A new factor in the *Aedes aegypti* immune response: CLSP2 modulates melanization

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Microbial infections in the mosquito *Aedes aegypti* activate the newly identified *CLSP1* and *CLSP2* genes, which encode modular proteins composed of elastase-like serine protease and C-type lectin domains. These genes are predominantly regulated by the immune deficiency pathway, but also by the Toll pathway. Silencing of *CLSP2*, but not *CLSP1*, results in the activation of prophenoloxidase (PPO), the terminal enzyme in the melanization cascade, suggesting that *CLSP2* is a negative modulator of this reaction. Haemolymph PPO activation is normally inhibited in the presence of *Plasmodium* parasites, but in *CLSP2*-depleted mosquitoes, the *Plasmodium*-induced block of melanization is reverted, and these mosquitoes are refractory to the parasite. Thus, *CLSP2* is a new component of the mosquito immune response.

Keywords: IMD; Toll; prophenoloxidase

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INTRODUCTION

Melanization is a unique defence mechanism in arthropods involved in wound healing and pathogen encapsulation. Phenol-oxidases are key enzymes of melanization, which mediate the enzymatic conversion of tyrosine to eumelanin. A serine-protease (SP) cascade, similar to the blood-clotting cascade of vertebrates, proteolytically activates prophenoloxidases (PPOs) to phenol-oxidases. The spontaneous activation of the melanization reaction is tightly controlled; otherwise, excessive amounts of quinones generated as a result of systemic hyperactivation of the phenol-oxidase system would be deleterious to arthropods. Thus, arthropods have developed a series of negative modulators of melanization. Before infection or injury, the SP cascade involved in PPO activation is inhibited by several serpins specifically controlling SPs (Kanost, 1999; Salzet *et al.*, 1999). Other melanization inhibitors from moth (*Manduca sexta*) and crayfish (*Pacifastacus leniusculus*) have been shown to prevent

formation of melanin from phenoloxidase-oxidized phenol substrates (Zhao *et al.*, 2005; Söderhäll *et al.*, 2009).

We study two related genes (*CLSP1* and *CLSP2*) that encode modular proteins consisting of C-type lectin (CTL) and elastase-like SP (ESP) domains from *Aedes aegypti*. Both genes are induced by microbial infections and controlled by the nuclear factor (NF)- κ B factors of the immune deficiency (IMD) and Toll pathways. RNA interference (RNAi) depletion experiments showed that *CLSP2* is essential for preventing spontaneous haemolymph melanization. The PPO was proteolytically processed in the haemolymph of mosquitoes 24 h post-blood meal (PBM), but this response was not observed in mosquitoes infected with the avian malaria parasite, *Plasmodium gallinaceum*. The *CLSP2* gene was activated in *P. gallinaceum*-infected mosquitoes; however, RNAi depletion of *CLSP2* in infected mosquitoes resulted in the appearance of proteolytically processed PPO. Moreover, these mosquitoes showed a high level of resistance to *P. gallinaceum*. This suggests that *CLSP2* is involved in modulation of melanization.

RESULTS AND DISCUSSION

Characterization of *CLSP1* and *CLSP2*

We cloned full-length complementary DNAs (cDNAs) corresponding to *CLSP1* and *CLSP2* open reading frames by combining the methods of cDNA library screening, PCR cloning and rapid amplification of cDNA ends, followed by sequencing (supplementary Fig S1,S2 online). Sequences of both genes can be found in the VectorBase—*CLSP1* (AAEL011622) and *CLSP2* (AAEL011616). *CLSP1* and *CLSP2* genes encode related proteins, each composed of an amino-terminal ESP domain and a carboxy-terminal CTL domain (Fig 1A). C-termini of both *CLSP1* and *CLSP2* contain the three-amino-acid sequence QPD, which is a signature of the galactose-specific CTL. The presence of a signal peptide indicates the secretory nature of these protein factors (Fig 1A). Proteins with a composite structure such as those in *CLSP1* and *CLSP2* have not been previously reported for mosquitoes. The horseshoe-crab factor C contains SP and CTL domains, and is involved in the haemolymph clotting system (Nakamura *et al.*, 1986). Factor C functions as a pattern-recognition receptor (PRR); in the presence of diaminopimelic acid (DAP)-positive peptidoglycan bacteria, it undergoes autoproteolytic cleavage, and the resulting activated protein, containing a SP domain, initiates a haemolymph-coagulation cascade (Ariki *et al.*, 2004). On the basis of the

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Fig 1 | Characterization of *CLSP1* and *CLSP2* genes. (A) Predicted domain structure of CLSP factors. The black box indicates a putative signal peptide, SP and CTL are serine protease and CTL domains, respectively. (B) Immune-inducible expression of *CLSP* genes after infection with either bacteria *Enterobacter cloacae* (Ec) or fungi *Beauveria bassiana* (Bb; left panel). *CLSP* genes were highly expressed in the fat body on infection (right panel). (C) Regulation of *CLSP* gene expression by the immune deficiency pathway. Induction of *CLSP* genes by *E. cloacae* infection was blocked in RNA interference *REL2*-depleted mosquitoes. (D) Regulation of *CLSP* genes by Toll pathway. Left panel: *Cactus* depletion activates transcription of *CLSP* genes; this activation was compromised by simultaneous depletion of *REL1*. Right panel: Induction of *CLSP* genes by *B. bassiana* (2D Bb) was compromised in *REL1*-depleted mosquitoes. Depletion of *TOLL5A* had no effect. B–D are Northern blot analyses. 8 h, 1D or 2D Ec, UGAL mosquitoes 8 h, 1 day or 2 days after septic injury with *E. cloacae*; 1D or 2D Bb, UGAL mosquitoes 1 day or 2 days after septic injury with *B. bassiana*; FB, fat body; Naive, previtellogenic UGAL; OV, ovary; iLuc, iCactus, iREL1, iREL2 indicate luciferase-, *Cactus*-, *REL1*-, *REL2*- and double-stranded RNA-treated mosquitoes, respectively.

similarity with horseshoe-crab factor C, we expected that mosquito CLSPs were immune proteins with dual functions, as pathogen sensors and signal transducers. Both CLSPs contain putative cleavage sites, further emphasizing similarities with factor C (supplementary Fig S1,S2 online).

The N-terminal SP domains of *Aedes* CLSPs are similar to the SP domains of *Drosophila melanogaster* gastrulation defective (DGD), *D. melanogaster* ModSP and *M. sexta* HP14 (Han *et al*, 2000; Ji *et al*, 2004; Buchon *et al*, 2009). The primary specificity pockets of these SPs do not contain the three-amino-acid sequence DGG, which is a signature of the trypsin-like SPs (supplementary Fig S3 online). This suggests that these SPs belong to a subgroup of chymotrypsin/ESPs (Jiang *et al*, 2005). Arthropod ESPs are involved in the initiation of SP cascades by proteolytically activating other SPs. The Toll pathway establishes the dorsal–ventral polarity during *Drosophila* embryonic development. Activation of DGD (Han *et al*, 2000) in the extracellular perivitelline space leads to sequential cleavage of two SPs: Snake (DeLotto & Spierer, 1986) and Easter (Chasan & Anderson, 1989). *M. sexta* HP14 is autoactivated by an interaction with a peptidoglycan to initiate HP21 CLIP SP in the melanization SP cascade (Ji *et al*, 2004). HP14 is a modular SP, which in addition to an ESP domain contains four LDLrA repeats, one Sushi domain and one unique wonton domain in the N-terminal (Wang & Jiang, 2006).

Drosophila ModSP (CG31217), an orthologue of *M. sexta* HP14, mediates signals from PRRs and leads to the activation of Grass CLIP SP in the Toll immune pathway (Buchon *et al*, 2009).

The C-termini of *CLSP1* and *CLSP2* factors are related to CTL-type molecules that recognize microbial pathogens through lectin–carbohydrate interaction and act as PRRs. A *M. sexta* CTL, IML-2, agglutinates *Escherichia coli* in the presence of calcium and enhances melanization and encapsulation (Yu & Kanost, 2003). Two *Anopheles* CTLs, CTL4 and CTLMA2, have been shown to have a role in defence against DAP-positive peptidoglycan bacteria, as well as an antagonist role in the melanization response to *Plasmodium berghei* ookinetes (Osta *et al*, 2004; Schnitger *et al*, 2009).

Regulation of expression of *Aedes* CLSP genes

We performed northern blot analyses after infections with DAP-positive peptidoglycan bacteria *Enterobacter cloacae* and fungi *Beauveria bassiana*, which are known to activate the IMD and Toll pathways, respectively. Transcripts of approximately 1.4 Kb in size for each *CLSP* gene were highly elevated after infection with *E. cloacae*, but to a lesser degree after *B. bassiana* (Fig 1B). The transcripts of both *CLSP* genes were enriched in the fat body after infection (Fig 1B). These transcripts were not visible in the ovaries and barely detectable in haemocytes (Fig 1B).

Fig 2 | The effect of CLSP2 on haemolymph activation of prophenoloxidase. (A) Acute transient activation of haemolymph PPO after septic infections with either *Enterobacter cloacae* or *Staphylococcus aureus*. A 20-kDa band is present in haemolymph samples at 5 h, but absent at 24 h, after infection. Immunoblot using *Manduca* PPO1/2 antibodies; the antibodies against *Aedes* lipophorin II (LPII) were used as the loading control. (B) Progression of *CLSP2* gene expression over the course of infection with *E. cloacae*. Northern blot analysis. (C) Spontaneous PPO activation in *CLSP2*-depleted mosquitoes. The 20-kDa cleavage product is present in haemolymph samples from *CLSP2*-depleted mosquitoes, but not in those treated with iLuc or iCLSP1. (D) The haemolymph PPO activation in *CLSP2*-depleted mosquitoes is compromised by a simultaneous depletion of IMP2, showing that *CLSP2* suppresses the PPO cleavage mediated by IMP2. Depletion of IMP1 had no effect. Immunoblot using *Manduca* PPO1/2 antibodies; the antibodies against *Aedes* LPII were used as the loading control. (E) Reverse-transcription polymerase chain reaction analysis showing the specificity of *CLSP*-gene knockdowns 5 days after double-stranded RNA (dsRNA) treatment. 5 h, 24 h *Ec*, mosquitoes 5 or 24 h after infection with *E. cloacae*; 5, 24 h *Sa*, mosquitoes 5 or 24 h after infection with *S. aureus*; iCLSP1 and iCLSP2 indicate *CLSP1* and *CLSP2* dsRNA-treated mosquitoes, respectively. Naive, previtellogenic UGAL.

To further investigate the regulatory network responsible for controlling expression of *CLSP* genes, we evaluated the transcript abundance of these genes after RNAi silencing of key factors of the IMD and Toll pathways. Depletion of the mosquito IMD pathway NF- κ B factor REL2, but not the mosquito Toll pathway NF- κ B factor REL1, blocked upregulation of *CLSP* genes elicited by *E. cloacae*, which is consistent with the role of the IMD pathway in mediating *CLSP* gene activation (Fig 1C). RNAi silencing of *Cactus*—a gene encoding an inhibitor of Toll NF- κ B factor REL1—resulted in upregulation of *CLSP* genes. The activation of *CLSP* genes by *Cactus* depletion was compromised by simultaneous depletion of REL1, suggesting involvement of the Toll pathway in regulation of *CLSP* genes (Fig 1D). In an additional experiment, upregulation of *CLSP* genes activated by infection with *B. bassiana* was compromised as a result of RNAi depletion of REL1, further supporting involvement of the Toll pathway in regulation of the *CLSP* genes (Fig 1D). *Aedes* TOLL5A has been proposed to be the receptor of the Toll immune pathway (Shin et al, 2006). However, depletion of TOLL5A did not yield a response similar to REL1 RNAi in compromising upregulation of *CLSP* gene expression after

fungal infection (Fig 1D). The reason for this discrepancy is unclear. Overall, these experiments showed the dual regulation of *CLSP* genes by the IMD and Toll pathways.

CLSP2 as a modulator of melanization

Proteolytic cleavage of haemolymph PPO, which suggests its activation, was observed in mosquitoes after fungal infection with *B. bassiana*, as well as in *Cactus*- and *Serpin-1*-depleted mosquitoes (Zou et al, 2010). This was demonstrated by the appearance of a 20-kDa protein band, detected by western blot analysis of mosquito haemolymph with antibodies against *M. sexta* PPO1/2 (Zou et al, 2010). By using the same approach, we observed PPO haemolymph cleavage 5 h after infections with either *E. cloacae* or *Staphylococcus aureus* bacteria (Fig 2A). Haemolymph PPO cleavage was not detectable 24 h later, suggesting that it is an acute response to bacterial infection. As the activation of *CLSP* genes was linked to bacterial infection, we investigated the abundance of the *CLSP2* transcript over the time course of *E. cloacae* infection; the transcript continued to accumulate up to 24 h post-infection, when acute activation of

Fig 3 | Interaction of CLSP2 with *Plasmodium* parasite in mediating PPO activation. (A) Haemolymph prophenoloxidase activation was observed in mosquitoes 24 h post-blood meal (PBM); the 20-kDa cleavage band was not present in haemolymph samples from mosquitoes blood-fed on chickens infected with the avian malaria parasite, *Plasmodium gallinaceum*. (B) The 20-kDa cleavage product was present in haemolymph samples from CLSP2-depleted mosquitoes fed on *P. gallinaceum*-infected mosquitoes, but not in those treated with iLuc control or iCLSP1. (A,B) Immunoblot using *Manduca* PPO1/2 antibodies; the antibodies against *Aedes* lipophorin II (LPII) were used as the loading control. (C) Northern blot analyses of activation of CLSP genes in mosquitoes fed on either uninfected or *P. gallinaceum*-infected chickens. The CLSP2 gene was upregulated at 24 and 48 h PBM on *P. gallinaceum*-infected chickens. Naive, previtellogenic UGAL mosquitoes; PBM, post-blood meal; PPO, prophenoloxidase.

PPO cleavage was cleared (Fig 2B). Furthermore, we tested whether CLSP factors were involved in controlling PPO haemolymph activation; RNAi depletion of CLSP2, but not CLSP1, resulted in PPO cleavage of haemolymph (Fig 2C), suggesting that the CLSP2 factor functions as a negative modulator of haemolymph melanization.

In *A. aegypti*, two CLIP SPs, immune melanization protease (IMP)1 and IMP2, have been implicated in mediating haemolymph PPO cleavage (Zou et al, 2010). We investigated the relationship between CLSP2 and these two proteases in haemolymph melanization. Simultaneous depletion of CLSP2 with IMP2, but not with IMP1, stopped the PPO cleavage triggered by depletion of CLSP2 (Fig 2D). This suggests that CLSP2 is involved in the suppression of IMP2-mediated immune melanization, functioning either upstream from or directly on this CLIP SP. In accordance with these data, we observed no melanotic tumours in CLSP2-depleted mosquitoes (supplementary Fig S4 online). As a control, we used depletion of *Serpin 2*, which is involved in tissue melanization, and which depletion by RNAi results in the formation of melanotic tumours (Zou et al, 2010).

Taken together, our results indicate that *A. aegypti* CLSP2 factor is a negative modulator of haemolymph melanization that is probably responsible for its timely inhibition following an acute phase of infection, as well as for preventing its spontaneous activation. Moreover, we suggest that, similar to the horseshoe-crab factor C, the mosquito CLSP2 factor is a modular protein containing the CTL domain—which probably acts as a PRR pathogen sensor—and the ESP domain, which is involved in control of melanization. It seems that, similar to *D. melanogaster* DGD and ModSP, as well as *M. sexta* HP14 (Han et al, 2000; Ji et al, 2004; Buchon et al, 2009), *Aedes* CLSP2 functions by interacting with a CLIP SP, in this case IMP2. Further studies are required to understand the mechanistic basis of CLSP2 action.

CLSP2 is required for *Plasmodium* development

We observed an increase in the haemolymph PPO activation in the mosquitoes at 24 h PBM. However, this PPO activation did not occur when the blood meal contained the malaria parasite *P. gallinaceum* (Fig 3A). Depletion of CLSP2, but not of CLSP1, reversed the effect of *Plasmodium* infection and resulted in haemolymph PPO cleavage in infected mosquitoes at 24 h PBM (Fig 3B). Expression of both CLSP genes was elevated in *P. gallinaceum*-infected mosquitoes at 24 h PBM; however, the CLSP2 gene was upregulated to a higher level and its transcript was still present at 48 h PBM (Fig 3C; supplementary Fig S5 online). This indicated that the CLSP2 gene was responsive to an unknown stimuli linked to *Plasmodium* infection. Further studies are required to explain the mechanism of CLSP gene activation by *Plasmodium* infection.

Importantly, RNAi depletion of CLSP2 resulted in a significantly reduced number of oocysts in midguts of *P. gallinaceum*-infected mosquitoes, as compared with iLuc controls. (Fig 4; supplementary Fig S6 online). At the same time, several melanized ookinetes increased in these mosquitoes (Fig 4). RNAi depletion of CLSP1 had no effect on oocyst levels in midguts of *P. gallinaceum*-infected mosquitoes (Fig 4). Thus, CLSP2 was required for *P. gallinaceum* survival in *Aedes* mosquitoes. It has been reported that RNAi-mediated depletion of CTLs, CTL4 and CTLMA2 (mannose-specific CTL2) functioned as protective agonists on the development of ookinete to oocysts in *P. berghei* in *A. gambiae* (Osta et al, 2004).

Mosquitoes have an effective defence system that is finely tuned to detect various pathogens, including malaria parasites. An integral part of the anti-*Plasmodium* response involves targeting of the parasite by complexes of leucine-rich repeat proteins, LRIM1, APL1A and APL1C, and thioester-containing protein 1 (Riehle et al, 2006; Povelones et al, 2009; Baxter et al, 2010). In turn, the parasite has evolved effective means to

Fig 4 | Reduction of *Plasmodium gallinaceum* oocyst number in CLSP2-depleted mosquitoes. Data were collected from three independent experiments with CLSP double-stranded-RNA-treated mosquitoes (supplementary Fig S6 online) and pooled; sample sizes are shown in parentheses. The number of fully developed oocysts in each midgut is shown as a coloured circle. The mean number of parasite oocysts for each group is indicated with a black line and the number on the right. Mean values of melanized ookinetes are indicated by black columns.

overcome mosquito defences. The ability for tremendous proliferation at the sporozoite developmental stage is an evolutionary adaptation that allows the parasite to overcome losses during the main bottleneck of its developmental cycle from an ookinete to an oocyst. However, *Plasmodium* evades mosquito immunity by other mechanisms that allow ookinetes to successfully develop to oocysts, which are poorly defined. Lambrechts *et al* (2007) showed that *P. falciparum* evades the melanization immune response of *A. gambiae*. Our data indicate that CLSP2 is required for parasite survival in the mosquito host and might be involved in the parasite-mediated evasion of the haemolymph melanization response.

METHODS

Experimental animals. The colony of *A. aegypti* mosquitoes was maintained in the laboratory and fed continuously on water and 10% sucrose solution. To initiate egg development, mosquitoes were blood-fed on white rats. All procedures using vertebrate animals were approved by the University of California Riverside Institutional Animal Care and Use Committee (#A20100016; 05/27/2010). The avian malaria parasite *P. gallinaceum* was maintained in the laboratory by natural transmission between *A. aegypti* and chickens. Chickens were housed in cages in the vivarium, and all procedures were pre-approved by the Institutional Animal Care and Use Committee. Midguts from mosquitoes 8 days after a parasite-harboring infectious blood-meal were dissected and stained with 1% mercurochrome solution. The parasites were viewed and counted under a Nikon E400 light microscope.

Synthesis and microinjection of double-stranded RNA, septic injury, reverse transcription–polymerase chain reaction and RNA blot analysis. The experiments were conducted as described previously (Zou *et al*, 2010). Primers are listed in the supplementary information online.

Immunoblot analysis. The experiments were conducted as described previously (Zou *et al*, 2010). The primary antibody against *M. sexta* PPO1 and PPO2 (provided by Dr H. Jiang) was used for the immunoblot analysis. Antibodies against *Aedes* lipophorin II were used as loading controls.

Statistical analysis. Data on *Plasmodium* refractoriness from three independent experiments were collected and analysed using the Kolmogorov–Smirnov goodness-of-fit test and pooled. Statistical significance between samples was evaluated using the Mann–Whitney *U*-test (Graphpad 5.0).

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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