Plasmodium berghei calcium-dependent protein kinase 3 is required for ookinete gliding motility and mosquito midgut invasion

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Summary

Apicomplexan parasites critically depend on a unique form of gliding motility to colonize their hosts and to invade cells. Gliding requires different stage and species-specific transmembrane adhesins, which interact with an intracellular motor complex shared across parasite stages and species. How gliding is regulated by extracellular factors and intracellular signalling mechanisms is largely unknown, but current evidence suggests an important role for cytosolic calcium as a second messenger. Studying a *Plasmodium berghei* gene deletion mutant, we here provide evidence that a calcium-dependent protein kinase, CDPK3, has an important function in regulating motility of the ookinete in the mosquito midgut. We show that a *cdpk3⁻* parasite clone produces morphologically normal ookinetes, which fail to engage the midgut epithelium, due to a marked reduction in their ability to glide productively, resulting in marked reduction in malaria transmission to the mosquito. The mutant was successfully complemented with an episomally maintained *cdpk3* gene, restoring mosquito transmission to wild-type level. *cdpk3⁻* ookinetes maintain their full genetic differentiation potential when microinjected into the mosquito haemocoel and *cdpk3⁻* sporozoites produced in this way are motile and infectious, suggesting an ookinete-limited essential function for CDPK3.

Introduction

To complete their life cycle, malaria parasites require three invasive 'zoite'-stages. (i) Merozoites invade erythrocytes in the bloodstream of the vertebrate host. (ii) Ookinetes are the motile zygotes that form in the blood meal of the mosquito vector, penetrate the peritrophic matrix and the mosquito midgut epithelium and then replicate as an oocyst on the gut wall. (iii) Sporozoites are highly motile parasite stages that emerge from the cyst and penetrate cells of the mosquito salivary gland and various tissues of the vertebrate host, before invading and differentiating inside parenchymal cells of the liver. Malarial invasive life cycle stages differ in morphology, size, target tissues and modes and speed of movement, but the molecular mechanisms driving motility and invasion are nevertheless thought to be very similar (Baum *et al.*, 2006).

Much of our molecular understanding of apicomplexan gliding motility comes from studies on tachyzoites of *Toxoplasma gondii* and on malaria sporozoites [recently reviewed by Kappe *et al.* (2004) and Keeley and Soldati (2004)]. Motility and invasion require the regulated apical release of adhesive proteins or protein complexes from secretory organelles, such as micronemes, rhoptries and dense granules, which typically form part of the apical complex that characterizes all apicomplexan invasive stages. The actomyosin-based motor complex that powers motility is located between the parasite's plasma membrane and the underlying inner membrane complex (IMC). The bridge between the motor and the extracellular substrate is provided by a family of micronemal adhesins typified by the thrombospondin-related anonymous protein (TRAP) of the sporozoite. Its translocation to the posterior end of the parasite generates the force for gliding motility and invasion (Sultan *et al.*, 1997). In malaria parasites, each invasive stage uses a different member of the TRAP family, probably reflecting the diverse extracellular substrates and host tissues, with which the malarial life cycle stages interact. In ookinetes the circumsporozo-

ite/TRAP-related protein (CTRP) fulfils this function in the mosquito midgut (Dessens *et al.*, 1999; Yuda *et al.*, 1999; Templeton *et al.*, 2000), while erythrocyte invasion is thought to involve the merozoite TRAP homologue, MTRAP (Baum *et al.*, 2006).

In marked contrast to the surface adhesins, intracellular components of the motor complex are thought to be highly conserved across all invasive life cycle stages and apicomplexan parasite species (Baum *et al.*, 2006). The conserved cytoplasmic tails of all TRAP-family adhesins engage the motor indirectly, by binding tetramers of the glycolytic enzyme fructose-1,6-bisphosphate aldolase, which in turn is capable of nucleating short actin filaments (Buscaglia *et al.*, 2003; Jewett and Sibley, 2003). The force for parasite gliding and invasion is generated by the dynamic interaction of these actin filaments with a class XIV myosin, MyoA, which is essential for gliding (Meissner *et al.*, 2002). MyoA is anchored to the IMC by a MyoA tail-interacting protein (MTIP; Bergman *et al.*, 2003), which has properties of a myosin light chain (Herm-Gotz *et al.*, 2002), and by two glideosome-associated proteins, GAP45 and GAP50 (Gaskins *et al.*, 2004; Baum *et al.*, 2006).

Studies on *T. gondii* tachyzoites point towards an important role of calcium as a second messenger regulating gliding. Microneme secretion, motility and invasion occur independently of extracellular or host cell calcium levels, but require the release of calcium from intracellular stores, most likely the endoplasmic reticulum (Carruthers and Sibley, 1999; Lovett *et al.*, 2002). Artificially raising cytosolic calcium levels by pharmacological agents, such as calcium ionophores, is sufficient to trigger microneme release in *T. gondii* tachyzoites (Carruthers and Sibley, 1999) and *Plasmodium falciparum* sporozoites (Gantt *et al.*, 2000). Studying individual gliding tachyzoites, Lovett and Sibley (2003) observed rapid increases in cytosolic calcium accompanying or immediately preceding bursts of motility *in vitro*, illustrating the key role calcium plays as a second messenger.

Protein kinases are emerging as important downstream effectors of the calcium signal(s) regulating gliding (Dobrowolski *et al.*, 1997; Carruthers *et al.*, 1999; Wiersma *et al.*, 2004). Candidates include members of the family of calcium-dependent protein kinases (CDPK), one of which, TgCDPK2, was identified as a prominent target for a kinase inhibitor, KT5926 (IC$_{50}$ *c.* 100 nM), that blocks tachyzoite motility in *T. gondii* (Kieschnick *et al.*, 2001). Members of the CDPK family have been identified in plants, green algae, ciliates and apicomplexan parasites (see Harper and Harmon, 2005 for review). They characteristically combine within the same protein a serine/threonine protein kinase domain with a regulatory carboxy-terminal calmodulin-like domain composed of four calcium-binding EF hands. The *P. falciparum* genome encodes five typical members of the CDPK family but none of these have so far been implicated in regulating gliding motility. CDPK1 is secreted from asexual blood stage parasites by an unusual, acylation-dependent mechanism (Moskes *et al.*, 2004), but its molecular function remains unknown. Another family member, CDPK4, plays a key role in cell cycle regulation during male gamete formation (Billker *et al.*, 2004). CDPK3 was also speculated to have a function in gametocytes, based on the transcriptional upregulation of the *cdpk3* gene in sexual stages of *P. falciparum* (Li *et al.*, 2000). However, most recently a *Plasmodium berghei* mutant with a disrupted *cdpk3* gene has provided strong evidence for an essential function of CDPK3 in the ookinete (Ishino *et al.*, 2006). The authors of that study conclude CDPK3 has no fundamental function in gliding, but defining a novel transition point in the midgut invasion process, they suggest that CDPK3 is required for ookinetes to traverse a gel-like layer surrounding the blood meal of *Anopheles stephensi* mosquitoes, in which *cdpk3⁻* ookinetes migrating out of the blood meal were concluded to become trapped.

Studying a similar *cdpk3* gene knock-out mutant in *P. berghei* we here confirm an important function of CDPK3 in malaria transmission. Importantly, and in marked contrast to the previous study, we show that *cdpk3⁻* ookinetes are severely compromised in productive gliding motility *in vitro*. The crucial role of CDPK3 in regulating gliding motility provides a satisfactory explanation for the strongly reduced transmission of the *cdpk3⁻* mutant. We argue that the developmental defect of *cdpk3⁻* ookinetes can be understood within the traditional model of mosquito midgut invasion by the ookinete, and that no revision of this model is thus required.

Results

Ookinetes need CDPK3 to penetrate the mosquito midgut epithelium

We generated a *cdpk3* gene knock-out clone in *P. berghei* by replacing the protein-coding regions corresponding to amino acids 181–543 with a resistance marker (Fig. 1A). Integration of the targeting construct and deletion of the *cdpk3* gene was verified by diagnostic PCR and Southern blot analysis (Fig 1B and C). *cdpk3⁻* parasites had no detectable defect in asexual erythrocytic development in mice and normal numbers of male and female gametocytes were formed (data not shown). When cultured *in vitro*, *cdpk3⁻* gametocytes differentiated into gametes, fertilized, and the zygotes developed into morphologically mature ookinetes. Conversion rates from female gametes to ookinetes were equally high in wild-type (80.3 ± 7.8%, *n* = 3) and *cdpk3⁻* parasites (76.0 ± 5.6%, *n* = 3), showing that CDPK3 has no essential function up to the stage of ookinete formation. However, when *A. stephensi* mosqui-

A

Wild-type

ol56 ol44 ol62 *cdpk3* ol46

ol45

ol63

ol47

X   X
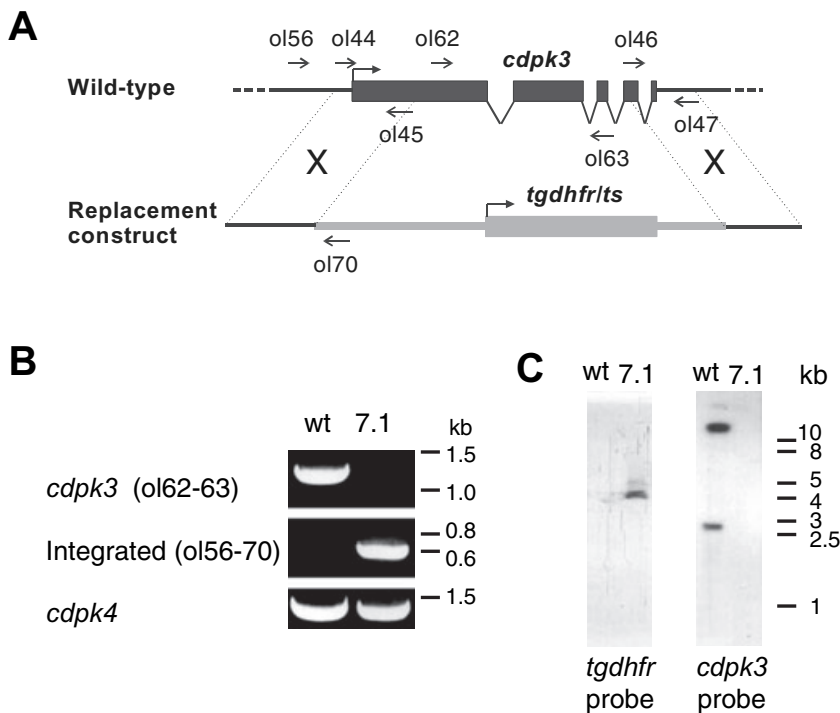
Replacement construct

tgdhfr/ts

ol70

Fig. 1. Targeted disruption of the *cdpk3* gene.
A. Illustration of the *cdpk3* gene locus and the disruption construct used.
B. Diagnostic PCR showing absence of a *cdpk3*-specific product and presence of an integration-specific product in *cdpk3⁻* clone 7.1.
C. Southern blot analysis of EcoRI/HindIII-digested genomic DNA, showing presence of the *tgdhfr/ts* resistance gene in clone 7.1 and two bands diagnostic of the intact *cdpk3* locus in wild type. The presence of two bands is due to HindIII restriction sites within the *cdpk3* gene.

B

wt 7.1 kb

cdpk3 (ol62-63) — 1.5 / 1.0

Integrated (ol56-70) — 0.8 / 0.6

cdpk4 — 1.5

C

wt 7.1   wt 7.1   kb

— 10 / 8
— 5
— 4
— 3 / 2.5
— 1

tgdhfr probe   *cdpk3* probe

toes were allowed to feed on mice carrying *cdpk3⁻* gametocytes, the number of oocysts present on the midgut epithelium 10 days later was reduced by about 98% compared with wild type (Table 1), a marked decrease that is in good agreement with the 99% blockade observed by Ishino *et al.* (2006) with a separately derived *cdpk3⁻* parasite. We conclude that CDPK3 is important in ookinetes to give rise to oocyst infections. To test this hypothesis, equal numbers of wild-type or *cdpk3⁻* ookinetes from *in vitro* cultures were offered to mosquitoes in membrane feeders and oocyst numbers counted 10 days later. Infectivity of *cdpk3⁻* ookinetes was reduced by 99% (Table 1, Experiments 8 and 9). When we re-introduced the *pbcdpk3* gene into the knock-out clone on an episomally maintained plasmid (Fig. 2A), ookinetes expressed a CDPK3 protein with a carboxy-terminal epitope tag (Fig. 2B), and their infectivity was restored to wild-type levels (Fig. 2C). These results confirm that a CDPK3-

dependent defect at or after the ookinete stage is responsible for the dramatic reduction in oocyst numbers.

To test whether *cdpk3⁻* ookinetes retained the genetic potential to undergo ookinete-oocyst development we microinjected equal numbers of *in vitro* cultured wild-type and *cdpk3⁻* ookinetes into the haemocoel of *A. stephensi*. When given direct access to the mosquito in this way, both wild-type and *cdpk3⁻* ookinetes matured ectopically into oocysts, gave rise to heavy salivary gland infections within 21 days, and produced salivary gland sporozoites that were infectious to mice (Table 2). We conclude that *cdpk3⁻* ookinetes retain their full developmental potential but have a specific defect in overcoming the physical barrier posed by the midgut epithelium. We next asked whether *cdpk3⁻* ookinetes could bind and invade midgut epithelial cells. Equal numbers of wild-type and *cdpk3⁻* ookinetes (800 per µl) were fed to *A. stephensi* in a membrane feeding apparatus. Midguts were dissected within

Table 1. Oocyst counts from *A. stephensi* mosquitoes fed either directly on infected mice (experiments 1–7) or on membrane feeders containing uninfected mouse blood mixed with different numbers of ookinetes from cultures (experiments 8 and 9).

	Clone	Oocysts per mosquito[a]	Prevalence[b]	n[c]
Experiments 1–7 (fed on infected mice)	Wild type	375.41	97.14 (3.80)	7
	cdpk3⁻	8.25	77.81 (21.99)	8
Experiment 8 (fed on 800 ookinetes per µl)	Wild type	38.96	100.00	1
	cdpk3⁻	0.15	15.00	1
Experiment 9 (fed on 2200 ookinetes per µl)	Wild type	148.36	100.00	1
	cdpk3⁻	0.07	10.00	1

a. Geometric mean oocyst numbers per mosquito are given.
b. Prevalence of infections is given as the percentage of mosquitoes infected (standard deviation).
c. *n* gives the number of independent infectious feeds. For each feed 20–30 mosquitoes were dissected.

A

2.7 kb   *cdpk3*   2 x myc

pISK217

hdhfr

B

anti   anti
c-myc   CDPK4

cdpk3⁻ *cdpk3-myc*   *cdpk3⁻* *cdpk3-myc*
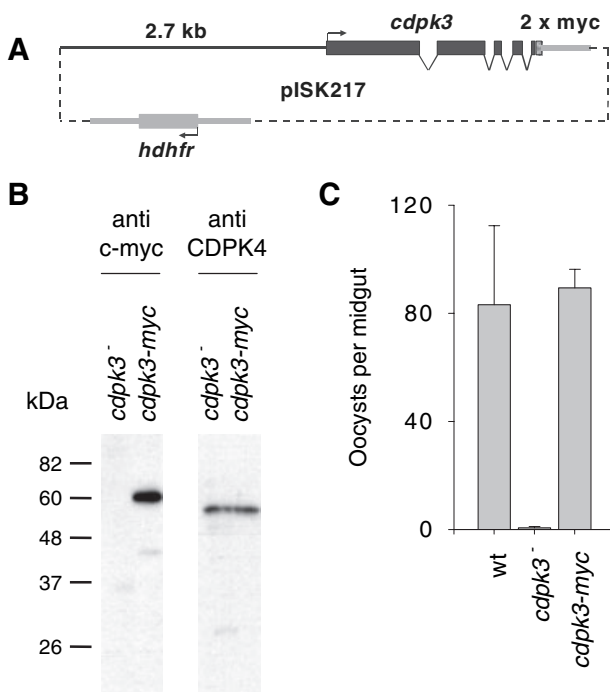
kDa

82 —
60 —
48 —
37 —
26 —

C

Fig. 2. Functional complementation of the *cdpk3⁻* mutant.
A. Schematic illustration of the complementation plasmid, showing the *pbcdpk3* gene fused to an N-terminal double c-myc epitope tag under the control of 2.7 kb upstream sequence and a generic 3′UTR from the *pbdhfr/ts* gene.
B. Western blot analysis of protein extracts from 10^5 ookinetes of the *cdpk3⁻* clone 7.1 and a CDPK3 myc-complemented parasite population. The blot was first probed with the anti-myc mouse monoclonal antibody 9E10 (Sigma, UK). As a loading control the membrane was then stripped and re-probed with an rabbit polyclonal serum raised against *T. gondii* CDPK2 (Kieschnick *et al.*, 2001) that recognizes CDPK4 in *P. berghei* (Billker *et al.*, 2004).
C. Oocyst numbers counted 10 days after mosquitoes were allowed to feed on blood containing 800 ookinetes per μl. Averages oocyst numbers and standard deviations from two experiments using independent ookinetes culture are shown. Twenty-five midguts were dissected in each replicate experiment.

2–4 h and processed for immunostaining to visualize ookinetes associated with the gut epithelium (Fig. 3). On average, 34.9 wild-type ookinetes (±14.3; $n = 15$ midguts) were associated with the midgut cells. In marked contrast,

only 1.1 *cdpk3⁻* ookinetes (±0.9; $n = 14$ midguts) had accessed the gut.

CDPK3 is required for productive gliding motility of ookinetes

Recognizing that the reduced infectiousness of *cdpk3⁻* ookinetes could result from a fundamental defect in motility, we compared the ability of wild-type and *cdpk3⁻* ookinetes to glide on glass slides *in vitro*. We exploited the fact that *P. berghei* ookinetes purified from cultures are usually found in aggregates. We find that their dispersal relies on gliding motility and is sensitive to the anti-cytoskeletal agent cytochalasin D. When cocultured with insect cells, aggregates of wild-type ookinetes dispersed rapidly (Fig. 4A and Movie S1). In marked contrast, *cdpk3⁻* ookinetes showed a strongly reduced ability to glide productively and aggregates thus failed to disperse (Fig. 4B and Movie S2). Motility of *cdpk3⁻* ookinetes was abnormal, showing frequent flexing, bending, twirling and pendular motions, but only rare bouts of translocation over short distances (Movie S2). These observations were confirmed in a quantitative analysis, in which the proportion of *cdpk3⁻* ookinetes that dispersed from aggregates was scored and compared with wild type, either in the absence or in the presence of cytochalasin D (Fig. 4C). Prompted by the reported conservation among the motor components across invasive parasites stages (Baum *et al.*, 2006), we asked whether sporozoite motility was also CDPK3-dependent. Twenty to 25% of wild-type and *cdpk3⁻* sporozoites obtained from ookinete-injected mosquitoes produced typical circular motility traces on glass slides (Fig. 4E) that were indistinguishable from wild type in shape and size (Fig. 4D). Motility traces were of similar length, corresponding to on average 1.7 ± 0.5 ($n = 23$) complete revolutions in wild type and 1.9 ± 0.8 ($n = 23$) in the *cdpk3⁻* sporozoites. Motility in *cdpk3⁻* sporozoites was consistent with their normal infectivity for mice. Together with the absence of a *cdpk3⁻* phenotype in asexual parasite development, these data suggest a key role

Table 2. Salivary gland sporozoites in *A. stephensi* that were either fed on or injected with *in vitro* cultured ookinetes.

	Clone	Sporozoites in glands[a]	Infectious to mice[b]	
			By bite	By injection
Fed on 800 ookinetes per μl	Wild type	19 800 (±4 900)	2/2	n.d.
	cdpk3⁻	0 (±0)	0/2	n.d.
Injected with 600 ookinetes	Wild type	13 000 (±3 200)	2/2	n.d.
	cdpk3⁻	4 800 (±200)	2/2	4/4

a. Arithmetic mean sporozoites per mosquito are given ($\pm$ standard deviation from repeat experiments with different ookinete cultures). For each data point 18–30 pairs of glands were examined.
b. Infectivity to mice was determined by allowing mosquitoes to feed on naïve mice or by injection of isolated sporozoites into a tail vein. Whenever sporozoites were observed in the glands, these were highly infectious, resulting in blood infections from day 4 post infection. Data are given as mice positive/mice infected; n.d., not done.
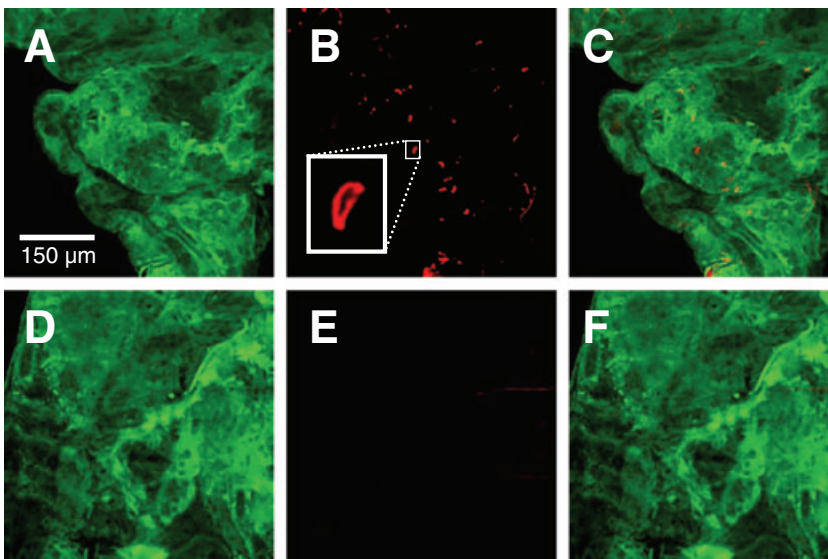
Fig. 3. *cdpk3⁻* ookinetes fail to associate with the mosquito midgut epithelium. Representative midgut epithelial sheets are shown that were dissected 2 h after mosquitoes had fed on equal numbers of cultured wild-type (A–C) or *cdpk3⁻* (D–F) ookinetes. Midguts were fixed, permeabilized and immunostained with an antibody against *A. stephensi* annexin to visualized the epithelium (panels A and D), and against the ookinete surface antigen P28 (panels B and E). All images are stacks of confocal sections. A higher magnification inset in (B) shows an ookinete associated with the midgut epithelium.
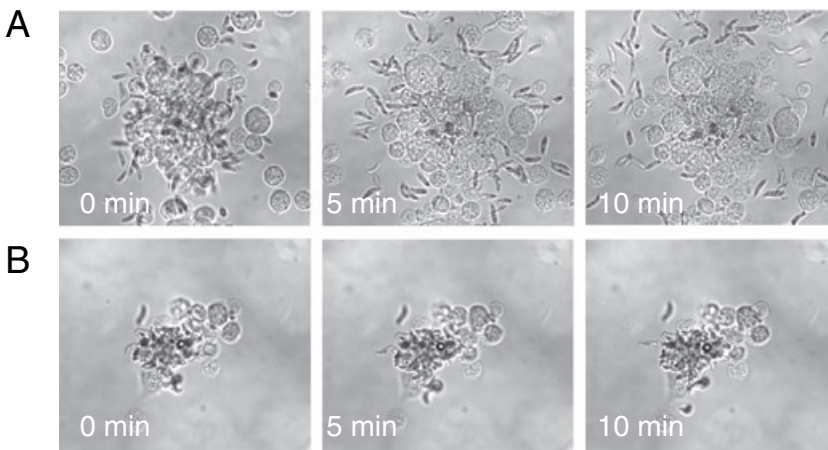

Fig. 4. Motility of wild-type and *cdpk3⁻* ookinetes and sporozoites.
A. Wild-type ookinetes purified from *in vitro* cultures disperse from aggregates after being incubated with insect cells.
B. *cdpk3⁻* ookinetes fail to show productive gliding motility. Each sequence of images is representative of at least three independent experiments with ookinetes cultured from different infected mice.
C. Quantification of ookinete dispersal. The proportion of dispersed ookinetes was determined after 24 h of culture in the absence and presence of cytochalasin D. Arithmetic means ± standard deviations from three independent experiments are shown.
D and E. Motility trails of wild-type (D) and *cdpk3⁻* sporozoites (E) obtained from salivary glands of ookinete-injected mosquitoes. Trails were visualized using a monoclonal antibody directed against the circumsporozoite protein, which labels both the sporozoites (arrows) and the trails of surface proteins shed by gliding sporozoites. The upper row of each panel shows corresponding immunofluorescence and phase contrast images of the same sporozoites. The bottom row of each panel illustrates additional representative circumsporozoite trails.
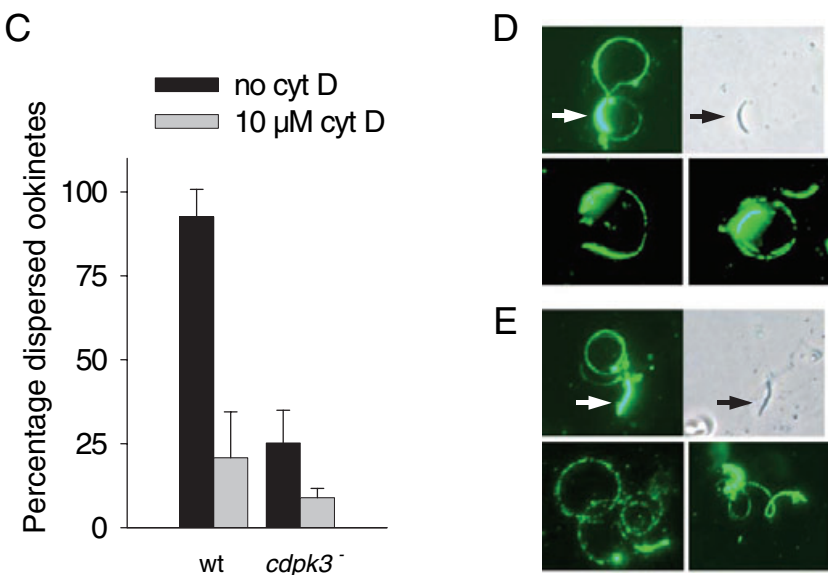
for CDPK3 in regulating productive gliding motility specifically at the ookinete stage.

Discussion

Due to their stage-specific expression patterns, the plant-like CDPK of apicomplexan parasites have the potential to translate the ubiquitous second messenger calcium into different, stage-specific cellular responses in the parasite. We have previously identified a specific essential role for CDPK4 in the initiation of cell cycle events in male gametocytes of *P. berghei* (Billker *et al.*, 2004). Our current analysis of a *cdpk3⁻* deletion mutant shows that this protein kinase has no essential function during asexual and sexual intraerythrocytic parasite development and that fertilization and the differentiation of the zygote into morphologically normal ookinetes do not rely on CDPK3. Furthermore, when ookinetes are given direct access to the mosquito haemocoel, CDPK3 is not required for their further differentiation into oocyst and infectious sporozoites. However, our analysis concurs with recent work studying a similar mutant (Ishino *et al.*, 2006) in concluding that *cdpk3⁻* ookinetes are severely affected in their ability to establish an infection in the mosquito, because they fail to access the midgut epithelium. Our functional analysis is supported by a complementation experiment, which shows that re-introducing a *cdpk3* allele into the *cdpk3⁻* mutant on an episomally maintained plasmid restores infectivity.

Using video microscopy and a quantitative ookinete spreading assay, we show here for the first time that *cdpk3⁻* ookinetes have a severely reduced ability to move productively on glass slides and to disperse from aggregates *in vitro*. This motility defect is sufficient to explain the reduced ability of *cdpk3⁻* ookinetes to engage and invade the mosquito midgut epithelium. Importantly, *cdpk3⁻* ookinetes are still capable of some bending, twirling and pendular movements and limited translocation, which may account for their residual infectivity of 1–2% of wild type. How CDPK3 regulates ookinete gliding remains to be defined. Gliding in other apicomplexan zoites can be regulated through the apical secretion of microneme proteins that form the bridge between the extracellular substrate and the intracellular motor. Microneme secretion is calcium-dependent in many apicomplexan zoites, including malarial sporozoites (Gantt *et al.*, 2000) and in *T. gondii* tachyzoites it is a precondition for motility and host cell invasion (Carruthers *et al.*, 1999). Our unpublished observations suggest ookinete motility is stimulated in the presence of insect cells and – consistent with the regulatory function of CDPK3 – is inhibited by a chelator of cytosolic calcium. However, whether the secretion of the microneme adhesin CTRP is controlled by cytosolic calcium levels and CDPK3 is unknown. Looking at two

micronemal proteins of the ookinete, SOAP and CTRP, we have been unable with currently available reagents to establish a suitable microneme secretion assay to examine this possibility.

Recent *in vivo* imaging studies showed that ookinetes and sporozoites not only initiate and terminate gliding, but display twirling and bending actions, circular and straight-segment movements, and shape changes, in addition to invasive motility (Frischknecht *et al.*, 2004; Vlachou *et al.*, 2004; Amino *et al.*, 2006). Furthermore, oocyst-derived sporozoites exhibit chemotaxis towards a heat-stable factor from mosquito salivary glands (Akaki and Dvorak, 2005) and it is tempting to speculate that ookinetes may be able to similarly direct their motility towards the midgut epithelium. This surprisingly large behavioural repertoire probably requires other layers of regulation in addition to microneme secretion. Potential phosphorylation-dependent control points include (i) the nucleation, stabilization or destabilizing of actin filaments by aldolase or other actin-binding molecules, such as toxofilin, which is subject to dynamic phosphorylation in *T. gondii* (Delorme *et al.*, 2003), or (ii) the activity of myosin, which may involve the myosin light chain-like MTIP protein (Bergman *et al.*, 2003). It will be interesting to examine whether molecular components of the motor complex are substrates for CDPK3. The ookinete-specific essential function for CDPK3 is unexpected in view of the universal role of calcium in zoite motility and considering that all invasive stages of *Plasmodium* are thought to use the same molecular motor (Baum *et al.*, 2006). We speculate that in merozoites and sporozoites other members of the CDPK family may have functions similar to CDPK3 in the ookinete.

Our quantitative assay showing that productive gliding in *cdpk3⁻* ookinetes is fundamentally disturbed is in marked disagreement with a recent report investigating ookinete gliding in a similar mutant, but on a gel-like substrate (Matrigel™) that contains mouse basement membrane proteins (Ishino *et al.*, 2006). Gliding of *cdpk3⁻* ookinetes on the gel surface is reported (but not quantified) as being normal and is contrasted with the failure of *cdpk3⁻* ookinetes to penetrate the gel matrix. Analysing mosquito blood meals 20 h after an infectious feed by transmission electron microscopy, Ishino *et al.* (2006) further show that *cdpk3⁻* ookinetes accumulate in an electron-lucent layer surrounding the blood meal. Likening this layer to the Matrigel™ used *in vitro*, a novel, CDPK3-dependent transition point in the midgut invasion process, is proposed, at which ookinetes would have to switch from CDPK3-independent gliding through the blood meal to a different, CDPK3-dependent mode of motility to penetrate the layer surrounding the blood meal, before eventually gaining access to the epithelium. In an attempt to reconcile our data with the previous report, we performed a quantitative estimate of ookinete

gliding in the two movies published by Ishino *et al.* (2006). Wild-type ookinetes migrating in Matrigel™ cover a distance corresponding to on average 5.61 times their own length (standard deviation = 1.25, *n* = 16 ookinetes) in 45 min. During the same period *cdpk3⁻* ookinetes on the gel surface, while clearly showing some movement, progress by only 0.83 times their own length (standard deviation = 1.33, *n* = 20 ookinetes), and many ookinetes not included in our estimate fail to disperse from an aggregate on the gel surface, just as we have observed with our *cdpk3* KO clone*.*

As we show here that *cdpk3⁻* ookinetes have a general motility defect, it is no longer necessary to assume CDPK3 has a more specific function for ookinetes when they penetrate the digested periphery of the blood meal. It is well established that haemolysis and digestion proceed centripetally from the periphery of the blood mass inwards (Clements, 1992) and immotile objects resistant to digestive enzymes, such as *cdpk3⁻* ookinetes, must therefore accumulate in the narrow lysis zone that forms between the undigested blood and the midgut epithelium. Finally, our membrane feeding assays showing that mature cultured *cdpk3⁻* ookinetes still fail to infect when brought in immediate intimate contact with the midgut epithelium, rule out any specific function for CDPK3 at the hypothetical transition point between the undigested erythrocyte mass and the peripheral layer as suggested by Ishino *et al.* (2006). We conclude that a fundamental motility defect is entirely sufficient to explain the phenotype of *cdpk3⁻* ookinetes within the traditional model of ookinete interactions with the blood meal. Future studies should focus on dissecting the exact molecular functions of CDPK3 and other CDPK family members to assess the importance of this group of plant-like protein kinases as targets for pharmacological intervention.

Experimental procedures

Parasite maintenance, in vitro *culture and transmission to mosquitoes*

The *P. berghei* ANKA wild-type strain 2.34 and transgenic lines were maintained in phenyl hydrazine-treated Theiler's Original outbred mice and transmitted by *A. stephensi*, strain SD500, as described previously (Sinden *et al.*, 2002). The course of infections and gametocyte production were monitored on Giemsa-stained blood films. Ookinetes were grown *in vitro* by culturing of gametocyte-infected mouse blood, quantified and then purified by selective lysis of erythrocytes as described previously (Sinden *et al.*, 2002). Macrogamete-to-ookinete conversion rates were determined in 24 h cultures by live immunofluorescence microscopy with a Cy3-conjugated monoclonal antibody against the macrogamete/zygote/ookinete surface antigen p28, as previously described (Tewari *et al.*, 2005a). For transmission experiments, batches of 50 mosquitoes, which had been starved overnight, were either fed directly on anaesthetized infected mice on day 4 of a blood-induced infection, or were allowed to feed on a membrane feeder apparatus loaded with a suspension of cultured ookinetes in blood from an uninfected mouse. All feeds were done for 20 min at 19°C. Unfed mosquitoes were removed the following day. Oocysts were counted on dissected midguts on day 10 after feeding. Alternatively, adult female mosquitoes were infected by microinjection of 60 nl of cultured ookinetes into the thorax, using a Nanoject II hand-held microinjector (Drummond, USA). Wild-type and *cdpk3⁻* ookinetes for injection were cultured *in vitro* from infected blood, from which leucocytes had been removed (Sinden *et al.*, 2002). Ookinete numbers in 20–24 h cultures were determined in a haemocytometer, ookinete density adjusted to 10^4 per μl, and cultures back-filled into borosilicate injector needles.

Deletion and complementation of the cdpk3 *gene*

A targeting vector for *pbcdpk3* was constructed in plasmid pBS-DHFR, in which polylinker sites flank a *T. gondii dhfr/ts* expression cassette conveying resistance to pyrimethamine. A 608 bp fragment comprising 5′ upstream sequence followed by the first 543 bp of exon1 of *cdpk3* was PCR amplified from *P. berghei* genomic DNA and inserted into KpnI and ApaI restriction sites upstream of the *dhfr/ts* cassette of pBS-DHFR. A 686 bp fragment comprising the last two exons and 3′ flanking region of *pbcdpk3* was then inserted downstream of the *dhfr/ts* cassette. The replacement construct was excised as a KpnI/BamHI fragment and used for the electroporation of cultured *P. berghei* schizonts as described (Menard and Janse, 1997; Billker *et al.*, 2004). Following dilution cloning of drug-resistant parasites, genotyping of a *cdpk3⁻* clone, named 7.1, was carried out by Southern blot analysis and diagnostic PCR across the predicted integration site in principle as described previously for other gene deletions (Billker *et al.*, 2004). A complementation vector was constructed in two steps in plasmid p141, a generic version of the myc-tagging vector p142 described previously (Billker *et al.*, 2004). First a 2.7 kb fragment representing the *cdpk3* 5′ upstream intergenic region was PCR-amplified from *P. berghei* genomic DNA using oligonucleotides ol175 (3′-GAGA<u>GGTACC</u>GGGAAAATGAT GTAACTATAAGATG, restriction site underlined) and ol176 (3′-CAT<u>GCTAGC</u>TTTTACGTATTAAACTATTTCCAAAAT) and inserted into KpnI abd NheI digested p142, giving rise to p142/11. Next the complete *cdpk3* genomic sequence, including all introns, but excluding the TAA stop codon, was amplified from genomic DNA using primers ol177 (3′-CAT<u>GCTAGC</u>ATGAATCAATTATGTGTAGAAAG) (restriction site underlined) and ol178 (3′-GAGA<u>GGGCCC</u>ACACTT TAGTTTCATCATTTCGCAA) and inserted into the NheI and ApaI restriction sites of 142/11, downstream of the putative *cdpk3* promoter and 5′UTR sequence, in frame with a double c-myc epitope tag, followed by the stop codon and 0.5 kb of the *P. berghei dhfr/ts* 3′UTR. The resulting plasmid, p217, was confirmed by sequencing. p217 was introduced into the *cdpk3⁻* clone 7.1 by electroporation and maintained as an episome by selection for the *hdhfr* gene as described previously (Billker *et al.*, 2004).

Staining of infected midguts

Midguts from fed mosquitoes were dissected at the indicated times in 1.85% paraformaldehyde in phosphate- buffered saline (PBS), pH 7.3. The blood meal was removed and the midgut epithelia were fixed for 1 h in 3.7% paraformaldehyde, washed once in PBS and then incubated for 30 min in PBS, 0.1% TritonX-100, 5% normal goat serum (NGS, Jackson Immunoresearch). Gut epithelial cells were stained with a mouse monoclonal antibody against mosquito annexin (Kotsyfakis *et al.*, 2005), diluted in PBS, 5% NGS, for at least 1 h at room temperature. Three 10 min washes in PBS, 5% NGS, were followed by an incubation with Alexa488-conjugated goat anti-mouse antibody (Molecular Probes) for 1 h. Following another three washes, ookinetes were visualized with a Cy3-conjugated mouse monoclonal antibody 13.1 directed against the ookinete surface protein P28 (Winger *et al.*, 1988). Stained midgut epithelial sheets were mounted in Vectashield (Vector Laboratories) and analysed by confocal microscopy.

Motility assays

Ookinetes purified from *in vitro* cultures were mixed on a microscope slide with *Aedes aegytpi* Mos20 cells in M199 medium (Sigma, UK), supplemented with 10% bovine calf serum. Ten microlitres of cell suspension was placed under Vaseline-rimmed coverslips and digital images recorded every 30 s for 10 min. Wild-type and mutant ookinetes were analysed in parallel. To quantify motility, aggregated ookinetes purified from *in vitro* cultures were seeded in duplicates into cocultures with Mos20 cells in LabTek 8-well chamber slides (Nunc, UK). 10 μM cytochalasin D (Calbiochem, UK) was added to one of the two wells for each sample. After 20–24 h ookinetes were visualized by staining with the Pb70 antibody against an ookinete cytoskeletal protein (Siden-Kiamos *et al.*, 2000). Dispersed ookinetes and those that remained in aggregates were counted separately in randomly selected fields under the microscope. At least 200 ookinetes were counted in each sample. The proportion of all ookinetes that had dispersed is a measure of the motility of the ookinetes, while the sample containing cytochalasin D indicates the random occurrence of single ookinetes, as ookinetes are non-motile under these conditions (I. Siden-Kiamos and C. Louis, unpublished). To examine sporozoite gliding, we visualized motility trails using the 3D11 mouse monoclonal antibody (Yoshida *et al.*, 1980), generously provided by Laurent Renia, against the circumsporozoite protein, using a previously described protocol (Tewari *et al.*, 2005b).

References

Akaki, M., and Dvorak, J.A. (2005) A chemotactic response facilitates mosquito salivary gland infection by malaria sporozoites. *J Exp Biol* **208:** 3211–3218.

Amino, R., Thiberge, S., Martin, B., Celli, S., Shorte, S., Frischknecht, F., and Menard, R. (2006) Quantitative imaging of *Plasmodium* transmission from mosquito to mammal. *Nat Med* **12:** 220–224.

Baum, J., Richard, D., Healer, J., Rug, M., Krnajski, Z., Gilberger, T.W., *et al.* (2006) A conserved molecular motor drives cell invasion and gliding motility across malaria life-cycle stages and other apicomplexan parasites. *J Biol Chem* **281:** 5197–5208.

Bergman, L.W., Kaiser, K., Fujioka, H., Coppens, I., Daly, T.M., Fox, S., *et al.* (2003) Myosin A tail domain interacting protein (MTIP) localizes to the inner membrane complex of *Plasmodium* sporozoites. *J Cell Sci* **116:** 39–49.

Billker, O., Dechamps, S., Tewari, R., Wenig, G., Franke-Fayard, B., and Brinkmann, V. (2004) Calcium and a calcium-dependent protein kinase regulate gamete formation and mosquito transmission in a malaria parasite. *Cell* **117:** 503–514.

Buscaglia, C.A., Coppens, I., Hol, W.G., and Nussenzweig, V. (2003) Sites of interaction between aldolase and thrombospondin-related anonymous protein in *Plasmodium*. *Mol Biol Cell* **14:** 4947–4957.

Carruthers, V.B., and Sibley, L.D. (1999) Mobilization of intracellular calcium stimulates microneme discharge in *Toxoplasma gondii*. *Mol Microbiol* **31:** 421–428.

Carruthers, V.B., Giddings, O.K., and Sibley, L.D. (1999) Secretion of micronemal proteins is associated with *Toxoplasma* invasion of host cells. *Cell Microbiol* **1:** 225–235.

Clements, A.N. (1992) *The Biology of Mosquitoes*, Vol. I. London: Chapman & Hall.

Delorme, V., Cayla, X., Faure, G., Garcia, A., and Tardieux, I. (2003) Actin dynamics is controlled by a casein kinase II and phosphatase 2C interplay on *Toxoplasma gondii* toxofilin. *Mol Biol Cell* **14:** 1900–1912.

Dessens, J.T., Beetsma, A.L., Dimopoulos, G., Wengelnik, K., Crisanti, A., Kafatos, F.C., and Sinden, R.E. (1999) CTRP is essential for mosquito infection by malaria ookinetes. *EMBO J* **18:** 6221–6227.

Dobrowolski, J.M., Carruthers, V.B., and Sibley, L.D. (1997) Participation of myosin in gliding motility and host cell invasion by *Toxoplasma gondii*. *Mol Microbiol* **26:** 163–173.

Frischknecht, F., Baldacci, P., Martin, B., Zimmer, C., Thiberge, S., Olivo-Marin, J.C., *et al.* (2004) Imaging movement of malaria parasites during transmission by *Anopheles* mosquitoes. *Cell Microbiol* **6:** 687–694.

Gantt, S., Persson, C., Rose, K., Birkett, A.J., Abagyan, R., and Nussenzweig, V. (2000) Antibodies against thrombospondin-related anonymous protein do not inhibit *Plasmodium* sporozoite infectivity *in vivo*. *Infect Immun* **68:** 3667–3673.

Gaskins, E., Gilk, S., DeVore, N., Mann, T., Ward, G., and Beckers, C. (2004) Identification of the membrane receptor of a class XIV myosin in *Toxoplasma gondii*. *J Cell Biol* **165:** 383–393.

Harper, J.F., and Harmon, A. (2005) Plants, symbiosis and parasites: a calcium signalling connection. *Nat Rev Mol Cell Biol* **6:** 555–566.

Herm-Gotz, A., Weiss, S., Stratmann, R., Fujita-Becker, S., Ruff, C., Meyhofer, E., *et al.* (2002) *Toxoplasma gondii*

myosin A and its light chain: a fast, single-headed, plus-end-directed motor. *EMBO J* **21:** 2149–2158.

Ishino, T., Orito, Y., Chinzei, Y., and Yuda, M. (2006) A calcium-dependent protein kinase regulates *Plasmodium* ookinete access to the midgut epithelial cell. *Mol Microbiol* **59:** 1175–1184.

Jewett, T.J., and Sibley, L.D. (2003) Aldolase forms a bridge between cell surface adhesins and the actin cytoskeleton in apicomplexan parasites. *Mol Cell* **11:** 885–894.

Kappe, S.H., Buscaglia, C.A., Bergman, L.W., Coppens, I., and Nussenzweig, V. (2004) Apicomplexan gliding motility and host cell invasion: overhauling the motor model. *Trends Parasitol* **20:** 13–16.

Keeley, A., and Soldati, D. (2004) The glideosome: a molecular machine powering motility and host-cell invasion by Apicomplexa. *Trends Cell Biol* **14:** 528–532.

Kieschnick, H., Wakefield, T., Narducci, C.A., and Beckers, C. (2001) *Toxoplasma gondii* attachment to host cells is regulated by a calmodulin-like domain protein kinase. *J Biol Chem* **276:** 12369–12377.

Kotsyfakis, M., Vontas, J., Siden-Kiamos, I., and Louis, C. (2005) The annexin gene family in the malaria mosquito *Anopheles gambiae*. *Insect Mol Biol* **14:** 555–562.

Li, J.L., Baker, D.A., and Cox, L.S. (2000) Sexual stage-specific expression of a third calcium-dependent protein kinase from *Plasmodium falciparum*. *Biochim Biophys Acta* **1491:** 341–349.

Lovett, J.L., and Sibley, L.D. (2003) Intracellular calcium stores in *Toxoplasma gondii* govern invasion of host cells. *J Cell Sci* **116:** 3009–3016.

Lovett, J.L., Marchesini, N., Moreno, S.N., and Sibley, L.D. (2002) *Toxoplasma gondii* microneme secretion involves intracellular Ca(2+) release from inositol 1,4,5-triphosphate (IP(3))/ryanodine-sensitive stores. *J Biol Chem* **277:** 25870–25876.

Meissner, M., Schluter, D., and Soldati, D. (2002) Role of *Toxoplasma gondii* myosin A in powering parasite gliding and host cell invasion. *Science* **298:** 837–840.

Menard, R., and Janse, C.J. (1997) Gene targeting in malaria parasites. *Methods* **13:** 148–157.

Moskes, C., Burghaus, P.A., Wernli, B., Sauder, U., Durrenberger, M., and Kappes, B. (2004) Export of *Plasmodium falciparum* calcium-dependent protein kinase 1 to the parasitophorous vacuole is dependent on three N-terminal membrane anchor motifs. *Mol Microbiol* **54:** 676–691.

Siden-Kiamos, I., Vlachou, D., Margos, G., Beetsma, A., Waters, A.P., Sinden, R.E., and Louis, C. (2000) Distinct roles for pbs21 and pbs25 in the *in vitro* ookinete to oocyst transformation of *Plasmodium berghei*. *J Cell Sci* **113:** 3419–3426.

Sinden, R.E., Butcher, G., and Beetsma, A. (2002) Maintenance of the *Plasmodium berghei* life cycle. In *Methods in Molecular Medicine, Vol. 72: Malaria Methods and Protocols.* Doolan, D.L. (ed.). Totowa, NJ: Humana, pp. 25–40.

Sultan, A.A., Thathy, V., Frevert, U., Robson, K.J., Crisanti, A., Nussenzweig, V., *et al.* (1997) TRAP is necessary for gliding motility and infectivity of plasmodium sporozoites. *Cell* **90:** 511–522.

Templeton, T.J., Kaslow, D.C., and Fidock, D.A. (2000) Developmental arrest of the human malaria parasite *Plasmodium falciparum* within the mosquito midgut via CTRP gene disruption. *Mol Microbiol* **36:** 1–9.

Tewari, R., Dorin, D., Moon, R., Doerig, C., and Billker, O. (2005a) An atypical mitogen-activated protein kinase controls cytokinesis and flagellar motility during male gamete formation in a malaria parasite. *Mol Microbiol* **58:** 1253–1263.

Tewari, R., Rathore, D., and Crisanti, A. (2005b) Motility and infectivity of *Plasmodium berghei* sporozoites expressing avian *Plasmodium gallinaceum* circumsporozoite protein. *Cell Microbiol* **7:** 699–707.

Vlachou, D., Zimmermann, T., Cantera, R., Janse, C.J., Waters, A.P., and Kafatos, F.C. (2004) Real-time, *in vivo* analysis of malaria ookinete locomotion and mosquito midgut invasion. *Cell Microbiol* **6:** 671–685.

Wiersma, H.I., Galuska, S.E., Tomley, F.M., Sibley, L.D., Liberator, P.A., and Donald, R.G. (2004) A role for coccidian cGMP-dependent protein kinase in motility and invasion. *Int J Parasitol* **34:** 369–380.

Winger, L.A., Tirawanchai, C.S., Nicholas, J., Carter, H.E., Smith, J., and Sinden, R.E. (1988) Ookinete antigens of *P. berghei*. Appearance on the zygote surface of a Mr 21 kD determinant identified by transmission blocking monoclonal antibodies. *Parasite Immunol* **10:** 193–207.

Yoshida, N., Nussenzweig, R.S., Potocnjak, P., Nussenzweig, V., and Aikawa, M. (1980) Hybridoma produces protective antibodies directed against the sporozoite stage of malaria parasite. *Science* **207:** 71–73.

Yuda, M., Sakaida, H., and Chinzei, Y. (1999) Targeted disruption of the *Plasmodium berghei* CTRP gene reveals its essential role in malaria infection of the vector mosquito. *J Exp Med* **190:** 1711–1716.

Supplementary material

The following supplementary material is available for this article online:

Movie S1. Motility of wild-type ookinetes; the parasites move out of a central aggregate of ookinetes. Purified wild-type ookinetes were mixed with Mos20 cells under a Vaseline-rimmed coverslip. Images were captured every 30 s for 10 min.

Movie S2. Motility of *cdpk3⁻* ookinetes; the ookinetes show some residual motility but the majority of the ookinetes do not display translocational motility and thus are not able to move out of the aggregate. Purified mutant ookinetes were mixed with Mos20 cells under a Vaseline-rimmed coverslip. Images were captured every 30 s for 10 min.

This material is available as part of the online article from http://wwww.blackwell-synergy.com