egress from infected cells. This machinery, called the glideosome, is structured around the acylated gliding-associated protein GAP45 that recruits the motor complex composed of myosin A and two associated myosin light chains (TgMLC1 and TgELC1). This motor is presumably firmly anchored to the inner membrane complex underneath the plasma membrane via an interaction with two integral membrane proteins, GAP50 and GAP40. To determine if the previously mapped phosphorylation sites on TgGAP45 and TgMLC1 have a direct significance for glideosome assembly and function, a series of phospho-mimetic and phospho-null mutants were generated. Neither the overexpression nor the allelic replacement of TgMLC1 with phospho-mutants impacted on glideosome assembly and parasite motility. TgGAP45 phosphorylation mutants were functionally investigated using a complementation strategy in a TgGAP45 inducible knockout background. The loss of interaction with TgGAP50 by one [...]
Assessment of phosphorylation in *Toxoplasma* glideosome assembly and function

Damien Jacot,1 Karine Frénal,1 Jean-Baptiste Marq,1 Pushkar Sharma2 and Dominique Soldati-Favre1*

1Department of Microbiology & Molecular Medicine, CMU/University of Geneva, Rue Michel-Servet 1, CH-1211 Geneva 4, Switzerland.
2Eukaryotic Gene Expression Laboratory, National Institute of Immunology, New Delhi 110067, India.

**Summary**

Members of the phylum Apicomplexa possess a highly conserved molecular motor complex anchored in the parasite pellicle and associated with gliding motility, invasion and egress from infected cells. This machinery, called the glideosome, is structured around the acylated gliding-associated protein GAP45 that recruits the motor complex composed of myosin A and two associated myosin light chains (TgMLC1 and TgELC1). This motor is presumably firmly anchored to the inner membrane complex underneath the plasma membrane via an interaction with two integral membrane proteins, GAP50 and GAP40. To determine if the previously mapped phosphorylation sites on TgGAP45 and TgMLC1 have a direct significance for glideosome assembly and function, a series of phospho-mimetic and phospho-null mutants were generated. Neither the over-expression nor the allelic replacement of TgMLC1 with phospho-mutants impacted on glideosome assembly and parasite motility. TgGAP45 phosphorylation mutants were functionally investigated using a complementation strategy in a TgGAP45 inducible knockout background. The loss of interaction with TgGAP50 by one previously reported GAP45-mutant appeared to depend only on the presence of a remaining competing wild type copy of TgGAP45. Accordingly, this mutant displayed no phenotype in complementation experiments. Unexpectedly, GAP45 lacking the region encompassing the cluster of twelve phosphorylation sites did not impact on its dual function in motor recruitment and pellicle integrity. Despite the extensive phosphorylation of TgMLC1 and TgGAP45, this post-translational modification does not appear to be critical for the assembly and function of the glideosome.

**Introduction**

Members of the Apicomplexa are responsible for a wide range of diseases in both humans and animals. This large group of obligate intracellular parasites includes *Toxoplasma gondii* which affects humans and warm-blooded animals and causes toxoplasmosis (Montoya and Liesenfeld, 2004), and *Plasmodium* species, the causative agents of malaria that lead to hundreds of thousands of deaths per year (Murray et al., 2012). Most invading stages of the Apicomplexans are capable of migrating across biological barriers and actively penetrate and egress from host cells by a mechanism that relies on substrate-dependent motion. This gliding motility requires actin polymerization (Dobrowolski and Sibley, 1996; Wetzel et al., 2003) and is powered by a large molecular machine called the glideosome. This complex is highly and exclusively conserved across the phylum and positioned at the parasite pellicle, within the narrow space that separates the plasma membrane (PM) from the inner membrane complex (IMC) composed of flattened cisternae (Morrissette and Sibley, 2002).

In *T. gondii*, the glideosome has been extensively characterized and is composed of a myosin heavy chain (TgMyoA) (Herm-Gotz et al., 2002; Meissner et al., 2002), two associated light chains TgMLC1 (myosin light chain 1) (Herm-Gotz et al., 2002) and TgELC1 (essential light chain 1) (Nebi et al., 2011), the peripheral membrane protein TgGAP45 (gliding associated protein 45) (Gaskins et al., 2004) and two integral IMC proteins TgGAP50 and TgGAP40 (Gaskins et al., 2004; Frenal et al., 2010). The motor complex composed of TgMyoA/TgMLC1/TgELC1 is recruited via the C-terminal part of TgGAP45 at the pellicle where it interacts with TgGAP40 and/or TgGAP50 (Gaskins et al., 2004; Frenal et al., 2010). In addition, TgGAP45 is anchored to the PM via N-terminal acylations and constitutes a fluid bridge between the two membranes of the pellicle to maintain their cohesion (Rees-Channer et al., 2006; Frenal et al., 2010). During
motility and invasion, microneme contents are discharged apically in response to a rise in intracellular calcium and the adhesins released onto the parasite surface form complexes with the extracellular matrix or the host cell receptors (Carruthers and Boothroyd, 2007). Presumably, the glideosome generates a forward motion by translocation of the adhesin-receptor complexes to the posterior pole of the parasite (Opitz and Soldati, 2002; Keeley and Soldati, 2004). TgGAP45 and TgMyoA have been reported to be central for all three experimentally assessable functions of the glideosome: gliding, invasion and egress (Meissner et al., 2002; Frenal et al., 2010; Andenmatten et al., 2013).

The glideosome is predicted to be tightly controlled, both temporally and spatially and several studies point towards an important role for phosphorylation in its assembly and, or function. Phosphorylation on residues S163 and S167 of TgGAP45 was reported to play a role in the final assembly of the glideosome, as transient expression of a phosphomimetic mutant failed to associate with TgGAP50. Specifically, this indicated that dephosphorylation is required for the final anchoring step at the IMC and this loss of interaction was speculated to be critical (Gilk et al., 2009). P. falciparum late schizont stage parasites treated with a broad-range serine/threonine kinase inhibitor (staurosporine) showed a significant reduction in phosphorylated forms of PIGAP45 resulting in an important decrease in interaction with the actomyosin motor (Jones et al., 2009). Furthermore, PIGAP45 was reported to interact with and to be phosphorylated in vitro by the protein kinase B (PKB) (Vaid et al., 2008; Thomas et al., 2012). Additionally, in vitro phosphorylation assays also identified PICDPK1 (calcium-dependent protein kinase 1), which is localized to the PM of P. falciparum merozoites and is the potential kinase phosphorylating PIGAP45 and PIMTIP (MyoA tail-interacting protein), the homologue of TgMLC1 in the malaria parasite (Green et al., 2008; Kato et al., 2008; Winter et al., 2009; Tewari et al., 2010; Azevedo et al., 2013). Phosphorylation of PIGAP45 at S103 and S149, two of the PICDPK1 target sites, was also observed in the parasite (Ridzuan et al., 2012; Thomas et al., 2012). Finally, PKA also phosphorylates PIGAP45 and PIMyosin in vitro (Lasonder et al., 2012).

Microneme secretion is triggered by changes in intracellular calcium levels (Carruthers and Sibley, 1999; Lovett and Sibley, 2003; Blikker et al., 2009; Lourido et al., 2012). Invasion and egress from infected cells are tightly connected to microneme secretion and demonstrated to depend on the action of TgCDPK1 and TgCDPK3 in T. gondii (Lourido et al., 2010; 2012; Garrison et al., 2012; McCoy et al., 2012). Using comparative phosphoproteome analyses in T. gondii, several residues in TgGAP45, TgMLC1 and TgMyoA, for which phosphorylation was selectively increased upon calcium stimulation, were identified (Nebl et al., 2011). Finally, global phosphoproteome analyses of T. gondii and P. falciparum (Solyakov et al., 2011; Tyler et al., 2011; Lasonder et al., 2012; Pease et al., 2013) revealed extensive phosphorylation of various components of the glideosome with up to 35 and 25 sites respectively (Jacot and Soldati-Favre, 2012). While the identity of phosphorylation sites on these proteins is clear, the functional implications of phosphorylation remain unknown.

Here we functionally assessed the importance of recently mapped phosphorylation sites on TgMLC1 and TgGAP45 via the generation of parasite lines expressing phosphorylation mutants. Results indicated that the phosphorylation of most sites on TgGAP45 and TgMLC1 is dispensable for glideosome assembly and parasite motility. The GAP45-S163/7E mutant previously reported to lose interaction with TgGAP50 was confirmed only in the presence of the wild type (wt) inducible copy of TgGAP45, which likely competes for association with GAP50. In contrast, GAP45-S163/7E interaction with GAP50 was restored in the absence of GAP45 and allelic replacement of TgGAP45 with this mutant had no impact on parasite fitness.

Results

Phosphorylation of TgMLC1

To investigate the impact of TgMLC1 phosphorylation on the glideosome (Figs 1A and S1A), a first strategy was based on overexpression of TgMLC1 mutated second copies. A destabilization domain (DD) and a Myc tag were fused to the N-terminus of TgMLC1 for rapid and tight control of TgMLC1 expression upon addition of shield (Shld-1) (Herm-Gotz et al., 2007). Residues S55, S98 and T132 have previously been reported to be phosphorylated with the latter two showing a significant increase in phosphorylation when parasites were stimulated with calcium ionophore (Nebl et al., 2011). We first generated mutants at each individual site where the amino acid residue was mutated to either an alanine (phospho-null) or to a glutamic acid (phospho-mimetic). No phenotype was observed by plaque assay and no alteration of the glideosome composition was monitored by co-immunoprecipitation (co-IP) upon conditional stabilization of the mutants (data not shown). We then generated triple mutants where phosphorylation sites S55, S98 and T132 were mutated to alanine (DDMycMLC1-AAA) or to glutamic acid (DDMycMLC1-EEE). The construct DDMycMLC1-wt was included as a control for comparison. Expression of all the mutants was undetectable unless Shld-1 was added to the culture. Upon stabilization of the DDMycMLC1 second copies, the level of endogenous MLC1 was dramatically reduced (Fig. 1B). It was
Fig. 1. Phosphorylation of TgMLC1.
A. Schematic representation of TgMLC1 phosphorylation sites (Treeck et al., 2011). The sites investigated in this study are highlighted in red and the calcium-dependent phosphorylation sites are indicated by a star. Nt, N-terminus; DR, disordered region (Nebl et al., 2011).
B. Western blot analyses using anti-MLC1 antibodies of DDMycMLC1 and RH strains treated for 24 h ± Shld-1. Expression of DDMycMLC1 (38 kDa) was only observed in the presence of Shld-1 and resulted in destabilization of the endogenous TgMLC1 (24 kDa). Detection of T. gondii actin (TgACT1) was used as a loading control.
C. Metabolic labelling with 35S-methionine/cysteine followed by co-IP using anti-Myc antibodies revealed no alteration in the glideosome composition.
D. Western blots performed with anti-MLC1 antibodies on the different strains of TgMLC1 generated by knock-in. A slight molecular-weight shift was observed compared with the wild type (ΔKu80) due to the presence of the Myc tag. TgACT1 detection is included as a loading control.
E. Immunofluorescence analyses (anti-Myc) revealed no alteration of the localization at the pellicle. Scale bar 2 μm.
F. Metabolic labelling with 35S-methionine/cysteine followed by co-IP using anti-Myc antibodies showed no alteration of the glideosome composition. No reduction of co-IP MyoA was observed for MycMLC1-S113E.
G–I. No phenotype was observed in calcium ionophore-induced egress, plaque assay, or competition experiments for all the phosphorylation sites investigated by knock-in compared with the parental strain (ΔKu80).
previously reported that the level of MLCs are regulated by their capacity to associate with their corresponding myosin heavy chain (Polonais et al., 2011). In consequence, the drop in the endogenous level of TgMLC1 suggested that the mutated copies were assembled with TgMyoA. All the mutants localized to the pellicle and displayed no defect by plaque assay when compared with DDMycMLC1-wt (Fig. S1B and C). The composition of the glideosome in the different parasite lines was assessed by co-IP with anti-Myc antibodies following metabolic labelling with 35S-methionine and cysteine (Fig. 1C). The band migrating at 130 kDa corresponds to myosin C (TgMyoC), which associates with TgMLC1 and composes the MyoC-glideosome (K. Frénal, J.B. Marq, V. Polonais and D. Soldati-Favre, unpublished). Only the mutant DDMycMLC1-EEE displayed a reduction in its ability to bind TgMyoC while the other mutants were not affected when compared with DDMycMLC1-wt.

Since residual expression of endogenous MLC1 was still detectable in transgenic parasites upon stabilization of MLC1 mutants, we opted for an allelic replacement approach to obtain a definitive answer. The composition of the TgMLC1 locus includes an unusually long 5′ UTR that was used to introduce mutated forms of TgMLC1 downstream of the promoter by single homologous recombination (knock-in). This resulted in displacement of the endogenous open reading frame (Fig. S1D). The same AAA, EEE and wt mutants tagged in their N-terminus with a Myc tag were generated (MycMLC1-AAA, -EEE, -wt). In addition, we have generated the single mutants MycMLC1-S113A and MycMLC1-S113E, based on the report that the interaction of PfMTIP and PfMyoA might be specifically modulated by phosphorylation at residues S107 and/or S108 of PfMTIP. Specifically, phosphomimetic mutants at both residues were reported in vitro to weaken the tight clamp around PfMyoA (Douse et al., 2012). These two residues align with residues S113 and Y114 of TgMLC1 (Fig. S1A) that have also been identified in vivo to be phosphorylated (Tyler et al., 2011). As a negative control, we replaced MycMLC1-wt in the transfection plasmid with DsRed and despite several transfections; we failed to obtain any positive pools proving that the TgMLC1 locus cannot be disrupted unless an additional copy of TgMLC1 is integrated.

All MycMLC1 constructs were integrated accurately at the TgMLC1 genomic locus as demonstrated by genomic PCR analysis (Fig. S1E). Western blot analyses using anti-MLC1 antibodies confirmed the gene displacement diagnosed by the slight shift in molecular weight accounted for by the Myc tag compared with endogenous TgMLC1 (Fig. 1D). The MycMLC1 mutants showed the same localization as MycMLC1-wt (Fig. 1E). In addition, the single mutations S113A and S113E did not affect the association of TgMLC1 with TgMyoA or with the rest of the complex (Fig. 1F). Transgenic parasites displayed no impairment in calcium ionophore-induced egress (Fig. 1G), formed plaques of similar sizes compared with the parental strain (Fig. 1H) and showed no defect in intracellular growth (Fig. S1F). To assess the contribution of more subtle changes that could not be detected in the previous assays, we performed competition experiments with all MycMLC1 strains. The different transgenic lines were co-cultured with a parental strain over an extended period of time but significant alteration in the ratio of parental versus transgenic strains was not observed (Fig. 1I).

To determine if mutation of the three phosphorylation sites S55, S98 and T132 resulted in compensatory phosphorylation elsewhere in the molecule, MycMLC1-AAA and MycMLC1 were co-immunoprecipitated using anti-Myc antibodies and analysed by mass spectrometry after phosphopeptides enrichment. Two of the nine previously reported phosphorylation sites in MLC1 were identified in the MycMLC1-wt sample (T98 and S124) as well as two additional ones at residues S92 and S97 (Fig. S3A and B). In MycMLC1-AAA analyses, the two sites S97 and S124 were also found phosphorylated as well the serine S57 close to the mutated S55 (Fig. S3C).

Taken together, these results indicate that the phosphorylation sites investigated here do not critically impact TgMLC1 function and its assembly within the glideosome although cryptic phosphorylation on neighbouring site might have a functional compensatory effect.

### Phosphorylation of TgGAP45

The importance of phosphorylation for TgGAP45 function was investigated by complementation of the inducible knockout strain of TgGAP45 (ΔGAP45e/MycGAP45i) (Frenal et al., 2010) with phosphorylation mutants. These mutants were Ty-tagged between the conserved N-terminus and the coiled-coil region to distinguish them from the inducible copy MycGAP45i (Figs 2A and S2, TyGAP45c-wt). Stable clones of four double and four single mutants were generated with either an alanine or a glutamic acid substitution (TyGAP45c-S163/7A, S163/7E, S184/5A, S184/5E, T189A, T189E, S239A and S239E). All residues were reported to be phosphorylated in vivo (Tyler et al., 2011) and residues S184/5 and T189 showed increased phosphorylation upon stimulation of calcium signalling (Nebl et al., 2011). Additionally, a strain complemented with a wild type version of TgGAP45 was generated (TyGAP45c-wt). The complementing copies were expressed at comparable levels in ΔGAP45e/MycGAP45i and the tight regulation of MycGAP45i by ATc was preserved (Fig. 2B). The mutants and the wt showed proper localization to the pellicle either in the presence or absence of ATc (Fig. 2C). No phenotype was observed by plaque assay upon addition of ATc for the complemented
Fig. 2. Complementation of the inducible knockout strain of TgGAP45 with phosphorylation mutants.

A. Schematic representation of TgGAP45 post-translational modifications (Frenal et al., 2010; Nebi et al., 2011; Treeck et al., 2011). The phosphorylation sites investigated in this study are highlighted in red, sites not investigated are highlighted in black and the stars indicate the calcium-dependent phosphorylation sites. The putative myristoylation (G) and palmitoylation (C) sites are highlighted in blue, Nt, conserved N-terminus; Ct, conserved C-terminus; DR, disordered region. The Ty tag was not included in the amino acid numbering.

B. All complemented TyGAP45 strains are expressed at similar levels by western blot analyses using anti-Ty antibodies. Regulation of MycGAP45i was assessed with anti-Myc antibodies after 48 h ± ATc. Detection of T. gondii catalase was used as a loading control.

C. Immunofluorescence analyses (anti-Ty) revealed no alteration in the localization at the pellicle. Scale bar 2 μm.

D. The complemented strains formed plaques of similar sizes ± ATc. In contrast, no plaque was formed in ΔGAP45e/MycGAP45i in the presence of ATc.

E. Only mutant TyGAP45c-T189A showed a 30% reduction in calcium ionophore-induced egress after 48 h of ATc treatment. Parasites lacking TgGAP45 (ΔGAP45e/MycGAP45i + ATc) were severely affected in egress.

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strains while ΔGAP45e/MycGAP45i formed no visible plaques (Fig. 2D). Calcium ionophore-induced egress assays revealed that the mutant TyGAP45c-T189A displayed only a slight reduction in egress from infected host cells in the presence of ATc while the control ΔGAP45e/MycGAP45i was severely impaired (Fig. 2E).

Unexpectedly, the mutant TyGAP45c-S163/7E previously reported to lose interaction with TgGAP50 (Gilk et al., 2009) was readily generated and displayed no phenotype in both RH (data not shown) and in the ΔGAP45e/MycGAP45i background. Co-IPs using anti-Ty antibodies in the presence of ATc resulted in no alteration of glideosome composition, hence contrasting with the previously reported findings (Fig. 3A). In order to resolve this discrepancy, we repeated the experiment in the presence or absence of ATc. In the absence of ATc, TyGAP45c-S163/7E was partially impaired in its ability to interact with the IMC resident proteins TgGAP40 and TgGAP50.

Western blot analyses of the GAP45Ty-wt, -S163/7A and -S163/7E revealing a slight molecular weight shift compared with the endogenous TgGAP45 (ΔKu80) due to the presence of the Ty tag. Anti-GAP45 antibodies were used for probing. Detection of TgACT1 was used as a loading control.

Immunofluorescence analyses (anti-Ty) revealed no alteration of the localization at the pellicle. Scale bar 2 μm.

E–G. All GAP45Ty parasites formed plaques of similar sizes to ΔKu80, were not affected in egress from infected cells and displayed no significant phenotypes in competition experiments.

H. Co-IPs using anti-GAP45 antibodies with 35S-methionine/cysteine metabolically labelled TyGAP45c-wt, -S163/7E and -S163/7A revealed no alteration in glideosome composition.
fluctuate in the mutant S163/7A cultivated in the presence or absence of ATc. Accordingly, the level of co-immunoprecipitated TgGAP40 mirrored the level of TgGAP50 (Fig. 3B). To further consolidate this observation, we substituted TgGAP45 by single homologous recombination (knock-in) at the endogenous locus with either TyGAP45c-wt or the two corresponding phospho-null or phospho-mimetic-mutants, Ty-tagged just upstream of the predicted disorganized region (GAP45Ty-wt, -S163/7A, -S163/7E). This caused a small shift in the apparent molecular weight observed by western blot using anti-GAP45 antibodies (Fig. 3C). Both GAP45Ty mutants and TyGAP45c-wt localized to the pellicle (Fig. 3D) and no phenotype was observed by plaque assay or by calcium-ionophore induced egress (Fig. 3E and F). In addition, both phospho-mutants were not overgrown by the parental strain in competition experiments although TyGAP45c-wt parasites appeared to grow very modestly better (Fig. 3G). Concordantly, the composition of the glideosome and specifically the association with TgGAP50 and TgGAP40 were not affected in co-IPs using anti-GAP45 antibodies (Fig. 3H).

In the calcium ionophore-induced egress assays only the TyGAP45c-T189A expressing mutant displayed a slight reduction in egress from infected host cells in the presence of ATc (Fig. 2E). The assembly of the glideosome was further examined for this mutant and it was found that all the components were assembled properly (Fig. 4A). Of relevance, the majority of TyGAP45c-T189A expressing clones showed lower levels of expression compared with TyGAP45c-T189E that resulted in a strong phenotype both by plaque assay and induced egress. We did however succeed in obtaining clones such as the one reported here, which exhibited a level of expression comparable to the other mutants. To complement the characterization of these mutants, we performed pulse-chase experiments and found no difference in protein turnover suggesting that the absence of phosphorylation did not significantly affect the stability of the protein (data not shown). To rule out the possibility that the slight phenotype observed in the complementation experiment using the mutant TyGAP45c-T189A was due to an insufficient protein level, we substituted the TgGAP45 locus with the two mutants as previously described (GAP45Ty-T189A, -T189E). Western blot analyses using anti-GAP45 antibodies revealed the expected small shift in molecular weight...
compared with the parental strain (ΔKu80) (Fig. 4B). Genomic PCR analyses were performed and sequencing of the products confirmed the presence of the mutation at the modified TgGAP45 locus (data not shown). These GAP45Ty mutants displayed a correct localization to the pellicle (Fig. 4C) and no phenotype was observed by plaque and competition assays when compared with GAP45Ty-wt (Fig. 4D and E). In contrast to the complementation experiments using TyGAP45c-T189A in ΔGAP45e/MycGAP45i, no impairment in egress was observed with the GAP45Ty-T189A strain generated by knock-in (Fig. 4F).

Twelve of the 13 phosphorylation sites mapped on TgGAP45 are clustered in the predicted disordered region (DR) (Nebl et al., 2011) (Fig. 2A). Since individual point mutations had no impact on the assembly and function of the glideosome, we complemented ΔGAP45e/MycGAP45i with a TgGAP45 deletion mutant lacking this entire disordered region (TyGAP45c-ΔDR; Fig. S2). TyGAP45c-ΔDR localized to the pellicle (Fig. 5A), was wt + ΔDR

Egress assay TyGAP45c

Fig. 5. TgGAP45 disordered region deletion mutant.
A. TyGAP45c-ΔDR is correctly localized to the pellicle. Scale bar 2 μm.
B. TyGAP45c-ΔDR is found with the expected molecular weight shift compared with TyGAP45c-wt by western blot analyses using anti-Ty antibodies. Regulation of MycGAP45i was assessed with anti-Myc antibodies after 48 h in the presence of ATc. Detection of T. gondii catalase was used as a loading control.
C. No alteration of the glideosome composition was observed for TyGAP45c-ΔDR by 35S-methionine/cysteine metabolic labelling followed by co-IP using anti-Ty antibodies.
D. Western blot analyses of the same samples using anti-GAP40 and anti-GAP50 antibodies revealed that both proteins co-immunoprecipitate equally in both TyGAP45c-wt and TyGAP45c-ΔDR.
E–G. In contrast to ΔGAP45e/MycGAP45i, TyGAP45c-ΔDR parasites are not affected in calcium ionophore-induced egress (E), plaque formation (F) or competition experiments (G) ± ATc.

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expressed at a similar level compared with the TyGAP45c-wt and migrated at the predicted weight on SDS-PAGE. Importantly, MycGAP45i remained tightly regulated by ATc in this mutant (Fig. 5B). The assembly of the glideosome was not affected in TyGAP45c-ΔDR based on co-IP experiments on metabolically labelled parasites (Fig. 5C). The presences of TgGAP50 and TgGAP40 in the co-IP were further confirmed by western blot analyses on the same samples using the corresponding antibodies (Fig. 5D). Phenotypic investigation of this mutant in the presence of ATc revealed no defect in plaque formation or calcium ionophore-induced egress, while the control ΔGAP45e/MycGAP45i failed to form visible plaques and to egress in the presence of ATc as previously reported (Fig. 5E and F). No significant change was observed in competition experiments for TyGAP45c-ΔDR and control TyGAP45c-wt parasites. To rule out the possibility that deletion of the DR results in compensatory phosphorylation elsewhere in the molecule, TyGAP45c-ΔDR and TyGAP45c-wt were co-immunoprecipitated using anti-Ty antibodies and analysed by mass spectrometry. Seven of the eleven previously reported phosphorylation sites in the DR as well as an additional phosphorylation site at residue T199 were identified in the TyGAP45c-wt samples (Fig. S4A and B). TyGAP45c-ΔDR analyses revealed no additional phosphorylation in the vicinity of the DR but one cryptic phosphorylation was found in the Ty tag (Fig. S4B). Importantly, in both analyses residue S239 was found to be phosphorylated.

Taken together, these data establish that phosphorylation of TgGAP45 is dispensable for glideosome assembly and function.

Discussion

Since the components of the glideosome are abundantly phosphorylated both in T. gondii and P. falciparum (Nebel et al., 2011; Solyakov et al., 2011; Tyler et al., 2011; Lasonder et al., 2012; Pease et al., 2013), it was anticipated that this post-translational modification could regulate the assembly and impact on the activity of the motor complex. Using two independent approaches that involved either overexpression or allelic replacement with phosphorylation mutants, we demonstrated that several of the reported sites on TgMLC1 were non-critical for the glideosome functions. In addition to TgMyoA, TgMLC1 also associates with TgMyoC (K. Frénal, J.B. Marq, V. Polonais and D. Soldati-Favre, unpublished). This myosin localizes to the anterior and posterior polar rings and overexpression of the alternatively spliced variant TgMyoB was associated with pellicle integrity during replication (Delbac et al., 2001). In consequence, we assessed the intracellular growth but recorded no defect with the MLC1-EEE mutant that exhibited a loss of interaction with TgMyoC. Importantly, competition experiments that are best suited to score minor loss of fitness also failed to reveal any defect.

Out of the four sites that were not investigated, two (S57 and S124) are poorly conserved in the TgMLC1 sequence across the phylum, and the other two (T182 and/or T183) are surrounded by negatively charged residues conserved in other Apicomplexa (Fig. S1A). In the case of PMTIP, the only residues found to be phosphorylated in vivo are the S107 and/or S108. In vitro studies suggested that these phosphorylation sites might modulate the interaction between PMTIP and PMMyoA (Douse et al., 2012). The corresponding serine residue in TgMLC1 (S113) was mutated however no change in the level of TgMyoA was observed in the co-IP. It is not possible to exclude that the two myosin light chains are differentially regulated by phosphorylation in the two organisms. Interestingly the phosho-peptides analysis performed in this study revealed two phosphorylation sites on TgMLC1 that were not described in the literature before S92 and S97. Since the analysis performed here cannot be claimed to be exhaustive, no strong conclusion can be drawn however, no cryptic phosphorylation site compensating for the mutations was identified in the MycMLC1-AAA. Given that not all the phosphorylation sites reported on MLC1 have been mutated here, we cannot exclude that this PTM impacts on the glideosome function.

In parallel, a similar mutagenesis approach was applied to TgGAP45 and the mutants were assessed for functional complementation in ΔGAP45e/MycGAP45i. Again the results revealed no significant impact of phosphorylation, except for the alanine substitution at residue T189 that resulted in a modest defect during calcium ionophore-induced egress. However, allelic replacement of the endogenous TgGAP45 locus with this mutant showed no phenotype suggesting either a compensatory effect during the selection process or an adapted level of expression that allowed full complementation. During the generation of the different strains, we observed that the level of expression of the complemented copies critically contributed to the phenotype. Indeed, and logically, mutant copies expressed at low level failed to complement. Since none of the point mutations made in TgGAP45 had a phenotypic consequence for glideosome functions, the cluster of phosphorylation located in the DR was deleted entirely and the truncated TgGAP45 was assessed for functional complementation. Despite this substantial deletion, TyGAP45c-ΔDR was capable of fully replacing MycGAP45i. Mass spectrometry analyses revealed no additional compensatory
phosphorylation except in the N-terminal Ty tag. Given that the Ty tag is not part of the endogenous protein this phosphorylation is incidental and unlikely to have significant functional impact.

The interaction of the mutant S163/7 with IMC resident proteins TgGAP50 and TgGAP40 appeared to depend to some extent on the presence or absence of a wt copy of TgGAP45. It is possible that phosphorylation at residues S163/7 prevents a premature insertion of the glideosome within the IMC during daughter cell formation (Gilk et al., 2009). The pre-complex formed by TgGAP45/TgMyoATgMLC1 is first assembled in the cytoplasm and only recruited to the IMC in mature parasites (Gaskins et al., 2009). The wt form of TgGAP45 apparently has a selective advantage to be inserted with the appropriate timing and hence competes with the mutant S163/7E. Additionally, the essential N-terminal acylation of TgGAP45 was also reported to prevent association of the pre-complex with the nascent IMC (Frenal et al., 2010). These findings suggest that different post-translational modifications contribute to the insertion of the motor at the pellicle in a temporally controlled manner; however, phosphorylation of the known modified sites is not absolutely required for this process. Concordantly, mutant S163/7A was not prematurely inserted in the IMC of nascent daughter cells.

In comparison to TgGAP45, PIGAP45 contains several additional phosphorylation sites in the coiled-coil region that were not reported to be modified on the Toxoplasma protein (Jacot and Soldati-Favre, 2012). Since PIGAP45 can functionally substitute for TgGAP45 (Frenal et al., 2010), these sites are either phosphorylated by T. gondii kinase(s) or not crucial. Supporting the second hypothesis, none of the episomally expressed phospho-mutants of PIGAP45 that have been analysed so far, revealed any significant impact on the assembly of the glideosome or PIGAP45 localization in blood stage malaria parasites (Ridzuan et al., 2012; Thomas et al., 2012).

How the glideosome is timely harnesssed for concerted action during motility remains to be further investigated, however phosphorylation of the glideosome itself does not appear to play a central role. MyoA and GAP40 also contain phosphorylation sites that remain to be investigated and a cumulative effect of multiple phosphorylation events on one or more components of the glideosome cannot be excluded (at least 35 phosphorylation sites are associated with the glideosome) (Jacot and Soldati-Favre, 2012). This postulate is experimentally very complicated to address due to the vast number of possible combinations. It could also be conceivable that there is a large amount of noise in the phosphorylation process where numerous sites may have negligible consequences. Consistent with the results presented in this study, all the recent kinases implicated in egress affect microneme secretion (Lourido et al., 2010; 2012; Garrison et al., 2012; McCoy et al., 2012; Collins et al., 2013) and none so far have been attributed selectively to the glideosome function. Given the essential contribution of microneme proteins in egress, it was not possible to measure the impact on glideosome activation in situations where microneme secretion is simultaneously impaired.

Experimental procedures

T. gondii culture

T. gondii tachyzoites were grown in confluent human foreskin fibroblasts (HFFs) maintained in Dulbecco’s modified Eagle’s medium (DMEM; GIBCO, Invitrogen) supplemented with 10% fetal calf serum, 2 mM glutamine and 25 μg ml⁻¹ gentamicin. Conditional expression was regulated with 0.5 μM Shd-1 for DD-fusion stabilization (Herm-Gotz et al., 2007) or with 1 μg ml⁻¹ antibiotic-tetracycline (ATc) for the Tet-inducible system (Meissner et al., 2001).

Parasite transfection and selection of stable transgenic parasites

Parasite transfections were performed as previously reported (Soldati and Boothroyd, 1993). Selection of transgenic parasites was performed with both mycophenolic acid and xanthine for HXGPRT selection (Donald et al., 1996) and with phleomycin for bleomycin (BLE) selection (Messina et al., 1995). All strains were cloned by limiting dilution. The RH strain (Donald et al., 1996) was transfected with 60 μg of the following plasmids: pT8DDmyc-MLC1-mutants-HXGPRT (linearized with NotI). ΔKu80 (Huynh and Carruthers, 2009) was transfected with 40 μg of the following plasmids: MycMLC1cDNA-mutants-HXGPRT (linearized with EcoRV), GAP45Ty-HXGPRT (T189A, T189E, S163/7A, S163/7E and wt, linearized with BglII). ΔGAP45e/MycGAP45i (Frenal et al., 2010) was transfected with 60 μg of pT8-TgGAP45c-mutant-BLE and pT8-TgGAP45c-ΔDR-BLE (linearized with ScaI), pT8, tubulin promoter.

Cloning of DNA constructs

Genomic DNA was isolated using the Wizard SV genomic DNA purification system (Promega). RNA was isolated using Trizol (Invitrogen) extraction. cDNA was generated by RT-PCR performed with Superscript II reverse transcriptase (Invitrogen) according to the manufacturer’s protocol. Site directed mutagenesis was performed with the QuickChange II Site-Directed Mutagenesis Kit (200524, Agilent technologies) following the manufacturer’s protocol. Primers used are presented in Table S1. Mutagenesis was performed in pGEM®-T Easy backbones and subcloned into the final vectors. All mutants were cloned following the same procedure. Mutants S239A and S239E were generated using reverse primers 4732 and 4733 respectively and forward primer 2258 with pT8-TgGAP45c-wt-BLE as template.

pT8DDmyc-TgMLC1-HXGPRT: TgMLC1 was amplified from RH cDNA using primers 2993-2995, digested with Nael and Pacl and cloned into pT8DDmycFH2-HX (Daher et al., 2010).

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MycTgMLC1cDNA-HXGPRT: the homology fragment for recombination was amplified with primers 3458–3459, digested with KpnI and SfiI and cloned into pT8DDmyc-TgMLC1-HXGPRT. pT8-TyGAP45c-BLE: the Ty-tag was introduced by PCR by amplifying pT8 and NiGAP45 with primers T7–2786 with pT8MycGAP45 as template (Frenal et al., 2010) and by subcloning this insert between KpnI and NsiI sites in the same vector. The BLE cassette was then amplified with primers 2787/2642, digested with SacII and cloned. pT8-TyGAP45c-ΔDR-BLE: NiGAP45Ty-CC and GAP45CT were amplified with primers 2258–3961 and 3960–2383 respectively with TyGAP45 as template. GAP45CT was digested with PstI and PacI and cloned into the same vector. NiGAP45Ty-CC was cloned into the PstI site of the resulting vector. GAP45Ty-HXGPRT: NiGAP45 was amplified from gDNA of ΔKu80 using primers 3993–3994 and primers 3995–3996. These two PCR products were first ligated to introduce a unique BglII site (between 3994–3995) and a Ty tag after 3996. This fragment was digested with KpnI and EcoRV and cloned into MycTgMLC1cDNA-HXGPRT. GAP45CT was amplified using primers 3997–2383, digested with EcoRV and PacI, and cloned into the same vector. NiGAP45Ty-CC was cloned into the PstI site of the resulting vector. GAP45Ty-HXGPRT: NiGAP45 was amplified from gDNA of ΔKu80 using primers 3993–3994 and primers 3995–3996. These two PCR products were first ligated to introduce a unique BglII site (between 3994–3995) and a Ty tag after 3996. This fragment was digested with KpnI and EcoRV and cloned into MycTgMLC1cDNA-HXGPRT. GAP45CT was amplified using primers 3997–2383, digested with EcoRV and PacI, and cloned into the same vector. The corresponding pT8-TyGAP45c-BLE plasmids were used as templates for these PCRs.

All the primers used in this study are listed in Table S1.

Plaque assay

A confluent monolayer of HFFs was infected with freshly egressed parasites and treated ± ATc or ± Shld-1 for 7 days before the cells were fixed with paraformaldehyde/glutaraldehyde (PFA/GA). The host cell layer was then stained for 10 min at RT with Giemsa or crystal violet (Sigma-Aldrich). Data are representative of three independent biological experiments.

Egress assay

Freshly egressed parasites were allowed to invade HFF monolayers on coverslips and incubated for 2 h before washing of the host cells. A pre-treatment of 12 h (± ATc) was performed for parasites in the ΔGAP45e/MycGAP45i background. After 30 h of growth, parasites were treated for 7 min at 37°C with DMEM containing 0.06% DMSO or 3 μM calcium ionophore A23187 from Streptomyces chartreusensis (Calbiochem). Coverslips were fixed with PFA/GA and stained with anti-GAP45 antibodies. Two hundred vacuoles were counted for each strain and the number of egressed vacuoles is represented. Data are mean values ± SD from three independent biological experiments.

Intracellular growth assay

MycMLC1 parasites were grown for 30 h prior to fixation with PFA/GA. Immunofluorescence analyses using anti-GAP45 antibodies were performed and the number of parasites per vacuole was scored. Two hundred vacuoles were counted for each condition. Data are mean values ± SD from three independent biological experiments.

Competition assays

The different mutants were mixed with a parental strain at day 0 and the ratio of transgenic parasites was assessed by immuno-fluorescence analyses using anti-Ty or anti-Myc antibodies every 2 or 4 days. ΔKu80 was used as the parental strain for the GAP45 Ty and MycMLC1 strains and RH was used for the TyGAP45c and ΔGAP45e/MycGAP45i strains. Regulation of MycGAP45i by ATc was assessed at days 6 and 16.

Antibodies

The antibodies used in this study are as follows: rabbit polyclonal; α-CAT (Ding et al., 2000), α-GAP45 (Plattner et al., 2008), α-MLC1 (Herm-Gotz et al., 2002), α-GAP40, α-GAP50 (Jones et al., 2009); mouse monoclonal, α-ACT (Herm-Gotz et al., 2002), α-Ty (BB2), α-Myc (9E10). For Western blot analyses, secondary peroxidase-conjugated goat α-rabbit/mouse antibodies (Molecular Probes) were used as well as Alexa Fluor 694-conjugated goat α-mouse/rabbit antibodies. For immunofluorescence analyses, the secondary antibodies Alexa Fluor 488 and Alexa Fluor 594-conjugated goat α-mouse/rabbit antibodies (Molecular Probes) were used.

To generate the anti-GAP40 antibodies, two peptides (H2N-GGENYADVCDEAHSS-COOH and AcNH-KNIVSNRLIRTTGQAP-COHNH2) were synthesized and used to immunize two rabbits according to the Eurogentec standard protocol.

Metabolic labelling and co-immunoprecipitation

Freshly egressed parasites were harvested and incubated for 15 min in methionine/cysteine-free DMEM (Sigma). Parasites were harvested and re-suspended in DMEM containing 10 μCi [35S]-labelled methionine/cysteine (Hartmann analytic GmbH) per ml for 4 h. Parasites were lysed in Cold buffer [1% (v/v) Triton X-100, 50 mM Tris-HCl, pH 8, 150 mM NaCl] in the presence of a protease inhibitor cocktail (Roche). Cells were incubated for 10 min on ice, frozen and thawed, sonicated and centrifuged at 14 000 rpm for 30 min. Supernatants were incubated with corresponding antibodies as previously described (Gaskins et al., 2004). All samples were boiled prior to SDS-PAGE. DDMycMLC1 were treated with Shld-1 24 h prior to the co-IP. TyGAP45c was treated ± ATc for 48 h.

Immunofluorescence assay and confocal microscopy

HFFs seeded on coverslips in 24-well plates were inoculated with freshly released parasites. After 2–5 rounds of parasite replication, cells were fixed with 4% paraformaldehyde (PFA) or 4% PFA/0.05% glutaraldehyde (PFA/GA) in PBS, neutralized 3–5 min in 0.1 M glycine/PBS, and processed as previously described (Plattner et al., 2008). Confocal images were generated with a Zeiss laser scanning confocal microscope (LSM700, objective apochromat 63×/1.4 oil) at the bioimaging facility of the Faculty of Medicine, University of Geneva.

Western blot analyses

Parasites were lysed in RIPA buffer (150 mM NaCl, 1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris pH 7.5) using standard procedures and mixed with SDS-PAGE loading buffer under reducing conditions. Suspension was subjected to two
sonication cycles. SDS-PAGE was performed using standard methods. Separated proteins were transferred to nitrocellulose membranes and probed with appropriate antibodies in 5% non-fat milk powder in 1× PBS-0.05% Tween 20. Bound secondary peroxidase conjugated antibodies were visualized using either the ECL system (GE healthcare) or SuperSignal (Pierce).

**Mass spectrometry (MS)**

In gel digestion. Gel fragments obtained after one-dimensional electrophoresis (1-DE) were first destained by incubation in 50 mM ammonium bicarbonate and 30% acetonitrile (AcN) for 30 min at room temperature. Destaining solution was removed and fragments were then incubated for 35 min at 56°C in 10 mM DTT in 50 mM ammonium bicarbonate. DTT solution was then replaced by 55 mM iodoacetamide in 50 mM ammonium bicarbonate and the gel fragments were incubated for 30 min at room temperature in the dark. Gel pieces were then washed for 30 min with 50 mM ammonium bicarbonate and for 30 min with 50 mM ammonium bicarbonate and 30% AcN. Gel pieces were then dried for 45 min in a Centrivap vacuum centrifuge (Labconco, Kansas City, USA). Dried pieces of gel were rehydrated for 45 min at 4°C in 50 mM ammonium bicarbonate containing trypsin at 6.25 ng μl⁻¹. Extraction of the peptides was performed with 1% TFA for 30 min at room temperature with occasional shaking. The TFA solution containing the proteins was transferred to a polypropylene tube. A second extraction of the peptides was performed with 0.1% TFA in 50% AcN for 30 min at room temperature with occasional shaking. The second TFA solution was pooled with the first one. The volume of the pooled extracts was completely dried by evaporation under vacuum.

**Phosphopeptides enrichment.** Performed following Larsen protocol (Jensen and Larsen, 2007). Briefly: stage-tips were homemade prepared using a small plug made of Glass Microfibre Filters (Whatman), and 5 μm TiO₂ beads (GL SCIENCES) to make a 5-mm-long column. TiO₂ beads were suspended in 0.1% TFA in 50% AcN, 0.1% FA for MS analysis.

**MS analysis.** LC-ESI-MS/MS was performed on a linear trap quadrupole (LTQ) Orbitrap Velos (Thermo Electron, San Jose, CA, USA) equipped with a NanoAcuity system (Waters). Peptides were trapped on a home-made 5 μm 200 Å Magic C18 AQ (Microm) 0.1 × 20 mm pre-column and separated on a commercial 0.075 × 150 mm Nikkyo (Nikkyo Technology) analytical nanocolumn (C18, 5 μm, 100 Å). The analytical separation was run for 17 min using a gradient of H₂O/FA 99.9%/0.1% (solvent A) and CH₃CN/FA 99.9%/0.1% (solvent B). The gradient was run as follows: 0–1 min 95% A and 5% B, then to 65% A and 35% B for 15 min, and 20% A and 80% B for 17 min at a flow rate of 220 nl min⁻¹. For MS survey scans, the orbitrap (OT) resolution was set to 60000 and the ion population was set to 5 × 10⁵ with an m/z window from 400 to 2000. For protein identification, up to 5 precursor ions were selected for collision-induced dissociation (CID) in the LTQ. Multistage activation (MSA) of frequencies corresponding to neutral loss of phosphoric acid (−98, −49 and −32.6 Th relative to the precursor ion) was performed. The ion population was set to 1 × 10⁶ (isolation width of 2 m/z). The normalized collision energies were set to 35% for CID.

**Protein identification.** Peak lists (MGF file format) were generated from raw orbitrap data using the EasyProtConv conversion tool from the EasyProt software platform (Gluck et al., 2013). The peaklist files were searched against the Toxoplasma gondii GT1 database (Toxoplasma Genomics Resource, release 10.0 of 31-Jan-2014, 8461 entries, 6648588 residues) using Mascot (Matrix Sciences, London, UK). Sequences of modified GAP45 and MLC1 (tagged WT and mutants) proteins were inserted in Toxoplasma database. The parent ion tolerance was set to 10 ppm. Variable amino acid modifications were oxidized methionine and phosphorylated serine, threonine and tyrosine. Fixed amino acid modification was carboxymethyl cystein. Trypsin was selected as the enzyme, with one potential missed cleavage, and the normal cleavage mode was used. The mascot search was validated using Scaffold 4.0.4 (Proteome Software, Portland, OR). Only proteins matching with two different peptides with a minimum probability score of 95% were considered identified.

Raw data of the TiO₂ enriched fractions are presented in Table S2 for MLC1 and GAP45.

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**Conflict of interest**

The authors declare to have no conflict of interest.

**References**


Azvedo, M.F., Sanders, P.R., Krejany, E., Nie, C.Q., Fu, P., Bach, L.A., et al. (2013) Inhibition of *Plasmodium falciparum* CDPK1 by conditional expression of its


eupathDB (Aurrecoechea et al., 2007): Toxoplasma gondii (Tg), TGME49_223940; Neospora caninum (Nc), NCLIV_048570; Plasmodium falciparum (Pf), PF3D7_1222700; Theileria annulata (Ta), TA13510; Babesia bovis (Bb), BBOV_II005470.

**Fig. S3.** (A) Schematic representation of TgMLC1 highlighting the previously reported phosphorylation sites (Treeck et al., 2011). The sites investigated in this study are in red. Schematic representation of MycMLC1-wt (B) and MycMLC1-AAA (C) phosphorylation sites identified by mass spectrometry in this study and presentation of the corresponding spectra. The Myc tag was not included in the amino acid numbering.

**Fig. S4.** (A) Schematic representation of TgGAP45 highlighting the previously reported phosphorylation sites (Nebl et al., 2011; Treeck et al., 2011). In red are the sites investigated in this study. Schematic representation of TyGAP45c-wt (B) and TyGAP45c-ΔDR (C) phosphorylation sites identified by mass spectrometry in this study and presentation of the related most relevant spectra. Phosphorylation in the Ty tag is highlighted in red. The Ty tag was not included in the amino acid numbering.

**Table S1.** Primers used in this study.

**Table S2.** Raw data of the TiO2 enriched fractions for identification of the phosphorylation sites of MLC1 and GAP45.