Distinct signalling pathways control *Toxoplasma* egress and host-cell invasion

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Calcium signalling coordinates motility, cell invasion, and egress by apicomplexan parasites, yet the key mediators that transduce these signals remain largely unknown. One underlying assumption is that invasion into and egress from the host cell depend on highly similar systems to initiate motility. Using a chemical-genetic approach to specifically inhibit select calcium-dependent kinases (CDPKs), we instead demonstrate that these pathways are controlled by different kinases: both TgCDPK1 and TgCDPK3 were required during ionophore-induced egress, but only TgCDPK1 was required during invasion. Similarly, microneme secretion, which is necessary for motility during both invasion and egress, universally depended on TgCDPK1, but only exhibited TgCDPK3 dependence when triggered by certain stimuli. We also demonstrate that egress likely comes under a further level of control by cyclic GMP-dependent protein kinase and that its activation can induce egress and partially compensate for the inhibition of TgCDPK3. These results demonstrate that separate signalling pathways are integrated to regulate motility in response to the different signals that promote invasion or egress during infection by *Toxoplasma gondii*.

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Introduction

Toxoplasma gondii belongs to the phylum Apicomplexa, a group of single-celled, obligate intracellular parasites of animals, including *Plasmodium spp.*, the aetiological agents of malaria. Apicomplexans are characterized by a specialized complex of apical organelles used to penetrate and modify the host cells they replicate within (Besteiro *et al.*, 2011). Host-cell invasion is powered by parasite motility and relies on the posterior translocation of adhesins from their site of secretion at the apical end of the parasite (Sibley, 2010). The signalling pathways that regulate parasite motility are poorly

understood, although transient increases in cytosolic calcium are known to be essential for microneme secretion, motility, and host-cell invasion (Carruthers and Sibley, 1999; Lovett and Sibley, 2003). In *T. gondii*, it was first demonstrated using the calcium ionophore A23187 that direct manipulation of the intracellular calcium concentrations could trigger motility and induce egress (Endo *et al.*, 1982). However, these calcium transients can be triggered naturally by the accumulation of abscisic acid produced by parasites (Nagamune *et al.*, 2008), or exposure to a low potassium environment, which occurs upon host-cell rupture (Moudy *et al.*, 2001). Artificially, intracellular calcium levels can be increased and microneme secretion induced by incubating *T. gondii* parasites with ethanol (Carruthers *et al.*, 1999), which occurs through IP₃ generation (Lovett *et al.*, 2002). In *Eimeria tenella*, a related apicomplexan, microneme secretion can be triggered by serum albumin (Bumstead and Tomley, 2000; Wiersma *et al.*, 2004), which also stimulates motility in *Plasmodium berghei* sporozoites, in a manner dependent on intracellular calcium (Kebaier and Vanderberg, 2010). At a different point in the *Plasmodium* life cycle, xanthurenic acid in the mosquito midgut can also trigger calcium transients in male gametocytes and induce their differentiation (McRobert *et al.*, 2008). Taken together, these observations suggest that calcium acts as a second messenger in response to a variety of extracellular signals, in many cases converging on the regulation of motility.

The wide range of cellular processes regulated in most eukaryotes by intracellular calcium occurs in part through the activation of calcium-regulated protein kinases. In animals, these kinases belong to two families: protein kinase C (PKC) and related kinases, and calmodulin-dependent kinases (CaMKs) (Barclay *et al.*, 2005). However, plants and alveolates lack obvious PKC homologues, and calcium-dependent protein kinases (CDPKs) represent the dominant calcium-responsive kinases in these organisms (Harper and Harmon, 2005; Nagamune and Sibley, 2006). CDPKs are characterized by a kinase domain followed by a calmodulin-like domain, which directly binds calcium to activate the enzyme. CDPKs have a novel mechanism of activation that is triggered by binding of calcium to the EF hands, causing a reorganization of the calcium-binding domain that releases the kinase domain from inhibition (Wernimont *et al.*, 2010). In *Arabidopsis*, as many as 42 CDPK isoforms regulate myriad transcriptional and cellular processes, leading to effects in diverse phenotypes including pollen tube formation, closure of stomata, and a wide range of stress responses (Harper and Harmon, 2005).

Apicomplexan parasites contain an expanded family of CDPKs and several of these have been shown to control important functions (Billker *et al.*, 2009). *Plasmodium spp.* and *T. gondii* genomes each code for 5 and 6 canonical CDPKs, respectively, and an additional 2–6 related kinases with different domain architectures (Billker *et al.*, 2009). In *P. berghei*, disruption of CDPKs that are not essential in

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Figure 1 Localization of TgCDPK3 depends on putative acylation sites. Immunofluorescence analysis of parasites expressing different alleles of HA9-tagged TgCDPK3. The region within the dotted line represents an optical slice through the centre of the gliding parasite, collapsed onto the slice showing its trail. Graph represents the relative fluorescence intensity across the apical end, marked by the white arrows, of three parasites; mean $\pm$ s.e.m.; red, HA9; green, SAG1.

the asexual phase leads to developmental blocks at later stages, including male gamete exflagellation (*PbCDPK4*), ookinete motility (*PbCDPK3*), and sporozoite invasion of hepatocytes (*PbCDPK6*) (Billker *et al*, 2004; Ishino *et al*, 2006; Coppi *et al*, 2007). However, this approach is limited to kinases dispensable for the asexual cycle, and the block caused by disruption of a particular kinase precludes examination of its function beyond the stage of developmental arrest. This limitation was recently circumvented by tagging the endogenous *Plasmodium falciparum* CDPK5 with a destabilization domain, which allowed investigators to regulate its degradation, demonstrating a role for PfCDPK5 in merozoite egress from erythrocytes (Dvorin *et al*, 2010). In *T. gondii*, the availability of a regulatable promoter (Meissner *et al*, 2002) allowed us to generate a conditional knockout of *TgCDPK1*, thus revealing its requirement for microneme secretion, and hence motility and invasion (Lourido *et al*, 2010). We also exploited the unique ATP-binding pocket of TgCDPK1, which has a glycine at a key position termed as the ‘gatekeeper’, to specifically inhibit TgCDPK1 using bulky pyrazolo [3,4-*d*] pyrimidine (PP) derivatives (Lourido *et al*, 2010), confirming the role of TgCDPK1 using chemical genetics.

The function of CDPKs has also been probed *in vitro* using kinase assays that suggest that components of the motor complex, which is essential for motility (Meissner *et al*, 2001), are phosphorylated by PfCDPK1 in *P. falciparum* (Green *et al*, 2008; Ridzuan *et al*, 2012). However, PfCDPK1 remains refractory to genetic disruption in *P. falciparum*, and there is no test of whether the motor complex is an essential target of PfCDPK1 *in vivo*. Here, we have extended the chemical-genetic approach to probe the function of TgCDPK3, which is the closest homologue of PfCDPK1 based on phylogenetic comparison of the kinase domain, as well as putative lipid modifications and cellular localization (Billker *et al*, 2009). Our results suggest a much broader role for TgCDPK3 in responding to signals that govern motility, and yet likely does not involve direct regulation of the motor complex.

Results

TgCDPK3 localizes to the parasite membrane

PfCDPK1 has been reported to localize to the membrane in *P. falciparum* merozoites (Green *et al*, 2008). To localize

its orthologue TgCDPK3 in *T. gondii*, we introduced a c-terminally HA9-tagged allele of TgCDPK3, under the regulation of a modified *TetO7SAG1* promoter, into the TATi strain (Meissner *et al*, 2002). Immunofluorescence analysis of the localization of TgCDPK3 showed that it localizes to the membrane of the parasite in a pattern similar to the surface antigen SAG1 (Figure 1). The N-terminal residues of TgCDPK3 and its orthologue PfCDPK1 are predicted to be acylated and therefore likely required for its localization. We mutated the predicted myristoylation site (G²) and palmitoylation site (C³) to alanine, alone or in combination. We observed that mutation of either putative acylation site was sufficient to mislocalize TgCDPK3 to the parasite cytosol (Figure 1). In all the mutants, there was also reduced deposition of TgCDPK3 in the trails of gliding parasites, also consistent with a lack of membrane localization (Figure 1). Unfortunately, we were unable to disrupt the endogenous copy of TgCDPK3, despite the presence of a second wild-type regulatable copy, pointing to one potential limitation of this strategy.

Chemical genetic manipulation of CDPKs

The ATP-binding pockets of many eukaryotic protein kinases can be engineered to be susceptible to PP derivatives, which are normally excluded due to the presence of a bulky residue at a key position in the binding pocket, the aforementioned gatekeeper (Bishop *et al*, 2000). An alignment of the kinase domains from the 109 *T. gondii* kinases that are predicted to be active (Peixoto *et al*, 2010) shows that >80% contain a large hydrophobic amino acid as the gatekeeper residue (green bars; Figure 2A). Unique among the active kinases, TgCDPK1 harbours a glycine at this key position. Previous studies demonstrated that susceptibility of TgCDPK1 to 3-methyl-benzyl pyrazolo [3,4-*d*] pyrimidine (3-MB-PP1) and related compounds, both *in vivo* and *in vitro*, depends on the gatekeeper residue (Lourido *et al*, 2010; Murphy *et al*, 2010; Sugi *et al*, 2010). To determine whether the related kinase TgCDPK3 could be rendered susceptible to selective inhibition by PP derivatives, we generated recombinant proteins for full-length TgCDPK3 with either a glycine or a methionine at the gatekeeper positions (designated by a superscript G or M, respectively) and compared them to similar allelic forms of TgCDPK1. All four enzymes were

Figure 2 Chemical genetic strategy to inhibit CDPKs *in vitro* and *in vivo*. **(A)** Frequency of gatekeeper residues in the 109 active kinases of *T. gondii*. **(B)** *In vitro* kinase activity against syntide-2. Recombinant TgCDPK1 or TgCDPK3 carrying a methionine (M) or glycine (G) gatekeeper was assayed in the presence of different concentrations of 3-MB-PP1. Means $\pm$ s.e.m., $n = 3$ experiments. **(C)** Genotypes of the strains used in this study at the *TgCDPK1* and *TgCDPK3* loci. Exons are designated by boxes, and introns by lines. The codon and amino acid for the gatekeeper residue are designated in red, as well as epitope tags introduced in the process of manipulation. **(D)** Immunoblot for c-myc-tagged TgCDPK1^M, or Ty-tagged TgCDPK3 following allelic replacement. Aldolase was used as a loading control.

similarly able to phosphorylate the synthetic substrate peptide syntide-2, but showed dramatically different susceptibilities to 3-MB-PP1. As previously shown, wild-type TgCDPK1 (TgCDPK1^G) showed sub-nanomolar susceptibility to the inhibitor (IC₅₀ = 0.8 nM, 95 % CI (0.3–2.3)), and mutation of the gatekeeper to a methionine (TgCDPK1^M) rendered the enzyme insensitive to inhibitor concentrations of up to 1 μM (green lines; Figure 2B). Conversely, wild-type TgCDPK3 (TgCDPK3^M) was insensitive to the inhibitor unless its gatekeeper residue was mutated to a glycine (TgCDPK3^G), in which case the enzyme became sensitive, similarly to wild-type TgCDPK1 (IC₅₀ = 19.2 nM, 95 % CI (8.5–43.5); red lines; Figure 2B).

In order to study the function of TgCDPK1 and TgCDPK3 *in vivo*, we introduced similar gatekeeper residues into the genes encoding these enzymes in parasites. These manipulations were made easier by the high levels of homologous recombination in strains lacking *Ku80*, an essential component of the non-homologous end-joining DNA repair pathway (Fox *et al.*, 2009; Huynh and Carruthers, 2009). Using the *Ku80* knockout as the parental strain, henceforth designated as CDPK1^G, we replaced the wild-type *TgCDPK1* with a partial cDNA coding for a methionine at the gatekeeper position and c-terminally tagged with c-myc (CDPK1^M; Figure 2C). The CDPK1^M strain was further used to manipulate the *TgCDPK3* locus. The later manipulations were performed using a vector that lacked a promoter and contained the second intron of *TgCDPK3* fused to the cDNA starting at the third exon and

ending with a c-terminal Ty tag (Figure 2C). By selecting for drug resistance conferred by the vector, and screening for homologous recombination at the second intron by PCR, we were able to isolate parasites that expressed a single functional Ty-tagged copy of *TgCDPK3*, downstream of which the endogenous allele was left without a promoter or the first two exons, and was therefore silent. In this manner, we isolated strains where the Ty-tagged *TgCDPK3* resembled the wild type and carried a methionine at the gatekeeper position (CDPK3^M; Figure 2C), or where the position had been mutated to a glycine (CDPK3^G; Figure 2C). Both alleles of *TgCDPK3* were expressed to similar levels, as demonstrated by western blotting against the Ty tag (Figure 2D), in the otherwise isogenic background of the CDPK1^M strain.

TgCDPK3 is dispensable for invasion but required for egress

The isogenic parasite lines expressing either sensitive or resistant alleles were used to investigate whether inhibition of TgCDPK3 affected host-cell invasion, a process previously shown to depend on the related enzyme TgCDPK1 (Lourido *et al*, 2010). Consistent with our previous results, the strain carrying the wild-type *TgCDPK1* allele was significantly impaired in its ability to invade in the presence of 3-MB-PP1 (CDPK1^G; Figure 3A). Allelic replacement of the endogenous allele for one carrying a methionine at the gatekeeper position rendered the CDPK1^M strain completely resistant to inhibition (Figure 3A). In contrast to the observations with

Figure 3 TgCDPK1 and TgCDPK3 play different roles in A23187-triggered egress and host-cell invasion. **(A)** Invasion of fibroblasts by different strains in the presence of 5 μM 3-MB-PP1 or vehicle alone (DMSO). Extracellular and intracellular parasites were stained differentially and counted relative to the number of host-cell nuclei in each field. Student's *t*-test; ****P* > 0.0005; means ± s.e.m., *n* = 3 experiments. **(B)** Egress following 20 min incubation with different concentrations of 3-MB-PP1, and treatment with 2 μM A23187 for 5 min. Egress was measured as a function of lactate dehydrogenase released from host cells, and normalized to the levels resulting from parasites treated with DMSO instead of 3-MB-PP1. Means ± s.e.m., *n* = 3 experiments. **(C)** A23187-induced vacuole permeabilization detected by vacuolar DsRed leakage monitored by fluorescence video microscopy of strains in the presence of 5 μM 3-MB-PP1. The time stamps represent minute:second after the addition of A23187, the circle in the first frame of each movie represents the area quantified for **(D)**. **(D)** Relative fluorescence in a circular area with a diameter of 6 μm, within the vacuole. The three lines for each genotype represent measurements from three independent experiments.

CDPK1^G, 3-MB-PP1 had no effect on invasion by the CDPK3^G strain carrying the sensitive allele of the kinase (Figure 3A). CDPK1^G parasites showed a dose-dependent inhibition of invasion by 3-MB-PP1, while CDPK3^G expressing parasites were unaffected at even high doses (i.e., 50 μM) of 3-MB-PP1 (Supplementary Figure 1). Treatment of parasites with 3-MB-PP1 prior to egress, followed by mechanical harvest, also did not affect invasion by CDPK3^G parasites, although it significantly inhibited the CDPK1^G strain (Supplementary Figure 2).

To determine whether TgCDPK3 was also dispensable for other phenotypes previously associated with TgCDPK1, we examined the stimulation of egress from infected host cells by the calcium ionophore A23187, which causes a generalized collapse of calcium gradients and greatly increases intracellular calcium. As previously shown, treatment with 3-MB-PP1 significantly decreased the ability of the CDPK1^G strain to egress from host cells in response to A23187 (Figure 3B). In contrast to the lack of a role during invasion, inhibition of TgCDPK3 within intracellular parasites dramatically reduced their ability to respond to A23187, with an IC₅₀ for 3-MB-PP1 of 0.2 μM (95% CI (0.1, 0.5)), a concentration more than one hundred times lower than that which showed no effect on invasion by the same strain (CDPK3^G; Figure 3B). When the time period for induction of egress was increased from 5 min

to 30 min, both CDPK1^G and CDPK3^G expressing strains were still significantly inhibited by 3-MB-PP1 (Supplementary Figure 3), indicating that the failure to egress was not simply a delay, but a long-term block.

To further investigate the role of TgCDPK3 during egress, we monitored the permeabilization of the parasitophorous vacuole (PV) membrane following stimulation of intracellular parasites with A23187. This was achieved by transiently expressing a constitutively secreted form of DsRed, which accumulates in the PV and is released prior to egress, following membrane permeabilization that occurs due to microsome secretion and release of a perforin-like pore forming protein called TgPLP1 (Kafsack *et al*, 2009). Ionophore stimulation of the CDPK3^M strain treated with 3-MB-PP1 resulted in normal permeabilization of the PV membrane and egress within a few minutes, consistent with this strain being resistant to PP inhibitors (Figure 3C; Supplementary Movie 1). In contrast, the CDPK3^G strain treated with 3-MB-PP1 failed to permeabilize the PV membrane and remained immobile for the entire recorded period, up to 10 min following the addition of ionophore (Figure 3C; Supplementary Movie 2). To further quantify the differences in timing and magnitude of this response, we measured the relative fluorescence of a circular area within the PV. DsRed fluorescence

Figure 4 Gliding and microneme secretion following inhibition of TgCDPK1 or TgCDPK3. (A) Types of gliding motility recorded over 4 min for each strain in the presence of 5 μ M 3-MB-PP1. Student's *t*-test; ****P* < 0.0005; ***P* < 0.005; means $\pm$ s.e.m., *n* = 3 experiments, corresponding to 2–3 videos each. (B) Speed of gliding observed in the presence of 5 μ M 3-MB-PP1. Means $\pm$ s.e.m., *n* = 3 experiments, corresponding to 2–3 videos each. (C) Effect of 5 μ M 3-MB-PP1 on MIC2 secretion in response to 2% ethanol or 2 μ M A23187. Student's *t*-test; ****P* < 0.0005; ***P* < 0.005; means $\pm$ s.e.m., *n* = 3 experiments. (D) Surface staining for MIC2 following stimulation of secretion in the presence of 5 μ M 3-MB-PP1 or vehicle alone (DMSO). The ROM4 cKO was incubated with ATc where noted, and used as a control for the surface accumulation of MIC2. The histogram was generated for the intensity of surface staining of different strains in a representative FACS experiment.

was completely lost from CDPK3^M vacuoles within 3 min, but remained stable in CDPK3^G strains for the entire 10 min period of recording (Figure 3D). Together, these findings highlight the striking requirement for both TgCDPK1 and TgCDPK3 in egress, while only TgCDPK1 is essential during invasion.

Inhibition of TgCDPK1 or TgCDPK3 leads to defects in gliding motility and microneme secretion

Having uncovered a distinction between the effects of TgCDPK1 versus TgCDPK3 on egress and invasion, we asked whether the processes of motility and microneme secretion underlying these phenotypes were also differentially regulated. Analysis of gliding motility in all four strains

in the presence of 3-MB-PP1 demonstrated a requirement for both TgCDPK1 and TgCDPK3 in this process. Inhibition of either kinase led to an ~50% reduction in the number of parasites moving during the period observed (Figure 4A). Similarly to the previous report that TgCDPK1 is required for motility (Lourido *et al*, 2010), all types of motility were less frequent when TgCDPK3 was inhibited. However once movement was initiated, the speed of parasite gliding was normal for both sensitive and resistant strains tested in the presence of 3-MB-PP1 (Figure 4B).

Parasite motility also requires release of adhesins from micronemes (Carruthers and Sibley, 1999), a process governed by TgCDPK1 (Lourido *et al*, 2010). Hence, we examined the role of TgCDPK3 and TgCDPK1 in release of

the micronemal protein MIC2 following stimulation with ethanol, which induces formation of IP₃ and leads to elevation of calcium (Lovett *et al*, 2002). We compared ethanol-stimulated secretion of MIC2 in strains treated with or without 3-MB-PP1. Although ethanol-stimulated secretion by the CDPK1^G strain was significantly inhibited by 3-MB-PP1, the CDPK3^G strain was only marginally impaired in the presence of 3-MB-PP1 (Figure 4C). In contrast, when A23187 was used as a secretagogue, microneme secretion by both CDPK1^G and CDPK3^G strains was strongly inhibited by 3-MB-PP1 (Figure 4C), consistent with the results observed following stimulation of egress. Intriguingly, both CDPK1^M and CDPK3^M strains showed slightly enhanced secretion in the presence versus absence of 3-MB-PP1, although the basis of this stimulation is unknown. Importantly, although the data for each strain were normalized to compare secretion in the presence versus absence of 3-MB-PP1, the differences between TgCDPK1 and TgCDPK3 were not due to strain-specific changes in baseline secretion (Supplementary Figure 4).

To confirm that the secretion defect observed following TgCDPK3 inhibition was a result of microneme secretion and not due to inhibition of proteolytic activity leading to reduced shedding of protein into the supernatant, we measured the amount of MIC2 on the parasite surface by FACS. Surface accumulation was not observed for either CDPK3^G or CDPK3^M strains, regardless of whether TgCDPK3 was inhibited or not (Figure 4C). In contrast, shutdown of the ROM4 protease, which cleaves MIC2 from the surface (Buguliskis *et al*, 2010), led to an accumulation of MIC2 on the parasite surface (ROM4 cKO + ATc; Figure 4C). These findings confirm that the observed defect in MIC2 secretion caused by TgCDPK3 inhibition is a consequence of reduced microneme secretion, and not loss of protein shedding from the parasite surface.

Activation of PKG can overcome the inhibition of TgCDPK3 during egress

Given our observations that microneme secretion required TgCDPK3 under certain conditions but not others, we hypothesized that another kinase might compensate for TgCDPK3. In *T. gondii* and other apicomplexans, use of a potent cyclic GMP-dependent protein kinase (PKG) inhibitor, known as Compound 1, has been shown to affect microneme secretion, motility, and invasion of tachyzoites (Gurnett *et al*, 2002; Wiersma *et al*, 2004; Donald *et al*, 2006). Prior studies have concluded that although TgCDPK1 shows some sensitivity to Compound 1 *in vitro*, the primary target of its action *in vivo* is PKG (Donald *et al*, 2006). Nonetheless, to rule out any secondary effect on TgCDPK1, we used the CDPK1^M strain to assess the specific inhibition of PKG by Compound 1. We first sought to establish whether PKG was playing a role in A23187-induced egress, by comparing the ability of strains to egress following incubation with different concentrations of Compound 1. Consistent with a role for PKG in egress that is independent of TgCDPK1, both CDPK1^G and CDPK1^M strains showed marked susceptibility to inhibition by Compound 1 (Figure 5A).

Having established a role for PKG in egress, we wondered whether stimulation of the PKG pathway could on its own induce egress. In *P. berghei*, male gametocyte exflagellation can be artificially triggered by the cGMP-specific phosphodiesterase inhibitor Zaprinast, which presumably increases

cGMP and leads to PKG activation (Billker *et al*, 2004; McRobert *et al*, 2008). We tested the ability of Zaprinast to trigger egress by monitoring parasite behaviour by video microscopy. We observed that within minutes of adding Zaprinast, parasites became motile and egressed from host cells, with similar kinetics to A23187-induced egress (Figure 5B; Supplementary Movie 3). To determine whether Zaprinast-induced egress works through the activation of PKG, we tested whether Compound 1 could inhibit this process. Compound 1 completely blocked the ability of Zaprinast to trigger egress, with an IC₅₀ of 0.6 μM (95% CI (0.5, 0.7); Figure 5C), suggesting that activation of PKG is both necessary and sufficient for egress.

Studies in *P. berghei* ookinetes have shown that stimulation of the PKG pathway using Zaprinast can partially compensate for loss of ookinete motility controlled by PbCDPK3 (note: not a direct orthologue despite the similar numbering) (Moon *et al*, 2009). To determine whether Zaprinast could overcome the inhibition of TgCDPK3, we treated intracellular parasites with 3-MB-PP1 and examined egress after 20 min. We observed that Zaprinast was able to overcome the inhibition of TgCDPK3 (CDPK3^G; Figure 5D), suggesting that stimulation of PKG can compensate for the role of TgCDPK3 in egress of *T. gondii*. Comparing the levels of egress triggered by either Zaprinast or A23187 showed that inhibition of CDPK3^G by 3-MB-PP1 was largely rescued by activation of the PKG pathway with Zaprinast (Figure 5E). Furthermore, Zaprinast was more effective in triggering egress of 3-MB-PP1-insensitive CDPK3^M parasites (EC₅₀ = 93 μM, 95% CI 82–106) versus the sensitive CDPK3^G parasites (EC₅₀ 246 μM, 95% CI 173–349), suggesting that the PKG and TgCDPK3 pathways work together. In contrast, 3-MB-PP1 inhibited egress of CDPK1^G parasites to a similar extent regardless of whether triggering was conducted with A23187 or Zaprinast (Figure 5E).

Discussion

We tested the previously proposed model that TgCDPK3 regulates the motor complex required for motility using a highly specific chemical-genetic approach. Our findings are consistent with a role for TgCDPK3 in microneme secretion and parasite motility, rather than acting on the motor complex directly. Specific chemical inhibition of sensitized alleles revealed that TgCDPK1 was required for both egress and invasion, while TgCDPK3 was required only for egress. Similarly, microneme secretion, which is necessary for both egress and invasion, required TgCDPK1 under all circumstances but only exhibited TgCDPK3 dependence under specific circumstances. We also revealed a likely role for PKG in egress from host cells, and provide pharmacological evidence that activation of PKG can overcome the inhibition of TgCDPK3. These results suggest that all three kinases contribute to the control of microneme secretion and hence impact egress, motility, and invasion to various degrees. The fact that under certain conditions the functions of TgCDPK3 can be circumvented by the activation of PKG, suggests that the parasite modulates input from different signals to fine-tune responses using a complex network of signalling proteins.

We previously reported that TgCDPK1 is specifically inhibited by PP analogues due to the natural occurrence of

Figure 5 PKG activation compensates for the inhibition of TgCDPK3 during egress. (A) Egress following 20 min incubation with different concentrations of Compound 1, and treatment with 2 μM A23187 for 5 min. Egress was measured as a function of lactate dehydrogenase released from host cells, and normalized to the levels resulting from parasites treated with DMSO instead of Compound 1. Means ± s.e.m., $n = 3$ experiments. (B) Strain CDPK3^M stimulated by 0.5 mM Zaprinast. Egress monitored by video microscopy. The time stamps represent minute:second after the addition of Zaprinast. (C) Egress following 20 min incubation with different concentrations of Compound 1, and treatment with 0.5 mM Zaprinast for 20 min. Means ± s.e.m., $n = 3$ experiments. (D) Egress triggered by different Zaprinast concentrations after treating strains for 20 min with 5 μM 3-MB-PP1. Egress was measured as described above. Means ± s.e.m., $n = 3$ experiments. (E) Comparison of egress following treatment for 20 min with or without 5 μM 3-MB-PP1, and triggered for 5 min with either 2 μM A23187 or 0.5 mM Zaprinast alone or in combination. Student's t -test; *** $P < 0.0005$; ** $P < 0.005$; * $P < 0.05$; n.s., $P > 0.05$; means ± s.e.m., $n = 3$ experiments.

a glycine at its gatekeeper position, a feature unique among all the active kinases in *T. gondii* (Lourido *et al*, 2010). In budding yeast, the bio-orthogonality of these PP analogues has allowed researchers to study the function of various kinases by mutating their gatekeeper residues to render them sensitive to inhibition (Bishop *et al*, 2000; Snead *et al*, 2007). Seeking to extend this chemical-genetic approach to parasite kinases, we replaced the endogenous *TgCDPK1* with an allele harbouring a methionine at the gatekeeper position to generate a resistant genetic background in which to sensitize other kinases. Using this background, we replaced the *TgCDPK3^M* allele with *TgCDPK3^G*, which is sensitive to 3-MB-PP1, in order to study its function. This manipulation of the *TgCDPK3* allele did not affect parasite replication or viability, demonstrating that this is a valid strategy for studying essential parasite kinases. By preserving the endogenous promoter, this strategy does not significantly alter the timing or level of expression of the kinase, which is unavoidable with the regulatable promoters currently available for *T. gondii*. In the case of *TgCDPK3*, it is possible that such differences in protein expression between the endogenous and regulatable alleles might have led to fitness costs that precluded the isolation of conditional

knockouts from mixed populations. Another advantage of the chemical-genetic approach over conventional conditional alleles is that it allows for rapid inhibition of these essential kinases, excluding the possibility of pleiotropic effects from loss of the kinase during development. The rarity of kinases containing small gatekeeper residues in the genome of *T. gondii* reduces the chance of off-target effects, although intermediate sensitivity is expected for kinases containing A, S, or T in the gatekeeper position, thus potentially limiting this approach in some cases. Nonetheless, our results suggest that this strategy could be generalizable to other parasite kinases given the conservation of the ATP-binding pocket in most eukaryotic serine/threonine protein kinases.

Using this chemical-genetic strategy, we were able to compare the functions of *TgCDPK1* and *TgCDPK3* at different stages of the *T. gondii* life cycle. Inhibition of either *TgCDPK1* or *TgCDPK3* led to a sustained block in egress in response to the calcium ionophore A23187, while only *TgCDPK1* was required for invasion. The magnitude of inhibition in egress was also greater for *TgCDPK3* than for *TgCDPK1*, although both showed a sustained block in egress. Together, these data provide the first evidence in *T. gondii* that the signalling requirements for invasion differ from those for egress, and

suggest that different targets are likely controlled by these distinct kinases. In contrast, most cellular processes governing motility are thought to do so globally, such as the actomyosin motor complex (Soldati and Meissner, 2004) or the secretion of adhesins from micronemes (Lourido *et al*, 2010). A similar observation of the requirement of *P. falciparum* CDPK5 in egress but not invasion of red blood cells (Dvorin *et al*, 2010) indicates an emerging theme distinguishing the signalling events required for these two processes in apicomplexans. Whether the signalling differences during egress and invasion reflect the regulation of known processes by different pathways, or the regulation of still unknown cellular responses required for egress but not invasion, will be the focus of future research. Such studies may be facilitated by the use of sensitized kinases, which we have demonstrated successfully for CDPKs, and techniques for downstream analysis of their specific targets, as described previously (Allen *et al*, 2005; Snead *et al*, 2007).

Inhibition of TgCDPK1 or TgCDPK3 reduced gliding motility of extracellular parasites to the same extent. We had previously proposed that because TgCDPK1 controls microneme secretion, it is universally required for motility during egress and invasion. In contrast, the phenotypes described for TgCDPK3 are an exception to this all-or-none model of microneme secretion. Intracellular parasites demonstrated a strong requirement for TgCDPK3 in microneme secretion as shown by the lack of PV membrane permeabilization in response to A23187. Release of DsRed from the PV normally occurs when TgPLP1 is secreted from micronemes, an early event that is necessary for ionophore induced egress (Kafsack *et al*, 2009). Our findings indicate that both TgCDPK1 and TgCDPK3 are required for microneme release when parasites are intracellular. However, the ability of parasites to invade host cells normally following TgCDPK3 inhibition argued against a general defect in microneme secretion. Direct measurement of MIC2, one of the adhesins released from micronemes, demonstrated that the two kinases were also differentially required for microneme secretion in response to different stimuli, similarly displaying a universal requirement for TgCDPK1 but a stimulus-specific requirement for TgCDPK3.

In *P. falciparum*, the orthologue of TgCDPK3, known as PfCDPK1, has been implicated in the phosphorylation of GAP45 and MTIP (Green *et al*, 2008), components of the actomyosin motor complex that are conserved in apicomplexans (Frenal *et al*, 2010) and required for motility and host-cell invasion. However, these studies only establish that sites phosphorylated by PfCDPK1, under permissive conditions *in vitro*, can also be identified in parasites, while failing to exclude the potential role of other kinases in this process. Additionally, recent studies have failed to demonstrate a functional role for some of the phosphorylation sites on GAP45, showing that they are not required for the localization or assembly of the motor complex, functions previously attributed to these modifications (Ridzuan *et al*, 2012). Moreover, knockdown of PbCDPK1 during sexual development of *P. berghei* revealed a role in ookinete maturation prior to demonstrated requirements for the motor components MyoA and MTIP (Sebastian *et al*, 2012). From our own results, three observations argue against a role for TgCDPK3 in the regulation of the motor complex:

(i) microneme secretion is independent of the motor complex (Meissner *et al*, 2002; Kafsack *et al*, 2009) yet under some conditions it was dependent on TgCDPK3, (ii) although the fraction of parasites moving was reduced upon TgCDPK3 inhibition, the speed of gliding was normal once initiated, and (iii) inhibition of TgCDPK3 did not affect invasion, which requires a functioning motor complex (Dobrowolski and Sibley, 1996; Meissner *et al*, 2002). Although we cannot rule out that under certain conditions components of the motor complex are phosphorylated by TgCDPK3 *in vivo*, such an activity would have to be either dispensable or redundant during invasion. Instead, our results indicate that TgCDPK3 regulates other cellular targets to activate motility.

Our results also support a role for PKG in egress of *T. gondii* and indicate that PKG can compensate for TgCDPK3 under some circumstances. Previous studies demonstrated that PKG contributes to microneme secretion and host-cell invasion in *T. gondii* (Wiersma *et al*, 2004) based on the use of Compound 1 that specifically targets PKG *in vivo* (Donald *et al*, 2002). Here, we show that the cGMP-specific phosphodiesterase Zaprinast induces egress of *T. gondii* from host cells. Although we have not directly monitored cGMP levels, we interpret this result to indicate that PKG activation leads to egress. This conclusion was further supported by the finding that Compound 1 completely blocked Zaprinast-induced egress. Treatment with Zaprinast also overcame the inhibition of TgCDPK3 during egress. Furthermore, the shift in EC₅₀ for Zaprinast when TgCDPK3 was active versus inactive suggests that the two pathways likely operate cooperatively. Although these studies suggest a role for PKG in compensating for TgCDPK3, further work is needed to confirm the interaction between these two kinases, including whether their roles are additive or synergistic. PKG and CDPKs belong to related groups of kinases, whose targets consist of sites enriched in basic amino acids preceding S/T, opening the possibility that they might display overlapping substrate specificities (Hanks and Hunter, 1995). The ability of different kinases to converge on a single target may allow different stimuli to regulate a single pathway, and this is a known feature of the nucleotide-activated family of kinases including PKG (Pearce *et al*, 2010). Taken together, these observations suggest that PKG acts in parallel to various CDPK-regulated pathways, providing multiple layers of regulation on a single cellular process, and allowing the parasite to respond differently to intracellular signals that govern egress versus extracellular signals that control invasion.

Materials and methods

Parasite growth and selection

T. gondii tachyzoites were grown in human foreskin fibroblasts (HFF) cultured in Dulbecco's Modified Eagles Medium (DMEM; Invitrogen) supplemented with 10% tetracycline-free FBS (HyClone), 2 mM glutamine, 10 mM HEPES (pH 7.5), and 20 µg/ml gentamicin, as previously described (Lourido *et al*, 2010). When noted, parasites were selected using growth media containing any of the following: chloramphenicol (20 µg/ml; Sigma), phleomycin (5 µg/ml; Invitrogen), ATc (1 µg/ml; Clontech), pyrimethamine (3 µM; Sigma), 25 µg/ml mycophenolic acid, and 50 µg/ml xanthine. Negative selection of *HXPRT* was performed by growing parasites in DMEM supplemented with 1% dialysed FBS (Invitrogen), 10 mM HEPES (pH 7.4), and 340 µg/ml 6-thioxanthine (Sigma).

Plasmid and strain generation

For a full description of the plasmids and strains generated for this study, see the Supplementary Methods and Supplementary Table 1. Attempts to generate a conditional knockout of TgCDPK3 were done in the TATi strain (provided by D Soldati-Favre, University of Geneva, Switzerland) following the methods previously described (Lourido *et al*, 2010). All other strains were generated in the Δ ku80 Δ hxp_{prt} background, referred to for clarity in this study as CDPK1^G (provided by V Carruthers, University of Michigan, USA). The CDPK1^M strain was generated in two stages. First, we introduced, by single site homologous recombination into the endogenous *TgCDPK1* locus, a plasmid carrying the first intron of *TgCDPK1* fused to the cDNA starting at the second exon and ending with a c-terminal c-myc tag. This plasmid contained both a mutation methionine gatekeeper and an hxp_{prt} cassette. Negative selection with 6-thioxanthine was used to remove the sequences between the c-myc tag and the endogenous 3'-UTR of *TgCDPK1*, by double homologous recombination with a construct bearing homology to both sites. The latter strain was named CDPK1^M, lacked *HXGPRT*, and contained the mutant version of *TgCDPK1* between the endogenous 5'- and 3'-UTRs. The CDPK1^M strain was transfected with a vector containing the second intron of *TgCDPK3* fused to the cDNA starting at the third exon and ending with a c-terminal Ty tag. Parasites were selected with mycophenolic acid and xanthine for *HXGPRT* present in the vector. Clones were screened by PCR for homologous recombination at the second intron such that they carried the Ty-tagged copy of *TgCDPK3*, downstream of which the endogenous allele was left without a promoter or the first two exons, and was therefore silent. The alleles were genotyped as coding for a methionine or a glycine at the gatekeeper position by allele-specific PCR. Clonal lines were maintained without selection, and no reversion to the wild-type locus was observed during the course of the study.

Immunofluorescence microscopy

Immunofluorescence staining was performed as described previously (Starnes *et al*, 2006) following permeabilization with 0.1% saponin (Sigma) with rabbit anti-HA9 (Invitrogen) and mouse anti-SAG1 (mAb DG52), followed by Alexa564-goat anti-rabbit IgG (Invitrogen) and Cy5-goat anti-mouse IgG (Jackson). Images were collected on a Zeiss LSM 510 confocal microscope and analysed using the LSM 510 Examiner software.

Sequence alignment

The published alignment of the *T. gondii* active kinases (Peixoto *et al*, 2010) was used to identify the gatekeeper residues based on the position of subdomain V (Hanks and Hunter, 1995) and the previously identified gatekeeper residue of TgCDPK1 (Lourido *et al*, 2010).

Protein purification

Full-length *TgCDPK1* and *TgCDPK3* were PCR amplified from a *T. gondii* RH cDNA library generated using the SMART cDNA synthesis kit (Clontech). The primers used (*TgCDPK1*: 5'-GCGCATATGATGGG GCAGCAGGAAAGCAC-3' and 5'-GCGCTCGAGGTTTCCGACAGAGCT TCAAGAGC-3'; *TgCDPK3*: 5'-GCGCATATGATGGGTCGCTCCACTCC AAG-3' and 5'-GCGGCGCGCGCTGCTTCACTTTGACGTCGCAG-3') contained restriction sites that were used to directionally clone the PCR product, *NdeI* or *XhoI* or *NotI*, into the pET-22b(+) vector, in frame with a c-terminal hexahistidine tag. Mutation of the gatekeeper codon, corresponding to G¹²⁸M for TgCDPK1 and M¹⁵³G for TgCDPK3, was achieved using the QuikChange II Site-Directed Mutagenesis Kit (Agilent Technologies), with specific primers designed according to manufacturer instructions. Plasmids were transformed into BL21(DE3)V2RpAcYc-LIC + LamP *Escherichia coli*, as described previously (Wernimont *et al*, 2010). Following overnight growth in Terrific Broth at 37°C, cells were cooled to 15°C, induced by addition of 1 mM isopropylthio- β -D-galactopyranoside, and returned to culture overnight. Cells were lysed in CellLyticB solution (Sigma), and proteins purified using HIS-select Nickel Affinity Gel following manufacturer's instructions (Sigma). Purified proteins were dialysed (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.125% Chelex 100) and stored in 20% glycerol at -20°C. Protein purity and concentration were determined by SDS-PAGE followed by staining with SYPRO Ruby (Invitrogen).

Kinase assays

Kinase assays were conducted using a peptide-based ELISA. Syntide-2 peptide (10 mg/ml; Calbiochem) was used to coat 96-well plates by overnight incubation in carbonate coating buffer (pH 9.6) at 4°C. Following washing in Tris-tween (50 mM Tris-HCl, pH 7.5, 0.2% Tween-20), plates were blocked with 3% BSA in Tris-tween for 2 h at room temperature, and all further washes were done with Tris-tween. Kinase reactions were conducted at 30°C for 20 min in kinase buffer (20 mM HEPES, pH 7.5, 10 mM MgCl₂, 1 mM DTT, 2.5 mM CaCl₂, 0.1 mM EGTA, 0.005% Tween-20) containing appropriate amounts of ATP (*K_m* for each enzyme) and enzyme dilutions (see below). Phosphorylation was detected with mAb MS-6E6 (MBL), followed by peroxidase-conjugated goat-anti-mouse IgG, developed with the substrate 3,3',5,5'-tetramethylbenzidine and detected by absorbance at 450 nm.

Each kinase preparation was individually tested in the assay to establish its half-maximal activity from a dose-response curve plotted in Prism (GraphPad). The *K_m* for ATP was determined for each enzyme tested at its half-maximum, by serial dilution of ATP and plotting in Prism (GraphPad). The sensitivity of each enzyme to 3-MB-PP1 was tested at its individual half-maximal activity and *K_m* for ATP. Triplicate samples were run for all assays. Data were analysed using Prism (GraphPad) to determine IC₅₀ values by plotting normalized, log-transformed data (x axis), using non-linear regression analysis as a sigmoidal dose-response curve with variable slope.

Western blotting

Lysates from 1 × 10⁷ parasites per lane were resolved by SDS-PAGE, transferred onto nitrocellulose membranes, and blotted with rabbit anti-TgALD1, mouse anti-c-myc (Santa Cruz Biotechnology, mouse anti-Ty (mAb BB2) (Bastin *et al*, 1996), or mouse anti-MIC2 (mAb 6D10). The signal was detected using IRDye 680CW conjugated donkey anti-rabbit IgG (LI-COR Biosciences) and IRDye 800CW conjugated goat anti-mouse IgG (LI-COR Biosciences) on the Odyssey infrared imager (LI-COR Biosciences). Images were processed and analysed using the Odyssey infrared imaging system software.

Invasion assays

Invasion was measured as previously described (Huynh *et al*, 2003). Briefly, parasites harvested in invasion medium (DMEM containing 20 mM HEPES pH 7.5, supplemented with 3% FBS) were incubated in 5 μ M 3-MB-PP1 or media containing an equivalent amount of vehicle (DMSO), for 20 min at 37°C, before invasion. Subconfluent HFF monolayers in 24-well plates were infected with the treated parasites (5 × 10⁶ per well) and allowed to invade for 20 min. Monolayers were then fixed and stained to distinguish intracellular from extracellular parasites. Samples were performed in triplicate for each experiment and parasite numbers per field were normalized to host-cell nuclei.

Video microscopy of egress and PV permeabilization

Egress and PVM permeabilization were analysed by video microscopy as described previously (Håkansson *et al*, 1999). Sample dishes were allowed to equilibrate for 5 min on the heated stage before the addition of either 2 μ M A23187 (EMD) or 0.5 μ M Zaprinast (EMD). Vacuoles were imaged for up to 10 min after stimulant addition. To quantify vacuole permeabilization, parasites were transfected with p30-DsRed (Kafsack *et al*, 2009) (using a plasmid provided by F Dzierzinski, McGill University, Canada), allowed to infect HFF monolayers, and imaged 24 h post infection. The fluorescence intensity within a 6- μ m-diameter circular region within each vacuole was measured using the Openlab software. The values for each vacuole were normalized against the starting (100%) values for that particular vacuole. When noted, 5 μ M 3-MB-PP1 was added 20 min prior to imaging.

Gliding assays

Gliding was monitored by video microscopy as described above. Parasites were harvested in intracellular buffer (5 mM NaCl, 142 mM KCl, 1 mM MgCl₂, 2 mM EGTA, 5.6 mM glucose, 25 mM HEPES, pH 7.2 adjusted with KOH) containing 5 μ M 3-MB-PP1, and allowed to settle onto glass chamber slides (Lab-Tek). The supernatant was carefully removed and replaced again with intracellular buffer containing 5 μ M 3-MB-PP1 and the dishes were kept at 37°C

for up to 1 h. Prior to imaging the media in each dish was exchanged for extracellular buffer (141.8 mM NaCl, 5.8 mM KCl, 1 mM MgCl₂, 1 mM CaCl₂, 5.6 mM glucose, 25 mM HEPES, pH 7.2 adjusted with NaOH) containing 5 µM 3-MB-PP1, the dish was allowed to equilibrate 4 min on the heated stage, and imaged 4 min, at 1 frame per second. Images were collected and analysed with Openlab v. 4.1 (Improvision). Two to three movies were taken for each strain during a given experiment. The numbers of parasites performing each type of motility were normalized to the total number of parasites in each movie. Gliding speed was determined by measuring the distance travelled in a given time by three motile parasites for each movie.

Egress assays

Egress was quantified by measuring lactate dehydrogenase (LDH) release from host cells as a consequence of rupture. In a 96-well format, HFF monolayers were infected with 5×10^5 parasites per well. Twenty-four hours post infection, monolayers were washed once with mammalian Ringer's buffer supplemented with 1% FBS, and then incubated 20 min in 50 µl of the same buffer containing the specified amount of 3-MB-PP1, Compound 1 (obtained from MERCK & CO., Inc.) or vehicle control (DMSO). To induce egress, 50 µl of the same solution was added to each well, but additionally containing either 4 µM A23187 or 1.0 mM Zaprinast, unless otherwise noted. Supernatants were collected after further incubation for 5 or 30 min and LDH measured using the CytoTox 96 assay (Promega) according to manufacturer's instructions. Values were normalized to total lysis (100%) and uninfected (0%). None of the compounds tested caused significant LDH release in uninfected cells. Samples were assayed in triplicate for each experiment. Data were analysed using Prism (GraphPad) to determine IC₅₀ values by plotting normalized, log-transformed data (x axis), using non-linear regression analysis as a sigmoidal dose-response curve with variable slope.

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Secretion assays and FACS analysis

Microneme secretion was assayed by monitoring the release of MIC2 into the culture medium, as described previously (Carruthers *et al*, 1999). Parasites were pretreated for 15 min with 5 µM 3-MB-PP1 or vehicle control (DMSO) at 37°C before stimulation. Secretion was stimulated by treatment for 5 min with either 2% ethanol or 2 µM A23187, at 37°C. Parasite lysis was monitored by the release of actin into the medium: this level remained undetectable in all experiments presented. Samples were resolved by SDS-PAGE and western blots were quantified as described above. Following secretion, parasites were stained for surface accumulation of MIC2, as previously described (Buguliskis *et al*, 2010). FACS data were analysed using FloJo 7.4 software (Tree Star Inc.), gated on the parasites by forward and side scatter, and used to generate histograms.

Supplementary data

Supplementary data are available at *The EMBO Journal* Online (<http://www.embojournal.org>).

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Conflict of interest

The authors declare that they have no conflict of interest.

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