

SOAP, a novel malaria ookinete protein involved in mosquito midgut invasion and oocyst development

Johannes T. Dessens,^{1*} Inga Sidén-Kiamos,² Jacqui Mendoza,¹ Vassiliki Mahairaki,^{2,3} Emad Khater,¹ Dina Vlachou,^{2,4} Xiao-Jin Xu,⁵ Fotis C. Kafatos,⁴ Christos Louis,^{2,3} George Dimopoulos⁵ and Robert E. Sinden¹

¹Department of Biological Sciences, Sir Alexander Fleming Building, Imperial College London, South Kensington Campus, SW7 2AZ, UK.

²Institute of Molecular Biology and Biotechnology, FORTH, Heraklion, Crete, Greece.

³Department of Biology, University of Crete, Heraklion, Crete, Greece.

⁴European Molecular Biology Laboratories, Meierhofstrasse 1, Heidelberg, Germany.

⁵Department of Biological Sciences, Centre for Molecular Microbiology and Infection, The Flowers Building, Imperial College London, South Kensington Campus, SW7 2AZ, UK.

Summary

An essential, but poorly understood part of malaria transmission by mosquitoes is the development of the ookinetes into the sporozoite-producing oocysts on the mosquito midgut wall. For successful oocyst formation newly formed ookinetes in the midgut lumen must enter, traverse, and exit the midgut epithelium to reach the midgut basal lamina, processes collectively known as midgut invasion. After invasion ookinete-to-oocyst transition must occur, a process believed to require ookinete interactions with basal lamina components. Here, we report on a novel extracellular malaria protein expressed in ookinetes and young oocysts, named secreted ookinete adhesive protein (SOAP). The SOAP gene is highly conserved amongst *Plasmodium* species and appears to be unique to this genus. It encodes a predicted secreted and soluble protein with a modular structure composed of two unique cysteine-rich domains. Using the rodent malaria parasite *Plasmodium berghei* we show that SOAP is targeted to the micronemes and forms high molecular mass complexes via disulphide bonds. Moreover, SOAP interacts strongly with mos-

quito laminin in yeast-two-hybrid assays. Targeted disruption of the SOAP gene gives rise to ookinetes that are markedly impaired in their ability to invade the mosquito midgut and form oocysts. These results identify SOAP as a key molecule for ookinete-to-oocyst differentiation in mosquitoes.

Introduction

Malaria transmission by mosquitoes starts with the uptake of male and female gametocytes from parasite-infected blood. Following rapid gametogenesis and fertilisation, ookinetes develop in the blood bolus in the mosquito midgut. Newly developed ookinetes avoid digestion by traversing the midgut wall, where they transform into oocysts. Thousands of sporozoites subsequently develop in each oocyst, which invade the mosquito salivary glands and are introduced into the vertebrate host by mosquito bite to complete the malaria transmission cycle (Sinden, 1997).

Ookinete-to-oocyst development constitutes a bottleneck in the malaria parasite life cycle (Sinden, 1999). Successful oocyst development requires specific interactions between ookinete and mosquito molecules, interactions of which still little is known. Putative carbohydrate-like molecules from the mosquito midgut have been identified that may act as receptors or ligands for ookinete invasion (Rudin and Hecker, 1989; Zieler *et al.*, 1999), whereas midgut basal lamina components such as laminin and collagen are strongly implicated in oocyst transition (Weathersby, 1952; Warburg and Miller, 1992; Adini and Warburg, 1999; Schneider and Shahabuddin, 2000; Vlachou *et al.*, 2001; Arrighi and Hurd, 2002). Only a handful of extracellular ookinete proteins have been reported that are either conclusively or putatively involved in ookinete-to-oocyst development. These include (i) chitinases, secreted enzymes involved in penetrating the peritrophic membrane, a chitin-containing sac that forms around the blood meal (Shahabuddin *et al.*, 1993; Vinetz *et al.*, 2000; Dessens *et al.*, 2001); (ii) circumsporozoite- and TRAP-related-protein (CTRP), a transmembrane protein involved in ookinete motility and invasion (Dessens *et al.*, 1999; Yuda *et al.*, 1999; Templeton *et al.*, 2000); (iii) P25 and P28, two GPI-anchored surface proteins which have multiple and partially redundant functions involving ookinete development/survival, midgut invasion and

Accepted 28 March, 2003 *For correspondence. E-mail j.dessens@imperial.ac.uk; Tel. (+44) 207 5945424; Fax (+44) 207 5945424.

none of the PxSOAP molecules displayed clear homologies with proteins in other organisms, indicating that these molecules constitute a new class of cysteine-rich proteins unique to *Plasmodium*.

PbSOAP is expressed in ookinetes and targeted to the micromeres

The identification of the *PbSOAP* gene from our ookinete gene-enriched subtraction cDNA library (Dessens *et al.*, 2000) pointed to its expression in ookinetes. Indeed, Northern blot analysis using the *PbSOAP*-specific cDNA as a probe detected an abundant messenger RNA of approximately 1 kb in total RNA derived from *in vitro* cultured ookinetes, whereas no signal was obtained in RNA samples from purified asexual blood stage parasites or

gametocytes (Fig. 2A). After prolonged exposure a signal corresponding to *PbSOAP* was detectable in the gametocyte sample (data not shown), which we suspect may be the result of partial gametocyte activation during purification. As such, the transcription pattern of *PbSOAP* is very similar to that of *PbCTRP* (Dessens *et al.*, 1999; Yuda *et al.*, 1999).

To further investigate the expression and subcellular localization of *PbSOAP*, antiserum was raised against recombinant GST/SOAP fusion protein (see *Experimental procedures*). Immunofluorescent antibody staining of parasites from *in vitro* ookinete cultures with the anti-SOAP immune serum obtained clearly labelled ookinete stages (Fig. 2B), whereas a control immune serum raised against recombinant GST gave no labelling (data not shown). Staining of other parasite stages was not

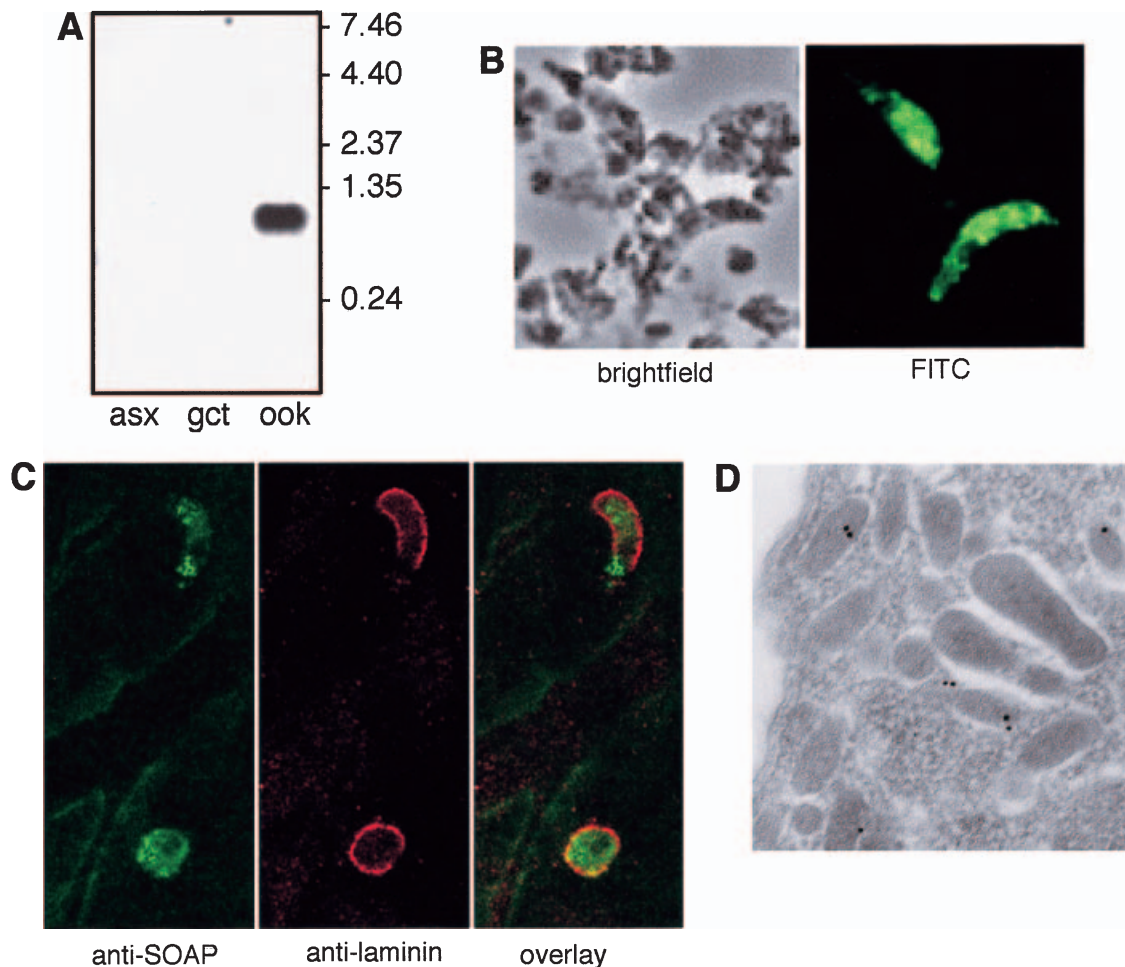


Fig. 2. Expression and localization of *PbSOAP*.

- A. Northern blot hybridization of purified total RNA from asexual blood stages (asx), gametocytes (gct) and ookinetes (ook).
 B. Immunofluorescent antibody staining of *in vitro* cultured ookinetes with anti-SOAP immune serum (FITC-labelled).
 C. Immunofluorescent antibody staining of 24 h parasite infected midgut with anti-SOAP immune serum (FITC-labelled) and anti-laminin immune serum (Texas red-labelled) showing ookinete and young oocyst.
 D. Immunogold antibody staining of ookinete thin section with anti-SOAP immune serum, showing labelled micronemes.

observed with the exception of retort-like stages (i.e. immature ookinetes), indicating that PbSOAP expression starts early in ookinete development. This expression pattern is fully consistent with the transcription pattern (Fig. 2A). Staining for PbSOAP appeared intracellular with the exception of the nucleus. Non-permeabilized ookinetes did not stain, indicating PbSOAP is not present on the parasite surface. In the majority of ookinetes examined, the staining pattern showed a clear polarization towards the apical end of the cells (Fig. 2B), indicative of the protein being targeted to the micronemes as previously described for PbCTRP (Dessens *et al.*, 1999; Yuda *et al.*, 1999). PbSOAP was also detected in 24 h parasite-infected mosquito midguts, staining ookinetes as well as young oocysts (i.e. rounded-up ookinetes on the basal side of the gut) (Fig. 2C).

Subsequent immunogold labelling of PbSOAP in thin sections of 24 h *in vitro* cultured ookinetes resulted in a large majority of gold particles associated with the micronemes (Fig. 2D). The signal obtained by immunogold labelling was relatively weak compared to that obtained in immunofluorescence (Fig. 2B), most likely due to the use

of thin sections versus whole mount. Quantitative assessment of gold distribution in a sample of 10 representative cells showed 58 particles (85%) associated with micronemal structures, five particles associated with the endoplasmic reticulum, and five particles not obviously associated with organellar structures. Control anti-GST immune serum gave no labelling (data not shown). These results are consistent with micronemal targeting of PbSOAP.

PbSOAP gene disruption

To investigate the function of PbSOAP we generated transgenic parasites by insertion of a modified *Toxoplasma gondii* dihydrofolate reductase/thymidylate synthase (DHFR/TS) gene cassette, conferring resistance to the antimalarial drug pyrimethamine, into the *PbSOAP* gene by double homologous recombination (Fig. 3A) (van Dijk *et al.*, 1995; Waters *et al.*, 1997). The DHFR/TS cassette was inserted at nucleotide position 400 of the *PbSOAP* sequence, upstream of domain II (Fig. 3A). The integrity of two cloned transgenic parasite populations

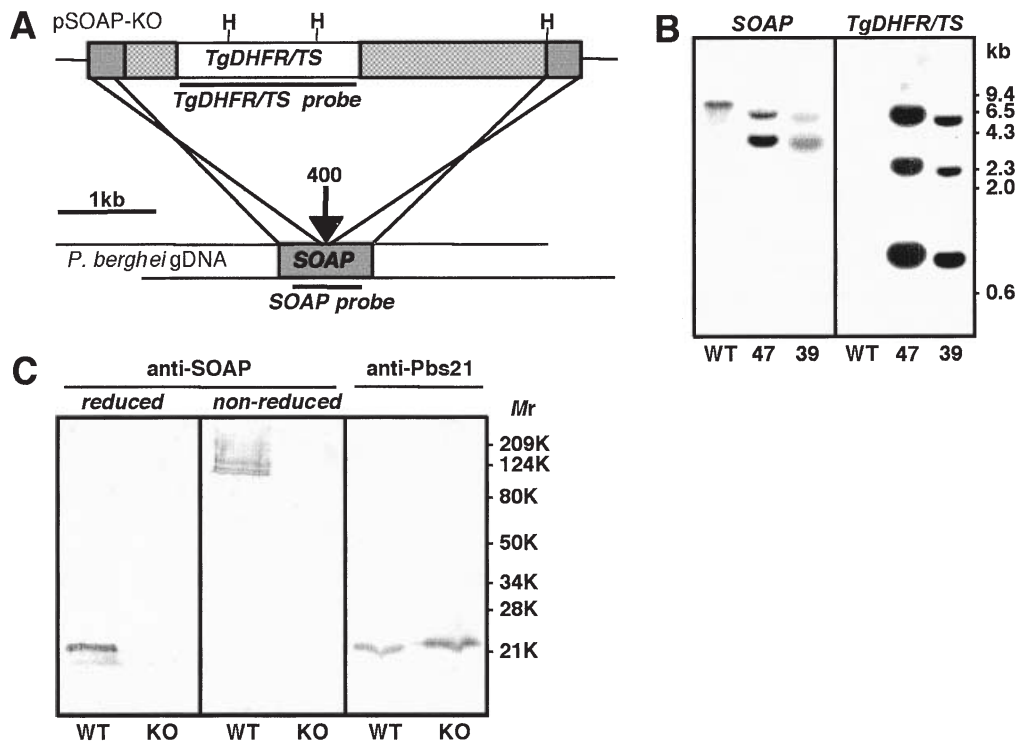


Fig. 3. Construction of *SOAP*-disrupted parasites.

A. Schematic diagram of targeted gene disruption strategy. Indicated are the DHFR/TS cassette flanked by *PbSOAP* crossover sequences (dark grey) and the relative positions of the *HincII* sites (H) in the transfection vector pSOAP-KO.

B. Southern blot of *HincII*-digested genomic DNA of WT and *SOAP*-KO parasites clones 47 and 39, hybridized with *SOAP* probe and *DHFR/TS* probe.

C. Western blot of WT and *SOAP*-KO ookinete homogenates with anti-*SOAP* immune serum and monoclonal antibody 13.1, recognising Pbs21 (P28).

Table 1. Effect of PbSOAP knockout on *Plasmodium berghei* oocyst development in vector-competent *Anopheles* mosquitoes.

| Expt | Mosquito | Type of feed (clone) ^a | Mean oocyst no. ± sem (no. of midguts scored) ^b of mosquitoes infected with: | | % of WT |
|------|--------------------------------|-----------------------------------|-----------------------------------------------------------------------------------------|-------------------------------|---------|
| | | | WT | SOAP-KO | |
| 1 | <i>A. stephensi</i> | gct (49) | 60.1 ± 3.5 (140) | 13.4 ± 1.1 ^c (139) | 22 |
| 2 | <i>A. stephensi</i> | gct (39) | 61.3 ± 4.7 (150) | 13.6 ± 1.5 ^c (150) | 22 |
| 3 | <i>A. stephensi</i> | gct (49) | 79.7 ± 3.7 (145) | 27.4 ± 1.4 ^c (150) | 34 |
| 4 | <i>A. stephensi</i> | gct (39) | 66.2 ± 4.5 (128) | 21.8 ± 1.8 ^c (125) | 33 |
| 5 | <i>A. stephensi</i> | ook (49) | 56.9 ± 3.1 (150) | 23.1 ± 2.0 ^c (121) | 41 |
| 6 | <i>A. stephensi</i> | ook (39) | 10.6 ± 0.77 (99) | 4.4 ± 0.49 ^c (101) | 40 |
| 7 | <i>A. stephensi</i> | ook (49) | 31.6 ± 1.8 (102) | 4.6 ± 0.41 ^c (104) | 15 |
| 8 | <i>A. stephensi</i> | ook (39) | 2.2 ± 0.24 (100) | 0.49 ± 0.07 ^c (85) | 22 |
| 9 | <i>A. gambiae</i> ^d | gct (49) | 6.5 ± 2.4 (19) | 1.3 ± 0.57 ^c (20) | 20 |

a. Gametocyte (gct) or ookinete (ook) feed, and SOAP-KO clone used.

b. Each experiment is based on pooled data from three mice (gct) and three membrane feeders (ook).

c. Significantly different ($P < 0.01$) from value for WT parasite-infected control group as calculated by Mann-Whitney *U*-test.

d. *A. gambiae* G3 *P. berghei* susceptible strain.

(named SOAP-KO, clones 39 and 47) was assessed by Southern blot analysis of *HincII*-digested genomic DNA. As expected, a probe corresponding to *PbSOAP* (no *HincII* sites present) gave rise to a single band in the parental wild-type (WT) parasites, but two bands in the SOAP-KO parasites (Fig. 3B), demonstrating disruption of the *PbSOAP* gene. Conversely, a probe corresponding to the *DHFR/TS* gene (two *HincII* sites present) gave rise to three DHFR/TS-specific bands in the SOAP-KO parasites, but no signal in the WT parasite sample (Fig. 3B). These combined results confirmed correct integration of the DHFR/TS cassette into the *PbSOAP* locus.

PbSOAP knockout phenotypes

SOAP-KO parasites were morphologically indistinguishable from WT parasites in Giemsa-stained blood smears and normal differentiation of gametocytes into ookinetes both *in vitro* and *in vivo* was observed (data not shown). To test PbSOAP expression in the transgenic parasites, homogenates of *in vitro* cultured ookinetes were fractionated in SDS-containing polyacrylamide gels and subjected to Western blot analysis using the anti-SOAP immune serum. Under reducing conditions a single band of approximately M_r 21 000 was obtained in WT parasites; in contrast, no signal was detected in the SOAP-KO ookinete sample (Fig. 3C), confirming successful disruption of PbSOAP expression. Under non-reducing conditions a large number of high molecular mass bands were detected, which were absent in the SOAP-KO sample (Fig. 3C). The presence of these bands points to a high level of disulphide bonding of the PbSOAP molecules. Both WT and SOAP-KO parasite samples gave comparable signals with anti-Pbs21 (P28) antibodies (Fig. 3C). These results demonstrate that the SOAP-KO parasites are PbSOAP null mutants.

To assess the effects of PbSOAP knockout on mosquito infection, SOAP-KO and WT parasites were fed to vector-competent *Anopheles* mosquitoes and compared for their ability to form oocysts, a measure of parasite infectivity. Reductions in oocysts numbers ranging between 60 and 85% were consistently observed in SOAP-KO parasite-infected mosquitoes, and similar transmission phenotypes were observed in two independent clonal populations of the SOAP-KO parasite (Table 1). Comparable levels of oocyst reduction were observed when mosquitoes were infected by gametocyte or ookinete feeds (Table 1). In gametocyte feeds ookinetes must first develop in the midgut lumen over a period of 20–24 h. During this period of development, blood meal ingestion triggers a cascade of reactions leading to the expression and activation of digestive proteases and the formation of a peritrophic membrane (Berner *et al.*, 1983; Müller *et al.*, 1993). In ookinete feeds controlled numbers of ookinetes are fed to the mosquitoes that proceed immediately with midgut invasion in the absence of a developed peritrophic matrix and in low levels of digestive enzymes (Dessens *et al.*, 2001). The comparable phenotypes obtained from gametocyte and ookinete feeds thus indicate that neither the number of ookinetes developing in the midgut, nor the effects of the peritrophic membrane or digestive midgut enzymes are likely to be factors contributing to the reductions in oocyst development observed.

Although oocyst numbers were markedly reduced in SOAP-KO parasite-infected mosquitoes (Table 1), those oocysts that developed were morphologically indistinguishable from WT oocysts of comparable age, and formed large numbers of sporozoites similar to WT oocysts. Sporozoites from the SOAP-KO parasite appeared morphologically normal and successfully invaded the salivary glands (data not shown). Indeed,

mosquitoes that were infected with SOAP-KO sporozoites successfully transmitted the parasite to mice. Parasites from such sporozoite-induced infections retained their phenotype in subsequent mosquito infections, an observation consistent with stable disruption of the gene. These observations combined with a lack of PbSOAP staining in sporozoites indicate the protein is not involved in sporozoite development, nor in sporozoite infectivity to the insect or vertebrate host. Instead, our results show that PbSOAP plays an important role during ookinete-to-oocyst development in mosquitoes.

Mosquitoes generate an array of innate immune responses to ookinete invasion including antimicrobial peptides and nitric oxide production (Dimopoulos *et al.*, 1997; 1998; Luckhart *et al.*, 1998; Han *et al.*, 2000). We investigated whether PbSOAP played a role involving the mosquito immune response. At 24 h post infection SOAP-KO parasites elicited an upregulation in the transcription of defensin and cecropin genes, molecular markers for innate immune response in ookinete-infected midguts (Dimopoulos *et al.*, 1997; Vizioli *et al.*, 2000) (Fig. 4A). We also used *A. gambiae* L3-5 mosquitoes, which are refrac-

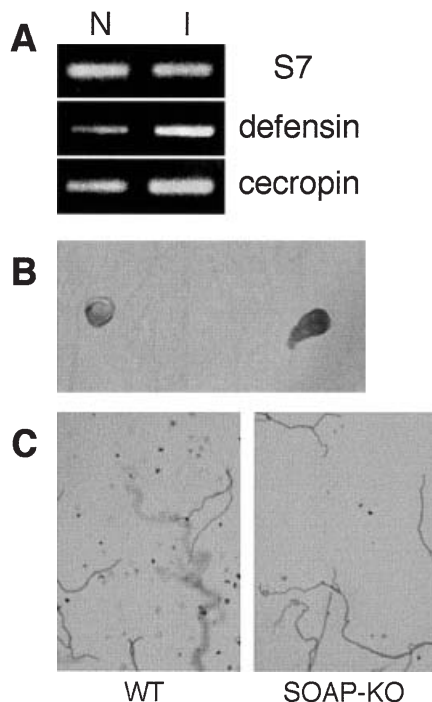


Fig. 4. PbSOAP knockout and mosquito immune response. A. PCR amplification of S7, defensin and cecropin genes from cDNA generated from naïve (N) or SOAP-KO parasite-infected (I) mosquitoes. B. Close-up view of SOAP-KO parasites melanised in refractory *A. gambiae* L3-5 at six days postinfection. C. Overview of a typical *A. gambiae* L3-5 midgut infected with WT or SOAP-KO parasites at six days post infection. Melanised parasites, visible as black dots, are markedly more numerous in the WT parasite-infected mosquitoes.

tory for malaria transmission through melanotic encapsulation of the ookinetes on the basal side of the midgut epithelium (Collins *et al.*, 1986). When these mosquitoes were infected with our SOAP-KO parasites normal melanisation was observed (Fig. 4B). These observations indicate that PbSOAP is not involved in the induction of, nor the recognition by the innate immune system in mosquito midguts.

Although SOAP-KO ookinetes underwent normal melanotic encapsulation in L3-5 mosquitoes, the observed numbers of melanised parasites were noticeably lower in the majority of SOAP-KO parasite-infected insects (Fig. 4C), indicating that fewer ookinetes had crossed the midgut wall. To verify this, we assessed ookinete numbers inside *A. stephensi* midgut epithelia at 24 h post infection by immunofluorescence using antibodies to the abundant ookinete surface protein P28. Indeed, at this early time point numbers of invaded SOAP-KO ookinetes were reduced by 87% compared to mosquitoes infected with WT parasites [mean number of ookinetes per gut \pm sem for WT parasites: 69 ± 5.8 ($n = 25$) and for SOAP-KO parasites: 9 ± 1.9 ($n = 25$)]. These results show that SOAP-KO ookinetes are impaired in their ability to invade the mosquito midgut. This is likely to be responsible, at least in part, for the reduced numbers of oocysts developing (Table 1).

Recently, an *in vitro* ookinete-to-oocyst development system was reported using a Mos20 *Aedes aegypti* cell line (Sidén-Kiamos *et al.*, 2000). In this system ookinetes invade the unpolarised insect cells and develop into intracellular oocysts that undergo several rounds of nuclear division before development is halted. Comparison of *in vitro* cultured SOAP-KO and WT ookinetes in this system revealed apparently normal ookinete motility and attachment to the insect cells for both parasites (data not shown). Surprisingly however, oocyst development of SOAP-KO ookinetes was significantly more efficient than that of WT ookinetes ($P < 0.01$, ANOVA two-factor analysis), a result that was reproducible over several experiments (Table 2). These observations contrast with those made *in vivo* (Table 1), indicating that the function of PbSOAP involves factors that are specific to its environment in the mosquito midgut.

PbSOAP interacts with mosquito laminin

The extracellular ookinete proteins P25, P28 and CTRP have been shown to interact with laminin, a prominent component of basal lamina (Adini and Warburg 1999; Vlachou *et al.*, 2001; Arrighi and Hurd, 2002). To test whether PbSOAP might also interact with laminin we employed the yeast-two-hybrid system, an assay commonly used to detect protein–protein interactions (Fields

Table 2. Effect of PbSOAP knockout on *Plasmodium berghei* oocyst development in Mos20 cells *in vitro*.

| Expt | Scored on day | No. of circumsporozoite protein positive oocysts ^a in Mos20 cells co-cultured with ookinetes from: | | % of WT |
|------|---------------|---------------------------------------------------------------------------------------------------------------|---------|---------|
| | | WT | SOAP-KO | |
| 1 | 7 | 64 | 278 | 434 |
| | 14 | 15 | 72 | 480 |
| 2 | 7 | 69 | 400 | 578 |
| | 11 | 40 | 77 | 193 |
| 3 | 14 | 15 | 187 | 1247 |
| 4 | 7 | 135 | 475 | 352 |
| | 13 | 193 | 165 | 86 |
| 5 | 6 | 160 | 355 | 222 |
| 6 | 7 | 148 | 148 | 100 |
| | 14 | 67 | 83 | 124 |
| 7 | 7 | 94 | 147 | 156 |
| | 14 | 28 | 275 | 982 |

a. Values are shown as no. oocysts per 25 fields at 400× magnification.

and Song, 1989). As 'bait' we used different amino-terminal subdomains (IV-VI) of *A. gambiae* laminin γ 1 (Vlachou *et al.*, 2001) fused to the DNA binding domain of LexA in vector pEG202-NLS. As 'prey' we used the full-length predicted extracellular domain of PbSOAP fused to the B42 activation domain in vector pJG4-5. Protein interactions were assayed qualitatively by observing blue colour on Gal/Raff/X-gal plates and quantitatively with liquid *LacZ* assays, as described (Vlachou *et al.*, 2001). In these assays, co-expression of pBlamV and B42-Soap gave rise to *LacZ* activation levels 15-fold higher than background (i.e. empty pJG4-5 activation vector) (Table 3), demonstrating that domain V of laminin γ 1 interacts strongly with PbSOAP in yeast. No significant activation with respect to background was observed when using domains IV or VI of laminin γ 1, or using empty DNA binding vector (pEG202-NLS) as bait. Moreover, when using pBlamV and B42-SOAP with unrelated prey or bait constructs, containing the *Drosophila melanogaster E(spl) m8* gene (B42-m8) and *groucho* gene (LexA-Gro), respectively, no

significant activation was observed in β -galactosidase assays (Table 3). These observations show that PbSOAP has specific binding properties to laminin γ 1 in yeast cells.

Discussion

The recent completion of the *Plasmodium* genome revealed that some 60% (3208) of all predicted genes encode hypothetical proteins because of insufficient homology to proteins in other organisms (Gardner *et al.*, 2002). The allocation of functional assignments to these genes will be a clear challenge for the future. In this paper we describe and characterize one of these genes, *SOAP*, in the rodent malaria parasite *P. berghei*. We show that the *PbSOAP* gene product constitutes a predicted soluble protein that is expressed and secreted by ookinetes via the micronemes. The high level of sequence conservation between *Plasmodium* orthologues of SOAP indicates that the protein may serve similar roles in different *Plasmodium* species. Moreover, the absence of structural relatives in other organisms points to a function unique to malaria parasites and possibly limited to the ookinete.

We have generated PbSOAP null mutant parasites in *P. berghei* by targeted gene disruption to investigate the roles of the protein in mosquito infection and malaria transmission. The results obtained from gametocyte and ookinete feeds show that PbSOAP null mutants have a markedly reduced capacity to complete ookinete-to-oocyst development in vector-competent *Anopheles* mosquitoes. We further show that this reduction, at least in part, stems from a diminished ability of SOAP-KO ookinetes to invade the mosquito midgut. This scenario is consistent with the general view that *Plasmodium* micronemal proteins are involved in cell invasion (Dubremetz *et al.*, 1993). We did not find evidence of invaded SOAP-KO ookinetes suffering increased losses after entry of the midgut epithelium, indicating that the impairment of midgut invasion is caused by a reduced ability to enter the epithelial cells. However, it should be noted that *P. berghei* ookinete-invaded *A. stephensi* midgut cells have been shown to undergo cell death and

Table 3. PbSOAP-laminin interactions in yeast-two-hybrid assays.

| | Bait | Prey | Activation (<i>LacZ</i> units) ^a |
|--------------|------------------------------------------------------------------|-------------------------------------------------------|----------------------------------------------|
| Experimental | pBlamIV (<i>laminin</i> γ 1 gene domain IV) ^b | B42-Soap (<i>PbSOAP</i> gene) ^c | 8 ± 3 |
| | pBlamV (<i>laminin</i> γ 1 gene domain V) ^b | B42-Soap (<i>PbSOAP</i> gene) | 527 ± 97 |
| | pBlamVI (<i>laminin</i> γ 1 gene domain VI) ^b | B42-Soap (<i>PbSOAP</i> gene) | 10 ± 4 |
| Control | pBlamV (<i>laminin</i> γ 1 gene domain V) | pJG4-5 (empty vector) ^b | 35 ± 4 |
| | pEG202-NLS (empty vector) ^b | B42-Soap (<i>PbSOAP</i> gene) | 6 ± 2 |
| Specificity | pBlamV (<i>laminin</i> γ 1 gene domain V) | B42-m8 (<i>D. melanogaster m8</i> gene) ^b | 25 ± 4 |
| | LexA-Gro (<i>D. melanogaster groucho</i> gene) ^b | B42-Soap (<i>PbSOAP</i> gene) | 11 ± 3 |

a. Mean ± sem of three independent measurements.

b. As described in Vlachou *et al.* (2001).

c. As described herein.

subsequent removal through a purse-string mechanism (Han *et al.*, 2000). Thus, it cannot be ruled out that invaded ookinetes killed by the mosquito might be removed by this process.

Ookinete-to-oocyst development in Mos20 cells *in vitro* was not adversely affected, and even enhanced, by the PbSOAP disruption (Table 2). This observation implies that PbSOAP is not important for ookinete-to-oocyst development *in vitro*. One explanation is that PbSOAP may be required for ookinete penetration of structures such as the microvillar network (Zieler *et al.*, 2000) that are present in the midgut wall, but are absent in the mosquito cells *in vitro*. The enhanced oocyst development *in vitro* in the absence of PbSOAP is more difficult to explain, albeit an observation worth reporting. One possibility is that PbSOAP promotes parasite invasion by interacting with specific ligands on the surface of the midgut epithelium or associated structures. If the invasion of Mos20 cells were achieved by an alternate ligand interaction, it would be conceivable that the presence of PbSOAP might hinder invasion of these cells.

Striking analogies exist between PbSOAP and the recently reported PbWARP protein (Yuda *et al.*, 2001). Both proteins are encoded by highly conserved single copy genes, are abundantly expressed in malaria ookinetes and are targeted to the micronemes. Moreover, both are small, secreted, soluble proteins that form high molecular mass complexes via disulphide bonds. Whereas the role of WARP in ookinete infectivity awaits elucidation, its predicted structure is indicative of an adhesive protein. As such, WARP is speculated to play a role in ookinete midgut invasion (Yuda *et al.*, 2001). A large number of both membrane-anchored and soluble micronemal proteins with adhesive properties have already been identified in Apicomplexa parasites including *Toxoplasma* and *Eimeria* (Soldati *et al.*, 2001; Tomley and Soldati, 2001; Claudianos *et al.*, 2002). These proteins typically contain one or more adhesive domains. Different types of adhesive domains have been identified, including: (i) thrombospondin type I-like domains; (ii) von Willebrand Factor type A-like domains; (iii) epidermal growth factor (EGF)-like domains; (iv) apple domains; and (v) scavenger receptor cysteine-rich domains. These domains contain conserved cysteine residues in structurally important positions. The cysteine-rich, modular structure of SOAP combined with its micronemal expression and putative binding to laminin are indicative of a new class of adhesive protein not previously identified in Apicomplexa parasites. The biological significance of forming high molecular mass complexes, as shown for both PbSOAP and PbWARP is unclear, but it could be a way for these small proteins to enhance their adhesive properties, mimicking multidomain adhesive molecules like *Plasmodium* CTRP (Trottein *et al.*, 1995) or *Eimeria* MIC4 (Soldati *et al.*, 2001).

The strong interaction of PbSOAP with mosquito laminin $\gamma 1$ in the yeast-two-hybrid assays suggests that the native protein may interact with the mosquito basal lamina and, hence, may play a role in ookinete-to-oocyst transition in addition to its demonstrated role in midgut invasion. This is not unprecedented: previously, the ookinete proteins P25, P28 and CTRP were shown to interact with basal lamina components as well as having functions in midgut invasion. P25 and P28 were shown to interact with laminin, while the ookinete micronemal protein CTRP has been shown to interact with both laminin and collagen IV (Adini and Warburg, 1999; Vlachou *et al.*, 2001; Arrighi and Hurd, 2002). Immuno-electron microscopic observations of PbCTRTP in ookinete-invaded *A. stephensi* midgut epithelia localized the protein at the site of contact between the ookinete and the basal lamina, suggesting that PbCTRTP functions as an adhesion molecule for ookinete movement into the midgut lumen and epithelial cell and for ookinete association with the midgut basal lamina and transformation into an oocyst (Limviroj *et al.*, 2002). The interactions in yeast of the extracellular domains of P25 and P28 were shown to be restricted to domain V of *Anopheles* laminin subunit $\gamma 1$ (Vlachou *et al.*, 2001). This domain is composed of four EGF-like domains, and as such is similar in structure to both P25 and P28 which have four and 3.5 EGF-like domains respectively. Interestingly, PbSOAP interacted with the same domain of laminin $\gamma 1$ (Table 3). Laminin EGF-like domains are known to be involved in protein-protein interaction and in mouse laminin $\gamma 1$ harbour the nidogen binding site (Stetefeld *et al.*, 1996). In this context it is worth noting that SOAP contains short sequence motifs homologous to EGF-like domains of a variety of extracellular matrix proteins including laminins. This could indicate that SOAP has a distant structural relationship to EGF domains, which in turn could be responsible for its interaction with laminin EGF-like domains.

Accepting the various interactions with basal lamina components, the precise roles of CTRP, P25, P28 and SOAP in the process of ookinete-to-oocyst transition remain unclear. Null mutant parasites for P25 and P28 are capable of reduced levels of oocyst development both *in vivo* and *in vitro*, but their multiple as well as mutually redundant roles before and during midgut invasion make it difficult to assess their potential contribution to oocyst transition (Sidén-Kiamos *et al.*, 2000; Tomas *et al.*, 2001). Similarly, studies using CTRP null mutants have not resolved the possible involvement of this protein in ookinete-to-oocyst transition, because these parasites form ookinetes that are not motile and can not invade the midgut wall (Dessens *et al.*, 1999; Yuda *et al.*, 1999; Templeton *et al.*, 2000). The increasing number of ookinete proteins that can interact with the basal lamina indicates that the molecular mechanisms that underly ookinete-to-

oocyst transition may possess a significant level of functional redundancy.

An actively pursued strategy to block the transmission of malaria parasites is the immunization of the human host with extracellular proteins that are expressed in the mosquito midgut stages of the parasite. This generates a transmission-blocking immune response mainly through the action of antibodies that are carried into the midgut with the blood meal (reviewed in Carter 2001; Richie and Saul, 2002). The extracellular nature of SOAP and its restricted expression in the mosquito midgut stages of *Plasmodium* make this protein a potential transmission-blocking antibody target and vaccine candidate. Indeed, in a pilot transmission blocking experiment we obtained significantly reduced oocyst numbers in mosquitoes fed on SOAP-immunised mice infected with *P. berghei* (J. T. Dessens, unpubl. obs.). These results indicate that SOAP could prove useful as a component in a multiantigen-based malaria transmission blocking vaccine, and should stimulate further research on this topic.

Experimental procedures

Parasite maintenance, culture and purification; parasite RNA extraction and purification; Southern, Northern and Western blotting; transmission experiments in *Anopheles*; immune induction and melanisation experiments; were carried out as previously described (Dessens *et al.*, 1999; 2001). *In vitro* ookinete motility, attachment and oocyst development assays were as previously described (Sidén-Kiamos *et al.*, 2000).

Construction of transgenic parasites

A 5'-portion of the *PbSOAP* gene was amplified with primers SOAP-Bam (GGATCCTCTTCTGAAAAACAACGTAAT) and SOAP-ERI (GAATTCTGGTAGGCATTTACACAC), digested with *Bam*HI and *Eco*RI and ligated into *Bam*HI/*Eco*RI-digested pBS-DHFR (Dessens *et al.*, 1999) to give pSOAP-BE. A 3'-portion of *SOAP* was amplified with primers SOAP-Kpn (GGTACCGATATATGTATTATATGGTATATG) and SOAP-Hind (AAGCTTGTGAATGTGAATGCAGTTGT), digested with *Kpn*I and *Hind*III and ligated into *Kpn*I/*Hind*III-digested pSOAP-BE to give the transfection plasmid pSOAP-KO. Fifty µg of *Kpn*I/*Bam*HI-digested pSOAP-KO was transfected into purified schizonts followed by pyrimethamine selection and limiting dilution cloning as described (Waters *et al.*, 1997).

Antibody production

The *PbSOAP* coding region minus the signal peptide sequence was PCR-amplified with primers (GGATCCAGGAAAAATCCATAAGAAATGTA) and (TCTAGATTAA CAATAACATGAACAGCTAC) and ligated into pGEM-T Easy (Promega). The insert was excised with *Bam*HI and *Eco*RI and ligated into *Bam*HI/*Eco*RI-digested pGEX-4T-1 (Amersham) to give pGEX-SOAP. This construct gives rise to the

expression of PbSOAP as a carboxy-terminal fusion with glutathione S transferase (GST). Protein expression was induced in transformed *Escherichia coli* strain BL21(DE3) by adding IPTG to a final concentration of 0.4 mM, and the fusion protein was purified from soluble protein fractions by affinity chromatography on glutathione affinity resin following the manufacturer's instructions (Amersham). Antiserum was raised in mice. Briefly, 100 µg of purified recombinant GST/SOAP protein emulsified in Freund's incomplete adjuvant was injected subcutaneously in female BALB/c mice. Similarly expressed and purified GST from empty pGEX-4T-1 was used to raise control immune serum. A total of three booster injections were administered at monthly intervals. Sera were collected two weeks after the last booster injection and were stored refrigerated with 0.01% sodium azide.

Immunofluorescent antibody staining

In vitro cultured ookinetes (24 h) were spotted on microscope slides, air dried, and fixed in 1% formaldehyde for 5 min. PbSOAP staining was carried out with anti-SOAP immune serum (1 in 100) as primary antibody, and goat anti-mouse FITC conjugated secondary antibody (Sigma) as described (Dessens *et al.*, 1999). Midguts from infected mosquitoes were dissected at 24 h post infection, cut open in phosphate-buffered saline and the blood meal removed. After three washes in PBS guts were fixed in 1% paraformaldehyde for 1 h, permeabilised with 1% Triton-X100 for 1 h, incubated with primary and secondary antibodies, and examined by confocal microscopy as described (Tomas *et al.*, 2001). Laminin staining was carried out with rabbit anti-laminin immune serum (Abcam) 1 in 500, followed by goat anti-rabbit Texas red-conjugated secondary antibody (Sigma). To quantify and compare numbers of invaded ookinetes, 13.1 monoclonal antibody (recognising Pbs21) was used as described (Tomas *et al.*, 2001). Results for each parasite are based on pooled data from three pots of mosquitoes fed on three infected mice with similar parasitemia.

Immunogold electron microscopy

Purified *in vitro* cultured ookinetes were fixed and embedded in LRWhite. Thin sections (100 Å) were incubated with anti-SOAP or control immune serum as primary antibody, 15 nm gold-conjugated goat anti-mouse IgG as secondary antibody, and examined in a Zeiss electron microscope.

Yeast-two-hybrid assays

The full-length coding sequence of *PbSOAP* excluding the amino-terminal 24 amino acid signal peptide was PCR-amplified with primers (CCCCGAATTCTCCATAAGAAATG TAGTAAGCGCATAT) and (GGGGCTCGAGTTAACAATAA CATGAACAGCTACATTC), digested with *Eco*RI and *Xho*I, and ligated into *Eco*RI/*Xho*I-digested pJG4-5 (DupLexA system, OriGene Technologies) to give the prey construct B42-Soap. Bait constructs are described in (Vlachou *et al.*, 2001). The LexA yeast-two-hybrid assays were performed in yeast host strain EGY48 as described (Vlachou *et al.*, 2001).

Acknowledgements

We wish to thank the scientists and funding agencies comprising the international Malaria Genome Project for making sequence data from the genome of *P. falciparum* (3D7) public before publication of the completed sequence. The Sanger Centre (UK) provided sequence for chromosomes 1, 3–9, and 13, with financial support from the Wellcome Trust. A consortium composed of The Institute for Genome Research, along with the Naval Medical Research Center (USA), sequenced chromosomes 2, 10, 11 and 14, with support from NIAID/NIH, the Burroughs Wellcome Fund and the Department of Defense. The Stanford Genome Technology Center (USA) sequenced chromosome 12, with support from the Burroughs Wellcome Fund. The Plasmodium Genome Database is a collaborative effort of investigators at the University of Pennsylvania (USA) and Monash University (Melbourne, Australia), supported by the Burroughs Wellcome Fund. Preliminary sequence and/or preliminary annotated sequence data from the *Plasmodium yoelii* genome was obtained from The Institute for Genomic Research website (<http://www.tigr.org>). This sequencing program is carried on in collaboration with the Naval Medical Research Center and is supported by the U.S. Department of Defense. *P. knowlesi* sequence data were produced by the *P. knowlesi* Sequencing Group at the Sanger Institute and can be obtained from http://www.sanger.ac.uk/Projects/P_knowlesi. *P. chabaudi* sequence data were produced by the *P. chabaudi* Sequencing Group at the Sanger Institute and can be obtained from http://www.sanger.ac.uk/Projects/P_chabaudi.

This work was supported by a project grant from the Wellcome Trust and TMR/RTN grants from the European Union. E. K. was supported by a grant from the Islamic Development Bank Merit Scholarship Program in High Technology, Jeddah, Saudi Arabia.

The sequence reported in this paper has been deposited in the GenBank database (Accession No. AY046918).

References

- Adini, A., and Warburg, A. (1999) Interactions of *Plasmodium gallinaceum* ookinetes and oocysts with extracellular matrix proteins. *Parasitology* **119**: 331–336.
- Arrighi, R.B., and Hurd, H. (2002) The role of *Plasmodium berghei* ookinete proteins in binding to basal lamina components and transformation into oocysts. *Int J Parasitol* **32**: 91–98.
- Berner, R., Rudin, W., and Hecker, H. (1983) Peritrophic membranes and protease activity in the midgut of the malaria mosquito, *Anopheles stephensi* (Liston) (Insecta: Diptera) under normal end experimental conditions. *J Ultrastruct Res* **83**: 195–204.
- Carter, R. (2001) Transmission blocking malaria vaccines. *Vaccine* **19**: 2309–2314.
- Claudianos, C., Dessens, J.T., Trueman, H.E., Arai, M., Mendoza, J., Butcher, G., et al. (2002) A malaria scavenger receptor-like protein essential for parasite development. *Mol Microbiol* **45**: 1473–1484.
- Collins, F.H., Sakai, R.K., Vernick, K.D., Paskewitz, S., Seeley, D.C., Miller, L.H., et al. (1986) Genetic selection of a *Plasmodium*-refractory strain of the malaria vector *Anopheles gambiae*. *Science* **234**: 607–610.
- 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 infectio by malaria ookinetes. *EMBO J* **18**: 6221–6227.
- Dessens, J.T., Margos, G., Rodriguez, M.C., and Sinden, R.E. (2000) Identification of differentially regulated genes of *Plasmodium* by suppression subtractive hybridization. *Parasitol Today* **16**: 354–356.
- Dessens, J.T., Mendoza, J., Claudianos, C., Vinetz, J.M., Khater, E., Hassard, S., et al. (2001) Knockout of the rodent malaria chitinase PbCHT1 reduces infectivity to mosquitoes. *Infect Immun* **69**: 4041–4047.
- van Dijk, M.R., Waters, A.P., and Janse, C.J. (1995) Stable transfection of malaria parasite blood stages. *Science* **268**: 1358–1362.
- Dimopoulos, G., Richman, A., Müller, H.-M., and Kafatos, F.C. (1997) Molecular immune responses of the mosquito *Anopheles gambiae* to bacteria and malaria parasites. *Proc Natl Acad Sci USA* **94**: 11508–11513.
- Dimopoulos, G., Seeley, D., Wolf, A., and Kafatos, F.C. (1998) Malaria infection of the mosquito *Anopheles gambiae* activates immune-responsive genes during critical transition stages of the parasite life cycle. *EMBO J* **17**: 6115–6123.
- Dubremetz, J.F., Garcia-Reguet, N., Conseil, V., and Fourmaux, M.N. (1993) Apical organelles and host cell invasion by Apicomplexa. *Int J Parasitol* **28**: 1007–1013.
- Fields, S., and Song, O. (1989) A novel genetic system to detect protein–protein interactions. *Nature* **340**: 245–246.
- Gardner, M.J., Hall, N., Fung, E., White, O., Berriman, M., Hyman, R.W., et al. (2002) Genome sequence of the human malaria parasite *Plasmodium falciparum*. *Nature* **419**: 498–511.
- Han, Y.S., Thompson, J., Kafatos, F.C., and Barillas-Mury, C. (2000) Molecular interactions between *Anopheles stephensi* midgut cells and *Plasmodium berghei*: the time bomb theory of ookinete invasion of mosquitoes. *EMBO J* **19**: 6030–6040.
- Langer, R.C., Hayward, R.E., Tsuboi, T., Tachibana, M., Torii, M., and Vinetz, J.M. (2000) Micronemal transport of *Plasmodium* chitinases to the electron-dense area of the apical complex for extracellular secretion. *Infect Immun* **68**: 6461–6465.
- Limviroj, W., Yano, K., Yuda, M., Ando, K., and Chinzei, Y. (2002) Immuno-electron microscopic observations of *Plasmodium berghei* CTRP localization in the midgut of the vector mosquito *Anopheles stephensi*. *J Parasitol* **88**: 664–672.
- Luckhart, S., Vodovotz, Y., Cui, L., and Rosenberg, R. (1998) The mosquito *Anopheles stephensi* limits malaria parasite development with inducible synthesis of nitric oxide. *Proc Natl Acad Sci USA* **95**: 5700–5705.
- Müller, H.-M., Crampton, J.M., della Torre, A., Sinden, R.E., and Crisanti, A. (1993) Members of a trypsin gene family in *Anopheles gambiae* are induced in the gut by blood meal. *EMBO J* **12**: 2891–2900.
- Richie, T.L., and Saul, A. (2002) Progress and challenges for malaria vaccines. *Nature* **415**: 694–701.
- Rudin, W., and Hecker, H. (1989) Lectin-binding sites in the

- midgut of the mosquitoes *Anopheles stephensi* Liston and *Aedes aegypti* L. (Diptera: Culicidae). *Parasitol Res* **75**: 268–279.
- Schneider, D., and Shahabuddin, M. (2000) Malaria parasite development in a drosophila model. *Science* **288**: 2376–2379.
- Shahabuddin, M., Toyoshima, T., Aikawa, M., and Kaslow, D.C. (1993) Transmission-blocking activity of a chitinase inhibitor and activation of malarial parasite chitinase by mosquito protease. *Proc Natl Acad Sci USA* **90**: 4266–4270.
- Sidén-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. (1997) Infection of mosquitoes with rodent malaria. In *The Molecular Biology of Insect Disease Vectors*. Crampton, J.M., Beard, C.B., and Louis, C., (eds). London, UK: Chapman & Hall, pp. 261–267.
- Sinden, R.E. (1999) *Plasmodium* differentiation in the mosquito. *Parassitologia* **41**: 139–148.
- Soldati, D., Dubremetz, J.F., and lebrun, M. (2001) Microneme proteins: structural and functional requirements to promote adhesion and invasion by the apicomplexan parasite *Toxoplasma gondii*. *Int J Parasitol* **31**: 1293–1302.
- Stetefeld, J., Mayer, U., Timpl, R., and Huber, R. (1996) Crystal structure of three consecutive laminin-type epidermal growth factor-like (LE) modules of laminin gamma1 chain harboring the nidogen binding site. *J Mol Biol* **257**: 644–657.
- Templeton, T.J., Kaslow, D.C., and Fidock, D. (2000) Developmental arrest of the human malaria parasite *Plasmodium falciparum* within the mosquito midgut via CTRP gene disruption. *Mol Microbiol* **36**: 1–9.
- Tomas, A.M., Margos, G., Dimopoulos, G., van Lin, L.H., de Koning-Ward, T.F., Sinha, R., *et al.* (2001) P25 and P28 proteins of the malaria ookinete surface have multiple and partially redundant functions. *EMBO J* **20**: 3975–3983.
- Tomley, F.M., and Soldati, D.S. (2001) Mix and match modules: structure and function of microneme proteins in apicomplexan parasites. *Trends Parasitol* **17**: 81–88.
- Trottein, F., Triglia, T., and Cowman, A.F. (1995) Molecular cloning of a gene from *Plasmodium falciparum* that codes for a protein sharing motifs found in adhesive molecules from mammals and plasmodia. *Mol Biochem Parasit* **74**: 129–141.
- Vinetz, J.M., Valenzuela, J.G., Specht, C.A., Aravind, L., Langer, R.C., Ribeiro, J.M.C., and Kaslow, D.C. (2000) Chitinases of the avian malaria parasite *Plasmodium gallinaceum*, a class of enzymes necessary for parasite invasion of the mosquito midgut. *J Biol Chem* **14**: 10331–10341.
- Vizioli, J., Bulet, P., Charlet, M., Lowenberger, C., Blass, C., Müller, H.-M., *et al.* (2000) Cloning and analysis of a cecropin gene from the malaria vector mosquito, *Anopheles gambiae*. *Insect Mol Biol* **9**: 75–84.
- Vlachou, D., Lycett, G., Siden-Kiamos, I., Blass, C., Sinden, R.E., and Louis, C. (2001) *Anopheles gambiae* laminin interacts with the P25 surface protein of *Plasmodium berghei* ookinetes. *Mol Biochem Parasit* **112**: 229–237.
- Warburg, A., and Miller, L.H. (1992) Sporogonic development of a malaria parasite *in vitro*. *Science* **225**: 448–450.
- Waters, A.P., Thomas, A.W., van Dijk, M.R., and Janse, C.J. (1997) Transfection of malaria parasites. *Methods* **13**: 134–147.
- Weathersby, A.B. (1952) The role of the stomach wall in the exogenous development of *Plasmodium gallinaceum* as studied by means of heamocoel injections of susceptible and refractory mosquitoes. *J Inf Dis* **91**: 198–205.
- 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.
- Yuda, M., Yano, K., Tsuboi, T., Torii, M., and Chinzei, Y. (2001) von Willebrand factor A domain-related protein, a novel microneme protein of the malaria ookinete highly conserved throughout *Plasmodium* parasites. *Mol Biochem Parasit* **116**: 65–72.
- Zieler, H., Nawrocki, J.P., and Shahabuddin, M. (1999) *Plasmodium gallinaceum* ookinetes adhere specifically to the midgut epithelium of *Aedes aegypti* by interaction with a carbohydrate ligand. *J Exp Biol* **202**: 485–495.
- Zieler, H., Garon, C.F., Fischer, E.R., and Shahabuddin, M. (2000) A tubular network associated with the brush-border surface of the *Aedes aegypti* midgut: implications for pathogen transmission by mosquitoes. *J Exp Biol* **203**: 1599–1611.