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Two serine proteases from *Anopheles dirus* haemocytes exhibit changes in transcript abundance after infection of an incompatible rodent malaria parasite, *Plasmodium yoelii*

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Abstract

Serine proteases are involved in regulation of innate immune responses, such as haemolymph coagulation, melanization reaction and antimicrobial peptide synthesis. Although several serine proteases have been characterized in *Anopheles gambiae* (*A. gambiae*), few were cloned from other malaria vectors. In this study, we identified three cDNA fragments of serine proteases (AdSp1, AdSp2 and AdSp3) from haemocytes of an oriental malaria vector, *Anopheles dirus* (*A. dirus*), by cloning of fragments amplified with degenerate primers into the T-vector. RT-PCR analysis demonstrated that both AdSp1 and AdSp3 genes were also expressed in salivary gland. Basic local alignment search tool (BLAST) search found that both AdSp1 and AdSp3 were highly similar in sequence to *A. gambiae* Sp14A and Sp14D2, insects prophenoloxidase activating enzyme (PPAE) and *Drosophila* protease easter. Semi-quantitative RT-PCR indicated the transcription level of both AdSp1 and AdSp3 in haemocytes of *A. dirus* infected with *Plasmodium yoelii* (*P. yoelii*) was significant higher than that fed on 5% glucose or normal mouse blood at 7 days after the infectious meal ($p < 0.05$), when *P. yoelii* oocysts began to be melanized by *A. dirus*. Our results indicated that both AdSp1 and AdSp3 might play an important role during melanotic encapsulation of *P. yoelii* by *A. dirus*.

Keywords: *Plasmodium yoelii*; *Anopheles dirus*; Serine protease; Melanization

1. Introduction

Malaria is one of the most devastating infectious diseases caused by protozoan parasites of the genus *Plasmodium*. *Plasmodium* requires completion of their sporogonic cycle within the *Anopheles* mosquitoes to

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be transmitted to vertebrate hosts. During their development, however, *Plasmodium* will encounter robust innate immune responses of mosquitoes. Even in susceptible mosquito, *Plasmodium* experiences severe losses due to the induced expression of antimicrobacterial peptides (Lowenberger et al., 1999) and nitric oxide synthase (Luckhart et al., 1998). In genetically selected mosquito, *A. gambiae* L35, development of several species of *Plasmodium* can be completely blocked due to lysis in midgut epithelium (Vernick et al., 1995) and melanization of early oocysts (Collins et al., 1986).

Melanization, also called melanotic encapsulation, is characterized by isolation and killing of invaded microorganisms in a layer of melanin produced by cross-linking of host as well as microbial proteins. Melanization is regarded as the main phenotype of the refractory strain of *A. gambiae*, and three quantitative trait locis (Pen1, Pen2 and Pen3) were reported to be responsible for this phenotype (Zheng et al., 1997). Although the specific genes that are responsible for melanization of malaria parasites have not been identified, sequencing of the Pen1 genomic region has revealed clusters of sequence polymorphisms that may be related to the melanotic reaction of *A. gambiae* (Thomasova et al., 2002). Broad physiological differences between these refractory and other susceptible mosquitoes were related to the production and detoxification of reactive oxygen, and an elevated level of reactive oxygen species was also account for the *Plasmodium* melanotic encapsulation (Kumar et al., 2003). Recently, dsRNA knock-out of a complement-like protein TEP1 (thioeaster-containing protein) was reported to reduce the melanization rate of *Plasmodium* by the refractory *A. gambiae*, indicating a pivotal role of recognition step preceding melanization reaction (Blandin et al., 2004). The regulation of melanotic encapsulation, however, is still largely unknown.

Accumulating evidence demonstrates invertebrate defense responses, including haemolymph coagulation, antimicrobial peptide synthesis, and melanization of pathogen, were regulated by serine proteases. For example, horseshoe crab haemolymph coagulation (Iwanaga et al., 1998) was initiated by autoactivation of serine protease Factor C upon binding to LPS, and then followed by activation of other two serine proteases, Factor B and proclotting enzyme (PCE). The Toll pathway, responsible for the induced synthesis of antimicrobial peptides, also involves cleavage activation of spätzle by a cascade of serine proteases (Lemaitre et al., 1996). During melanotic encapsulation (Cerenius and Soderhall, 2004), prophenoloxiase activating enzyme (PPAE) converts prophenoloxidase (PPO) to active phenoloxidase (PO). Serine proteases inhibitors (serpins) negatively regulate all of the three defense responses. For example, *Limulus* intracellular clotting inhibitors (LICIs) inhibit horseshoe crab clotting pathway (Agarwala et al., 1996), mutations of *Spn43Ac* result in constitutive expression of drosomycin in *Drosophila melanogaster* (*D. melanogaster*) (Levashina et al., 1999), and serpin-3 through -6 regulate *Manduca sexta* (*M. sexta*) PPO activation (Tong and Kanost, 2005; Zou and Jiang, 2005).

So far, five serine proteases have been characterized in a major Africa malaria vector, *A. gambiae* (Gorman et al., 2000a; Paskewitz et al., 1999). Of those, Sp14A, Sp14D2 and Sp22D exhibited change in transcript abundance in response to bacteria injections. Sp14A and Sp14D2, as well as Sp14D1, were induced by infection with the malaria parasite, *Plasmodium berghei*. Phylogenetic analysis grouped Sp14A, Sp14D1 and Sp14D2 with three insect PPAEs and *D. melanogaster* easter. Interestingly, Sp14D1 was also identified as a refractory strain-specific serine protease (Chun et al., 2000), mapped together with one melanotic encapsulation phenotype-related quantitative trait loci *Pen3* (Zheng et al., 1997). Similarity to easter and PPAEs, together with immune inducibility has suggested that Sp14A, Sp14D1 or Sp14D2 might activate spätzle-like ligand or PPO in melanotic encapsulation of *Plasmodium*. Existence of melanization preventing factor, a putative serine protease inhibitor in haemolymph of susceptible mosquito, indicated that regulation of *Plasmodium* melanization was also through serpin (Paskewitz and Riehle, 1998).

Few serine proteases have been identified in other malaria vectors so far. *A. dirus* is a major human malaria vector in China and Southeast Asia, which is naturally refractory to *P. yoelii* by melanization of most oocysts on the midgut. Here, we reported the cloning of three serine proteases from *A. dirus* haemocytes and expression profiles of their transcript