Identification and Characterization of Bioactive Molecules from the Tick *Haemaphysalis longicornis*

2009

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Contents

Abbreviations

A APE: apurinic/apyrimidinic endonuclease

APTT: activated partial thromboplastin assay

B BLAST: basic local alignment search tool

C CD: circular dichroism

cDNA: complementary DNA

COPII: coatomer protein complex-II

CPA: carboxypeptidases A

CPB: carboxypeptidases B

CPs: carboxypeptidases

D dsRNA: double-stranded RNA

E ECF: East Coast fever

eEF: elongation factor

EGFR: epidermal growth factor receptor

ELISA: enzyme-linked immunosorbent assay

ER: endoplasmic reticulum

G GFP: green fluorescent protein

GST: glutathione *S*-transferase

H HlP0: ribosomal P0 protein from *Heamaphysalis longicornis*

HlTCI: tick carboxypeptidase inhibitor from the *Heamaphysalis longicornis*

I

IgG: immunoglobulin G

IPTG: isopropyl β-D-1-thiogalactopyranoside

L

LCI: leech carboxypeptidase inhibitor

LD: longin domain

M

MCPI: tomato metallocarboxypeptidase inhibitor

mRNA: messenger RNA

MYA: million years ago

N

NMR: nuclear magnetic resonance

O

ORF: open reading frame

P

PBS: phosphate-buffered saline

PCI: potato carboxypeptidase inhibitor

PCR: polymerase chain reation

PDI: protein disulfide isomerase

PGE: prostaglandin E

PPIase: peptidyl-prolyl isomerase

PVDF: polyvinylidene difluoride membranes

R

RER: rough endoplasmic reticulum

RMSD: root mean square deviation

RNAi: RNA interference

RPs: ribosomal proteins

RT-PCR: reverse transcription-PCR

S

SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

SNARE: soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors

SPF: specific pathogen-free

T TAFI: thrombin-activatable fibrinolysis inhibitor

TCI: tick carboxypeptidase inhibitor

TM: thrombomodulin

tPA: tissue-type plasminogen activator

TrX: thioredoxin

U UTR: untranslated region

V VAMP2: synaptobrevin

Vg: vitellogenin

General introduction

1. Biology of ticks

Tick is a kind of ectoparasite that ranks the second to the mosquito as a vector to transmit pathogens to animals and humans worldwide. They are considered to be of the earlier lineages of terrestrial arachnids and the earliest organisms to evolve blood-feeding capabilities (Mans and Neitz, 2004) with proposed origins in the late Silurian (443–417 MYA) (Lindquist, 1984), Devonian (417–362 MYA) (Oliver, 1989), late Permian (290–248 MYA) (Hoogstraal and Aeschlimann, 1982), Triassic (248–206 MYA) (Hoogstraal, 1985; Balashov, 1989, 1994) and Cretaceous (90-94MYA) (de La Fuente, 2003). The superfamily Ixodoidea includes two major families, hard ticks and soft ticks, and a minor family of Nuttalliellidae. Among the total 867 currently known ticks species, approximately 80% (683 species) were ixodid ticks (hard ticks) and with exception of one species in the family Nuttalliellidae, the remainders are argasid ticks (183 ticks) (Horak et al, 2002). However, a total of 899 species of ticks worldwide has been recorded in another report (Barker and Murrell, 2004). The most important genera of hard ticks are *Amblyomma*, *Dermacentor*, *Haemaphysalis*, *Hyalomma*, *Ixodes* and *Rhipicephalus*. The genus *Haemaphysalis* contains 168 species. The tick *Haemaphysalis longicornis* as well as other *Haemaphysalis* species, is eyeless, three-host ticks, with 4 developmental stages, an embryonated egg and the three active stages, a larva, a nymph and an adult (Tenquist, 1977).

Ticks are a greatly successful family based on their wide geographical distribution, adaptation to the climatic extremes (i.e. feeding on penguins in Antarctica and lizards in the tropics), and the diversity of hosts on which they feed (mammals, birds, and reptiles) (Wang and Nuttall, 1999). *H. longicornis* mainly exists in northeastern areas of Russia and China, the Korean peninsular, Japan, Australia, New Zealand, New Caldonia, and Fuji Islands (Hoogstraal et al., 1968). In Japan, *H. longicornis* is one of the most important ectoparasites in pasturing cattle, human, and wild animals (Hoogstraal et al., 1968; Yamaguti et al., 1971).

Ticks not only cause tick paralysis, irritation, tick bite allergies, various tick toxicoses and immune responses through biting the hosts, but also transmit a variety of pathogens including protozoa, rikettsiae, viruses, bacteria, and even fungi to human, livestock and wild animals (Sonenshine, 1991; Makala et al., 2003; Uilenberg, 2006). Furthermore, 15 previously unrecognized bacterial pathogens have been described since 1982 (Parola and Raoult, 2001). *H. longicornis* is the vector to transmit *Theileria*, *Babesia*, rickettsia that causes Q-fever and virus that leads to the encephalitis (Hoogstraal et al., 1968; Higuchi, 1986).

2. Economic significance

It is estimated that 80% of the world's cattle are infested with ticks. From this aspect, ticks are the most economically important ectoparasite for global livestock production. In southern Africa, Taylor and Plumb (1981), using fully tick-susceptible cattle, demonstrated a large difference (48 kg) of group mean-weight gain between

heavily tick-infested and tick-free animals. de Castro (1987) also demonstrated that high tick numbers caused proportionally greater live-weight losses in tick-susceptible Boran cattle (*Bos indicus*) than in tick-resistant animals of the same breed. For each fully engorged brown ear tick (*R. appendiculatus*), a loss of 4.4 g in live-mass of a cattle gain is recorded (Norval et al., 1988). *R.* (*Boophilus*) spp. in Australia resulted in losses of about 0.6–1.5 g, and *A. hebraeum* in Zimbabwe about 10 g per tick. Moreover, ticks *per se* are not a major problem, but the pathogens they transmit are the real threat. Field trials in Kenya using Boran heifers immunized against East Coast fever (ECF, tick-borne disease) showed that animals dipped weekly gained an average of 78 g per day more than undipped animals over a 30-week period (de Castro et al., 1985). McCosker (1979) estimated global costs of control and productivity losses to be some US$7000 million annually (=US$7/head/year). In Australia, losses caused by the blue tick *R.* (*Boophilus*) *microplus* are estimated to be 100 million Australian dollars per year (Lee and Opdebeeck, 1999). It is clear that ticks, and the diseases that they transmit, have been a major constraint to the improvement of livestock industries, particularly in the developing countries for the past 100 years (Peter et al., 2005).

3. Tick control measures and shortcomings

For the sake of reducing the economic losses caused by ticks, chemical acaricides are overwhelmingly used today. However, the use of acaricides is leading to a limited efficacy because of the resistance of ticks. The environmental contamination and contamination of milk and meat products with drug residues are

also harmful drawbacks of this measure. Moreover, the development of new drugs to control ticks is a long-term and expensive process. As an alternative method, to control ticks with the natural enemies of ticks (i.e. bacteria, fungi, nematodes and wasps) appears to be feasible (Samish et al., 2004). It has been shown that the use of entomopathogenic fungi, such as *Beauveria bassiana* and *Metarhizium anisopliae*, may have the potential for controlling populations of certain tick species (Kirkland et al., 2004; Samish et al., 2004), for example, they were demonstrated to be harmful to fed nymphs and to unfed adults of *R. sanguine*us (Reis et al., 2005). Nevertheless, it is a big challenge to devise effective strategies to bring the appropriate entomopathogenic strains to practical use (Samish et al., 2004).

Using anti-tick vaccine is a promising measure since the use of Bm86-based vaccines led to the 55 - 100% efficacy in the control of *B. microplus* infestations in grazing cattle for up to 36 weeks in controlled field trials in Cuba, Brazil, Argentina and Mexico (de la Fuente et al., 1998, 1999). However, there is no effective vaccine for some other tick species (i.e. *R. appendiculatus* and *A. variegatum*; de Vos et al., 2001) on cattle, yet available vaccines for dogs or other hosts of ticks. Inspiringly, recent advances in molecular biology, protein chemistry and computational biology have accelerated the isolation, sequencing and analysis of biological proteins from the ticks. The identification of the biological activities of proteins encoded by newly isolated genes from the tick may provide great help to discover potential targets for vaccines against ticks and the tick-borne diseases (Valenzuela, 2004).

4. Molecules identified from ticks

In the last decade, the studies about the identification of biological molecules from ticks have been reported in rapid succession like bamboo shoots after a spring rain, including the molecules responsible for the ingestion, digestion, anticoagulation, immunomodulation, the oocyte formation, and the apoptosis (Mans and Neitz, 2004; Hovius et al., 2008; Scopinho et al., 2008). Besides of these molecules, researchers have endeavored to explore proteins from ticks that are associated with the protein synthesis, modification and transport in the cells (Karim et al., 2004a, b; Liao et al., 2007; Boldbaatar et al., 2008b).

In details, the proteins identified from ticks include enzymes, i.e. acetylcholinesterase (Baxter and Barker, 1998), acid phosphatase (Gough and Kemp, 1995), alpha-amylase (Mohamed, 2000), apyrase (Mans et al., 1998), aspartic proteinases (Boldbaatar et al., 2006), aspartate aminotransferase (Mohamed, 2001), beta-N-acetylhexosaminidase (del Pino et al., 1999), calreticulin protein (Ferreira et al., 2002), cAMP-dependent protein kinase (Tabish et al., 2006), cathepsin L-like proteinase (Renard et al., 2000), chitinase (You et al., 2003), cubilin-related serine proteinase (Miyoshi et al., 2004a), esterase and lipase (Fahmy et al., 2004), glutathione peroxidase (Cossio-Bayugar et al., 2005), glutathione *S*-transferase (Rosa de Lima et al., 2002), hyaluronidase (Mohamed, 2005), immune-responsive lysozymes (Simser et al., 2004), isocitrate lyase (Kamel and Fahmy, 1982), metalloprotease (Decrem et al., 2008), monoamine oxidase (Kaufman and Sloley, 1996), Na, K-ATPase (Rutti et al., 1980), nitric oxide synthase (Bhattacharya et al.,

2000), phenol oxidase (Zhioua et al., 1997), phosphodiesterase (McMullen and Sauer, 1978), phospholipase A2 (Zhu et al., 1998), serine proteinases (Miyoshi et al., 2008), prostaglandin H synthase (Pedibhotla et al., 1995), stearoyl CoA desaturase (Luo et al., 1997), and thiol-activated metalloendopeptidase (Bastiani et al., 2002).

Inhibitors were also widely found in ticks, i.e. thrombin inhibitor (Lai et al., 2004), Complement inhibitor of C5 (Nunn et al., 2005), factor Xa inhibitor (Rezaie, 2004), platelet aggregation inhibitor (Keller et al., 1993), peroxiredoxin (Tsuji et al., 2001), plasma kallikrein-kinin system inhibitor (Kato et al., 2005a), RGD-containing antagonist of glycoprotein IIb-IIIa and platelet aggregation inhibitor (Variabilin) (Wang et al., 1996), serine protease inhibitor (serpin) (Prevot et al., 2006), tissue factor pathway inhibitor (TFPI) (Francischetti et al., 2002), and trypsin inhibitors (Sant'Anna Azzolini et al., 2003; Hovius et al., 2008).

The identified immunomodulant proteins comprise antioxidant (Salp25D) (Das et al., 2001), B-cell inhibitory protein factor (Hannier et al., 2004), Complement inhibitor of C5 (Nunn et al., 2005), Histamine-binding protein (Paesen et al., 1999), IgG-binding proteins (Wang and Nuttall, 1995), IL-2 binding protein (Gillespie et al., 2001), *I. rinicus* immunosupressor (Iris) (Leboulle et al., 2002), *I. scapularis* salivary anticomplement protein (Isac) (Valenzuela et al., 2000; Titus et al., 2006).

In addition, some antibacterial peptides (cysteine-rich antimicrobial peptides, Ixodidin, defensin-like protein) (Fogaca et al., 2004, 2006; Zhou et al., 2007), anti-inflammation protein (chemokine-binding protein) (Deruaz et al., 2008), some receptors (ergot alkaloid receptor, octopamine receptor, prostaglandin E2 receptor,

vitellogenin receptor) (Qian et al., 1997; Baxter and Barker, 1999; Kaufman and Minion, 2006; Boldbaatar et al., 2008a), membrane proteins (synaptobrevin, Bm86, Bm95) (de Vos et al., 2001; Karim et al., 2004b; Boue et al., 2004) and other molecules (insect kinins, testis-associated protein) (Taneja-Bageshwar et al., 2006; Yamada et al., 2008) were isolated from the ticks.

5. RNA interference

RNA interference (RNAi), the sequence-specific degradation of mRNA mediated by homologous dsRNA, has become a valuable tool for determining the biological role of genes. To date, RNAi of genes has been performed in several kinds of arthropods, including mosquito (Attardo et al., 2003; Boisson et al., 2006), fruit flies (Boutros et al., 2004), silkworm (Ohnishi et al., 2006), and ticks (Zhou et al., 2006; Hatta et al., 2007).

6. Aims of the present study

To obtain a great amount of blood from the host, ticks have evolved a variety of proteins to counterwork with the coagulantion and immune system of host. However, it comes to the questions that what is responsible for the synthesis of these proteins and what is the scene upstream of the protein pool when tick is feeding on the host. Base on this consideration, two proteins caught my attention because of their multifunctions and vital roles in the synthesis and secretion of proteins, the ribosomal P0 protein, associating with protein synthesis, and a SNARE molecule (Ykt6),

attending the protein transport. The significance of these two molecules will be exhaustively described in the following chapters, respectively. In order to describe the significance of the genes for the blood-feeding and survival of the ticks, I tried RNA interference of the two genes mentioned above. In addition, a tick carboxypeptidase inhibitor (TCI) from another tick species *R. burse*, showed inhibitory activity to carboxypeptidases involved in the digestion, and carboxypeptidase called CPU or thrombin-activable fibrinolysis inhibitor (TAFI), and hence significantly accelerates the clot lysis (Arolas et al., 2005a). Is TCI involved in the ingestion and digestion of the tick? Keep this in mind, I performed an investigation on the distribution and function of TCI in *H. longicornis*. The whole study encircling the three molecules provided a novel sight along the protein secretion process in ticks and the crucial information for the development of anti-tick vaccines.

Chapter 1

Gene silencing of ribosomal protein P0 is lethal to the tick *Haemaphysalis longicornis*

1-1. Introduction

Ticks are hematophagous arthropods that infest many host species. Once they attach to the host, their blood sucking can cause irritation and infection of the skin, anemia, and paralysis (Vedanarayanan et al., 2004). More importantly, they transmit a variety of pathogens, which has been a major constraint to the improvement of the livestock industries. Moreover, 14 tick-borne diseases have been reported in international travelers, and the incidence of travel-associated tick-borne diseases is increasing as more people travel (Jensenius et al., 2006). To control ticks and tick-borne diseases, the frequent use of chemicals is not sustainable for environmental, medical, and economic reasons. Vaccination is, thus, considered to be a promising alternative measure to control ticks. With respect to tick vaccine development, the molecular and biochemical mechanisms of tick activities, i.e., feeding, oviposition, and molting, need to be characterized. To date, a large array of studies has been carried out on the function of bioactive molecules from different tissues of ticks (Willadsen, 2004). Simultaneously, a series of proteins responsible for the secretion of proteins has been characterized (Karim et al., 2004a, b, 2005). Nevertheless, proteins associated with protein synthesis in ticks remain unknown. It has been reported that

the overall mass and protein in the salivary glands increased about 25-fold during tick feeding in female *A. americanum* (Shipley et al., 1993). New gene expression was observed in the salivary glands of female ticks after attachment, mating, and feeding (Oaks et al., 1991). This change in the protein expression level is directly related to the function of ribosomes.

As an important component of the translation of protein, the ribosome is universal to all organisms. Ribosomes in mammals consist of four RNA species and a vast number of ribosomal proteins (RPs). Among the RPs in eukaryotic cells, acidic phosphoproteins P0, P1, and P2 form a complex, presumably as the pentameric form $(P1)_2$ /$(P2)_2$ /P0 (Shimizu et al., 2002). P0 protein herein is a multifunctional protein. It contributes RNA-binding domain to the binding of $(P1)_2$ /$(P2)_2$ /P0 complex to 28S rRNA (Uchiumi and Kominami, 1992), and is associated with the interaction of the complex with the elongation factor, eEF2 (Justice et al., 1999). Moreover, the dephosphorylated P0 detaches from the ribosome and is transported to the nucleus, where it showed apurinic/apyrimidinic endonuclease (APE) activities that are involved in DNA repair (Sanchez-Madrid et al., 1981; Yacoub et al., 1996). In order to describe the function and significance of ribosomal protein P0, the disruption of protein P0 was previously performed using P-element insertion (Frolov and Birchler, 1998) and C-terminal truncation (Griaznova and Traut, 2000; Hagiya et al., 2005). In addition to the above methods, interference with the gene by double-stranded RNA (dsRNA) transfection has been employed in the study of P0 in mosquito cells (Jayachandran and Fallon, 2003).

In this chapter, I isolated a ribosomal protein P0 from the adult female tick *Haemaphysalis longicornis* and investigated its transcription profiles in different developmental stages and tissues. Moreover, through RNAi of ribosomal protein P0 gene, I determined that normal expression of P0 is essential for the feeding and survival of ticks. The down regulation of P0 led to the failure of blood sucking and subsequent death of ticks.

1-2. Materials and methods

Ticks. The Okayama strain of hard tick *H. longicornis* (Fujisaki, 1978) was infested on the ears of Japanese white rabbits (SPF, Japan Laboratory Animals, Japan). To obtain desired tissues from partially fed ticks, unfed adult ticks were allowed to feed for 4 days on rabbits and then manually detached. The recovered ticks were immediately subjected to dissection under a microscope, and tissues were collected in a cold PBS buffer. The collected tissues were stored at -80℃ until use.

Sequencing and analysis of the gene. A full-length salivary gland cDNA library was constructed using the vector-capping method as described previously (Kato et al., 2005b), and a total of 10,000 recombinant transformants from the library were randomly selected and partially sequenced to form the database of expressed sequence tags (Harnnoi et al., 2007). From the database, 2 sequences containing a ribosomal P0 encoding insert were selected and fully sequenced using an automated sequencer (ABI prism 310 Genetic Analyzer, Applied Biosystems, USA) (Boldbaatar et al., 2006) by three primers, T7 forward, P0 gene-specific (HlP0-con, Table 1), and

T3 reverse primers. For sequencing, BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA) was employed and the PCR reaction conditions for sequencing were: 96℃ 2 min followed by 25 cycles of 96℃ for 10 s, 50℃ for 5 s and 60℃ for 4 min. The obtained full-length cDNA sequence of ribosomal P0 was then analyzed using a basic local alignment search tool (BLAST) (NCBI: http://www.ncbi.nlm.nih.gov).

In vitro **expression of recombinant HlP0 and preparation of the anti-rHlP0/TrX serum.** The HlP0 gene was PCR-amplified using primers HlP0-E-F and HlP0-E-R (Table 1), and non-directionally cloned into the *Bam*HI site of the expression vectors pET32a and pGEX-4T-3 (Amersham Pharmacia Biotech, USA) and used to transform *E. coli* DH5α strain. The resultant clones, designated HlP0/GST and HlP0/TrX, respectively, were sequenced and used to transform *E. coli* BL21 strain for protein expression. Fusion proteins were expressed according to the description of McBride et al. (2000). After identification of the positive clones, recombinant HlP0/GST (rHlP0/GST) protein was solubilized in 2% (W/V) *N*-Lauroylsarcosine sodium (Sigma, USA) and purified with glutathione sepharose 4B according to the manufacturer's instructions (Pharmacia Biotech, USA). Recombinant HlP0/TrX (rHlP0/TrX) protein was expressed in the form of an inclusion body, which was primarily purified by sonication for immunization, as described elsewhere (Kukkonen et al., 2004). Briefly, inclusion bodies of rHlP0/TrX were completely mixed with an equal volume of Freund's complete adjuvant (Sigma, USA) and intraperitoneally injected into mice (ddY, 8 weeks old; SCL, Japan). The mice were immunized twice

again with the same dose of recombinant protein in the incomplete adjuvant at days 14 and 28. Eight days after the third injection, the titer of the antibodies was evaluated using ELISA, employing rHlP0/GST as an antigen (5 μg/ml, 50 μl/well). Subsequently, all the sera were collected from the blood of the mice.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Salivary glands, midgut, cuticle, ovary, synganglion, and fat body isolated from 4-day-fed adult ticks were sonicated and Western blotting was performed as follows. The same amount of protein lysates (about 1,000 ng/lane) were mixed with 2 x concentrated sample buffer (containing 5% 2-mercaptoethanol) and boiled at 100℃ for 5 min. All samples were electrophoresed on 15% polyacrylamide gel and then transferred to Immobilon P^{SQ} polyvinylidene difluoride membrane (PVDF membrane; Millipore, USA). The successful transfer of the proteins to the membrane was confirmed using SeeBlue Pre-Stained Protein marker plus 2 (Invitrogen, USA). The membrane was then incubated with the serum (1:300) collected as described above. The peroxidase-conjugated goat anti-mouse immunoglobulin G (IgG, 1: 2,000) was used as the secondary antibody for the detection of the native protein.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis of HlP0 transcriptional profiles. To investigate the HlP0 expression pattern at different developmental stages (eggs, larvae, nymphs, and adult females), partially fed ticks (except for eggs) were detached by hand for RNA extraction. Simultaneously, 4-day-fed adult ticks were manually detached and immediately dissected under the

microscope for the various tick tissues (midgut, salivary glands, cuticle, ovary, synganglion, and fat body) (You et al., 2001). Total RNA from these samples was extracted using the TRIZOL reagent (Invitrogen, USA). The mRNA abundance of HlP0 in the extracted RNA samples was evaluated by RT-PCR analysis using a one-step RNA PCR kit (Takara, Japan) by HlP0 (HlP0-E-F and HlP0-E-R, Table 1) and actin gene-specific primers (HlActin-F and HlActin-R, Table 1). The RT-PCR amplification was carried out in a 10 μl reaction liquid including 200 ng RNA from each sample, and the RT-PCR program for RNA of developmental stages was 40 min at 50℃, followed by the PCR program of 2 min of denaturation at 94℃ and 30 cycles of 94℃ for 40 s, 58℃ for 40 s, and 72℃ for 1 min, and finally 72℃ for 7 min. The RT-PCR amplification for RNA from different tissues was performed for 60 min at 50℃, followed by the similar PCR reaction for 40 cycles.

Generation of dsRNA and injection of ticks with dsRNA. Gene silencing of HlP0 in adult ticks was carried out as reported previously (Hatta et al., 2007). In brief, one of the extracted plasmids (see above) was subjected to PCR amplification using two pairs of primers: HlP0i-U1 and HlP0i-D1, HlP0i-U2 and HlP0i-D2 (Table 1). The amplification was performed using a program described above. The resulting PCR products were purified from agarose gel and employed as templates for double-stranded RNA (dsRNA) synthesis using the T7 RiboMax™ Express Large-scale RNAi Kit (Promega, USA). The quality of dsRNA was checked using 1.5% agarose gel, and the concentration was quantified by spectrometry. To obtain a negative dsRNA control, plasmid pCX-EGFP (Zhang et al., 2007a) was treated with

*Eco*RI, and the recovered product was subjected to similar PCR amplification to that described above employing GFP-specific primers (Table 1) and synthesis of GFP dsRNA. dsRNA or PBS was injected into ticks using a method described by Hatta et al. (2007). Injected ticks were stored at 25℃ for 24 h in a moist chamber, and the alive ticks were allowed to feed on rabbits. Two rabbits were involved in the tick infestation, one for HlP0 dsRNA-injected and PBS-injected ticks (on the left and right ears, respectively), and the other one for HlP0 dsRNA-injected and GFP dsRNA-injected ticks.

Analysis to confirm HlP0 gene silencing. Four days post-infestation, 4 ticks each were randomly collected from the PBS- and dsRNA-injected groups. The ticks were homogenized using a mortar and pestle, one half for RNA extraction and the other half for protein preparation. The extracted RNA samples were subjected to the same RT-PCR amplification for 30 cycles mentioned above. The products of RT-PCR amplification were fractionated by 1.5% agarose gel and the densities of the bands were analyzed by computerizing densitometry using Image Master Program (Luminous Imager Version 2.0G; Aisin Cosmos, Japan) as described elsewhere (Tanaka et al., 2004). Moreover, to confirm that the translation product of the HlP0 gene decreased due to disruption of HlP0 mRNA, the endogenous protein of the HlP0 from each group was subjected to Western blotting, employing antibodies against HlP0 recombinant protein as primary antibodies. Expression of actin protein was investigated simultaneously as an internal control, using anti-actin antibody (1: 500; Sigma, USA). The density of Western blot bands from each lane was measured with

Shimadzu Phoretix software (Shimadzu-biotech, Japan) as described previously (Takenaka et al., 2006). The relative expression ratio of HlP0 was calculated as: relative density ratio of HlP0 protein = (HlP0 signal density/actin signal density). Statistical significant of difference between controls and HlP0 dsRNA-treated group was calculated by Student's *t* test. Both RT-PCR and Western blotting experiments were performed at least three times.

Evaluation of the RNAi effect of HlP0. The success of tick feeding was investigated by measuring the attachment rate at 2 days post-infestation, feeding periods, engorgement rate, body weight, and mortality (Zhou et al., 2006). Furthermore, two ticks were detached using forceps at 4 days post-infestation and dissected for cuticle and salivary gland collection, and the size of these tissues was compared under the microscope.

1-3. Results

Characterization of HlP0 cDNA. The full-length HlP0 cDNA consists of 1,141 nucleotides, with an open reading frame (ORF) of 963 bp from 76 bp to 1,038 bp (GenBank accession number, EU048401). The cDNA sequence contains a poly adenylation signal, AATAAA, located 14 bp upstream of the poly (A) tail (Fig. 1A). The presumptive 5'-end of the HlP0 cDNA sequence, ATCGTTT (marked with *stars* in Fig, 1A), shared six residues with the insect conserved cap site ATCA(G/T)T(C/T) (Hultmark et al., 1986). The ORF of the cDNA sequence encodes a putative protein of 320 amino acid residues, with a predicted molecular mass of 35 kDa. The deduced

amino acid sequence of the HlP0 gene did not show a signal sequence (SignalP3.0). In the primary structure of HlP0, three functional domains may be recognized (Krokowski et al., 2002), including the rRNA-binding domain (position 44-67; Rodriguez-Gabriel et al., 2000), the second functional domain (position 182-298) that is responsible for the interaction with P1/P2 proteins (Shimizu et al., 2002), and the third highly conserved region (position 304-319) that is implicated in the interactions with elongation factors during translation (Uchiumi et al., 1986; Zhang et al., 2007b). A database search using the BLAST program revealed a high sequence similarity of the HlP0 protein to P0 proteins from various species. The alignment of the deduced HlP0 protein sequence with that from *Ixodes scapularis* (AAY66850), *Aedes albopictus* (AAM97779), *Bombyx mori* (NP_001037123), *Tribolium castaneum* (XP_966610), and *Homo sapiens* (NP_000993) yielded identities of 94.7%, 70.64%, 71.56%, 74.0%, and 68.75%, respectively (Fig. 1B).

Identification of the endogenous HlP0 protein. rHlP0/Trx was expressed as inclusion bodies with a molecular mass of 57 kDa (Fig. 2A) and used to immunize mice three times. Simultaneously, rHlP0/GST was expressed as a GST fusion protein with a molecular mass of 61 kDa (Fig. 2B). Purified rHlP0/GST was used as an antigen to evaluate the titer of anti-rHlP0/Trx. Subsequently, the sera were collected and recruited to recognize native HlP0 in different tissues from 4-day-fed adult ticks. The result of Western blotting demonstrated that native HlP0 with an expected molecular mass of 35 kDa was ubiquitously detected in all dissected tissues (Fig. 3).

Expression pattern of HlP0 gene. The total RNA of eggs, larvae, nymphs, and adult ticks, as well as that of different tissues dissected from 4-day-fed ticks, including salivary glands, midgut, ovary, synganglion, fat body, and cuticle, was subjected to RT-PCR analysis. Fig. 4 shows that the HlP0 gene transcript was present in the whole life cycle of ticks and ubiquitously expressed in all tested tissues.

Confirmation of HlP0 gene silencing. The disruption of HlP0 mRNA due to an injection of dsRNA was confirmed by RT-PCR of total RNA from 4-day-fed adult ticks and Western blotting. The result indicated that the transcript of HlP0 gene was obviously detected in PBS-injected and GFP-injected ticks but showed a remarkable decrease in HlP0 dsRNA-injected ticks (Fig. 5A and B). The success of RNAi was further confirmed using Western blotting for the detection of endogenous HlP0 protein. Treatment with HlP0 dsRNA led to a significant reduction in the levels of HlP0 protein compared with ticks injected with PBS ($P< 0.0001$) and GFP dsRNA ($P< 0.005$, Fig. 5C and D).

Impact of HlP0 silencing on tick feeding and survival. Ticks injected with PBS, GFP dsRNA, and HlP0 dsRNA were placed on the ears of two rabbits; success of feeding and survival were investigated. PBS-injected ticks showed a more obvious increase in body size than HlP0 dsRNA-injected ticks 4 days post-infestation (Fig. 6A). Detached HlP0 dsRNA-injected ticks were much smaller than those in the PBS control group (Fig. 6B). The ticks from each group were weighed before and after feeding, and the results were summarized. The average weight of the unfed ticks from the HlP0 dsRNA-injected group (1.99 ± 0.13 mg) was not different from that in the

PBS-injected (2.00±0.17 mg) and GFP dsRNA-injected groups (1.99±0.10 mg). However, there was a significant variation in tick fed body weight (2.63±1.21 mg in the HlP0 dsRNA-injected group vs. 226.75±74.80 mg in the PBS-injected group and 231.15±51.32 mg in the GFP dsRNA-injected group; $P< 0.01$; Fig. 6C). Disruption of HlP0 mRNA did not affect the attachment rate of ticks in the PBS-, GFP dsRNA-, and HlP0 dsRNA-injected groups (74.38%, 87.18%, and 70.73%, respectively) 2 days post-infestation (Fig. 6D). The ticks from the PBS- and GFP dsRNA-treated groups fed on rabbits for 5 to 8 days until repletion and then detached. Nevertheless, HlP0 dsRNA-treated ticks fed on rabbits for 6 to 12 days until they detached or died on the ear of rabbits without reaching repletion. As a result, 100% of attached ticks treated by PBS and 94.11% treated by GFP dsRNA were engorged, while only 4% of HlP0 dsRNA-injected ticks were engorged. After feeding, 2.5% of PBS- and 10.4% of GFP dsRNA-treated ticks died; in contrast, 96% of HlP0 dsRNA-treated ticks died (Fig. 6D). PBS- and GFP dsRNA-injected ticks started to lay eggs 5 days post-engorgement, while no data could be obtained from HlP0 dsRNA-treated ticks because of the death of the ticks.

In order to investigate the impact of RNAi on the morphology of tissues from ticks, salivary glands and cuticle were dissected out and compared under the microscope. As shown in Fig. 7A, salivary glands from HlP0 dsRNA-injected ticks obviously exhibited a smaller size of acini than those of PBS-injected ticks. Moreover, the cuticle of HlP0 dsRNA-injected ticks was thinner and less symmetrical than that of the control group (Fig. 7B).

1-4. Discussion

In the present chapter, a full-length of the 1,141 bp ribosomal protein (HlP0) gene was isolated from the tick *H. longicornis*. This is the first report to date of P0 from a tick or from any other arachnid. The gene encodes a putative 320 amino acid residue with a molecular mass of 35 kDa. The predicted HlP0 protein was supposed to contain three domains: the rRNA-binding domain, the P1/P2 protein-binding domain, and a highly conserved C-terminal region. It was reported that vaccination with a peptide of C-terminal domain of the P0 protein from *Plasmodium falciparum* protected mice against a malaria parasite challenge (Rajeshwari et al., 2004). In a similar way, it is possible that the inoculation of hosts with the C-terminal domain of HlP0 from *H. longicornis* may also provide some protection against tick infestation.

In this chapter, rHlP0/Trx rather than rHlP0/GST was used to produce antibodies for the detection of native HlP0 protein in different tissues from ticks. This decision was based on the consideration that anti-rHlP0 sera developed from the rHlP0/GST protein can recognize the GST protein in the tissues, since GST is ubiquitously expressed in ticks (da Silva Vaz Jnr et al, 2004). The results of both Western blotting and RT-PCR indicated that the HlP0 gene transcript was expressed in all tested tissues.

In a previous study, it was indicated that P1/P2 could be knocked out in yeast and the cells were still viable with a remaining copy of P0, while removal of P0 was lethal (Santos and Ballesta, 1994). This lethality of the disruption of HlP0 mRNA was also

demonstrated in ticks by dsRNA treatment in this chapter. RNAi of the HlP0 gene blocked the feeding of ticks and led to the death of ticks; even the silencing of the HlP0 gene was incomplete. Incomplete silencing of the gene seems to take place frequently in dsRNA interference experiments (Gotta and Ahringer, 2001). In order to exclude the possibility of a toxic effect of dsRNA, GFP dsRNA was employed as a negative control (Ghanim et al., 2007), and similar body size of partially fed and engorged ticks as well as similar size of salivary glands and cuticle to those of PBS-treated ticks was observed (data not shown). Interestingly, GFP dsRNA-treated ticks indicated a little faster migration of actin protein and lower relative expression of HlP0 protein than those from PBS-treated group (Fig. 5C) because of unknown reason, which deserve further investigation. Moreover, 10 HlP0 dsRNA-injected ticks were kept at 25℃ in a moist chamber for more than 12 days, and none died (data not shown). The death of fed ticks in the HlP0-silenced group was probably due to the disability of a large subunit in cell growth or apoptosis. After P0 repression in yeast, a dramatic reduction in the amount of polysomes was observed in the cell extracts, and half-mers in the polysome population appeared, which indicated a deficit in the amount of active large subunits available (Santos and Ballesta, 1994). As described, the cleavage of 28S RNA is thought to be an important step in the process of apoptosis, and there is a positive, though incomplete, correlation between rRNA cleavage and internucleosomal DNA fragmentation (Houge and Doskeland, 1996). Since P0 directly binds 28S RNA, it is possible that repression of HlP0 resulted in the same effect as rRNA cleavage. Furthermore, the P0 protein has both 3' and 5' APE

activity, acts on abasic DNA, and shows strong DNase activity for both single- and double-stranded DNA (Yacoub et al., 1996; Frolov and Birchler, 1998), which is important for repair of damaged DNA. The knockdown of HlP0 gene may thus lead to the accumulation of damaged DNA. Primary observations in mosquito cells indicated that silencing of P0 resulted in the fragmentation of DNA, a sign of apoptosis (Jayachandran and Fallon, 2003).

After feeding, the HlP0 dsRNA-injected ticks achieved a conspicuously lower body weight (1.32-fold vs. 113.38-fold and 116.16-fold, calculated by the fed body weight/unfed body weight) and a remarkably smaller size of salivary gland acini and cuticles than that of the two control groups. This suggests that the function of the salivary gland was impacted and synthesis of the new cuticle was obviously blocked by the knockdown of HlP0 gene. Salivary gland is an important organ for the feeding of ticks, where various pharmacoactive molecules are synthesized and secreted into saliva during feeding, i.e., anti-platelet factors, anticoagulant proteins, and anti-inflammatory proteins (Valenzuela, 2004). In cells from acinus II of unfed ticks, many free ribosomes were observed, and cisternae of rough endoplasmic reticulum (RER) were not distended, while, in 2-day-fed ticks, the cells became larger and contained dilated cisternae of RER (Sonenshine, 1991). I can suspect that binding of 40S and 60S particles occurred during this period, which could be attributed to the 28S RNA-binding activity of P0. However, this binding was supposed to be impeded by the disruption of HlP0 mRNA, which led to the weak synthesis of pharmacoactive molecules and the consequent failure of tick feeding. In previous study, the tick

cuticle was demonstrated to increase in thickness during the first few days after attachment for the accommodation of a large amount of blood meal during the later phase of rapid engorgement. In phase of rapid repletion, synthesis of the new cuticle slows down or ceases (Obenchain and Galun, 1982; Sonenshine, 1991). However, in the present chapter, the silencing of HlP0 gene was supposed to impede the rapid cuticle synthesis in the first phase. Furthermore, it is possible that HlP0 expression in other tissues may be impaired as well. In any case, it will be interesting to continue this investigation for a further understanding of the effects in ticks induced by RNAi of HlP0 gene.

1-5. Summary

A full-length gene termed HlP0 has been isolated from the tick *H. longicornis*. The deduced amino acid sequence of the HlP0 gene contains three presumptive domains. Transcripts of HlP0 exist in all developmental stages of ticks and different tissues from partially fed adult ticks. The RNAi of HlP0 gene led to the failure of blood sucking and tick death.

Table 1. Gene-specific primers used in sequencing, expression, and RT-PCR amplification.

Name	Sequence
HlActin-F	5'-CCAACAGGGAGAAGATGAACG-3'
HlActin-R	5'-ACAGGTCCTTACGGATGTCC-3'
HlP0-con	5'-CAAGATCTCCAAGGGCACG-3'
HlP0-E-F	5'-CCGGATCCATGGTCAGGGAGGACAAGACC-3'
HlP0-E-R	5'-CAGGATCCGAGCTCAGTCGAAGAGTCCG -3'
HlP0i-U1	5'-GAGACATCCTTGGCTCACTCTC-3'
HlP0i-U2	5'-GGATCCTAATACGACTCACTATAGGGAGACATCCTTGGCTCACTCTC-3'
HlP0i-D1	5'-GGATCCTAATACGACTCACTATAGGAGAGGCGGAGGGAGCTCAGTC -3'
HlP0i-D2	5'-AGAGGCGGAGGGAGCTCAGTC-3'
GFPi-U1	5'-ATGGTGAGCAAGGGCGAGGAGC-3'
GFPi-U2	5'-GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGGAGC-3'
GFPi-D1	5'-GGATCCTAATACGACTCACTATAGGACTTGTACAGCTCGTCCATGCCG-3'
GFPi-D2	5'-ACTTGTACAGCTCGTCCATGCCG-3'

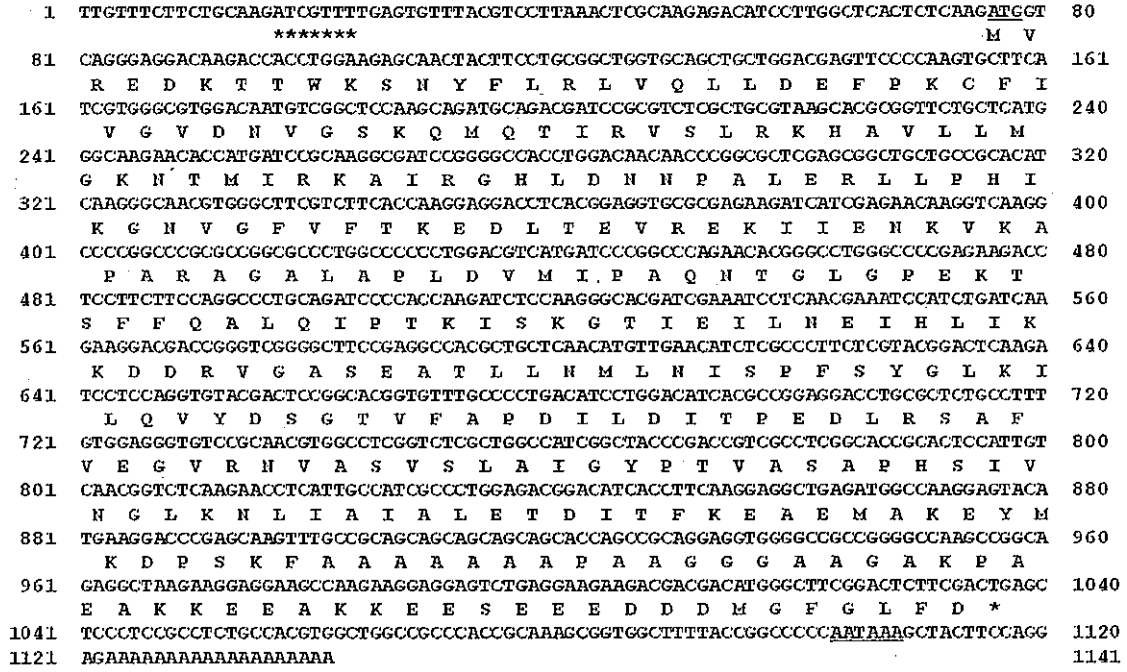

Fig. 1A. Analysis of the HlP0 gene sequence.

Nucleotide and deduced amino acid sequence of HlP0. The 5'-end nucleotide sequence that shares similar residues with insect conserved cap site is marked with *stars*. The start codon (ATG) and AATAAA are *underlined*. The final star indicated the stop codon of the putative HlP0 protein sequence.

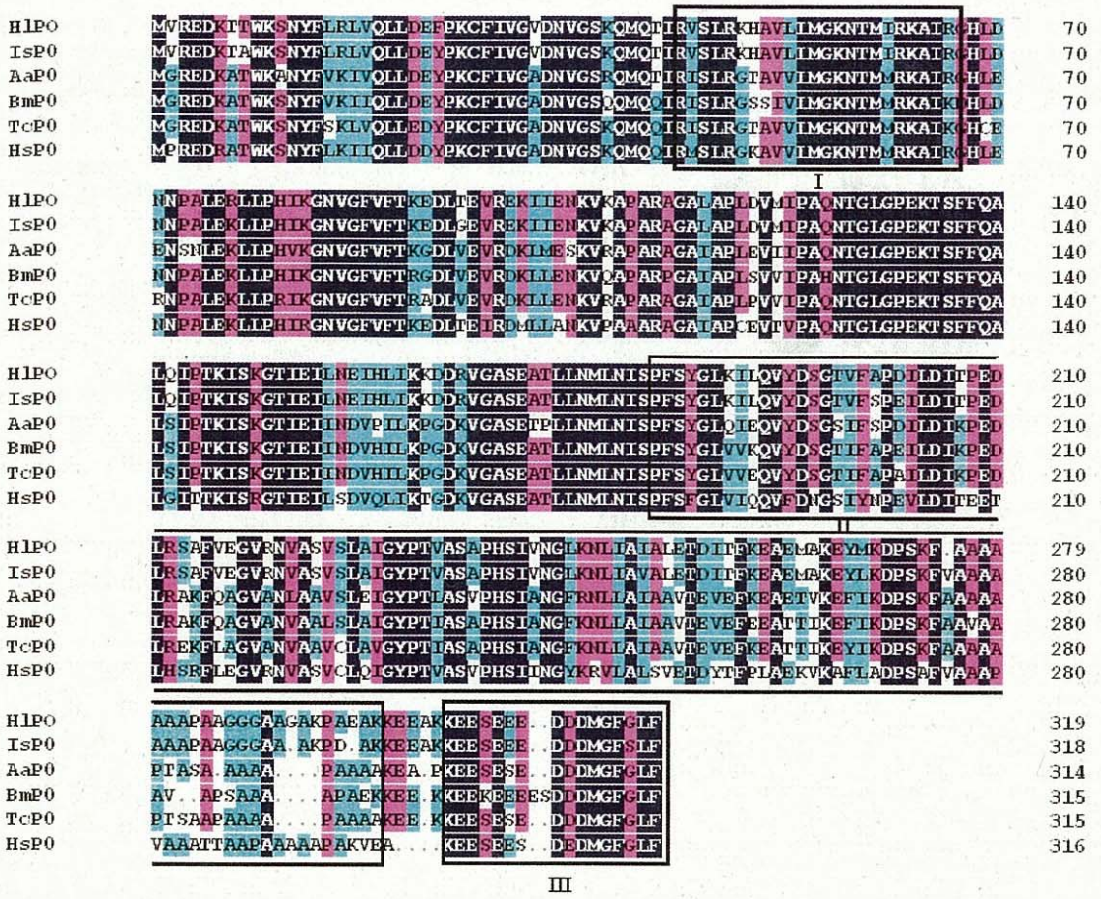

Fig. 1B. Alignment of the deduced protein sequence of HlP0 from *H. longicornis* tick with those from other species.

Sequences included in the alignment and identities: IsP0 (*Ixodes scapularis*, AAY66850, 94.7%), AaP0 (*Aedes albopictus*, AAM97779, 70.64%), BmP0 (*Bombyx mori*, NP_001037123, 71.56%), TcP0 (*Tribolium castaneum*, XP_966610, 74.0%), and HsP0 (*Homo sapiens*, NP_000993, 68.75%). The predicted rRNA-binding domain (position 44-67), the P1/P2-binding domain (position 182-298), and the conservative C-terminal region (position 304-319) are marked with *boxes* and indicated as domains I, II, and III, respectively.

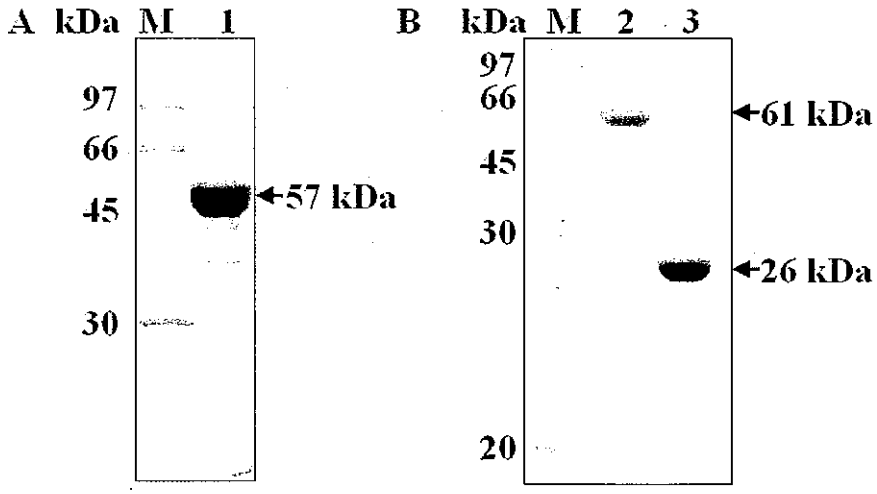

Fig. 2. Purification of recombinant HlP0 proteins.

A, Purification of rHlP0/TrX protein. B, Purification of rHlP0/GST protein. M, Protein Molecular Weight Marker (Low); lane 1, purified insoluble inclusion bodies of rHlP0/TrX protein; lane 2, purified rHlP0/GST protein; lane 3, GST protein. The expressed protein bands with expected molecular size are shown with *arrows*.

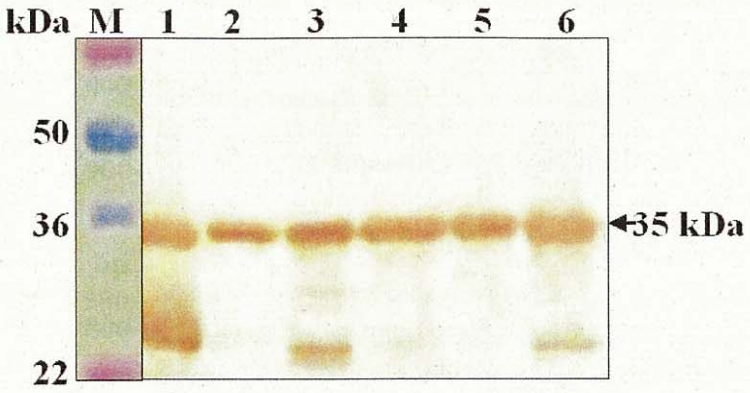

Fig. 3. Identification of endogenous HlP0 by Western blot analysis.

The anti-rHlP0/TrX serum was used as the first antibody to detect the native protein in various tissues from partially fed ticks. M, pre-stained protein ladder; lane 1, midgut; lane 2, salivary glands; lane 3, cuticle; lane 4, ovary; lane 5, synganglion; lane 6, fat body.

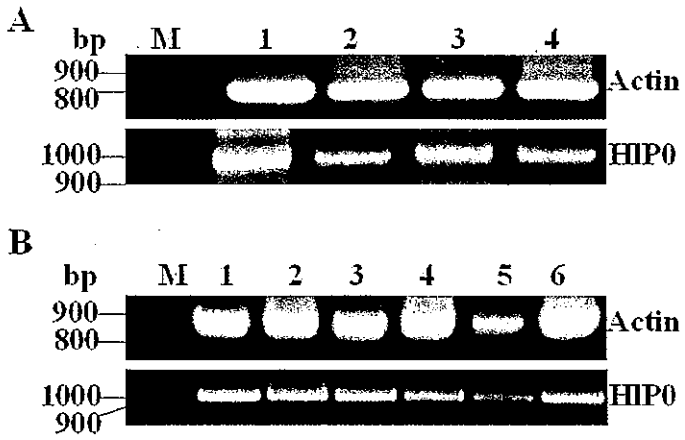

Fig. 4. Transcription profiles of the HlP0 gene in different developmental stages and tissues of partially fed ticks by RT-PCR.

A, Total RNA extracted from different developmental stages of ticks was subjected to RT-PCR. Lane 1, eggs; lane 2, larvae; lane 3, nymphs; lane 4, adult ticks. B, Total RNA extracted from different tissues was subjected to RT-PCR. Lane 1, midgut; lane 2, salivary glands; lane 3, cuticle; lane 4, ovary; lane 5, synganglion; lane 6, fat body.

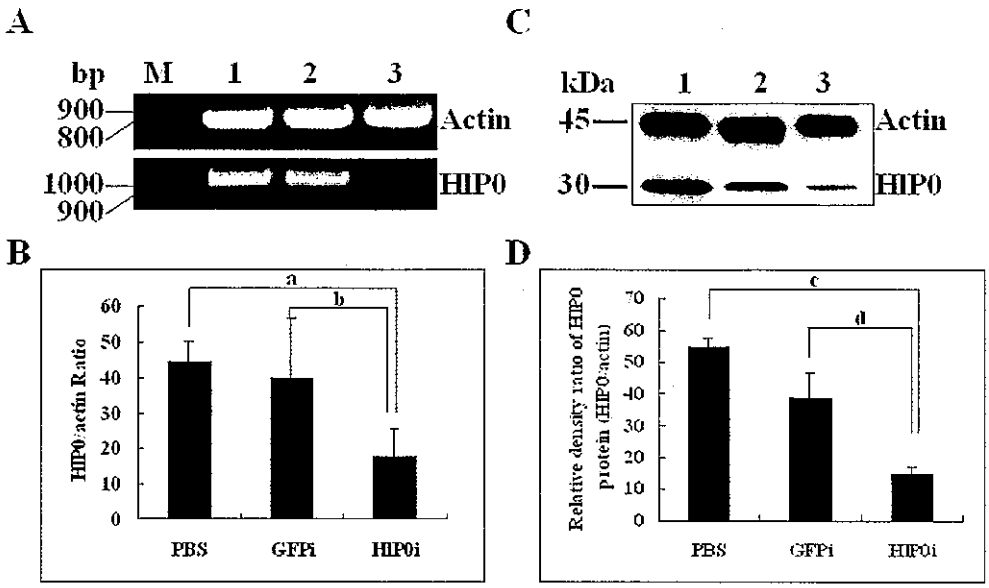

Fig. 5. Confirmation of silencing of the HlP0 gene by reverse transcription PCR (RT-PCR) and Western blotting.

A, HlP0 transcription products in RNA from 4-day-fed ticks were investigated using RT-PCR (30 cycles) with actin and HlP0 gene-specific primers. B, Quantification of the band density obtained by RT-PCR. HlP0 band density is shown relative to actin. C, Western blotting of native HlP0 protein from 4-day-fed ticks was carried out using anti-actin sera and anti-rHlP0/TrX sera. M, 100 bp DNA ladder; lanes 1, PBS-injected ticks; lanes 2, GFP dsRNA-injected ticks; lanes 3, HlP0 dsRNA-injected ticks. D, Density of HlP0 protein bands is shown as a ratio to actin protein. Values represent mean ± s.d. a, $P< 0.01$; b, $P< 0.05$; c, $P< 0.0001$; d, $P< 0.005$ (Student's *t*-test).

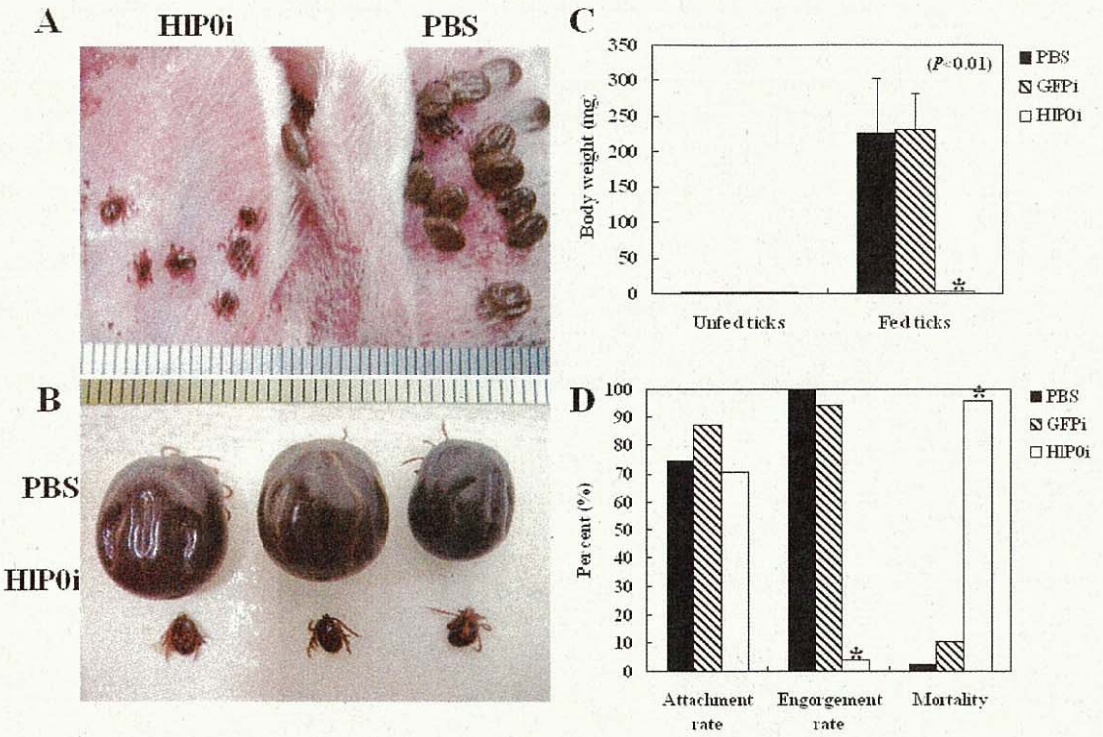

Fig. 6. Effect of HlP0 RNAi on feeding and survival of adult ticks.

Comparison of the size of 4-day-fed (A) and detached ticks (B) between the control and HlP0 dsRNA-treated groups, showing that both partially fed ticks and finally detached ticks in the HlP0 dsRNA-treated group were much smaller than those in the control group. C, Comparison of the body weight of unfed and detached ticks in each group. The fed body weight of HlP0 dsRNA-injected ticks was remarkably lower (shown with a *star*) than that of ticks in the control groups. D, Comparison of the attachment rate 2 days post-infestation, engorgement rate, and mortality in each group. HlP0 dsRNA-treated ticks showed lower engorgement rate (4%) and higher mortality (96%) (shown *with stars*) than that of the PBS- and GFP dsRNA-treated ticks.

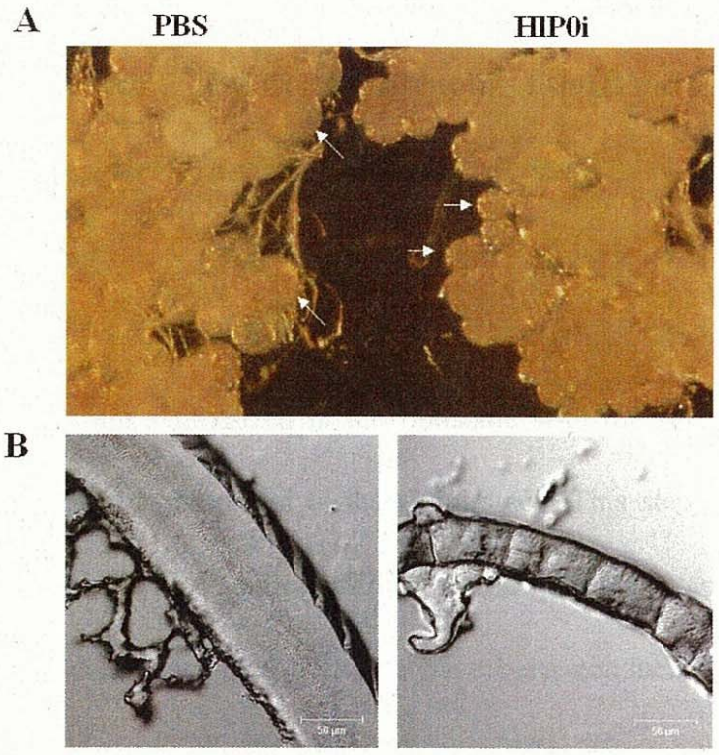

Fig. 7. Morphological effect of HlP0 RNAi on the salivary glands and cuticle of ticks.

A, Difference of the salivary glands between PBS- and HlP0 dsRNA-treated ticks. The granular salivary gland acini are shown with *arrows*. B, Difference of the cuticle between PBS- and HlP0 dsRNA-treated ticks.

Chapter 2

Blocking the secretion of saliva by silencing the HlYkt6 gene in the tick *Haemaphysalis longicornis*

2-1. Introduction

Almost proteins are synthesized in the ribosome with the involvement of P0 protein, and subsequently transported to the Golgi stack. This step depends on the SNAREs, soluble *N*-ethylmaleimide-sensitive fusion protein attachment protein receptors. Many studies concerning SNAREs have been conducted since the first characterization of the proteins in the late 1980s. SNAREs comprise a superfamily of small, mostly membrane-anchored proteins that share a 'SNARE' motif of 60-70 amino acids (Jahn et al., 2003), which functions in the docking of vesicles/containers with the target compartment and catalyzes the fusion of the apposing membranes of the transport intermediate and the target compartment. Fusion of the membrane happens between different organelles, such as lysosomes, the *trans*-Golgi network, the endoplasmic reticulum (ER), and the plasma membrane, leading the exocytosis and endocytosis (Jahn and Scheller, 2006). Exocytosis and endocytosis are of great importance for ticks. First, the fluid secretion of the salivary gland, associated with exocytosis, is greatly increased to facilitate attachment and feeding. Karim et al. revealed the presence and key roles of a member of SNAREs in the tick salivary gland (Karim et al., 2004b, 2005). Second, neurotransmitters, such as dopamine,

which stimulates fluid secretion, are also released by exocytosis (Maritz-Olivier et al., 2005). Third, during oogenesis, vitellogenin (Vg), the protein precursors to form yolk, is synthesized in the fat body trophocytes, secreted into the hemolymph, and taken up by oocytoes through micropinocytosis (Sonenshine, 1991). It is conceivable that exocytosis and endocytosis are involved in the whole process of feeding and oviposition in ticks. Furthermore, the regulation and mechanism of vesicle fusion are probably important for the transmission of pathogens. *Borrelia burgdorferi*, the Lyme disease spirochetes, was believed to move across cells by endocytosis and exocytosis (Hechemy et al., 1992; Kurtti et al., 1994). Therefore, based on the critical roles of SNAREs in membrane fusion and the likelihood that the tick SNARE cycle is conserved across tick species, targeting the SNARE cycle could provide a panacea for controlling the tick and tick-borne diseases (Jaworski, 2003).

SNAREs can be functionally classified into v-SNAREs, which are associated with the vesicle/container, and t-SNAREs, which belong to the target compartment. Structurally, SNAREs can be distinguished into the Q-SNARE and R-SNARE types according to the central Gln (Q) or Arg (R) residue in the 0 layer of the SNARE motif respectively (Hong, 2005). Q-SNAREs are further divided into Qa (for the Syn subfamily), Qb (for the S25N subfamily), and Qc (for the S25C subfamily) SNAREs. One member of each subfamily composes the Qa: Qb: Qc: R four-helical bundle configuration of a SNARE complex. Eukaryotic cells contain a series of SNAREs (Pratelli et al., 2004). So far, 21 members in *Saccharomyces cerevisiae*, 35 in humans, 68 in *Arabidopsis thaliana*, and 20 in *Drosophila* have been isolated (Pratelli et al.,

2004), and about 25 members in *Leishmania major* have been classified as SNAREs according to the phylogenetic analysis by bioinformatic searches of the *L. major* genome (Besteiro et al., 2006). As for ticks, the sequence of a v-SNARE in *Amblyomma americanum*, synaptobrevin (VAMP2), has been described, and knockdown of the gene led to a salient decrease of the engorgement rate and body weight (Karim et al., 2004b). However, there seems to be far more than one molecule of SNAREs in the ticks. According to a previous report, Ykt6 (A v-SNARE or R-SNARE) is the most versatile SNARE in eukaryotes involved in the multiple fusion reactions at the Golgi, endosomal, and vacuolar membranes *in vivo* (Rossi et al., 2004; Meiringer et al., 2008). This led to a hypothesis, i.e., that damage of the Ykt6 mRNA would cause an abnormal intracellular transport of vacuoles or failure of membrane fusion and, hence, the collapse of exocytosis, which are vital for salivation. In the present chapter, I characterized a Ykt6-like protein gene from *H. Haemaphysalis longicornis* and clarified its function on saliva secretion by using an RNA interference technique. A decrease in the engorged body weight and death of the replete ticks in the HlYkt6 dsRNA-injected group were observed.

2-2. Materials and methods

Materials. The one-step RNA PCR kit (AMV) and DNA ligation kit Ver 2.1 came from TaKaRa (Japan). An AmpliTaq Gold® with geneAmp® was obtained from Roche (USA). A Geneclean® kit was bought from QBIOgene (USA). DADE Actin FSL Activated PTT (APTT) Reagent and standard human plasma were bought from

DADE BEHRING (Japan). The T7 RiboMax™ Express Large-scale RNAi Kit was bought from Promega (USA).

Ticks and tissues. The pathenogenetic Okayama strain of *H. longicornis* was reared in the National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan. To obtain the partially fed ticks, larvae and nymphs were infested on the ears of rabbits for 4 days and then manually detached. Contaminated tissues from the host were removed using forceps. Subsequently, the ticks were homogenized in nitrogen and stored in Trizol for RNA extraction. In addition, six kinds of tissues, i.e., midgut, salivary gland, ovary, synganglion, hemolymph, and fat bodies, were isolated from 4-day-fed female ticks immediately after detachment and then put in a PBS buffer for SDS-PAGE. All samples were stored at -80℃ until use.

Sequencing and analysis of the gene. Two clones encoding the Ykt6 protein were selected from the cDNA library previously constructed from the tick *H. longicornis* (Zhou et al., 2006). Plasmids were extracted from the clones and subsequently subjected to an automated sequencer (ABI prism 310 Genetic Analyzer, USA) using plasmid vector pGCAP1 special primers. To obtain the full length, a gene-specific primer (HlYkt6-con, Table 2) was designed according to the sequence determined in the first turn. The full-length sequence of the Ykt6 protein (named HlYkt6 protein) was subjected to analysis employing ScanProsite and BlastP software.

***In vitro* expression of HlYkt6.** Gene-specific primers (HlYkt6-E-F and

HlYkt6-E-R, Table 2) were designed according to the open reading frames (ORF) of the HlYkt6 gene and the restriction digestion sites in the sequence of vectors pET32a (+) and pGEX-4T-3. The expression and purification of the recombinant protein were performed as described in chapter 1. Briefly, recombinant HlYkt6/TrX (rHlYkt6/TrX) was expressed in the form of an inclusion body, which was primarily purified by sonication for immunization. Meanwhile, the HlYkt6/GST (rHlYkt6/GST) protein was solubilized in 2% (W/V) *N-Lauroylsarcosine* sodium (Sigma, USA) and purified with glutathione sepharose 4B according to the manufacturer's instructions.

Preparation of the anti-serum in mice. As described by Kukkonen et al. (2004), inclusion bodies of rHlYkt6/TrX were completely mixed with an equal volume of Freund's complete adjuvant (Sigma, USA) and intraperitoneally injected into mice (ddY, 8 weeks old; SCL, Japan). The mice were immunized twice again with the same dose of recombinant protein in the incomplete adjuvant at days 14 and 28. Eight days after the third injection, the titer of the antibodies was evaluated using ELISA, employing rHlYkt6/GST as an antigen. Subsequently, all the sera were collected from the blood of the mice.

Identification of the native HlYkt6 in ticks by Western blot analysis. Midgut, salivary gland, ovary, synganglion, hemolymph, and fat bodies from 4-day-fed female ticks were dissected, sonicated, and electrophoresed on a 15% polyacrylamide gel. The proteins were then transferred to Immobilon P polyvinylidene difluoride membranes (PVDF membrane; Millipore, USA) and subjected to Western blot analysis as described in chapter 1. The first antibody was diluted 100 times, and the

second antibody, peroxidase-conjugated goat anti-mice immunoglobulin G (IgG), was diluted 2,000 times for native HlYkt6 protein detection.

Expression pattern analysis of HlYkt6 in ticks by reverse-transcription PCR (RT-PCR). Total RNA was extracted from the eggs and 4-day-fed larvae, nymphs, and female adult ticks using a Trizol reagent (Sigma, USA). RT-PCR amplification was carried out using a one-step RNA PCR kit (TaKaRa, Japan) according to the manufacturer's procedures. The reaction conditions were 50℃ for 60 min to synthesize the first-strand cDNA followed by 94℃ for 2 min and 30 cycles of 94℃ for 30 s, 55℃ for 30 s, and 72℃ for 60 s, finally 72℃ for 7 min, in which the gene-specific primers for expression were used (HlYkt6-E-F and HlYkt6-E-R, Table 2). In addition, primers designed for the actin gene of *H. longicornis* were adopted to check the integrality of RNA (GenBank accession number, AY254898; HlActin-F and HlActin-R, Table 2). In order to investigate the distribution of HlYkt6 transcripts in different tissues from the female adult ticks, RNA of the midgut, salivary gland, synganglion, ovary, hemolymph, and fat bodies from 4-day-fed female adult ticks was analyzed by the same RT-PCR amplification.

Generation of dsRNA and injection of ticks. Silencing of the HlYkt6 gene in adult ticks was carried out as reported previously (Hatta et al., 2007). In brief, a template encoding the ORF of HlYkt6 was amplified by PCR using oligonucleotide primer sets (HlYkt6i-U1, U2, D1, and D2; Table 2). The amplification was performed using a PCR program of 95℃ for 5 min, 35 cycles of 95℃ for 40 s, 55℃ for 40 s, and 72℃ for 1 min, followed by elongation for 7 min at 72℃. The dsRNA of the HlYkt6

gene was synthesized, and the quality was checked according to the previous description (Chapter 1). The GFP dsRNA was similarly generated employing GFP-specific primers (GFPi-U1, U2, D1, and D2; Table 2). Injection of ticks was carried out by the method described by Hatta et al. (2007). Control ticks were injected with a PBS buffer or GFP dsRNA. Injected ticks were stored in a moist chamber at 25℃ for 24 h, and the living ticks were allowed to feed on rabbits. Two rabbits were involved in tick infestation according to the method described by Miyoshi et al. (2004b), one for HlYkt6 dsRNA-injected and PBS-injected ticks (on the left and right ears, respectively), and the other one for HlYkt6 dsRNA-injected and GFP dsRNA-injected ticks. Moreover, the performance of RNAi was evaluated twice.

Confirmation of HlYkt6 gene silencing. Four days post-infestation, 4 female ticks were randomly collected from each group and homogenized using a mortar and pestle for RNA extraction. Two-hundred nanograms of extracted RNA was subjected to RT-PCR amplification using a one-step RNA PCR kit with HlYkt6 primers (HlYkt6-RNAi-U and HlYkt6-RNAi-D, Table 2) and actin gene-specific primers (Table 2). The RT-PCR was performed by 60 min at 50℃ followed by the same PCR program as mentioned above. The products of RT-PCR amplification were fractionated by 1.5% agarose gel, and the densities of the bands were analyzed by computerizing densitometry using an Image Master Program (Luminous Imager Version 2.0G; Aisin Cosmos, Japan) (Tanaka et al., 2004). In addition, the salivary glands were dissected from the 4-day-fed female ticks injected with PBS or dsRNA for Western blot analysis, and the HlP0 protein was identified as an internal control.

Evaluation of the effect of HlYkt6 gene silencing on saliva secretion, tick feeding, and survival. The success of tick feeding was investigated by measuring the attachment rate 2 days post-infestation, the feeding periods, engorgement rate, engorged body weight, mortality, and oviposition rate (Zhou et al., 2006). Furthermore, 4 female ticks from each group were detached using forceps 4 days post-infestation. The salivary glands of one tick from each group were dissected for size comparison of the control and test groups. The other ticks were stored in a glass bottle until the cement part was detached. Subsequently, the ticks were wiped with 75% alcohol and dorsally fixed on a sticky tape. Near the genital aperture, 1-2 μl of 10% pilocarpine (Sigma, USA) in PBS was ventrally injected into the tick to stimulate saliva secretion (Shirafuji-Umemiya, 2008). Then, 0.5 cm × 0.5 cm filter paper was employed to absorb the secreted liquid for 30 min and dried at room temperature. Finally, these papers with saliva secreted by PBS-, GFP dsRNA-, or HlYkt6 dsRNA-injected ticks were immersed in a 20 μl PBS buffer, and the same volume of 2 × sample buffer was added for SDS-PAGE. The gel was then silver-stained (Celis et al., 2006). Two days post-engorgement, the ovaries of two ticks from each group were dissected to observe the formation of oocytes.

***In vivo* and *in vitro* RNAi of salivary glands and the anti-coagulation assay.** The *in vivo* RNAi of salivary glands was performed as follows. The PBS-, GFP dsRNA-, or HlYkt6 dsRNA-injected ticks were infested on rabbit ears for 4 days. Subsequently, four adult female ticks from each group were detached out manually and their salivary glands dissected under the microscope. The isolated glands were

stimulated with PGE2 (0.1 μM) for 5 min, and then an activated partial thromboplastin assay (APTT; intrinsic pathway assay) was performed as described by Karim et al. (2004b) and Zhu et al. (1997). As for the RNAi study *in vitro*, eight 4-day-fed female ticks without injection were subjected to dissection, and their left and right salivary glands were collected for PBS treatment and HlYkt6 dsRNA treatment, respectively. The isolated salivary glands were incubated with 15 μg HlYkt6 dsRNA (1 μg/μl) or the same volume of PBS in an M199/Mops buffer (Sigma, USA) containing penicillin and streptomycin sulfate for 6 h at 37℃, respectively. Simultaneously, another group of eight ticks was subjected to the same dissection, and left glands were immersed in 15 μg GFP dsRNA (1 μg/μl) for 6 h as the control. After incubation, the glands were treated with PGE2 and subjected to the same APTT assay as described above.

RNA interference of HlYkt6 gene in nymphs. I tried to knock down the HlYk6 gene in nymphs by immersing the ticks in dsRNA liquid. Briefly, 60 nymphs were kept at room temperature and normal humidity for 24 h and then immersed in 60 μl HlYkt6 dsRNA (1 μg/μl) and stored at 4℃ for 24 h to prevent the ticks from moving out of the liquid. The surviving ticks were subsequently infested on rabbits (Miyoshi et al., 2004b) to monitor the engorgement rate and engorged body weight.

2-3. Results

Sequence analysis of the HlYkt6 gene. As shown in Fig. 8A, the HlYkt6 gene consists of a 600 bp ORF coding a putative protein of 199 amino acids (22.6 kDa),

and the complete 5' untranslated region (UTR) was 110 bp (GenBank accession number, EU795692). In the 3'UTR, AATAAA, the eukaryotic consensus polyadenylation signal, is present upstream from the polyadenylation $(A)^{+}$. The predicted protein contains no signal peptide. Analysis of the protein sequence using ScanProsit (http://www.expasy.ch/tools/scanprosite/) indicated that it contains two main domains, a longin domain (LD) and a v-SNARE domain. LD encompassing residues 9-131 is only present in v-SNAREs (marked with an *underline*), conserved in all eukaryotes, and seems to be essential for regulating membrane trafficking (Zhang and Hong, 2001). The other domain, the v-SNARE motif, contains residues 139-199 at the C-terminal (shown with *arrows*). The CAAX motif (where C = cysteine, A = an aliphatic amino acid, and X = any amino acid) in the C-terminus of the protein sequence is a unique feature of Ykt6 (for prenylation; shown with a *box*). The predicted HlYkt6 protein showed higher homology to mammals (*Mus. musculus*, 64%) than to yeast (42.8%), but the v-SNARE domain seemed highly conserved (Fig. 8B). Arginine (R) on residue 164 is supposed to be the "0" layer of the v-SNARE centers to react with the glutamine residue of t-SNAREs (Jahn and Sheller, 2006).

Purification of the recombinant proteins. Overexpression of the gene in the pET32a vector resulted in insoluble bodies in the bacteria. rHlYkt6/GST and rHlYkt6/Trx with expected molecular masses of 48.6 kDa and 42.6 kDa, respectively, were purified from *E. coli* BL21 strain (Fig. 9A). rHlYkt6/Trx was used as an antigen to raise the antibody in mice, and rHlYkt6/GST was used as an antigen to determine the antibody titer by ELISA.

Detection of the native HlYkt6 protein by Western blot analysis. Western blot analysis was performed employing the serum from the immunized mice, which was collected after determining the titer by ELISA. As shown in Fig. 9B, the polypeptide of about 26 kDa was detected in all tested tissues, including midgut, salivary glands, ovary, synganglion, hemolymph, and fat bodies. The difference in the size of the native HlYkt6 protein from the predicted (22.6 kDa) suggests that this protein may be post-translationally modified by the addition of a 15- or 20-carbon isoprenoid group. This modification (prenylation) is critical for yeast Ykt6 to execute its biological function in membrane fusion (McNew et al., 1997). This molecular difference was also observed in the Ykt6 protein of *M. musculus* (Zhang and Hong, 2001).

Expressing pattern of the HlYkt6 gene in *H. longicornis*. Total RNA extracted from eggs, partially fed larvae, nymphs, and adult female ticks were subjected to RT-PCR amplification. Similarly, the RNA of the midgut, salivary glands, ovary, synganglion, hemolymph, and fat bodies from 4-day-fed female ticks was subjected to RT-PCR amplification using actin-specific and HlYkt6 expression primers. As observed in Fig. 10, the HlYkt6 gene was ubiquitously expressed in all the developmental stages of ticks and in all the tested tissues.

Effect of HlYkt6 gene silencing on the feeding, phenotype of the salivary gland, and saliva secretion of ticks. Results of RT-PCR amplification demonstrated that an injection of dsRNA led to an obvious decrease of HlYkt6 mRNA (a, $P< 0.001$; b, $P< 0.01$; Fig. 11A). Furthermore, the Western blot analysis also showed a significant decrease of the HlYkt6 protein in the salivary glands from the

gene-silenced ticks (c, $P< 0.0005$; d, $P< 0.0001$; Fig. 11B). HlYkt6 dsRNA-injected female ticks showed an obviously lower engorged body weight than the control groups (82.9 ± 26.8 mg vs. 178.7 ± 57.0 mg in the PBS-injected group and 232.17 ± 59.1 mg in the GFP dsRNA-injected group; e, $P< 0.001$; f, $P< 0.001$; Fig. 11C). The attachment rate and engorgement rate of the test group did not exhibit a significant difference from those of the controls; however, the mortality of engorged ticks increased strikingly (100% in the test group vs. 4.8% and 20.4% in the control groups; Fig. 11D). The engorged female ticks in the HlYkt6 dsRNA-injected group showed a smaller and rounder appearance than those in the PBS-injected group, with no wrinkles on the cuticle surface, as were found in control ticks (Fig. 12A). A discrepancy in fed tick size between the tested and control groups began to be observable after 4 days of infestation (Fig. 12B). Moreover, the engorged ticks treated with HlYkt6 dsRNA failed to generate oocytes and died during 7 days post-engorgement; on the other hand, the control groups developed oocytes 2 days post-engorgement (Fig. 12C) and began to lay eggs 4-5 days post-engorgement. Since the HlYkt6 protein is closely associated with the secretion process, the secretion of saliva from female ticks and the content in the saliva were investigated. As shown in Fig. 12D, saliva drops accumulated fast on the mouthparts of the control ticks after injection of pilocarpine, while no observable liquid drops appeared during the entire investigation time (30 min) in the test group. However, no remarkable difference in size in the salivary glands was detected between the control and test groups (Fig. 12E). The filter papers that absorbed the saliva secretion were solved in the same amount of

PBS, and the liquid was subjected to SDS-PAGE. Silver staining revealed a lack of proteins in the saliva of the HlYkt6 dsRNA-injected ticks; in contrast, the saliva from the control ticks was composed of various proteins (Fig. 12F).

Impact of silencing of the HlYkt6 gene on the anticoagulant activity of ticks. The salivary glands were dissected from the partially fed female ticks injected with dsRNA or PBS before feeding. After stimulation with PGE2, the supernatant from the mixture was subjected to the APTT assay. As shown in Fig. 13A, the disruption of HlYkt6 mRNA was confirmed by RT-PCR. The secretion from the gene-silenced salivary glands showed a significantly short APTT time (25.25 ± 1.50 s) compared to the control groups (39.25 ± 0.50 s in the PBS-treated group; c, $P< 0.001$, and 40.0 ± 1.41 s in the GFP dsRNA-treated group; d, $P< 0.00001$; Fig. 13B).

Interestingly, I detected neither the obvious silencing of HlYkt6 transcripts (Fig. 14A) nor a difference in the APTT time (Fig. 14B) by *in vitro* RNAi of the HlYkt6 gene in the salivary glands of ticks.

Silencing of the HlYkt6 gene in nymphs. Sixty nymphs of each group were stored at room humidity for 24 h and then completely immersed in a liquid containing a PBS buffer, GFP dsRNA, or HlYkt6 dsRNA for 24 h. After infestation on rabbits, about one-third of the HlYkt6 dsRNA-soaked ticks obtained a low engorged body weight (Fig. 14E), and the disruption of HlYkt6 mRNA was observed, while the others ticks appeared normal (Fig. 14C, D).

2-4. Discussion

The Ykt6 protein from *S. cerevisiae* is a multifunctional R-SNARE that is required for yeast cell viability and participates in ER-Golgi transport (Rossi et al., 2004) as well as post-Golgi membrane traffic to the vacuole (Kweon et al., 2003). In the present chapter, I isolated, cloned, and expressed a HlYkt6 gene from the tick *H. longicornis*. HlYkt6 is a 199-residue protein that consists of an LD and a v-SNARE motif. LD adopts a profilin-like structure, which is implicated to bind to the C-terminal SNARE domain and results in a folded-back conformation. The folded-back conformation is essential for both the stability of the protein *in vivo* and its correct localization in several cell types (Hasegawa et al., 2003; Fukasawa et al., 2004). There is a carboxyl-terminal CAAX motif at the C-terminal of the predicted HlYkt6 protein. Ykt6 attaches to the membrane by prenylation of the CAAX motif, while the majority of the SNAREs are anchored to the membrane by the C-terminal hydrophobic region (31 out of 36 SNAREs) (Fukasawa et al., 2004). The native HlYkt6 protein detected in the tissues of ticks showed a higher molecular size than had been assumed, which may hint at the prenylation of the CAAX motif in the native HlYkt6 protein (Fig. 9B).

Both the Western blot analysis and RT-PCR amplification demonstrated that HlYkt6 was ubiquitously expressed in all the tissues isolated from the partially fed ticks (Fig. 9B and 10). Based on these results, it may be deduced that, first, despite its possible intracellular specificity and cell-type dependent specificity (Hasegawa et al., 2003), HlYkt6 is not selectively expressed in different tissues. The wide distribution

of HlYkt6, one of the SNARE molecules, in the tick enables its involvement in the secretion of saliva, release of regulators from the synganglion and digestive enzymes from the midgut, discharge of vitellogenin from the fat body, accumulation of vitellogenin in the hemolymph, and phagocytosis of oocytes. Secondly, HlYkt6 may be one of the core v-SNAREs that are sufficient to mediate most intracellular vesicle fusion events in the tick. The SNARE family has remained mostly unchanged in yeast, flies, and worms, but it has increased in humans to 35 members. Mammals appear to use additional SNAREs to obtain further tissue-specific specialization of membrane trafficking, but a set of core SNAREs is sufficient to mediate most intracellular vesicle fusion events (Bock et al., 2001). Those additional SNAREs are supposed to exhibit discrepancy in their expression patterns, while the core SNAREs will be expressed ubiquitously (Uemura et al., 2004). Based on this opinion, HlYkt6 may belong to these set of core SNAREs.

The present study is the first to investigate the interference of the Ykt6 gene in an arthropod. The RT-PCR and Western blot analysis demonstrated the disruption of the HlYkt6 mRNA and the decrease of the HlYkt6 protein in the HlYkt6 dsRNA-injected ticks, respectively. In the Western blot analysis, HlP0 was employed as an internal control, a substitute for actin protein, because the later has ever shown an abnormal migration on the gel. The use of ribosomal P0 protein as a loading control was supported by other reports (Wycoff et al., 1998).

HlYk6 dsRNA-injected ticks obtained less than half of the engorged body weight achieved by control ticks (Fig. 11C). To prove that this failure in the feeding of ticks

originates from the collapse of exocytosis caused by the diminution of the HlYkt6 protein, the secretion and components of saliva from PBS, GFP dsRNA, and HlYkt6 dsRNA-injected ticks were investigated. The result indicates that the secretion from HlYkt6 dsRNA-injected ticks contains few kinds of proteins (Fig. 12F). As is well known, ticks secrete many kinds of pharmacoactive proteins, such as anti-hemostatic, anti-inflammatory, and immunomodulatory molecules, to obtain a blood meal from the hosts (Valenzuela, 2004). Since the silencing of the HlYkt6 gene resulted in the decrease in the amount and kinds of proteins in the saliva from ticks, it can be easily deduced that the anticoagulant activity of the saliva decreased. As expected, the result of the APTT assay demonstrated the reduction of the coagulation time, suggesting that the anticoagulant activity of the saliva diminished significantly in the HlYkt6 dsRNA-injected ticks (Fig. 13B). Since the HlYkt6 dsRNA-injected ticks could not overcome the coagulation of the blood in the host, the amount of blood obtained by the tick was dramatically reduced; hence, the engorged body weight of gene-silenced ticks decreased significantly, as observed in this chapter. Nevertheless, I, for an unknown reason, was not able to detect the silencing of the HlYkt6 gene by *in vitro* immersion of the salivary glands in a dsRNA liquid.

The Ykt6 intramolecular interactions were predicted to create a compact, closed conformation of the SNARE that prevents promiscuous targeting interactions and premature insertion into membranes (Hasegawa et al., 2004). The HlYkt6 gene disruption was assumed to lead to the promiscuity or failure of membrane fusion, hence reducing or blocking the exocytosis of various molecules that facilitate blood

feeding. Simultaneously, we can imagine that transport vesicles and ER membranes may significantly accumulate in the cells of the salivary gland, midgut, ovary, and fat bodies, as observed in the HsYkt6p-depleted yeast (McNew et al., 1997). However, the amassment of proteins in the cells does not seem to have an obvious effect the morphology of the acini in the salivary gland (Fig. 12E). On the other hand, the normal development of oocytes in the ovary of engorged ticks was not detected in the HlYkt6 gene-silenced ticks (Fig. 12C). This may be easily explained by the involvement of the HlYkt6 protein in the transport of Vg from fat bodies to the hemolymph, and finally to the ovary (Sonenshine, 1991). Depletion of the H1Ykt6 protein may have caused the collapse of the transport process. The strikingly high mortality (100%, Fig. 11D) of the HlYkt6 dsRNA-injected ticks suggests that a variety of digestive enzymes in the gene-silenced ticks, including aspartic protease (Boldbaatar et al., 2006), leucine aminopeptidase (Hatta et al., 2006), serine carboxypeptidase (Motobu et al., 2007), and asparaginyl endopeptidases (Alim et al., 2008), were not transported and blocked in the cells of the midgut. As a result, the sucked blood could not be digested and accumulated in the midgut, which may have damaged the midgut and, ultimately, caused the death of the tick. However, further investigation is required to prove the hypothesis. Other SNAREs, such as synaptobrevin (Karim et al., 2004b, 2005) and syntaxins (Jantsch-Plunger and Glotzer, 1999), have been silenced in ticks and *C. elegans*, respectively. Silencing of synaptobrevin resulted in ~20% engorgement in the adult tick of *A. americanum*, while the other ticks died 2 days after detachment or on a rabbit (Karim et al., 2004b,

2005). The result of HlYkt6 gene silencing is consistent with the study performed by Karim et al. and suggests that both synaptobrevin and HlYkt6 execute vital functions in ticks and that depletion of either gene will lead to the death of ticks. This experiment supported once again the opinion that HlYkt6 was one of the core SNAREs in ticks.

In the cascade of protein synthesis, modification, and transport, a series of associated molecules has been identified from the tick *H. longicornis*. It can be hypothesized that the corresponding mRNA of proteins in the tick migrates from the nucleus to the cytoplasm and localizes on the ER. With the involvement of the HlP0 protein, the proteins are synthesized and then modified in the ER, in which protein disulfide isomerase (PDI), one of the most abundant proteins in the ER, may be responsible for the formation of disulfide bonds (Liao et al., 2007). On the other hand, cyclophilin members are possibly responsible for the correct folding of proteins due to their peptidyl-prolyl isomerase (PPIase) activity (Boldbaatar et al., 2008b). The synthesized proteins are transported from the ER, through coatomer protein complex-II (COPII)-coated vesicles, which may contain HlYkt6 protein, to the Golgi complex (Jahn and Scheller, 2006). With the assistance of synaptobrevin-2 (Karim et al., 2004b) and other molecules, the vacuoles detaches from the Golgi complex and fuses with the membrane, and the content in the vesicles is finally released out of the cell. However, further studies are needed to support this hypothesis.

In this chapter, I kept the nymphs at room temperature and room humidity for 24 h to engender lack of water in nymphs. Subsequent immersing of nymphs in the liquid

of dsRNA was supposed to facilitate the absorption of dsRNA by nymphs to obtain a similar effect by dsRNA-injection as obtained in adult ticks. The result indicated that the HlYkt6 gene in some of the nymphs was successfully silenced. However, the technique needs more modification, and optimization of the RNAi treatment condition in nymphs is ongoing. I expect that the success of this method will provide a convenient and labor-saving approach for the RNAi of ticks as well as other arthropods without the need for dsRNA-injection equipment.

2-5. Summary

A gene called HlYkt6 was isolated, coding a protein of 199 amino acids of about 22.6 kDa. The predicted protein contains an LD and a v-SNARE motif. This protein was expressed in all tested tissues. Interference of the HlYkt6 gene seriously affected the secretion of saliva from the ticks, and significantly decreased the anticoagulant activity of secretion from the HlYkt6 gene-silenced ticks. As a result, the HlYkt6 gene-silenced ticks obtained a lower body weight than that of the control groups. Injection of HlYkt6 dsRNA into unfed adult ticks also led to a strikingly high mortality of the ticks post-infestation. This study provided the first investigation on the impact of the silencing of SNAREs on saliva secretion in ticks.

Table 2. Gene-specific primers used in sequencing, expression, RT-PCR amplification, and RNA interference.

Name	Sequence
HlActin-F	5'-CCAACAGGGAGAAGATGAACG-3'
HlActin-R	5'-ACAGGTCCTTACGGATGTCC-3'
HlYkt6-con	5'-GACTAAGATTCAGGCCGAC-3'
HlYkt6-E-F	5'-GAGAATTCACAATGGTAAAGCTTTTCCACATAGC -3'
HlYkt6-E-R	5'-CAGAATTCCTTCACGTATCTACACAGGCAGC-3'
HlYkt6i-U1	5 '-TCTAAAAACTAGAGACCCGGAAGG-3'
HlYkt6i-U2	5'-GGATCCTAATACGACTCACTATAGGTCTAAAAACTAGAGACCCG GAAGG -3'
HlYkt6i-D1	5'-GGATCCTAATACGACTCACTATAGGTATCTGTACAGCAACTCCTG GAAC-3'
HlYkt6i-D2	5'-TATCTGTACAGCAACTCCTGGAAC-3'
HlYkt6-RNAi-U	5'-CCAACATTAC TGTGGTCGCTTG-3'
HlYkt6-RNAi-D	5'-GCACATCAAATCTCAGTGCCACA-3'
GFPi-U1	5'-ATGGTGAGCAAGGGCGAGGAGC-3'
GFPi-U2	5'-GGATCCTAATACGACTCACTATAGGATGGTGAGCAAGGGCGAGG AGC-3'
GFPi-D1	5'-GGATCCTAATACGACTCACTATAGGACTTGTACAGCTCGTCCATG CCG-3'
GFPi-D2	5'-ACTTGTACAGCTCGTCCATGCCG-3'

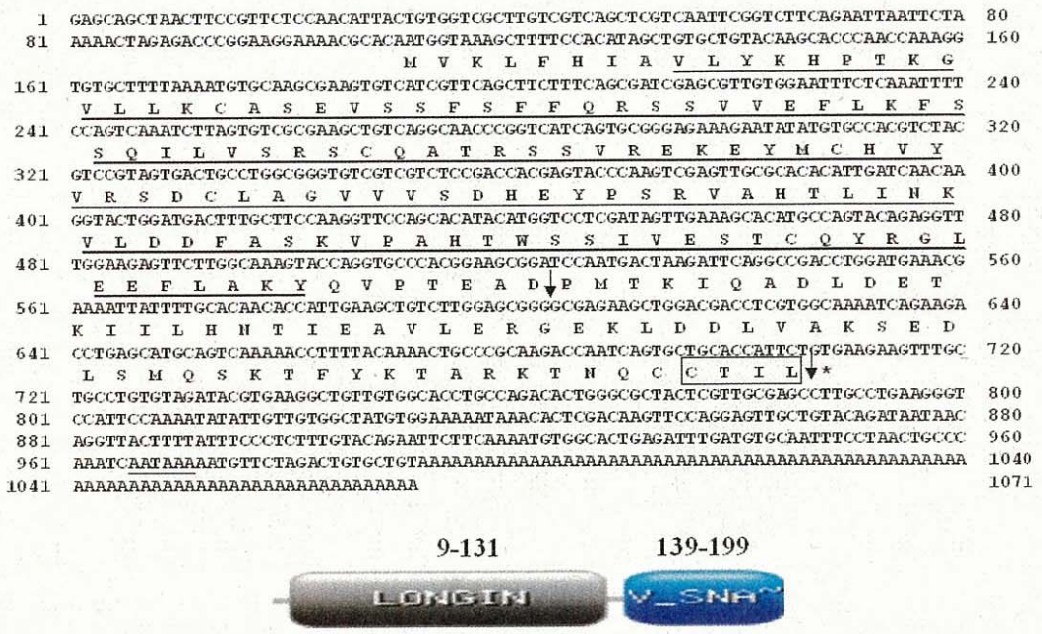

Fig. 8A. Sequence analysis of the HlYkt6 gene from the tick *H. longicornis*.

Nucleotide and predicted amino acid sequences of HlYkt6. The longin domain (LD) and consensus polyadenylation signal are *underlined*. The v-SNARE domain is marked with *arrows*. The CAAX box is marked with a *box*.

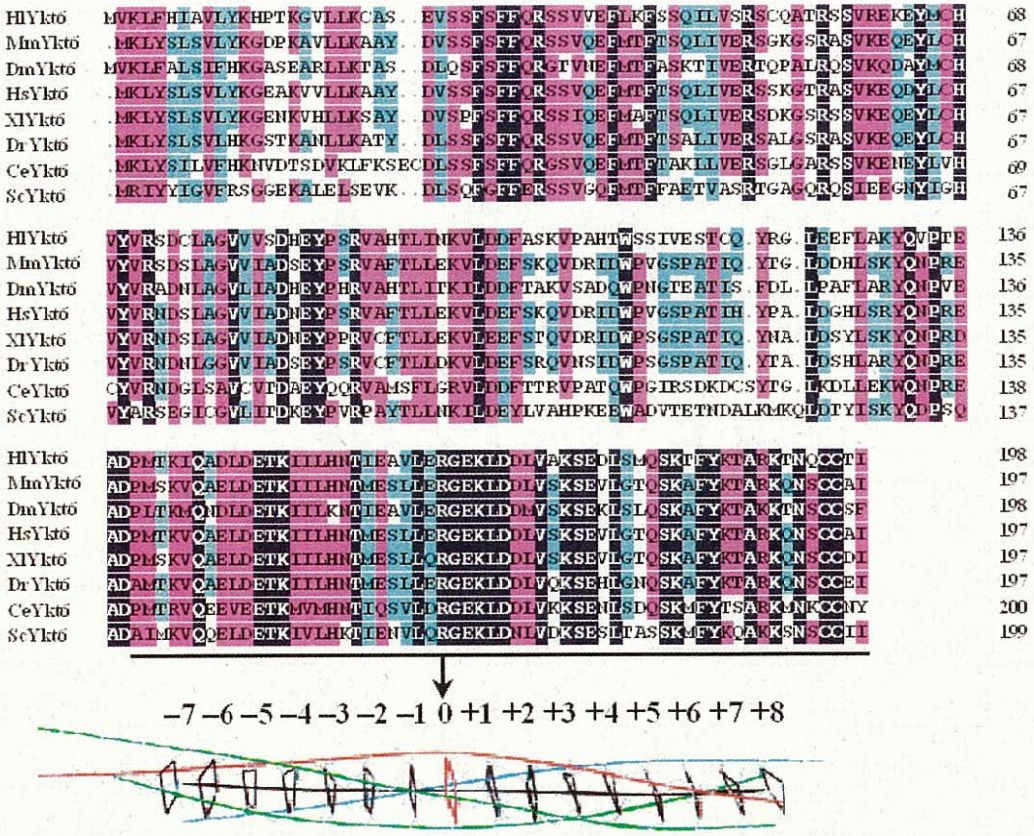

Fig. 8B. Alignment of the predicted protein sequence of the HlYkt6 gene to the sequences from other species.

The v-SNARE domain is *underlined*, and the highly conserved "R" is shown to compose the 0 layer in the conformation of the SNARE complex. Skeleton diagram that indicates the position of the central layers of interacting side chains (numbered) in the SNARE core complex. The '0' layer is red, and all of the other layers are black (cited from Jahn and Sheller, 2006). The complex contains four motifs, Qa in red; Qb and Qc in green, and R in blue. MmYkt6, *Mus musculus*; DmYkt6, *Drosophila melanogaster*; HsYkt6, *Homo sapiens*; XlYkt6, *Xenopus laevis*; DrYkt6, *Danio rerio*; CeYkt6, *Caenorhabditis elegans*; ScYkt6, *Saccharomyces cerevisiae*.

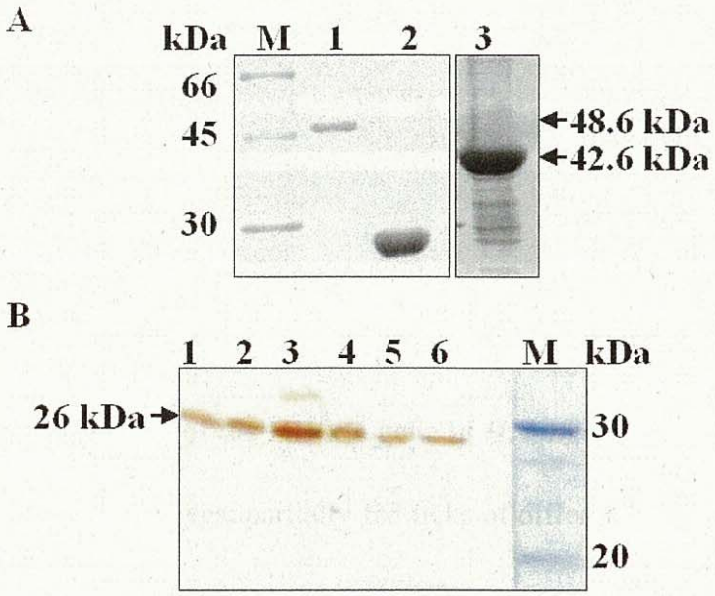

Fig. 9. Purification of the recombinant proteins and identification of the native protein.

A, SDS-PAGE analysis of purified recombinant proteins. Purified rHlYkt6/ GST (48.6 kDa) and rHlYkt6/ TrX (42.6 kDa) are indicated by *arrows*. M, Protein molecular weight marker (Low); lane 1, purified rHlYkt6/GST protein; lane 2, GST protein; lane 3, purified insoluble inclusion bodies of rHlYkt6/TrX protein. B, Western blot analysis of the native HlYkt6 protein (26 kDa) in different tissues from 4-day-fed female ticks using an antibody against rHlYkt6/ Trx. The molecular mass is indicated on the left. The midgut (lane 1), salivary gland (lane 2), ovary (lane 3), synganglion (lane 4), hemolymph (lane 5) and fat bodies (lane 6) were obtained from 4-day-fed female ticks. M, Protein molecular weight marker (Low).

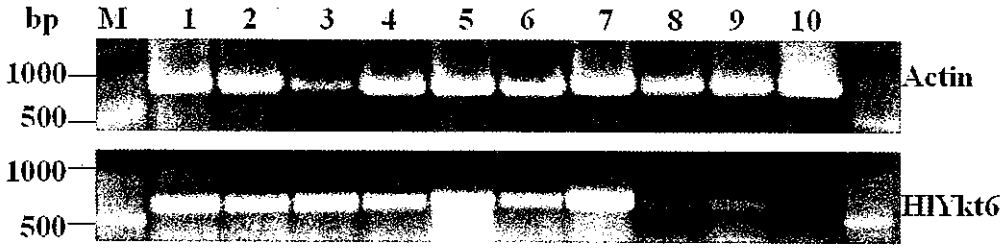

Fig. 10. Expression pattern of the HlYkt6 gene in *H. longicornis*.

Total RNAs extracted from eggs, partially fed ticks of different developmental stages (larvae, nymph, and adult female ticks) and different tissues of partially fed female ticks (including midgut, salivary glands, ovary, synganglion, hemolymph, and fat bodies) were subjected to RT-PCR analysis with HlYkt6-gene-specific primers, and an actin gene was amplified from panel samples as control. DNA markers in the base pair are indicated on the left. Lane 1, eggs; lane 2, larvae; lane 3, nymphs; lane 4, adult ticks; lane 5, midgut; lane 6, salivary glands; lane 7, ovary; lane 8, synganglion; lane 9, hemolymph; lane 10, fat body.

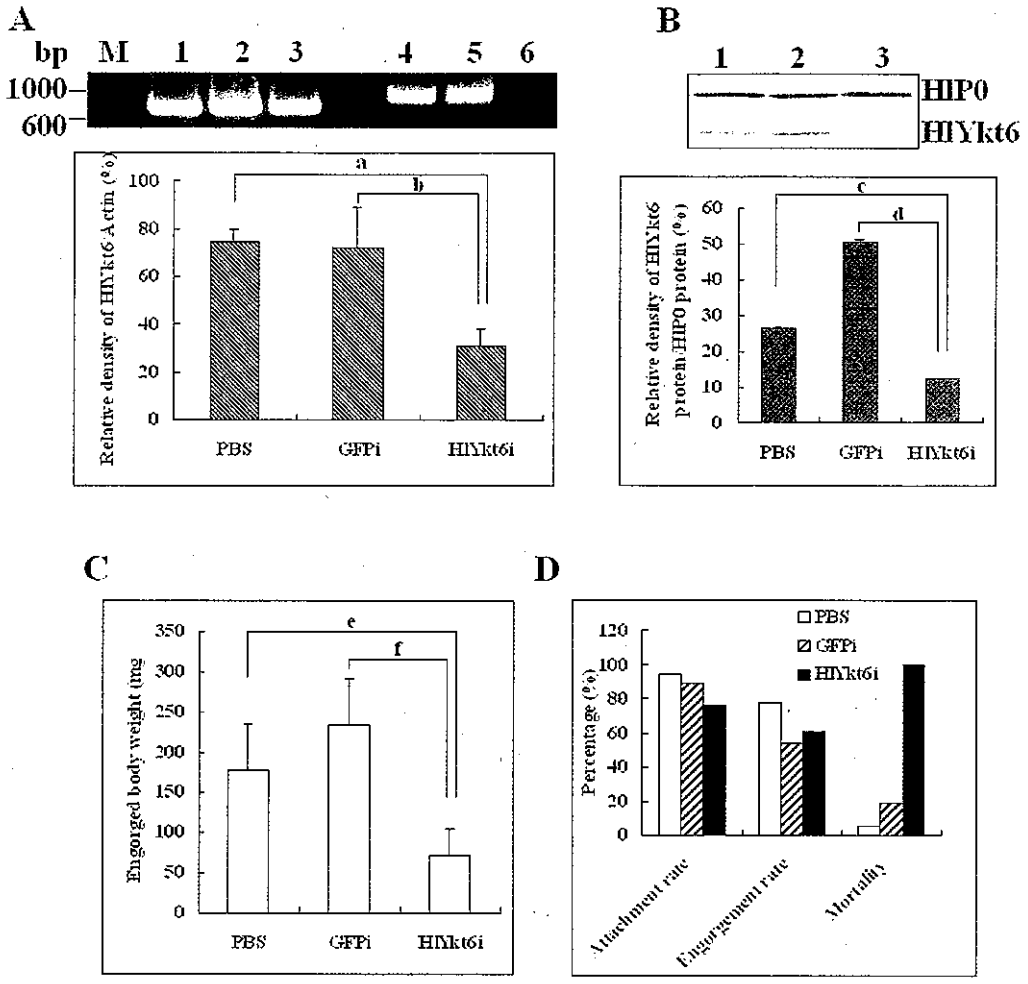

Fig. 11. Confirmation of RNAi of the HlYkt6 gene by RT-PCR and effect of silencing on the feeding and survival of adult female ticks.

A, HlYkt6 transcription products in the mRNA from 4-day-fed female ticks were investigated using RT-PCR with actin-specific (lanes 1, 2, and 3) and HlYkt6-RNAi primers (HlYkt6-RNAi-U and HlYkt6-RNAi-D, lanes 4, 5, and 6). Quantification of the band density of HlYkt6 cDNA is shown relative to actin (3 repeats, Student's *t*-test, a, $P< 0.001$; b, $P< 0.01$). Lanes 1 and 4, PBS-injected ticks; lanes 2 and 5, GFP dsRNA-injected ticks; lanes 3 and 6, HlYkt6 dsRNA-injected ticks. B, Confirmation

of the HlYkt6 gene interruption by Western blot analysis. The density of the HlYkt6 protein bands is shown as a ratio to the HlP0 protein (Student's *t*-test, c, $P< 0.0005$; d, $P< 0.0001$). C, Comparison of the engorged body weight of female ticks in each group (Student's *t*-test, e, $P< 0.001$; f, $P< 0.001$). D, Comparison of the attachment rate 2 days post-infestation, the engorgement rate, and the mortality in each group. HlYkt6 dsRNA-treated ticks showed higher mortality (100%) than PBS- (4.8%) and GFP dsRNA-treated ticks (20.4%).

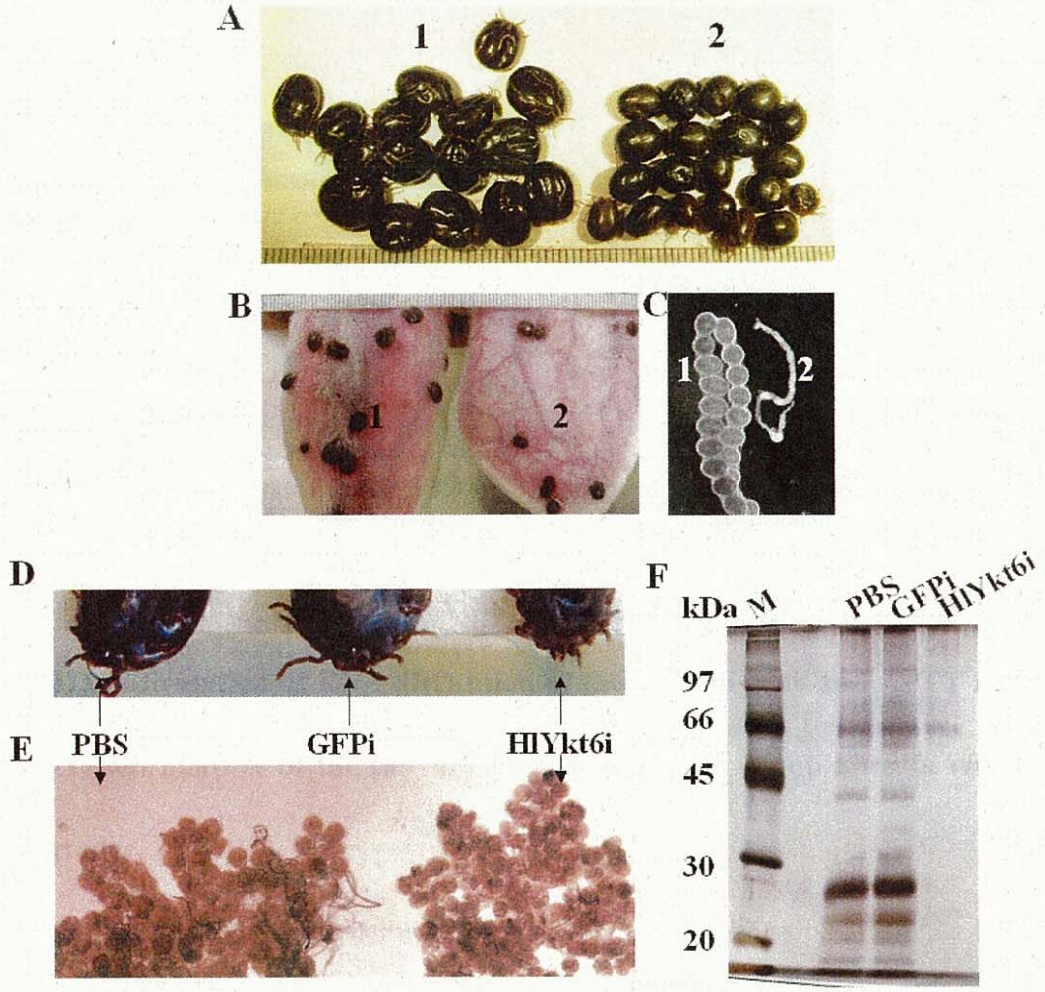

Fig. 12. Effect of HlYkt6 gene silencing on the morphology and saliva secretion of ticks.

A, Comparison of the size and shape of engorged adult female ticks in the PBS- and dsRNA-injected groups. B, Comparison of the size of the 4-day-fed female ticks in the PBS- and HlYkt6 dsRNA-injected groups. C, Comparison of the ovary from 2 days post-engorged female ticks. 1, PBS-injected ticks; 2, HlYkt6 dsRNA-injected ticks. D, Appearance of the saliva secretion on the mouth part of ticks from each group 20 min post-injection of pilocarpine liquid. E, Morphology comparison of the salivary glands from PBS- and HlYkt6 dsRNA-injected ticks. F, Silver staining of the proteins in the saliva collected from the dsRNA-injected and subsequent pilocarpine-injected ticks.

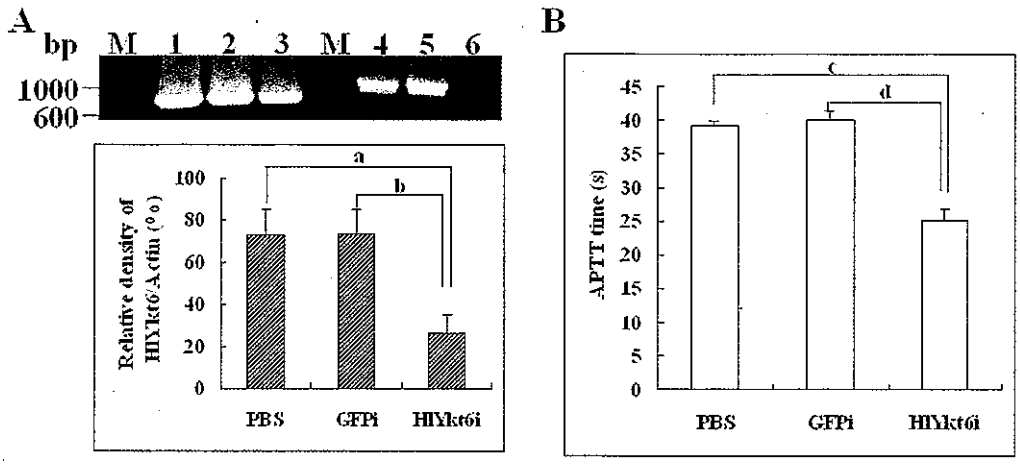

Fig. 13. Confirmation of RNA disruption of the HlYkt6 gene and APTT assay of the secretion mixture of the salivary glands from each group after *in vivo* RNA interference.

A, Confirmation of RNA silencing of the HlYkt6 gene in the salivary glands from the PBS- or dsRNA-injected adult female ticks by RT-PCR. The upper panel showed the RT-PCR result of the HlYkt6 mRNA from each group, and the lower panel demonstrated the quantification of the band density of HlYkt6 cDNA relative to actin. Lanes 1 and 4, PBS-injected ticks; lanes 2 and 5, GFP dsRNA-injected ticks; lanes 3 and 6, HlYkt6 dsRNA-injected ticks (a, $P< 0.0001$, b, $P< 0.0001$, Student's *t*-test). B, APTT assay of the secretion from salivary glands stimulated by PGE2. APTT time 39.25 ± 0.50 s, 40 ± 1.41 s, and 25.25 ± 1.50 s (c, $P< 0.001$, d, $P< 0.00001$, Student's *t*-test).

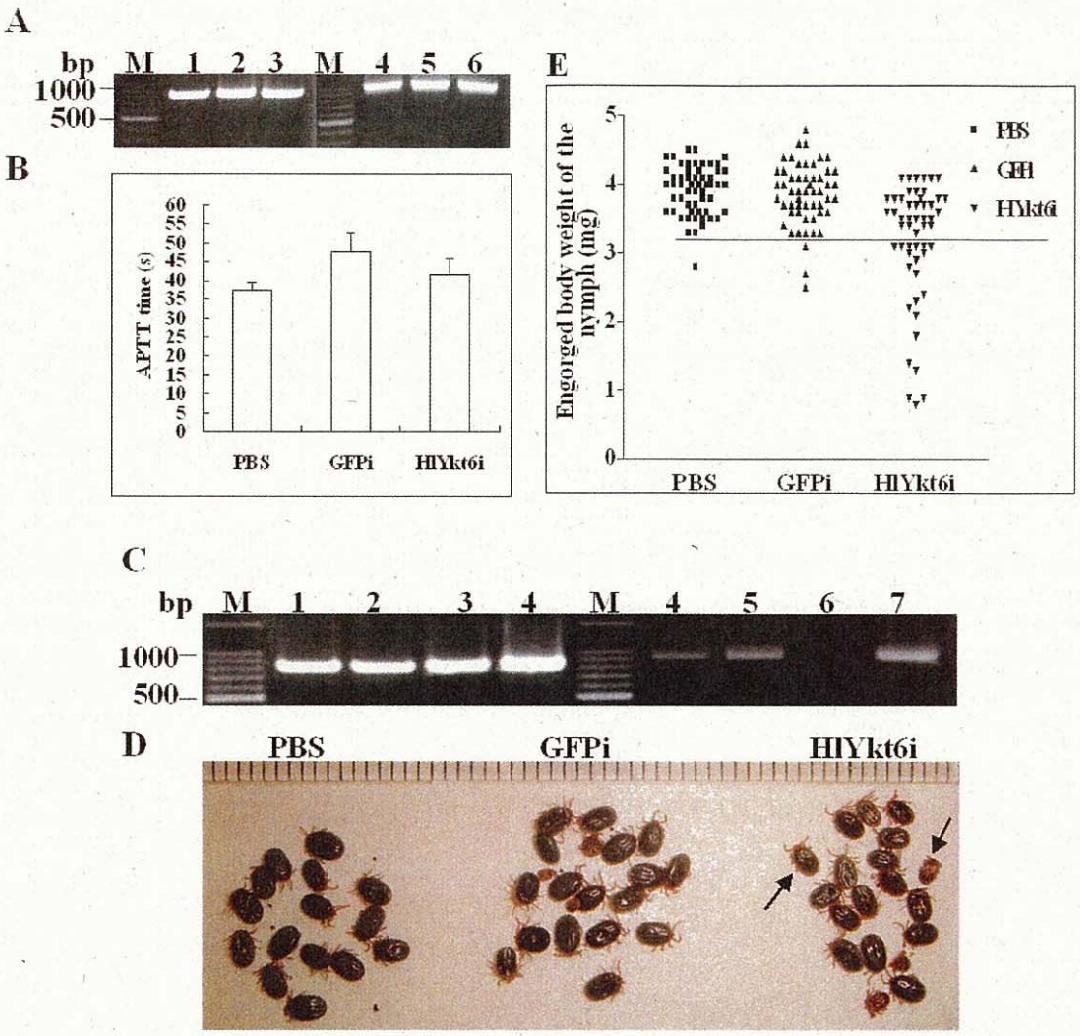

Fig. 14. *In vitro* RNAi of the HlYkt6 gene in the salivary glands and RNAi of the HlYkt6 gene in the nymphs.

A, Investigation of HlYkt6 transcripts in the mRNA from the salivary glands treated with PBS or dsRNA *in vitro*. Lanes 1 and 4, PBS-immersed salivary gland; lanes 2 and 5, GFP dsRNA-immersed salivary gland; lanes 3 and 6, HlYkt6 dsRNA-immersed salivary gland. B, APTT assay of the liquid from the PBS- or dsRNA-treated salivary glands *in vitro* was performed, and no significant difference

between the control and test groups was observed. C, RT-PCR of RNA from the engorged nymphs that had been immersed in PBS, GFP dsRNA, and HlYkt6 dsRNA. Lanes 1 and 4, PBS-immersed nymphs; lanes 2 and 5, GFP dsRNA-immersed nymph; lanes 3 and 6, HlYkt6 dsRNA-immersed nymphs (nymphs of low engorged body weight); lanes 4 and 8, HlYkt6 dsRNA-immersed nymphs (nymphs of normal engorged body weight). D, Size of the engorged nymphs immersed in PBS, GFP dsRNA, and HlYkt6 dsRNA. The smaller nymphs were marked with *arrows*. E, Engorged body weight of nymphs immersed in PBS, GFP dsRNA, and HlYkt6 dsRNA. Nymphs with low engorged body weight were separated from those with normal body weight by a *line*.

Chapter 3

Characterization of a carboxypeptidase inhibitor from the tick *Haemaphysalis longicornis*

3-1. Introduction

A secretory protein is synthesized in the ribosome, transported to the Golgi stack, and secreted out of the cell. Subsequently, it raises the questions that where it will localize and what function it will perform. To obtain a view of the function of the secretory proteins, a carboxypeptidases inhibitor associated with fibrinolysis is isolated for the further study in this chapter.

Carboxypeptidases (CP) are enzymes that play roles in the digestion process, i.e., carboxypeptidases A and B (CPA and CPB; Reynolds et al., 1989), the maturation of neuroendocrine peptide precursors (Fricker et al., 1989), and, probably, the control of peptide hormone activity. Inhibitors of CP bind to the active site groove of CPs by the C-terminal tail in a way that mimics substrate binding (Vendrell et al., 2000). The inhibitory functions of carboxypeptidase inhibitors to CPs suggest that they have functional significance in the regulation of the mechanisms described above. To date, only a few specific inhibitors that bind to metallo-carboxypeptidases have been identified, including inhibitors from potato and tomato (PCI; Hass et al., 1979), the intestinal parasite *Ascaris suum* (Homandberg et al., 1989), the medicinal leech (LCI; Reverter et al., 1998), and rat and human tissues (Normant et al., 1995). PCI has been

demonstrated to inhibit digestive CPs with inhibition constants in the nanomolar range; it was shown to inhibit a kind of regulatory carboxypeptidase called CPU or thrombin-activatable fibrinolysis inhibitor (TAFI), and hence it significantly accelerates the clot lysis induced by the tissue-type plasminogen activator (tPA) (Walker et al., 2003). Moreover, PCI from potato was indicated to block the development of carcinomas by binding to the epidermal growth factor receptor (EGFR), which plays a prominent role in the signal transduction pathway of carcinomas (Sitja-Arnau et al., 2005). Recently, a tick carboxypeptidase inhibitor (TCI) was isolated from *Rhipicephalus bursa*, and the structure and inhibitory functions of TCI against CPA, CPB and TAFI have been described in detail (Arolas et al., 2005a). However, the distribution of carboxypeptidase inhibitors in tick tissues and their significance in the feeding and reproduction of ticks have not been reported. In this chapter, a carboxypeptidase inhibitor from *H. longicornis* (HlTCI) was isolated from the ovary cDNA library of partially engorged female ticks. The expression pattern of the molecule and the inhibitory activity of the recombinant HlTCI protein (rHlTCI) were investigated.

3-2. Materials and methods

Ticks and tissues. The Okayama tick strain, *H. longicornis*, was provided by National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Japan. To obtain partially fed ticks, larvae and nymphs were infested on rabbit ears for 3 days and thereafter detached while adult ticks were

allowed to feed for 4 days. Any contaminated tissues of the host obtained by the ticks were removed using surgical forceps. The recovered adult ticks were then divided into three groups. One group was homogenized whole with liquid nitrogen, while another group was dissected and the desired organs collected and homogenized. All homogenized samples were stored in Trizol reagent (Sigma, USA) for RNA extraction. In the third group, 4-day fed adult ticks were dissected in PBS and the desired organs were sonicated to obtain the native proteins. All samples were stored at -80℃ until use.

Sequencing and analysis of HlTCI gene. A full-length ovary cDNA library was previously constructed from *H. longicornis* as described by Zhou et al. (2006). Briefly, the cDNA was synthesized from total RNA and ligated into the plasmid vector pGCAP1. The resulting plasmids were transformed into electrocompetent *E. coli* DH12S strain (Invitrogen, USA). A total of 10,000 recombinant transformants from the library were randomly selected and sequenced (Zhou et al, 2006). Based on the database of this constructed cDNA library, two clones with the inserts encoding HlTCI were selected and sequenced (ABI prism 310 Genetic Analyzer, USA) using T7 forward and T3 reverse primers. The resulting sequence was confirmed by sequencing four additional clones from the same library. The full-length cDNA sequence was then analyzed using basic local alignment search tool (BLAST) accessed through the national center for Biotechnology Information (NCBI: http://www.ncbi.nlm.nih.gov). The putative signal peptide cleavage site in the HlTCI amino acid sequence was predicted using the SignalP server

(http://www.cbs.dtu.dk/services/SignalP/). The three-dimensional model of HlTCI was constructed using the SWISS-MODEL software (Schwede et al., 2003). The template was TCI from *R. bursa* in a complex with bovine CPA (PDB code, 1ZLH). Whatcheck software was employed to evaluate the accuracy of the model.

***In vitro* expression of HlTCI.** Two expression primers were designed according to the open reading frame (ORF) of HlTCI nucleotide sequence excluding the signal peptide and including terminal restriction sites and used to perform PCR amplification. The PCR product was digested with *Bam*HI and *Eco*RI, subcloned into a pGEX-4T-3 expression vector (Amersham Pharmacia Biotech, USA), and then transformed into the *E. coli* BL21 (DE3) strain. One of the recombinant clones was sequenced to check for accurate insertion. This sequenced positive clone was induced by 1 mM IPTG with 0.1% glycerol in the medium. The *E. coli* were collected, lysed in 1% Triton X-100-PBS, sonicated, and centrifuged. Subsequently, the supernatant containing the GST-fused HlTCI was collected and purified with glutathione-Sepharose 4B beads (Amersham Pharmacia Biotech, UK) according to the manufacturer's instructions. The concentration of purified recombinant protein was determined using a BCA protein assay kit (Pierce, USA).

Preparation of the anti-rHlTCI serum. One hundred micrograms of rHlTCI for one mouse was completely mixed with an equal volume of Freund's complete adjuvant (Sigma, USA) and intraperitoneally injected into mice (ddy, 8 weeks old; SCL, Japan). The last two times of immunization were performed at days 14 and 28 with the same dose of recombinant protein in Freund's incomplete adjuvant. Eight

days after the third injection, the titer of the antibodies was evaluated using ELISA, and, subsequently, all the sera were isolated from the blood of the mice.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. Salivary gland, midgut, ovary, synganglion and hemolymph from 4-day-fed ticks were homogenized. The same amount of protein lysates (about 1,000 ng/lane) were mixed with 2 x concentrated sample buffer (containing 5% 2-mercaptoethanol) and boiled at 100℃ for 5 min. All samples were electrophoresed on 18% polyacrylamide gel and then transferred to Immobilon P^{SQ} polyvinylidene difluoride membrane (PVDF membrane; Millipore, USA). The BenchMark Pre-Stained Protein ladder (Invitrogen, USA) was employed to confirm the transfer. The membrane was then blocked using 3% skim milk in PBS-Tween 20 (PBST, 0.5% Tween 20 in PBS) for 1 h and subsequently incubated with anti-rHlTCI (1:50), which had been previously pre-incubated with bacterial lysate containing GST protein. After washing three times with PBST, the membrane was soaked in peroxidase-conjugated goat anti-mice immunoglobulin G (IgG, 1:2,000) and incubated at 37 ℃ for 1 h. Finally, the membrane was washed and 3,3'-diaminobenzidine tetrahydrochloride and H_2O_2 were added to the membrane to detect the native protein.

Expression analysis of HlTCI by reverse-transcription PCR (RT-PCR). Total RNA was extracted from eggs and unfed ticks including larvae, nymphs, and adult ticks using Trizol reagent. The RT-PCR was carried out at 50℃ for 60 min to synthesize the first-strand cDNA, followed by 94℃ for 2 min and 30 cycles of 94℃

for 30 s, 55℃ for 30 s, and 72℃ for 60 s, finally 72℃ for 7 min, in which the gene-specific primers (sense primer, 5'-CCGGATCCGACGTGAACGACTGTGTCAGC-3'; anti-sense primer, 5'-CTGAATTCGTGTCGTAATACTTCGGGCCAG-3') were used. In addition, primers designed for the actin gene (Zhou et al., 2006) in ticks were adopted to check the integrity of the extracted RNA. RNA from partially fed larvae, nymphs, and adult ticks was subjected to the same RT-PCR amplification. In order to investigate the distribution of HlTCI mRNA in tissues of adult ticks, RNA extracted from midgut, salivary gland, synganglion, ovary, and hemolymph from 4-day-fed adult ticks was analyzed by RT-PCR amplification. All the RNA samples were confirmed to be free of genomic DNA by performing PCR using the same primers under the same conditions mentioned above.

Inhibitory assay of rHlTCI. The inhibitory activity of GST-fused rHlTCI was evaluated by measuring the residual activity of CPA and CPB after incubation with the inhibitor. Hydrolysis of Hippuryl-L-Phenylalanine by CPA (EC 3.4.17.1; Sigma, USA) and of Hippuryl-L-Arginine by CPB (EC 3.4.17.2; Sigma, USA) was measured by monitoring absorbance at 254 nm. Substrates were freshly dissolved in a Tris-HCl buffer (20 mM, pH 7.5) containing 500 mM NaCl to make a 1 mM solution. Various concentrations of rHlTCI were pre-incubated with CPA or CPB for 3 min at 25℃, then added to the substrates, immediately mixed, and the increase of A_{254} was recorded. The effect of the temperature on the inhibitory activity of rHlTCI was measured by incubating rHlTCI at 25℃, 50℃, 75℃, and 95℃ for 10 min before

mixing with CPA and then checking the absorbance of the liquid at 254 nm. All these experiments were conducted with GST protein as the negative control and PCI (Sigma, USA) as the positive control.

Clot lysis assay. The clot lysis assays were carried out as described previously (Walker et al., 2003; Arolas et al., 2005a) with slight modifications. Briefly, the assay system consisted of three parts. The first part consisted of 25 μl of 0.02 M Hepes, pH 7.4, 0.15 M NaCl, 0.01% Tween 20 (HBST) plus $CaCl_2$ (final concentration, 10 mM), thrombin (5 nM), tPA (1.2 nM) and thrombomodulin (TM, 10 nM). rHITCI and PCI were diluted into variable concentrations (0-2,000 nM) and added into the mixture. The clot formation and lysis assay were started by the addition of 35 μl of human standard plasma (DADE BEHRING, Japan). The entire experiment was performed in 96-well microtiter plates, which were incubated at 37℃ and sealed with clear tape to avoid evaporation. The absorbance of the mixture at 415 nm was monitored every 2 minutes.

Anti-bacterial activity assay of rHITCI. Seven kinds of bacteria were employed to evaluate the defensin-like effect of rHITCI. Four Gram-positive bacteria (*Staphylococcus aureus* ATCC 6538P, *Micrococcus luteus* JCM 1464, *Bacillus megaterium* JCM 2506, and *Bacillus subtilis* JCM 1465), and three Gram-negative bacteria (*Pseudomonas aeruginosa* IFO 3080, *Salmonella typhimurium* ATCC 13311, and *E. coli* O157 ATCC 35150) were incubated in LB medium at 37℃ overnight, diluted by 1:100, and then distributed in 96-well plates with 100 μl per well. One hundred microliters LB containing 0, 0.1, 1, and 10 μM rHITCI or GST protein was

added to the plates respectively. After incubation for 0, 1, 2, 4, 6, 12, 36, and 48 h, OD_{600} of the liquid culture was recorded in each case.

3-3. Results

Cloning and sequence analysis of HlTCI. As shown in Fig. 15A, the full-length cDNA of HlTCI with a total length of 479 bp consists of a 59-nt 5' untranslated sequence, an ORF of 291 bp, and a 129-nt 3'-untranslated region (GenBank accession number, EF197978). The polyadenylation signal, AATAAA, is detected upstream of the poly (A)+ tail. The ORF encodes 96 amino acid residues including a predicted 19-residue signal peptide, and the predicted mature protein has a molecular mass of about 8.4 kDa. The putative HlTCI protein has an N-glycosylation site at amino acid residue Asn82. A database search using BlastP (WWW.ncbi.nlm.nih.gov/blast) showed 63.9% identity of the HlTCI-deduced protein sequence to TCI isolated from another tick species, *R. bursa* (GenBank accession number, AY794405; Arolas et al., 2005a) (Fig. 15B). The predicted protein is a cysteine-rich peptide in which 12 cysteines are assumed to be responsible for the formation of six disulfide bridges based on the homology of HlTCI to TCI. The predicted hydrophobicity of HlTCI is highly consistent with that of TCI from *R. bursa*. Based on the high homology, identical number and site of cysteines, and similar hydrophobicity, HlTCI was assumed to adopt a similar three-dimensional structure to the previously determined *R. bursa* TCI (Prevot et al., 2006). Therefore, a three-dimensional model of HlTCI was built according to the experimentally

determined structure of TCI from *R. bursa* using SWISS-MODEL software (Fig. 15C) (Schwede et al., 2003; Arnold et al., 2006). The model was tested using Whatcheck software; the final total energy was -2454.702 KJ/mol, and the root mean square deviation (RMSD) between the HlTCI model and its template was 0.26 (on 74C). The C-terminal amino acid sequence (Cys-Cys-Val-Trp-Leu-His75) of TCI from *R. bursa* was substituted by Cys-Cys-Val-Leu-Leu-Glu77 in HlTCI. The disulfide conformation of both HlTCI and TCI from *R. bursa* is similar to that of members of the β-defensin-fold family, which suggests an anti-bacterial activity (Arolas et al., 2006).

Expression of rHlTCI *in vitro*. The cDNA fragment encoding the HlTCI-deduced mature protein was ligated into the expression vector pGEX-4T-3. The plasmid from the identified positive clone was then transformed into *E. coli* BL21 strain. After induction by 1 mM IPTG and sonication, rHlTCI was predominantly detected in the supernatant. The soluble recombinant protein with an expected molecular size of 34.4 kDa was purified using Sepharose 4B columns, to produce a pure rHlTCI solution with a concentration of 2.308 mg/ml (Fig. 16).

Identification of native protein in the ovary. After immunizing mice three times with rHlTCI, anti-rHlTCI sera were collected and pre-absorbed by GST lysate, and then used as the primary antibodies for the detection of the native protein in different tissues from partially engorged female ticks fed for 4 days. As shown in Fig. 17, four bands in the ovary were recognized by anti-rHlTCI serum, in which the size of lowest band was about 8.4 kDa. However, in the lysate of midgut, salivary gland,

synganglion and hemolymph, no band was detected.

Expression pattern of HlTCI in *H. longicornis*. Total RNA was extracted from ticks in various stages of development, including partially fed and unfed, and, simultaneously, tissues from 4-day fed adult female ticks were extracted. RT-PCR using HlTCI-specific primers showed that HlTCI was only expressed in the partially engorged female ticks (Fig. 18A and B) and particularly localized in the ovary (Fig. 18C and D). Actin-specific primers were used as controls. Omission of the reverse transcriptase step, followed by PCR, confirmed that the detected bands were amplified from RNA, and not genomic DNA (data not shown).

Inhibitory activity of rHlTCI to digestive carboxypeptidase. To determine the inhibitory activity of rHlTCI , the enzymes CPA and CPB were separately incubated with the inhibitor for 3 min at 25℃, after which the residual enzyme activity was determined using the appropriate substrate. As shown in Fig. 19A, higher concentrations of rHlTCI led to lower residual CPA activity. When the concentration of rHlTCI was 100 μM, CPA was almost entirely inhibited. This inhibitory activity was temperature-dependent. The inhibitory activity decreased by 58% at 75℃, and, after incubation at 95℃ for 10 min, rHlTCI entirely lost its inhibitory activity, whereas PCI retained its inhibitory activity (Fig. 19B). The hydrolytic activity of CPB against substrate Hippuryl-L-Arginine was also inhibited by rHlTCI in a dose-dependent manner (Fig. 19C).

Effect of rHlTCI on clot lysis. The lysis of clots that formed from standard human plasma was carried out in the presence of various concentrations of PCI or

rHlTCI. As expected, 500 nM of both PCI (Fig. 20A) and rHlTCI (Fig. 20B) clearly accelerated clot lysis, namely fibrinolysis. In contrast, the same concentration of the negative control GST had no effect on clot lysis. A lower concentration (25 nM) of PCI significantly prolonged clot lysis (Fig. 20A); however, the prolongation effect of rHlTCI on clot lysis was much less pronounced (Fig. 20B). Varying concentrations of PCI and rHlTCI (0-2,000 nM) were used to evaluate the effect of inhibitor concentration on clot lysis time. Depending on the concentration used, PCI displayed prolongation or shortening or no effect on the clot lysis time (Fig. 21). Clot lysis time was prolonged 1.17 fold when a low concentration of PCI (25 nM) was used. The same concentration of rHlTCI had a slight delaying effect on clot lysis but was not as obvious as that with PCI. When the concentration of rHlTCI was increased ($\geqslant$250 nM), the clot lysis was accelerated by 1.1-2.1 fold. The effect of rHlTCI on clot lysis was consistent with that in previous studies using PCI (Walker et al., 2003) and TCI from *R. bursa* (Arolas et al., 2005a).

Anti-microbial activity assay of rHlTCI. According to earlier reports (Arolas et al., 2005b, 2006), the 3-D structure of TCI from *R. bursa* shares similar domains and disulfide patterns with molecules of the β-defensin-fold family, such as venom toxin and human β-defensin-2. Several anti-microbial defensins from vertebrates belong to this family. The pattern and number of cysteine residues of HlTCI were expected to be quite similar to those in TCI. Therefore, four strains of Gram-positive bacteria (*S. aureus*, *M. luteus*, *B. megaterium*, and *B. sublitis*) and three strains of Gram-negative bacteria (*P. aeruginosa*, *S. typhimurium*, and *E. coli* O157) were

employed to evaluate the possible anti-bacterial function of rHlTCI; however, the growth of bacteria was not inhibited by the addition of rHlTCI (data not shown).

3-4. Discussion

I have isolated and characterized a carboxypeptidase inhibitor HlTCI from *H. longicornis*. The cDNA sequence of HlTCI was predicted to encode a 19-residue signal peptide and a 77-residue mature protein. The putative amino acid sequence of HlTCI shows 63.9% homology to TCI isolated from *R. bursa*. The mature protein contains 12 cysteines, as does that of TCI from *R. bursa*, which are predicted to form six disulfide bridges. Based on the similar hydrophobicity, similar cysteine pattern, and high homology of HlTCI to TCI from *R. bursa*, a 3-D structure model of HlTCI was constructed using the SWISS-MODEL software. It has been demonstrated that when target and template share >50% identical residues, the automated sequence alignments are sufficiently reliable (Rost et al., 1999). In this chapter, deduced amino acid sequence of HlTCI exhibited 63.9% homology to the template, and hence the 3-D structure model of HlTCI was constructed by the automated mode. The structures of both molecules are similar to those of members of the β-defensin-fold family.

The rHlTCI protein was purified and used to generate anti-sera. Native proteins of various tissues from partially engorged ticks were subjected to Western blot analysis. Four bands in the lysate of ovary were detected by immunoblotting using anti-rHlTCI serum, while no band was observed in other tissues. This result suggests that HlTCI performs a special function in the ovary of ticks. However, the presence in

the gel of several bands in the ovary extract indicates that in addition to the monomeric form, native HlTCI protein may form oligomers. A similar case was also observed for other disulfide-rich proteins such as C-terminal portion of porcine submaxillary mucin (Perez-Vilar et al., 1996).

In previous studies, TCI was supposed to contribute to the maintenance of blood liquidity during tick feeding by stimulation of fibrinolysis (Arolas et al., 2005a). Host blood interacts directly with tick saliva, into which many potent pharmacologically active components are secreted by the tick's salivary gland (Wikel et al., 1994). However, in this chapter, RT-PCR demonstrated that HlTCI mRNA is found specifically in the ovary of fed ticks. Transcripts of HlTCI were too low to be detected in other tissues. It is difficult to speculate that HlTCI is synthesized in ovary and moves into saliva to play roles in fibrinolysis. In plants, it has been suggested that carboxypeptidase inhibitors were involved in plant defense against insect attack (Graham and Ryan, 1981). A tomato metallocarboxypeptidase inhibitor (MCPI) was found to be expressed in the anthesis stage ovaries at a very high level and decrease quite rapidly during fruit development (Martineau et al., 1991). Similarly, HlTCI may play protective roles in preventing tick ovaries from invasions by parasites, bacteria, and spirochetes as they accommodate and transmit these pathogens. However, further evidence will be required to support this suggestion.

In the present chapter, rHlTCI showed inhibitory activity to digestive CPA and CPB, which is in agreement with previous reports about carboxypeptidase inhibitors from leech (LCI; Reverter et al., 1998) and *R. bursa* (TCI; Arolas et al., 2005a).

However, GST appears to affect the stability of rHlTCI, and the inhibitory activity of rHlTCI decreased with the increase of the temperature treatment (Fig. 19B). When the temperature was raised to 95℃, the activity of rHlTCI completely disappeared. In contrast, the inhibitory activity of PCI maintained stable. Circular dichroism (CD) and nuclear magnetic resonance (NMR) spectroscopy measurements showed that recombinant TCI from *R. bursa* free of a vector protein retained a well-folded conformation over a wide range of temperatures (Arolas et al., 2005a), which might suggest that the inhibitory activity of recombinant TCI remained unaffected with the increase of temperature treatment. It is possible that the sensitivity of rHlTCI to temperature probably is due to the presence of GST in the fusion protein. As has been reported, TCI from *R. bursa* have two domains called Nt domain and Ct domain. These two domains can fold independently without any cooperation between them and without any fundamental role of the connecting loop in the overall folding. The Nt domain is more sensitive to temperature than Ct domain (Arolas et al, 2006). In this chapter, the presence of the GST amino acid sequence at the N-terminal of rHlTCI might affect the folding of the Nt domain, and hence affect its inhibitory activity and temperature resistance.

Activation of coagulation generates thrombin and subsequently results in the conversion of fibrinogen to fibrin. The C-terminal lysine residues of fibrin act as the ligands for plasminogen. Activation of plasminogen by a plasminogen activator (tPA) leads to the breakdown of fibrin, in a process called fibrinolysis. An active form of thrombin-activatable fibrinolysis (TAFIa) prolongs the clot lysis time by removing the

lysine-residue of fibrin (Bouma et al., 2003). In previous studies, PCI, LCI, and TCI from *R. bursa* were demonstrated to inhibit TAFIa, and hence, to accelerate or prolong fibrinolysis (Walker et al., 2003; Arolas et al., 2005a). In this chapter, rHLTCI showed a similar effect, accelerating lysis when the concentration of the protein was high (≥250 nM), but in contrast to the case for PCI, reducing the concentration of rHlTCI had no effect or only slightly increased the clot lysis time (Fig. 21). It is possible that rHlTCI may have therapeutic potential as a medicine to treat thrombotic disorders caused by abnormal TAFI function.

3-5. Summary

A gene named HlTCI, which showed highest homology to the carboxypeptidase inhibitor (TCI) was isolated from another ixodid tick, *R. bursa*. HlTCI was specifically expressed in the ovary from partially engorged adult ticks. The recombinant protein of HlTCI (rHlTCI) with glutathione *S*-transferase (GST) showed inhibitory activity against digestive metallocarboxypeptidases A and B, but the activity was affected by the increase of the temperature treatment. High concentrations of rHlTCI were shown to significantly accelerate fibrinolysis *in vitro*. This effect of rHlTCI on clot lysis suggests its promising potential for use in some thrombotic disorders.

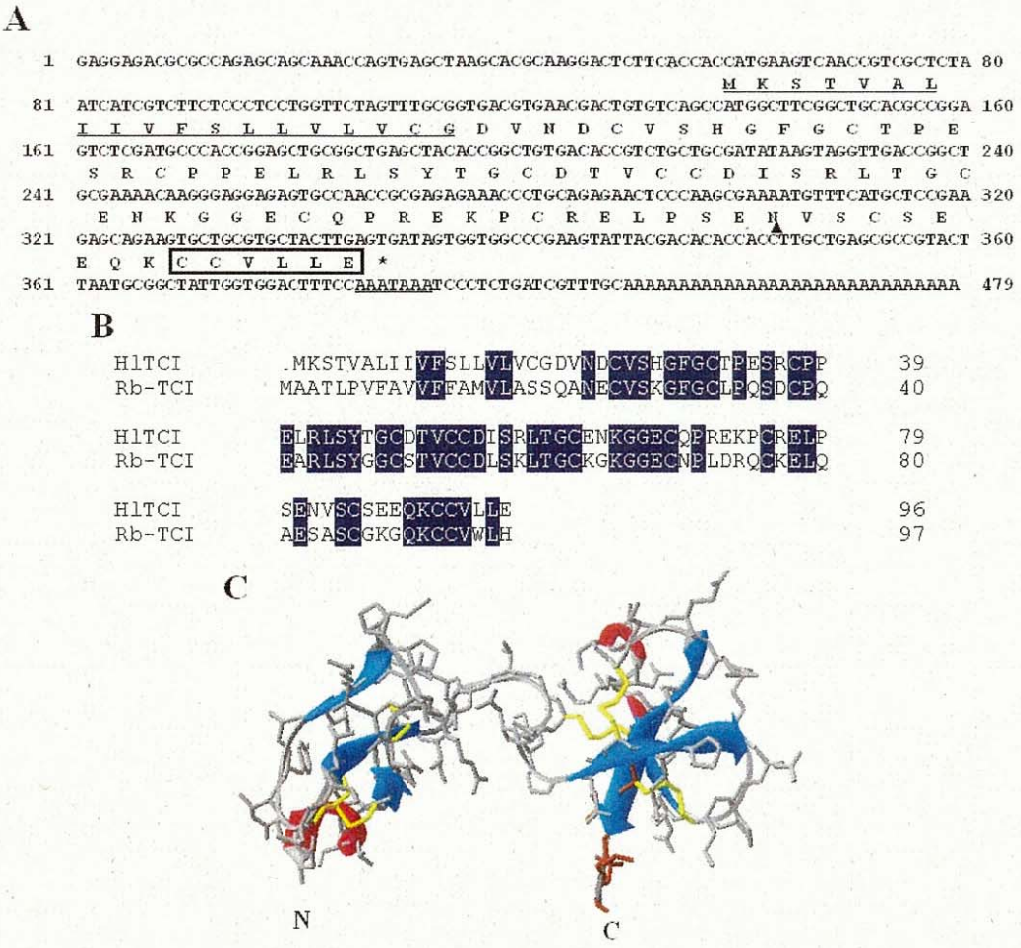

Fig. 15. Analysis of the HlTCI sequence.

A, Nucleotide and deduced amino acid sequence of HlTCI. The potential glycosylation site is marked with a *closed triangle*. The C-terminal residues with potential activity are marked with a *box*. The stop codon is marked with a *star*. The predicted signal peptide and polyadenylation signal are *underlined*. B, Alignment of the deduced protein sequence of HlTCI with that of TCI from *Rhipicephalus bursa*. The abbreviations are: HlTCI, TCI from *H. longicornis*; Rb-TCI, TCI from *R. bursa*. Identical amino acids are marked with *black boxes*. C, Three-dimensional structure model of HlTCI. Six disulfide bridges are shown in yellow. The coils are shown in red, and β-strands are shown in lightblue. The C-terminal residues are shown in brown.

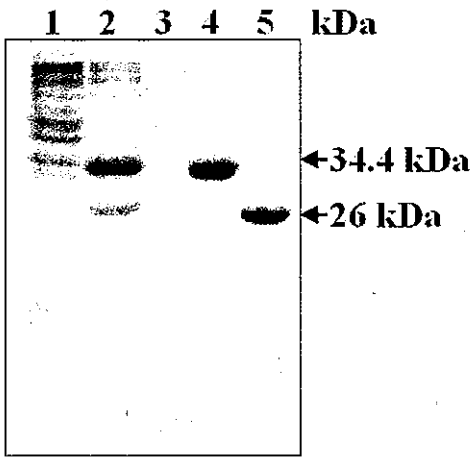

Fig. 16. Purification of rHlTCI protein.

Lane 1, bacteria protein without induction; lane 2, total protein of positive clone induced by IPTG; lane 3, Protein molecular weight marker (Low); lane 4, purified rHlTCI protein; lane 5, GST protein.

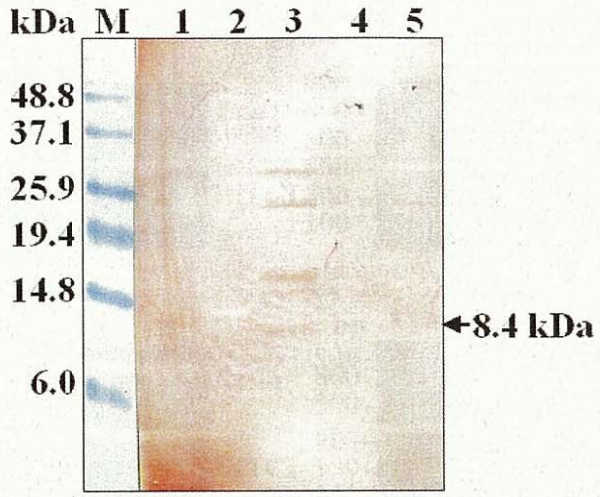

Fig. 17. Identification of native HlTCI by Western blot analysis.

The anti-rHlTCI serum was pre-absorbed by lysate of bacteria containing GST protein and then used as the first antibody to detect the native protein in various tissues from partially fed ticks. The lowest band (monomer of HlTCI) in the lysate of ovary was shown with an *arrow*. M, Pre-stained protein ladder; lane 1, salivary gland; lane 2, midgut; lane 3, ovary; lane 4, synganglion; lane 5, hemolymph.

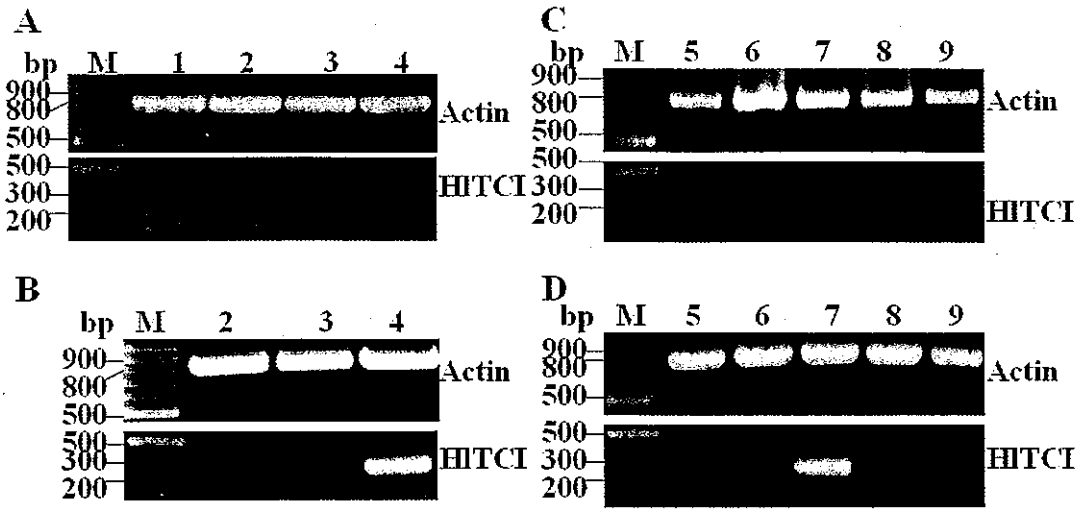

Fig. 18. Expression analysis of HlTCI in ticks by RT-PCR.

A, RT-PCR of HlTCI in egg, unfed larvae, unfed nymphs, and unfed adult female ticks. B, RT-PCR of HlTCI in partially fed larvae, nymphs, and adult female ticks; HlTCI was found to express in the 4-day-fed adult ticks. Lane 1, eggs; lane 2, larvae; lane 3, nymphs; lane 4, adult female ticks. C, RT-PCR of HlTCI in salivary gland, midgut, ovary, synganglion, and hemolymph from unfed adult female ticks. D, RT-PCR of HlTCI in different tissues from 4-day-fed female ticks; HlTCI was detected in the ovary. Lane 5, salivary gland; lane 6, midgut; lane 7, ovary; lane 8, synganglion; lane 9, hemolymph. 100 bp DNA Ladder (M) was used as a DNA marker.

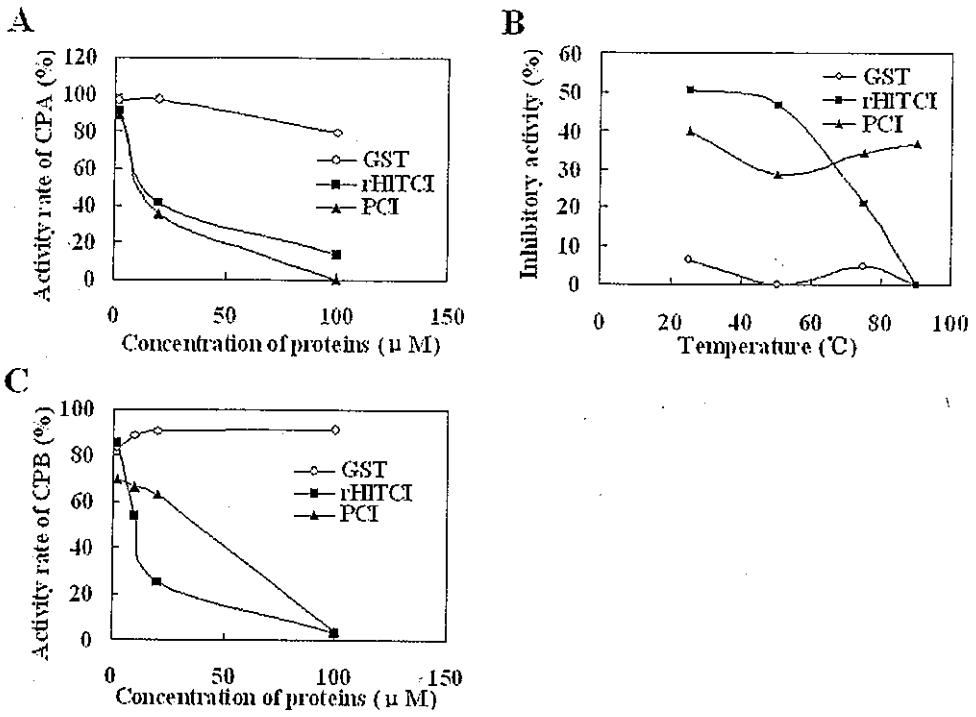

Fig. 19. Inhibitory effect assay of rHITCI.

A, 2 μM, 20 μM, 100 μM GST, rHITCI, or PCI was mixed with CPA and incubated at 25℃ for 3 min. Subsequently, the substrate was added, and the absorbance was immediately checked at 254 nm. The residue activity of CPA was calculated. B, The effect of the temperature on the inhibitory activity of rHITCI was investigated by incubating the GST, rHITCI, and PCI at 25℃, 50℃, 75℃, and 95℃ for 10 min respectively and then checking A_{254} of the mixture of above proteins, CPA and substrate. C, 2 μM, 10 μM, 20 μM, 100 μM GST, rHITCI, or PCI was mixed with CPB and incubated at 25℃ for 3 min. Subsequently, the substrate was added, and the absorbance was immediately checked at 254 nm. The residue activity of CPB was calculated.

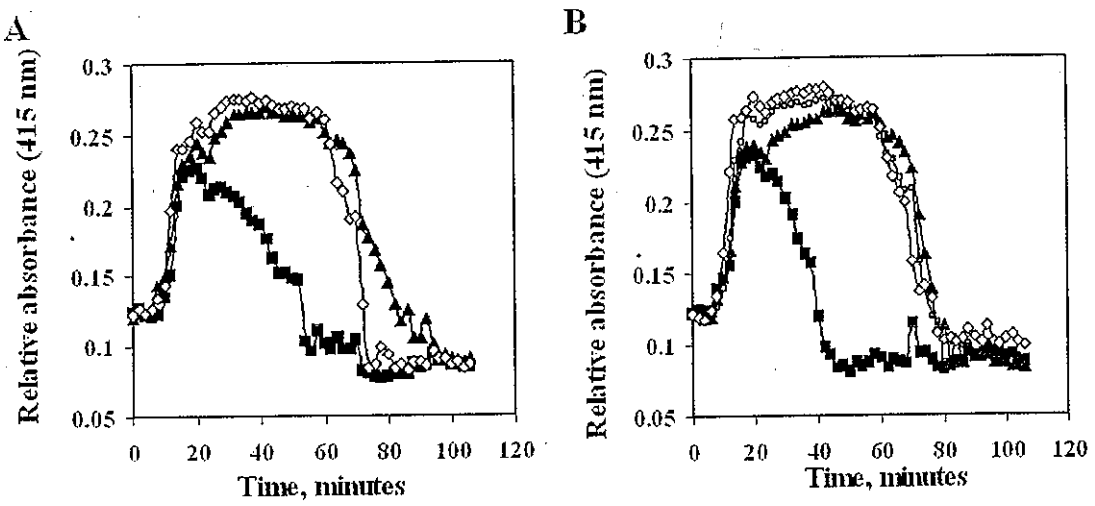

Fig. 20. Effect of rHITCI and PCI on clot lysis.

Plasma was added to the mixture composed of $CaCl_2$ (final concentration, 10 mM), thrombin (5 nM), tPA (1.2 nM), and thrombomodulin (TM, 10 nM) in the absence (*open diamond shapes*) or presence of PCI (A) or rHITCI (B). Both proteins enhanced clot lysis at high concentrations (500 nM, *closed squares*) and retarded the process at low concentrations (25 nM, *closed triangles*). However, the vector protein GST (*open circles*) showed no effect on clot lysis.

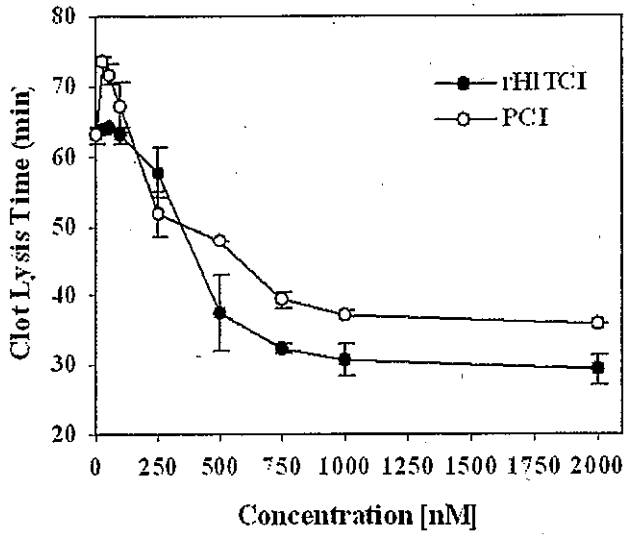

Fig. 21. Effects of rHlTCI and PCI on the clot lysis time.

Effect of rHlTCI (*closed circles*) and PCI (*open circles*) on clot lysis in the presence of tPA (1.2 nM) and TM (10 nM). The clot lysis time was defined as the time required for the degradation of clots by 50%.

General discussion

As a successful family that exists on the world for thousands of years, ticks are well evolved to adapt to a blood-feeding life (Mans and Neitz, 2004). This evolution can be elucidated as the development of the anticoagulant proteins (Maritz-Olivier et al., 2007) to block the coagulation of the host blood, the immunomodulant molecules to avoid the attack of the host immune system, the digestive enzymes to digest the blood meal for the nutrient (Miyoshi et al., 2008). Due to the perfect development of ticks and diversification of some molecules among the different tick species, it is difficult to find a unique effective molecule to control all kinds of ixodid tick species. Even through the Bm86 is a good vaccine for *R.* (*B.*) *microplus*, it has no significant effect on some other tick species (de Vos et al., 2001). Therefore, to explore some proteins or peptides conserved among the tick species but in heterology from the hosts appears to be an encouraging route for the development of vaccines to control various tick species. In this study, I have endeavored to identify some proteins from the ixodid tick *H. longicornis* that show high homology among different tick species (HlP0 and HlTCI) and a conserved molecule (HlYkt6) among eukaryote. Although they may show high homology to the host also, the variably encoded fragments in these molecules are the probable vaccine targets. In the eukaryote, the proteins are synthesized in the ribosome, then transported to the Golgi stock and finally secreted out of the tick cells. In this study, three proteins involved in this cascade, ribosomal HlP0 associating with protein synthesis, HlYkt6 attending the protein transport and a final protein product, HlTCI were isolated and identified.

HlP0 protein is a ribosomal protein that forms complex with P1 and P2 to execute their function in protein synthesis (Shimizu et al., 2002). In addition, dephosphorylated P0 detaches from the ribosome and is transported to the nucleus, and involved in the DNA repair (Sanchez-Madrid et al., 1981; Yacoub et al., 1996). The multi-functional characters of HlP0 protein provide a good explanation for the surprisingly high mortality (96%) of HlP0 dsRNA-injected ticks (Fig. 6D in Charpter 1). As for the other two members in the complex, P1 and P2 modulate cytoplasmic translation by influencing the interaction between subunits, thereby regulating the rate of cell proliferation. The loss of P1/P2 proteins produced a decrease in the growth rate of cells, as well as an altered polysome pattern with reduced translation efficiency, but without affecting the free 40 S/60 S subunit ratio (Martinez-Azorin et al., 2008). Nevertheless, the silencing of ribosomal P0 gene led to a disability of the large subunit, and subsequently the inhibition of the cell growth or apoptosis in the yeast (Santos and Ballesta, 1994). Even though I didn't make an investigation on the ribosome from the tick, I can hypothesize that the cell proliferation in ticks was impacted or even blocked based on the failure of the acini in the salivary gland for extending and the cuticle for proliferating in the HlP0 dsRNA-treated ticks (Fig. 7 in Chapter 1).

As a conserved gene, HlP0 showed high homology to other P0 gene from other species, but the C-terminal of the motif II showed variation among the different species except for another ixodid tick species (Fig. 1B in Chapter 1). Based on this, maybe I can obtain two hints. The first, the difference of the peptide at the C-terminal

may be utilized for vaccine development since the vaccination with a peptide of C-terminal domain from *P. falciparum* protected mice against a malaria parasite challenge (Rajeshwari et al., 2004). The second, HlP0 gene showed a remarkably high homology to the other tick species (94.7%), including the C-terminal, which hints that a good vaccine developed from the HlP0 protein may provide potent protection against other tick species also, thus resolve the problem that confine the use of the vaccine Bm86. In the same way, HlTCI may provide an excited effect against the infestation of various tick species benefiting from the high homology of inner-species and low homology of inter-species (Fig. 15B in Chapter 3). As well known, cattle are always infested by difference tick species (de Castro et al., 1989), and one vaccine with effect on one tick species won't give the same potent effective result against various tick species as acaricides. Therefore, studies on these inner-species conserved but inter-species varied proteins or peptide fragments will contribute to the development of vaccine against multi tick species.

After the proteins are synthesized, they will be transported from the ribosome to the Golgi stock, the SNAREs are responsible for this process, which include t-SNAREs and v-SNAREs. Each group is composed of a series of proteins, i.e. *Sed 5*, *Bet 1*, *Bos 1* (t-SNARE), and *Sec22*, *Ykt6* (v-SNARE) in *S. cerevisiae* (Jahn and Scheller, 2006). Ykt6 is proved to be a core constituent of membrane fusion machineries (Chen et al., 2005). The knock-down of the HlYkt6 gene resulted in the death of the adult ticks (Fig. 11D in Chapter 2) but not the nymphs, which hints that lack of HlYkt6 protein is lethal to the adult ticks but not to nymphs even though this

interference effected the engorged body weight of nymphs (Fig. 14E in Chapter 2). However, I failed to completely silence the HlYkt6 gene in the nymphs, which may be improved through modifying the injected buffer that suitable for dsRNA absorption or the dehydration condition. I executed a first trying to interfere a gene in nymphs by immersing ticks in liquid instead of injection.

Silencing of both the HlP0 gene and HlYkt6 gene decreased the engorged body weight of adult ticks, but it seems that the disruption of HlP0 protein blocked the blood-feeding from the original step, the synthesis of the regulatory proteins and enzymes necessary for the ingestion of blood, while destroying of HlYkt6 mRNA obstructed the cascade in the middle process, namely the transport of those proteins mentioned above. The appearance of the dsRNA-silenced adult ticks showed significant difference between the HlP0 (Fig. 6 in Chapter 1) and HlYkt6 genes (Fig. 12C in Chapter 2). This difference may be explained by the reason that there is a substitution for HlYkt6 molecule as described previously in *S. cerevisiae* (Jahn and Scheller, 2006) while none for HlP0 gene. However, the substitution is not complete because HlYkt6 has at least one additional non-SNARE function in homotypic vacuolar fusion (Rossi et al., 2004), which may lead to the failure of the secretion of a serious of digestive enzymes and, thus, the final death of the fed ticks. Nevertheless, the detailed mechanism needs more investigation.

The proteins secreted from the salivary gland were widely studied compared to the proteins from other organs. However, some molecules, including vitellogenin receptor (Boldbaatar et al., 2008a), HlTCI, follistatin (Zhou et al., 2006) have been

isolated from the ovary and identified. HlTCI, a molecule has the inhibitory activity to the digestive CPA and CPB, was expressed in the ovary but not in other tissues (Figs. 17 and 18 in Chapter 3). I suspected that HlTCI plays a role in the protection of the ovary or the oocyte from the infestation of some pathogens, i.e. *Babesia* (Uilenberg, 2006). Moreover, as a strong inhibitor of plasma carboxypeptidase B, also known as TAFI, TCI stimulates the fibrinolysis of blood clots, which renders it a promising adjuvant for use in thrombolytic therapies based tPA (Maritz-Olivier et al. 2007).

In addition, it is significant to perform a direct research further to reveal the relationship of the upstream molecules (HlP0 and HlYkt6) involved in the producing of proteins with the downstream of the final protein product (HlTCI), which may be concerned in our future work.

General summary

Three molecules, HlP0, HlYkt6 and HlTCI were isolated and characterized in the present study, the former two of which are involved in the cascade of the synthesis and transport of proteins, and the latter one is the terminal protein product. HlP0 and HlYkt6 were ubiquitously expressed in all the tissues from the 4-day-fed adult tick *Haemaphysalis longicornis*, while HlTCI was specially expressed in the ovary of partially fed ticks. Knockdown of HlP0 and HlYkt6 genes resulted in the dramatic decrease of the body weight and the death of the fed adult female ticks. The failure of the binding of the large and the small subunit or apoptosis may caused the unsuccessful blood-sucking and the death of the HlP0 dsRNA-injected adult ticks, while the disruption of the secretion of the saliva is supposed to be a major reason for the decrease of blood meal obtained by the HlYkt6 dsRNA-injected adult ticks. HlTCI was a dose-and temperature-dependent inhibitor to the CPA and CPB. rHlTCI protein accelerated the fibrinolysis when the high dose was used, which suggests its promising potential for use in some thrombotic disorders. This whole study not only provided the functional information for the cascade of protein production, but also served as a base for the vaccine and the new pharmaceuticals development.

Acknowledgements

This work was supported by the Bioorientated Technology Research Advancement Institution (BRAIN), Grants-in-Aid for Scientific Research (A) from the Japan Society for the Promotion of Science, and a grant from the 21st Century COE program (A-1) of the Ministry of Education, Sports, Science, and Technology of Japan.

I am deeply grateful to my supervisor, Prof. Kozo Fujisaki (Obihiro Univ. Agri. Vet. Med. and Kagoshima Univ.), for his technological, economical and spirit support. His detailed guidance, constructive suggestions, and tireless instruction will be a great memory in my future life. I should express my gratitude for his warm help when I encountered problems in the study, work and life and his patience in revising my dissertation.

I would like to express my deep acknowledgement to my other supervisor Prof. Xuenan Xuan (Obihiro Univ. Agri. Vet. Med.). Without his meticulously guidance in each step, his wholehearted support on the techniques, his analysis for my obtained results and helpful suggestions for further studies, his providing a good study environment, I can not finish my study successfully. Besides the work *per se*, Prof. Xuan always gave me a hand to remove the obstacles from my study and life, for which I should express my great appreciation here.

I would like to express my gratitude to Prof. Zhou (CAAS), who led me into the world of ticks and gave me many kinds of help on both my life and works. I would

like to express my appreciation to the Associate Prof. Yoshifumi Nishikawa (Obihiro Univ. Agri. Vet. Med.), who always provided beneficial comments and suggestions on my work results.

My sincere thanks to Prof. Ikuo Igarashi (Obihiro Univ. Agri. Vet. Med.), Prof. Hiroshi Suzuki (Obihiro Univ. Agri. Vet. Med.), Prof. Hirotaka Kanuka (Obihiro Univ. Agri. Vet. Med.), Prof. Panagiotis Karanis (Obihiro Univ. Agri. Vet. Med.), Prof. Shin-ichiro Kawazu (Obihiro Univ. Agri. Vet. Med.), Associate Prof. Naoaki Yokoyama (Obihiro Univ. Agri. Vet. Med.), Prof. Associate Prof. Noboru Inoue (Obihiro Univ. Agri. Vet. Med.), Associate Prof. Makoto Igarashi (Obihiro Univ. Agri. Vet. Med.), and Assistant Prof. Shinya Fukumotō (Obihiro Univ. Agri. Vet. Med.) for their constructive suggestions and comments.

I am sincerely grateful to Dr. Liao (Kagoshima Univ.), who warmly helped me on the technology and always sent me materials for study.

I would like to say thanks to all the staff and students of NRCPD for their kind assistance.

I am indebted to my family for their support on my spirit and their understanding.

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