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Laminin and the malaria parasite's journey through the mosquito midgut

Romanico B. G. Arrighi[1,*], Gareth Lycett[1,2,*], Vassiliki Mahairaki[1,3], Inga Siden-Kiamos[1] and Christos Louis[1,3,†]

[1]*Institute of Molecular Biology and Biotechnology, Foundation for Research and Technology – Hellas, Vassilika Vouton, PO Box 1527, 71110 Heraklion, Crete, Greece,* [2]*European Molecular Biology Laboratory, Meyerhofstr. 1, 69000 Heidelberg, Germany* and [3]*Department of Biology, University of Crete, 71110 Heraklion, Crete, Greece*

*These authors contributed equally to this work
[†]Author for correspondence (e-mail: louis@imbb.forth.gr)

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Summary

During the invasion of the mosquito midgut epithelium, *Plasmodium* ookinetes come to rest on the basal lamina, where they transform into the sporozoite-producing oocysts. Laminin, one of the basal lamina's major components, has previously been shown to bind several surface proteins of *Plasmodium* ookinetes. Here, using the recently developed RNAi technique in mosquitoes, we used a specific dsRNA construct targeted against the *LANB2* gene (laminin γ1) of *Anopheles gambiae* to reduce its mRNA levels, leading to a substantial reduction in the number of successfully developed oocysts in the mosquito midgut. Moreover, this molecular relationship is corroborated by the intimate association of developing *P. berghei* parasites and laminin in the gut, as observed using confocal microscopy. Our data support the notion of laminin playing a functional role in the development of the malaria parasite within the mosquito midgut.

Key words: *Anopheles gambiae*, basal lamina, ookinete, *Plasmodium berghei*, RNAi.

Introduction

Approximately 24 h after the ingestion of an infected blood meal, ookinetes of the genus *Plasmodium* initiate their journey through the peritrophic membrane and the midgut epithelium of the anopheline mosquito host. They come to rest in contact with the basal lamina and here they undergo transformation to oocysts and begin sporogony (reviewed by Siden-Kiamos and Louis, 2004). Oocyst development culminates in the rupture of the mature oocyst about 15 days later and the release of thousands of sporozoites into the haemocoel (see Kappe et al., 2003). Although very thorough descriptions exist for this process involving two different organisms, the specific molecular partners that make these interactions possible have not yet been identified (see Sinden, 2002). Several ookinete surface molecules, such as P25 and P28 (Siden-Kiamos et al., 2000), SOAP (secreted ookinete adhesive protein; Dessens et al., 2003) and CTRP (circumsporozoite protein and thrombospondin-related adhesive protein; Dessens et al., 1999), have been implicated in the process of midgut invasion but, to date, no putative receptor has been identified or proposed on the midgut epithelium. Direct or indirect binding to *Plasmodium* surface proteins has only been demonstrated for laminin (Vlachou et al., 2001; Arrighi and Hurd, 2002; Dessens et al., 2003; Adini and Warburg, 1999) and collagen IV (Arrighi and Hurd, 2002; Adini and Warburg, 1999), both components of all vertebrate (Miosge, 2001) and invertebrate (Hynes and Zhao, 2000) basal laminae, including the one surrounding the insect midgut on its haemocoel side.

In the context of vector–parasite interactions, the possibility that the *Anopheles* midgut basal lamina could be involved in additional functions other than the obvious one, namely that of a physical barrier that prevents ookinetes from continuing their passage, has often been considered. It has been known for several decades that parasites injected into the haemocoel can develop further upon binding onto the mosquito midgut wall (Weathersby, 1952; Warburg and Schneider, 1993), and this was confirmed in a dramatic way when it was shown that the injection of parasites into *Drosophila melanogaster* also leads to the development of infective sporozoites within oocysts attached to the basal lamina at multiple sites, not just the midgut (Schneider and Shahabuddin, 2000). Thus, binding of the ookinete to the basal lamina is a condition that supports its development into a functional oocyst, as demonstrated with the critical requirement of Matrigel in the successful culture of *Plasmodium berghei in vitro* (Al-Olayan et al., 2002).

Although laminin is only one of many constituents of the basal lamina, the fact that it has been shown to interact directly with invading parasites suggests a crucial role in the invasion process. To investigate this possibility, we temporally followed, using confocal microscopy and immunohistochemical techniques, the localisation of laminin during different stages of *P. berghei* parasite development in

the *Anopheles gambiae* midgut. Moreover, to observe whether the biochemical interactions can indeed have a functional role, we used the recently described RNAi technique (Fire et al., 1998) to determine whether a reduction in the transcription of mosquito laminin γ1 would lead to a novel infection phenotype. Our results indicate that laminin does indeed interact with the parasite and plays a crucial role in the parasite's development within the mosquito midgut.

Materials and methods

Immunohistochemistry

Midguts from *Plasmodium berghei*-infected *Anopheles gambiae* mosquitoes were dissected in phosphate-buffered saline (PBS) containing 2% formaldehyde at various time points after blood feeding. The midguts were cleaned of the food bolus when necessary and washed three times, fixed for 30 min in 4% formaldehyde, washed again three times for 20 min and incubated for at least 90 min in blocking buffer (PBS, 0.2% saponin, 5% normal goat serum). The midguts were incubated with primary polyclonal anti-laminin antibody (AbCam, Cambridge, UK) raised against laminin-1 from Engelbreth–Holm–Swam (EHS) tumour, for at least 4 h, washed three times, then incubated with fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit antibody (Molecular Probes, Eugene, OR, USA) or alternatively with an Alexa 568-conjugated anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA, USA) for 1.5 h, followed by three washes. The guts were then incubated with either (i) the *P. berghei* monoclonal antibody 12.1 conjugated to FITC (Siden-Kiamos et al., 2000) for 30 min and washed twice, (ii) the *P. berghei* monoclonal anti-CSP (circumsporozoite protein) antibody, washed three times and then incubated with the Alexa 555-conjugated anti-mouse antibody (Molecular Probes) or (iii) anti-SOAP (secreted ookinete adhesive protein) immune serum and goat anti-mouse FITC conjugated secondary antibody (Sigma, St Louis, MO, USA) as described previously (Dessens et al., 1999). In some cases, guts were finally stained with TO-PRO 3 nuclear stain (Molecular Probes), as recommended by the manufacturer. The entire process was performed at room temperature, the antibodies were diluted in blocking buffer, and PBS was used for all washes. The guts were mounted on microscope slides and examined with a confocal laser scanning microscope (Leica TCS NT/SP, Wetzlar, Germany). To examine specificity of the laminin antibody, western blot analysis of conditioned medium from mosquito cell line 4a3a indicated recognition of a single very high-molecular-mass protein under non-reducing conditions. The antiserum also recognised recombinant laminin-γ1 during similar analysis. In addition, immunofluorescent staining controls using the secondary antibody on its own did not give any specific staining (not shown).

Double-stranded RNA production and injection

Double-stranded (ds) RNA was produced as described previously using the plasmid pLL6ds for control dsGFP (ds green fluorescent protein; Levashina et al., 2001) and pLLlam for laminin (dsLANB2). In order to construct pLLlam, a 750-bp fragment (covering the 3′ UTR) was PCR-amplified from pLAM-*Bam*HI, which contains almost the entire *LANB2* gene that encodes laminin γ1 (Vlachou et al., 2001). The primers used were FPNotL (TTG CGG CCG CAA GCA GCA GCA CTA GCA GTA GTA) and RPBamL (CGG ATC CGG TTA TCT TCT GCG GCA CG). The fragment was cloned between the two T7 promoters of the plasmid pLL6, to create pLLlam. Sense and antisense RNAs were synthesized using the T7 Ambion (Austin, TX, USA) Megascript kit, annealed in water, and stored as dsRNA at –80°C until use. A nanoinjector (Nanoject; Drummond, Burton, OH, USA) was used to inject 69 nl of dsRNA (2 μg μl^{-1}) into the thorax of CO_2-anaesthetized 1–2-day-old female mosquitoes as described by Blandin et al. (2002). The mosquitoes were allowed to recover for 4 days before being fed on infected mice.

RNA analysis with quantitative real-time PCR

Total RNA was extracted from 10 injected mosquito guts using TRIzol reagent (Gibco BRL, Gaithersburg, MD, USA) 4 days post-injection, and 24 h after blood feeding. First-strand cDNA was synthesized using Superscript II Reverse Transcriptase (Invitrogen, Carlsbad, CA, USA). Plasmids to be used as standards for qPCR were constructed by inserting gene fragments from the coding regions of either the *LANB2* or *S7* gene into the pGEM T-easy vector (Promega, Madison, WI, USA). The concentration of the plasmids used as standards ranged from 1 ng to 10 fg to determine copy numbers of the target inserts. Laminin-specific primers were selected that do not overlap with the region of the *LANB2* gene used to construct the dsRNA: FPLAM2, 5′-GCTAAGACGGACAA-CCGACTG-3′; RPLAM2, 5′-TCTCGGCAGCACTCAG-ACG-3′. *S7* primers (forward, 5′-GTGCGCGAGTTGGAGA-AGA-3′; reverse, 5′-ATCGGTTTGGGCAGAATGC-3′) were used as internal standards (Salazar et al., 1993). Using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA), PCR reactions were carried out in a continuous fluorescence detector gradient cycler (OPTICON; MJ Research, Waltham, MA, USA) over 35 cycles of 95°C for 15 s, 60°C (*LANB2* – 235 bp product) or 55°C (*S7* – 76 bp product) for 30 s and 72°C for 30 s, followed by one cycle of continuous monitoring of fluorescence from 60°C to 94°C to generate the melting curve of the PCR products. Different primers and conditions were used for semi-quantitative PCR: FPLAM, 5′-CAAACAGCCCAGGACAAGTA-3′; RPLAM, 5′-TTACGGTTCCAGATCGT-3′. The primers produce a 551-bp fragment using a standard PCR program (30 s at 95°C; 45 s at 56.5°C; 45 s at 72°C) for 29 cycles. The *S7* standard (Salazar et al., 1993) was amplified with 19 cycles using the same conditions. Each experiment was repeated three times.

Plasmodium *infections*

Within each experiment, dsGFP- and dsLANB2-injected mosquitoes (4 days after the injection) were simultaneously fed on the same mouse that was infected with a GFP-expressing

Plasmodium berghei clone (Vlachou et al., 2004). Mosquito guts were dissected 7 days post-blood meal and fixed. Oocysts were counted using a UV-light fluorescent microscope. Thirty mosquitoes were injected per treatment (i.e. GFP or LAM), and each series of injections was repeated four times.

Statistical analysis

Analysis was carried out using the MINITAB 13 statistical package. Since none of the data were normally distributed, they were analysed using the Mann–Whitney *U*-test for non-parametric data.

Results

Laminin attaches to the developing parasite in the midgut

In order to describe in more detail the localization of laminin and the parasite as it penetrates the midgut epithelium and develops into the rounded oocyst, we followed the process by confocal microscopy after immunostaining infected midguts at different times after a blood meal containing *P. berghei* parasites. The mosquito laminin was localised using a commercial antiserum directed against laminin. This antiserum did not show any staining of *in vitro* cultured ookinetes (data not shown). The parasite was detected using the 12.1 antibody that recognizes the P28 surface protein, an antibody directed against the micronemal protein SOAP or a monoclonal antibody against the CSP. The results are shown in Fig. 1. Fig. 1A,B shows ookinetes in the midgut epithelium, 20–24 h after the ingestion of the blood meal; the ookinetes are located close to the basal lamina. They are identified by staining for the SOAP protein, which results in a green punctuated cytoplasmic labelling since this is a micronemal protein. This allows for a better visualization of the anti-laminin staining in red, which is shown to coat the entire surface of the parasites. The extracellular matrix around the muscle fibres is brightly stained for the laminin protein, as expected. The insets in Fig. 1B show the intense red fluorescence of the anti-laminin antibody, indicating that the mosquito protein envelops the parasite in substantial amounts, while the SOAP antibody stains the cytoplasm of the ookinete.

When the ookinetes reach the basal lamina, they transform into the rounded oocysts that will subsequently initiate sporogony. Fig. 1C shows one young oocyst, 3 days after the ingestion of the blood meal, counterstained with the green fluorescing anti-P28 antibody. Under higher magnification (two insets), laminin is observed coating the parasite. At day 10 (Fig. 1D), a thin line of anti-laminin antibody is still seen to label the oocyst surface, although it is difficult to determine whether there is an intimate contact between the surface of the parasite and the extracellular matrix. Finally, 15 days after the ingestion of the blood meal, the by now much larger oocysts are covered by a thin layer of laminin, but this may not be bound to the oocyst as such. Probably, this represents the stretching out of the basal lamina as the oocyst grows in size. A representative oocyst at this stage is shown protruding from the muscles in Fig. 1D.

The intimate association of mosquito laminin and the parasites ends upon rupture of the oocyst approximately 15 days after the blood meal. In a low magnification of the midgut (Fig. 1F), green fluorescing sporozoites can be seen on the

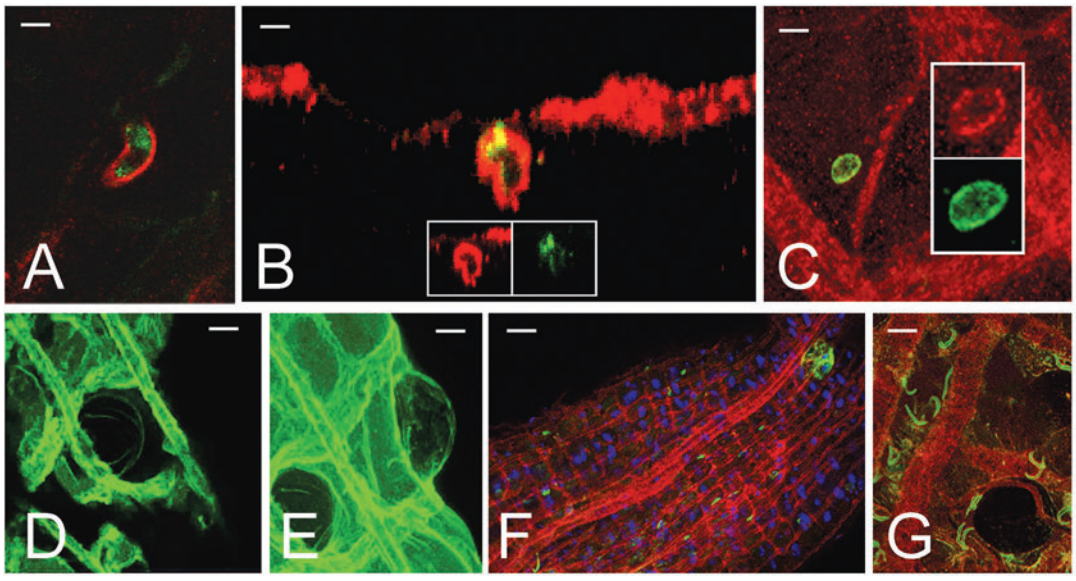

Fig. 1. Association of laminin and developing *Plasmodium berghei* in the mosquito midgut. Mosquitoes were given a blood meal containing *P. berghei* parasites. At different time points, the midguts were dissected and immunostained using the antibodies described in detail in Materials and methods. (A,B) A parasite is seen reaching the basal lamina 20–24 h after blood feeding. Green, SOAP protein; red, laminin; insets in B show details of the parasite staining showing only the red or green colour channel. Scale bars: 2 mm in A, 1 mm in B. (C) A young oocyst localized close to the muscle layer surrounding the midgut 3 days after the blood meal; green, P28 protein; red, laminin. Insets in C: details of the parasite staining showing only the red or green colour channel. Scale bar: 10 mm. (D) 10 days after the blood meal laminin (green) is seen concentrated around the larger oocyst. Bar: 10 mm. (E) 15 days after blood feeding, a thin coat of laminin (green) is seen on a mature oocyst protruding through the muscle layer shortly before rupturing. Scale bar: 10 mm. (F) A midgut 15 days after the blood meal. Individual sporozoites can be seen (green: CSP) along the muscles (red: laminin) while an almost mature oocyst is seen on the right; blue T-PRO stained nuclei of immature oocysts or epithelial cells. Scale bar: 100 mm. (G) Higher magnification of the midgut shown in F. Individual sporozoites can be seen (green: CSP) along the muscles (red: laminin). Scale bar: 10 mm.

haemocoel-side of the midgut musculature, while a very late-stage oocyst can be seen at the right-hand side, defined by the strong CSP staining (blue: nuclei of still immature oocysts and epithelial nuclei). Lastly, in Fig. 1G, the sporozoites are clearly seen on the muscles, while a circular black area within a rectangle of laminin-covered muscles represents an immature (non CSP-expressing) oocyst.

Invasion and oocyst formation are influenced by expression of laminin γ1 mRNA

In a previous report (Vlachou et al., 2001), we had determined that one of the mosquito laminins, laminin γ1, could specifically bind the *Plasmodium* surface proteins P25 and P28. Moreover, it was later shown that the same laminin could also bind to the SOAP (Dessens et al., 2003) and CTRP (Mahairaki et al., 2005) proteins of the ookinete. In order to investigate whether these biochemical laminin–parasite interactions and the association described above also serve a biological role in the survival of the parasite during the invasion of the mosquito midgut by *P. berghei* parasites, we took advantage of the dsRNA gene silencing technology (Fire et al., 1998). This method has previously been used to assess gene function both in *Anopheles* cells (Levashina et al., 2001) and in whole mosquitoes (Blandin et al., 2002, 2004; Osta et al., 2004). Double-stranded RNA corresponding to the gene to be silenced is produced *in vitro* and injected into adult mosquitoes. The mosquitoes are then fed on infected mice, and oocyst numbers are counted after one week. The construct that was used to silence *LANB2* contained a 756 bp fragment derived from the 3′ UTR region of the laminin γ1 transcript (nucleotides 5269–6025; see Fig. 2A). BLAST analysis determined that this is a single-copy segment of the genome of *An. gambiae* (not shown). As a control, dsRNA transcribed *in vitro* from a construct containing the gene for GFP (green fluorescent protein) was injected in parallel.

We found it important to first verify that *LANB2* mRNA expression was reduced using this method. Therefore, the mRNA was extracted from a number of mosquitoes that had been injected with the dsRNA (either GFP or laminin dsRNA). mRNA was extracted 4 days post-injection, and 24 h post-blood meal, and was used for both quantitative real-time PCR (Fig. 2C) and semi-quantitative RT-PCR (Fig. 3). In Fig. 2C, as an additional control, we used the copy number of the constitutively expressed ribosomal protein *S7* gene as a standard. It is obvious that mRNA accumulation decreased in all cases following dsLANB2 injection. The effect of dsLANB2 is somewhat more pronounced in sugar-fed (83% decrease) than in blood-fed mosquitoes (45% decrease). It should be noted, though, that the fall in laminin γ1 mRNA levels after blood feeding is assayed against a background of induced transcription following haematophagy. We therefore conclude that the dsRNA does indeed suppress the expression of the *LANB2* gene and that this effect is evident both in sugar-fed and in blood-fed mosquitoes. In contrast to a reduction of the mRNA levels, we could not detect a reduction of the laminin γ1 protein in western analyses (not shown). This was most probably due to the fact that the antibody used is not specific for this protein but, rather, recognizes more laminin proteins.

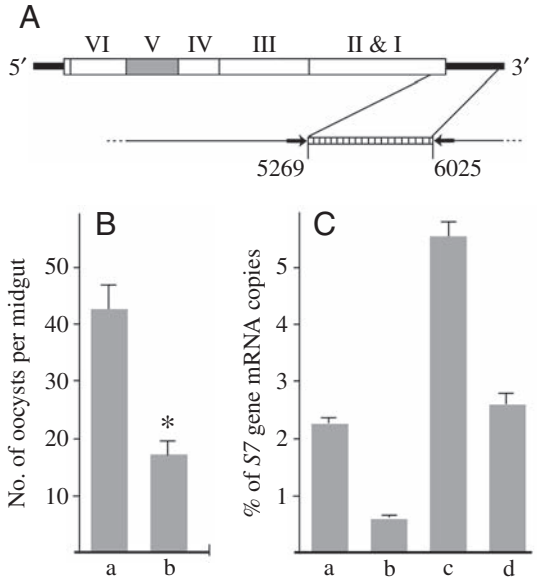

Fig. 2. (A) Schematic representation of the *Anopheles gambiae LANB2* gene. The coding area is represented by boxes corresponding to the domains of the protein (grey: domain V, containing EGF repeats and shown previously to interact with P25 and P28). The black line indicates non-coding segments, while the small map below shows the extent of the segment used to obtain the dsRNA used in the experiments spanning the nucleotides indicated. (B) Effects of dsRNA injections. The bars indicate the mean number of oocysts found in the midguts of mosquitoes that had previously been injected with dsRNA directed against the *GFP* gene as a control (a) or the *LANB2* gene (b). Error bars represent standard error of the mean (N=85 and N=79, respectively). The asterisk indicates a significant difference in oocyst burden (P<0.0001). See Results for details. (C) Estimates of the *LANB2* gene mRNA copy number expressed in percent of the *S7* gene mRNA copies after injection of dsRNA determined by quantitative real-time PCR. a, sugar-fed mosquitoes injected with dsRNA against *GFP*; b, sugar-fed mosquitoes injected with dsRNA against *LANB2*; c, blood-fed mosquitoes injected with dsRNA against *GFP*; d, blood-fed mosquitoes injected with dsRNA against *LANB2*. Error bars represent the standard error of the mean (N=3). See Materials and methods for experimental details.

We then proceeded to test the effect of knocking down the laminin γ1 expression on parasite development. Double-stranded RNA was injected as described above and, after recovery for 4 days, the injected mosquitoes (both experimental and control) were fed on the same *P. berghei*-infected mice. Several mosquitoes from each experiment did not survive the injections, leading to a reduction in the total number of midguts analysed. It is important to note, though, that there was no significant difference in the survival of the mosquitoes injected with the GFP dsRNA (85/120) or the laminin γ1 (79/120). Midguts were dissected 7 days later and the number of oocysts was counted under the microscope. The results of these experiments are shown in Fig. 2B. The mean

number of oocysts observed in 85 mosquitoes injected with GFP dsRNA was 42.5 per midgut. By contrast, in 79 females injected with laminin γ1 dsRNA, the mean number was 17.5 per midgut, a significant reduction in oocyst levels ($P<0.0001$). Taken together, this indicates that there was an approximately 60% reduction in oocyst formation when *LANB2* expression was suppressed.

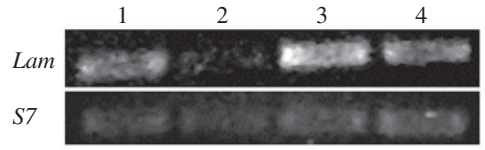

Fig. 3. *LANB2* mRNA accumulation in the midgut. Semi-quantitative PCR was performed to determine *LANB2* mRNA accumulation in isolated midguts (*Lam*) using *S7* mRNA as a standard (*S7*). 1, sugar-fed mosquitoes injected with dsRNA against GFP; 2, sugar-fed mosquitoes injected with dsRNA against *LANB2*; 3, blood-fed mosquitoes injected with dsRNA against GFP; 4, blood-fed mosquitoes injected with dsRNA against *LANB2*. See Materials and methods for experimental details.

Discussion

Here, we show that the previously described capacity of *An. gambiae* laminin γ1 to bind surface proteins of *P. berghei* ookinetes (Vlachou et al., 2001) or micronemal proteins (Dessens et al., 2003; Mahairaki et al., 2005) correlates with the co-localization of laminin with ookinetes and early oocysts in the midgut. Moreover, using dsRNA technology, we demonstrate that a reduction in *LANB2* expression results in a significantly decreased transmission efficiency of the parasites through their mosquito hosts. This loss-of-function phenotype is evident as a ~60% drop in the number of oocysts formed in mosquitoes in which laminin γ1 transcription has been reduced.

The fact that laminin is an integral component of the *An. gambiae* basal lamina (Vlachou et al., 2001), a structure that is reached by ookinetes only after the primary invasion of, and exit from, an epithelial cell, implies that its role is different from that of a (putative) receptor that would be mediating entry into the epithelium. However, the idea of laminin acting as a receptor to 'trigger' ookinete transformation into an oocyst has been suggested by Arrighi and Hurd (2002). Furthermore, the fact that laminin is found coating the young oocysts at day 3 of development, and is seen in close vicinity to older oocysts 10 days after the uptake of the infected blood meal, suggests an ongoing role, at least for the first half of sporogony. Previous studies have also shown a close association between both ookinetes and oocysts with the basal lamina during sporogonic development (Meis et al., 1989, 1992). One potential function of the interaction (or association) that fits these time constraints would be a masking of the developing parasite from possible attacks by the insect's immune system. Studies by Rizki and Rizki (1984) in *Drosophila* suggest that foreign bodies lacking a 'coating' of basal lamina are attacked and melanized. In addition, Paskewitz and Riehle (1998) demonstrated that pre-incubation of Sephadex beads in a susceptible strain of *An. gambiae* led to a significant reduction in melanization when the beads were transferred to a refractory strain. Obviously, our results cannot provide a definite answer on the 'masking theory', yet this remains an attractive hypothesis.

The effect of knocking-down the *LANB2* gene on parasite development indicates that the basal lamina and its integrity are vital for parasite development. It is likely that the effects observed are due to an overall reduction of fully functional basal lamina and not that the decrease of laminin γ1 *per se* has a direct effect on the development of *P. berghei,* although this latter possibility cannot be excluded. Unfortunately, it is impossible to use the antibody stains to make either a quantitative or a qualitative statement in this respect.

The basal lamina is a structure that is formed early in development during the pupal stages when the imaginal structures are differentiated (Clements, 1992). Since the midgut is formed quasi *de novo*, the majority of the surrounding basal lamina is also synthesized during the same period. Thus, a reduction in expression of the gene was not necessarily expected in the adult stage. On the other hand, the blood meal is accompanied by an extreme extension of the midgut, a concomitant change in the epithelium to become squamous (Billingsley, 1990) and, as we have shown here, an increase in midgut-specific laminin expression. It is obvious that this extension includes the surrounding basal lamina, as a previous report by Reinhardt and Hecker (1973) showed that the basal lamina is thicker following digestion of a blood meal. Therefore, a new synthesis of some or all of its components could be considered to be normal in order to facilitate the reorganisation of cellular morphology and to repair possible physical damage. Such a model for basal lamina protein expression during parasite development has been proposed by Gare et al. (2003). This phenomenon of continuous production and reorganization has also been described in vertebrate extracellular matrices (Schwarzbauer, 1999). In insects, it has been suggested that a specific type of hemocytes may be able to synthesize and secrete basal lamina components (Fessler and Fessler, 1989). In fact, it may be worth considering the possibility that the binding of laminin to the surface of ookinetes and oocysts may be due to an indirect effect of basal lamina protein processing. Perhaps *Plasmodium* parasites exploit this synthesis of laminin in order to achieve a more efficient infection of the insect host.

This report adds further evidence to the list of observations that suggest that laminin is a component that is indeed involved in the invasion and development of the malaria parasite within the mosquito midgut, thereby supporting the notion that components of the basal lamina play an important role in the malaria parasite's sporogonic cycle. This was suggested earlier in two *in vitro* systems (Warburg and Schneider, 1993; Al-Olayan et al., 2002), although it was not necessary in others (Siden-Kiamos et al., 2000). Nevertheless, it remains to be seen what the exact role of the basal lamina is in this process.

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References

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

Al-Olayan, E. M., Beetsma, A. L., Butcher, G. A., Sinden, R. E. and Hurd, H. (2002). Complete development of mosquito phases of the malaria parasite *in vitro*. *Science* **295**, 677-679.

Arrighi, R. B. G. and Hurd, H. (2002). The role of *Plasmodium berghei* ookinete proteins in binding to basal lamina components and transformation into oocysts. *Int. J. Parasitol.* **32**, 91-98.

Billingsley, P. F. T. (1990). The midgut ultrastructure of hematophagous insects. *Annu. Rev. Entomol*. **35**, 219-248.

Blandin, S., Moita, L. F., Kocher, T., Wilm, M., Kafatos, F. C. and Levashina, E. A. (2002). Reverse genetics in the mosquito *Anopheles gambiae*: targeted disruption of the *Defensin* gene. *EMBO Rep.* **3**, 852-856.

Blandin, S., Shiao, S. H., Moita, L. F., Janse, C. J., Waters, A. P., Kafatos, F. C. and Levashina, E. A. (2004). Complement-like protein TEP1 is a determinant of vectorial capacity in the malaria vector *Anopheles gambiae*. *Cell*. **116**, 661-670.

Clements, A. N. (1992). Metamorphosis. In *The Biology Of Mosquitoes*, vol. 1, pp. 171-193. New York: Chapman and Hall.

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

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

Fessler, J. H. and Fessler, L. I. (1989). *Drosophila* extracellular matrix. *Annu. Rev. Cell. Biol.* **5**, 309-339.

Fire, A., Xu, S., Montgomery, M. K., Kostas, S. A., Driver, S. E. and Mello, C. C. (1998). Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* **391**, 806-811.

Gare, D. C., Piertney, S. B. and Billinglsey, P. F. (2003). *Anopheles gambiae* collagen IV genes: cloning, phylogeny, and midgut expression associated with blood feeding and Plasmodium infection. *Int. J. Parasitol.* **33**, 681-690.

Hynes, R. O. and Zhao, Q. (2000). The evolution of cell adhesion. *J. Cell Biol.* **150**, F89-F95.

Kappe, S. H., Kaiser, K. and Matuschewski, K. (2003). The *Plasmodium* sporozoite journey: a rite of passage. *Trends Parasitol.* **19**, 135-143.

Levashina, E. A., Moita, L. F., Blandin, S., Vriend, G., Lageux, M. and Kafatos, F. C. (2001). Conserved role of a component-like protein in phagocytosis revealed by dsRNA knockout in cultured cells of the mosquito, *Anopheles gambiae*. *Cell* **104**, 709-718.

Mahairaki, V., Voyatzi, T., Siden-Kiamos, I. and Louis, C. (2005). The *Anopheles gambiae* gamma1 laminin directly binds the *Plasmodium berghei* circumsporozoite- and TRAP-related protein (CTRP). *Mol. Biochem. Parasitol.* **140**, 119-121.

Meis, J. F., Pool, G., van Gemert, G. J., Lensen, A. H., Ponnudurai, T. and Meuwissen, J. H. (1989). *Plasmodium falciparum* ookinetes migrate intercellularly through *Anopheles stephensi* midgut epithelium. *Parasitol. Res.* **76**, **1**, 13-19.

Meis, J. F., Wismans, P. G., Jap, P. H., Lensen, A. H. and Ponnudurai, T. (1992). A scanning electron microscopic study of the sporogonic development of *Plasmodium falciparum* in *Anopheles stephensi*. *Acta Trop.* **50**, 227-236.

Miosge, N. (2001). The ultrastructural composition of basement membranes *in vivo*. *Histol. Histopathol.* **16**, 1239-1248.

Osta, M. A., Christophides, G. K. and Kafatos, F. C. (2004). Effects of mosquito genes on *Plasmodium* development. *Science* **303**, 2030-2032.

Paskewitz, S. M. and Riehle, M. (1998). A factor preventing melanization of Sephadex CM-25 beads in *Plasmodium*-susceptible and refractory *Anopheles gambiae*. *Exp. Parasitol.* **90**, 34-41.

Reinhardt, C. and Hecker, H. (1973). Structure and function of the basal lamina and of the cell junctions in the midgut epithelium (stomach) of female *Aedes aegypti* L. (Insecta, Diptera). *Acta Trop.* **30**, **4**, 213-236.

Rizki, T. and Rizki, R. M. (1984). The cellular defense system of *Drosophila melanogaster*. *Insect Ultrastruct.* **2**, 579-603.

Salazar, C. E., Mills-Hamm, D., Kumar, V. and Collins, F. H. (1993). Sequence of a cDNA from the mosquito *Anopheles gambiae* encoding a homologue of human ribosomal protein S7. *Nucleic Acids Res.* **21**, 4147.

Schneider, D. and Shahabuddin, M. (2000). Malaria parasite Development in a *Drosophila* model. *Science* **288**, 2376-2379.

Schwarzbauer, J. (1999). Basement membrane: Putting up the barriers. *Curr. Biol.* **9**, R242-R244.

Siden-Kiamos, I. and Louis, C. (2004). Interactions between malaria parasites and their mosquito hosts in the midgut. *Insect Biochem. Mol. Biol.* **34**, 679-685.

Siden-Kiamos, I., Vlachou, D., Margos, G., Beetsma, A., Waters, A. P., Sinden, R. E. and Louis, C. (2000). Distinct roles for Pbs21 and Pbs25 in the *in vitro* ookinete to oocyst transformation of *Plasmodium berghei*. *J. Cell Sci.* **113**, 3419-3426.

Sinden, R. E. (2002). Molecular Interactions between *Plasmodium* and its insect vectors. *Cell. Microbiol.* **4**, 713-724.

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. *Mol. Biochem. Parasitol.* **112**, 229-237.

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

Warburg, A. and Schneider, I. (1993). *In vitro* culture of the mosquito stages of *Plasmodium falciparum*. *Exp. Parasitol.* **76**, 121-126.

Weathersby, A. B. (1952). The role of the stomach wall in the exogenous development of *Plasmodium gallinaceum* as studied by means of haemocoel injections of susceptible and refractory mosquitoes. *J. Infect. Dis.* **91**, 198-205.