Conserved peptide sequences bind to actin and enolase on the surface of *Plasmodium berghei* ookinetes

J. HERNÁNDEZ-ROMANO[1,3], M. H. RODRÍGUEZ[1], V. PANDO[1], J. A. TORRES-MONZÓN[2], A. ALVARADO-DELGADO[1], A. N. LECONA VALERA[1], R. ARGOTTE RAMOS[1], J. MARTÍNEZ-BARNETCHE[1] *and* M. C. RODRÍGUEZ[1]*

[1] *Centro de Investigación sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Avenida Universidad No. 655, Col. Santa María Ahuacatitlán, Cuernavaca, Morelos, CP62508 México*
[2] *Centro Regional de Investigación en Salud Pública, Instituto Nacional de Salud Pública, 4ª. Av. Norte y 19 calle Poniente, Tapachula, Chiapas CP30700 México*
[3] *Universidad Politécnica del Estado de Morelos, Boulevard Cuauhnáhuac No. 566, Col. Lomas del Texcal, Jiutepec, Morelos CP62550 México*

(*Received 1 June 2011; revised 4 July 2011; accepted 5 July 2011; first published online 5 August 2011*)

SUMMARY

The description of *Plasmodium* ookinete surface proteins and their participation in the complex process of mosquito midgut invasion is still incomplete. In this study, using phage display, a consensus peptide sequence (PWWP) was identified in phages that bound to the *Plasmodium berghei* ookinete surface and, in selected phages, bound to actin and enolase in overlay assays with ookinete protein extracts. Actin was localized on the surface of fresh live ookinetes by immunofluorescence and electron microscopy using specific antibodies. The overall results indicated that enolase and actin can be located on the surface of ookinetes, and suggest that they could participate in *Plasmodium* invasion of the mosquito midgut.

Key words: actin, ookinetes, midgut invasion.

INTRODUCTION

The ookinete is a motile stage of malaria parasites that forms in the midgut lumen of the mosquito vector within the infective bloodmeal bolus. It invades the intestinal epithelium and upon arriving at the basal lamina it transforms into an oocyst in which sporozoites, the parasite stage infective for vertebrates develop (Sinden, 1999). Sporozoites and ookinetes move by gliding, a locomotion form characteristic of the invasive stages of apicomplexan parasites (Kappe *et al*. 1999; Ménard, 2001; Fowler *et al*. 2004).

Gliding is a substrate-dependent movement driven by an actin-myosin motor that translocates surface thrombospondin-related adhesive protein (TRAP)-like molecules from the apical to the rear extreme of the parasite. As the ookinete moves, the TRAP-like CTRP and Pbs25 (Lecona *et al*. 2010) are shed on the substrate. Aldolase acts as a metabolic enzyme as well as a bridge between the actomyosin system and the transmembrane adhesins (Daher and Soldati, 2009; Baum *et al*. 2006; Soldati-Favre, 2008).

Several proteins have been described in *Plasmodium* ookinetes: P25, P28, CTRP, WARP, SOAP, MAOP, chitinase, myosin A, CDPK3, PbGCbeta, IMC1b, etc. (Huber *et al*. 1991; Tsuboi *et al*. 1997; Naitza *et al*. 1998; Dessens *et al*. 1999; Yuda *et al*. 1999; Margos *et al*. 2000; Siden-Kiamos *et al*. 2000; Yuda *et al*. 2001; Tomley and Soldati, 2001; Dessens *et al*. 2003; Kadota *et al*. 2004; Siden-Kiamos *et al*. 2006; Hirai *et al*. 2006; Tremp *et al*. 2008). These molecules participate in motility, invasion (Yuda *et al*. 1999; Templeton *et al*. 2000; Limviroj *et al*. 2002; Dessens *et al*. 2003; Hirai *et al*. 2006; Tremp *et al*. 2008), peritrophic matrix degradation (Huber *et al*. 1991), basal lamina recognition (Adini and Warburg 1999; Vlachou *et al*. 2001) and defence against proteolysis. However, the description of ookinete surface proteins and their participation in the complex process of mosquito midgut invasion is still incomplete.

In this work, we used a phage display strategy to identify a consensus sequence (PWWP) in peptides that bind to the surface of *Plasmodium berghei* ookinetes. Assuming that these peptides display the structural features of ookinete ligands in the vector mosquito midgut, we used them to search for new molecules on the ookinete surface. Two proteins were identified, actin and enolase.

MATERIALS AND METHODS

Parasites

Ookinetes of *P. berghei* strain Antwerpen Katanga (ANKA) clone 2.34 (kindly provided by

* Corresponding author: Centro de Investigación sobre Enfermedades Infecciosas, Instituto Nacional de Salud Pública, Avenida Universidad No. 655, Col. Santa María Ahuacatitlán, Cuernavaca, Morelos, CP62508 México. Tel: +52 777 3293075. Fax: +52 777 3175485. E-mail: mrodri@insp.mx; 2mrodri@gmail.com

doi:10.1017/S0031182011001296

R. E. Sinden, Imperial College, UK) were obtained as previously described (Rodríguez *et al*. 2000). Briefly, 6-week-old BALB/c male mice were inoculated with 200 μl of phenylhydrazine (PH) (6 mg/ml) in PBS, by the intraperitoneal route (IP). Three days after PH treatment, mice were inoculated IP with 1×10^8 *P. berghei* parasites. Three days post-infection, the presence of gametocytes was determined in thin blood films stained with Giemsa. The blood containing gametocytes was diluted 1:5 in RPMI containing 20% heat-inactivated bovine fetal serum (Hyclone, Thermo Scientific, Utah, USA). The suspension was incubated for 24 h at 20 °C to allow ookinete development. Red blood cells were lysed by adding NH_4Cl (0·17 M). Red blood cell debris was removed and ookinetes purified by centrifugation using a Nycodenz™ gradient.

Selection of phages that bind to P. berghei *ookinete surfaces*

The interaction of *P. berghei* ookinete surface molecules with a collection of peptides with random sequences was carried out using a phage display commercial kit (Phage Display Peptide Library Kit Ph.D.–12™, New England Biolabs, #cat. E8110S) following the manufacturer's instructions. This phage collection displays linear peptides of 12 amino acids in length. Using freshly prepared ookinetes, 5 selection cycles were carried out. Before and after each selection cycle, the phage titres in the working solution and the eluted solution were determined. For each selection cycle, 4×10^{11} plaque-forming units (pfu) were used diluted in 1 ml of Tris-Buffered Saline (TBS, Tris-base 0·5 M, NaCl 9%, pH 7·6). Phages were incubated with ookinetes for 1 h at room temperature (RT) with gentle shaking. The ookinete-phage suspension was centrifuged for 3 min at 1500 ***g*** and washed 10 times with TBS to remove the unbound phages. Ookinete-bound phages were eluted using glycine 0·2 M HCl, pH 2·2 in 1 mg/ml of bovine serum albumin (BSA). The sample was centrifuged for 3 min at 1500 ***g***, and the supernatant recovered and neutralized with 1 M Tris-HCl, pH 9·1. The eluted phages were amplified, purified and titrated. In the second and third selection cycle, 0·01% Tween-20 (v/v) in TBS was used as washing solution. For the fourth and fifth selection cycles, 0·05% Tween-20 in TBS was used. After the fifth cycle, eluted phages were titrated and individual phage clones were amplified. The corresponding DNA from each clone was obtained and sequenced at the DNA Sequencing Unit of the National Institute of Public Health in Cuernavaca, Morelos, Mexico.

Ookinete binding pattern of selected phages

The binding patterns of 2 randomly selected phage clones (5 and 14) to *P. berghei* ookinetes were determined by immunofluorescence assays. Ookinetes and selected clones were mixed in a 1:12 500 ratio, incubated for 20 min at RT, washed 4 times with 1x phosphate buffered saline (PBS), twice with PBS-0·05% Tween-20, and a final wash with PBS. The samples were centrifuged for 3 min at 1500 ***g*** and the pellets containing the ookinetes and phages were suspended in 100 μl of PBS. An aliquot of each sample was placed on a slide and allowed to air dry. Ookinetes incubated with non-selected phage clones and/or secondary antibody only were included as control. The samples were incubated for 1 h with a murine monoclonal antibody anti-M13 (which recognizes the gp8 protein of M13) (Amersham, 27-9420-01) diluted 1:250 in PBS-2% BSA. After incubation, the unbound antibody was removed by washing 3 times with PBS-0·05%, Tween-20 and once with PBS, and incubated 1 h in the dark with an anti-mouse IgG coupled to FITC (American Qualex, A106FN) diluted 1:100 in PBS-2% BSA. Unbound secondary antibody was removed by washing 3 times with PBS-0·05% Tween-20 and once with PBS. Each sample was incubated with propidium iodide (0·5 μg/ml) and ribonuclease (to avoid staining of RNA) (500 μg/ml) in PBS for 5 min, washed twice with PBS, and covered with a drop of SlowFade Light Antifadant (Molecular Probes, S-7461, Invitrogen, UK) to preserve fluorescence. The preparations were analysed under a UV fluorescence microscope (Nikon, E600 Eclipse).

Detection of phage ligands by overlay assays

Overlay assays were used to detect interactions between the selected phages and ookinete proteins (Crawford *et al*. 1992). Ookinete proteins were separated by one-dimensional (1D) and two-dimensional (2D) SDS-PAGE (Laemmli, 1970; O'Farrell, 1975). For 1D electrophoresis, proteins were extracted from 50×10^6 ookinetes using 600 μl of lysis buffer (Tris-HCl 50 mM, NaCl 150 mM, EDTA 15 mM, Tween-20 0·1%, glycerol 10%) containing protease inhibitors (AEBSF 104 mM, aprotinin 0·08 mM, leupeptin 2 mM, bestatin 4 mM, pepstatin 1·5 mM, E-64 1·4 mM) (Sigma P8340). The sample was centrifuged for 15 min at 10 000 ***g***, the supernatant recovered and the protein concentration determined by Bradford methodology (Sigma, B6916). Then 50 μg of ookinete proteins were separated using 10% polyacrylamide preparative running gels, and transferred to nitrocellulose membranes (Towbin *et al*. 1979).

For 2D electrophoresis, 60×10^6 ookinetes were treated with lysis buffer (urea 9·5 M, NP-40 2%, β-mercaptoethanol 5%, and ampholytes 5–7/3–10; GE Healthcare, Denmark) for 15 min at RT. The sample was centrifuged for 5 min at 13 000 ***g*** and the supernatant proteins were separated in an isoelectric focusing gel for 15 h at 400 V and 2·5 h at 800 V. After

https://doi.org/10.1017/S0031182011001296 Published online by Cambridge University Press

pI separation, proteins were resolved by molecular mobility using SDS-PAGE and transferred onto nitrocellulose membranes at 120 V for 1·5 h.

The nitrocellulose membrane obtained from the 1D gel was cut into 3 mm strips for overlay assays. The membrane obtained from the bi-dimensional gel was used intact in one overlay assay. Nitrocellulose membranes were blocked with 5% skimmed-milk in PBS for 1 h, then incubated at RT for 1 h with selected phage clones at a concentration of 1×10^9 pfu/ml diluted in 2% skimmed-milk in PBS and 1% Tween-20. After incubation, membranes were washed for 4 h with Tween-20 1% in PBS and for 1 h with Tween-20 0·5% in PBS, with changes of washing solution every 30 min, and then given 2 final washings of 5 min each with PBS. The strips and the membrane were incubated for 1 h at RT with anti-M13 (Amersham, UK, 27-9420-01) at 1:5000 dilution, followed by 3 washings with 0·05% Tween-20 in PBS and 1 with PBS. The membranes were incubated with an anti-mouse IgG coupled to horseradish peroxidase (Amersham, UK, NIF 825) in a 1:20000 dilution of 2% skimmed milk in PBS for 1 h at RT. The unbound secondary antibody was removed by 3 washings with 0·05% Tween-20 with PBS and 3 washings with PBS. The reactions were visualized by chemiluminescence (ECL, Amersham, UK, RPN2209), after which membranes were stained with Coomassie blue to visualize protein spots.

Identification by mass spectrometry of ookinete proteins recognized by selected phages

Ookinete proteins were separated by 2D electrophoresis as described above. The gel was stained with Coomassie blue and the spots corresponding to proteins recognized by the selected phages were identified and excised from the gel. All steps of MALDI-sample were performed manually, including preparation, picking of gel spots, destaining of the gel pieces, in-gel trypsin digestion, and sample loading onto MALDI plates. The gel pieces were dehydrated by washing 3 times (~10 min) with 25 mM NH_4HCO_3/50% acetonitrile (ACN), once with ACN, then dried in a Concentrator Plus (Eppendorf). Dried gel plugs were treated with freshly prepared 10 mM DTT in 50 mM NH_4HCO_3 for 45 min at 56 °C. After incubation, the DTT was replaced by the same volume of freshly prepared 55 mM iodoacetamide in 50 mM NH_4HCO_3 for 30 min and dehydrated with 100% ACN. The dried gel pieces were incubated for 12 h at 37 °C with 25 mM NH_4HCO_3 containing 0·02 μg/μl of mass spectrometry grade trypsin (Promega, Madison, WI, USA). Extraction of tryptic peptides was performed by the addition of 5% formic acid (FA)/50% ACN 3 times. The extracts were pooled and dried completely by centrifugal lyophilization. Tryptic peptides were suspended in 20 μl of 0·1% TFA, desalted and concentrated on C-18 ZipTips (Millipore, Billerica, MA, USA) following the manufacturer's protocol. ZipTips were eluted with 10 μl of 50% ACN/0·1% TFA solution and were mixed (1:1) with a matrix consisting of a saturated solution of alpha-cyano-4-hydroxycinnamic acid prepared in 50% ACN/0·1% TFA. Aliquots (0·7 μl) were spotted onto the MALDI sample target plate. Peptide mass spectrometry analyses were obtained on a MALDI-TOF-MS Voyager DE Pro (Applied Biosystems, Foster City, CA, USA). Peptide mass spectra were acquired in positive reflection mode, averaging 1500 laser shots with a resolution of 10000. Spectra were processed and analysed by the MASCOT software against NCBInr 20080616 database and PlasmoDB release 5.4 (Matrix Science, London, UK) for peptide mass fingerprints. Taxonomy was restricted to *Alveolata*. NCBInr 20080616 database was used for all searches under Alveolata (148527 sequences) and PlasmoDB release 5.4. Database search parameters were as follows: carbamidomethylation as a fixed modification, oxidation of methionine as a variable modification and 1 missed tryptic cleavage. The peptide mass tolerance was 100 ppm. A confident identification had a statistically significant ($P < 0·05$) protein score.

Co-culture of insect cells with P. berghei *ookinetes pre-incubated with selected phages*

To evaluate the effect of selected phages on mosquito cell invasion by the parasites, interaction assays (Varma and Pudney, 1969) were carried out using ookinetes and Mos20 cells (kindly provided by I. Sidén-Kiamos, IMBB, Greece). The Mos20 cells originated from *Aedes aegypti* and can be invaded by *P. berghei* ookinetes (Sidén-Kiamos *et al*. 2000). Parasites were pre-incubated with the selected phage clones (clones 5 and 14) at $1:5 \times 10^5$ ookinete:phage ratio, washed once to eliminate excess phages and co-cultivated with Mos20 cells at 1:1 ookinetes:cells ratio. Ookinetes and cells were placed in a 24-well plate containing circular cover slips, allowed to interact for 3 h, and fixed with 1% formaldehyde in PBS for 1·5 h. The formaldehyde excess was blocked with a solution of PBS-2% BSA. To visualize phages, the preparations were incubated for 1 h with mouse anti-M13 antibody diluted 1:250 in PBS-2% BSA, washed 3 times with PBS, incubated for 1 h at RT with anti-mouse Alexafluor 594-conjugated antibody, diluted 1:300 in PBS-2% BSA, and washed 3 times with PBS.

As a control for surface protein (Rodríguez *et al*. 2000), the samples were incubated for 1 h with a mouse anti-Pbs25 monoclonal antibody (Rodríguez *et al*. unpublished). Samples were washed 3 times with PBS, and incubated in the dark with anti-mouse FITC-conjugated antibody (Zymed, 816511) (1:250 dilution in PBS-2% BSA) for 1 h at RT, and finally washed 3 times with PBS. The preparations were

https://doi.org/10.1017/S0031182011001296 Published online by Cambridge University Press

covered with a drop of SlowFade Light Antifadant (Molecular Probes) and observed as above. In these preparations, phages were identified by red signal and Pbs25 on the ookinete surface was identified by green signal.

To identify the intracellular parasites that had invaded Mos20 cells, a permeabilization step after incubation with the primary antibody was performed as follows. After 3 h, co-cultures were fixed with paraformaldehyde 1% in PBS and incubated with the anti-Pbs25 monoclonal antibody for 1 h, unbound antibody was removed by 3 washes with PBS, and the co-cultures were then incubated for 1 h with a goat anti-mouse FITC-conjugated IgG diluted 1:250 in PBS-2% BSA and washed 3 times with PBS. Cells were permeabilized by adding 250 μl of cold (4 °C) alcohol:acetone mix (1:1) for 2 min. Samples were allowed to air dry and were then washed with PBS. After permeabilization, the samples were incubated again with the anti-Pbs25 monoclonal antibody for 1 h, washed 3 times with PBS, incubated 1 h with an anti-mouse Alexafluor 594-conjugated IgG antibody, diluted 1:300 in PBS-2% BSA, and finally washed 3 times with PBS. Preparations were covered with a drop of SlowFade Light Antifadant (Molecular Probes) and assessed as above. In these preparations, intracellular parasites showed red fluorescence, while extracellular parasites, visualized by green and red fluorophores, were detected as yellow.

Western blot of P. berghei *ookinetes with anti-actin antibodies*

Plasmodium berghei ookinetes were produced as previously described (Rodríguez *et al*. 2002) and used for Western blot analysis using the anti-actin antibody (Actin I-19:sc-1616-R, Santa Cruz Biotechnology, CA, USA), diluted 1:1000. Conditions used were as described previously (Rodríguez *et al*. 2000). Incubation with a mouse irrelevant IgG was included as negative control.

Immunofluorescence assays of live-fresh, NH_4Cl purified and permeabilized ookinetes probed with anti-actin antibody

To investigate the presence of actin on the surface of *P. berghei* ookinetes, immunofluorescence assays (IFI) were carried out using fresh-live ookinetes and those purified with NH_4Cl. Ookinetes were obtained as previously described (Rodríguez *et al*. 2002). Briefly, 5×10^6 parasites were obtained and centrifuged for 10 min at 300 ***g*** and 4 °C, washed twice with cold PBS and incubated in 400 μl of PBS containing anti-actin antibody (1:100) for 1 h at 4 °C with gentle shaking.

For NH_4Cl purification, ookinete cultures were centrifuged for 10 min at 300 ***g*** and 4 °C, the cell pellet was diluted 1:20 with cold 0·17 M NH_4Cl for 20 min at 4 °C and constant shaking to eliminate red blood cells and *P. berghei*-infected red blood cells (Winger *et al*. 1988). Purified ookinetes were washed 3 times with cold PBS and incubated with the anti-actin antibody as above. Samples were incubated in the dark at 4 °C with anti-FITC. propidium iodide (PI) (0·5 μg/ml in PBS), diluted 1:200 was added 10 min before the end of the 1 h incubation period. Samples were washed 3 times with PBS and centrifuged for 5 min at 300 ***g*** and 4 °C. Samples were fixed with 2·5% formaldehyde in PBS for 15 min at 4 °C, washed as above, placed on a glass slide, allowed to air dry and covered with a drop of SlowFade Light Antifadant (Molecular Probes) and examined under a fluorescence microscope (Nikon E600 Eclipse). Assays were carried out in triplicate and the number of ookinetes showing fluorescence with anti-actin antibody was recorded. A rabbit anti-β-tubulin antibody (Santa Cruz, Biotechnology) was included as control for intracellular labelling. Incubation of ookinetes with an irrelevant rabbit IgG was included as negative control.

To permeabilize ookinetes, NH_4Cl purified parasites were fixed for 15 min with 2·5% formaldehyde and treated with 0·1% Triton X-100 in PBS for 15 min. After washing they were blocked with 1% BSA-50 mM NH_4Cl and incubated with anti-actin and or anti-tubulin antibody as described above.

Analysis of P. berghei *ookinetes treated with anti-actin antibody, by transmission electron microscopy*

Purified ookinetes (8×10^6) were mixed with 1 ml of RPMI, pH 8·3, and samples were placed on nickel grids covered with formvar-carbon and incubated for 15 min at 21 °C in a wet chamber. Excess liquid was absorbed with filter paper and the samples were fixed with 2·5% paraformaldehyde in PBS 1X for 15 min. Slides were washed with distilled water and blocked overnight with 1% BSA in 50 mM PBS-NH_4Cl at 4 °C in a wet chamber. Samples were probed with rabbit anti-actin antibody (Actin I-19:sc-1616-R, Santa Cruz Biotechnology) diluted 1:20 in PBS containing 50 mM NH_4Cl, and then incubated for 1 h at RT in a wet chamber. After incubation, samples were washed once with PBS-0·05% Tween 20 and 3 times with PBS containing 50 mM NH_4Cl, 5 min each wash. Samples were incubated with a goat anti-rabbit antibody coupled to 10 nm colloidal gold particles (EMS, cat. 25109) for 1 h at RT in a wet chamber. The same wash was carried out, with an additional PBS wash. To stabilize the secondary antibody binding, samples were fixed with 2·5% glutaraldehyde in PBS for 10 min, washed once with water and allowed to air dry. Uranyl acetate (2·5% aqueous solution) was used as a contrast solution for 3 min, after which samples were rinsed abundantly with H_2O and allowed to dry.

https://doi.org/10.1017/S0031182011001296 Published online by Cambridge University Press

Table 1. Peptide sequences displayed by selected phages

(The 21 sequenced clones that bound to ookinetes after 5 selection cycles displayed peptides with a motif PWP or similar (bold). This motif was not present in the 5 phages sequenced from the non-selected collection. Three different sequences were repeated at least twice (shaded), suggesting a higher affinity by ookinete ligands.)

Selected phagic clones		Non-selected phagic clones	
Clone	Sequence	Clone	Sequence
1	---**LPWWLP**YRGESN	A	VVTYLGKPLYST
2	---**LPWWLP**YRGESN	B	TSYGPMPLTTES
3	---**LPWWLP**YRGESN	C	SLSSGAKEFHSA
4	---**LPWWP**QASISPP	D	SDYQAMAFGPPP
5	---**LPWWP**IQRVSHL	E	TSSSNSKFLTRP
6	---**LPWW**I**P**KEGWAV		
7	---**LPWWLP**PSLSRV		
8	--YG**PWW**YSSNAES-		
9	--YG**PWW**YSSNAES-		
10	--AG**PWW**HQTSVHV-		
11	--NI**PWWP**FSLHAP-		
12	--GGA**WWP**TSLVMY-		
13	-APYS**WWP**YSAYN--		
14	-SP**L**S**WWP**HATVG--		
15	-SP**L**S**WWP**HATVG--		
16	SSVKS**WWP**AFTP--		
17	WYPQ**P**F**WP**YRQA---		
18	-YGK**P**F**WP**SSLWW--		
19	---I**P**Y**WP**FLPDTSM		
20	---**LP**Y**WLP**YSSGNK		
21	---V**P**Y**W**M**P**PPTVIP		
Consensus	----**PWWP**----------		

Ookinetes treated with an irrelevant rabbit IgG were included as control. All samples were examined under an electron microscope (JEOL 1011).

In silico *analysis and hydrophobicity profiles of phage peptides*

The BioEdit software (Hall, 1999) was used to compare hydrophobicity profiles between phage displayed peptides and *Anopheles gambiae* proteins. The prediction of transmembrane domains was made using the TMpred (Hofmann and Stoffel, 1993) and TMHMM (Sonnhammer, 1998) programmes.

RESULTS

The selected phage clones share similar peptide sequences

After 5 selection cycles, 21 phage clones positive for binding to the surface of *P. berghei* ookinetes were sequenced and their DNA sequences translated *in silico* into amino acid sequences. The 21 selected phage peptides had adjacent residues of tryptophan (W) and proline (P) within their sequence (Table 1), with proline residues flanking tryptophan. Prolines (8/21) were frequently associated with leucine (L) residues. Three groups of complete 12-amino acid peptides were repeatedly observed: LPWWLPYRG-ESN in clones 1, 2 and 3; YGPWWYSSNAES in clones 8 and 9; and SPLSWWPHATVG in clones 14 and 15 with a consensus sequence PWWP. The latter motif was not present in the 5 sequenced clones from the initial phage pool (Table 1), indicating that this sequence feature was positively selected.

The selected phage peptide sequences showed identity with extracellular proteins and or the peritrophic matrix in the *An. gambiae* genome (Table 2), and with domains present in a wide variety of proteins that participate in protein-protein interactions (Stec *et al*. 2000).

Selected phages bind to the ookinete surface

Specific peripheral immunofluorescence was uniformly observed on ookinetes incubated with selected phages (clones 5 and 14, Fig. 1A and B, arrows). In some cases the signal was observed only at one end of the parasite (Fig. 1B, inset and arrow). Ookinetes incubated with non-selected phage clone (C) and/or incubated with secondary antibody only showed no signal (D).

Phage distribution during ookinete invasion of Mos20 cells

In experiments with ookinetes pre-incubated with phage clones 5 and 14 added to Mos20 cells, samples stained before and after cell permeabilization confirmed that ookinetes pre-incubated with selected

https://doi.org/10.1017/S0031182011001296 Published online by Cambridge University Press

Table 2. Peptide sequences displayed by selected phages show homology with proteins with domains typical of mosquito midgut components (intestinal lumen and basal lamina)

Phagic clone	Sequence of the displayed peptide	Domains present in protein with which share similarity
4	LPWWPQASISPP	Perlecan (AAA39911·1)
5	LPWWPIQRVSHL	Laminin (NP_501970)
10	AGPWWHQTSVHV	Fibrinogen (AGAP006790-PA)
11	NIPWWPFSLHAP	PWWP Domain (Stec, 2000)
14 and 15	SPLSWWPHATVG	Peritrophin (FBpp0074629)
17	WYPQPFWPYRQA	Peritrophin (FBpp0088744)
19	IPYWPFLPDTSM	Spacrcan (AAF13154·1)
21	VPYWMPPPTVIP	Peritrophin (AGAP004851-PA)

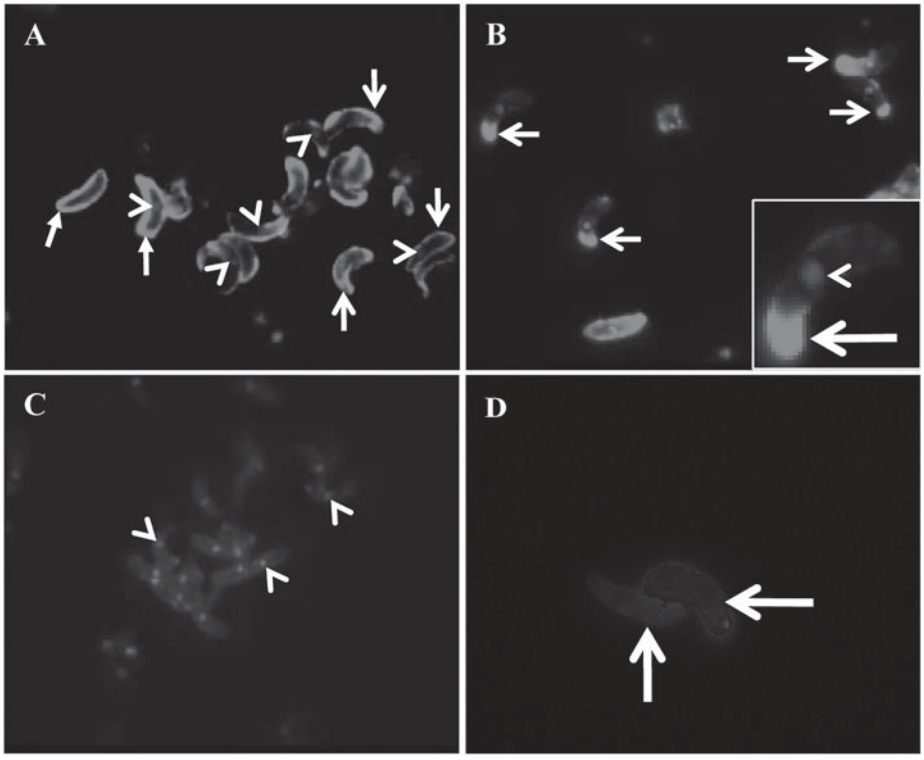

Fig. 1. Immunofluorescence detection of selected phages bound to the surface of *Plasmodium berghei* ookinetes. (A) Phage clone 5 stained with anti-M13 FITC (green, big arrows) at the periphery of the ookinete; nuclei were stained with propidium iodide (red, small arrows). (B) Phage clone 14 binds preferentially to one end of the ookinete as shown by arrows. (C) Negative control, ookinetes incubated with non-selected phage clones. (D) Background fluorescence.

phages invaded Mos20 cells (Fig. 3). The parasites interacting with Mos20 cells were devoid of phages, as evidenced by the anti-M13 staining (Fig. 2B', C'and D', arrows). A double staining using an anti-Pbs25, and anti-M13 was performed to track the location of bound phages during Mos20 invasion by ookinetes (Fig. 2). The Pbs25 protein and phages co-localized at one end of these parasites (Fig. 2A', arrow).

The selected phages recognized four ookinete proteins

Overlay assays using ookinete proteins separated by 1D gel electrophoresis showed that different selected phage clones (clones 2–6, 8, 10, 12, 14 and 20) recognized the same 2 main bands between ~37 and ~50 kDa (Fig. 4A). Using 2D electrophoresis, clone 14 recognized at least 4 proteins in the range of ~50 kDa (Fig. 4B, 1–4). Only 2 of these proteins were visualized in the 2D gel (1 and 4) (Fig. 4C, arrows). The analysis of these spots by mass fingerprint identified proteins gi|68070065 enolase from *Plasmodium berghei* and PFL2215w actin I from *Plasmodium falciparum* 3D7. The orthologue in Plasmo DB version 7.1 is the protein PBANKA_103010 actin II, putative from *Plasmodium berghei* str. ANKA. The observed masses that matched with actin I were 1961·07, 1193·60, 1177·70, 1123·54, 905·48, 767·44, and 644·07. The masses used for enolase identification were 840·43, 1180·60,

https://doi.org/10.1017/S0031182011001296 Published online by Cambridge University Press

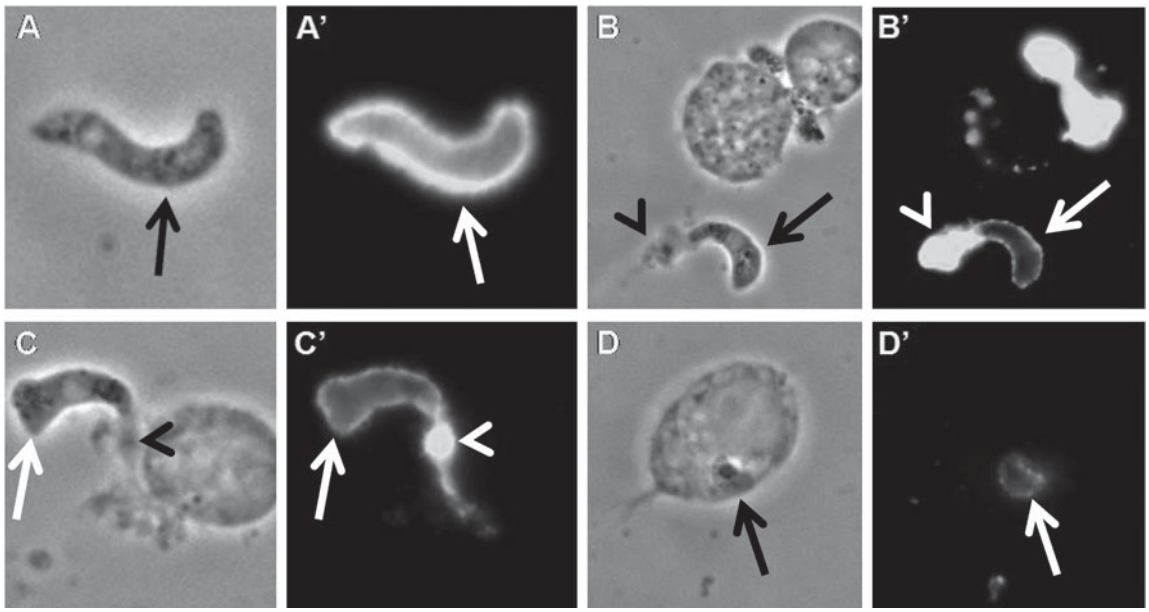

Fig. 2. Immunofluorescence detection of selected phages (clones 5 and 14) bound to *Plasmodium berghei* ookinetes after co-culture of Mos20. The phase-contrast images (capitals without apostrophe) have their corresponding fluorescence images (capitals with apostrophe). (A) An ookinete covered with phage clone 5 detected with anti-M13 and anti-Pbs25 (arrow). In (B′) and (C′) ookinetes are shown devoid of phages but positive for Pbs25 (arrows). An intense co-location of both phage clones and Pbs25 can be observed at one pole of the ookinete (arrow heads). In (D) a Mos cell is invaded by an ookinete (arrow).

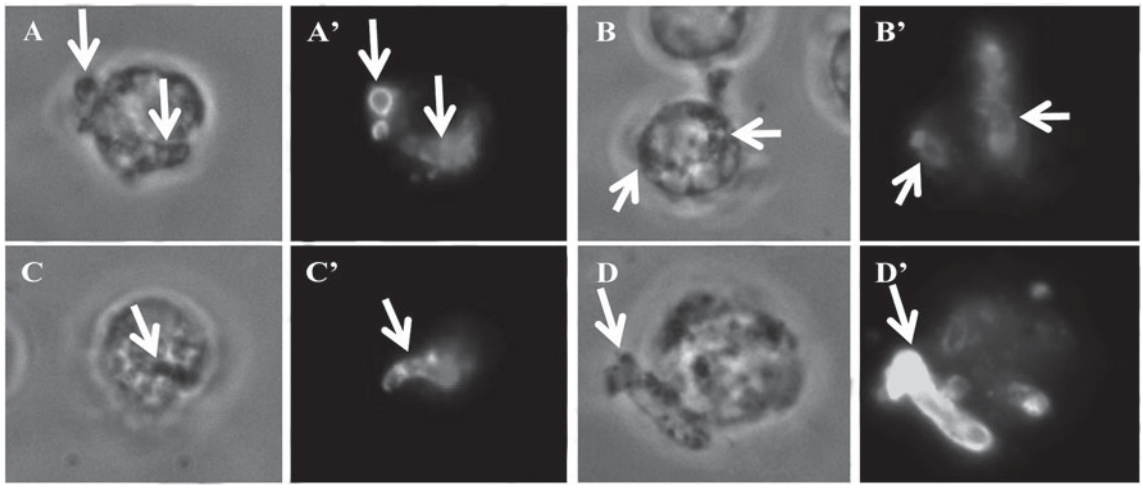

Fig. 3. Ookinetes pre-incubated with selected phages invade Mos20 cells. The phase-contrast images (capitals without apostrophe) have their corresponding fluorescence images (capitals with apostrophe). Ookinetes were pre-incubated with selected phage clone 14 and afterwards with Mos20 cells. A double staining was applied to identify intracellular and extracellular parasites, both probed with anti-Pbs25 antibody. (A) Transverse view of an extracellular ookinete (left arrow) next to a longitudinal view of an intracellular ookinete (right arrow). (B) Two intracellular ookinetes invading the same cell (left and right arrows). (C) An ookinete in the process of invading a Mos cell (arrow). (D) A Mos-20 cell with an extracellular ookinete (arrow).

1199·57, 1619·85, 1754·94, 1801·87, 1833·9498 and 1896·94.

In Western blot analysis of ookinete protein extracts, the anti-actin antibody recognized a band of approximately 47 kDa, corresponding to actin (Fig. 5A, lane 2, arrow head), no signal was observed when ookinete protein extracts were incubated with a mouse irrelevant IgG (Fig. 5A, lane 3). We further characterized actin in *P. berghei* purified ookinetes, permeabilized and unpermeabilized, and by electron microscopy (EM).

Actin is detected on the surface of live, fresh P. berghei *ookinetes*

In immunofluorescence assays of live-fresh (intact) ookinetes treated with the anti-actin antibody, 99·37% of parasites depicted a patchy pattern signal

https://doi.org/10.1017/S0031182011001296 Published online by Cambridge University Press

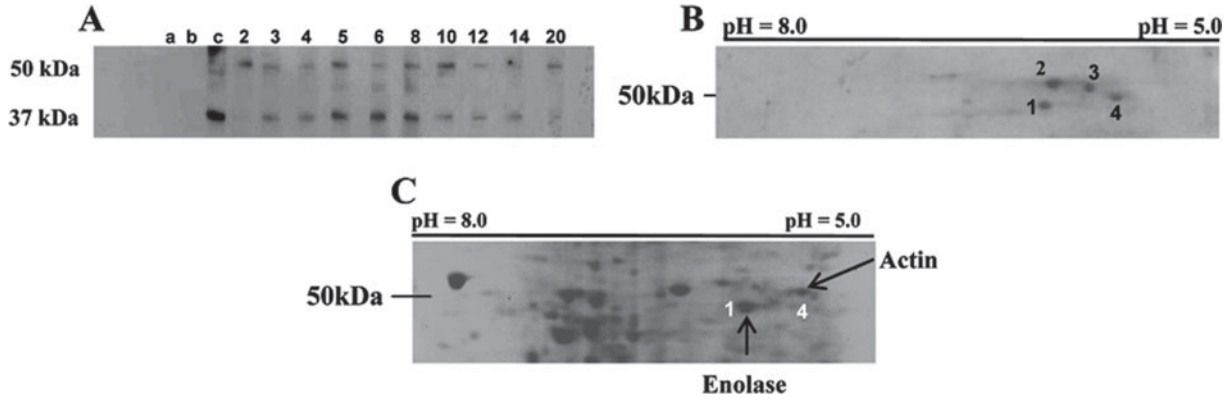

Fig. 4. Western blot of phage clones bound to ookinete proteins using anti-M13 antibody. (A) SDS–PAGE electrophoresis, selected phages recognized 2 protein bands of 37 and ~50 kDa. Lanes: (a) no phage; (b) native phage (without peptide); (c) pool of random-selected phages. Numbered lanes correspond to clone number. (B) In 2D electrophoresis, phage clone 14 bound to 4 proteins around 50 kDa. (C) Coomassie-stained 2D gel of ookinete proteins. The arrows indicate 2 proteins recognized by phage clone 14 that were identified as actin and enolase by mass spectrometry.

(Table 3 and Fig. 5B, panel A, arrow) but were negative to the anti-tubulin antibody. While only 68·37% of purified (NH_4Cl-exposed) ookinetes incubated with the same antibody presented this patchy anti-actin signal (Table 3 and Fig. 5B, panel D, arrow heads), 27·71% presented a uniform surface pattern (Fig. 5B, panel D, arrows). Of the NH_4Cl exposed ookinetes, 83·3% were stained with the anti-tubulin antibody (see Supplementary Fig. S1, online version only, and Table 4). Live-fresh and purified ookinetes were not stained with propidium iodide (Fig. 5B, panels B and E, respectively). Permeabilized ookinetes stained completely with the anti-actin antibody and propidium iodide (Fig. 5B, panels G and H, respectively), and with the anti-tubulin antibody (Supplementary Fig. S2, online version only). No fluorescence was detected on ookinetes incubated only with an irrelevant rabbit IgG (Fig. 5B, panel K) or an anti-rabbit IgG-FITC (S2). A positive signal (anti-actin gold-labelled antibody) was detected on the surface of ookinetes (Fig. 6A and D, square and arrows), but no signals were detected on ookinetes probed with secondary anti-rabbit IgG (Fig. 6, B and E, respectively) or with an irrelevant primary rabbit IgG (Fig. 6, C and F).

Selected peptides share features with An. gambiae *midgut proteins*

Several peptides displayed by selected clones, despite not having identical sequences, shared a hydropathicity profile (values based on Kyte and Doolittle, 1981) characterized by 2 maximums separated by a valley of hydropathicity (data not shown). When a homology analysis was conducted in the *An. gambiae* gene database, searching for proteins with similarity with the selected peptides, a protein with Peritrophin-A domains (AGAP004851-PA) was found (Table 2). This protein (AGAP004851-PA) had a 274 amino acid sequence and a region (positions 213–224) homologous to the peptide displayed by clone 21. The similarity between both profiles was significant (Pearson correlation = 0·926, $P = 0{\cdot}00004$) in a window of 11 amino acids.

DISCUSSION

Using a phage display we identified a PWWP peptide consensus sequence, which bound to the *P. berghei* ookinete surface. Given that selection was carried out using live ookinetes with presumed unaltered membrane permeability, it was expected that the selected phages bind to the ookinete surface. All selected phage clones that bound to ookinetes share structural features that could reflect some of the structural requirements of native mosquito ligand molecules. The peptide sequences displayed by these phages have sequences and hydropathicity profiles similar to proteins with domains typical of insect peritrophic membrane and basal lamina, such as peritrophin-A (AGAP004851-PA). Therefore, these peptide sequences seem to indicate structural similarities of the phages to the mosquito digestive tract molecules, which interact with ookinete surfaces during their migration. The peritrophin-A domain is found in chitin-binding proteins, particularly in midgut peritrophic matrix proteins of mosquitoes and other insects (Shen and Jacobs-Lorena, 1998). This structure represents a potential barrier with which the ookinete needs to interact and through which it needs to pass during its journey to the basal lamina.

The displayed peptides have tryptophan and proline residues with relative positions that might be functionally important. Several protein-protein interactions involve sequences less than 10 residues long

https://doi.org/10.1017/S0031182011001296 Published online by Cambridge University Press

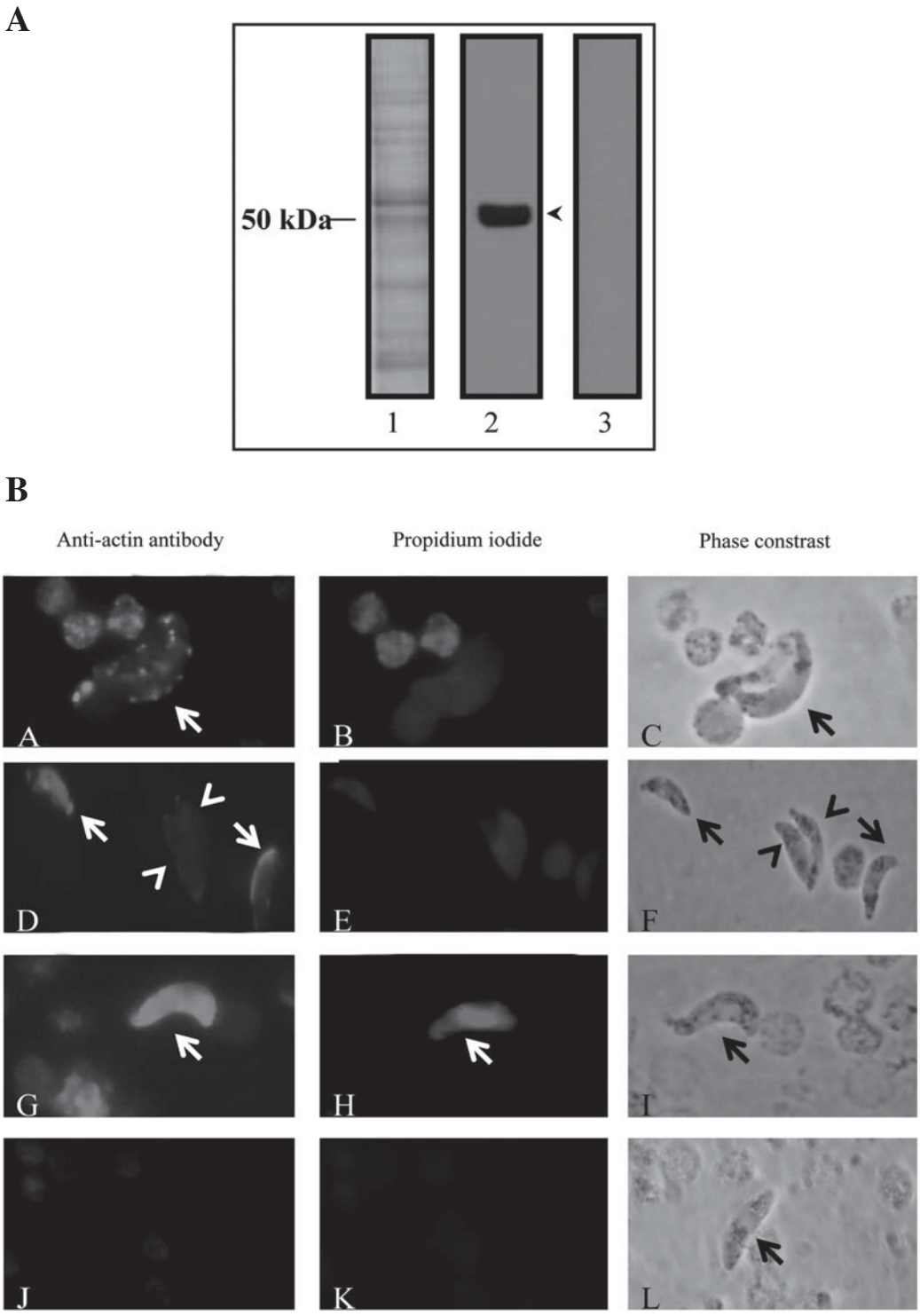

Fig. 5. (A) Western blot of ookinetes and anti-actin antibody. Lane 1: ookinete extract stained with Coomassie. Lane 2: ookinete extract probed with anti-actin antibody (1:1000) recognizing a band of approx. 50 kDa (arrow). Lane 3: ookinete extract probed with an anti-rabbit IgG only. Molecular weights are expressed in kilodaltons (kDa). Blot was developed with a chemiluminescence kit. (B) Immunofluorescence analysis of *Plasmodium berghei* ookinetes with anti-actin antibody. (Ba) Live-fresh ookinetes show patchy actin on their surface (arrows) and do not stain with propidium iodide (Bb). Ookinetes exposed to NH_4Cl show patchy actin (Bd, arrow heads), uniform actin (Bd, arrows), and do not stain with PI (Be). Permeabilized ookinetes stained with anti-actin (Bg, arrow) and PI (Bh, arrow). Ookinetes are negative to an irrelevant rabbit IgG (Bj), to incubation with an anti-rabbit-FITC only (data not shown) and to PI (Bk). Images on Bc, f, i and l are the phase-contrast images of Ba, d, g and j, respectively.

https://doi.org/10.1017/S0031182011001296 Published online by Cambridge University Press

Table 3. Percentage of live-fresh and purified ookinetes expressing actin

(Results are from three independent experiments.)

Condition	No. of ookinetes counted	% ookinetes expressing actin	% ookinetes expressing patchy actin	% ookinetes expressing uniform surface actin	% negative ookinetes
Live-fresh	321	99·38	99·37	0	0·62
Purified NH_4Cl	332	96·09	68·37	27·71	3·91

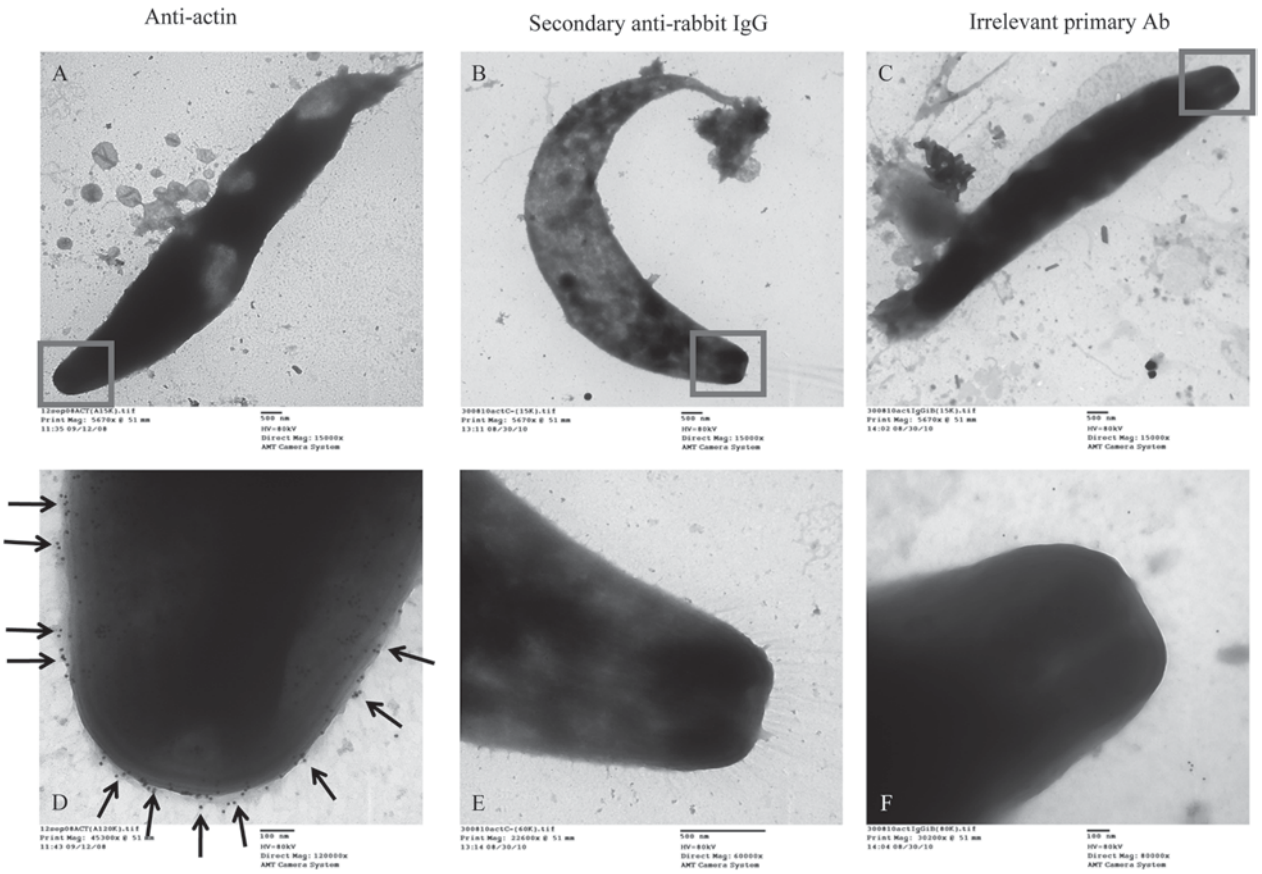

Fig. 6. *Plasmodium berghei* ookinetes probed with anti-actin antibody and evaluated by electron microscopy analysis. (A) *P. berghei* ookinetes probed with anti-actin. (B) *P. berghei* ookinetes probed with an anti-rabbit IgG conjugated to gold particles (incubation with a primary antibody omitted). (C) *P. berghei* ookinetes probed with an irrelevant rabbit IgG. Images in D, E and F are magnifications of A, B and C, respectively.

(Geysen *et al*. 1985) and within these the proline residues are essential for the interaction (Kay *et al*. 2000).

Selected phages bound to ookinetes presented various patterns. In some cases phages were bound only to one end of parasites, indicating that phages interact with components from the apical end of live ookinetes. During ookinete interaction with Mos20 insect cells, tagged phages were displaced from the anterior to the posterior parasite end and shed along with Pbs25. This indicates that the parasite molecules recognized by the phages may be involved in parasite gliding. However, as interacting and intracellular parasites were devoid of phages, it is also possible that the parasite-mosquito cell interaction does not require the molecules recognized by phages.

Two parasite proteins were identified by mass spectrometry assays of the ookinete protein spots: enolase and actin I. Because these are typically intracellular proteins, this finding was unexpected. However, identification has been made in several organisms of surface enolases with what are most likely non-enzymatic functions (Pancholi and Fischetti 1998; Bergmann *et al*. 2001; Pancholi, 2001; Pitarch *et al*. 2006), including apicomplexan parasites (Labbé *et al*. 2006; Pal-Bhowmick *et al*. 2007*b*). Enolase has been documented in asexual and sexual stages (merozoites, schizonts, gametocytes and sporozoites) of *P. falciparum* and *P. yoelli* (Pal-Bhowmick *et al*.

https://doi.org/10.1017/S0031182011001296 Published online by Cambridge University Press

Table 4. Percentage of live-fresh and purified ookinetes expressing tubulin

(Results are from one experiment.)

Condition	No. of ookinetes counted	% ookinetes expressing tubulin
Live-fresh	1500	0
Purified NH_4 CI	1487	83·3

2007*a*), and our findings indicate that it is also present on the surface of *P. berghei* ookinetes. Interestingly, this molecule was localized on the merozoite surface, and anti-enolase antibodies blocked erythrocyte infection, indicating that it participates in invasion (Pal-Bhowick *et al*. 2007*b*). The participation of enolase in invasion has been documented in *Eimeria tenella*, where the molecule was localized on the sporozoite apex and appears to be secreted into the medium (Labbé *et al*. 2006). The ability of enolase to bind human plasminogen opens the possibility of tethering this molecule (Labbé *et al*. 2006), and suggests that it is able to activate matrix metalloproteases (Gong *et al*. 2008) on the merozoite surface, enabling cell invasion. Similarly, the possibility that enolase recruits vertebrate plasminogen (from the bloodmeal bolus) to the ookinete surface and that it participates in the mosquito midgut invasion warrants investigation.

The interaction of actin with myosin A and myosin light chain (MLC 1) to form a molecular motor for gliding has been well documented in Apicomplexa (Keeley and Soldati, 2004). In the current gliding model, the actin-myosin complex (located under the parasite plasma membrane) is anchored through GAP45 (glideosome associated protein 45) to the inner membrane complex. Aldolase functions as a bridge between the submembranous actin and transmembrane adhesins (Jewett and Sibley, 2003). These adhesins in *Plasmodium* belong to the thrombospondin-related Anonymous Protein family (Buscaglia *et al*. 2003), represented in ookinetes by CTRP (Lecona *et al*. 2010). The polymerization of actin filaments drives the motile process (Wetzel *et al*. 2003; Ganter *et al*. 2009).

Besides the submembranous actin, our results (phage overlay, IFI, immuno-electron microscopy) documented the presence of this molecule on the ookinete surface. To rule out the possibility that this atypical location resulted from parasite plasma membrane damage, anti-actin fluorescence was compared on fresh live, NH_4Cl treated, and detergent treated parasites. The integrity of fresh parasites was tested by their negative staining with an anti-tubulin antibody and propidium iodide. Fresh parasites presented a patchy pattern of anti-actin staining, while treatment with NH_4Cl and permeabilization increased the proportion of parasites with a uniform signal.

Actin has been observed on the surface of different cells, such as lymphocytes, monocytes and endothelial cells (reviewed by Smalheiser, 1996), and various functions have been attributed to the surface location of this protein in different systems (Moroianu *et al*. 1993; Liu *et al*. 2005). The interaction of cells with extracellular matrix components could determine cellular phenotype induction and maintenance by cytoskeleton proteins and integrins (Cody and Wicha, 1986; Kikkawa *et al*. 2000). Accordingly, it has been proposed that the interaction of ookinetes with components of the midgut basal lamina provides possible cues for their transformation into oocysts (reviewed by Sinden, 1999). The fact that *Plasmodium* ookinetes interact with laminin, a major component of the mosquito basal lamina (Arrighi *et al*. 2008), and integrin, probably from midgut epithelial cells, has been documented (Mahairaki *et al*. 2005; Nacer *et al*. 2008). It is possible that the integrin that coats invading ookinetes does so by binding to laminin (Sonnenberg 1993). The ookinete surface proteins P25 (Vlachou *et al*. 2001), SOAP (Dessens *et al*. 2003) and CTRP (Mahairaki *et al*. 2001) with affinity for laminin have been proposed as the parasite counterparts for these interactions. However, SOAP and CTRP are not essential for ookinete transformation into oocysts (Nacer *et al*. 2008), and P25 is shed off as the parasite moves, indicating that they are unlikely to function as mediators for the cues leading to oocyst transformation. On the other hand, we could not detect actin on the tracks left by *P. berghei* ookinetes on a solid substrate during gliding, suggesting that surface actin is not shed off. We propose that actin on the parasite surface could anchor the parasite to the basal lamina and transmit signals for parasite transformation, as could possible interactions of the parasite with transmembrane proteins. Further investigation is needed regarding these possible interactions, as well as the specific routes followed by enolase and actin to the parasite surface.

The identification of molecules involved in *Plasmodium-Anopheles* interactions is crucial to the development of new strategies that could interrupt malaria transmission by disrupting the establishment of the parasite in the vector. Identification of *Plasmodium* proteins with functions related to the motility and invasion of the ookinete could enable the development of new transmission blocking strategies capable of controlling malaria.

ACKNOWLEDGEMENTS

The authors thank Robert E. Sinden for critically reviewing the manuscript and for useful suggestions. Thanks to Fidel de la Cruz Hernández-Hernández and Adriana Mendoza Rodriguez for technical help. This work was supported by a CONACYT grant (g-37186M) to M.H.R. and a CONACYT grant (CB200801107006) to M.C.R.

REFERENCES

Adini, A. and Warburg, A. (1999). Interaction of *Plasmodium gallinaceum* ookinetes and oocysts with extracellular matrix proteins. *Parasitology* **119**, 331–336.

Arrighi, R. B., Ebikeme, C., Jiang, Y., Ranford-Cartwright, L., Barrett, M. P., Langel, U. and Faye, I. (2008). Cell-penetrating peptide TP10 shows broad- spectrum activity against both *Plasmodium falciparum* and *Trypanosoma brucei brucei*. *Antimicrobial Agents and Chemotherapy* **52**, 3414–3417.

Baum, J., Richard, D., Healer, J., Rug, M., Krnajski, Z., Gilberger, T. W., Green, J. L., Holder, A. A. and Cowman, A. F. (2006). A conserved molecular motor drives cell invasion and gliding motility across malaria life cycle stages and other apicomplexan parasites. *Journal of Biological Chemistry* **281**, 5197–5208.

Bergmann, S., Rohde, M., Chhatwal, G. S. and Hammerschmidt, S. (2001). Alpha- Enolase of *Streptococcus pneumoniae* is a plasmin(ogen)-binding protein displayed on the bacterial cell surface. *Molecular Microbiology* **40**, 1273–1287.

Buscaglia, C. A., Coppens, I., Hol, W. G. J. and Nussenzweig, V. (2003). Sites of interaction between aldolase and Trombospondin-related anonymous protein in *Plasmodium*. *Molecular Biology of the Cell* **14**, 4947–4957.

Cody, R. L. and Wicha, M. S. (1986). Clustering of cell surface laminin enhances its association with the cytoskeleton. *Experimental Cell Research* **165**, 107–116.

Crawford, A. W., Michelsen, J. M. and Beckerle, M. C. (1992). An interaction between zyxin and alfa-actinin. *Journal of Cell Biology* **116**, 1381–1393.

Daher, W. and Soldati, D. (2009). Mechanisms controlling glideosome function in apicomplexans. *Current Opinion in Microbiology* **12**, 408–414.

Dessens, J. T., Beetsma, A. L., Dimopoulus, G., Wengelnick, K., Crisanti, A., Kafatos, F. C. and Sinden, R. E. (1999). CTRP is essential for mosquito infection by malaria ookinetes. *European Molecular Biology Organization Journal* **18**, 6221–6227.

Dessens, J. T., Sidén-Kiamos, I., Mendoza, J., Mahairaki, V., Khater, E., Vlachou, D., Xu, X. J., Kafatos, F. C., Louis, C., Dimopoulos, G. and Sinden, R. E. (2003). SOAP, a novel malaria ookinete protein involved in mosquito midgut invasion and oocyst development. *Molecular Microbiology* **49**, 319–329.

Fowler, R. E., Margos, G. and Mitchell, G. H. (2004). The cytoskeleton and motility in apicomplexan invasion. *Advances in Parasitology* **56**, 213–263.

Ganter, M., Schüler, H. and Matuschewski, K. (2009). Vital role for the *Plasmodium* actin capping protein (CP) beta subunit in motility of malaria sporozoites. *Molecular Microbiology* **74**, 1356–1367.

Geysen, H. M., Barteling, S. J. and Meloen, R. H. (1985). Small peptides induce antibodies with a sequence and structural requirement for binding antigen comparable to antibodies raised against the native protein. *Proceedings of the National Academy of Sciences, USA* **82**, 178–182.

Gong, Y., Hart, E., Shchurin, A. and Hoover-Plow, J. (2008). Inflammatory macrophage migration requires MM-9 activation by plasminogen in mice. *Journal of Clinical Investigation* **118**, 3012–3024.

Hall, T. A. (1999). BioEdit: a user-friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series* **41**, 95–98.

Hirai, M., Arai, M., Kawai, S. and Matsuoka, H. (2006). PbGCbeta is essential for *Plasmodium* ookinete motility to invade midgut cell and for successful completion of parasite life cycle in mosquitoes. *Journal of Biochemistry* **140**, 747–757.

Hofmann, K. and Stoffel, W. (1993). TMbase: a database of membrane spanning protein segments. *Biological Chemistry Hoppe Seyler* **347**, 166–171.

Huber, M., Cabib, E. and Miller, L. H. (1991). Malaria parasite chitinase and penetration of the mosquito peritrophic membrane. *Proceedings of the National Academy of Sciences, USA* **88**, 2807–2810.

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

Kadota, K., Ishino, T., Matsuyama, T., Chinzei, Y. and Yuda, M. (2004). Essential role of membrane-attack protein in malarial transmission to mosquito host. *Proceedings of the National Academy of Sciences, USA* **101**, 16310–16315.

Kappe, S., Bruderer, T., Gantt, S., Fujioka, H., Nussenzweig, H. and Ménard, R. (1999). Conservation of a gliding motility and cell invasion machinery in Apicomplexan parasites. *Journal of Cell Biology* **147**, 937–943.

Kay, B. K., Williamson, M. P. and Sudol, M. (2000). The importance of being proline: the interaction of proline-rich motifs in signaling proteins with their cognate domains. *FASEB Journal*. **14**, 231–241.

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

Kikkawa, Y., Sanzen, N., Fujiwara, H., Sonnenberg, A. and Sekiguchi, K. (2000). Integrin binding specificity of laminin-10/11: laminin-10/11 are recognized by alpha 3β1, α6beta 1 and alfa 6 beta 4 integrins. *Journal of Cell Sciences* **113**, 869–876.

Kyte, J. and Doolittle, R. F. (1982). A simple method for displaying the hydrophobic character of a protein. *Journal of Molecular Biology* **157**, 105–142.

Labbé, M., Péroval, M., Bourdieu, C., Girard-Misguich, F. and Péry, P. (2006). *Eimeria tenella* enolase and pyruvate kinases: A likely role in glycolysis and in other functions. *International Journal for Parasitology* **36**, 1443–1452.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature, London* **227**, 680–685.

Lecona, A., Rodriguez, M. H., Argotte-Ramos, R. S., Alvarado-Delgado, A. and Rodriguez, M. C. (2010). *Plasmodium berghei* ookinetes glide and release Pbs25 and CTRP on solid surface substrates. *Journal of Parasitology* **96**, 216–218.

Limviroj, W., Yano, K., Yuda, M., Ando, K. and Chinzei, Y. (2002). Immuno-electron microscopic observation of *Plasmodium berghei* CTRP localization in the midgut of the vector mosquito *Anopheles stephensi*. *Journal of Parasitology* **88**, 664–672.

Liu, D. Y., Clarke, G. N. and Baker, H. W. (2005). Exposure of actin on the surface of the human sperm head during *in vitro* culture relates to sperm morphology, capacitation and zona binding. *Human Reproduction* **20**, 999–1005.

Mahairaki, V., Lycett, G., Sidén-Kiamos, I., Sinden, R. E. and Louis, C. (2005). Close association of invading *Plasmodium berghei* and β integrin in the *Anopheles gambiae* midgut. *Archives of Insect Biochemistry and Physiology* **60**, 13–19.

Mahairaki, V., Voyatzi, T., Siden-Kiamos, I. and Louis, C. (2001). The *Anopheles gambiae* gamma 1 laminin directly binds the *Plasmodium berghei* circumsporozoite- and TRAP- related protein (CTRP). *Molecular and Biochemical Parasitology* **140**, 119–121.

Margos, G., Sidén-Kiamos, I., Fowler, R. E., Gillman, T. R., Spaccapelo, R., Lycett, G., Vlachou, D., Papagiannakis, G., Eling, W. M., Mitchell, G. H. and Louis, C. (2000). Myosin A expressions in sporogonic stages of *Plasmodium*. *Molecular and Biochemical Parasitology* **111**, 465–469.

Ménard, R. (2001). Gliding motility and cell invasion by Apicomplexa: insights from the *Plasmodium* sporozoite. *Cell Microbiology* **3**, 63–73.

Moroianu, J., Fett, J. W., Riordan, J. F. and Vallee, B. L. (1993). Actin is a surface component of calf pulmonary artery endothelial cells in culture. *Proceedings of the National Academy of Sciences, USA* **90**, 3815–3819.

Nacer, A., Underhill, A. and Hurd, H. (2008). The microneme proteins CTRP and SOAP are not essential for *Plasmodium berghei* ookinete to oocyst transformation *in vitro* in a cell free system. *Malaria Journal* **7**, 82. doi:10.1186/1475-2875-7-82.

Naitza, S., Spano, F., Robson, K. J. H. and Crisanti, A. (1998). The thrombospondin- related protein family of Apicomplexan parasites. The gears of the cell invasion machinery. *Parasitology Today* **14**, 479–484.

O'Farrell, P. H. (1975). High resolution two-dimensional electrophoresis of proteins. *Journal of Biological Chemistry* **250**, 4007–4021.

Pal-Bhowmick, I., Mehta, M., Coppens, I., Sharma, S. and Jarori, G. K. (2007*a*). Protective properties and surface localization of *Plasmodium falciparum* enolase. *Infection and Immunity* **75**, 5500–5508.

Pal-Bhowmick, I., Vora, H. K. and Jarori, G. K. (2007*b*). Sub-cellular localization and post-translational modifications of the *Plasmodium yoelii* enolase suggest moonlighting functions. *Malaria Journal* **6**, 45.

Pancholi, V. (2001). Multifunctional alpha-enolase: its role in diseases. *Cellular Molecular Life Sciences* **58**, 902–920.

Pancholi, V. and Fischetti, V. A. (1998). Alpha enolase, a novel strong plasmin(ogen) binding protein on the surface of pathogenic streptococci. *The Journal of Biological Chemistry* **273**, 14503–14515.

Pitarch, A., Jimenez, A., Nombela, C. and Gil, C. (2006). Decoding serological response to *Candida* cell wall immunome into novel diagnostic, prognostic, and therapeutic candidates for systemic candidiasis by proteomic and bioinformatics analyses. *Molecular Cell Proteomics* **5**, 79–96.

Rodriguez, M. C., Gerold, P., Dessens, J., Kurtenbach, K., Schwartz, R. T., Sinden, R. E. and Margos, G. (2000). Characterisation and expression of pbs25, a sexual and sporogonic stage specific protein of *Plasmodium berghei*. *Molecular and Biochemical Parasitology* **110**, 147–159.

Rodríguez, M. C., Margos, G., Compton, H., Ku, M., Lanz, H., Rodríguez, M. H. and Sinden, R. E. (2002). *Plasmodium berghei*: routine production of pure gametocytes, extracellular gametes, zygotes, and ookinetes. *Experimental Parasitology* **101**, 73–76.

Shen, Z. and Jacobs-Lorena, M. (1998). A type I peritrophic matrix protein from the malaria vector *Anopheles gambiae* binds to chitin. Cloning, expression, and characterization. *Journal of Biological Chemistry* **273**, 17665–17670.

Siden-Kiamos, I., Ecker, A., Nyback, S., Louis, C., Sinden, R. E. and Billker, O. (2006). *Plasmodium berghei* calcium-dependent protein kinase 3 is required for ookinete gliding motility and mosquito midgut invasion. *Molecular Microbiology* **60**, 1355–1363.

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*. *Journal of Cell Science* **113**, 3419–3426.

Sinden, R. E. (1999). *Plasmodium* differentiation in the mosquito. *Parassitologia* **41**, 139–148.

Smalheiser, N. R. (1996). Proteins in unexpected locations. *Molecular Biology of the Cell* **7**, 1003–1014.

Soldati-Favre, D. (2008). Molecular dissection of host cell invasion by apicomplexans: the glideosome. *Parasite* **15**, 197–205.

Sonnenberg, A. (1993). Integrins and their ligands. *Current topics in Microbiology and Immunology* **184**, 7–35.

Sonnhammer, E. L. L., von Heijne, G. and Krogh, A. (1998). A hidden Markov model for predicting transmembrane helices in protein sequences. In *Proceedings of the Sixth International Conference on Intelligent Systems for Molecular Biology* (ed. Glasgow, J., Littlejohn, T., Major, F., Lathrop, R., Sankoff, D. and Sensen, C.), pp. 175–182. AAAI Press, Menlo Park, CA, USA.

Stec, I., Nagl, S. B., van Ommen, G. B. and den Dunnen, J. T. (2000). The PWWP domain: a potential protein-protein interaction domain in nuclear proteins influencing differentiation? *FEBS Letters* **473**, 1–5.

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. *Molecular Microbiology* **36**, 1–9.

Tomley, F. M. and Soldati, D. S. (2001). Mix and match modules: structure and function of microneme proteins in apicomplexan parasites. *Trends in Parasitology* **17**, 81–88.

Towbin, H., Staehelin, T. and Gordin, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proceedings of the National Academy of Sciences, USA* **76**, 4350–354.

Tremp, A. Z., Khater, E. I. and Dessens, J. T. (2008). IMC1b is a putative membrane skeleton protein involved in cell shape, mechanical strength, motility, and infectivity of malaria ookinetes. *Journal of Biological Chemistry* **283**, 27604–27611.

Tsuboi, T., Cao, Y., Kaslow, D. C., Shiwaku, K. and Torii, M. (1997). Primary structure of a novel ookinete surface protein from *Plasmodium berghei*. *Molecular and Biochemical Parasitology* **85**, 131–134.

Varma, M. G. R. and Pudney, M. (1969). The growth and serial passage of cell lines from *Aedes aegypti* (L) larvae in different media. *Journal of Medical Entomology* **6**, 432–439.

Vlachou, D., Lycett, G., Sidén-Kiamos, I., Blass, C., Sinden, R. E. and Louis, C. (2001). *Anopheles gambiae* laminin interacts with the P25 surface protein of *Plasmodium berghei* ookinetes. *Molecular and Biochemical Parasitology* **112**, 229–237.

Wetzel, D. M., Håkansson, S., Hu, K., Roos, D. and Sibley, L. D. (2003). Actin filament polymerization regulates gliding motility by apicomplexan parasites. *Molecular Biology of the Cell* **14**, 396–406.

Winger, L. A., Tirawanchai, N., Nicholas, J., Carter, H. E., Smith, J. E. and Sinden, R. E. (1988). Ookinete antigens of *Plasmodium berghei*. Appearance on the zygote surface of an Mr 21 kD determinant identified by transmission-blocking monoclonal antibodies. *Parasite Immunology* **10**, 193–207.

Yuda, M., Sawai, T. and Chinzei, Y. (1999). Structure and expression of an adhesive protein- like molecule of mosquito invasive-stage malarial parasite. *Journal of Experimental Medicine* **189**, 1947–1952.

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. *Molecular and Biochemical Parasitology* **116**, 65–72.