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Legumains from the hard tick *Haemaphysalis longicornis* play modulatory roles in blood feeding and gut cellular remodelling and impact on embryogenesis

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ABSTRACT

The biology and vectorial capacity of haematophagous ticks are directly related to effective blood feeding and digestion. The midgut-associated proteases in ticks are involved in the blood (Hb) digestion cascade, the molecular mechanisms of which are yet poorly understood. Our previous studies indicated that *Haemaphysalis longicornis* midgut-specific asparaginyl endopeptidases/legumains, HlLgm and HlLgm2, act in the Hb digestion cascade. Here, we investigated the potential of these enzymes in blood feeding and digestion, midgut remodelling and reproduction of ticks by employing RNA interference (RNAi) techniques. Injection of *HlLgm*- and *HlLgm2* gene-specific double-stranded RNAs into unfed adult female *H. longicornis* caused gene-specific transcriptional and translational disruptions. RNAi impacted on tick blood feeding leading to death of the feeding ticks, failure of ticks to reach repletion and significant reductions in engorged tick body weight. Histological examination revealed that deletion of legumains resulted in damage to the midgut tissues and disruption of normal cellular remodelling during feeding. Gene knock-down also caused significantly delayed onset of oviposition, reduced number of eggs and, most strikingly, structurally deformed eggs that failed to hatch suggesting imperfect embryogenesis. Synergistic impacts of RNAi were reflected on all parameters evaluated when *HlLgm* and *HlLgm2* were silenced together. These findings suggest that legumains may play modulatory roles in blood feeding and digestion, midgut cellular remodelling and embryogenesis in *H. longicornis*. Deletion of legumains in *H. longicornis* would help in controlling the tick population and thereby transmission of diseases to their hosts.

1. Introduction

The life cycles of the three-host non-nidicolous tick species are characterised by feeding for a few days to a few weeks on a host and a long period of diapause in habitats where either they moult into the next stage or the adult females lay eggs and die. The newly moulted stage or the newly hatched larva repeats the process of blood feeding on a different host (Ostfeld et al., 2006). This unique feature of the life cycle history of the non-nidicolous ticks provides an opportunity for many bacterial, viral, protozoal and rickettsial species to use ticks as effective vehicles for dispersal from one vertebrate host to another (Soulsby, 1986; Grubhoffer et al., 2005; Klompen, 2005).

Host blood feeding and digestion of blood (Hb) provides nutrition and energy for the moulting, development and vitellogenesis of ticks (Grandjean, 1984). Also, in ticks, the microbial pathogens generally do not reproduce and typically do not cause obvious disease until the ticks begin feeding on a host (Klompen, 2005; Ostfeld et al., 2006). The ingested blood meal supplies the energy reserves, especially proteins, required for yolk formation on a massive scale, and more than 50–60% of the engorged female body weight is converted into eggs (Sonenshine et al., 2002; Hatta et al., 2007). The high fecundity and the unique capacity of ticks for transmitting various viruses, bacteria, rickettsiae and protozoa to their progeny by way of transovarial transmission (Klompen, 2005; Oliveira et al., 2005) make ticks highly successful as disease vectors. *Haemaphysalis longicornis* is a common, prevalent three-host tick in East Asia and Australia (Hoogstraal et al., 1968; Fujisaki et al., 1994). *H. longicornis* sucks a considerable amount of blood and causes damage to the host skin. It transmits many bacterial, viral, protozoal and rickettsial diseases of humans and animals (Hoogstraal et al., 1968). In particular, *H. longicornis* is the primary vector for *Babesia* sp. parasites in Japan (Tsuji et al., 2007) and also serves as a major vector of *Coxiella burnetti* in cattle, dogs and humans (Ho et al., 1995). To combat ticks and tick-borne diseases, a variety of control measures, including chemical and biological strategies have been attempted worldwide. However, to avoid

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toxicity to humans and animals and to avoid wide-spread multi-acaricide resistance development (Zaim and Guillet, 2002; Bianchi et al., 2003), vaccination or the knock-down of the vital tick genes seems to be the most promising alternative to chemical pesticide application for controlling tick and tick-borne diseases. Recently, some bioactive molecules of vaccine and drug-target importance have been identified and characterised in the ixodid ticks *H. longicornis* (Miyoshi et al., 2004; Boldbaatar et al., 2006; Hatta et al., 2006) and *Boophilus microplus* (Mendiola et al., 1996; Renard et al., 2000). Previously, we cloned and partially characterised two cDNAs from the midgut of adult *H. longicornis* that encode the asparaginyl endopeptidases/legumains, HlLgm (Alim et al., 2007) and HlLgm2 (Alim et al., 2008). Endogenous legumains were localised in the midgut and the recombinant proteins efficiently digested the blood proteins, Hb and bovine serum albumin (BSA) Alim et al., 2007, 2008). Legumain from *Ixodes ricinus* (IrAE) has been reported to digest Hb and to *trans*-process and activate the cathepsin B1 zymogen, an enzyme in the Hb digestion cascade in the gut of the blood fluke, *Schistosoma mansoni* (Sojka et al., 2007).

Here, we investigated the biological roles of *H. longicornis* legumain genes (*HlLgm* and *HlLgm2*) on tick blood feeding, gut remodelling and reproduction by using the nucleic acid-based reverse genetic approach, RNA interference (RNAi). In recent years, RNAi has successfully been employed to investigate and identify the role of proteins hypothesised to be involved in blood feeding by different ticks (Aljamali et al., 2003; Narasimhan et al., 2004; Pal et al., 2004; Karim et al., 2005; Ramamoorthi et al., 2005; Hatta et al., 2007; Huang et al., 2007) and also for screening tick protective antigens (de la Fuente et al., 2005, 2006; Nijhof et al., 2007)and *HlLgm2* genes by RNAi treatment resulted in reduced tick survival, blood feeding, disruption of midgut cellular development and differentiation, reduced oviposition and strikingly, impaired embryogenesis suggesting their critical roles in development and reproduction of ixodid ticks.

2. Materials and methods

2.1. Ticks and experimental animals

The parthenogenetic Okayama strain of *H. longicornis* has been maintained in the Laboratory of Parasitic Diseases, National Institute of Animal Health (NIAH), Tsukuba, Ibaraki, Japan by feeding on rabbits as described previously (Alim et al., 2007). The infested rabbits were checked daily and the ticks were collected when engorged or after the indicated period of attachment. The animals were adapted to the experimental conditions for 2 weeks prior to the experiment and were treated in accordance with the protocols approved by the Animal Care and Use Committee, NIAH (Approval nos. 441, 508, 578).

2.2. In vitro transcription of HlLgm and HlLgm2 gene double-stranded RNA

The open reading frames (ORF) of *HlLgm* and *HlLgm2* genes inserted into *Escherichia coli* expressed pTrcHisB/*HlLgm* and pTrcHisB/*HlLgm2* plasmids (Alim et al., 2007, 2008) were used as templates and cloned into pBluescript II SK+ plasmid (Toyobo, Osaka, Japan). In parallel, PBS and double-stranded RNA (dsRNA) complementary to the non-functional portion of the *E. coli* malE gene (*malE*) for ticks that encodes the maltose-binding protein was used as a negative control (Cheon et al., 2006). mRNA from *E. coli* (BL21 strain, Invitrogen, Carlsbard, CA, USA) was isolated using an mRNA isolation kit (QIAGEN Sciences, Germantown, MA, USA) and following the manufacturer's protocol. The mRNA template was employed to prepare single-stranded cDNA by reverse transcriptase (RT)-PCR using a Takara RNA PCR Kit (AMV) Ver.3.0 (Takara, Shiga, Japan) and following the protocol provided by the manufacturer. The cDNA of *malE* was cloned into pBlueskript II SK+ plasmid using the oligonucleotides 5′-CCGCTCGAGCGGTTAT GAAAATAAAAACAGGTGCA-3′ and 5′-GAATTCGCTTGTCCTGGAACG CTTTGTC-3′ as forward and reverse primers, respectively. The inserted sequences for HlLgm, HlLgm2 and malE were amplified by PCR using the oligonucleotide T7 (5′-GTAATACGACTCACTA TAGGGC-3′) and CMo422 (5′-GCGTAATACGACTCACTATAGGGAA CAAAAGCTGGAGCT-3′) as primers to attach T7 promoter recognition sites on both the 5′ and 3′ ends (T7-HlLgm-T7, T7-HlLgm2-T7 and T7-malE-T7). The PCR products were purified by agarose gel electrophoresis using the QIAquick® Gel Extraction kit (QIAGEN Sciences, Germantown, MA, USA) and following the manufacturer's protocol. Using approximately 2 μg T7-HlLgm-T7, T7-HlLgm2-T7 and T7-malE-T7 as templates, 50–100 μg dsRNA complementary to the sequences encoding ORFs of *HlLgm*, *HlLgm2* and *malE*, respectively, was synthesised by an in vitro transcription method using T7 RNA polymerase (Ribomax™ Express Large Scale RNA Production System, Promega, Madison, WI, USA) in accordance with the manufacturer's instructions. The purity of the synthesised dsRNAs was checked by 1% agarose gel electrophoresis and their concentration was determined by spectrophotometer (GE Healthcare Biosciences KK, NJ, USA).

2.3. Injection of ticks with dsRNA and tick feeding

The ticks were microinjected with dsRNA as described previously (Tsuji et al., 2007). Briefly, 1 μg of *HlLgm* dsRNA and 1 μg of *HlLgm2* dsRNA either separately or in combination in 0.5 μl of PBS was injected into the haemocoel through the fourth coxa in 10–14 day post-moult adult unfed female ticks fixed on a glass slide with adhesive tape. The control ticks were injected with 0.5 μl of PBS alone or 0.5 μl of PBS containing 1 μg dsRNA of *malE*. We used 75, 110, 73, 65 and 115 ticks which were injected with PBS alone, *malE* dsRNA, *HlLgm* dsRNA, *HlLgm2* dsRNA and with a combination of *HlLgm* and *HlLgm2* dsRNA, respectively. Following treatment, the ticks were allowed to rest for 18–24 h at 25 °C and then placed on the ears of rabbits for attachment. The ticks that dropped on repletion were picked up and those that did not engorge on day 7 post-infestation were removed forcibly using forceps.

2.4. Analysis to confirm gene silencing by reverse transcriptase (RT)-PCR and quantitative RT-PCR

The midguts from three randomly collected ticks from control and RNAi groups during feeding (24 h, 48 h, 72 h and 96 h), repletion, pre-oviposition (4 days post-engorgement (PE)) and oviposition period (10 days PE) were dissected and used for mRNA detection by RT-PCR and quantitative RT-PCR as described previously (Alim et al., 2008). Immediately after collection, the midgut tissues were stored in RNA*later* RNA Stabilisation Reagent (QIAGEN). The total RNA from these samples was extracted by using an RNeasy Mini Kit (QIAGEN) in accordance with the manufacturer's protocol and either used immediately or stored at −80 °C until used. RT-PCR was carried out with a template of 500 ng of total RNA for each 10-μl reaction using a Takara RNA PCR Kit (AMV) Ver.3.0 (Takara, Shiga, Japan) and following the manufacturer's instructions. PCRs were performed using 500 ng of each cDNA synthesis reaction and oligonucleotides specific for either *HlLgm* (forward primer 5′-CGACGAGCAAATCGTAGTCA-3′ and reverse primer 5′-ACTTTTCCGCTTCCTCCATT-3′) or *HlLgm2* (forward primer 5′-CCTTCGCAACAAGCTAAAGG-3′ and reverse primer 5′-TCAGAA GTCCTTCGGTGCTT-3′) or primers specific for positive control cDNA encoding β-actin in a final volume of 20 μl. PCR was performed for