in vivo *24*: 271-280 (2010)

A Possible Key Molecule for the Invasion of the *Plasmodium berghei* Ookinetes into the Midgut Epithelium of *Anopheles gambiae* Mosquitoes

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Abstract. *Background: In order to identify molecules necessary for the invasion of the mosquito midgut epithelium by plasmodia, interaction assays between both these structures were devised. Materials and Methods: Enrichment of Plasmodium berghei ookinetes was first carried out using a double 5-17% gradient Nycodenz cushion, which resulted in an enrichment factor of over 800%. Viability of these ookinetes was confirmed by membrane feeding mosquito infection assays, showing no decrease in infection prevalence or intensity, and suggesting that putative surface proteins necessary for the invasion were unaffected. Results: Protein interaction assays between mosquito midgut and ookinete extracts were optimized yielding Anopheles gambiae mosquito midgut proteins of >220, 200 and 48 kDa that could bind biotinylated P. berghei ookinete extracts, and P. berghei ookinete proteins of 48-45 kDa that could bind biotinylated An. gambiae midgut proteins. Using an An. gambiae midgut biotinylated extract linked to a streptavidin-agarose matrix, P. berghei ookinete proteins of approximately 116, 45 and 21 kDa were obtained. This protein chromotography pull-down assay was reproducibly repeated and spots from 2D eletrophoretic separation were analysed by mass spectrometry. For one spot, a significant match with a putative erythrocyte binding protein from P. falciparum (Pf EBA-165) was obtained. This protein belongs to the erythrocyte binding superfamily of the merozoite stage that is involved in the invasion process of the erythrocytes. Conclusion: Our findings suggest that there is a possibility that a homologue of Pf EBA-165 takes part in the ookinete recognition and invasion process of the mosquito midgut by plasmodia ookinetes.*

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Key Words: Malaria, mosquito, *Anopheles gambiae, Plasmodium berghei,* ookinete, midgut.

Malaria is still one of the great burdens of world health, with 40% of the population at risk, 247 million cases, nearly a million deaths, mostly of children under 5 years, as estimated in 2006 (1). Malaria control faces important challenges such as parasite and mosquito resistance to drugs and insecticides, respectively. Effective vaccines, despite continued research efforts, are not yet available (2).

After mosquitoes have taken a bloodmeal on an infected host *Plasmodium* ookinetes penetrate the epithelial cell layer and transform into oocysts upon reaching the basal lamina. The luminal surface of the midgut epithelium exhibits a dense microvillar brush border with a microfibrillar network that is required for successful invasion by ookinetes (3). Although the identity of the receptor or target on the midgut epithelium for ookinete proteins have not yet been achieved, a number of studies have pointed to their postulated existence and role in ookinete penetration. SM1, a 12-amino acid peptide selected from a phage display library, binds to midgut epithelium blocking its invasion by *Plasmodium* parasites (4) and transgenic mosquitoes secreting this peptide into the lumen are incompetent for parasite transmission (5). The function and nature of the putative receptor for this peptide in the mosquito midgut is yet to be discovered. Likely candidates are membrane-bound microvilli glycoproteins as antibodies against these, blocked *P. yoelii* development in a dose-dependent way, and deglycosylation

assays pointed to oligosaccharide epitopes (6). Ookinete attachment has also been inhibited by the lectin Jacalin binding to *An. gambiae* aminopeptidase N, antibodies against which inhibited both *P. berghei* and *P. falciparum* development by approximately 70% (7). Similarly, antibodies against *An. gambiae* carboxypeptidases B have drastically reduced *P. falciparum* and *P. berghei* development (8). This was not observed for *An. gambiae* annexins. Although these are considered putative receptors for ookinete invasion, their blockage by antibodies reduced transmission only by about a third, suggesting that their role is partially redundant (9).

Extracts of ookinetes were observed to bind to laminin and collagen IV, putative constituents of the basal lamina of the mosquito midgut epithelium (10-12). However, laminin could not be considered a receptor for ookinete penetration into epithelial cells (13). Ookinete invasion is blocked by a phospholipase A2 which binds irreversibly to membranes without causing damage (14). Transgenic mosquitoes secreting these enzymes on the surface of the midgut epithelium are incompetent vectors for plasmodia (15), stressing the likely existence of a receptor.

Several ookinete proteins have been implicated in midgut invasion: i) the micronemal CTRP (16-18); ii) the secreted ookinete adhesive protein (SOAP) also targeted to the micronemes and interacting with mosquito laminin (19); iii) and the ookinete surface proteins P25/P28 (20). Disruption of either of these proteins, or of both in the case of the latter, impairs the development of ookinetes in the midgut. In addition, ookinetes release a subtilisin-like protease as they cross the epithelial cells suggesting their involvement in the invasion (21).

Proteases, extensively studied in the blood stages and implicated in penetration of erythrocytes (22) have yet to be fully assessed for their role during invasion of the mosquito epithelium. *P. berghei* metacaspase knockout parasites were found to infect normally *An. stephensi* mosquitoes suggesting that this particular protein is functionally redundant (23).

Despite the enormous amount of work, the exact nature of molecules on the surface of the ookinete or on the midgut epithelial cell that function as ligand and receptor, respectively, remain largely unknown (24). Furthermore, single molecules are unlikely to be responsible for invasion, rather multiple and redundant pathways are the rule (7, 25).

In this work, molecules derived from the epithelial cells of the midgut of the mosquito *An. gambiae* and of *P. berghei* ookinetes, have been assessed for their possible participation in the invasion process as receptor/ligand relationships. To maximize the chances of their finding, techniques for enrichment of ookinetes by separating them from remaining asexual parasites in ookinete cultures, were developed. These were then followed by Far-Western and affinity chromatography assays between protein extracts of the mosquito midgut epithelium and *Plasmodium* ookinete extracts in order to isolate molecules functioning in such interaction.

Materials and Methods

Mosquito rearing and midgut extracts. Anopheles gambiae Giles (Diptera: Culicidae) Suakoko strain, were reared under a photoperiod of 12:12 h light:dark, 26˚C, 75-80% RH. Larvae were fed with powdered and adults were maintained on 8% fructose containing 0.05% PABA *ad libitum* and bloodfed on CD1 mice anaesthetized with xylazine (4 mg/kg) and ketamine hydrochloride (100 mg/kg).

Sugar-fed 4-6 day old females were dissected in 100 mM Tris pH 7.2, 300 mM mannitol, and midguts removed and frozen. Midguts were homogenized in a tight fitting glass-glass Dounce homogenizer on ice with tris-mannitol 10 mM and $CaCl_2$ 10 mM supplemented with a cocktail of protease inhibitors (1 μg/ml antipain; 1 μg/ml aprotinin; 1 mM EDTA; 100 μM TPCK; 1 μg/ml leupeptin; 1 mg/ml Pefabloc; 1 μg/ml pepstatin), then sodium-dodecyl sulphate (SDS) was added to a 0.6% final concentration and vortex 5 times, 20 s (about 100 midguts in 100 μl buffer). The samples were centrifuged for 10 minutes at 9,000 ×g, 4˚C, and the supernatant was transferred to a fresh tube. Protein content was quantified by the Bradford method, separated in aliquots and stored at –20˚C.

Mice, malarial parasites and ookinete cultures. Female CD1 mice were infected with *Plasmodium berghei* ANKA gametocyte producer strain (clone 2.34) maintained by cyclic passage. Mice were injected intra-peritoneally (*i.p.*) with 0.2 ml of 6 mg/ml phenylhydrazine in 0.9% NaCl, 3-4 days prior to infection. Sequentially, mice were inoculated *i.p.* with 0.2 ml of infected blood containing at least 10^6 parasites. On day 3 after infection, mice were tail bled and parasitaemia was determined in Giemsa (pH 7.2) stained blood smears, and examined in 20 μl of ookinete medium for exflagellating microgametocytes. When 5-10 exflagellating centres per field were observed in all mice, these were bled by cardiac puncture under terminal anesthesia. Blood was passed through a cellulose powder CF11 (Whatman, Maidstone, UK) column pre equilibrated with cold complete ookinete medium (RPMI 1640® (Sigma, St. Louis, MO, USA), 2g/l sodium bicarbonate, 0.5 g/l hypoxanthine, 50 mg/l penicillin, 50 mg/l streptomycin, 100 mg/l neomycin (PSN® (Gibco, Invitrogen, San Diego, CA, USA)), 10% (v/v) heat-inactivated foetal calf serum, pH 8.3). Eluate was diluted 1:10 with complete ookinete medium into a wide flask to a maximum depth of 1 cm and kept at 19˚C for 16-18 h (26) after which the culture was checked for the presence of ookinetes.

Ookinete enrichment and extracts. Ookinete cultures were spun at 500 ×g for 10 min at 4˚C, the pellet diluted in 45 ml 0.17 M NH_4Cl, for 10 min on ice to lyse erythrocytes, and spun at 1000 ×g for 5 min at 4˚C. Nycodenz® (NycoPrep Universal, Greiner Bio-One, Stuttgart, Germany) was diluted (v/v) in complete ookinete medium. In a 50 ml centrifuge tube, a cushion of 15 ml 17% Nycodenz® was laid, onto which another cushion of 10 ml 5% Nycodenz® was gently laid, on ice. The pellet from the culture after erythrocyte lysis was layered onto this second cushion and spun at 1000 ×g for 25

min, at 4˚C. Ookinetes were removed from the 5-17% interface with a Pasteur pipette and washed in cold PBS, and the pellet after the enrichment was recovered from the bottom of the tube.

Enriched ookinetes were homogenized in a tight fitting glass-glass Dounce homogenizer in 100 mM Tris pH 7.2, 300 mM mannitol, and 10 mM CHAPS–3 [(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, supplemented with a cocktail of protease inhibitors as above.

Biotinylation of midgut and ookinete extracts. Extracts were biotinylated using an EZ-Link Sulpho-NHS-LC Biotinylation kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions. Five mg of midgut protein was diluted in 1 ml of phosphate-buffered saline (PBS) and incubated on ice during two hours with 0.5 mM of biotin. The excess of non-reacted biotin was removed using a desalting column and the confirmation of biotin incorporation was performed by HABA assay (Pierce Biotechnology).

Electrophoresis and Western blot. Mosquito midgut and ookinete extracts prepared as above, were separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) in a discontinuous buffer system on 5-20% gradient or 12% polyacrylamide slab gels, and run at 100 V, in a Mini-protean II gel system (Bio-Rad, Hercules, CA, USA). Samples were mixed (1:1) with sample buffer (80 mM Tris pH 6.8, 2% SDS, 20% glycerol, 0.24% bromophenol blue, and 100 mM DL-dithiothreitol was used as reducing agent), heated at 100˚C for 2-4 min, and then centrifuged for 5 min at 3000× g. Prestained standards (Bio-Rad, Hercules) were used. Proteins were stained with GelCode Blue Stain Reagent (Pierce Biotechnology) according to the manufacturer's instructions.

For Western blotting, proteins were transferred from gels onto PVDF (polyvinylidene difluoride) membranes using an LKB 2117-250 Pharmacia (GE Healthcare, Uppsala, Sweden) semi-dry blotter. Nonspecific binding was blocked with 5% (w/v) dried skimmed milk (DSM) and 5% (v/v) normal goat serum (NGS) in PBS for 2 h at RT. Monoclonal antibody (MAb) 13.1 anti-*P. berghei* PbS 21 (27), was used diluted 1:1000 in 1% (w/v) DSM in PBS, NMS was used as negative control, while incubation with just 1% (w/v) DSM in PBS served as control for the secondary antibody. Strips were washed 5-6 times, 10-15 min each in 0.5% (w/v) DSM in PBS-Tween 20 with constant shaking at RT. Horseradish peroxidase-conjugated goat antimouse IgG diluted 1:1000 in 1% (w/v) DSM in PBS was incubated for 45-60 min at RT, then strips washed as above. Membranes were revealed with chemoluminescent substrate (Lumi-Phos WB; Pierce Biotechnology) and exposed to CL-Xposure film for 20 s.

Assessment of the viability of ookinetes after enrichment. Equal volumes of ookinete culture, ookinete enriched fraction and pellet, washed with PBS, were resuspended in fresh blood from naive mice (1:1) and placed into water jacketed glass feeders, at a volume of 0.5 ml/feeder, covered with stretched Parafilm® and maintained at 37˚C. Five- to seven-day-old female mosquitoes were deprived of fructose 6-12 h before feeding and allowed to feed for 1 h, after which unfed females were removed, kept at 19˚C and 80% RH, and provided with 8% fructose, 0.05% PABA *ad libitum*. Midguts were dissected at the 10th day post infective feed, stained with 0.05% mercurochrome in PBS and examined for oocysts.

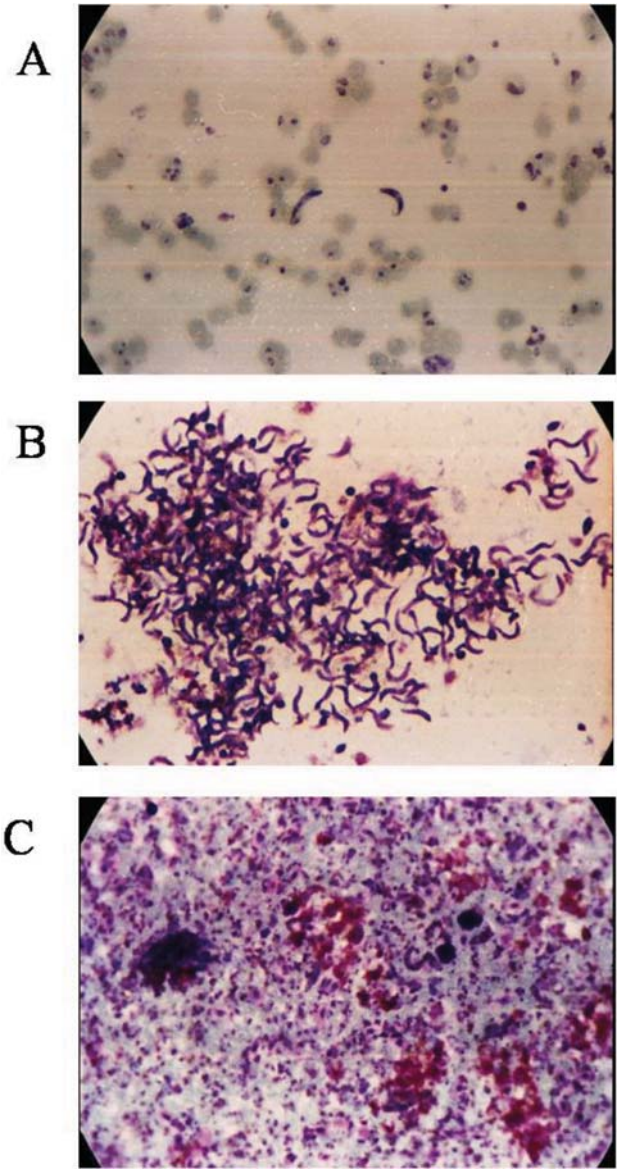

Figure 1. *Giemsa stained smears of P. berghei ookinete cultures: A: ookinete culture (pre-enrichment), B: ookinete enriched fraction, C: pellet after separation. Ookinete cultures were centrifuged at 1000 xg for 25 min, at 4˚C, on a Nycodenz® discontinuous 5-17% gradient. Magnification ×1000.*

Midgut and ookinete interaction Far-Western (protein-protein) assays. Interaction between mosquito midgut (and ookinete) extracts was assayed looking for protein:protein interactions. Midgut or ookinete extracts, prepared as above, were separated on a gradient 5-20% SDS-PAGE and electro-transferred to a PVDF membrane. Laminin (Sigma L2020; Sigma St. Louis, MO, USA) was used as a positive control for midgut proteins. Membranes were blocked with non fat dry milk 5% in PBS for 1 hour at room temperature and incubated with biotinylated ookinetes (or mosquito midgut) proteins of 5 µg/ml overnight. The detection of protein interaction was performed using streptavidin-HRP according to ProFound™ Far-Western Biotinylated-Protein:Protein Interaction Kit (Pierce Biotechnology).

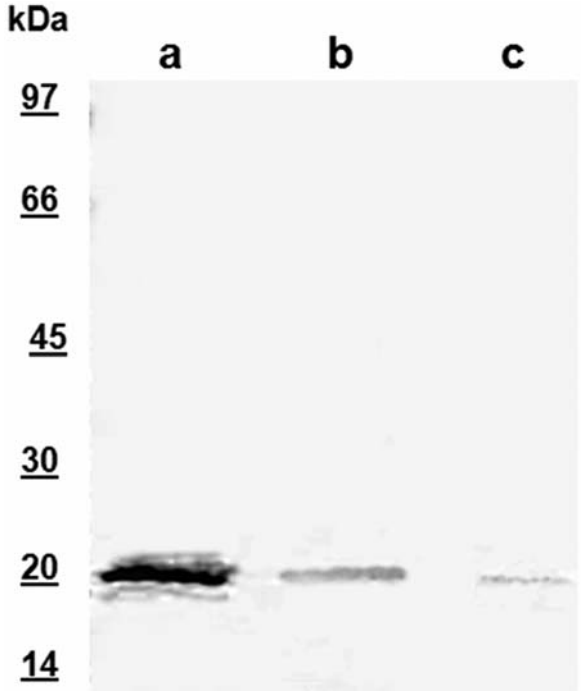

Figure 2. *Western blot of a) 5-17% Nycodenz® enriched P. berghei ookinete fraction, b) ookinete culture pre-enrichment, c) pellet after enrichment. Five micrograms of protein in each lane were separated on a 5-20% gradient SDS-Page, transferred onto nitrocellulose membrane and probed with MAb 13.1 anti-Pbs21, and revealed by chemoluminiscence, and exposed to X-ray films.*

Protein affinity chromatography – Pull-down assay. Protein chromatography was performed using a ProFound™ pull-down biotinylated protein:protein interaction kit (Pierce Biotechnology), which allows an efficient pull-down assay to be performed using immobilized streptavidin to capture a biotinylated protein, with high protein recovery and reproducibility.

The immobilized streptavidin was transferred to a spin column and incubated with 50 μg of biotinylated midgut proteins during 60 min at 4˚C in tris buffer, 150 mM NaCl (TBS) with gentle rocking motion. The excess of bait biotinylated proteins was discarded and free biotin was added to block available streptavidin sites. Subsequently, 100 μg of ookinete protein homogenate was added to the columns with midgut-streptavidin-bait and incubated overnight at 4˚C with slow rotary motion. Columns were washed with acetate buffer, 0.5 M NaCl at pH 5.0 to remove the non-bound or soft linkage proteins and the ookinete bound proteins (ookinete-prey-proteins) were eluted with 0.1 M glycine, pH 2.8. After elution, the sample was immediately neutralized with 1.5 M tris buffer at pH 8. Due to the very low yield, 4.34 μg/ml from a starting concentration of 1500 μg/ml in the ookinete extract, this protein pull-down assay was repeated through 90 chromatography cycles, corresponding to 90 ookinete cultures and enrichment procedures. Eluates of repeated pull-down procedures were pooled and concentrated in an Amicon® Ultra-15 Centrifugal Filter Unit Ultracel-5 membrane (Ref: UFC900508; Sigma), spun for 1 hour at 4˚C at 4000 ×g in a swinging rotor.

2D Electrophoresis. Protein extracts after Amicon® concentration as described above were used at 100μg per gel. Substances that could influence the first dimensional isoelectric focusing (IEF) were removed from the sample by the 2-D Clean-Up Kit protocol (GE Healthcare, Uppsala, Sweden). The cleaned pellet was then resuspended in IEF solubilization solution (9 M urea, 2% (w/v) CHAPS, 2% (v/v) β-mercaptoethanol, 0.8% (v/v) Pharmalyte pH 3–10, 0.01% bromophenol blue), mixed with 3 volumes of a gel slurry (30 mg Sephadex IEF/ml solubilization solution) and loaded on a previously rehydrated 13 cm Immobiline™ DryStrip pH 3-11 NL (GE Healthcare) (rehydration solution: 8 M urea, 0.5% (w/v) CHAPS, 0.2% (w/v) DTT, 0.25% (v/v) Pharmalytes pH 3-10, 0.5% (v/v) Triton X-100, 0.01% bromophenol blue). After IEF, the strip was incubated in equilibration buffer (6 M urea, 2% SDS, 0.1 mM EDTA, 50 mM Tris pH 6.8, 30% glycerol, 0.01% bromophenol blue) containing 10 mg/ml DTT for 15 minutes and in equilibration buffer containing 48.1 mg/ml iodoacetamide for 15 minutes. For SDS-PAGE, the strip was placed in 1% (w/v) low-melting point agarose in 1.5 M Tris pH 8.8, 0.4% SDS on the top of a 12.5% polyacrylamide gel. SDS-PAGE was performed at 25 mA/gel at 4˚C. Proteins were visualized by silver staining and the gel was scanned on a densitometer (GS-800; Bio-Rad, Hercules, CA, USA). The selected spots were excised for mass spectrometry analysis.

MALDI-TOF-MS analysis. MALDI-TOF-MS analysis was performed in a single spot excised from the polyacrylamide gel of the 2DE after being dehydrated using a speed vacuum and re-hydrated with 6.7 μg/ml of sequencing grade trypsin (Promega, Fitchburg, Wisconsin, USA) in 50 mM ammonium carbonate buffer, pH 8, for 30 min. The excess buffer was discarded, 20 μl of ammonium carbonate buffer were added, and the sample was incubated for 16 h at 37˚C. The digested peptides were concentrated and desalted using a microcolumn Poros R2 and directly eluted into a MALDI plate using α-cyano-4-hydroxycinnamic acid (CHCA) in 70% (v/v) acetonitrile with 0.01% (v/v) trifluoracetic acid.

Monoisotopic masses were obtained using a MALDI-TOF-MS model Voyager-DE-STR (Applied Biosystems, Foster City, CA, USA). External mass calibration was performed using a mixture of peptide standards, PepMix1 (Laserbio Labs, Sophia-Antipolis, France).

Contaminant peaks (peaks from contaminating human keratin or from the trypsin added for digestion of proteins, and peaks present in all mass spectra) were identified and eliminated from the MS spectra using the software PeakErazor (http://www.welcome.to/GPMAW). Protein identification was finally performed entering the mass list into the Mascot PMF database (MASCOT Peptide Mass Fingerprint, http://www.matrixscience.com). Searches were carried out against the MSDB database using the following parameters: one allowed missed cleavage; carbamidomethyl cysteine as fixed modification and oxidation of methionine as variable modification; mass tolerance 50-100 ppm.

Results

P. berghei ookinete enrichment and viability assessment. Enriched ookinetes were recovered from the 5-17% Nycodenz® interface (Figure 1 and Table I), presenting good reproducibility. Western blot with Mab 13.1 anti-Pbs 21 shows a stronger reactivity on the enriched ookinete fraction than on the original ookinete culture (Figure 2).

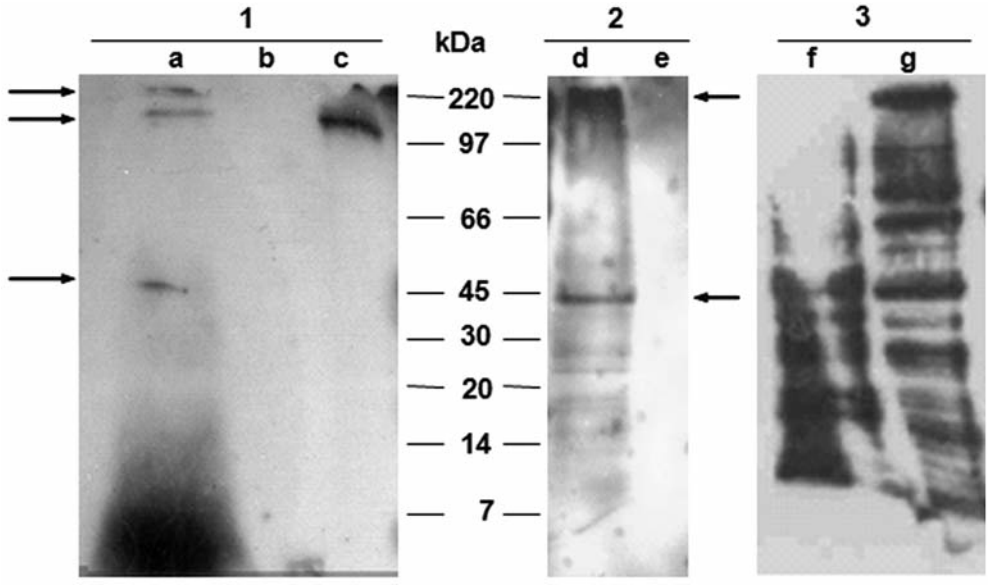

Figure 3. *Far-Western protein-protein interaction between An. gambiae midgut and P. berghei ookinetes. Midgut extracts (a, g), BSA (b, e), laminin (c) and ookinete extracts (d, f) were separated in a 12% SDS-PAGE and transferred onto PVDF membranes and probed with (1) biotinilated P. berghei ookinete extracts, (2) An. gambiae biotinilated midgut extracts, or (3) biotin, and incubated with streptavidin-HRP, luminol, and exposed to X-ray films.*

P. berghei oocyst infection of mosquitoes with ookinete culture before and after Nicodenz® 5-17% gradient enrichment and resulting pellet (Table II) indicates that this procedure did not affect the viability of the ookinetes, originating infections of similar prevalence and intensity as the ookinete culture. Conversely, the pellet yielded an infection with lower prevalence and intensity. The oocyst frequency distribution (data not shown) reveals a trend for higher proportion of positive (>1 oocyst) mosquitoes in the group that fed on enriched ookinetes, and on the contrary a trend for higher negative and lower positive proportions in the group that fed on the pellet.

Table I. *P. berghei ookinete enrichement factor after Nycodenz® discontinuous 5-17% gradient separation. Giemsa-stained smears were counted for five different optical fields for at least 100 parasites (asexual or ookinetes).*

	Ookinete density	Enrichment factor
A) Ookinete culture (pre-enrichment)	10.6%	
B) Ookinete enriched fraction	92.0%	8.68
C) Pellet	0.002%	0.00019

Midgut and ookinete interaction assays. Protein interaction assays between mosquito midgut and ookinete extracts were optimized yielding reproducible results (Figure 3). With mosquito midgut as the fixed phase, probed with biotinylated ookinete extract, ookinete binding could be detected with one band just above 220 kDa, another at 200 kDa and a third band at 48 kDa (Figure 3, lane a – arrows). Positive control with laminin yielded a band at 200 kDa similar to the second band obtained with midgut extract (Figure 3, lane c). No protein binding of ookinetes was detected to BSA (Figure 3, lane b).

When *P. berghei* ookinetes were the fixed phase, binding of biotinylated mosquito midgut detected two prominent bands at apparent MW 220 and 45 kDa (Figure 3, lane d - arrows), while there was no binding of ookinetes to BSA (Figure 3, lane e).

When the ligand was biotin numerous bands could be perceived in either midgut or ookinete extracts (Figure 3, lane f and g, respectively), suggesting that the binding of ookinete and midguts, whichever was the fixed or ligand phase, was specific.

Protein affinity chromatography–Pull-down assay. Affinity chromatography was used to purify ookinete proteins putatively binding to mosquito midgut. For this, *An. gambiae* midgut biotinylated extract linked to a streptavidin-agarose matrix was used as the fixed phase and *P. berghei* ookinete extract as the mobile phase (Figure 4, lane a). The recovered eluate was separated on a SDS-PAGE gel (Figure 4, lane b). From the multitude of protein bands present in the ookinete extract, only two become detectable in the eluate. After 90

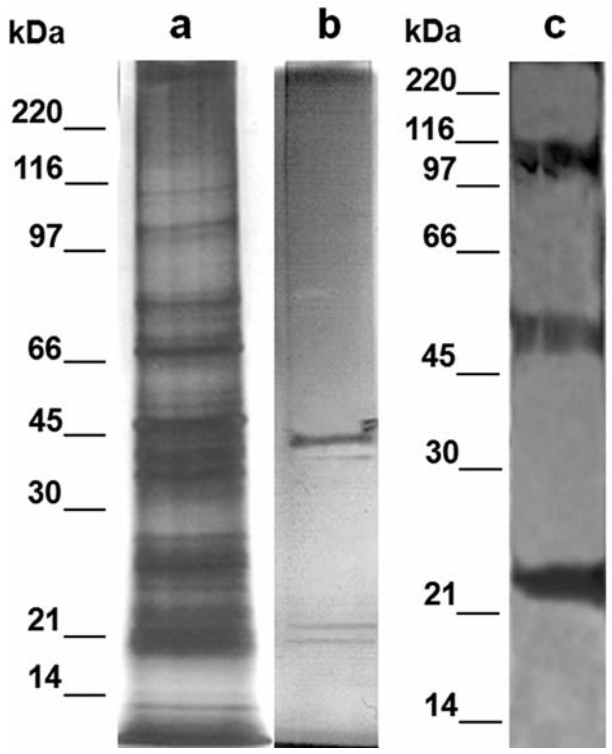

Figure 4. *Results of pull down assay of P. berghei ookinete extracts with immobilised An. gambiae midgut extract. a: P. berghei ookinete extract, b: eluate (pH 3) after pull down assay, c: flow through after Amicon® concentration. SDS-PAGE gel a and b at 5%-17% gradient and gel c at 12%.*

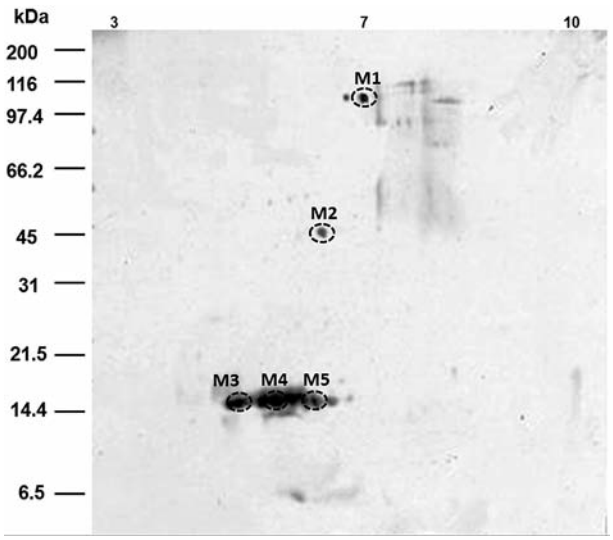

Figure 5. *Bidimensional 12.5% SDS-PAGE with pH 3-11 of P. berghei ookinete proteins after Amicon® concentration of protein chromatography. SDS-PAGE standard Broad Range molecular weight markers (Bio Rad), revealed with silver staining. Spots M1 to M5 were excised for mass spectrometry analysis.*

chromatography cycles 15 ml of eluate were obtained, and after concentration yielded 300 μl. The result of this procedure revealed consistency and reproducibility with the previous pull-down assay, yielding two bands of approximately 45 kDa and 21 kDa. A third band about 116 kDa was evidenced in this concentrated fraction (Figure 4, lane c).

2D electrophoresis and mass spectrometry after Pull-down assay. Figure 5 shows the result of 2D electrophoresis of the concentrated proteins from the eluate of the pull-down assay and the spots excised for mass spectrometry analysis. The obtained monoisotopic peptide masses were searched against *P. falciparum*, Alveolata and mouse *Mus musculus* or Eukaryota (as a source of possible contaminants) databases using Mascot® (http://www.matrixscience.com/home.html).

For spot M1 a significant match with a putative erythrocyte binding protein from *P. falciparum*, was obtained, in either Eukaryota, Alveolata or *P. falciparum* databases (Table III). However, mass and isoeletric point did not coincide with those of spot M1. Search in mouse database, as a control for possible contaminants, failed to give a significant score. Peptide mass fingerprinting from spot M1 matched in three different domains of Q8IFM3, characteristic of adhesive protein–related domains (Table IV).

For the other spots M2-M5 no significant matches were obtained.

Discussion

Techniques that improve the enrichment of *Plasmodium* ookinetes by separating these from remaining asexual parasites in ookinete cultures have been described using either Percoll (28), 12.5% or 17% Nycodenz gradients (29, 30) and magnetic isolation (31). The method hereby described uses a double 5-17% gradient Nycodenz cushion, allowing ookinetes to settle between these layers, with an enrichment factor of over 800%. This level of efficiency is higher than those described previously. The viability of these ookinetes was confirmed by membrane feeding mosquito infection assays and showed no decrease in prevalence or intensity of infection compared to the ookinete culture. This suggests that the enrichment procedure did not affect the capacity of the ookinetes to invade the mosquito midgut, thus confirming the presence and intact status of the putative surface proteins necessary for this process to occur. The other innovation of this process is that Nycodenz was diluted in ookinete RPMI-1640 complete culture medium. This may have contributed to maintain the healthy status of these cells after enrichment.

Table II. *Prevalence and intensity of P. berghei oocysts in An. gambiae membrane fed with ookinete culture before and after Nicodenz® discontinuous 5-17% gradient enrichment and resulting pellet.*

P. berghei oocyst	Ookinete culture	Ookinete enriched fraction	Pellet after enrichment
Prevalence of infected mosquitoes (N)	31% (16)	36% (22)	18% (11)
Intensity of infection (oocysts/midgut, mean, SD)	1.5±3.2	1.7±2.6	0.7±1.7

Table III. *Results of peptide mass fingerprinting using data from MALDI-TOF of protein from spot M1 against NCBI-Eukaryota, Alveolata and P. falciparum databases.*

Spot	Protein ID (Accession No.)	Theoretical Mr/pIa	Observed Mr/pI (b)	Matched peptide	Coverage (%)	Score (a) (p<0.05)
M1	erythrocyte binding antigen-165 (Q8IFM3_PLAF7)	162.2/5.7	116/7	13	14%	72

(a) MASCOT individual ions scores >62 was used to indicate identity or wide homology; (b) observed molecular weight (Mr) and experimental pI correspond roughly to their position on the 2D-E gel.

Table IV. *Peptide mass fingerprinting from spot M1 matched in three different domains of Q8IFM3, characteristic of adhesive protein–related domains.*

Domain[a]	Closest domain	Domain coverage
Erythrocyte ligand invasion pebl/eba-165 binding antigen (2 domains)	411-479/905-1173	68%/9.3%
Erythrocyte membrane pfemp1 binding	285-344	73.3%
Erythrocyte binding precursor receptor signal transmembrane duffy glycoprotein membrane antigen	109-284	10.2%

[a]Align subsequence with ProDom domains software using ncbi-blastp. (http://prodom.prabi.fr/prodom/current/html/home.php).

The objective of this work was to identify putative candidate molecules derived from the epithelial cells of the mosquito midgut and of *Plasmodium* ookinetes that participate in the invasion process as receptor/ligand. Protein interaction assays revealed *An. gambiae* midgut molecules of 220 kDa, 200 kDa and 48 kDa as capable of binding *P. berghei* ookinete extracts, and *P. berghei* ookinete proteins of 116 kDa, 48-45 kDa and 21 kDa as capable of binding *An. gambiae* midgut extracts. Ookinete extracts have previously been observed to bind to laminin and collagen IV, putative constituents of mosquito midgut epithelium basal lamina (10-12). In this work, ookinete proteins also were observed to bind to a laminin fraction (200-220 kDa). It must be stated that this interaction does not imply a role in invasion of the midgut epithelium itself. More likely, interaction with laminin only occurs upon reaching the basal lamina, and is thus involved in their transformation into oocysts (13).

After finding midgut-ookinete interactions using protein-protein binding assays with both mosquito midgut and ookinete extracts as the fixed phase, the second part of this study concentrated on searching for ookinete ligands to midgut cells. For this, an affinity chromatography "Pull-Down" assay was carried out with *An. gambiae* midgut as the fixed phase and *P. berghei* as the mobile phase. This led the isolation of ookinete proteins of approximately 116, 48-45 and 21 kDa. The results of the protein chromatography pull-down procedure are similar to those obtained with the Far-Western protein:protein assay, suggesting that the interaction that occurs in both procedures, between midgut and ookinetes is likely to be specific.

Mass spectrometry indicated that one of the spots from the 2D eletrophoresis of the eluted ookinete proteins (M1) matched with a significant score of peptide masses with the Erythrocyte binding antigen-165 from *P. falciparum* (*PfEBA-165*) (Q8IFM3_PLAF7) (32) in either Eukariota or Alveolata NCBI database. Three different domains of Q8IFM3 characteristic of adhesive protein–related domains were matched in PMF. These domains are highly conserved thus suggesting that the purified protein belongs to the adhesive protein family. *PfEBA-165* belongs to the erythrocyte binding-like (EBL) superfamily of the merozoite stage (33)

which possesses Duffy binding-like domains that are expressed by organisms of the phylum Apicomplexa (ex. *Plasmodium vivax*, *P. knowlesi* and *P. falciparum*). This family of proteins are described as being involved in the invasion recognition process of the erythrocytes as a ligand which in *P. vivax* are critical for erythrocyte receptor recognition (34) and that can be blocked by antibodies directed against the Duffy domains (35).

On the other hand, the protein *PfEBA-165* or *PfPEBL* has 86% similarity with the erythrocyte invasion ligand *PrBAEBL* (NCBI; AAT77189) from *P. reichenowi*, which although unable to infect humans is morphologically and biologically very close to *P. falciparum*, evidencing how conserved these molecules are in the invasion process (36). Therefore, a NCBI Blast-tblastn (http://blast.ncbi.nlm.nih.gov/ Blast.cgi) searching a translated nucleotide database was performed looking for gene/sequences from *P. berghei* with significant alignments to the *PfEBA-165* protein. This yielded 6 sequences with significant alignments from *P. berghei*, the first of which is the MAEBL protein family with an E value of 1e-24. MAEBL is a family of highly conserved erythrocyte binding proteins identified in the rodent malarial parasites *P. yoelii* and *P. berghei* that possess chimeric features of both Duffy binding-like domains of the micronemes and apical antigen 1 (AMA-1) of the rhoptry protein family, coexpressed and localized at the rhoptries, which possess erythrocyte binding activity and suggesting a role in erythrocyte invasion (37). Therefore, it is possible that it is also expressed by ookinetes, or that the ookinetes protein eluted is a close homologue involved in binding and/or invasion of the mosquito midgut epithelium. In fact, clustal consensus analysis has revealed a good similarity between PMF peptides from spot M1 with MAEBL (E value of 2E-24, data not shown).

The possibility that the purified molecules were contaminants from asexual parasites present in the ookinete culture can not be completely discarded, although this was tentatively avoided through the ookinete enrichment process which was highly productive and reproducible.

CTRP, a protein expressed only in midgut stage forms, would be another likely candidate for epithelial invasion. Its disruption blocks invasion of the midgut epithelium indicating that it is implicated in the ookinete motility and invasion (16, 17). However, a clustal consensus analysis has revealed low similarity of either the PMF peptides from spot M1 or *PfEBA-165* with CTRP (data not shown).

Evidence has been accumulating that malaria parasites have evolved multiple and redundant host cell invasion pathways (for revision see 25). This has been observed either in the asexual stages with lack of different phenotypes after the knockout of the merozoite surface proteins MSP-3 of *P. knowlesi* or with sexual stages with P25 and P28 proteins, where the knockout of either was not translated phenotypically, but only the double knockout was impaired for midgut invasion (20). This is even more striking as while *P. vivax* is dependent on the Duffy antigen for erythrocyte invasion, *P. falciparum* can invade RBC by multiple pathways (38). Therefore, the putative candidate molecules for ookinete invasion may face a similar hindrance and be just a part of a wider group of molecules in multiple invasion pathways.

This work used total midgut extracts. Similar studies using as "bait" midgut microvilli extracts may provide a more sensitive and specific means of identification of parasite proteins implicated in the binding/invasion of the epithelial cells. In fact, the whole midgut extracts may be masking basal lamina components not directly involved in the invasion process as seen with laminin (13). Furthermore, carbohydrate binding activities should be studied as their presence has been demonstrated in the microvilli (39), antibodies to which, or to midgut enzymes such as aminopeptidases or carboxypeptidases were found to block or reduce several plasmodia development in mosquitoes (6-8, 40).

This work has identified putative ligands involved in midgut-ookinete binding and invasion interactions. Proof that these molecules do indeed play such a role will require further studies, such as antibody inhibitions, knockouts or RNAi.

Conflict of interest

The authors declare no conflict of interest.

Acknowledgements

This work was funded by Project ref. POCTI/ESP/46271/2002, Portugal and FEDER.

We acknowledge Professor R.E. Sinden for kindly supplying MAb 13.1 and Professor V. Do Rosário for *P. berghei*.

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Received November 2, 2009
Revised March 22, 2010
Accepted March 22, 2010