Interaction of *Plasmodium gallinaceum* ookinetes and oocysts with extracellular matrix proteins

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SUMMARY

Plasmodium ookinetes are elongate, motile and invasive while inside the mosquito gut but promptly metamorphose into spherical immobile oocysts upon coming in contact with the basement membrane surrounding the midgut. There they begin a prolonged growth period characterized by massive DNA synthesis for the production of sporozoites. Living *Plasmodium gallinaceum* ookinetes attached avidly to the murine extracellular matrix proteins, laminin and collagen type IV. In ELISA-type assays, the main ookinete surface protein, Pgs28 was implicated as a mediator of parasite attachment to these basement membrane constituents. Laminin and collagen IV adhered to ookinete and oocyst lysates spotted onto nitrocellulose membranes. Receptor–ligand blot assays demonstrated that Pgs28 and an oocyst-specific antigen recognized by the mAb 10D6 interact with murine collagen IV and laminin. 10D6 antigen was also recognized by monospecific antiserum against the human epidermal growth factor receptor. Mosquito-derived laminin was incorporated into oocyst capsules of *P. gallinaceum* growing in *Aedes aegypti*. We hypothesize that contact with the mosquito basement membrane triggers the transformation of ookinetes into oocysts. Coalescence of basement membrane proteins onto the capsules masks developing oocysts from the mosquito's immune system and facilitates their prolonged extracellular development in the mosquito body cavity.

Key words: *Plasmodium gallinaceum*, oocyst, ookinete, basement membrane, laminin, collagen type IV.

INTRODUCTION

Sporogony in malaria parasites begins with the ingestion of gametocytes by a vector mosquito during blood feeding. Gametes undergo fertilization within the bloodmeal and zygotes transform into motile ookinetes that traverse the peritrophic matrix and the midgut epithelium. Upon contact with the basement membrane (BM), ookinetes metamorphose into spherical oocysts that develop extracellularly separated from the body cavity by the midgut BM. Oocyst growth and maturation is characterized by the formation of a thick capsule that is ultrastructurally similar to BM (Mehlhorn, Peters & Haberkorn, 1980). The oocyst stage is the longest stage in the life-cycle of *Plasmodium* and culminates in the production of sporozoites that invade the salivary glands and are injected into the vertebrate host during subsequent blood feeding (Sinden, 1984; Beier, 1998).

Recognition of mosquito BM and adhesion to it are crucial stages in the sporogonic cycle of *Plasmodium* spp. Here we identify 2 BM components and provide evidence for the probable role of 2 parasite proteins in this interaction. *P. gallinaceum* ookinete surface protein Pgs28 and an oocyst-membrane specific component, adhere to murine collagen type IV (CIV) and laminin.

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MATERIALS AND METHODS

Parasites

Plasmodium gallinaceum was maintained in 2-week-old chickens by weekly serial blood passage from infected donor chickens. Zygotes were prepared from parasitized blood (30% parasitaemia) of infected chickens and transformed *in vitro* into ookinetes and oocysts as previously described (Kaushal *et al*. 1983; Warburg & Miller, 1992).

Parasite lysates

Cultured ookinetes and oocysts were lysed in Tris buffer (20 mM Tris–HCl, 40 mM NaCl, pH 7·4) supplemented with a cocktail of protease inhibitors (final concentrations: 10 mM EDTA, 40 μg/ml 4-(2-aminoethyl)-benzensulphonyl fluoride (AEBSF), 7 μg/ml leupeptin, 1 mM iodoacetamide, 2 mM phenanthroline (Sigma). The lysate (100 μl) was placed in microcentrifuge tubes and sonicated for 10

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sec using a probe sonicator (Heat Systems–Ultrasonics, set to level 5). Complete lysis was verified microscopically.

Antibodies

The anti-*P. gallinaceum* surface protein 28 kDa (α-Pgs28) mAb designated B3B3 was a gift from Dr D. Kaslow, LPD/NIAID/NIH, Bethesda, MD. B3B3 recognizes the major surface protein of *P. gallinaceum* ookinetes (Duffy, Pimenta & Kaslow, 1993). Pgs28 is also a major surface component of cultured oocysts (Warburg unpublished observations). The mAb 10D6 was raised against cultured *P. gallinaceum* oocysts (Warburg & Miller, 1992). It is oocyst specific and recognizes a surface antigen of approximate molecular weight 180–200 kDa. Anti-murine collagen type IV (CIV) and anti-laminin were produced by us in rabbits using Engleberth Holm Swarm tumour-derived CIV and laminin (Sigma). Sheep anti-human epidermal growth factor receptor (EGFR) was purchased from Gibco–BRL (Cat. no. 3287SA).

Binding of living ookinetes to murine CIV and laminin-coated surfaces

Sterile-filtered laminin in Tris buffer or CIV in 0·25 % (v/v) acetic acid/H_2O (Sigma) were used to coat 24-well plates (250 μl of 100 μg/ml protein solution/well). Cultured *P. gallinaceum* ookinetes in medium M-199 (Gibco–BRL Cat. no. 31150-014) without fetal bovine serum (FBS) were seeded (10^6/ml/well) and allowed to adhere for 12 h at 26 °C. Excess medium was removed, wells were washed gently with M-199 and adhering ookinetes were counted under phase-contrast illumination using an inverted microscope. For each well, adhering ookinetes in 10 microscope fields (0·6 mm^2 each) were counted.

Dot blot assay on nitrocellulose

Biotinylated laminin and collagen IV were prepared by mixing 0·9 ml of 1 mg/ml protein solution with 100 μl of $NaHCO_3$ (1 M) and adding 120 μl of biotin solution (1 mg/ml in dimethylsulphoxide). Mixtures were incubated at room temperature for 2 h and dialysed overnight against PBS and azide (0·02 % w/v) with 3 changes.

Parasite lysate (1 μl of 50 μg protein/ml) was spotted onto nitrocellulose membranes and allowed to dry. Membranes were blocked with 3 % bovine serum albumin (BSA) in PBS overnight at 4 °C and subsequently washed with PBS supplemented with 0·05 % Tween (PBS–T). After incubation for 3 h at room temperature with biotinylated laminin or CIV (50 μg/ml in PBS–T), the membranes were washed 3 times and incubated with peroxidase-conjugated streptavidin (Jackson ImmunoResearch Lab 1 : 5000 in PBS–T) for 1 h at 37 °C. After washing 3 times in PBS–T blots were incubated in enhanced chemiluminescence (ECL) substrate (Sigma) according to manufacturer's instructions and then exposed to film.

Adhesion of ookinete and oocyst lysates to laminin- and CIV-coated surfaces

Acid-soluble CIV and laminin solutions (100 μg/ml protein) were used to coat wells of 96-well, flat-bottomed microtitre plate (50 μl/well, overnight at 4 °C). Plates were washed twice with PBS–T and blocked with 200 μl/well of 3 % skimmed powder milk overnight at 4 °C. The parasite lysate was calibrated to a concentration of 100 μg/ml, and 100 μl were added to each well and incubated for 3 h at 37 °C. The plate was washed 4 times in PBS–T and incubated in a 10^{-3} dilution of B3B3 ascites and 3 % skimmed milk in PBS–T for 1 h at 37 °C and washed 4 times. Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (Promega), diluted 1/2000 in PBS–T and 3 % skimmed milk was added to each well and incubated at 37 °C for 1 h, after which the plates were washed 4 times. Standard procedure for the colour reaction was followed using 2,2-azino-*bis* (3-ethylbenzthiaolinesulphonic) acid (ABTS) and the optical density was read at 405 nm using a microtitre plate reader.

Western and receptor–ligand blotting

Parasite lysates were electrophoresed on 10 % or 7·5 % SDS–PAGE under non-reducing conditions and electroblotted onto nitrocellulose membranes. The membranes were blocked with 0·05 % Tween, 150 mM NaCl and 10 mM Tris (TNT) + 5 % skimmed milk for 1 h at 37 °C. For Western blotting, membranes were incubated with appropriate dilutions of anti-malarial antibodies and washed. For ligand blots, membranes were first incubated with laminin or CIV solutions (100 μg/ml), for 24 h at 4 °C with gentle agitation. Following washes and blocking, the membranes were incubated with rabbit anti-mouse CIV or laminin (1 : 200 in TNT + 50 % skimmed milk) for a further 24 h at 4 °C with gentle agitation. Labelling was achieved by incubation at 37 °C for 1 h with HRP (1 : 2000)- or alkaline phosphatase (1 : 5000)-conjugated secondary antibodies and the enzymatic reaction was developed with appropriate chromogens and substrates. In some cases biotinylated laminin or CIV were used instead of antibodies.

Immunofluorescent staining

P. gallinaceum-infected *Aedes aegypti* were dissected 7 days post-infective feed. Midguts with attached oocysts were incubated with anti-laminin serum

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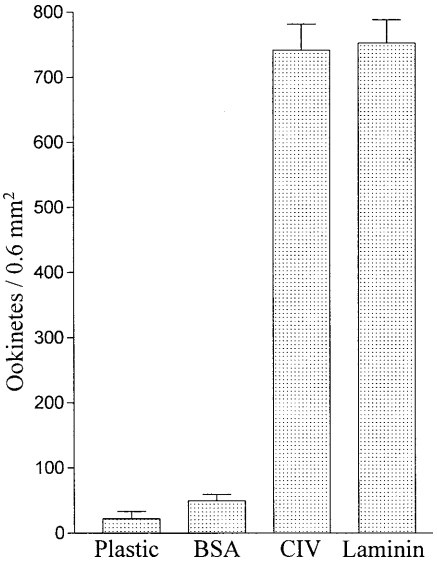

Fig. 1. Attachment of living *Plasmodium gallinaceum* ookinetes to murine CIV and laminin. Error bars represent average and standard deviation for ookinete numbers per 1 microscope field (=0·6 mm²). For details see Materials and Methods section.

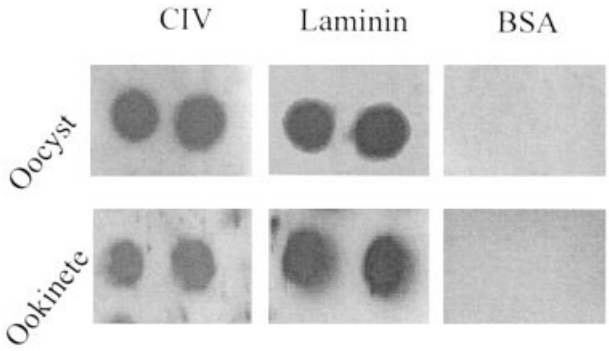

Fig. 2. Interaction of murine laminin and CIV with ookinete/oocyst lysates. Lysates spotted on nitrocellulose membranes were incubated with biotinylated laminin, CIV or BSA and developed with avidin-HRP and enhanced chemiluminescence (ECL). Representative results from 3 experiments.

(1:100 dilution) and fluorescein-conjugated secondary antibody (both incubations for 1 h). Controls were stained with secondary antibody alone.

RESULTS

P. gallinaceum *ookinetes bind to murine CIV- and laminin-coated surfaces*

Cultured *P. gallinaceum* ookinetes adhered avidly to BM-coated plastic. The average number of ookinetes that adhered to murine CIV or laminin-coated plastic was at least 10–15 times higher than the average that adhered to BSA or uncoated plastic (Fig. 1). For each experimental group, parasites were counted in 10 microscope fields per well. The experiment was repeated 3 times. Adhesion was not affected by pre-incubation of ookinetes with B3B3 (data not presented).

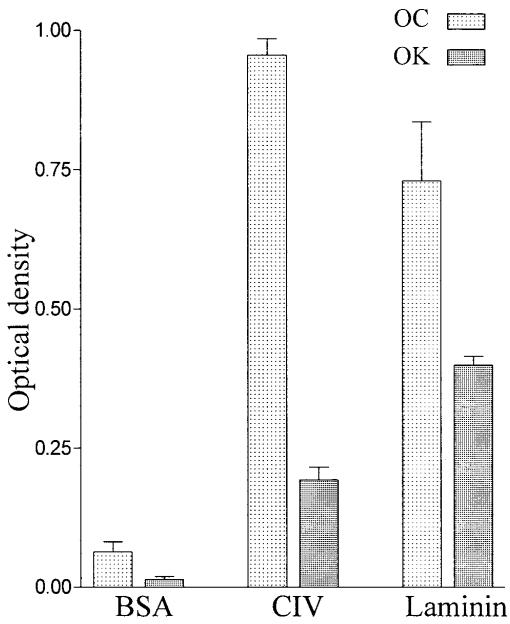

Fig. 3. Affinity of Pgs28 for laminin and CIV. Wells in a 96-well plate were coated with laminin, CIV or BSA and ookinete (OK) or oocyst (OC) lysates (100 μg/ml: 50 μl/well). Adhesion of Pgs28 was measured by incubation with the specific mAb, B3B3 and anti-mouse HRP. Optical density was read on a plate reader.

Ookinete and oocyst lysates specifically bind laminin and CIV

Dot blot assays of parasite lysates on nitrocellulose membrane that were incubated with biotinylated laminin or CIV confirmed that mosquito stages of *Plasmodium* display binding affinity for both these proteins (Fig. 2). There was no detectable adhesion of parasite lysates to bovine serum albumin (BSA) and no detectable HRP activity in parasite lysates (data not shown).

Pgs28 binds to CIV- and laminin-coated surfaces

A microtitre-plate ELISA-type assay was used to monitor the interaction of the *P. gallinaceum* ookinete and oocyst surface protein, Pgs28 with BM components. Ookinete and oocyst lysates were added to laminin- or CIV-coated microtitre wells and the interaction was monitored using the anti-Pgs28 mAb, B3B3. Pgs28 bound to both extracellular membrane proteins to a much higher extent than to BSA controls (Fig. 3). Results were not affected by the absence of Ca^{2+} ions in the reaction buffer (data not shown).

The P. gallinaceum *oocyst-specific bands recognized by the mAb 10D6 are also recognized by anti-human EGFR*

Oocyst and ookinete lysates were electrophoresed under non-reducing conditions and blotted onto nitrocellulose membranes. Blots were incubated with

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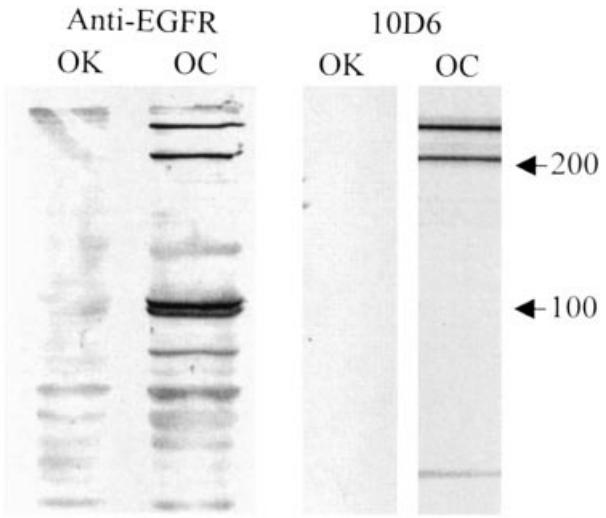

Fig. 4. Western blots of *Plasmodium gallinaceum* cultured ookinetes and oocysts using the oocyst-specific mAb, 10D6 and anti-human EGFR monospecific antiserum. Note, recognition by both antibodies of 2 high molecular weight (200–180 kDa) oocyst proteins that are absent from ookinetes.

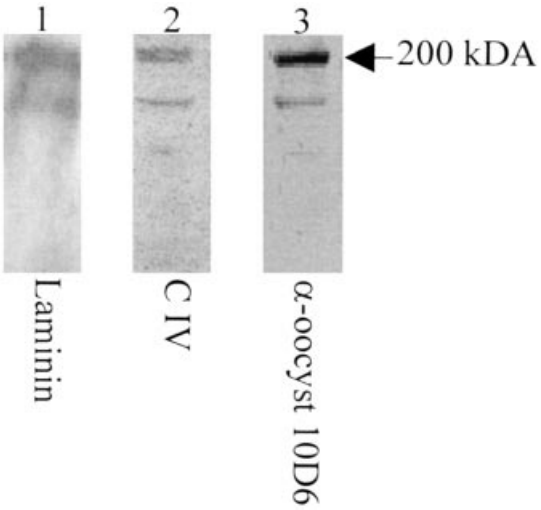

Fig. 5. CIV and laminin adhere to oocyst membrane-specific proteins. Oocyst lysate blots. Lane 1 incubated with biotinylated laminin solution. Lane 2 incubated with CIV solution, anti-CIV and anti-rabbit alkaline phosphatase (AP). Lane 3 developed using the oocyst-specific mAb 10D6 and anti-rabbit AP. Both BM proteins adhere to this component of oocyst membranes.

10D6 (1:1000 ascites) or anti-EGFR (5 μg/ml). 10D6 specifically recognized 2 bands of about 200–180 kDa, which were also labelled heavily with the α-EGFR serum. These bands were absent from ookinete lysates (Fig. 4).

Murine laminin and CIV bind to Pgs28 and to high MW bands recognized by the oocyst-specific mAb 10D6

Oocyst lysates were electrophoresed under non-reducing conditions and blotted onto nitrocellulose membranes. Blots were incubated with biotinylated CIV or laminin and specific adhesion of these BM proteins to at least 2 high MW bands was demonstrated. The same bands were also labelled by the oocyst-specific mAb 10D6 (Fig. 5). Adhesion of laminin to a 28 kDa band that probably corresponds to Pgs28 was also observed in separate experiments (Fig. 6).

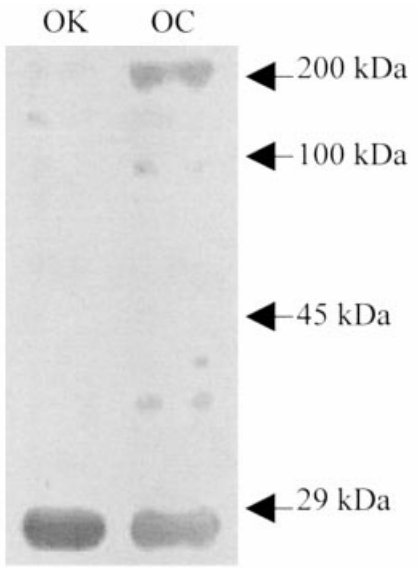

Fig. 6. Laminin adheres to an abundantly expressed *ca* 28 kDa protein (probably Pgs28) of ookinetes and oocysts. Cultured ookinetes (OK) and oocysts (OC) were electrophoresed under non-reducing conditions and electroblotted onto nitrocellulose membranes. Blots were incubated with biotinylated laminin and developed using ECL.

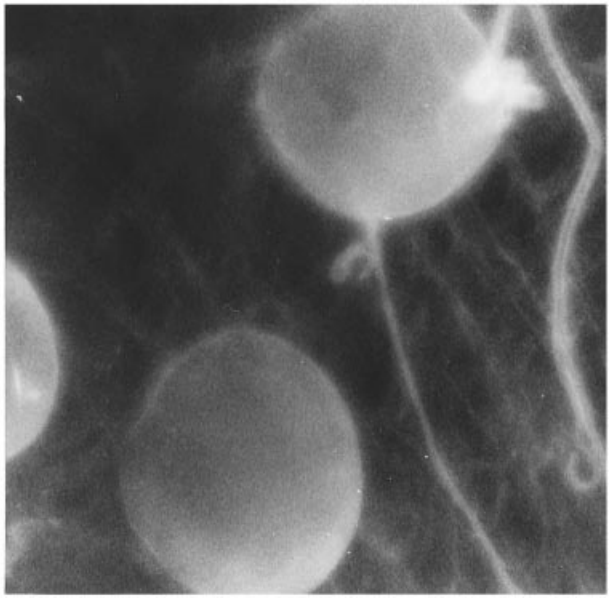

Fig. 7. *Plasmodium gallinaceum* oocysts 7 days post-infection, labelled with anti-mouse laminin serum and anti-rabbit FITC. Note, heavy labelling of oocyst capsule and some on mosquito BM.

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Oocyst capsules contain laminin

Immunostaining of *P. gallinaceum* oocysts with anti-laminin antibody indicated the presence of laminin in oocyst capsules (Fig. 7).

DISCUSSION

Ookinetes migrate through the peritrophic matrix and the epithelial wall of the mosquito midgut but stop at the BM on its basal side. There they transform into spherical, immobile, rapidly growing oocysts. In previous studies, a crucial association was observed between *Plasmodium* ookinetes and BM. Cultured *P. gallinaceum* and *P. falciparum* ookinetes transformed into oocysts upon contact with Matrigel, a murine BM-like gel (Warburg & Miller, 1992; Warburg & Schneider, 1993). The main vertebrate BM components, laminin and CIV are highly conserved amongst all metazoa including *Drosophila*, the only insect in which BM has been studied in detail (Fessler & Fessler, 1989). Therefore, we used murine-derived laminin and CIV to study the interaction of ookinetes and oocysts with BM. We determined that cultured ookinetes adhere to CIV and laminin and that components of lysed parasites also maintain specific recognition of BM. Adhesion was independent of Ca^{2+} ion concentration indicating that it is not mediated by integrins. Our results also indicate that Pgs28 is involved in this interaction. Pgs28, the main surface protein of ookinetes, is attached to the membrane via a glycosylphophatidylinositol (GPI) anchor (Duffy *et al*. 1993). There is a growing body of evidence showing that perturbation of malarial GPIs can trigger signal transduction events (Schofield & Hackett, 1993). Therefore, specific recognition of mosquito BM components by GPI-anchored Pgs28 may generate the trigger signal for ookinete transformation into oocyst.

We also identified an oocyst-specific membrane component that may be involved in attachment to CIV and laminin. It is a high molecular weight protein recognized by the *P. gallinaceum* oocyst specific mAb, 10D6. This oocyst-specific antigen was also recognized by a monospecific antiserum generated against a fragment of human EGFR. All laminin subunits contain highly conserved, cysteine-rich EGF-like motifs in several protein domains (reviewed by Hortsch & Goodman, 1991; Kusche-Gullberg *et al*. 1992). If the 10D6 oocyst antigen is indeed an EGFR-like receptor, mosquito laminin may function to promote growth and differentiation of *Plasmodium* oocysts (Engel, 1989). A phenomenon observed to be crucial *in vitro* (Warburg & Miller, 1992).

Plasmodium oocyst capsules differ from those of most other Apicomplexan parasites in their fibrous, BM-like, appearance and in lacking a defined operculum for the release of sporozoites. For this reason it has long been debated whether these unique extracellular envelopes are true oocyst capsules or mosquito-derived structures (Mehlhorn *et al*. 1980). A strong case for their parasite origin is made by the presence of *Plasmodium*-derived circumsporozoite (CS) protein on the internal side of immature oocyst capsules (Hamilton, Davies & Sinden, 1988; Boulanger *et al*. 1995; Warburg, unpublished observations). Our results indicate that oocyst capsules also contain laminin. Laminin is most probably of mosquito origin since single-celled organisms do not make BM and oocysts cultured *in vitro* lack a capsule (Pedersen, 1991; Warburg & Miller, 1992). In insects, BM components are mesodermal and produced by circulating haemocytes that secrete them on to surfaces lining the haemocoel (Lackie 1988; Fessler & Fessler 1989). Thus secreted laminin and CIV may coalesce onto oocyst surfaces where they interact with parasite antigens such as CS proteins that display an affinity for BM components (Frevert *et al*. 1993). Here we show that Pgs28 and the 10D6 antigen also bind laminin and CIV. Such associations may contribute to the construction of oocyst capsules, chimeric mosquito–parasite structures.

The entire haemocoel of insects is lined with BM that is recognized as 'self' by their immune systems. Foreign bodies without BM are attacked and melanized via phenoloxidase-mediated immune responses (Rizki & Rizki, 1984). Capsules containing host BM components may mask oocysts from the mosquito immune system. Perhaps thus *Plasmodium* parasites evade immune recognition and oocysts develop in the body cavity of susceptible vectors for extended periods of time.

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