The Melanization Reaction Is Not Required for Survival of *Anopheles gambiae* Mosquitoes after Bacterial Infections*

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The melanization reaction of insects requires activation of pro-phenoloxidase by a proteolytic cascade leading to melanin production. Studies in adult mosquitoes have shown that bacteria are efficiently melanized in the hemocoel, but the contribution of melanization to survival after bacterial infections has not been established. Here we show that the *Anopheles gambiae* noncatalytic serine protease CLIPA8, an essential factor for *Plasmodium* ookinete melanization, is also required for melanization of bacteria in adult mosquitoes. CLIPA8 silencing by RNA interference inhibits pro-phenoloxidase activation and melanization of bacteria in the hemolymph following microbial challenge. However, CLIPA8 is not required for wound melanization nor for melanotic pseudotumor formation in *serpin2* knockout mosquitoes, suggesting a specific role for pathogen melanization. Surprisingly, CLIPA8 knockout mosquitoes are as resistant to bacterial challenge as controls, indicating that melanization is not essential for defense against bacteria and questions its precise role in mosquito immunity.

Melanization is a prominent defense mechanism in arthropods and has received considerable attention in the context of a genetically selected refractory strain of *Anopheles gambiae* that melanizes several *Plasmodium* species, including New World strains of *Plasmodium falciparum* (1). Biochemical studies in large insects such as *Manduca sexta*, *Bombyx mori*, and *Holotrichia diomphalia* (for reviews see Refs. 2, 3) have led to the current model of the melanization response, in which recognition of nonself-molecular patterns eventually activates a clip-domain serine protease cascade, culminating in limited proteolysis and conversion of inactive pro-phenoloxidase (PPO) into active phenol oxidase (PO) by pro-phenoloxidase-activating enzymes. Activated PO catalyzes the rate-limiting step in the melanization reaction, the oxidation of tyrosine to dopaquinone that ultimately leads to production and cross-linking of melanin with proteins on the surfaces of microbes or wounds. Our recent genetic studies in *A. gambiae* (4–7) identified several proteins that regulate *Plasmodium berghei* ookinet melanization and highlighted the underlying genetic complexity of this immune module. However, field-caught *A. gambiae* (8) and most laboratory strains rarely utilize melanization to kill *Plasmodium* parasites, although certain mutants do so (4, 7). Although melanization does not seem to be a major parasite-killing mechanism, it remains unclear whether it is important for elimination of other pathogens such as bacteria. In this study we address specifically this question.

To date the role of melanization in defense against bacteria in mosquitoes remains controversial. Although melanized bacteria have been detected in the hemocoel of mosquitoes, including *Aedes aegypti* (9, 10) and *Anopheles albimanus* (11), no genetic studies have investigated whether this defense mechanism promotes survival of mosquitoes to bacterial infections. Using RNA interference, we have previously identified a noncatalytic clip-domain serine protease (serine protease homolog), CLIPA8, which is essential for ookinete melanization in a refractory *A. gambiae* strain as well as in certain reverse genetic mutant backgrounds (4–7). Here we show that CLIPA8 is also essential for melanization of both Gram-positive and Gram-negative bacteria in *A. gambiae*; however, melanization was not required for mosquito survival after bacterial infections. Furthermore, CLIPA8 is not required for melanization of wound surfaces nor for the formation of circulating melanotic pseudotumors.

**MATERIALS AND METHODS**

*Anopheles gambiae* Strain and Gene Silencing by RNA Interference—All experiments were performed with the *A. gambiae* G3 strain. *In vivo* gene silencing by RNA interference was performed as reported (12). Double-stranded RNAs (dsRNA) for *lacZ* (control), CLIPA8, and the Rel homology domain (RHD) of Rel2 were synthesized as described in Refs. 7, 13, 14, respectively.

**RNA Isolation and Quantitative Real Time PCR**—Mosquitoes were challenged with *Escherichia coli* or *Staphylococcus aureus* either by injecting bacterial suspensions at *A*600 = 0.4 or by prickling with overnight bacterial cultures. Mosquitoes injected or pricked with sterile PBS were used as controls. Total RNA was isolated from whole mosquitoes at the indicated time points using TRIzol reagent (Invitrogen) according to the supplier’s instructions, and contaminant genomic DNA was removed by DNase I treatment. First strand cDNA synthesis
and CLIPA8 primers used for quantitative real time PCR are as described previously (7).

**Generation of Monoclonal Antibodies against CLIPA8**—A 719-bp fragment corresponding to the protease domain of CLIPA8 was amplified using primers CLIPA8expF, 5′-CATGGAATTCCTTTGGTTGCGATTT-3′, and CLIPA8expR, 5′-GGGAGCCCCTACAGTTATTCCCTGATTGTAGCAT-3′, containing the restriction sites NcoI and NotI, respectively. The amplicon was first subcloned in pGEM®-T Easy vector (Promega) and then cloned between the NcoI and NotI sites of the pETM11 expression vector. Fusion protein containing an N-terminal His tag was expressed in *E. coli* strain BL21 (DE3) and extracted from inclusion bodies using 8 M urea, pH 8, as described (15). Monoclonal antibodies against CLIPA8 were produced at the EMBL Monoclonal Antibody Core Facility as described (16).

**Western Blot Analysis**—Bacteria from overnight cultures were washed and resuspended in PBS; suspensions of 69 nl (*E. coli*, *Enterobacter cloacae*, *Enterococcus faecalis*, and *S. aureus*, each at A$_{600}$ = 0.8) were injected into mosquitoes using a Nanoject II injector (Drummond). For immunoblotting, hemolymph proteins were collected in a reducing SDS buffer by proboscis clipping from 10 mosquitoes 6 h post-challenge, separated using 12% SDS-PAGE, and transferred as described (17). Blots were incubated with mouse anti-CLIPA8 monoclonal antibody (1/20), rat anti-PPO (1/100; a kind gift from H. M. Müller), and rabbit anti-SRPN3 (1/1000) polyclonal antibodies (18). Anti-mouse, anti-rabbit, and anti-rat IgG horseradish peroxidase-conjugated secondary antibodies (Promega) were used at 1/15,000, 1/30,000, and 1/15,000 dilutions, respectively. Relative protein band quantitation was done using the Phoretix 1D software.

**Measurements of PO Activity**—PO activity was assayed 6 h post-challenge with a mixture of *S. aureus* and *E. coli* (each at A$_{600}$ = 0.4) or PBS (control). Hemolymph was collected in ice-cold phosphate-buffered saline (PBS) containing protease inhibitors, and protein concentration was determined using the protein assay kit from Bio-Rad. Five μg of total hemolymph proteins in 40 μl of PBS containing protease inhibitors were mixed with 120 μl of saturated 1-3,4-dihydroxynaphthalene solution; absorbance at 492 nm was measured after incubation at room temperature for 30 min.

**Survival Experiments**—*E. coli* and *S. aureus* were cultured to A$_{600}$ = 0.7; pelleted, washed, and resuspended in phosphate-buffered saline to the indicated concentrations. Bacterial infections and survival assays were done as described (12).

**RESULTS**

**CLIPA8, a Hemolymph Protein, Is Cleaved by Wounding and Bacterial Infections**—Immunoblotting of hemolymph from naive mosquitoes (Fig. 1A, lane 1) revealed a full-length CLIPA8 protein (CLIPA8-F) with an apparent molecular mass of 47 kDa. However, soon after *S. aureus* injection into mosquitoes, a cleaved form of CLIPA8 (CLIPA8-C) corresponding to the noncatalytic protease domain appeared at 38 kDa in immunoblotted hemolymph extracted in reducing SDS buffer (Fig. 1A). Quantitative analysis of band intensity (Fig. 1A, bar graph) revealed that the CLIPA8-C levels increased after *S. aureus* infection, peaked at 6 h, and returned to the basal level by 24 h post-infection. Furthermore, CLIPA8 mRNA expression peaked at 12 h post-infection with *S. aureus* and *E. coli* (2.5- and 1.7-fold, respectively, relative to PBS-injected controls) and remained relatively high at 24 h post-infection (Fig. 2). In contrast, CLIPA8-F did not increase in response to infection (data not shown), suggesting that the observed transcriptional induction during this immune response may serve protein replenishment rather than accumulation. Both CLIPA8-F and CLIPA8-C are absent from hemolymph of mosquitoes treated...
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with double-stranded (ds) CLIPA8 RNA (Fig. 1B, lane 6). Further analysis revealed that CLIPA8 cleavage in the hemolymph is induced robustly by additional bacterial species, including E. coli, E. cloacae, and E. faecalis, but is relatively minor after sterile PBS injection (Fig. 1B). Quantitation of CLIPA8-C band intensities revealed a 17-, 43-, 26-, 40-, and 5-fold increase in the abundance of CLIPA8-C in hemolymph in response to the injection of E. coli, E. cloacae, E. faecalis, S. aureus, or sterile PBS alone, respectively, as compared with control noninjected mosquitoes. Hence, CLIPA8 cleavage is induced much more effectively by infection rather than by injury per se.

Cleavage of serine protease homologs (SPHs) involved in PPO activation has been reported in other insect species, including M. sexta (19) and H. diomphalia (20), where it occurs at a specific site within the clip-domain; however, the clip and protease domains remain linked by a disulfide bond. Immuno-blotting of mosquito hemolymph, extracted under nonreducing conditions, revealed that both CLIPA8-C and CLIPA8-F migrated as a single band (Fig. 1B, lane 7), indeed indicating the presence of a disulfide bond that bridges both domains.

CLIPA8 Is Essential for Melanization of Bacteria—The facts that CLIPA8 is essential for P. berghei ookinete melanization (7) and is cleaved in the hemolymph following bacterial challenge prompted us to investigate the potential involvement of CLIPA8 in bacterial melanization. We injected E. coli or S. aureus in wild type, dslacZ, or dsCLIPA8-treated adult A. gambiae and utilized light microscopy to score for the presence of melanized bacterial clumps in the mosquito abdomens, after dissection 3 days post-infection. Such clumps were detected in wild type mosquitoes injected with E. coli (Fig. 3B, arrows) or S. aureus (Fig. 3C, arrows) but not in noninjected (Fig. 3A) or PBS-injected mosquitoes (data not shown). These clumps appear as easily identifiable black bodies clustering mainly on both sides of the dorsal blood vessel. Studies in another mosquito species established that the large melanotic clumps appearing after bacterial infections are formed by aggregation of singly melanized bacteria in the hemocoel (11). Interestingly, after challenge with E. coli or S. aureus, melanized clumps were present in dslacZ-treated controls (Table 1) but absent from dsCLIPA8-treated mosquitoes (Fig. 3, D and E, respectively). The same clear difference was observed (data not shown) with two additional bacteria, E. cloacae (Gram-negative) and E. faecalis (Gram-positive), indicating that CLIPA8 is indeed required for melanization of bacteria in A. gambiae. If so, silencing CLIPA8 should also abolish hemolymph PPO activation by bacteria. We measured PO enzy-
downstream of effective antibacterial response, other than melanization (14). This comparison strongly suggests the existence of an immune system in A. gambiae mosquitoes, which succumb to infections as efficiently as did dsClipa8 controls (Fig. 3, F and G), suggesting that CLIPA8 is not essential for this process. CLIPA8 is also not required for the formation of spontaneous, large melanotic pseudotumors (Fig. 3, H and I), which develop in the absence of injected bacteria in mosquitoes silenced for the major melanization inhibitor, SRPN2 (5).

**DISCUSSION**

The phenol oxidase cascade is an integral part of insect physical and innate immune defenses, including cuticle sclerotization and melanization of pathogens. Melanization requires the limited proteolysis of inactive PPO to PO by pro-phenoloxidase-activating enzymes and their associated cofactors, including the serine protease homologs. Microscopy studies in different mosquito species revealed that bacteria are efficiently melanized in the hemocoel (9–11); however, no genetic analysis has yet been conducted to assess the contribution of melanization to mosquito survival after bacterial infections. Here we have addressed this question using in vivo functional genetic analysis based on RNA interference.

We show that CLIPA8, an SPH essential for the melanization of P. berghei ookinetes in the mosquito midgut, is required for PPO activation and the melanization of bacteria in the mosquito hemolymph. The fact that CLIPA8 is essential for the melanization of both Plasmodium and bacteria indicates the existence of at least a partial overlap in the genetic modules that regulate the melanization response of A. gambiae to both microorganisms. We also demonstrate by immunoblotting that CLIPA8 is cleaved following bacterial challenge; however, both the protease and clip domains remain tethered by a disulfide bond as has been shown for other insect SPHs (19, 21). Cleaved
CLIPA8 is most probably the functional form of the protein, and recent structural analysis of the *H. diomphalia* pro-phenoloxidase-activating factor (PPAF)-II, which belongs to the SPH family, revealed that uncleaved PPAF-II is a monomer, whereas cleaved PPAF-II is a large oligomer that binds tightly to PO (22). The CLIPA8-C band detected in immunoblots is often weak compared with CLIPA8-F, and a similar observation has been reported for *M. sexta* SPH1 and SPH2 (19). This imbalance could be due either to rapid clearance of the cleaved functional forms from the hemolymph to contain the melanization response or to their entrapment together with PO and possibly other proteins in the melanin capsule that forms around pathogens. Interestingly, even though CLIPA8 cleavage in the hemolymph was also detected after injection of sterile PBS and 3 days post-silencing of the major melanization inhibitor SRPN2 (data not shown), CLIPA8 was not required for melanization of wound surface nor for the formation of spontaneous melanotic pseudotumors in dsSRPN2 mosquitoes. These observations suggest the existence of distinct mechanisms for PPO activation during wound healing and melanotic pseudotumor formation.

Genome sequence comparisons had shown that the PPO family encompasses many more genes in *A. gambiae* than in *Drosophila* (23); moreover, no orthologs of the essential CLIPA8 melanization factor have been identified in *Drosophila* or other insects to date (Ref. 23 and data not shown). These observations suggested the possibility that hemolymph phenol oxidase activity may be essential for antibacterial defense in *Anopheles*, although it is dispensable in *Drosophila* (24, 25). Nevertheless, our data do not support this hypothesis; dsCLIPA8-treated mosquitoes were as resistant to bacterial infections as dslacZ controls, although they failed to activate hemolymph PPO. However, these results do not exclude a potential role for melanization in enhancing the effectiveness of different immune reactions as demonstrated recently in *Drosophila* (25). Broader elucidation of the functional roles of the evolutionary well conserved melanization immune response will require future genetic studies in non-Dipteran insects.

Acknowledgments—We thank H.-M. Müller for the PPO antibodies, K. Michel for SRPN3 antibodies and SRPN2 dsRNA, and G. K. Christophides for Rel2-RHD dsRNA. We also thank the EMBL-Monoclonal Antibody Facility for production of CLIPA8 monoclonal antibodies. We are grateful to Tiboeb Habtewold for mosquito rearing.

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