ABSTRACT

The Effects of the *Anopheles gambiae* CLIPC3 and CLIPC1 Genes on Melanization of an Abiotic Target.

Melanization is a mechanism of innate immunity in arthropods wherein a thick capsule of melanin is formed around foreign objects. Little is known about this pathway biochemically though serine proteases are known to play a critical role. In this paper we began to address the roles of CLIPC3 and CLIPC1. Both are potential components of the melanization pathway in *A. gambiae*. Knockouts are achived through injections of dsRNA. After 4 days, sephadex beads were injected and after 24 hours, mosquitoes were dissected and beads were scored for degree of melanization. Knockout of CLIPC1 showed little variation in the melanization levels observed compared with the control GFP injections. CLIPC3 knockouts showed an increase in lack of melanization and a decrease in complete melanized of bead comparison with the control. We conclude CLIPC3 is involved in the melanization pathway of *A. gambiae* and it is unlikely CLIPC1 plays a critical role.

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COVER SHEET

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The Effects of the *Anopheles gambiae* CLIPC3 and CLIPC1 Genes on Melanization of an Abiotic Target.

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Abstract:

Melanization is a mechanism of innate immunity in arthropods wherein a thick capsule of melanin is formed around foreign objects. Little is known about this pathway biochemically though serine proteases are known to play a critical role. To better understand the interactions between mosquitoes and parasites, such as *Anopheles gambiae* and *Plasmodium*, we are working on classifying serine proteases identified as potential components of the melanization pathway in *A. gambiae.* In this paper we began to address the roles of CLIPC3 and CLIPC1, potential homologues of an enzyme that mediates melanization in another insect, *Manduca sexta*. Injections of dsRNA were used to knockout the mosquito genes. After a 4 day recovery period, sephadex beads were injected and after another recovery period of 24 hours, the mosquitoes were dissected and the beads were scored for degree of melanization. Knockout of the genes was verified by a reverse transcription reaction followed by a PCR-amplification of the target gene in the carcasses of the dissected mosquitoes. Knockout of CLIPC1 showed little variation in the melanization levels observed compared with the control GFP injected mosquitoes. CLIPC3 knockouts showed an increase in beads that were not melanized and a decrease in the number of beads that were completely melanized in comparison with the GFP injected mosquitoes. We conclude CLIPC3 is a serine protease involved in the melanization pathway of *A. gambiae* whereas it is unlikely CLIPC1 plays a critical role in the pathway.

Introduction:

Melanization is a mechanism of innate immunity (Christophides GK et al., 2002), employed by mosquitoes as well as other arthropods, where a thick acellular capsule of melanin is created around the foreign object (Paskewitz and Gorman, 1999). In the short period of time after the foreign object is exposed to the hemolymph, small spots of a flexible, adhesive, non-pigmented material form at the surface of the object (Vey, 1993) . The spots increase in size to eventually cover the entire surface of the object at which point the coating darkens and hardens.

Melanization restricts movement of materials in and out of the capsule as well as movement of the foreign object itself.

From a biochemical perspective, based on studies involving large lepidopteran insects such as *Manduca sexta* and *Bombyx mori* (Ashida and Brey, 1997; Jiang and Kanost 2000; Volz J et al., 2004; Gorman et al., 2007), the process of melanization relies on an enzymatic cascade (Ashida and Yamazaki, 1990). There are currently two models for the activation of the pathway: (1) pathogen surface molecules such as LPS, ß-1,3-glucan, and peptidoglycan are first bound by specific recognition molecules (Ochiai and Ashida, 1988, 1999; Yu et al,. 1999) or (2) subtle interactions occur between critical enzymes and inhibitors and the foreign surfaces (Paskewitz and Gorman, 1999). The immediate next step is unknown but eventually leads to the cleavage and activation by a serine protease (SP) of prophenoloxidase activating protease (proPAP) which in turn cleaves and actives prophenoloxidase (Ashida, 1990) which then catalyzes the hydroxylation of tyrosine to DOPA and the oxidation of DOPA to DOPA-quinone (Ashida and Yamazaki, 1990). The pathway becomes complicated following this step but eventually leads to the formation of a melanotic capsule around the foreign object.

We are particularly interested in the steps of melanization in the mosquito *A. gambiae* due to its ability to transmit malaria. The failure of the traditional control methods to halt transmission of *Plasmodium*, the protozoan parasite that causes malaria, has led to research into new methods that make use of immune reactions of the mosquito to the parasite to interrupt parasite development and transmission (Gorman and Paskewitz, 2001). It has been noted that *Plasmodium* infection induces melanotic encapsulation under some circumstances (Paskewitz and Gorman, 1999). An increased knowledge of the immune system of mosquitoes can also be used to study other pathogens that mosquitoes interact with and might be useful to enhance biocontrol strategies to reduce mosquito populations.

Research on the melanization pathway in *M. sexta* led to the implication of clip domain serine proteases (CLIPs), essential components of extracellular signaling cascades (Rawlings and Barrett, 1994), as key mediators of this reaction. CLIPs are characterized by a chymotrypsin-like

SP (serine protease) domain and one or more clip domains at the N-terminus. Potential homologues of the *Manduca* CLIP HP21 in *A. gambiae* are CLIPC3 (18D), CLIPC1, and CLIPC2 (Gorman and Paskewitz, 2001) which reside in subfamily C of CLIPs (Christophides et al., 2002). All three have similar amino acid sequences to each other and HP21. A recent study published on HP21 indicated that proHP21 is activated by HP14 and the active HP21 cleaves proPAP3 (Gorman et al., 2007). Thus any of the three CLIPs identified in mosquitoes as homologues of HP21 may play a similar role in the melanization pathway in *A. gambiae*.

As part of the long term goal of understanding the enzymatic cascade of melanization, our objective for this study was to identify whether CLIPC3 or CLIPC1 play a role in this cascade through use of dsRNA interference created knockouts. Double stranded RNA was created from cDNA, injected, and the effect on the melanization cascade was then observed through reactions to injected sephadex beads. The G3 strain of *Anopheles gambiae*, a widely used laboratory colony, was used for this study.

Materials and Methods:

Mosquito Rearing. The *A. gambiae* G3 strain was reared according to Paskewitz et al (1999).

Production of GFP (control) dsRNA. An in vitro transcription template was produced using a 624 bp portion of the GFP gene cloned into a LambdaZAP II vector. Primers GFP R (5' – TAA TAC GAC TCA CTA TAG GGC AGG ACT ATA AAG ATA CCA GGC GT) and GFP F (5' – TAA TAC GAC TCA CTA TAG GGG GAT CTA GGT GAA GAT CCT TTT TG) (XXIDT, Skokie, IL, USA) were used. PCR reactions consisted of 1 U of GoTaq (Promega, Madison, WI, USA), 0.3 nM MgCl2, primers at 1 µM each, 0.08 mM dNTPs, and the manufacturer's suggested buffer in 20µL total volume. PCR was performed in a PTC-100 thermocycler (MJ Research, Hercules, California, USA) using a 65ºC annealing step and 35 cycles. Quality of the reaction was assessed on a 1% agarose (Biorad, Hercules, California, USA) and ethidium bromide 1% (Fisher) gel with Blue/Orange 6x loading dye (Promega) and a

1Kb ladder (Promega). Following electrophoresis, the lower band (size 624 bp) was excised and the DNA was extracted from the gel using the Qiaquick gel extraction kit (Qiagen, Valencia, CA, USA). The quantity of the purified DNA was assessed with a ND-1000 spectrophotometer (NanoDrop, Wilmington, DE, USA).

Purified products were used in a second PCR reaction with a primer containing a full T7 site. The resulting product was again purified and 1-2 ug were used as a template for transcription. Next, the MegaScript RNAi Kit (Ambion Inc., Foster City, CA, USA), was used for transcription and the production of purified dsRNA and the product was quantified with the ND-1000 spectrophotometer.

Creation of experimental dsRNA. A procedure similar to the creation of GFP dsRNA was followed. The initial PCR amplified a section of the of a LambdaZAP II cDNA library made from larvae and pupae of the G3 strain of *A. gambiae* as described previously (Gorman et al., 2000). Primers 18d(CLIPC3) R (5' – TAA TAC GAC TCA CTA TAG GGC AGT CCA GGT ATG GGT GAA CTT) and 18d(CLIPC3) F (5' – TAA TAC GAC TCA CTA TAG GGA AAA TTA CGA CAT CTT GCG CTT) were used to amplify a 493bp region or CLIPC1 R (5' – TAA TAC GAC TCA CTA TAG GGT CCA CGA AAG ATA CGA ATA GAC G) and CLIP C1 F (5' – TAA TAC GAC TCA CTA TAG GGG AGT ACA AGC AAA CGT CCC ACT) primers (XXIDT) were used to amplify a 536bp region. Components and total volume of the reaction were the same with the exception of the substitution of 1 U EconoTaq (Lucigen) and Lucigen's suggested buffer for Promega's products. The PCR had a 62ºC anneal with 35 cycles and quality of the reaction was assessed on a gel as above. The single band DNA product was purified using Qiaquick PCR purification kit (Qiagen) and quantity was assessed with the ND-1000 spectrophotometer. A transcription reaction using MegaScript RNAi Kit (Ambion Inc., Foster City, CA, USA), was performed to obtain purified dsRNA and results were quantified with the ND-1000 spectrophotometer.

RNA interference. Using transcription reactions resulting in at least 3000 ng/µL dsRNA, 0.3µL of reaction product were injected into the thorax of anesthetized female mosquitoes. A

control cohort was injected with dsGFP. Both groups were allowed to recover for 4 days and survivors were then injected with single CM C-25 Sephadex beads (with diameters of 40 to 80μm) (Sigma) rehydrated in dilute methylene blue. Following a 24 hour recovery period, living experimental and control mosquitoes were dissected and the beads examined by light microscopy for level of melanization. Scoring was subjective and was based on the estimated percent of the bead surface that was covered by melanin. The categories were 0%, 10-30%, 40-60%, 70-90%, and 100% melanization.

Verification of Knockout. After the beads were dissected, mosquito carcasses were used to isolate RNA. RNA was extracted using the AquaPure RNA isolation kit (Biorad). Remaining ssDNA and dsDNA were removed through a reaction consisting of 1 U RQ1 RNase-Free DNase and Promega's suggested buffer in a total volume of 20μL for 30 min at 37ºC. Quantity was assessed with the ND-1000 spectrophotometer and a reverse transcription reaction was performed using ABI PRISM High Capacity cDNA archive kit. Using primers CLIPC1 F and CLIPC1 R, or CLIPC3 F and CLIPC3 R, a PCR was performed using of 1 U EconoTaq (Lucigen) and Lucigen's suggested buffer. The PCR used a 65ºC annealing temperature and 35 cycles. A positive control PCR that amplified the ribosomal protein S7 gene was done simultaneously with both experimental and control reverse transcription products with primers S7 F (5' – CGC TAT GGT GTT CGG TTC C) and S7 R (5' – TGC TGC AAA CTT CGG CTA T) (XXIDT) set to flank a 650bp region . This PCR used a 60ºC annealing temperature and 26 cycles. Results were analyzed on a 1% agarose gel.

Results:

Knockout of CLIPC1 gene. Two separate experiments with CLIPC1 injections in 4 day old females yielded a total of 48 survivors for the GFP control and 34 survivors for the experimental sample. A majority of the Sephadex beads injected into the GFP controls were 100% melanized (Figure 1) and this was similar for the target gene, CLIPC1. There is little difference between the control and target at any of the melanization levels. Verification of the

knockout can be seen in figure 2. Here amplification of the ribosomal protein S7 gene is used as a control to check for the success of the RNA extraction and to normalize the amounts of cDNA used in the reactions. Use of the CLIPC1 primers shows a lack of CLIPC1 amplified from the knockout mosquitoes indicating a successful knockout. CLIPC1 was successfully amplified in the GFP control, as expected, indicating that injection with an exogenous dsRNA did not have nonspecific effects on transcript abundance for CLIPC1.

Knockout of CLIPC3. In three separate experiments involving knockout of the CLIPC3 gene in 1 day old G3 *A. gambiae*, there were a total of 89 survivors of the dsCLIPC3 injections and 86 survivors of the dsGFP injections (Figure 3). Again, the GFP control mosquitoes showed a high degree of bead melanization. On the other hand, a significantly higher percentage of the experimentally injected mosquitoes showed low levels (0% to 30%) of melanization. Overall, there was a shift downward in the degree of bead melanization. Verification of the knockout produced gels similar to those presented in figure 2.

Discussion:

In the interest of identifying the members of the melanization cascade in mosquitoes, we targeted two serine proteases, CLIPC3 and CLIPC1, for analysis. Gene knockouts allowed us to estimate specific effects on melanization of Sephadex beads in G3 *A. gambiae* mosquitoes. These two genes were selected because both were closely related to the serine protease HP21 from *Manduca*, which has been implicated in the melanization pathway through biochemical studies. Thus, it is possible CLIPC3 or CLIPC1 may play a similar role in the mosquito melanization pathway.

Experiments with knockout of CLIPC1 lead us to conclude CLIPC1 is not likely to be critical in melanization. When comparing the results from CLIPC1 to GFP, we see little difference in the pattern of melanization. This does not mean CLIPC1 is not involved though. It is possible the melanization pathway may include some redundancy, thus a knockout may have no effect. We conclude CLIPC1 does not play a critical role in the melanization pathway.

Knockout of CLIPC3 resulted in a change in the melanization profiles. The knockouts show a higher incidence of unmelanized beads and a lower incidence of completely melanized beads. Based on these results, we conclude that CLIPC3 is a serine protease involved in the melanization cascade. Due to its sequence similarities to HP21, it likely plays a homologous role in the *A. gambiae* melanization cascade.

In continuing our study of the melanization cascade in *A. gambiae*, more effort can now be focused on this single gene, CLIPC3, to understand exactly where it fits in the pathway. As for other serine proteases found to potentially have immunity related functions, they can be tested for their potential action in the melanization pathway through methods similar to those used in this study.

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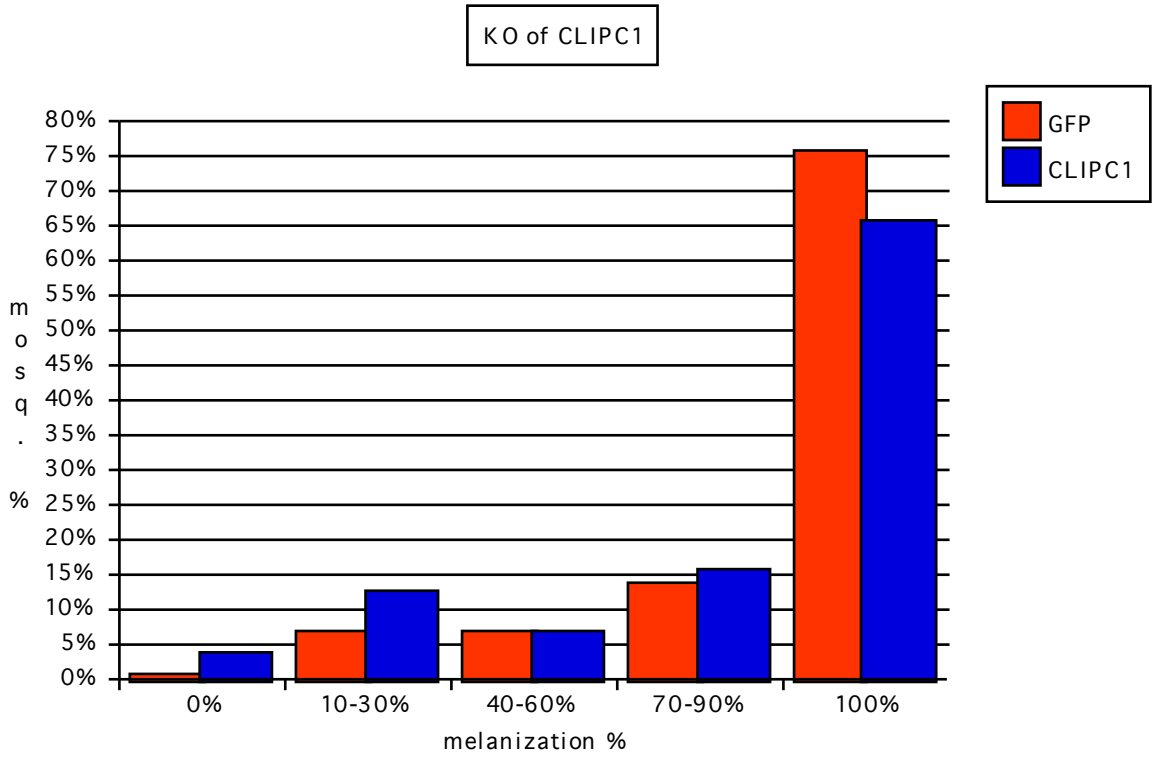
Tables and Figures:



	0%	10-30%	40-60%	70-90%	100%
GFP (48)	0% (0)	6% (3)	6% (3)	13% (6)	75% (36)
CLIPC1 (34)	3% (1)	12% (4)	6% (2)	15% (5)	65% (22)

Figure 1: Melanization results of Knockout (KO) of CLIPC1 versus GFP. In both the experimental and control groups, mosquitoes were grouped according to the percent of the Sephadex bead surface covered by melanin and then graphed as percents of the total mosquitoes that melanized beads at each respective level (0%, 10-30% etc.). The data for the graph can be found in the table where numbers in parenthesis are the raw numbers before conversion to percentage and those numbers next to the level are the total number of beads/mosquitoes in the group. Note that one bead was injected into one mosquito, so these numbers can be used interchangeably. Percentages were rounded to the nearest whole number.

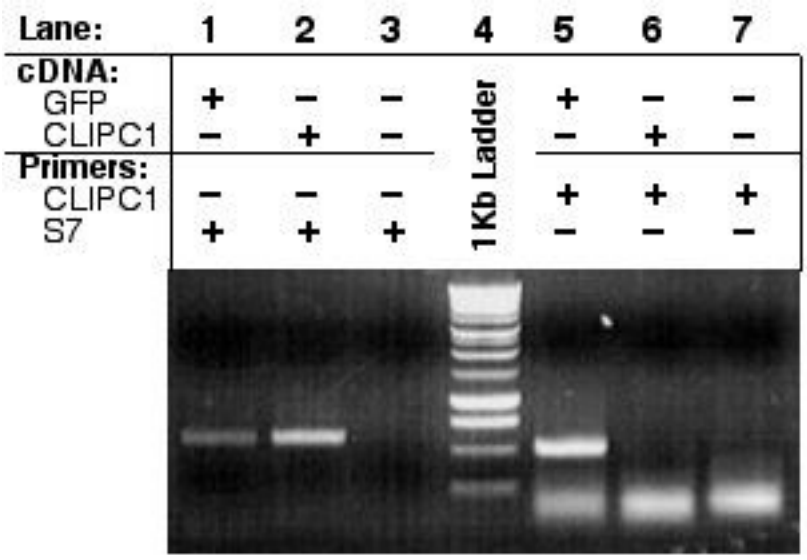
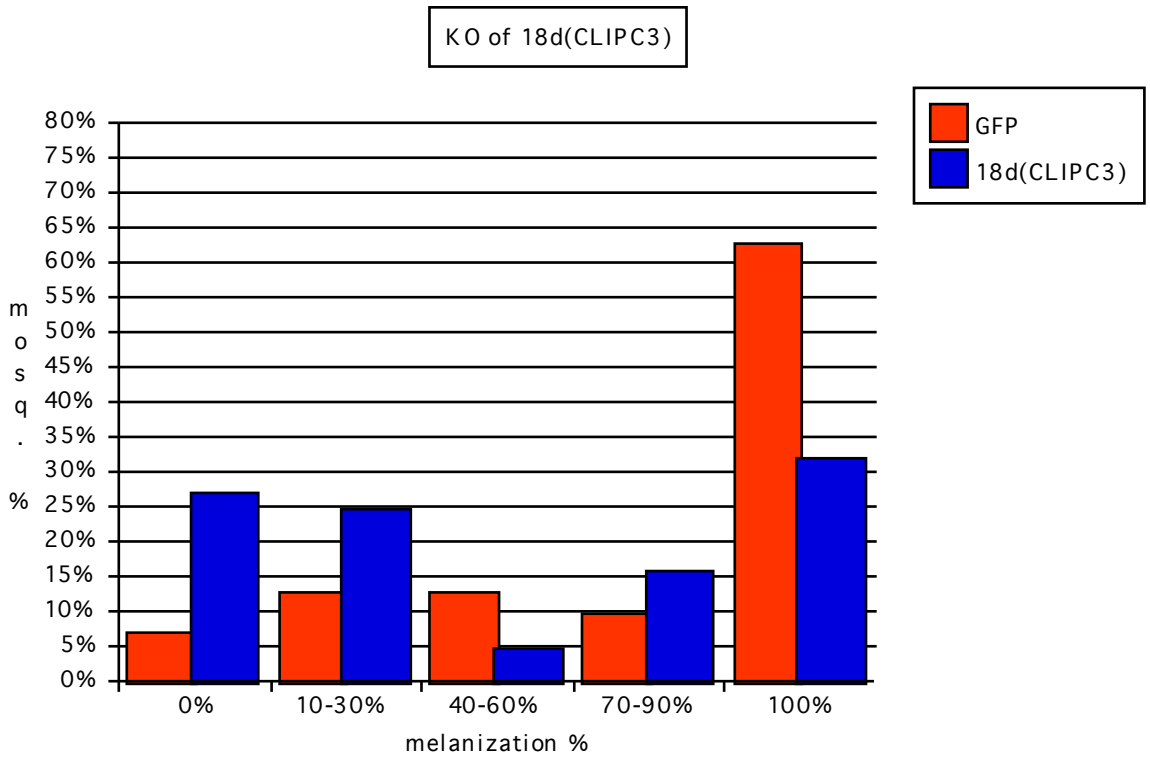


Figure 2: Gel Electrophoresis of verification of CLIPC1 Knockout (KO). To verify KO in mosquitoes, PCR following a reverse transcription reaction was performed. The symbol + indicates the cDNA or primer is present in the lane and – is not present. GFP=cDNA from GFP knockout mosquitoes; CLIPC1 = cDNA from CLIPC1 knockout mosquitoes. Lanes 3 and 7 are negative controls, thus no template DNA is present. The lower band of material in lanes 5 through 7 is a primer artifact. Note the absence of CLIPC1 product in lane 6 (CLIPC1 knockout) in comparison with the dsGFP knockout in lane 5.



	0%	10-30%	40-60%	70-90%	100%
GFP (86)	6% (5)	12% (10)	12% (10)	9% (8)	62% (53)
CLIPC3 (89)	25% (23)	24% (21)	4% (4)	15% (13)	31% (28)

Figure 3: Melanization results of Knockout (KO) of CLIPC3 versus GFP. In both the experiment and control groups, mosquitoes were grouped according to the percentage of the Sephadex bead surface covered by melanin (level 0%, 10-30%, 40-60% etc.) and then graphed as percentages of the total mosquitoes that melanized a bead to a given level. The data for the graph can be found in the table where numbers in parenthesis are the raw numbers before conversion to percentage and those numbers next to each level are the total number of mosquitoes/beads at that level. Note that one bead was injected into one mosquito, so these numbers can be used interchangeably. Percentages were rounded to the nearest whole number.