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Jacot, Damien; Soldati-Favre, Dominique

How to cite

JACOT, Damien, SOLDATI-FAVRE, Dominique. Does protein phosphorylation govern host cell entry and egress by the Apicomplexa? In: International journal of medical microbiology, 2012, vol. 302, n° 4-5, p. 195–202. doi: 10.1016/j.ijmm.2012.07.012

This publication URL: https://archive-ouverte.unige.ch/unige:23056

Publication DOI: 10.1016/j.ijmm.2012.07.012

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International Journal of Medical Microbiology 302 (2012) 195–202

Contents lists available at SciVerse ScienceDirect

International Journal of Medical Microbiology

journal homepage: www.elsevier.com/locate/ijmm

Mini Review

Does protein phosphorylation govern host cell entry and egress by the *Apicomplexa*?

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ARTICLE INFO

Keywords:
Apicomplexa
Kinase
Phosphorylation
Invasion
Gliding motility
Signaling

ABSTRACT

Members of the phylum *Apicomplexa* are responsible for a wide range of diseases in humans and animals. The absence of an effective vaccine or safe curing drugs and the continuous emergence of resistant parasites to available treatments impose a high demand on the identification of novel targets for intervention against the apicomplexans. Protein kinases are considered attractive potential therapeutic targets not only against cancers but also to combat infectious diseases. The scope and aim of this review is to report on the recent progress in dissecting the impact of protein phosphorylation in regulating motility and invasion.

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Introduction

Members of the phylum *Apicomplexa* are responsible for a wide range of diseases in humans and animals. This large group of obligate intracellular parasites includes *Plasmodium falciparum*, the causative agent of the most deadly form of malaria that leads to more than one million deaths per year (Murray et al., 2012); *Toxoplasma gondii*, responsible for severe congenital defects and life-threatening in immunocompromised individuals (Montoya and Liesenfeld, 2004); *Babesia*, tick-transmitted, hemoprotozoan parasites that cause disease in cattle, horses, dogs, and humans (Homer et al., 2000); *Cryptosporidium*, causing diarrhea (Xiao, 2010); and finally *Eimeria* and *Theileria* that account for a considerable economic burden in the poultry and cattle industry, respectively (Morrison, 2009; Shirley et al., 2007). The absence of an effective vaccine or safe curing drugs and the continuous emergence of resistant parasites to available treatments impose a high demand on the identification of novel targets for intervention against the apicomplexans. Protein kinases are considered attractive potential therapeutic targets not only against cancers but also to combat infectious diseases (Petersen et al., 2011).

Protein phosphorylation is a common and widespread post-translational modification that impacts all cellular processes with a prominent role in signal transduction. Similar to other organisms, *Apicomplexa* are in a constant "state of flux", where activation and regulation of cellular pathways have to be constantly modified in response to external stimuli (Pawson and Scott, 2005). Phosphorylation is a fast, reversible modification not simply to switch the activity of a given protein on or off but also to affect its stability, localization, or ability to interact with other proteins (Cohen, 2000). Kinases and phosphatases critically govern and fine-tune the function of large subsets of proteins among the huge background of potentially phosphorylated proteins (Ubersax and Ferrell, 2007).

Two independent genome database mining and phylogeny analyses led to the identification of the *P. falciparum* kinome, which comprised 86 (Anamika et al., 2005) and 99 (Doerig et al., 2008) kinases, respectively. In *T. gondii*, the kinome is slightly larger and consists of 108 predicted active kinases and 51 predicted pseudokinases, which include an important family of secreted enzymes that act as host effectors and play a critical role in virulence (Lim et al., 2012; Peixoto et al., 2010). All members of the *Apicomplexa* also possess a family of calcium-dependent protein kinases (CDPKs), which are normally found in plants. Calcium signaling is known to control several critical events in the parasite's life cycle including motility, invasion and egress, and because of their absence in mammalian hosts, the CDPKs represent conceivable drug targets (Billker et al., 2009).

The aim of this review is to report on the recent progress in dissecting the impact of protein phosphorylation in regulating motility and invasion. Host cell entry is a key event for survival and replication of any obligate intracellular pathogen. In apicomplexans, this vital multi-step process is initiated by the attachment and reorientation of the parasite such that the apical organelles (micronemes and rhoptries) sequentially discharge their content at the point of contact with the host cell. It is followed by an active penetration event that causes invagination of the host cell plasma membrane and leads to the formation of a parasitophorous vacuole membrane (PVM). During entry, the parasite forms a so-called moving junction (also described as tight junction (Bargieri et al., 2012)) that maintains close contact between the host cell

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http://dx.doi.org/10.1016/j.ijmm.2012.07.012

membrane and the parasite and is mediated by secreted parasite proteins. The rhoptry neck proteins (RONs) are targeted to the host plasma membrane and associated to the microneme protein AMA1 (apical membrane antigen 1), which remains anchored via its membrane-spanning domain to the parasite plasma membrane. Motility, host cell invasion, and egress from infected cells are powered by the glideosome, a large molecular machine firmly anchored in the parasite pellicle. The pellicle consists of the plasma membrane (PM) and the inner membrane complex (IMC) which is composed of flattened vesicles and closely opposed to the subpellicular microtubules (Morrissette et al., 1997). The glideosome is highly conserved across the phylum but best characterized in *T. gondii*, where it has been shown to be composed of a myosin heavy chain (TgMyoA), its two associated light chains (TgMLC1, TgELC1), two integral inner membrane proteins (TgGAP50 and TgGAP40), and an acylated protein (TgGAP45). The motor complex composed of TgMyoA/TgMLC1/TgELC1 (Bergman et al., 2003; Herm-Gotz et al., 2002; Nebl et al., 2011) powers motility and propels the parasite into the host cell (Meissner et al., 2002; Wetzel et al., 2003). TgGAP45 recruits and anchors the motor complex at the IMC, where it establishes contact with TgGAP40 and TgGAP50 (Frenal et al., 2010; Gaskins et al., 2004). The current model suggests that the myosin motor is anchored to the subpellicular cytoskeleton of the parasite, while actin (ACT) interacts via aldolase (ALD) with the cytoplasmic tails of microneme proteins (MICs), themselves interacting outside the parasite with host cell receptors or the extracellular matrix. This unique mode of substrate-dependent gliding critically assists the parasite's migration across biological barriers and spreading through tissues (Baum et al., 2006; Daher and Soldati-Favre, 2009). Gliding motility, more directly myosin ATPase activity, and actin polymerization need to be tightly regulated in a timely and spatial fashion. Several key regulators of actin dynamics including profilin (PRF), formins (FRMs), and actin depolymerization factor (ADF) were found to govern parasite motility (Baum et al., 2008; Daher et al., 2010; Mehta and Sibley, 2011; Plattner et al., 2008).

The importance of protein phosphorylation in controlling (i) motor assembly, (ii) actin polymerization, (iii) organelle secretion, and (iv) moving junction formation is starting to emerge through genome-wide phosphoproteomic analyses and functional investigations of key kinases and their targets.

Phosphoproteome

To evaluate the impact of protein phosphorylation in regulating a given biological process, it is important to first identify the repertoire of target proteins and the dynamic nature of the post-translational modifications. A global phosphorylation profile allows formulation of new hypotheses on which proteins or pathways might be selectively activated at a particular stage of the parasite. Ultimately, to dissect the mechanistic significance of this modification, it would be instrumental to map precisely the modified residues and identify the kinases implicated. Recent advances in phosphopeptide enrichment coupled with high-throughput mass spectrometry have considerably increased the power of global phosphoproteomic analysis. Phosphoproteomes for "intracellular" and "extracellular" *T. gondii* tachyzoites (Treeck et al., 2011) as well as for *P. falciparum* schizont stage have been recently reported (Solyakov et al., 2011; Treeck et al., 2011). Additionally, a phosphoproteome with thousands of phosphorylation sites in *P. falciparum* erythrocytic stages will be released shortly (Lasonder et al., 2012). The high reproducibility but also the complementary results obtained by Treeck, Solyakov, and Lasonder illustrate the importance of generating independent data sets in order to assure high coverage and high confidence in phosphorylation site assignment.

Treeck et al. (2011) identified 8463 phosphorylation sites localized in 1673 proteins in *P. falciparum*, meaning that 26% of predicted proteins are phosphorylated at least on one residue. 12,793 phosphorylation sites matching 2793 proteins were found for intracellular *T. gondii* tachyzoites. Due to the absence of host cell material, the analysis of purified "extracellular" parasites sample was considerably more sensitive, with 24,298 phosphorylation sites identified for 3506 proteins (42% of the predicted proteome is phosphorylated). These results are consistent with what was previously reported in other organisms, where it was estimated that 30% of all cellular proteins are modified on at least one residue in humans (Cohen, 2000). The invasome and exportome were ranked at the top of a classification of the most frequently phosphorylated groups of proteins, both in *T. gondii* and *P. falciparum*. Several key components of the glideosome, regulators of actin dynamics and proteins implicated in the tight junction formation were indeed abundantly phosphorylated (Tables S1 and S2).

In Solyakov et al. (2011), a kinome-wide reverse genetics approach was conducted in parallel to identify the essential malaria kinases (Solyakov et al., 2011). Some of the predicted essential kinases were previously implicated in motility such as cAMP-dependent protein kinase A (PKA) (Kurokawa et al., 2011; Leykauf et al., 2010), calcium-dependant protein kinase 1 (CDPK1) (Green et al., 2008; Kato et al., 2008; Winter et al., 2009), protein kinase B (PKB) (Vaid et al., 2008), and protein kinase G (PKG) (Hopp et al., 2012). These datasets therefore constitute an important resource for future hypotheses on the regulation of protein function by phosphorylation and their implications on host cell entry.

Glideosome: myosin motor assembly and function

The phosphoproteome revealed that all components of the glideosome are phosphorylated, and to a large extent, the sites are in conserved regions across the proteins of the phylum *Apicomplexa* (Fig. 1, Table S1). Moreover, a comparative analysis of the phosphoproteome following a pulse of calcium stimulation revealed a selective increase in phosphorylation of residues in TgGAP45, TgMLC1, and TgMyoA (Table S1) (Nebl et al., 2011).

In higher eukaryotes, regulation of the myosin ATPase activity is critically controlled by phosphorylation of myosin light chains (Scruggs and Solaro, 2011; Stull et al., 2011), but very little is known about MyoA. A conserved phosphorylation site between *P. falciparum* and *T. gondii* was identified in the N-terminal part of MyoA outside of the head domain. Several additional sites were found in *T. gondii*, including one located close to the IQ motif, responsible for binding to TgMLC1. TgMLC1 and its homolog in the malaria parasite PfMTIP (MyoA tail-interacting protein) are phosphorylated on several residues, including one conserved in both proteins (Fig. 1, Tables S1 and S2). Despite the importance of phosphorylation for the regulation of MLCs in other organisms, the allelic replacement of TgMLC1 with phospho-mimetic or phospho-null mutants did not affect the localization of the protein; its assembly with the rest of the glideosome and the motility and invasion of the transgenic parasites were unaltered (Jacot et al., unpublished data).

Assembly of the myosin motor at the pellicle is critically dependent upon GAP45, a protein extensively modified by acylation and phosphorylation both in *T. gondii* and *P. falciparum* (Fig. 1, Table S1). TgGAP45 was shown to be phosphorylated on residues S163 and S167 in vivo (Gilk et al., 2009). These modifications appeared to play a role in the final step of glideosome assembly, since transient expression of double phospho-null/mimetic mutants followed by co-immunoprecipitation revealed that the phospho-mimetic mutant failed to associate with TgGAP50. In *T. gondii* the bulk of phosphorylation sites is clustered in a disordered region located between the coiled coil and the conserved

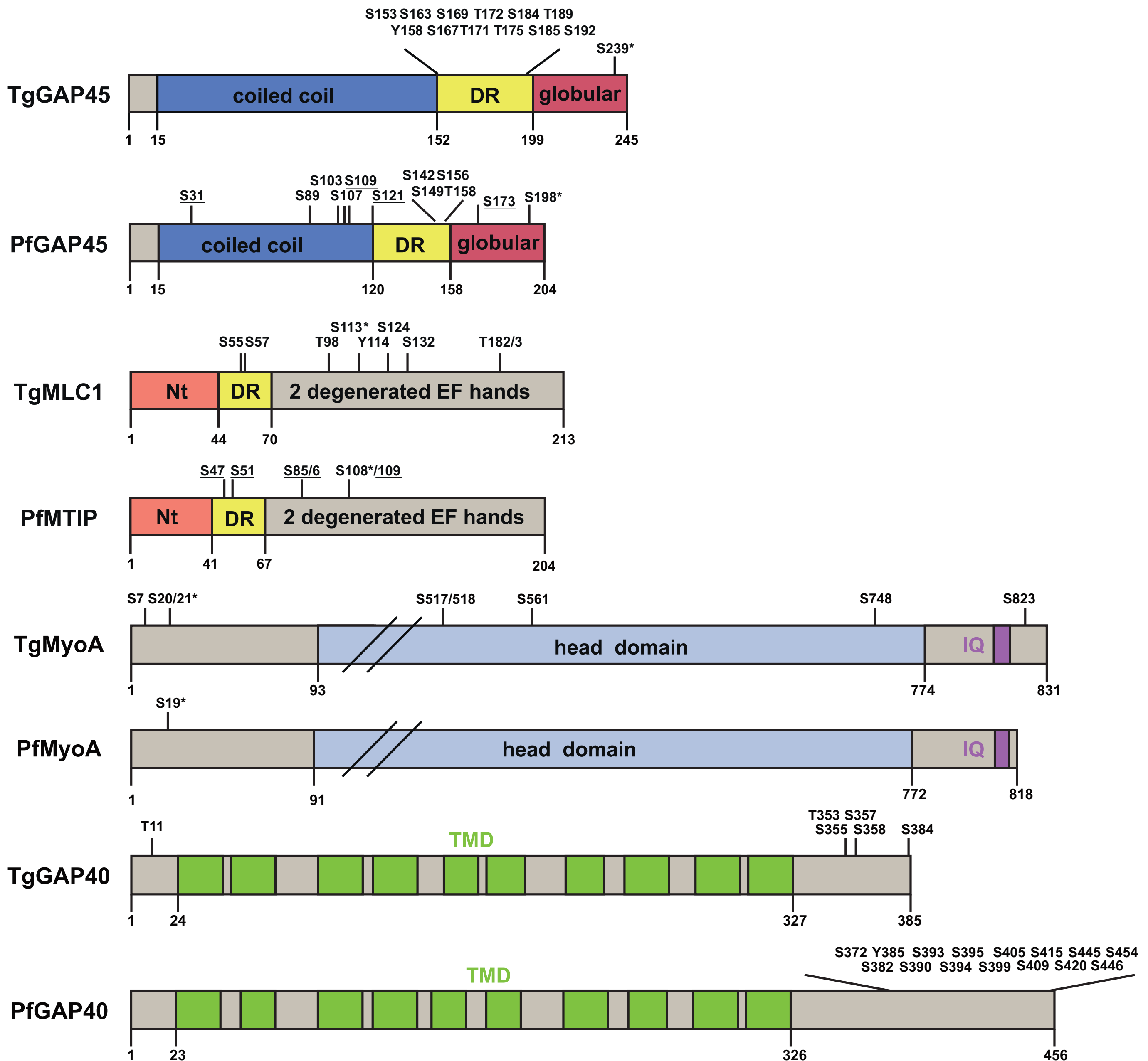

Fig. 1. Schematic representation of the phosphorylation site distribution in the glideosome proteins. All protein domains were predicted using SMART EMBL if not otherwise specified. Underlined phosphorylated sites were found only in silico. DR: disordered region (Nebl et al., 2011), Nt: N-terminal extension, TMD: transmembrane domain, Star: conserved phosphorylated residues. Proteins are presented with the N-terminus on the left. Phosphorylation site references are listed in Table S1.

C-terminal globular domain (Fig. 1). The globular domain alone was shown to be sufficient for assembly with the TgMLC1/TgMyoA complex and targeting to the IMC (Frenal et al., 2010). In consequence one can predict that phosphorylation in that region of TgGAP45 is unlikely to have a dramatic impact on the assembly of the complex. Moreover the complementation of TgGAP45 inducible knockout with a mutant version of TgGAP45 lacking this disordered region rescued gliding, invasion, and egress phenotypes (Jacot et al., unpublished data). In *P. falciparum*, two recent studies identified multiple phosphorylated residues on PfGAP45, but the expression of the corresponding phospho-mutants (null and mimetic) showed no apparent effect on PfGAP45 assembly into the motor or its subcellular location (Ridzuan et al., 2012; Thomas et al., 2012). Taken together, these results fail so far to identify a key contribution of phosphorylation to motor recruitment and function.

GAP40 is a polytopic IMC resident protein containing 10 transmembrane domains (TMDs) and a highly phosphorylated C-terminal tail predicted to be cytosolic. A phosphorylation site at the N-terminal region was also mapped on TgGAP40 (Fig. 1, Table S1). The second resident IMC protein known to be associated to the glideosome is GAP50. This predicted type I transmembrane protein is mainly localized to the lumen of the IMC, with only 6 amino acids facing the cytosol. A unique phosphorylation site has been identified exclusively on TgGAP50 and not in the malaria homolog. This site is located in the phosphatase domain that is predicted to be in the lumen of the IMC and inactive since key catalytic residues are lacking.

Fractal information is currently available on the identity of the kinases implicated in glideosome phosphorylation. In vitro phosphorylation assays of PfGAP45 and PfMTIP identified PfCDPK1 as

a potential kinase (Green et al., 2008; Kato et al., 2008; Winter et al., 2009). PfCDPK1 is localized at the periphery of the *P. falciparum* merozoite and is therefore correctly positioned to fulfill such a function (Green et al., 2008). The homolog of PfCDPK1 in *T. gondii*, TgCDPK3 can phosphorylate TgALD in vitro and is located at the apical pole of extracellular parasites (Sugi et al., 2009). Another enzyme, the protein kinase B (PfPKB), was reported to interact with PfGAP45 via immunoprecipitation assays and was also shown to phosphorylate PfGAP45 in vitro (Thomas et al., 2012; Vaid et al., 2008). PfPKB harbors a calmodulin binding domain that could make the link with an upstream activation cascade via calcium. Furthermore, Go 6983, a PKC inhibitor (PfPKB catalytic domain is closely related to PKC) is able to affect in vitro recombinant PfPKB activity and reduce the ability of *P. falciparum* to invade erythrocytes (Kumar et al., 2004). Consistent with a vital role in parasite development, both PKB and CDPK1 are refractory to gene disruption in *P. berghei* and *P. falciparum* (Kato et al., 2008; Solyakov et al., 2011; Tewari et al., 2010). Using a broad-range serine/threonine kinase inhibitor (staurosporine), a marked reduction of phosphorylation of PfGAP45 was observed (Jones et al., 2009). Surprisingly, when the phosphorylation of PfGAP45 is impaired, immunoprecipitation of the motor complex using GAP45 antibodies showed strong reduction of the immunoprecipitated PfMTIP and PfMyoA. This suggests a potential link between phosphorylation and the forming rate of the motor complex.

The extensive phosphorylation occurring on the components of the glideosome (Tg-36 and Pf-30, Table S2) is suggestive of a potentially important role in its assembly, stability, and function. Despite this, no kinase has yet been shown to selectively affect motility. It is worth mentioning that given the essential contribution of microneme proteins in gliding, it is experimentally not trivial to differentiate and measure the impact on gliding in situations where microneme secretion is concomitantly impaired. Preliminary observations on TgMLC1 and TgGAP45 suggest that phosphorylation is not essential for glideosome assembly and function, however the importance of phosphorylation on MyoA and GAP40 remains to be investigated.

Actin dynamics

In apicomplexan parasites, host cell entry usually takes 10–30 s, and therefore actin-based motility needs to be tightly controlled in space and time. Furthermore, parasites become readily motile upon induced egress and stop moving equally fast immediately after sealing the parasitophorous vacuole. A rather unique feature of apicomplexan actin is that it is largely maintained in an un-polymerized state (Angrisano et al., 2012; Dobrowolski et al., 1997). Filamentous actin is hence predictably highly dynamic, short, and the rate of polymerization and depolymerization must be tightly controlled. Several key players have been identified including profilin (PRF), formins (FRMs), and actin depolymerization factor (ADF). Profilin was found to critically contribute to egress, gliding, and host cell invasion (Plattner et al., 2008). In the absence of an ARP2/3 complex, the role of the actin nucleator is carried out by two formins in *Plasmodium* spp. and three in *T. gondii* (Baum et al., 2008; Daher et al., 2010, 2012). To promote motility, the two formins TgFRM1 and TgFRM2 act in concert at the parasite pellicle to generate presumably short, transient filaments (Daher et al., 2010). In *P. falciparum*, PfFRM1 was reported to localize at the apical pole of free merozoites and to follow the tight junction where the glideosome is predicted to power movement. The FH2 domains of these two formins enhance actin polymerization in the presence of *Toxoplasma* actin in vitro (Skillman et al., 2012). In those same assays, the biochemical properties of TgPRF indicate that the protein sequestrates actin monomers and does not contribute to polymerization (Skillman et al., 2012). TgADF is another highly potent factor that leads to actin depolymerization, and deletion of the gene results in the accumulation of actin filaments in *T. gondii* (Mehta and Sibley, 2011). This abnormal actin polymerization causes severe defects in egress, gliding, and host cell invasion. All of these regulators of actin dynamics were found to be phosphorylated with the exception of ADF (Table S2). Moreover, actin itself was found to be phosphorylated in both organisms (Table S2). To date, there is no report of the importance of phosphorylation in regulation of actin polymerization in apicomplexans.

In mammals, the actin nucleating activity of the formins is tightly controlled, and numerous formins act as Rho GTPase effectors. Rho binds to the formin N-terminal GTPase-binding domains (GBDs) and causes the adjacent Dia-inhibitory domain (DID) to release the C-terminal Dia-autoregulatory domain (DAD) that flanks the FH2 domain. This release allows the FH2 domain to nucleate actin (Schonichen and Geyer, 2010; Wallar et al., 2006). All apicomplexan parasites lack these regulatory domains, but there is abundant literature covering studies in mammals and yeast that highlight how phosphorylation regulates formins activity (Cheng et al., 2011; Iskratsch et al., 2010; Wang et al., 2009). In this context it is intriguing and likely relevant to notice the large cluster of phosphorylation sites on the C-terminal region of TgFRM2. A recent investigation of TgFRM3 has uncovered the importance of the C-terminal region in controlling the nucleating activity of this formin (Daher et al., 2012).

Microneme secretion and egress

Motility is triggered by signaling events involving an increase in intracellular calcium that leads to microneme secretion (Carruthers and Sibley, 1999; Moreno and Docampo, 2003; Wasserman and Chaparro, 1996; Wetzel et al., 2004) and also to conoid protrusion in *Coccidia*. The discharge of microneme proteins leads to the establishment of a bridge between the extracellular matrix or host cell receptors and the glideosome, hence allowing the parasite to move onto the substrate. In *T. gondii*, microneme secretion can be artificially triggered by ethanol or calcium ionophores (Carruthers et al., 1999; Carruthers and Sibley, 1999; Endo et al., 1982; Wetzel et al., 2004). During gliding motility cytosolic calcium levels undergo fast and dramatic changes that reflect multiple sequential microneme discharge events. However, when the parasite initiates host cell penetration, the intracellular calcium is rapidly quenched (Lovett and Sibley, 2003). In *P. falciparum*, invasion of red blood cells by merozoites is inhibited by intracellular calcium chelators (Jones et al., 2009; Wasserman and Chaparro, 1996). Free merozoites also exhibit a high calcium level, which drops following red blood cell attachment (Singh et al., 2010). The interaction of the transmembrane erythrocyte binding antigen-175 (EBA175, a microneme protein) with its receptor restores the basal calcium level and is believed to trigger the secretion of rhoptries. This interaction might also serve as a negative feedback loop to shut down the secretion of micronemes (Singh et al., 2010).

T. gondii calcium-dependent protein kinase 1 (TgCDPK1) was identified as a critical transducer downstream of calcium signaling that governs microneme exocytosis. A Tet-inducible knockout of TgCDPK1 blocks the secretion of micronemes, which results in impaired gliding motility, invasion, and egress (Lourido et al., 2010). Several specific inhibitors were designed against TgCDPK1, including pyrazolopyrimidine derivatives for which a gatekeeper mutant was shown to restore microneme secretion in the presence of the drug (Johnson et al., 2012; Kieschnick et al., 2001; Ojo et al., 2010; Sugi et al., 2010).

Inhibition of the *T. gondii* cGMP-dependent protein kinase G (PKG) by compound 1 was also found to inhibit microneme discharge with a minimal effect on intracellular parasite growth

(Wiersma et al., 2004). Moreover, the PKG locus was proved to be refractory to gene disruption, suggesting a vital function (Donald et al., 2002). TgPKG and TgCDPK1 could act independently and in parallel and might represent redundant signaling pathways for controlling this critical event (Lourido et al., 2010). In fact, many of the processes controlled by cGMP appear to be also regulated by calcium, and hence the two pathways are likely interconnected (Hopp et al., 2012). In *T. gondii*, PKG is believed to act downstream of calcium, as calcium ionophores are not able to induce microneme secretion in parasites treated with PKG inhibitors (Wiersma et al., 2004). In *Plasmodium* spp., the picture is more complicated due to the multiple effects resulting from PKG inhibition. In *P. berghei*, PKG is a key regulator of ookinete gliding, and the gene is refractory to disruption in intra-erythrocytic stages (Moon et al., 2009). A conditional disruption of PbPKG in sporozoites leads to a severe intracellular block in the liver stage, although the mutated sporozoites were still able to invade (Falae et al., 2010). PfPKG is also implicated in mediating differentiation of male and female gametocytes into mature gametes within the mosquito (McRobert et al., 2008). Treatment of *P. falciparum* ring stage with compound 1 allowed normal development up to 30 h post infection (pi), and segmented schizonts were able to form. Parasites treated only in the schizont stage became dysmorphic, were unable to rupture, and the merozoites released were not invasive (Taylor et al., 2010). Others have found that schizont treatment with compound 1 inhibited processing of the major surface protein 1 (PfMSP1) by the protease subtilisin 1 (PfSUB1), indicating that PKG acts upstream of the protease cascade (Dvorin et al., 2010). Egress of *Plasmodium* relies on protease activity in order to escape from the PVM (Blackman, 2008). PfCDPK5, a membrane-associated kinase expressed in late schizonts and merozoites was recently demonstrated to act downstream of this protease cascade (Dvorin et al., 2010). PfCDPK5-deficient parasites were arrested in mature schizonts with no apparent ultrastructure defects, and the processing of PfMSP1 by PfSUB1 was unaffected. Mechanically egressed PfCDPK5-deficient merozoites retained their ability to invade, excluding a block in microneme secretion. The authors therefore proposed a model of parasite egress where the protease cascade is necessary but not sufficient and where the final stages of the rupture of the PVM and the erythrocyte plasma membrane are downstream of PfCDPK5 (Dvorin et al., 2010).

In *T. gondii*, TgCDPK1 and TgPKG have clearly been implicated in microneme secretion, whereas in *P. falciparum* the exact contribution of PKG in parasite egress remains to be determined. The identification of the substrates will be instrumental in elucidating whether or not this kinase fulfills homologous functions in both parasites and other apicomplexans.

Moving junction formation

The identification of AMA1 complexed with several RON proteins provided the first grasp of the molecular nature of the moving junction and offered an elegant model to explain how the parasites hijack the host plasma membrane (Besteiro et al., 2011; Lamarque et al., 2011; Tonkin et al., 2011; Tyler et al., 2011). Intriguingly, the rhoptry proteins TgRON2, TgRON4, TgRON5, and TgRON8 are phosphorylated prior to secretion in *T. gondii* (Table S2), implying that a luminal kinase is responsible for the modification.

TgMIC2 is major determinant of gliding motility, connecting its cytoplasmic tail via TgALD to the actomyosin system (Brossier and David Sibley, 2005; Huynh and Carruthers, 2006; Jewett and Sibley, 2003). A phosphorylation site has been mapped in the highly acidic region of the TgMIC2 tail implicated in binding to TgALD; hence it is appealing to speculate that during invasion the phosphorylation of TgMIC2 might control the recruitment of the glycolytic enzyme in a timely, restricted fashion (Starnes et al., 2006) (Fig. 2). TgMIC8 was found to be dispensable for egress and gliding motility but essential for moving junction formation. TgMIC8 is presumably involved in a signaling cascade that leads to the secretion of rhoptries (Kessler et al., 2008). In *P. falciparum*, a similar function appears to be carried out by EBA175, the ligand for the erythrocyte surface protein glycophorin A. Intuitively, such a signaling function is likely to be coupled to phosphorylation, and indeed TgMIC8 cytosolic tail is phosphorylated, whereas EBA175 has not been reported to be modified (Fig. 2).

AMA1 is a key player for invasion and is the most conserved microneme protein across the phylum of *Apicomplexa*. (Hehl et al., 2000; Triglia et al., 2000). Analysis of a conditional knock-out of TgAMA1 established its important role in invasion and was found to be dispensable for gliding motility (Mital et al., 2005). In *T. gondii*, AMA1 is cleaved during invasion into its membrane-spanning domain by the rhomboid protease ROM4 (Buguliskis et al., 2010), whereas in *Plasmodium* a juxtamembrane cleavage is performed by the subtilisin protease (PfSUB2) (Harris et al., 2005; Howell et al., 2003; Olivieri et al., 2011). Both in *T. gondii* and *P. falciparum*, AMA1 was found to be phosphorylated (Fig. 2 and Table S1).

Using a complementation assay based on strain-specific inhibition, the cytosolic tail of PfAMA1 was found to be dispensable for correct trafficking but essential for the function (Treeck et al., 2009). A bioinformatics screen performed on the tail predicted 6 amino acids to be phosphorylated (2 of them were also found in Treeck et al., 2011: S590 and S610) and a phospho-mutant, generated by replacing all the residues by alanine, was unable to complement. Investigation of PfAMA1 phosphorylation was further conducted by Leykauf et al. (2010), where 2DE gel analysis of mature schizont stage revealed the presence of at least 3 phosphorylated residues dependent on the phosphorylation of residue S610. Time course experiments revealed that phosphorylation of PfAMA1 occurs mainly in late schizonts or even in merozoites. Moreover, ring stage parasites showed a simpler phosphorylation pattern, suggesting a dephosphorylation mechanism after or during invasion. Furthermore, an in vitro phosphorylation assay revealed a phosphorylation increase following cAMP stimulation, strongly indicating the implication of the cAMP-dependent protein kinase (PKA). Indeed, purified PfPKA was able to phosphorylate a recombinant PfAMA1 tail. Using the same strain-specific complementation assay, a phospho-null mutant S610A was unable to complement, and parasites were arrested after re-orientation. Moreover, a phospho-mimetic mutant was unable to complement, suggesting a dynamic turnover of phosphorylation for the function. Using a completely different approach in *T. gondii*, Santos et al. (2011) found a critical contribution of TgAMA1 as a rhomboid substrate triggering intracellular parasite replication. They used a dominant negative version of the rhomboid protease, TgROM4, in which the catalytic residue was mutated in order to block the cleavage of the substrates. The observed growth arrest phenotype could be overcome by conditional expression of TgAMA1 tail and PfAMA1 tail as a product of ROM4 cleavage. The phospho-null mutant S610A of the PfAMA1 tail was also able to complement (Santos et al., 2011). In the phospho-proteomes published by Treeck et al. (2011) and Solyakov et al. (2011), S610 was confirmed as well as 4 additional sites in the tail, and two new phosphorylated serine residues were identified in TgAMA1 (Fig. 1 and Table S1). Notably, there is no conservation of the phosphorylated residues between the 2 organisms. Further investigations into *P. falciparum* are needed in order to unravel how phosphorylation of S610 renders the merozoite competent for host cell entry. It is not known if the phosphorylation turnover of the PfAMA1 tail could impact intracellular growth via a signaling pathway.

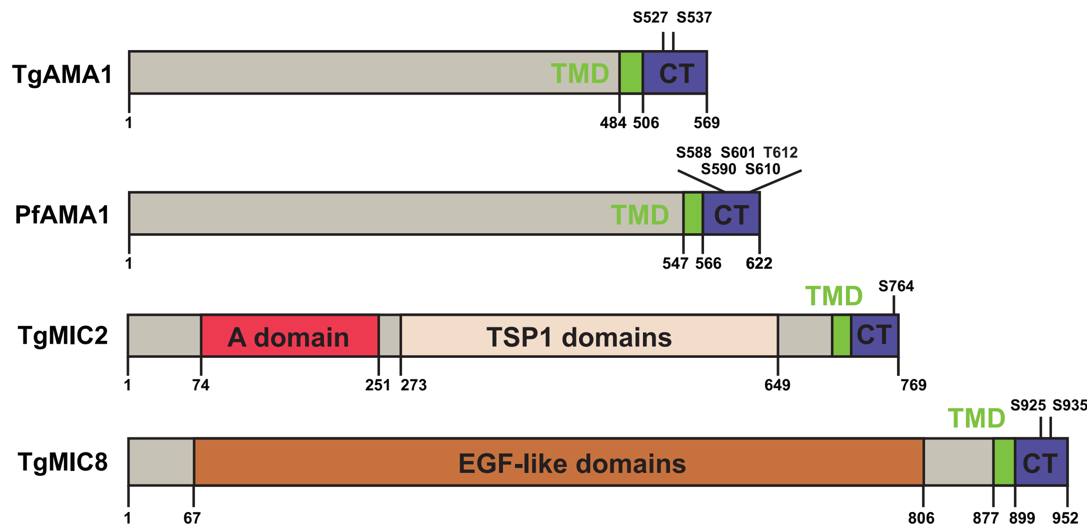

Fig. 2. Schematic representation of phosphorylation site distribution in AMA1 and TgMIC2/8. All protein domains were predicted using SMART EMBL. TMD: transmembrane domain, CT: cytosolic tail, EGF: epidermal growth factor-like domain, A domain: von Willebrand factor (vWF) type A domain, TSP1: thrombospondin type 1. Proteins are presented with the N-terminus on the left. Phosphorylation site references are listed in Table S1.

Conclusion and perspectives

In *T. gondii*, TgCDPK1 and TgPKG are implicated in microneme secretion while in *P. falciparum*, PfPKG, PfPKA, and PfPKB are associated with host invasion; however, in both organisms there is no direct evidence regarding an impact of phosphorylation on glideosome assembly or function. Nevertheless, the high level of phosphorylation identified on proteins participating in motility (150 for *T. gondii* and 57 for *P. falciparum*, Table S2) suggests that this posttranslational modification plays a role in controlling gliding motility.

A classic way to investigate site-specific phosphorylation is to generate a series of phospho-mutants to either suppress or mimic the phosphorylation. However, this technique has several limitations; each site or combinations of them have to be investigated one by one and represent again an infinite number of combinations, especially in the case of the glideosome where at least five differentially phosphorylated proteins are assembled in one large machine. Furthermore, by changing the amino acid composition, the structure/stability of the protein can be affected, and these changes might not be related to phosphorylation. Lastly and more importantly, the expression of an additional mutated copy could not be sufficient to identify phenotypes and assess the function; therefore gene replacement or generation of conditional knockout strains for complementation would be required. Large scale, quantitative MS-methods such as SILAC (stable isotope labeling in cell culture) will undoubtedly be further developed to get a more general picture of phosphorylation and identify the underlying networks and their interconnections. The next step would consist of interfering selectively and rapidly with a given kinase activity and record the changes in the phosphoproteome. The determination of phosphorylation sites is crucial for a complete understanding of protein kinases and kinase-substrate relationships, and we already have part of the answer. The current challenge is to further investigate which key steps (the glideosome, the actin polymerization, the organelle secretion and/or the moving junction) are governed by this modification and reveal how phosphorylation tailors the function(s) of the proteins implicated in these four key events.

Acknowledgments

We thank Dr. Karine Frenal and the other members of the laboratory for constructive discussions. Dominique Soldati-Favre is supported by the Swiss National Foundation (FN3100A0-116722) and Damien Jacot by the Indo-Swiss Joint Research Programme.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijmm.2012.07.012.

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