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Invited Review

Toxoplasma and *Plasmodium* protein kinases: Roles in invasion and host cell remodelling

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Some apicomplexan parasites have evolved distinct protein kinase families to modulate host cell structure and function. *Toxoplasma gondii* rhoptry protein kinases and pseudokinases are involved in virulence and modulation of host cell signalling. The proteome of *Plasmodium falciparum* contains a family of putative kinases called FIKKs, some of which are exported to the host red blood cell and might play a role in erythrocyte remodelling. In this review we will discuss kinases known to be critical for host cell invasion, intracellular growth and egress, focusing on (i) calcium-dependent protein kinases and (ii) the secreted kinases that are unique to *Toxoplasma* (rhoptry protein kinases and pseudokinases) and *Plasmodium* (FIKKs).
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1. Introduction

Protein kinases mediate the transfer of phosphate groups from ATP to specific residues on their target proteins, resulting in changes in the activity, stability, interactions with ligands or localisation of the phosphorylated substrates. Kinases themselves are often similarly regulated, and many of them function as signalling mediators that integrate upstream signals in the form of second messengers, post translational modifications or binding of regulatory proteins. It is therefore not surprising that many parasites have evolved distinct protein kinase families with novel domain structures and biochemical features to regulate key parasite-specific physiological processes that must be executed in a timely fashion during development or in response to external stimuli and physiological cues from the host. As signalling mediators, protein kinases are also well suited to function at the host–parasite interface, where they can perturb host signalling pathways, activate dormant mechanisms in the host cell, or disrupt the proper functioning of host proteins.

Both *Toxoplasma* and *Plasmodium* contain a family of calcium-dependent protein kinases (CDPKs), whose occurrence is restricted to plants and Alveolates (the superphylum that comprises the ciliates and apicomplexans), although trypanosomatids also possess kinases with an EF-hand calcium binding domain thought to be phylogenetically distinct from plant and Alveolate CDPKs (Parsons et al., 2005). CDPKs have a domain structure consisting of a calcium-binding domain fused to the kinase domain, such that kinase activity is stimulated upon calcium binding. Studies of apicomplexan CDPKs have revealed a conserved regulatory mechanism and highlighted the importance of calcium in regulating a number of key physiological processes including host cell attachment and invasion, gliding motility and parasite egress.

Toxoplasma gondii, a highly prevalent obligate intracellular protozoan parasite, seems to rely for a large part on protein kinases and pseudo kinases to modify the host cell. *Toxoplasma* is capable of infecting all nucleated cells of most warm-blooded animals. This ability to establish a chronic infection in such a wide range of host species and cell types is likely associated with its ability to modify many aspects of the host cell's normal physiology. It does this by secreting proteins from the rhoptry, a specialised secretory organelle that is found only in apicomplexan parasites, directly into the host cytosol where they can exert their function. Many rhoptry proteins have homology to kinases (Bradley et al., 2005) and a large proportion of them are predicted to be pseudokinases (Peixoto et al., 2010).

Malaria parasites (genus *Plasmodium*) also belong to the Apicomplexa, however, unlike *Toxoplasma*, they have a very restricted range of host cells which they can invade and in which they can

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replicate. After inoculation into the vertebrate host through the bite of an infected mosquito, *Plasmodium* sporozoites must first invade hepatocytes, where they undergo asexual proliferation (exoerythrocytic schizogony), generating several thousand merozoites in the process. This strict dependence on hepatocytes for the pre-erythrocytic stage of infection has, however, recently been challenged by in vivo observations of *Plasmodium berghei* parasites developing in dermal and epidermal cells at the site of inoculation and generating infective merozoites (Gueirard et al., 2010).

The only cell type permissive for invasion by *Plasmodium* merozoites is the erythrocyte (or the reticulocyte in some instances). Invasion involves release of the contents of rhoptries and micronemes (another type of specialised secretory organelle) and formation of a parasitophorous vacuole (PV). The parasite causes considerable modifications to its host red blood cell (RBC) that include the establishment of a complex trafficking system that mediates translocation of parasite-encoded proteins to the RBC membrane skeleton and surface (Cooke et al., 2004). In the case of *Plasmodium falciparum*, the most lethal of the five species of malaria parasites that can infect humans, such proteins include components of the so-called "knob" structures, through which parasite-infected RBCs adhere to vascular endothelial cells, thereby significantly contributing to pathogenesis (Cooke et al., 2001). There are no orthologs of the *Toxoplasma* rhoptry kinases in malaria parasites, however, the *P. falciparum* kinome includes a family (20 members) of related kinase-like sequences called FIKKs, due to the presence of a Phe-Ile-Lys-Lys motif they share in their kinase domain (Ward et al., 2004). Most notably, 18 of the 20 *fikk* genes in *P. falciparum* are predicted to encode fully functional kinases, 16 of which are predicted to be exported into the host RBC (Schneider and Mercereau-Puijalon, 2005), and at least some of these enzymes have been shown to be exported to the host RBC and to be associated with kinase activity (Nunes et al., 2007, 2010).

Many of the *Toxoplasma* and *Plasmodium* kinases are related to kinases with known functions in other eukaryotes and these will not be discussed in this review. Instead, we will discuss in more detail, kinases from these parasites that are known to be critical for host cell invasion, intracellular growth and parasite egress, focusing on (i) calcium-dependent protein kinases and (ii) the secreted kinases that are unique to *Toxoplasma* (ROPKs) and *Plasmodium* (FIKKs).

2. Calcium-dependent regulation of invasion and egress

Calcium levels in *T. gondii* play key roles in regulating microneme secretion-dependent processes including host cell invasion, gliding motility and parasite egress (Nagamune et al., 2008). Chelation of parasite intracellular calcium strongly inhibited both microneme release and invasion of host cells, and this effect was partially reversed by raising intracellular calcium using the ionophore A23187 (Carruthers et al., 1999). Additionally, evidence was provided in this study for the requirement of a staurosporine-sensitive kinase activity in regulating microneme discharge and for parasite invasion of host cells.

In *Toxoplasma* parasites, CDPKs were shown to be involved in host cell invasion through the use of KT5926, an inhibitor of CDPKs in other systems (Kieschnick et al., 2001). KT5926 blocks the motility of *Toxoplasma* tachyzoites and their attachment to host cells. In vivo, the phosphorylation of only three parasite proteins was found to be blocked by KT5926 (although there may be more), and in parasite extracts, a single KT5926-sensitive protein kinase activity was detected. This activity was calcium-dependent but did not require calmodulin. In a search for CDPKs in *Toxoplasma*, two members of the class of calcium-dependent protein kinases (CDPKs) were detected. TgCDPK2 was only expressed at the mRNA level in tachyzoites, with no detectable protein. TgCDPK1 protein present in *Toxoplasma* tachyzoites cofractionated precisely with the peak of KT5926-sensitive protein kinase activity. TgCDPK1 kinase activity was calcium-dependent but did not require calmodulin or phospholipids. TgCDPK1 was found to be inhibited effectively by KT5926 at concentrations that block parasite attachment to host cells. In vitro, TgCDPK1 phosphorylated three parasite proteins that migrated identically to the three KT5926-sensitive phosphoproteins detected in vivo. These observations suggested a central role for TgCDPK1 in regulating *Toxoplasma* motility and host cell invasion.

Subsequent chemical genetic and conditional knockout studies have shown that TgCDPK1 is the key transducer downstream of calcium fluxes in regulating these processes. Down-regulation of TgCDPK1 expression severely impaired the secretion of microneme proteins, including adhesins needed for gliding motility (Lourido et al., 2010). The resulting defect in gliding motility led to a significant decrease in host cell attachment and invasion. Conditional knockout of TgCDPK1 also impaired parasite egress from host cells upon induction with a calcium ionophore, with immotile parasites remaining within vacuoles. This coincided with a defect in PV membrane (PVM) permeabilisation upon calcium ionophore treatment, which likely resulted from impaired microneme secretion of the perforin-like TgPLP1 protein (Lourido et al., 2010).

The canonical CDPK domain structure consists of an N-terminal protein kinase domain, that is highly homologous to calmodulin-dependent kinases (CaMKs), and a C-terminal CDPK activation domain (CAD) with four calcium-binding EF-hands (EF1 to EF4). A recent biochemical and structural study of three apicomplexan CDPKs (TgCDPK1 and TgCDPK3 from *T. gondii*, and CpCDPK1 from *Cryptosporidium parvum*) has provided novel insights into how these CDPKs are activated upon calcium binding by the CAD (Wernimont et al., 2010). In in vitro assays, the kinase domain was activated by low micromolar calcium concentrations. Calcium 'in-out' experiments suggested a partial irreversibility of this calcium-dependent activation for TgCDPK1 and TgCDPK3, while CpCDPK1 activity was reduced to basal level upon calcium removal. Actual mapping of potential auto-phosphorylation sites may provide further insights into the reversibility of calcium-induced activation of these CDPKs.

Crystal structures have been solved for auto-inhibited (calcium-free) TgCDPK1 and TgCDPK3 and for calcium-bound TgCDPK1 and CpCDPK1 (Wernimont et al., 2010). The kinase domain adopts the bi-lobal structure characteristic of eukaryotic protein kinases (ePKs), with a smaller N-terminal lobe (consisting mainly of β-strands) and a larger C-terminal lobe (predominantly α-helical) connected together via a single stretch of polypeptide (hinge region). A deep cleft formed between the two lobes contains the ATP binding pocket and active site (Dar et al., 2005). In the calcium-free state, the CAD adopts an overall dumbbell shape (resembling calmodulin in the absence of calcium) with two EF-hands at each end of the dumbbell. The CAD packs against the front face of the kinase domain, making contacts with both the N- and C-terminal lobes. The N-terminal helix of EF1 extends into a long helix (CH1) that spans the length of the CAD and packs anti-parallel along a second long helix (CH2). The N-terminus of CH1 connects directly with the C-terminus of the kinase domain and packs against the kinase C-terminal lobe to block substrate binding, analogous to the pseudo-substrate segment of CAMKII (Rosenberg et al., 2005) (Fig. 1). Additional hydrophobic contacts with the N- and C-terminal lobes of the kinase domain are made by EF2 and EF3, respectively. Calcium binding to all four EF hands within the CAD causes conformational changes that expose hydrophobic surfaces and partial unwinding and bending of the CH1 and CH2 helices. This leads to a dramatic rearrangement of the CAD into a more compact structure. The calcium-bound CAD is also repositioned and binds to the back face of the kinase domain, which induces a widening of the active site cleft by altering the relative orientations

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