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CLIPB8 is part of the prophenoloxidase activation system in *Anopheles gambiae* mosquitoes

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ABSTRACT

In insects and other arthropods the formation of eumelanin (melanization) is a broad spectrum and potent immune response that is used to encapsulate and kill invading pathogens. This immune response is regulated by the activation of prophenoxidase (proPO), which is controlled by proteinase cascades and its serpin inhibitors, together forming the proPO activation system. While the molecular composition of these protease cascades are well understood in insect model systems, major knowledge gaps remain in mosquitoes. Recently, a regulatory unit of melanization in *Anopheles gambiae* was documented, comprised of the inhibitory serpin-clip-serine proteinase, CLIPB9 and its inhibitor serpin-2 (SRPN2). Partial reversion of SRPN2 phenotypes in melanotic tumor formation and adult survival by *SRPN2*/*CLIPB9* double knockdown suggested other target proteinases of SRPN2 in regulating melanization. Here we report that CLIPB8 supplements the SRPN2/CLIPB9 regulatory unit in controlling melanization in *An. gambiae*. As with *CLIPB9*, knockdown of *CLIPB8* partially reversed the pleiotropic phenotype induced by *SRPN2* silencing with regards to adult survival and melanotic tumor formation. Recombinant SRPN2 protein formed an SDS-stable protein complex with activated recombinant CLIPB8, however did not efficiently inhibit CLIPB8 activity *in vitro*. CLIPB8 did not directly activate proPO *in vitro* nor was it able to cleave and activate proCLIPB9. Nevertheless, epistasis analysis using RNAi placed CLIPB8 and CLIPB9 in the same pathway leading to melanization, suggesting that CLIPB8 either acts further upstream of CLIPB9 or is required for activation of a yet to be identified serine proteinase homolog. Taken together, this study identifies CLIPB8 as an additional player in proPO activation cascade and highlights the complexity of the proteinase network that regulates melanization in *An. gambiae*.

1. Introduction

Melanization, an arthropod-specific immune response, is used against a wide variety of pathogens (Cerenius and Soderhall, 2004; Cerenius et al., 2008). Indeed, insects deposit melanin on virtually all foreign surfaces naturally or experimentally introduced into their hemocoel, including nylon, epoxy, glass, and living tissues from other insects (Grimstone et al., 1967; Lackie, 1983; Salt, 1965, 1963). As all insects, the African malaria mosquito, *Anopheles gambiae* utilizes melanization as an anti-bacterial (Binggeli et al., 2014; Hillyer et al., 2003) and anti-fungal immune response (Yassine et al., 2012) that can also kill malaria parasites (Collins et al., 1986; Michel et al., 2005). Killing of pathogens through melanization is thought to occur through nutrient starvation (Chen and Chen, 1995) as well as direct toxic effects of reaction intermediates and byproducts (Nappi et al., 2009), although experimental support in *An. gambiae* is still lacking. Successful malaria transmission is therefore only ensured if *Plasmodium* parasites can escape the melanization response. Indeed, in transmission-permitting *Plasmodium* sp./mosquito species combinations, melanized parasites are rarely observed in the field (Cohuet et al., 2006; Lambrechts et al., 2007; Riehle et al., 2006; Schwartz and Koella, 2002), as *Plasmodium* sp. have evolved either active suppression (Boete et al., 2002) or avoidance mechanisms (Michel et al., 2006; Molina-Cruz et al., 2012).

Insect eumelanin is the product of oxidative polymerization of 5,6-dihydroxyindoles (DHIs). The substrate pathway that leads from tyrosine to DHI includes a number of enzymatic and nonenzymatic reactions, causing the initial production of the *o*-diphenols

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dopa and dopamine and their conversion to dopachrome and dopaminechrome, which form the precursors of DHI (Nappi and Christensen, 2005; Sugumaran, 2002). The key enzyme initiating melanization is phenoloxidase (PO), a monophenoloxygenase that catalyzes the initial hydroxylation of tyrosine to dopa as well as the oxidation of dopa and dopamine to their respective quinones (Nappi et al., 2009). POs are enzymes of about 80 kDa molecular weight that contain two binuclear copper binding sites (Hu et al., 2016). They are expressed as zymogens, called proPOs that require proteolytic cleavage at the N-terminus. While lepidopteran insect genomes encode only two, and *Drosophila melanogaster* encodes three proPOs, mosquito genomes encode between nine to ten proPOs, all with high sequence similarity (Bartholomay et al., 2010; Neafsey et al., 2015; Waterhouse et al., 2007), but it remains unclear if individual mosquito POs differ in their biological function. Several, but not all *An. gambiae* proPOs are upregulated by blood feeding, and expression of at least five proPOs can be altered by 20-hydroxy ecdysone (Ahmed et al., 1999; Muller et al., 1999), suggesting temporal and physiological specification of individual PO function.

The production of eumelanin generates a number of toxic byproducts including semiquinones and reactive oxygen species (Nappi and Christensen, 2005), which ultimately can be deleterious to the insect. Overstimulation of PO activity and ultimately melanization in *An. gambiae* causes a number of deleterious effects cumulating in shortened life span (An et al., 2011; Michel et al., 2005). It is therefore not surprising that melanization is tightly controlled, most prominently through regulation of the extracellular proteolytic cleavage of proPO to PO by the proPO activation cascade. Key enzymes in this cascade are clip-domain containing serine proteinases (CLIPs) that contain one or more amino-terminal clip domain that is separated by a linker region from a carboxyl-terminal S1A family serine proteinase domain (Smith and DeLotto, 1992). CLIPs are secreted into the hemolymph as zymogens, which require proteolytic activation by cleavage within the linker region. CLIPs are found in insects and other arthropods and can be subdivided into four evolutionarily distinct clades (A-D, Waterhouse et al., 2007; Kanost and Jiang, 2015), of which members of clade A have lost their proteolytic function. These proteinases are inhibited by serine proteinase inhibitors of the serpin family, the largest family of proteinase inhibitors in metazoans. Serpins act as suicide substrate inhibitors, forming covalent, stable inhibitory complexes with their target proteinases (Gettins, 2002), including CLIPs that are required for melanization in insects (Jiang and Kanost, 2000).

Our current understanding of the molecular make-up of the proPO activation system is derived from studies in a few model organisms such as *D. melanogaster* (Veillard et al., 2015), *Manduca sexta* (Kanost and Jiang, 2015), and *Tenebrio molitor* (Kan et al., 2008). A generalized view of the insect proPO activation system can be described as follows (Kanost and Jiang, 2015): The system consists of protease cascades that are triggered by the recognition of molecular patterns associated with pathogens or aberrant cells by soluble receptor molecules leading to the activation of a modular serine proteinase (MSP). MSP in turn activates a CLIPC that then activates the terminal CLIPB proteinase in this cascade, also called proPO activating proteinase (PAP) or proPO activating enzyme (PPAE). Active PAP then cleaves proPO to PO. In addition, the formation of the final active phenoloxidase complex on the foreign surface is mediated by one or more proteolytically inactive CLIPAs, which themselves require proteolytic activation in order to function. Furthermore, several protease cascades, characterized by specific PAPs and distinct upstream CLIPCs can act in parallel to regulate the melanization response in insects (An and Kanost, 2010). Under normal physiological conditions, proPO activation cascades are turned off, most prominently by a single highly conserved serpin (Park et al., 2000), referred to as Spn27A in *D. melanogaster* (De Gregorio et al., 2002; Ligoxygakis et al., 2002), Serpin-3 in *M. sexta* (Zhu et al., 2003), and SRPN2 in mosquitoes (Bartholomay et al., 2010; Christophides et al., 2002; Waterhouse et al., 2007; Zou et al., 2010). The only experimentally confirmed inhibitory target of these serpins in proPO activation cascades is the terminal PAP within each cascade (An et al., 2013, 2011; Zhu et al., 2003). The depletion of this serpin results in continuous activation of PO in the hemolymph, the formation of large melanotic pseudotumors, and increased mortality rates (Ligoxygakis et al., 2002; Michel et al., 2005; Zou et al., 2010).

In *An. gambiae*, CLIPB9 is currently the only known bona fide member of the proPO activation system. This proteinase functions as a PAP, directly cleaving and activating proPO *in vitro.* CLIPB9 and SRPN2 form a functional regulatory unit of a proPO activation system, as CLIPB9 is required for melanotic tumor formation in adult female *An. gambiae* that are depleted of SRPN2 and is inhibited directly by SRPN2 *in vitro* and *in vivo* (An et al., 2011). However, targeted reverse genetic screens have identified a number of additional CLIPBs required for melanization of foreign surfaces (Paskewitz et al., 2006; Volz et al., 2006). In addition, reversion of the SRPN2-depleted phenotype is incomplete after knockdown of *CLIPB9* suggesting additional proteinase cascades are required for complete activation of proPO in *An. gambiae*.

The current study was designed to test the specific hypothesis that CLIPB8 is part of the proPO activation system in *An. gambiae*. Previous studies indicate that CLIPB8 is required for ookinete melanization of the rodent malaria parasite, *Plasmodium berghei,* in *CTL4*kd *An. gambiae* mosquitoes (Volz et al., 2006), and also for melanization of Sephadex beads in *An. gambiae* hemolymph (Paskewitz et al., 2006). Phylogenetic analysis revealed that CLIPB8 is closely related to *M. sexta* PAP2 and 3 (An et al., 2010), suggesting a conserved role in mosquitoes. This is further substantiated by observations that the ortholog of *An. gambiae CLIPB8* in the Yellow fever mosquito, *Aedes aegypti,* binds SRPN2 *in vitro* and is required for melanotic tumor formation in this species (Zou et al., 2010). Using genetic and biochemical methodologies our laboratory established previously, we initially determined whether CLIPB8 is part of the proPO activation system that is controlled by SRPN2 in *An. gambiae*, and subsequently explored its position within this cascade, and in relation to its paralog, CLIPB9.

2. Results

2.1. CLIPB8 partially reverts the SRPN2-depletion phenotype

To test whether CLIPB8 is involved in *SRPN2*kd-mediated melanotic tumor formation, we performed double knockdown (dkd) analysis of *CLIPB8* and *SRPN2* in *An. gambiae* adult females using dsRNA injection. Kd efficiencies of *CLIPB8* and *SRPN2* were assessed at the transcript levels by RT-quantitative (q)PCR in each of the treatment groups (Fig. S1). *CLIPB8* transcript levels were reduced by 90–94% after treatment with *dsCLIPB8* or *dsCLIPB8*/*dsSRPN2* as compared to mosquitoes treated with only *dsGFP* or *dsSRPN2,* respectively. Transcript reduction of *SRPN2* was slightly lower at 71–73%, which is comparable to previously reported knockdown levels for this gene (An et al., 2011; Michel et al., 2005).

Kd of *CLIPB8* in a *SRPN2*-depleted genetic background decreased melanotic tumor formation caused by *SRPN2* depletion (Fig. 1A). Melanized area per abdomen of *dsSRPN2*/*dsCLIPB8*-treated female mosquitoes were significantly smaller than in SRPN2-depleted mosquitoes (Mann–Whitney U test, $P = 0.002$) (Fig. 1B). As observed previously, ds*GFP* or ds*CLIPB8* treatments alone did not result in melanotic tumors in adult mosquitoes.