Identification and Functional Characterization of Class B Scavenger Receptor CD36 from the Hard Tick, *Haemaphysalis longicornis*

(フタトゲチマダニ由来クラスBスカベンジャー受容体CD36の同定とその機能の解明)

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Abbreviations

BLASTp	**: Basic Local Alignment Search Tool (protein)**
cDNA	**: complementary DNA**
dsRNA	**: double stranded RNA**
EST	**: expressed sequence tag**
HlSRB	**: *Haemaphysalis longicornis* class B scavenger receptor CD36**
IPTG	**: isopropyl β-D-1-thiogalactopyranoside**
mRNA	**: messenger RNA**
Ni-NTA	**: nickel-nitrilotriacetic acid**
ORF	**: open reading frame**
PAGE	**: polyacrylamide gel electrophoresis**
PBS	**: phosphate buffered saline**
PCR	**: polymerase chain reaction**
PVDF	**: polyvinylidene difluoride**

PF	**: partially fed tick**
rHlSRB	**: recombinant HlSRB**
RNAi	**: RNA interference**
RNAi tick	**: *HlSRB* gene knockdowned tick**
RT-PCR	**: reverse transcription-polymerase chain Reaction**
SD	**: standard deviation**
SDS	**: sodium dodecyl sulfate**
SNMP	**: sensory neurone membrane protein**
SR	**: scavenger receptor**
SRB	**: class B scavenger receptor CD36**
TAE	**: tris-acetic acid-EDTA**
UF	**: unfed tick**
Vg	**: vitellogenin**

Unit abbreviations

hr	**: hour(s)**
mM	**: millimol/L**
ml	**: milliliter**
µl	**: microliter**
mg	**: milligram**
µg	**: microgram**
µm	**: micrometer**
kDa	**: kilodalton**
bp	**: base pair**

General introduction

Ticks are obligate hematophagous ectoparasites and are only second to mosquitoes as vectors of various pathogens that cause deadly diseases of human and animals (38, 72, 81). The ixodid ticks (Arthropoda: Ixodidae), popularly known as hard ticks, *Haemaphysalis longicornis*, serve as a unique vector of various pathogens that cause Lyme disease, tick-borne encephalitis, Rocky Mountain spotted fever, babesiosis, theileriosis, and anaplasmosis, during hematophagy (22, 38).

Most bacterial and viral diseases can be successfully controlled by vaccination and quarantine procedures. For the tick-borne diseases, a variety of methods, including the application of chemical acaricides have been employed to suppress tick vector population and tick-borne diseases. However, the development of resistance and environmental contamination by acaricides emphasizes the need to develop alternatives for tick vector and tick-borne diseases control. An anti-tick vaccine is considered to be one of the most promising methods; however, its development still depends on the identification, characterization and cloning of key tick molecules and the functional analysis of their roles in arthropod physiology (57).

Scavenger receptors (SRs) are multiligand-binding proteins expressed on the surface membrane of a variety of cells, including macrophages, platelets, mature monocytes and endothelial cells (43). Although not necessarily related structurally, the members of the SR family share a strong affinity for a broad range of specific ligands such as fatty acids, polyanions, phospholipids, and modified low-density lipoproteins (LDL) (43). The SRs known as ''pattern recognition receptors'' identify the conserved structure of the pathogen ligands and mediate the binding and uptake of microorganism antigens (25). Three major classes of macrophage SRs, designated type A (SR-AI, SR-AII, and SR-AIII), type B (SR-BI, SR-BII and SR-BIII), and type C SRs were also identified from *Drosophila* (69). In addition, two other macrophage receptors, MARCO (a macrophage receptor with a collagenous structure) and CD68 (macrosialin), may also contribute to the uptake of modified lipoproteins (17, 76). The SR class A, expressed on macrophages are involved in innate immunity by facilitating phagocytic activity especially the uptake and killing of bacteria (26). Other SRs belonging to class B are known to bind and internalize senescent neutrophils (44, 79) and to be involved in cell adhesion, aggregation (91) and signal transduction (63, 33).

The class B scavenger receptor CD36 (SRB) is involved in the first line of body defense and plays a pivotal role in innate immunity (32, 19), clearance of apoptotic cells, lipid transportation (62, 64, 18, 56, 54), macrophage foam cell formation, and the development of atherosclerosis (67), and regulation of angiogenesis (19, 80).

RNA interference (RNAi) has been proposed to have application possibilities for the autocidal control of tick populations (13) and the characterization of tick-borne pathogens

(12, 61). Double-stranded RNA (dsRNA)-mediated gene silencing, commonly referred to as RNAi, has been extensively used for the analysis of gene functions in ticks (14). Long dsRNAs have been successfully and regularly applied in *Haemaphysalis longicornis* (55) and other tick species (e.g., *Amblyomma*, *Ixodes*, *Rhipicephalus*, and *Dermacentor* spp.) for targeted gene knockdown in various stages of tick life, with evidence of systemic RNAi spread into subsequent stages (14, 61). Four different methods have been used to deliver dsRNA for RNAi in ticks to date: injection, soaking, feeding, and virus production of dsRNA (14). We have confirmed that RNAi can be a powerful tool for gene silencing of the hard tick, *H. longicornis*, by the injection (6–8, 49, 55) and soaking methods (24).

Direct injection of the dsRNA into target tissues or developmental stages is the most common method of delivering dsRNA to arthropods, such as insects and ticks (14, 74). Injection of the exogenous dsRNAs into the insect's hemocoel can provide transient knockdown of the target endogenous genes, since the dsRNA in the hemolymph can circulate systemically through the open circulatory system, in which dsRNA is taken up by a cell from the environment (89). It is known that there are at least two pathways for exogenous dsRNA uptake in insects (34). One is based on the transmembrane SID-1 channel protein, as well known from the *Caenorhabditis elegans* nematode. The second possible mechanism is based on the endocytosis-mediated pathway because it shares several components of its machinery with the dsRNA uptake mechanism. Herein, vacuolar H^+ATPase is considered to play an important role (87). However, the participation of SRs already known to play a key role in microbe phagocytosis as "pattern recognition receptors" (75) is not well-established in dsRNA uptake.

SRs are known to potentially act as receptors for dsRNA molecules in an endocytosis-mediated uptake mechanism in the *Tribolium castaneum* beetle (86) and *Drosophila melanogaster* fly (87). However, the involvement of SRs in dsRNA uptake and processing in the gene silencing of arthropods, including ticks, are not understood.

Insects have a well-developed innate immune system that allows a general and rapid response to infectious agents. Hemocytes are the primary mediators of cell-mediated immunity in insects, including phagocytosis, nodulation, encapsulation and melanization. Identification of hemocytes is essential to understand hemocyte-mediated immune responses in invertebrates. Interestingly, *Drosophila* SR macrophage/hemocytes are attractive candidates for insect immunity, and its expression and physiological functions have been reported (68). At least two types of phagocytic cells in ticks, plasmatocytes and granulocytes, have been reported (16, 20, 35, 46, 92). It is of current interest to look at the molecular scenario, in particular, the role of SR in hemocyte-mediated phagocytosis, inside vector ticks, which play an important role in first-line host defense against invading pathogens.

Phagocytosis refers to the recognition, engulfment, and intracellular destruction of invading pathogens and apoptotic cells by individual hemocytes (47). Phagocytosis in mammals is mainly achieved by mononuclear phagocytic cells, such as macrophages and dendritic cells and polynuclear neutrophils (83). In arthropods, such as insects (50) and ticks (35, 82), phagocytosis is achieved mainly by the circulating plasmatocytes and/or granulocytes, in the hemolymph. Since phagocytosis is a widely conserved cellular process that occurs in many protozoa and all metazoans, it could be hypothesized that arthropod

phagocytosis is also similar to mammalian phagocytosis (48, 50, 65). However, the molecular mechanisms of hemocyte-mediated phagocytosis in arthropods have not been intensively investigated (65).

Recent findings provide evidence for the essential role of SRB as a pattern-recognition receptor mediating innate immune responses of the mammalian (83) and insect hosts (50) to a range of exogenous pathogens. Other findings by Baranova et al. (6) indicated that human SRB serves as a phagocytic receptor for a variety of bacteria and mediates pathogen-induced JNK-mediated signaling (c-Jun NH_2-Terminal kinase-mediated signaling). However, the role of arthropod SRB documented in the hemocyte-mediated phagocytosis to invading pathogens has been very restricted to *Drosophila* (48, 50), and the precise functions of SRB in the uptake of various microbes into hemocytes are largely unknown, particularly in hematophagous and vector arthropods, such as ticks and mosquitoes.

In this study, I report the gene identification, isolation, sequence analysis, endogenous localization, and gene and protein expression pattern in developmental stages and different tissues of this SRB from *H. longicornis*. I elucidated the crucial role of *HlSRB* in the knockdown induction of other endogenous genes via microinjections of a different combination of dsRNAs into the hemocoel of female ticks. Also, I demonstrate that *HlSRB*-specific gene-knockdowned ixodid ticks completely lost the phagocytic ability of their hemocytes, in particular, granulocytes, to combat an exogenous bacterial pathogen, Gram-negative *E. coli*, and consequently failed to efficiently clear bacterial burdens in hemolymph and survive due to the profound bacteremia.

To the best of my knowledge, the identification and functional characterization of the first SRB molecule from the hard ixodid tick, *Haemaphysalis longicornis*, will provide a comprehensive contribution to studies linked with the development of control measures for ticks and tick-borne diseases.

Chapter 1:

Identification and characterization of class B scavenger receptor CD36 from the hard tick, *Haemaphysalis longicornis*

1. Introduction

The class B scavenger receptor CD36 (SRB) is a membrane glycoprotein present on platelets, mononuclear phagocytes, adipocytes, myocytes, some epithelia, and insect hemocytes (49, 61). The SRB has been identified in most orders of insects (Diptera, Hymenoptera, Coleoptera, and Lepidoptera) (31) as well as nematodes, sponges, and slime mold (59). Many insect species have an SRB ortholog, the sensory neuron membrane protein (SNMP), on dendrites of the specialized neural cells in antennae involved in pheromone detection (2, 61). On sensory cells, SRB is involved in insect pheromone signaling and rodent fatty food preference. On a microvascular endothelial cell, SRB is a receptor for thrombospondin-1 and related proteins and functions as a negative regulator of angiogenesis (81). The SRB-mediated signaling pathways are conserved, defined by certain

common themes, and involved in many critical cellular processes, but they are still relatively poorly understood. However, the identification and characterization of the SRB from blood-sucking ticks remain to be finalized.

Here, I describe the gene identification, isolation, sequence analysis, endogenous localization, and gene and protein expression pattern in developmental stages and different tissues of the SRB from the hard tick, *Haemaphysalis longicornis*.

2. Materials and methods

2.1. Ticks

Parthenogenetic (Okayama strain) ticks of *H. longicornis* have been maintained by feeding on ears of Japanese white rabbits (Kyudo, Kumamoto, Japan) for several generations at the Laboratory of Emerging Infectious Disease, Department of Frontier Veterinary Medicine, Faculty of Agriculture, Kagoshima University, Kagoshima, Japan (21, 22). Briefly, the ears of the rabbits were clipped and the ticks were attached supported by ear bags and a head collar. The ticks were collected when engorged or after the indicated period of attachment.

2.2. *Animals*

Rabbits and mice were cared for in accordance with the guidelines approved by Animal Care and Use Committee (Approval no. A08010) of Kagoshima University. These animals were maintained in a temperature- and humidity-regulated room under controlled lighting with free access to tap water and commercial regular chow throughout the experiments.

2.3. *Identification and characterization of the cDNA encoding scavenger receptor*

The full-length cDNA library was made using the vector capping method as previously reported (40). ESTs were constructed by random partial sequencing of the 5'-terminal of the cDNA clones from the cDNA libraries, and the similarities in the protein databases were then examined using the BLASTp program. The plasmids containing *HlSRB* gene-encoding inserts were extracted using the Qiagen DNA purification kit (Qiagen, Hilden, Germany). The deduced amino acid translation of the *HlSRB* sequence was determined using GENETYX, version 7 (Genetyx, Tokyo, Japan). Phylogenetic trees were generated according to the alignment of the SR amino acid sequences from different sources by the neighbor-joining method, and the confidence of the branching order was verified by using the fourth version of MEGA software (6). The tree was viewed and converted to a graphic format with TREEVIEW (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html). Homologous search of the full-length sequence of gene was performed using the BLAST program. The domain structure was determined using SMART (http://smart.embl-

heidelberg.de) and Prosite server (http://au.expasy.org/prosite). The theoretical pI (isoelectric point) and Mw (molecular weight) were determined by Compute pI/Mw (http://lcr.expasy.org/tools/pi-tool.html).

2.4. RNA isolation and reverse transcriptase-polymerase chain reaction (RT-PCR)

To investigate the expression patterns of the *HlSRB* gene, total RNA was extracted from different stages of ticks (eggs, larvae, nymphs, and adult females) and from tissues of 4-day-fed adult ticks, including midguts, salivary glands, ovaries, hemocytes and fat bodies. Ticks were homogenized using a mortar and pestle in TRIzol reagent (Invitrogen, Carlsbad, CA, USA). Tissues were collected from partially fed ticks by dissection. Hemolymphs were collected from the coxal-trochanteral tick joints and drawn into capillary tubes (20, 35) containing 100 µl of PBS, on ice. Hemocytes were obtained using a centrifuge at 100×*g* for 5 min at 4 °C. Prior to addition of the TRIzol reagent, each organ was rinsed briefly in phosphate-buffered saline (PBS). RNA was extracted from ticks and organs using the TRIzol reagent according to the manufacturer's protocol and stored at - 80 °C until use. Single-strand cDNA was generated by reverse transcription using the transcriptor first-strand cDNA synthesis kit (Roche, Mannheim, Germany) as recommended by the manufacturer. PCR was carried out with the appropriate dilutions of templates using SRB-specific primers (5' –TGTGCCCATATACCGGAAGT–3' and 5' –TGGTTGCAACTAGTGGGTCA–3').

Control amplification was carried out using the actin-specific primers (5'–CCAACAGGGAGAAGATGACG–3' and 5'– ACAGGTCCTTACGGATGTCC–3')

designed from *H. longicornis β-actin* gene (accession no. AY254898). A series of RT-PCR was performed in 50 μl of a mixture containing 0.5 μg of cDNA, a 10x PCR buffer containing 15 mM of $MgCl_2$, 2 mM of dNTPs, 1 U of RNase inhibitor, and 5 U of AMV-optimized Taq DNA polymerase. The reverse transcription reaction was carried out at 50 °C for 30 min, and then PCR was repeated for 37 cycles under the following conditions: 1 min of denaturation at 94 °C, 1 min of primer annealing at 65 °C, and 1 min of elongation at 72 °C; all subsequent amplifications were therefore carried out using this cycle range and conditions. The PCR products were subjected to electrophoresis in a 1.5% agarose gel in a TAE buffer; the DNA was visualized by ethidium bromide staining and analyzed using Quantity One 1-D Analysis Software (Quantity One Version 4.5, Bio-Rad Laboratories, Milan, Italy), in which band intensity was expressed in pixels. The *β-actin* gene of *H. longicornis* was used as an internal expression control.

2.5. Expression and purification of the recombinant protein

The *HlSRB* ORF was amplified by PCR using a forward primer (5' –ACCTCGAGATGGCTGTGAGTCGCGT–3') containing a recognition site for *Xho*I (underline letters) and a reverse primer (5' –ACGAATTCTGCTGTAGAACATGGGGTT –3') containing a recognition site for *Eco*RI (underline letters). The PCR was repeated for 40 cycles under the following conditions: 1 min of denaturation at 94 °C, 2 min of primer annealing at 74 °C, and 1 min of elongation at 72 °C, all subsequent amplifications were therefore carried out using this cycle range and conditions. The PCR products were purified using a gel purification kit (GENECLEAN II kit, MP Biomedical, Solon, OH, USA) and

subcloned in a frame into the pRSET-A vector (Invitrogen), which had been digested with *Eco*RI and *Xho*I. Recombinant plasmids were used to transform *E. coli* (DH 5α), and the transformed cells were grown to an optical density 1 at 600 nm (OD_{600}) at 37 °C in a Luria-Bertani broth medium (BD, Sparks, MD, USA) supplemented with 50 μg/ml of ampicillin. Histidine-tagged recombinant HlSRB synthesis was induced with 0.1mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the culture was grown for an additional 4h at 37 °C with shaking at 144 revolutions per minute (rpm). The *E. coli* lysate was centrifuged at 5,000 *g* for 30 min at 4 °C to use the pellet for *HlSRB* recombinant protein expression. SRB was expressed using the B-PER II Bacterial Protein Extraction Reagent (Thermo Scientific, Rockford, IL, USA) as recommended by the manufacturer. Purified recombinant HlSRB (rHlSRB) was isolated from Ni-NTA Agarose spin column (Ni-NTA Spin kit, Qiagen, Hilden, Germany) and dialyzed against PBS.

2.6. Preparation of the anti-rHlSRB serum

One hundred micrograms of rHlSRB for one mouse was completely mixed with an equal volume of Freund's complete adjuvant (Sigma-Aldrich, St. Louis, MO, USA) and intraperitoneally injected into mice (ddy, 6 weeks old, female). 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 (Sigma-Aldrich). Sera were collected from these mice 8 days after the last immunization.

2.7. *Western blot analysis*

To determine native HlSRB, protein expression was analyzed with lysates of eggs, larvae, nymphs, and adult ticks by Western blotting using mouse antiserum against rHlSRB. Total protein extracts from 3 day-fed adult ticks were prepared using an extraction method described elsewhere (6). Tick samples of *H. longicornis* were homogenized in liquid nitrogen and resuspended in PBS using a pellet pestle (Kontes, Osaka, Japan). The homogenized ticks were ultrasonicated on ice and then centrifuged at 5,000 *g* for 5 min at 4 °C. The supernatant was recovered and stored at - 30 °C until used for immunoblotting. The tick protein lysates were separated with 12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, pH 7.4, 0.05% Tween-20) and then incubated with a primary antibody (1:100 dilution). After the incubation of peroxide-conjugated sheep anti-mouse IgG (1:2000 dilution) (GE Healthcare, Little Chalfont, UK), a signal was detected using 0.5 mg/ml diaminobenzidine tetrahydrochloride.

2.8. *RNA interference*

Approximately, 570 bp fragments of *HlSRB* was amplified by PCR from the cDNA clone using oligonucleotides, including T7 forward and T7 reverse primers, to attach the T7 promoter recognition sites on both the forward and reverse ends (5' – ACGGATCCTAATACGACTCACTATAGGATGGCTGTGAGTCGCGT–3' and 5' –

ACGGATCCTAATACGACTCACTATAGGTGGTTGCAACTAGTGGGTCA–3', the T7 promoters are underlined). The cDNA of the fire fly *luciferase (luc)* (Promega, Madison, WI, USA) gene was amplified by PCR using oligonucleotides, including the T7 forward and T7 reverse primers (5' – GTAATACGACTCACTATAGGGCTTCCATCTTCCAGGGATACG–3' and 5' – GTAATACGACTCACTATAGGCGTCCACAAACACAACTCCTCC–3', the T7 promoters are underlined). The PCR was conducted for 40 cycles under the following conditions: 1 min of denaturation at 94 °C, 2 min of primer annealing at 62 °C, and 1 min of elongation at 72 °C; all subsequent amplifications were, therefore, carried out using this cycle range and conditions. The PCR products were purified using a gel purification kit (GENECLEAN II kit, MP Biochemical, OH, USA). The T7 RiboMax Express large-scale RNA kit (Promega) was used to synthesize RNA by *in vitro* transcription according to the manufacturer's protocol. Formation of dsRNA was confirmed by running 1μl of the reaction products in a 1.5% agarose gel. The dsRNA injection was followed as described previously (6). Briefly, 1 μg of the *HlSRB*-dsRNA and *luc*-dsRNA in 0.5 μl of an injection buffer (10 mM Tris, 1 mM EDTA, pH7.4) was injected into 50 unfed ticks in the experimental or control groups, through the fourth coxae into the haemocoel; the unfed ticks were fixed on a glass slide with adhesive tape. The injections were carried out using 50-μl microcapillaries (MICROCAP®, Drummond Scientific, Broomall, PA, USA) drawn to fine-point needles. The needles were connected to an air compressor. Injected ticks were left for 1 day at 25 °C in an incubator and then checked for mortality resulting from possible injury during injection. Ticks from both the experimental and control groups were simultaneously

fed on the same rabbit with two groups in different ears. Three days after attachment, a total of 10 ticks were detached from the host for subsequent experiments: five ticks for RNA extraction and five ticks for the preparation of tick protein lysate in each group. Thereafter, ticks were homogenized in TRIzol reagent (Invitrogen) and PBS for extraction of whole-tick RNA and lysate antigen preparation for gene-specific silencing confirmed by RT-PCR and Western blot analysis. The remaining ticks were allowed to feed until engorgement. The success of tick feeding was determined by measuring the total number of ticks engorged, the weight of engorgement, survival, and oviposition.

2.9. Indirect immunofluorescent antibody test (IFAT)

For endogenous localization of HlSRB, female *H. longicornis* adults were fed on the ears of rabbits (21) and recovered 4 days post-infestation. The midguts, salivary glands, and ovaries from partially fed ticks were immediately dissected out under the microscope (21). Dissected organs were separately fixed with 4% paraformaldehyde in PBS including 0.1% glutaraldehyde at 4 °C overnight. After washing with a sucrose series in PBS overnight, samples were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, Torrance, CA, USA) and frozen. Frozen sections (12 μm) were cut with a cryostat (Leica CM 1850, Leica Microsystems, Wetzlar, Germany) and placed on micro-glass slides and then blocked with 5% skim milk in PBS overnight at 4 °C. Sections were incubated for 30 minutes at 37 °C with 1:100 dilution of an anti-rHlSRB mouse serum. Normal mouse serum 1:100 was used as a negative control. After washing 3 times with PBS, Alexa 488-conjugated goat anti-mouse immunoglobulin (1:1000; Invitrogen) was applied as second antibody at 37 °C for 1

hour. After three washes with PBS, samples were mounted in a mounting medium (Vectashield, Vector Laboratories, Burlingame, CA, USA) and then covered with a cover glass, the images were photographed and recorded using a fluorescence microscope (Olympus, Tokyo, Japan).

2.10. Statistical analyses

All statistical analyses were done with the Student's *t*-test. Results are presented as the means ± SD. $P<0.05$ values were considered significant.

3. Results

3.1. Identification of cDNA encoding tick scavenger receptor

We identified the full-length *HlSRB* cDNA from EST clones (Fig. 1). Sequence analysis showed that the full length of *HlSRB* cDNA is 2,908 bp, with the predicted start codon at the 280-282 bases and the stop codon at 1,798-1,800 bases and an ORF extending from position 280-1,800 coding for 506 amino acid polypeptides with a predicted molecular mass of 50 kDa and a pI of 5.83. The 3' untranslated region contains 1,108 bp and ends with a 20 bp polyadenylation (A) tail that begins 13 bp downstream from AATAAA, the

eukaryotic consensus polyadenylation signal (Fig. 1). Blastx analysis revealed that HlSRB has high homology to a CD36-like protein known to be related to the SR class B family. Structural analysis further demonstrated the presence of a well-conserved SRB domain, which included a highly conserved proline, glycine, and cysteine region (aa 247-365) and hydrophobic domains located at the end of the predicted polypeptide sequence (Fig. 1). In addition to the conserved amino acid, nine asparagine [aa 44-46 (NNS), aa 67-69 (NLT), aa 105-107 (NGT), aa 121-123 (NAS), aa 211-213 (NGS), aa 241-243 (NLT), aa 253-255 (NGT), aa 271-273 (NYT), and aa 364-366 (NGS)] residues were identified as potential *N*-linked glycosilation sites. Furthermore, twelve glycine residues (aa 247, 248, 254, 257, 287, 293, 309, 327, 332, 337, 360, 365), ten proline residues (aa 261, 262, 288, 302, 314, 333, 339, 345, 352, 363), and four cysteine residues (C_1-249, C_2-278, C_3-317,C_4-334) were well-conserved in the proline, glycine, and cysteine-rich region. Four cysteine clustered residues (C_1-C_3, C_2-C_4) were recognized as potential sites for intrachain disulfide linkage. Finally, nine asparagine and four centrally clustered cysteine residues were linked as potential sites for *N*- linked glycosylation and intra-molecular disulfide bridges, respectively (77) (Fig. 1).

Alignment of the amino acid sequence of *SRB* gene from *H. longicornis* tick indicated that it shares 38% identity with the hard tick *Ixodes scapularis* (XP 002409323), 30% identity with the flies *Drosophila ananassae* (XP 001965367), 31% identity with the lice *Pediculus humanus corporis* (XP 002003657), and 31% identity with the human *Homo sapiens* (CAA80277). The *HlSRB* protein had an overall 30% identity to both mammalian vertebrate and insect invertebrate SRB membrane proteins, which is common for members of the SRB superfamily (45) (Fig. 2).

A phylogenetic tree using amino acid sequences of SRB from different sources by the neighbor-joining method, revealed that *HlSRB* and the mammalian *SRBs* represent a separate group from the invertebrates and insect *SRBs*. On the other hand, *HlSRB* is most closely related to a *SRB* like protease precursor from the ixodid tick, *Ixodes scapularis* (Fig. 3).

3.2. Expression profile of HlSRB

To determine whether the *HlSRB* transcription factor identified from the *H. longicornis* genome is expressed in adult females, we isolated total RNA from the following female developmental stages and body parts: adults, nymphs, larvae, eggs, midguts, salivary glands, ovaries, fat bodies, and hemocytes. The RT-PCR was performed using HlSRB-specific primers and was indexed to the levels obtained from the actin primers. As shown in Fig. 4, our preliminary data showed that *β-actin* did not change with the stage of the tick or across tissues. *HlSRB* was expressed in all developmental stages but adults and egg were higher than those of nymphs and larvae (Fig. 4A). All tissues, including the midgut, salivary glands, fat bodies, and hemocytes, from partially fed to fully engorged adult ticks showed similar expression of the *HlSRB* gene (Fig. 4B). A gradual increase in the expression of the *HlSRB* during feeding (day 2 to engorged) was observed in ovary. However, similar levels of expression of the gene were detected on all days examined in all different tissues except for what appears to be mRNA upregulation by ovary. I found that the transcript of the *HlSRB* was highly expressed in the midgut, fat body, salivary gland, and hemocytes (Fig. 4B).

3.3. Expression of recombinant HlSRB in E. coli

A recombinant protein carrying a tag of six histidine residues was produced in *E. coli.* The cDNA fragment encoding the *HlSRB* was amplified by PCR. The PCR product was inserted into the *Eco*RI site of the pRSET-A vector. The expression of the recombinant HlSRB in *E. coli* was confirmed by SDS-PAGE (Fig. 5A). Bacterial cells containing rHlSRB cDNA were induced for expression by addition of IPTG, which led to the synthesis of an approximately 50 kDa His_6-HlSRB fusion protein (Fig. 5A, lane 2). The his-tagged recombinant protein was purified by affinity chromatography on Ni-NTA resin columns (Fig. 5A, lane 3). Purified recombinant protein was used in the immunization of mice for the production of antiserum.

3.4. Identification of native protein in different developmental stages of ticks

To determine the molecular weight of endogenous native HlSRB protein corresponding to the cloned cDNA product, mouse anti-rHlSRB serum was used to probe the immunoblotting. Lysates of eggs and whole body lysates from partially fed larvae, nymphs and adult female ticks were used for Western blot analysis with mouse anti-rHlSRB serum. In this analysis, a strong band of 43 kDa was detected for the control anti-actin serum in any of the samples (Fig. 5B). HlSRB-specific 50 kDa strong band was detected for the anti-rHlSRB antibody in egg lysate and partially fed adult lysate, and a weak band was detected in partially fed nymphal and larval lysates (Fig. 5C). These results show that

all developmental stages of ticks express the HlSRB protein but the expression in adults and eggs was higher than that in nymphs and larvae.

3.5. HlSRB gene silencing

To confirm the gene silencing of *HlSRB* during tick feeding, ticks were injected with either *HlSRB* dsRNA for the experimental group or *luc* dsRNA for the control group before being fed on rabbits. Gene-specific primers were used for RT-PCR, and a parallel RNA sample with β-actin-specific primers were also amplified as a positive control. We found a considerable larger decrease in the *HlSRB* transcript in the experimental group than in the control group (Fig. 6A). Additionally, native HlSRB protein expression in the experimental and control groups were determined using Western blot analysis. The immunoblot showed that the HlSRB-specific band was not appearing in *HlSRB* dsRNA-injected tick lysates but was appearing in *luc* dsRNA-injected tick lysates, while mouse anti-actin serum (control) specifically reacted with the 43 kDa (Fig. 6B) and 50 kDa (Fig. 6C) bands of mouse anti-rHlSRB serum, respectively. These findings suggest that post-transcriptional gene silencing had been achieved in *H. longicornis* treated with sequence-specific dsRNA.

3.6. RNAi-mediated knockdown of HlSRB

RNAi-mediated knockdown of *HlSRB* was evaluated to determine its biological role in ticks. To confirm the RNAi of *HlSRB*, we used a dsRNA injection of the *HlSRB* gene and a control of the *luc* gene. The phenotypic features of the *HlSRB* dsRNA-injected and

control *luc* dsRNA-injected ticks are shown in Table 1. The average engorged body weight of *HlSRB* dsRNA-injected ticks was 141.6 ± 46.0 mg, while that of control group was 256.1 ± 55.0 mg. The average egg weight of *HlSRB* dsRNA-injected ticks was 61.8 ± 32.3 mg, while that of the control group was 131 ± 35.8 mg. The ratio of the body weight/egg weight in the *HlSRB* dsRNA-injected group was 42.45 ± 13.5 mg, whereas that of the control groups was 50.9 ± 7.3 mg. The percentage of hatching rate in the HlSRB dsRNA-injected group was 83.7 %, while that in the control group was 100 %. In the *HlSRB* dsRNA-injected group, 6.2% of the engorged ticks died. No dead ticks were observed in the *luc* dsRNA-injected control groups. However, significant differences were found between the two groups with regard to feeding duration, engorgement time, and survival rate. The results suggest that the knockdown of endogenous SRB by RNAi impacted on tick blood feeding and egg production.

3.7. Indirect immunofluorescent antibody test (IFAT)

To examine endogenous HlSRB localization in the midguts, salivary glands, and ovaries of 4-day post-engorgement adult females, an indirect fluorescent antibody test was performed using anti-rHlSRB immune serum as the test serum and normal mouse serum as a negative control for the primary antibody. Alexa 488-conjugated anti-mouse immunoglobulin was used as secondary antibody. As shown in Fig. 7, examination of the tissue sections demonstrated positive fluorescence in the midgut digestive cells, midgut undifferentiated cells, salivary gland granular acini and ovary oocytes, indicating that

endogenous HlSRB was expressed in these cells. Serum from normal mouse did not indicate any positive fluorescence.

Discussion

In this chapter, I describe the cloning, expression, localization, and characterization of HlSRB. Six full-length cDNA libraries of salivary glands, midgut, ovary, hemolymph, fatbody, and eggs of *H. longicornis* ticks were constructed, and the corresponding EST database was made in our laboratory. Several clones encoding putative *SRB* were picked up from the above libraries, and the plasmid DNA from the clones was prepared for sequencing. Therefore, the *SRB*-like gene was finally obtained and designed as *HlSRB*. An alignment of ESTs from the *H. longicornis* genome encoded a single assembled cDNA gene sequence with homology to an *SRB*-like cDNA, which, after cloning and sequencing of the complete cDNA from *H. longicornis*, revealed a predicted ORF of amino acids with 30% identity with CD36, a class B scavenger receptor protein found in vertebrates and invertebrates (27). As a member of the SRB superfamily, the HlSRB possessed several shared structural characteristics (27) including hydrophobic transmembrane regions in the carboxy- and amino-terminal ends, a highly conserved SRB domain containing well-conserved cysteine, glycine, and proline residues, and conserved asparagines that are presumed to serve as *N*-linked glycosylation sites.

Additionally, the peptides containing four centrally located cysteine residues, which have been suggested to be palmitoylated and to have a hydrophobic nature, in fractions from peptide maps of SRB (77). These cysteine residues (C1-C3 and C2-C4) are also linked by disulfide bonds, resulting the arrangement of disulfide bridges, demonstrating that the formation of an intra-molecular disulfide bridge in SRB is a prerequisite for intracellular processing and transport (28). Overall, based on these critical structural similarities, I conclude that the cloned *H. longicornis* SR-like protein belongs to the family of class B scavenger receptors CD36.

The molecular size of human SRB was estimated to be 53 kDa (64), which differed from the value of 88 kDa reported in other studies (51, 84). The difference was explained by the molecular conditions affected by post-translational modification in different tissues; moreover, there was glycosylated SRB and nonglycosylated SRB. A recent report on human SRB also showed an apparent molecular mass of 50 kDa in Sf9 cells infected with a recombinant baculovirus (90). I found a similar result, namely, that the His-tagged recombinant HlSRB (Fig. 5A) and also a native protein was recognized a 50 kDa (Fig. 5C); it is hypothesized that the HlSRB may represent SRB-like protein and may be similar to that of human SRB expressed in Sf9 cells infected recombinant baculovirus.

The expression of mRNA for the *HlSRB* gene was detected at four different developmental stages and in the major tissue of adult ticks by reverse transcription PCR (Fig. 4), indicating the important physiological role of this molecule throughout the tick life cycle as well as in different tissues of adult ticks. However, the expression levels of the *HlSRB* gene in different tissues are the same, but the different stages are not the same. As

shown in Fig. 4A, the *HlSRB* gene has much higher expression in the egg and adult stages than in the larval and nymphal stages, indicating that the *HlSRB* gene not only functions in the gut but also plays an important role in the lipoprotein-mediated lipid metabolism (36, 37). A similar case was also observed in *Drosophila*: SRB is expressed in the embryonic stage (41) during the third larval instar, late pupal, and imago stages, but no expression is observed in the early two larval instars and pupae of the *Drosophila* life cycle (88).

SRs are attractive candidates for pattern-recognition receptors that help confer the polyspecificity and self/nonself discrimination require for innate immunity in both vertebrates and invertebrates (1, 42, 11). The SR class B is expressed at high levels in the rat ovary, indicating that it plays a role in the delivery of cholesterol and also to be involved in the host defense against exogenous pathogens and in the recognition of damaged molecules and apoptotic cells (44, 85). In *Drosophila*, the expression pattern of *Drosophila* SR was found to gradually rise to all the embryonic macrophages/hemocyte development during embryonic development stage 10 to stage 14, suggest that *Drosophila* SR may participate in a variety of macrophage/hemocyte function and innate immunity (68). In this study, the expression pattern of *HlSRB* gene in hemocytes was well expressed and that in the ovary was up-regulated during blood feeding (day 2 to engorged) by RT-PCR (Fig. 4B); moreover, endogenous HlSRB protein was also detected in several tissues of ticks including ovary, by IFAT (Fig. 7). In addition, the recombinant rHlSRB protein was purified and used to generate anti-sera. Native proteins of various tissues from partially engorged ticks were subjected to Western blot analysis. The 50 kDa bands in different stages of tick lysates were detected along with a strong band in the lysate of egg and adult by

immunoblotting using anti-rHlSRB serum, while weak band was observed in the lysate of nymph and larvae (Fig. 5C). My result suggests that HlSRB appears to be involved in the functional activities of SRB, providing to next chapter of the functional conservation of innate immunity from the hard tick *H. longicornis* using hemocytes by IFAT. However, all different tissues including the midguts, salivary glands and fat bodies from partially unfed to fully engorged ticks showed similar expression by RT-PCR (Fig. 4B).

In the SRB family of membrane protein, a similar pattern of disulfide bridges and glycosylations is likely to be found in the sensory neurone membrane protein (SNMP) from mammalian and insect SRBs (77). Here, I have found that comprising nine glycosylations and two disulfide bridge in HlSRB protein (Fig. 1), indicates may be implicated pheromone detection in *H. longicornis* ticks. Furthermore, expression and internal localization are modified in an essential role for lipid and lipid metabolism in human SRB (4). In my experiment, I found that HlSRB was expressed in all developmental stages, in different tissues (Fig. 4), and in the endogenous native HlSRB protein of partially fed female *H. longicornis* ticks (Fig. 5C). In addition, the immunohistochemical localization of endogenous HlSRB was found to be expressed in several tissues, including the midguts, salivary glands, and ovaries, while the ticks were feeding on the host (Fig. 7). These results indicated that HlSRB may be involved in pheromone signaling and fatty food preference in sensory cells and may fulfill an essential role in lipid and the lipid metabolism.

Several reports have confirmed that RNAi can be a powerful tool for silencing the tick gene (3, 6, 49, 55, 59). I also applied RNAi with one part of the fragment of the *HlSRB* gene to *H. longicornis*. I found a considerable decrease in the *HlSRB* transcript in the

HlSRB dsRNA-treated group from that in the control, as detected by the RT-PCR (Fig. 6A) and Western blot analysis (Fig. 6B and C). These findings suggest that posttranscriptional gene silencing had been achieved in *H. longicornis* treated with *HlSRB* dsRNA. Next results showed that an injection of dsRNA of the *HlSRB* gene led to a larger reduction of the tick engorgement weight after blood feeding than an injection of the *luc* dsRNA control, suggesting that *HlSRB* may play an important role in metabolic default and physiological process including blood feeding, oviposition and also cuticle formation effect of *HlSRB* dsRNA treatment in tick *H. longicornis.* Similar result was observed in *S. mansoni*, in which a decrease in the length of the parasites and a change in the tegumental surface of the larval were noted after *S. mansoni SRB* dsRNA treatment (15). Similar to what has been reported in *Drosophila*, a reduction in infection was observed 3 days after dsRNA treatment for mycobacrerial infection (73).

In summary, I identified a scavenger receptor class B-like protein belonging to the CD36 superfamily in *H. longicornis*, the first to be structurally characterized in ticks. Even though the cloned molecule was found to be highly homologous to a class B scavenger receptor CD36 protein (SRB), the full-length of *HlSRB* contains a polypeptide, a hydrophobic *SRB* domain, and a highly conserved proline, glycine and cysteine region. HlSRB has been found to be expressed strongly in the egg and adult stages but weakly in the larval and nymphal stages and to locate on the midgut, salivary gland, and ovary of partially fed *H. longicornis*. However, subsequent RNAi experiments demonstrated a possible link between *H. longicornis* SRB-like transcript knockdown and disruption of the *HlSRB* gene, which led to a significant reduction of the engorged body weight. Therefore,

in next chapters, I focused on the details of the functional analysis of the class B scavenger receptor CD36 gene from *H. longicornis*.

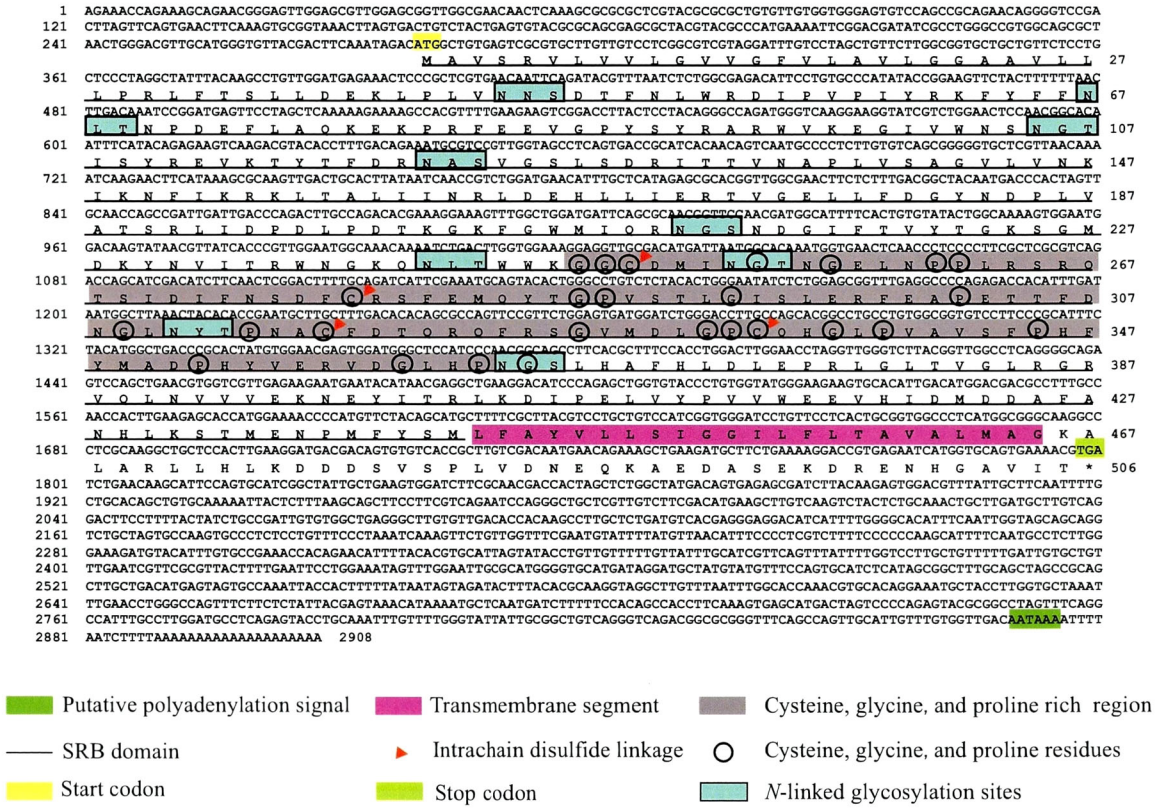


Fig. 1. Nucleotide and deduced amino acid sequence of class B scavenger receptor CD36 (*SRB*) gene from *H. longicornis* tick

Blastx analysis revealed an *HlSRB* domain (aa 1-442, underline showed), which included a highly conserved proline, glycine, and cysteine region (aa 247-365; cysteine, glycine, and proline residues are indicated in gray, underlined, and circled, respectively). Four cysteine clustered residues were recognized as potential sites for intrachain disulfide linkage (aa 249-334) and are indicated by red triangles. Amino acids are numbered on the right.

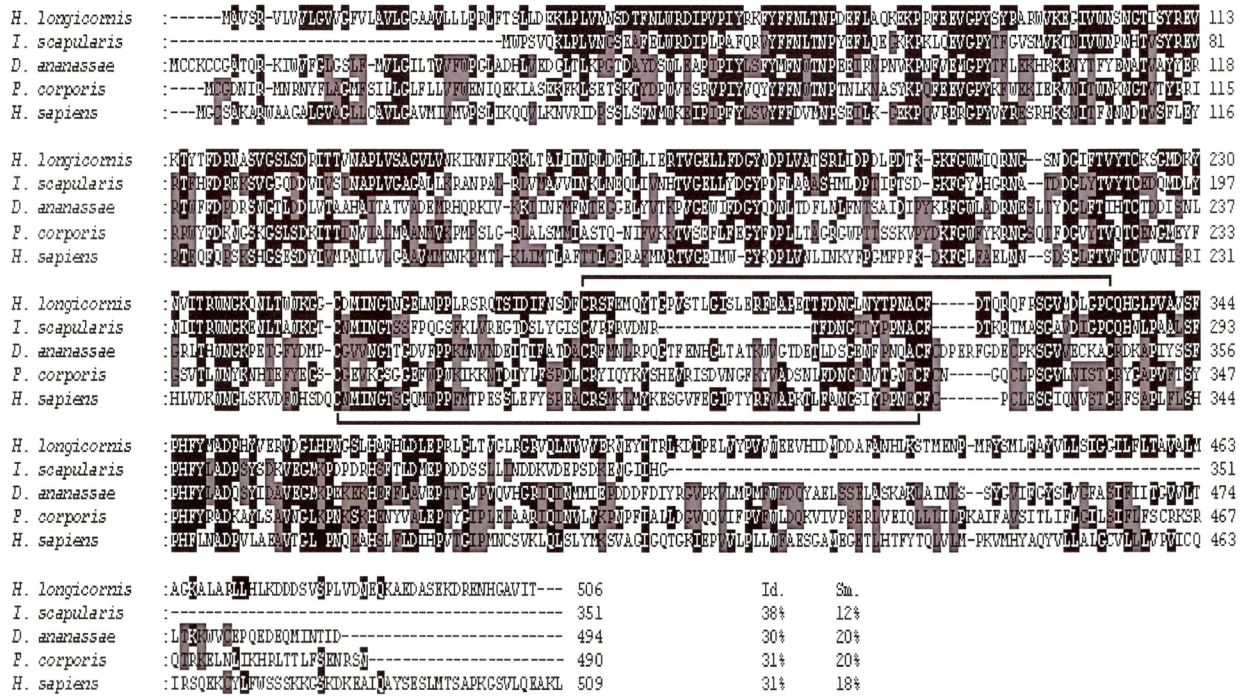


Fig. 2. The alignment of the amino acid sequence of HlSRB

The alignment of the amino acid sequence of class B scavenger receptor CD36 gene was compared with those of the class B scavenger receptor CD36 transcription factor of the ixodid tick *Ixodes scapularis* (XP 002409323), the flies *Drosophila ananassae* (XP 001965367), the lice *Pediculus humanus corporis* (XP 002003657), and the human *Homo sapiens* (CAA80277). Identical residues are dark shaded and similarity residues are grey shaded. Amino acids are numbered on the right. The disulfide bridges (C1-C3, C2-C4) are shown with a linked line.

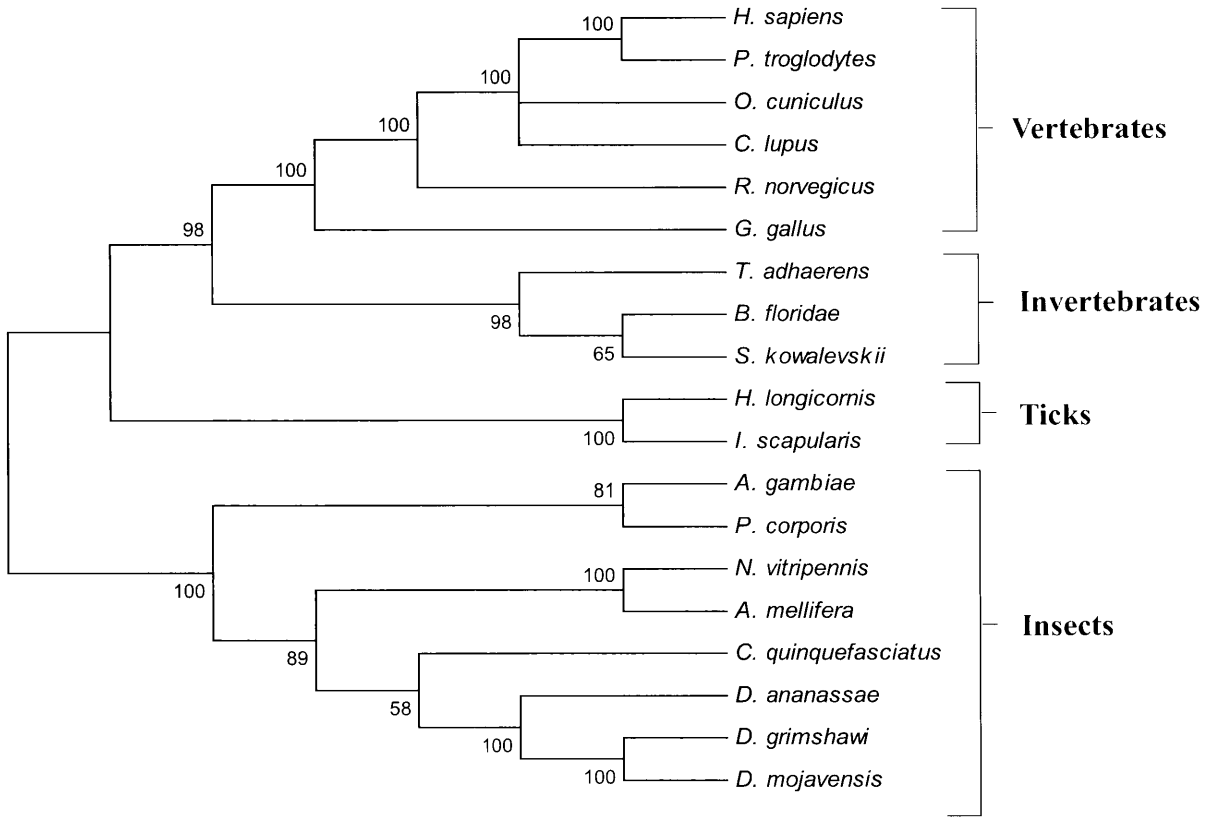


Fig. 3. Phylogenetic tree of the protein sequences of *HlSRB* genes.

The HlSRB amino acid sequences were used the bee *Apis mellifera* (GenBank accession No. XP392321), the wasps *Nasonia vitripennis* (XP001604561), the mosquitoes *Culus quinquefasciatus* (XP001844488) and *Anopheles gambiae* (XP314281), the flies *Drosophila ananassae* (XP001965967), *Drosophila mojavensis* (XP002003657), and *Drosophila grimshawi* (XP001988551), the lice *Pediculus humanus corporis* (XP002427891), the ixodid tick *Ixodes scapularis* (XP002409323), the bird *Gallus gallus* (XP415106), the human *Homo sapiens* (CAA80277), the monkey *Pan troglodytes* (XP509475), the rabbit *Oryctolagus cuniculus* (NP001076257), the dog *Canis lupus* (XP543366), the rat *Rattus norvegicus* (EDM13560), the Florida lancelet *Branchiostoma floridae* (XP002609178), the acorn worm *Saccoglossus kowalevskii* (XP002735902) and the Placozoa *Trichoplax adhaerens* (XP002112871).

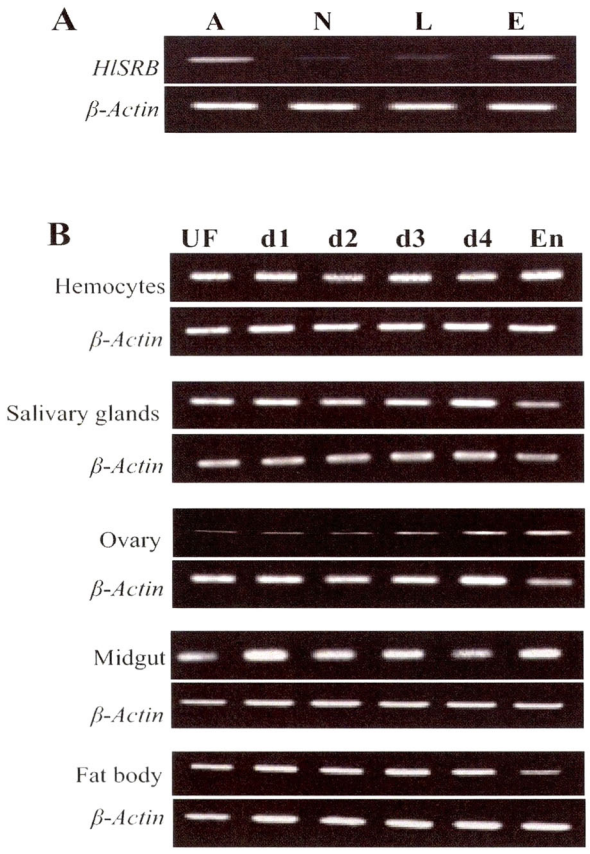


Fig. 4. Transcription profile of *HlSRB* in immature developmental stages and in different tissues of adult females.

Transcription profile of *HlSRB* in immature developmental stages and in different tissues of adult females. Total RNA was isolated from partially fed adults, larvae, nymphs, and eggs. Analysis of *HlSRB* gene expression in immature developmental stages (A). A, adult females; N, nymphs; L, larvae; E, eggs. Expression profile of the *HlSRB* gene at different days of hemocytes, salivary gland, ovary, midgut and fat body in *H. longicornis* female ticks during blood sucking (B). Uf, unfed ticks; d1, one-day-fed ticks; d2, two-day-fed ticks; d3, three-day-fed ticks; d4, four-day-fed ticks; En, engorged ticks. The transcription profiles of *β-actin* were used as an internal control.

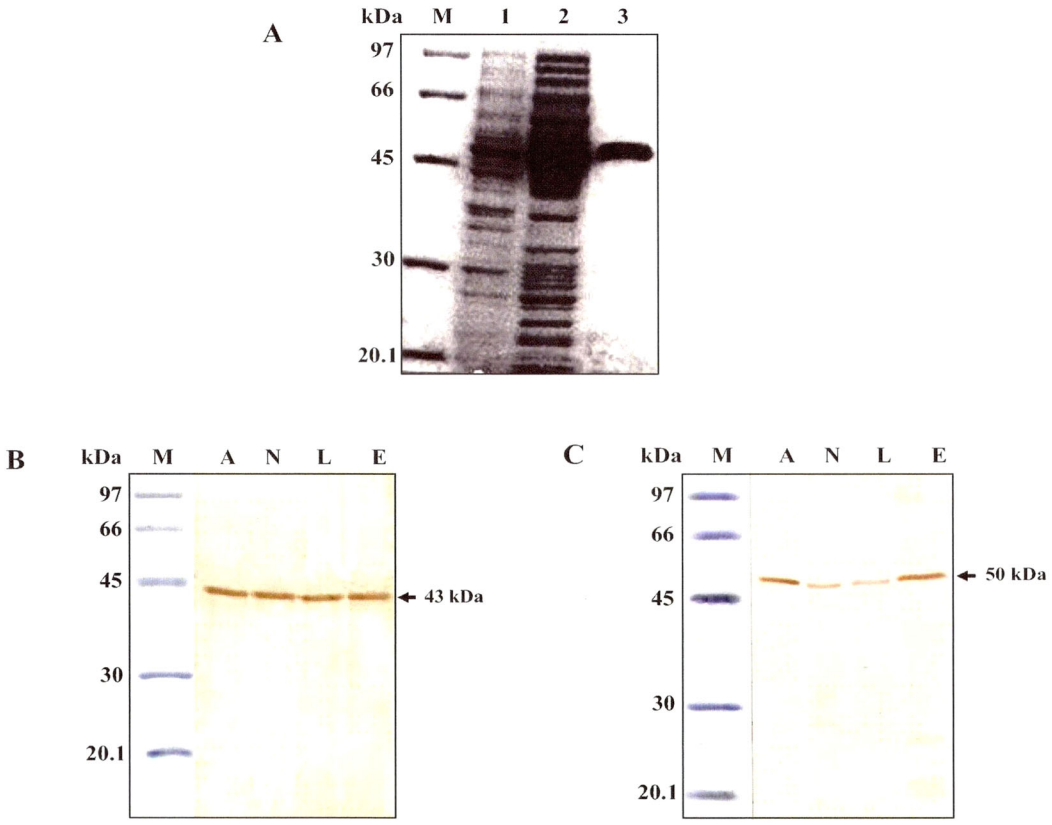


Fig. 5. Expression and purification of rHlSRB in *E. coli*

(A) Recombinant proteins or bacterial lysates were electrophoresed on a 12% SDS-polyacrylamide gel and then stained with Coomassie brilliant blue. M, molecular weight marker; lane 1, crude lysate of *E. coli* before induction; lane 2, IPTG-induced *E. coli* lysate; lane 3, purified recombinant HlSRB protein. Western blot analysis of endogenous HlSRB in *H. longicornis* tick lysates. Egg lysates and different stages (larval, nymphal and adult stages) of 3 day-fed tick lysates were subjected on 12% SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was probed with the mouse anti-actin serum (B) and the mouse anti-rHlSRB serum (C). M, molecular marker; A, lysate of 3-day fed adult females; N, lysate of 3-day fed nymphs; L, lysate of 3-day fed larvae; E, lysate of eggs.

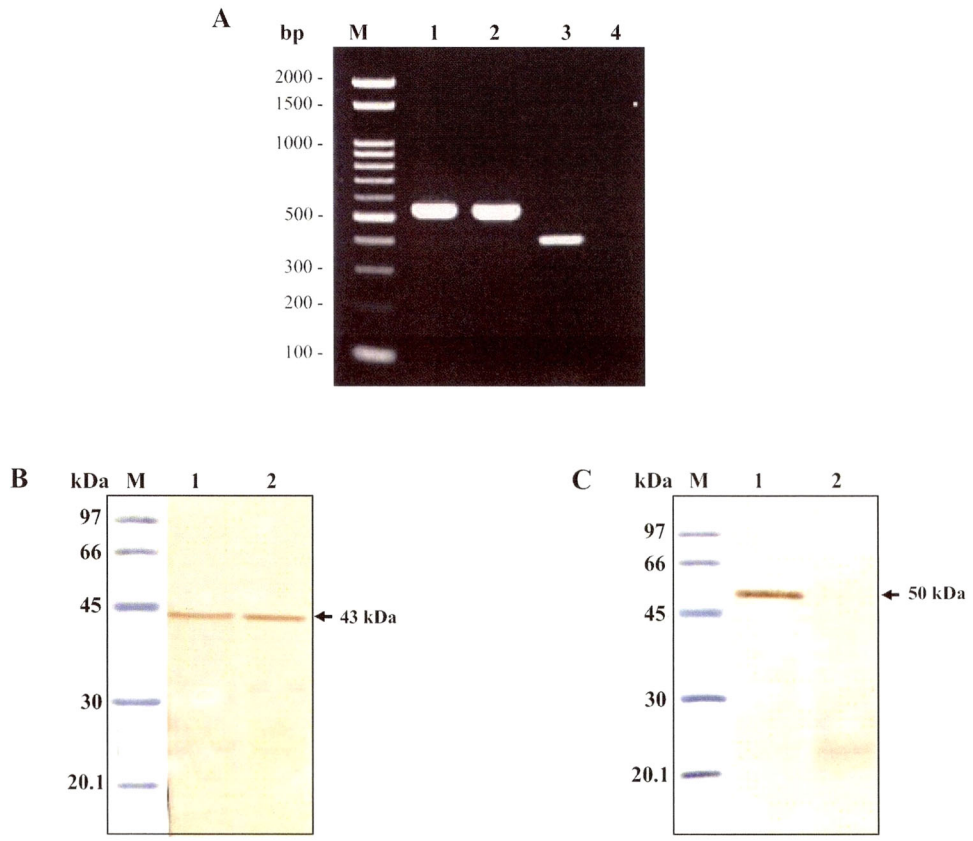


Fig. 6. Effect of dsRNA treatment on *HlSRB* gene disruption

dsRNA complementary to *HlSRB* was injected into *H. longicornis* adult females. The injected ticks (50 individuals for each group) were allowed to feed, and ticks were recovered from the *luciferase* (control) and *HlSRB* dsRNA-treated group, respectively, after 3 days of feeding. Reverse transcription PCR analysis (A). M, molecular marker; lane 1, *luc* dsRNA-treated control ticks with the primer set for actin; lane 2, *HlSRB* dsRNA-treated ticks with the primer set for actin, lane 3, *luc* dsRNA-treated control ticks with the primer set for scavenger receptor, lane 4, *HlSRB* dsRNA-treated ticks with the primer set for scavenger receptor. Western blotting analysis with mouse anti-actin serum (B) and anti-rHlSRB serum (C). M, molecular marker; lane 1, extract from *luc* dsRNA-treated control ticks, lane 2, extract from *HlSRB* dsRNA-treated ticks.

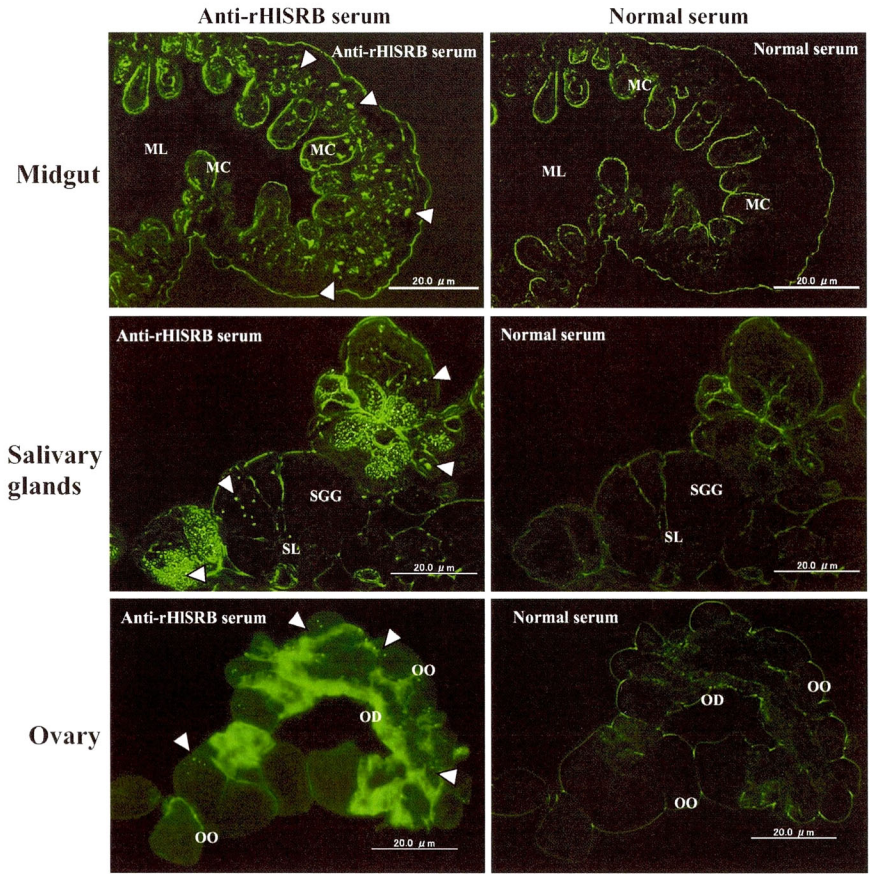


Fig. 7. Imunohistochemical localization of endogenous HlSRB in the midgut, salivary glands, and ovary of partially fed adult *H. longicornis* by IFAT

Staining pattern of anti-rHlSRB serum and normal mouse serum were used as primary antibodies with a fluorescence microscopy. The mouse anti-IgG conjugated with Alexa 488 was used as secondary antibody. ML, midgut lumen; MC, midgut cells; SL, salivary gland lumen; SGG, salivary gland granular acini; OO, oocyte; OD, oviduct. The scale bar represents 20μm.

Table 1 Effects of HlSRB RNA interference

Groups	No.	Engorged body weight (mg)[a,c]	Average egg weight (mg)[a,c]	Ratio of egg weight/body weight[a,c]	Mortality of engorged ticks (%)[b,c]	Hatching rate (%)[c]
HlSRB dsRNA	50	141.6 ± 46.0	61.8 ± 32.3	42.45 ± 13.5	6.2	83.7
luc dsRNA	50	256.1 ± 55.0	131 ± 35.8	50.9 ± 7.3	0	100

[a] Value is mean ± SD.

[b] The mortality was calculated as the ratio of dead engorged ticks to the total number of engorged ticks up to 20 days after ticks dropped off the host.

[c] Significantly different ($P<0.05$) as calculated by Students *t* test.

Chapter 2:

Scavenger receptor mediates systemic RNA interference in ticks

1. Introduction

RNA interference is an efficient method to silence gene and protein expressions. Injection of the exogenous dsRNAs into the insect's hemocoel can provide transient knockdown of the target endogenous genes, since the dsRNA in the hemolymph can circulate systemically through the open circulatory system, in which dsRNA is taken up by a cell from the environment (89). Long dsRNAs have been successfully and regularly applied in *Haemaphysalis longicornis* (55) and other tick species (e.g., *Amblyomma*, *Ixodes*, *Rhipicephalus*, and *Dermacentor* spp.) for targeted gene knockdown in various stages of tick life, with evidence of systemic RNAi spread into subsequent stages (14, 61). In our laboratory, we have confirmed that RNAi can be a powerful tool for gene silencing of the hard tick, *H. longicornis*, by the injection (55, 6–8, 49) and soaking methods (24). In this

chapter, I demonstrated for the first time that SRB may not only mediate the effective knock-down of gene expression by RNAi but also play essential roles for systemic RNAi of ticks by using injection method.

2. Materials and methods

2.1. Construction of dsRNA and microinjection of dsRNA into adult ticks

The dsRNA construction of *H. longicornis HlSRB* (Chapter-1), *H. longicornis HlVgR* (7), and *H. longicornis HlVg-1* (8) and firefly *luciferase* (*luc*) as a control was performed as described previously (Chapter-1). The dsRNAs were injected into the hemocoel of unfed female ticks as described by Chapter-1. The *HlSRB*-, *HlVgR*-, *HlVg-1*-, and *luc* dsRNA-injected ticks were allowed to rest at 25 °C and 90% humidity regulated in an incubator for 96 hours to complete knock-down of these genes (Chapter-1, 7, 8), and the mortality rate was then checked every 12 hours. Ninety-six hours after the first injection, three ticks were collected from the incubator in order to confirm gene-specific silencing by RT-PCR (Chapter-1, 7, 8). The remaining dsRNA-treated ticks were subjected to a second injection of dsRNAs.

Twelve tick groups injected with a single dsRNA or a combination of dsRNA(s) are as shown in Table 2. Each tick received a total of 0.5 μl dsRNA with a different concentration (for single dsRNA-injected groups, 1 μg/tick; for a combination of dsRNA(s)-

injected groups, 1 μg/gene for a dose equal to the injected dsRNA at 2 μg/tick). The ticks injected with these dsRNAs were infested on the ear of rabbits 12 hours after the first or the second dsRNA injection. Four days after infestation, a total of 16 attached ticks were removed and collected from rabbits for the subsequent experiments including four ticks for RNA extraction, four ticks for protein lysate preparation, and eight ticks for tissue collection. The remaining ticks were allowed to feed until engorgement. To assess the effects of RNAi in ticks after the first and the second injections, we measured the number of ticks attached on a rabbit 2 days after attachment, the engorged body weight of ticks 5-6 days after attachment, the mortality rates, fecundities, and oocyte development of engorged ticks 20 days after engorgement, and the hatching rate to larvae 60 days after the first dsRNA injections.

2.2. Collection of different tissues of dsRNA-injected ticks

Partially engorged female ticks 4 days post-infestation were removed from rabbits and dissected out for the subsequent tissue collection (21). Midguts were collected from ticks injected with a single *HlSRB* or *HlVg-1* dsRNA and a combination of *HlSRB/HlVg-1*, *HlVg-1/HlVgR*, *HlVg-1,HlSRB*, or *HlVgR/HlVg-1* dsRNAs, ovaries were collected from ticks injected with a single *HlSRB* or *HlVgR* dsRNA and a combination of *HlSRB/HlVgR*, *HlVg-1/HlVgR*, *HlVgR/HlSRB*, or *HlVgR/HlVg-1* dsRNAs, and salivary glands and the fat bodies were collected from ticks injected with a single *HlSRB* or *luc* dsRNA and a combination of *HlSRB/luc* or *luc/HlSRB* dsRNAs.

2.3. *Reverse transcriptase-polymerase chain reaction (RT-PCR)*

Whole bodies and dissected tissues from female ticks of each dsRNA-injected group fed for 4 days were subjected to total RNA extraction using a TRIzol reagent (Invitrogen, CA, USA). The RT-PCR analysis was performed using a one-step RNA PCR kit (Takara, Otsu, Japan) with the primer sets of *HlSRB* (Chapter-1), *HlVgR* (7), and *HlVg-1* (8) genes. For all experiments, control amplification was carried out using the *H. longicornis β-actin*-specific primers (accession no. AY254898). The PCR products were subjected to electrophoresis in a 1.5% agarose gel in a TAE buffer; the DNA was visualized by ethidium bromide staining and analyzed using Quality One 1-D Analysis Software (Quantity One Version 4.5, Bio-Rad Laboratories, Milan, Italy).

2.4. *Protein expression analysis by Western blotting*

The tick proteins from the lysates of whole bodies and the dissected tissues (about 1000 ng/lane) from female ticks of each dsRNA-injected group fed for 4 days were separated by 5-12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, MA, USA). The membrane was blocked with 5% skim milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween-20, pH 7.4) and then incubated with 1:100 dilution of anti-recombinant HlSRB (rHlSRB) (Chapter-1), 1:250 dilution of anti-rHlVgR (7), and 1:200 dilution of anti-rHlVg-1 mouse sera (8) or anti-actin serum (49) as a first antibody. After the incubation of peroxidase-conjugated sheep anti-mouse IgG as a second antibody (1:2000

dilution; GE Healthcare, Little Chalfont, UK), the specific protein bands were detected using 0.5 mg/ml 3,3'-diaminobenzidine tetrahydrochloride.

2.5. Indirect immunofluorescent antibody test (IFAT)

The midguts, ovaries, salivary glands, and fat bodies dissected out from female ticks of each dsRNA-injected group fed 4 days were separately fixed with 4% paraformaldehyde in PBS including 0.1% glutaraldehyde at 4 °C overnight. After washing with a sucrose series in PBS overnight, samples were embedded in Tissue-Tek O.C.T. Compound (Sakura Finetek, CA, USA) and frozen at - 80 °C. Frozen sections (12 μm) were cut with a cryostat (Leica CM 1850; Leica Microsystems, Wetzlar, Germany) and placed on micro-glass slides and then blocked with 5% skim milk in PBS overnight at 4 °C. Sections were incubated for 30 minutes at 37 °C with a 1:100 dilution of an anti-rHlSRB, a 1:200 dilution of anti-rHlVg-1, and a 1:250 dilution of anti-rHlVgR mouse sera. After washing three times with PBS, Alexa 488- or Alexa 594-conjugated goat anti-mouse immunoglobulin (1:1000; Invitrogen) was applied as a second antibody at 37 °C for 1 hour. After washing three times with PBS, samples were mounted in a mounting medium with DAPI or Propidium Iodide (Vectashield; Vector Laboratories, Burlingame, CA, USA) and then covered with a cover glass. The images were photographed and recorded using a fluorescence microscope (Olympus, Tokyo, Japan).

3. Results

3.1. dsRNA-mediated gene silencing of HlSRB, HlVg-1, and HlVgR

To investigate whether *HlSRB* knockdown has an effect on RNAi of other endogenous genes in ticks, we selected the *HlVg-1* and *HlVgR* genes. *HlVg-1* was transcribed only in the midgut (8), and the *HlVgR* gene, only in the ovary (7). It was previously demonstrated that HlVg-1, one of the tick multiple vitellogenins (Vgs), is a crucial yolk protein precursor for oocyte development in ticks (8) and that HlVgR, a receptor localized on the surface of oocytes, plays a critical role for the specific binding with Vgs and the resultant Vgs transfer from hemolymph into oocytes via receptor-mediated endocytosis (7). In this study, unfed female ticks were injected with a single *HlSRB*, *HlVgR*, or *HlVg-1* dsRNA for experimental groups as well as *luc* dsRNA as a control (Table 2). Ninety-six hours after the first injection, we observed apparent knockdown of the *HlSRB*, *HlVgR*, and *HlVg-1* transcriptions in ticks after the injection of respective dsRNAs by RT-PCR (Fig. 8A).

The mortality of ticks after dsRNA injections was examined every 12 hours. Forty-eight hours after injection, a slightly mortality was found in ticks of all groups (Fig. 8B). Seventy-two hours after injection, the mortality rate of ticks injected with the *luc* dsRNA was 10.0%, while those of the *HlSRB*-, *HlVgR*-, and *HlVg-1* dsRNA-injected tick groups were significantly higher, 14.4%, 20.0%, and 15.5%, respectively. Ninety-six hours after injection, the mortality rate of the *luc* dsRNA-injected ticks was 22.2%, while those of the *HlSRB*-, *HlVgR*-, and *HlVg-1* dsRNA-injected groups were significantly higher, 44.4%,

58.5%, and 62.2%, respectively. Four days after the first dsRNA injections, the live ticks were injected with second dsRNAs.

3.2. Gene silencing of HlSRB, HlVgR, and HlVg-1 in whole bodies of female ticks injected with a single dsRNA or a combination of dsRNA(s)

RT-PCR and Western blot analysis were performed to elucidate the gene transcription and protein translation of HlSRB, HlVgR, and HlVg-1 in whole bodies of female ticks injected with a single dsRNA or a combination of dsRNA(s) (Table 2). As shown in Fig. 9A and B, the β-actin gene and protein levels did not change in all dsRNA-injected groups. A clear mRNA and protein knock-down of HlSRB, HlVgR, or HlVg-1 was observed in ticks injected with a single dsRNA of *HlSRB*, *HlVgR*, or *HlVg-1* (Fig. 9A and B). In ticks injected with a combination of *HlSRB/HlVgR*, *HlSRB/HlVg-1*, *HlSRB/luc*, *HlVgR/HlSRB*, *HlVg-1/HlSRB*, or *luc/HlSRB* dsRNAs, apparent mRNA and protein knock-down of HlSRB was attained in all groups regardless of whether *HlSRB* dsRNA was used for the first or second injection (Fig. 9A and B). In ticks injected with a combination of *HlVg-1/HlVgR*, or *HlVgR/HlVg-1* dsRNAs, clear double knockdown of endogenous *HlVg-1* and *HlVgR* genes was observed in both groups (Fig. 9A and B). Similar double gene knockdown was detected in ticks injected with a combination of *HlVgR/HlSRB* or *HlVg-1/HlSRB* dsRNAs (Fig. 9A and B). However, in ticks injected with *HlSRB* dsRNA followed 96 hours later with *HlVg-1* or *HlVgR* dsRNAs, no knockdown of *HlVgR* and *HlVg-1* was achieved, although clear gene silencing of *HlSRB* was attained, as described above (Fig. 9A

and B), suggesting that RNAi mediated by the second *HlVgR* or *HlVg-1* dsRNA injection was inhibited by the first gene knockdown of *HlSRB*.

3.3. mRNA and protein knock-down of HlSRB, HlVg-1, and HlVgR in different tissues of female ticks injected with a single dsRNA or a combination of dsRNA(s)

RT-PCR and Western blot analysis were performed to elucidate whether the mRNA and protein level in major internal organs, such as midguts, ovaries, salivary glands, and fat bodies, of female ticks attained single or double gene knockdown after various dsRNA injections (Fig. 10). As a result, mRNA and protein knock-down of HlSRB, HlVg-1, and HlVgR in the individual organs showed the same pattern of single or double gene knockdown as that observed using the whole bodies of female ticks (Fig. 9).

In the midguts (Fig. 10A and B), apparent silencing of *HlSRB* was observed after dsRNA injections with *HlSRB*, *HlSRB/HlVg-1*, or *HlVg-1/HlSRB*. Similar evident knockdown of *HlVg-1* was detected after dsRNA injections with *HlVg-1*, *HlVg-1/HlVgR*, *HlVg-1/HlSRB*, or *HlVgR/HlVg-1*. Double gene knockdown with *HlVg-1/HlSRB* injection and the failure to knock-down *HlVg-1* with *HlSRB/HlVg-1* injection were also pronounced in the midguts.

In the ovaries (Fig. 10C and D), silencing of *HlSRB* was observed after dsRNA injections with *HlSRB*, *HlSRB/HlVgR*, or *HlVgR/HlSRB*. Knockdown of *HlVgR* was detected after dsRNA injections with *HlVgR*, *HlVg-1/HlVgR*, *HlVgR/HlSRB*, or *HlVgR/HlVg-1*. Double gene knockdown with *HlVgR/HlSRB* injection and the failure to knock-down *HlVgR* with *HlSRB/HlVgR* injection were demonstrated.

In the salivary glands and fat bodies, mRNA and protein knock-down of HlSRB was observed after dsRNA injections with *HlSRB*, *HlSRB/luc*, and *luc/HlSRB*, while mRNA and protein expression of HlSRB was observed after a single dsRNA injection of *luc* (Fig. 10E, F, G, and H).

3.4. Immunofluorescent staining of the midguts, ovaries, salivary glands, and fat bodies

An immunohistochemical examination using an indirect fluorescent antibody test (IFAT) was conducted to illustrate the localization of the endogenous protein in the midguts, ovaries, salivary glands, and fat bodies of female ticks that exhibited single or double gene knockdown after the various dsRNA injections (Fig. 11).

As shown in Fig. 11A, no localization of HlSRB protein in midguts was observed after dsRNA injections with *HlSRB*, *HlSRB/HlVg-1*, or *HlVg-1/HlSRB* (panel a, c, and e). Similarly, no localization of HlVg-1 in midguts was observed after dsRNA injections with *HlVg-1*, *HlVg-1/HlVgR*, *HlVg-1/HlSRB*, or *HlVgR/HlVg-1* (panel h, j, k, and l). Neither HlVg-1 nor HlSRB protein localized in the midguts of ticks exhibited double gene knockdown by an injection of a combination of *HlVg-1/HlSRB* dsRNAs (panel e and k). Normal localization of the HlVg-1 protein in midguts, attributed to the failure to knock-down *HlVg-1* in *HlSRB/HlVg-1* injection, was also confirmed by IFAT (panel i).

In the ovaries, no localization of the native HlSRB protein was observed after dsRNA injections with *HlSRB*, *HlSRB/HlVgR*, or *HlVgR/HlSRB* (Fig. 11B, panel a, c, and e). No expression of HlVgR in ovaries was detected after dsRNA injections with *HlVgR*, *HlVg-1/HlVgR*, *HlVgR/HlSRB*, or *HlVgR/HlVg-1* (Fig. 11B, panel h, j, k, and l). Neither HlVgR

nor HlSRB proteins localized in the ovaries of ticks exhibited double gene knock-down by an injection of a combination of *HlVgR/HlSRB* dsRNAs (panel e and k). The localization of HlVgR expressed in ovaries, thought to result from the failure to knock-down *HlVgR* with *HlSRB/HlVgR* injection, was also observed by IFAT (panel i).

In the salivary glands and fat bodies, no localization of native HlSRB protein was detected after dsRNA injections with *HlSRB*, *HlSRB/luc*, and *luc/HlSRB* (Fig. 11C and D, panels a, c, and d), while HlSRB localization was observed after a single dsRNA injection of *luc* (Fig. 11C and D, panels b).

3.5. Engorgement, mortality and fecundity of female ticks injected with a single dsRNA or a combination of HlSRB, HlVgR, HlVg-1, and luc dsRNA(s)

Table 3 shows the phenotypic changes of adult female ticks injected with *HlSRB*, *HlVgR*, and *HlVg-1* dsRNA individually or in combination as well as *luc* dsRNA as a control (Table 2).

There were no differences in the groups in the number of ticks attached on a rabbit 24 hours after infestation (Table 3). The engorged body weight of ticks injected with a single dsRNA or a combination of *HlSRB*, *HlVgR*, *HlVg-1*, *HlSRB/HlVgR*, *HlSRB/HlVg-1*, *HlSRB/luc*, *HlVgR/HlSRB*, *luc/HlSRB* dsRNA(s) were significantly lower than those of ticks injected with a single *luc* dsRNA as a control (Table 3). It was evident that the engorged body weights of *HlVg-1* knockdowned ticks was conspicuously lower (Table 3).

The mortality rates 20 days after engorgement in ticks injected with a combination of *HlVg-1/HlVgR*, *HlVgR/HlSRB*, *HlVg-1/HlSRB* and *HlVgR/HlVg-1* dsRNAs, in which a

double knockdown of targeted two genes was achieved (Fig. 9), were significantly higher, 86.4%, 60.5%, 71.0%, and 91.8%, respectively (Table 3). Most of the ticks from these groups died 18 hours after engorgement, and very few died within 5 days of engorgement. Mortality rates of 7.5%, 5.0%, and 27.5% were observed in ticks injected with a single dsRNA of *HlSRB*, *HlVgR*, and *HlVg-1* (Table 2). Similar lower mortality rates of 7.5%, 5.0%, 27.5%, 7.8%, 7.6%, 7.6%, and 7.8% were found in ticks injected with a single *HlSRB*, *HlVgR*, and *HlVg-1* dsRNA, respectively, or a combination of *HlSRB/HlVgR*, *HlSRB/HlVg-1*, *HlSRB/luc*, and *luc/HlSRB* dsRNAs, respectively (Table 3). These low mortality rates were observed in tick groups in which only a single knockdown of targeted genes was obtained (Fig. 9). No mortality was found in ticks injected with control *luc* dsRNA.

The fecundity of female ticks was estimated from the ratio of egg weight to engorged body weight. The ticks did not lay eggs in tick groups injected with a combination of *HlVg-1/HlVgR*, *HlVgR/HlSRB*, *HlVg-1/HlSRB*, and *HlVgR/HlVg-1* dsRNAs, in which double knockdown of targeted two genes was achieved, because the ticks could not achieve oviposition (Table 3). However, normal fecundity ratios were observed in tick groups with single gene knockdown, as described above (Table 3). The lower fecundity observed in ticks injected with a single *HlVg-1* dsRNA (Table 3) was attributed to the crucial involvement of Vgs in oocyte development in the ovaries.

The hatching rates from eggs to larvae were also examined at 25 °C in an incubator for 60 days (Table 3). All eggs from ticks injected with a single dsRNA of *luc* succeeded in hatching to larvae (Table 3). In tick groups injected with a single dsRNA of *HlSRB* and a

combination of *HlSRB/HlVgR*, *HlSRB/HlVg-1*, *HlSRB/luc*, and *luc/HlSRB* dsRNAs, in which single knockdown of *HlSRB* was achieved (Fig. 9), ca. 80% of hatching rates were observed (Table 3). The hatching rates of eggs laid by ticks showing single gene knockdown of *HlVgR* or *HlVg-1* after a respective dsRNA injection were evidently low, and eggs from the HlVgR dsRNA-injected ticks died without hatching (Table 3).

Discussion

There are at least two pathways for exogenous dsRNA uptake in insects, such as the transmembrane channel protein-mediated and the endocytosis-mediated mechanisms (34). In the latter endocytosis-mediated dsRNA uptake mechanism, the participation of SRs, known to be a key component of endocytosis (75), in dsRNA uptake was previously suggested in *T. castaneum* (86) and *D. melanogaster* insects (87). However, it was unknown if SRs could have an important role in the dsRNA-mediated RNAi of ticks. To elucidate the role of SRs in dsRNA uptake in ticks, we used dsRNA of *HlSRB*, the class B scavenger receptor of *H. longicornis* ticks (Chapter-1). In the current study, dsRNA of *HlSRB* was injected into female ticks individually or in combination with different exogenous dsRNAs of ticks, namely *HlVg-1*, a yolk protein precursor expressed only at the midgut of *H. longicornis* (8), and *HlVgR*, a receptor localized only in the oocyte surface of *H. longicornis* (7), as well as firefly *luciferase* (*luc*) as a control (Chapter-1).

As shown in Fig. 8A, the individual gene expression of the *HlSRB*, *HlVgR*, and *HlVg-1* was clearly inhibited in ticks 96 hours after a single dsRNA injection, indicating that mRNA knock-down had been successfully attained in *H. longicornis* ticks within 96 hours of the exogenous dsRNA injection. We found higher mortalities, from 44.4% to 62.2% in ticks 96 hours after a single dsRNA injection with *HlSRB*, *HlVgR*, or *HlVg-1* (Fig. 8B), while the mortality of control ticks injected with a *luc* dsRNA was 22.2%, which is not negligible. This result suggests that increased mortality in ticks injected with dsRNAs has been substantially attributed to the knockdown of targeted genes but partially associated with the possible external injuries, off-target effects associated with long dsRNA in RNAi screens, and functional impairments caused by microinjections in recipient ticks.

As shown in Fig. 9A and B, in the whole bodies of ticks injected with a combination of *HlVg-1/HlVgR* or *HlVgR/HlVg-1* dsRNAs, a clear and systemic double knockdown of endogenous *HlVg-1* and *HlVgR* genes was attained in both tick groups. Similar systemic double gene knockdown of targeted genes was observed in ticks injected with a combination of *HlVgR/HlSRB* or *HlVg-1/HlSRB* dsRNAs. However, in the whole bodies of ticks injected with a combination of *HlSRB/HlVgR* or *HlSRB/HlVg-1* dsRNAs, no knockdown of *HlVgR* and *HlVg-1* was achieved, although clear systemic gene silencing of *HlSRB* was attained. These results suggest that systemic RNAi mediated by the second *HlVgR* or *HlVg-1* dsRNA injection was dramatically inhibited by the first systemic gene knockdown of *HlSRB*. No conclusion can yet be drawn, but it is speculated that SID-1, SRs, vacuolar H^+ATPase, RDS-3, and RdRp could be possible components of the dsRNA uptake mechanism in several insects (34). With regard to SRs, two scavenger receptors of

D. melanogaster, SR-CI and Eater, account for more than 90% of dsRNA uptake by S2 cells (87). Our results clearly indicate that HlSRB, class B scavenger receptor of *H. longicornis* ticks is essential for systemic RNAi/effective knock-down of gene expression by RNAi.

Boldbaatar et al. (7, 8) demonstrated that the transcription and translation of HlVg-1 are midgut-specific and those of HlVgR are ovary oocyte-specific. In the current study, we examined the expression and localization of HlVg-1 in midguts and HlVgR in ovaries of female ticks showing a single or double systemic RNAi. The localization of HlSRB was also examined in midguts, ovaries, salivary glands, and fat bodies. As a result, normal expression of the gene and protein of HlVg-1 in midguts and HlVgR in ovaries were clearly shown in female ticks with a systemic RNAi of *HlSRB* (Figs. 10 and 11).

Systemic RNAi can only take place in multicellular organisms because it includes processes in which a silencing signal is transported from one cell to another or from one tissue type to another (34). In multicellular organisms, such as ticks, the *HlVg-1* or *HlVgR* dsRNA internalized through injection into hemocoel must be taken up from the hemolymph to the midgut cells or ovary oocytes in order to silence the target genes. We conclude that the dysfunction of receptor-mediated endocytosis was introduced in ticks by the systemic RNAi of *HlSRB*, resulting in the uptake abrogation of *HlVg-1* or *HlVgR* dsRNA in midguts or ovaries and leading to normal protein expression.

Most of our understanding of tick RNAi is mediated systemic delivering of RNAi effect and the literature demonstrated that a systemic RNAi silencing mechanism is active in ticks (6–8, 24, 49, 55, 61). Results obtained in this study might explain that the SR-mediated dsRNA uptake mechanism is evolutionarily conserved in ticks and plays a crucial

role in controlling the induction of systemic gene silencing in ticks. However, other factors, such as proteins of the vesicle-mediated transport, conserved oligomeric Golgi complex family, cytoskeleton organization and protein transport are directly and/or indirectly involved in endocytosis and known to play roles in dsRNA uptake and processing (78). Further studies are needed to examine these factors in *H. longicornis*. These results may contribute to the development of a control strategy for ticks and pathogen transmission.

The overall results presented in this study show that dsRNAs of *HlSRB*, *HlVg-1*, and *HlVgR* introduced into ticks individually or in combination resulted in different but significant phenotypic changes in them (Table 2). These phenotypes in engorgement, mortality, fecundity, and oocyte development of ticks were comparable with those in previous characterization (7, 8, Chapter-1) and were more prominent in ticks indicating double knockdown of *HlVg-1/HlVgR*, *HlVgR/HlSRB*, or *HlVg-1/HlSRB* genes than in those with single gene knockdown of *HlSRB*, *HlVg-1*, or *HlVgR* (Table 2), suggesting that systemic and specific double knockdown of target genes had been simultaneously attained in these ticks. This successful double gene knockdown might show promising application possibilities for combinational RNAi in the practical control measures of ticks and tick-borne diseases.

In summary, research in recent years has given new insights into the dsRNA uptake mechanism in the gene silencing of insects. However, the role of dsRNA uptake in ticks remains to be proven. I demonstrated for the first time in the current study, using *HlSRB*, a class B scavenger receptor CD36 of *H. longicornis*, that *SRB* mediates the effective knock-down of gene expression by RNAi and plays essential roles for the systemic RNAi of ticks.

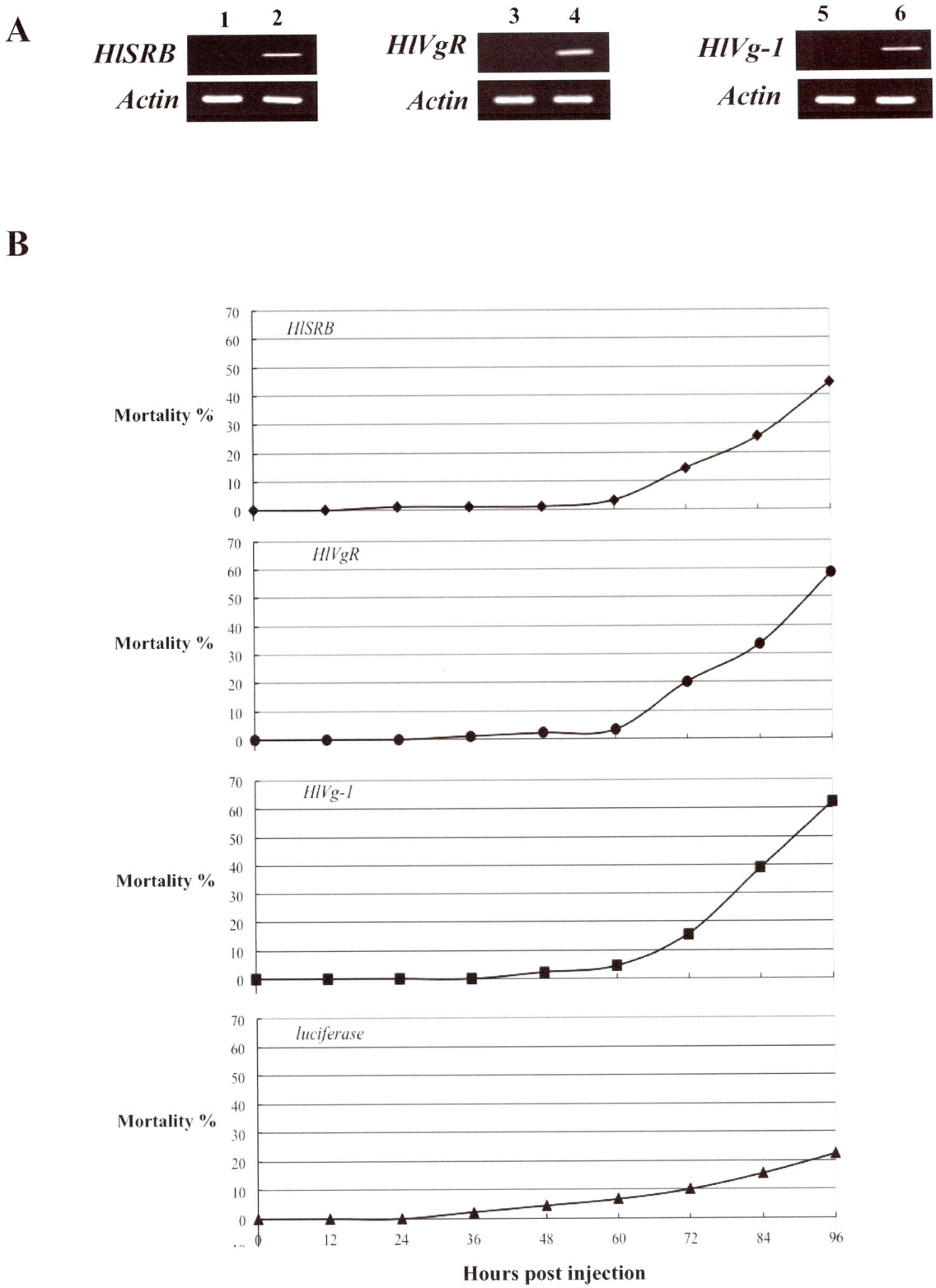

A
1
2
HlSRB
Actin
3
4
HlVgR
Actin
5
6
HlVg-1
Actin
B
HlSRB
HlVgR
HlVg-1
luciferase
Mortality %
Hours post injection
0
12
24
36
48
60
72
84
96

Fig. 8.

Fig. 8. Gene silencing and mortality rate of *H. longicornis* 96 hours after a single dsRNA injection

dsRNA complementary to *HlSRB*, *HlVgR*, and *HlVg-1* was injected into *H. longicornis* adult females. The injected ticks were allowed to rest at 25 °C in an incubator for four days to check mortality rates and gene silencing. RT-PCR analysis (A). PCR was performed using cDNA synthesized from three ticks injected with *HlSRB*, *HlVgR*, *HlVg-1*, or *luc* dsRNA with primer sets specific to *HlSRB*, *HlVgR*, *HlVg-1*, and the *β-actin* gene. Lane 1, *HlSRB* dsRNA-injected ticks; lanes 2, 4, and 6, *luc* dsRNA-injected ticks; lane 3, *HlVgR* dsRNA-injected ticks; lane 5, *HlVg-1* dsRNA-injected ticks. Mortality rates (B). Each panel represents treatment with one gene-specific dsRNA. Mortality rates were calculated by the percentage of number of dead ticks to the number of ticks used at the beginning of experiment in a different time course. *HlSRB*, *HlSRB* dsRNA-injected ticks; *HlVgR*, *HlVgR* dsRNA-injected ticks; *HlVg-1*, *HlVg-1* dsRNA-injected ticks; *luciferase*, *luciferase* dsRNA-injected ticks.

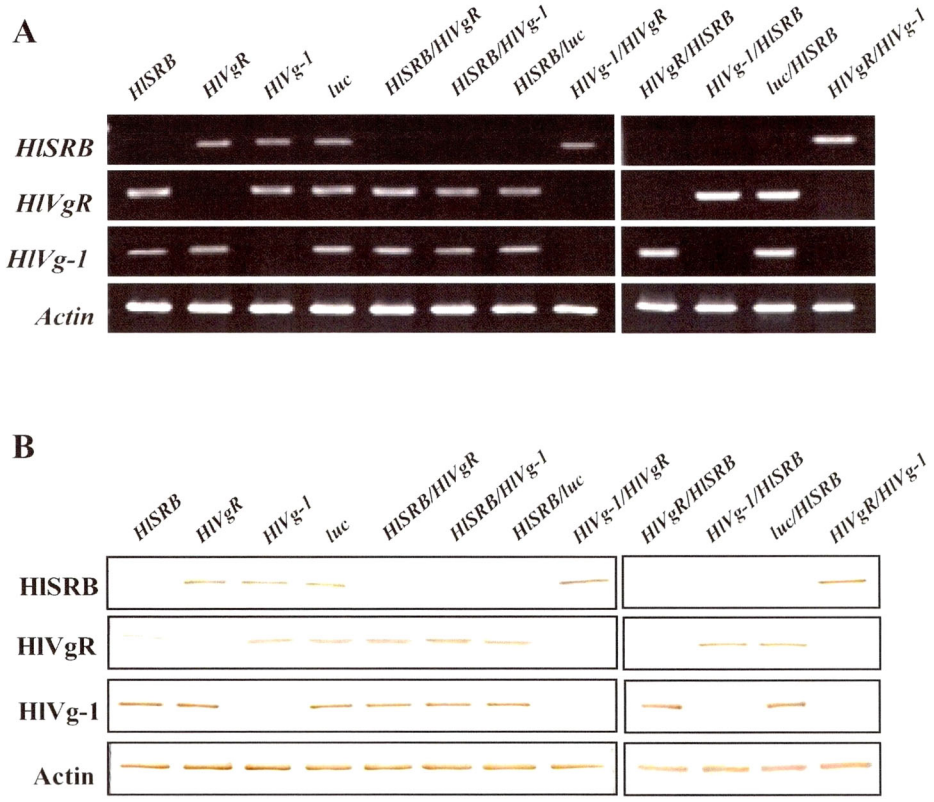


Fig. 9. Silencing of HlSRB, HlVgR, and HlVg-1 genes and proteins in the whole body of *H. longicornis*

Individually or in combination of *HlSRB*, *HlVgR*, *HlVg-1*, and *luc* dsRNA(s) were injected into *H. longicornis* adult ticks. The injected ticks were left for 12 hours at 25 °C and infested on the rabbits for four days and then ticks samples were collected for RNA extraction and the preparation of ticks protein lysates in each group. The name of each dsRNA group is indicated above. RT-PCR analysis (A). RT-PCR analysis was performed as shown in Fig. 8. (A). Western blot analysis (B). Tick lysates were subjected to SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was probed with mouse anti-rHlSRB, anti-rHlVgR, or anti-rHlVg-1 sera; mouse anti-actin serum was used as a control. The name of each dsRNA group is the same as that used in Fig. 8.

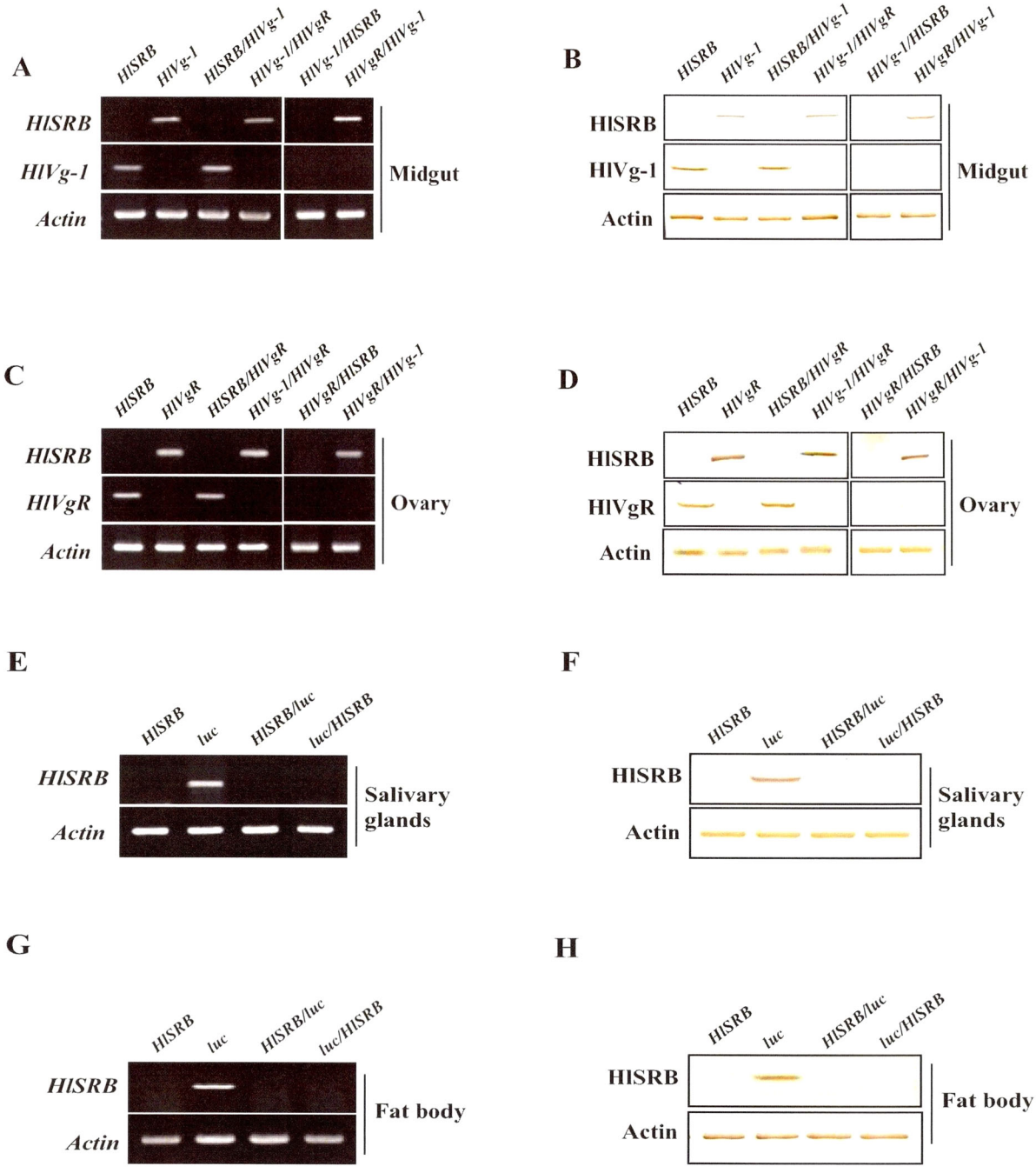

A
HlSRB
HlVg-1
HlSRB/HlVg-1
HlVg-1/HlVgR
HlVg-1/HlSRB
HlVgR/HlVg-1
HlSRB
HlVg-1
Actin
Midgut
B
HlSRB
HlVg-1
HlSRB/HlVg-1
HlVg-1/HlVgR
HlVg-1/HlSRB
HlVgR/HlVg-1
HlSRB
HlVg-1
Actin
Midgut
C
HlSRB
HlVgR
HlSRB/HlVgR
HlVg-1/HlVgR
HlVgR/HlSRB
HlVgR/HlVg-1
HlSRB
HlVgR
Actin
Ovary
D
HlSRB
HlVgR
HlSRB/HlVgR
HlVg-1/HlVgR
HlVgR/HlSRB
HlVgR/HlVg-1
HlSRB
HlVgR
Actin
Ovary
E
HlSRB
luc
HlSRB/luc
luc/HlSRB
HlSRB
Actin
Salivary glands
F
HlSRB
luc
HlSRB/luc
luc/HlSRB
HlSRB
Actin
Salivary glands
G
HlSRB
luc
HlSRB/luc
luc/HlSRB
HlSRB
Actin
Fat body
H
HlSRB
luc
HlSRB/luc
luc/HlSRB
HlSRB
Actin
Fat body

Fig. 10.

Fig. 10. Expression profiles of HlSRB, HlVg-1, and HlVgR genes and proteins in different tissues of *H. longicornis*

Individually or in combination of *HlSRB*, *HlVg-1*, *HlVgR*, and *luc* dsRNA(s) were injected into *H. longicornis* adult ticks. The midguts and ovaries of dsRNA-injected ticks at 4 days of feeding were dissected out in 0.1% diethylpyrocarbonate-treated 1 × PBS (-) under a microscope. The name of each dsRNA group is indicated above. RT-PCR analysis and Western blot analysis were conducted using the midguts (A and B), ovaries (C and D), salivary glands (E and F), and fat body (G and H), respectively.

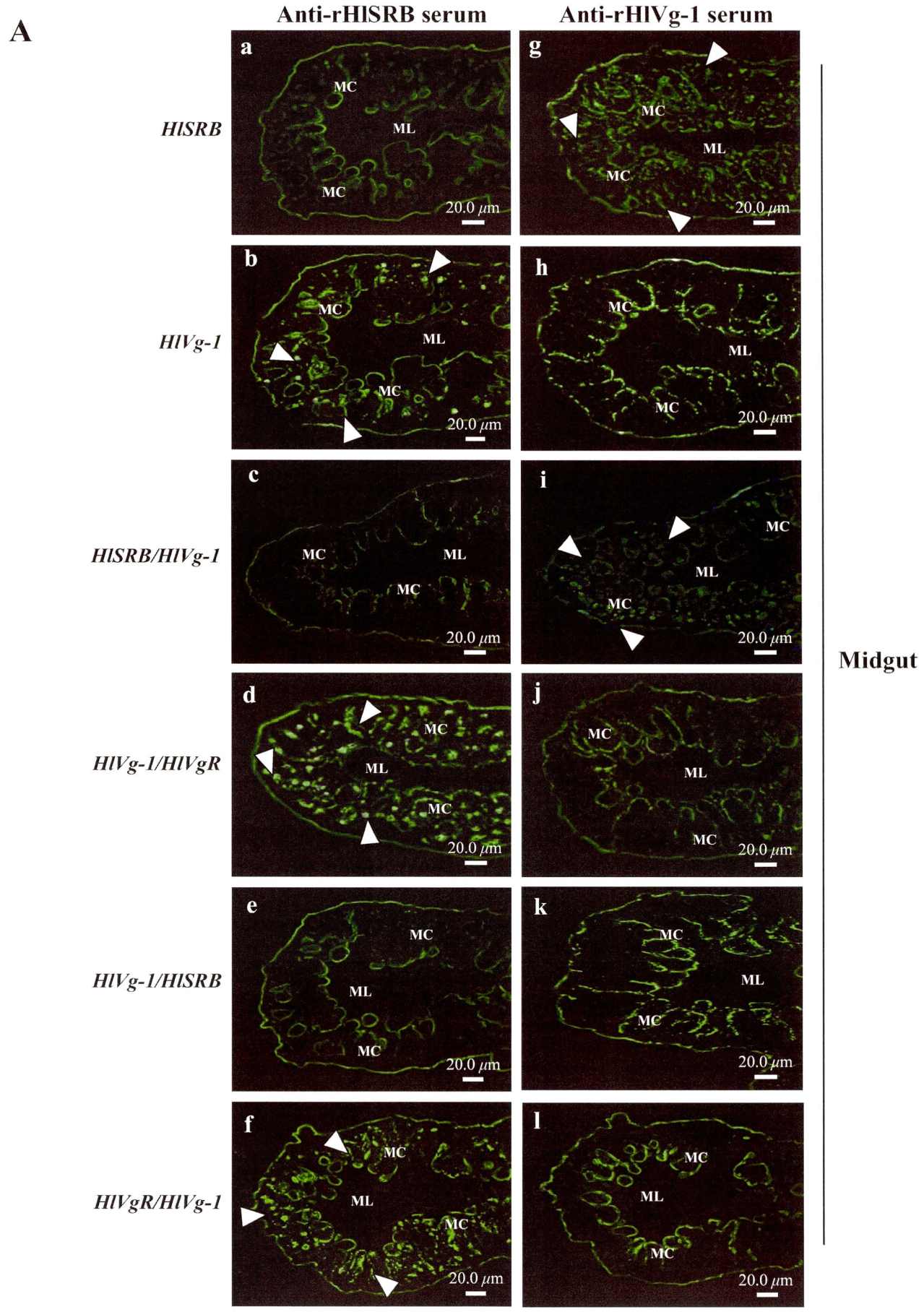

A
Anti-rHlSRB serum
Anti-rHlVg-1 serum
HlSRB
HlVg-1
HlSRB/HlVg-1
HlVg-1/HlVgR
HlVg-1/HlSRB
HlVgR/HlVg-1
Midgut
a
b
c
d
e
f
g
h
i
j
k
l
MC
ML
20.0 μm

Fig. 11.

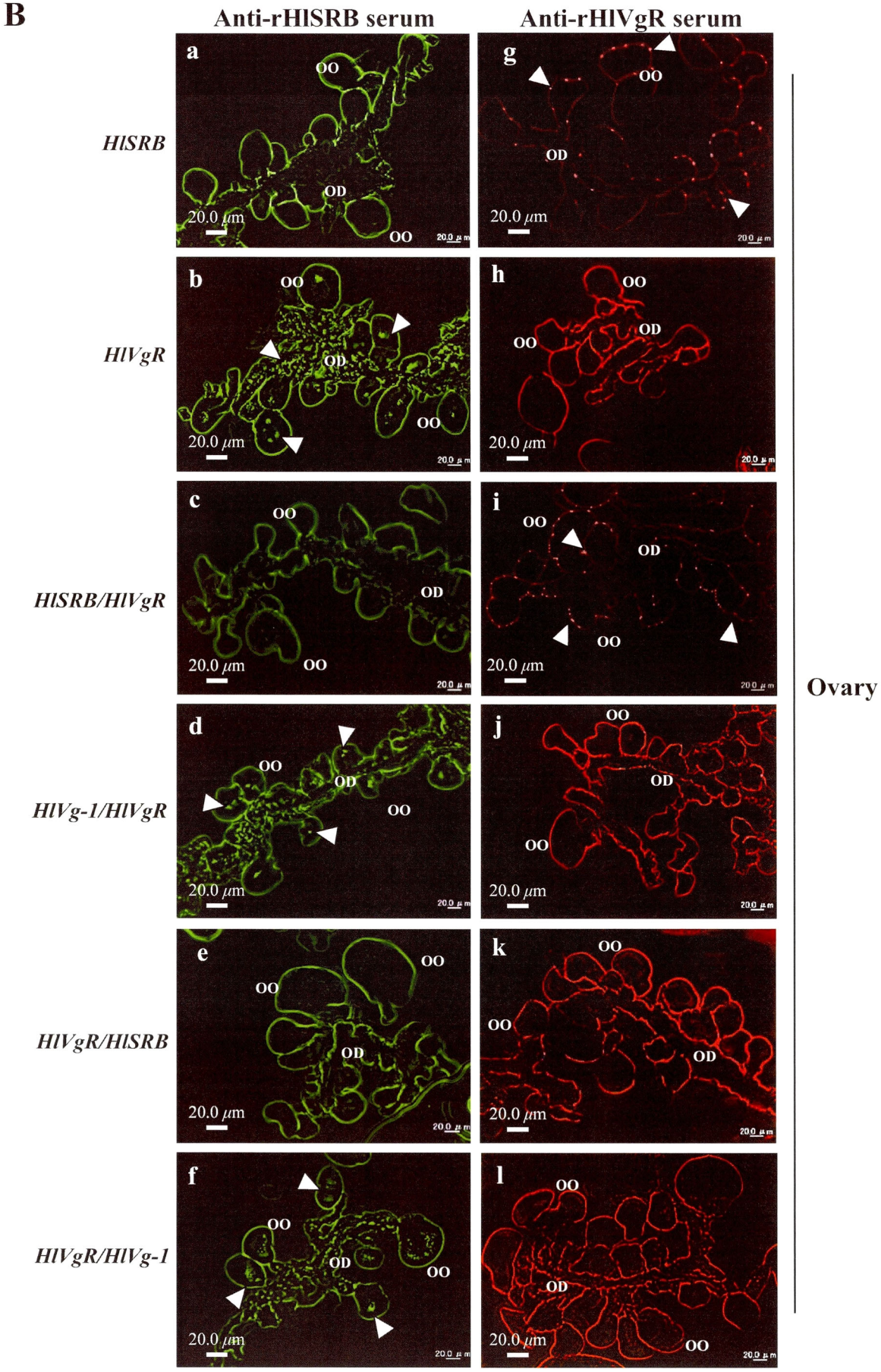

B
Anti-rHlSRB serum
Anti-rHlVgR serum
HlSRB
HlVgR
HlSRB/HlVgR
HlVg-1/HlVgR
HlVgR/HlSRB
HlVgR/HlVg-1
Ovary
a
b
c
d
e
f
g
h
i
j
k
l
OO
OD
20.0 μm

Fig. 11.

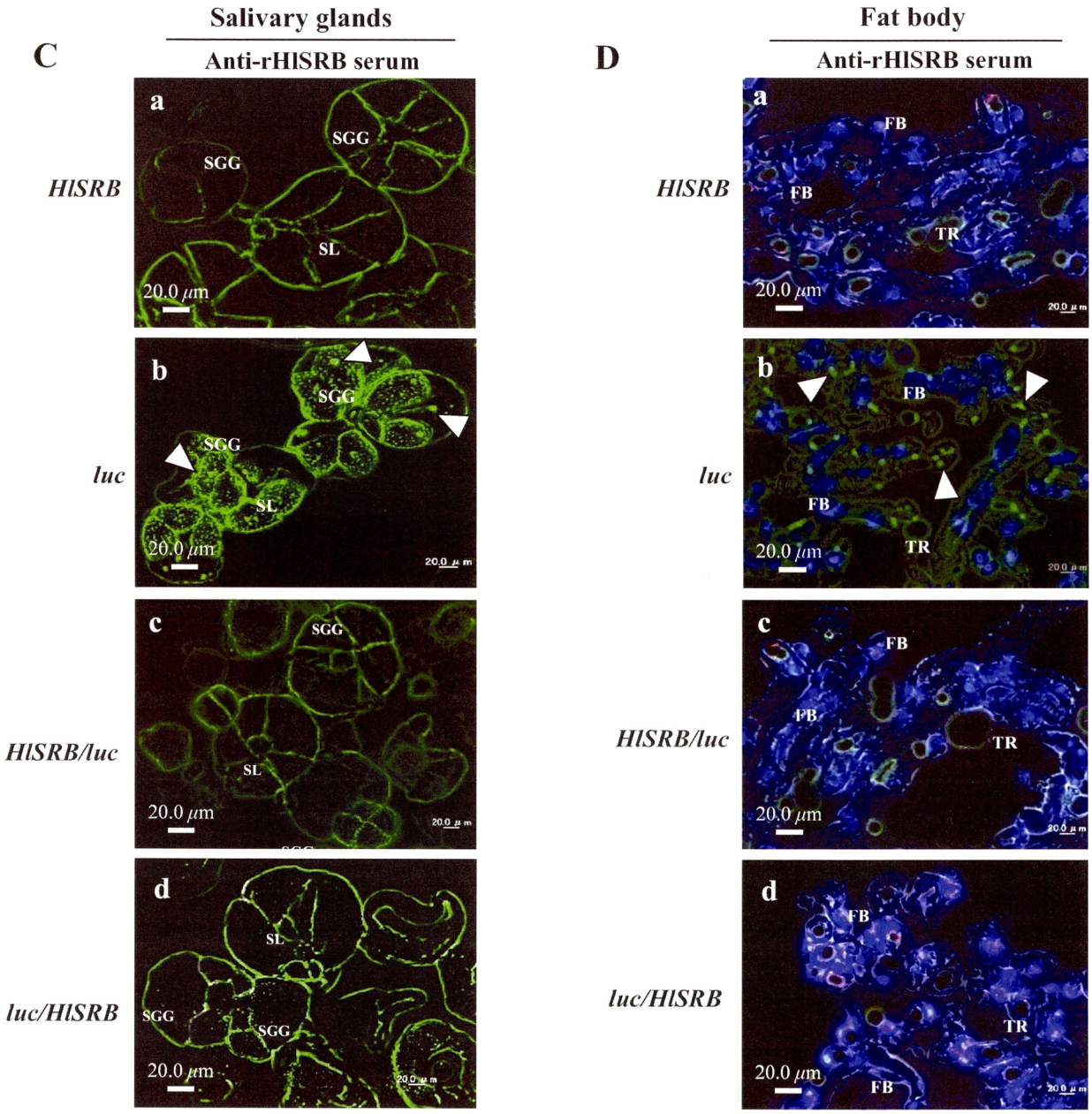

C
Salivary glands
Anti-rHlSRB serum
HlSRB
luc
HlSRB/luc
luc/HlSRB
a
b
c
d
SGG
SL
20.0 μm
D
Fat body
Anti-rHlSRB serum
FB
TR
20.0 μm

Fig. 11.

Fig. 11. Confirmation of RNAi on the endogenous HlSRB, HlVg-1, and HlVgR in the different tissues of *H. longicornis*

The dissected tissues from the dsRNA-injected 4-days-feeding ticks were observed under fluorescence microscopy. The name of each dsRNA group is indicated above. The midguts were stained with anti-rHlSRB and anti-rHlVg-1 antibodies followed by Alexa 488-conjugated mouse anti-IgG with DAPI (A). Arrowheads indicate the native HlSRB and HlVg-1 expressed in the midguts. ML, midgut lumen; MC, midgut cells. The ovaries, staining pattern of anti-rHlSRB and anti-rHlVgR serum were used as first antibodies (B). The mouse anti-IgG conjugated with Alexa 488 was used as a second antibody with DAPI for the left panels and Alexa 594 with Propidium Iodide for the right panels. Arrowheads indicate the native HlSRB and HlVgR expressed in the ovaries. OO, oocyte; OD, oviduct. The salivary glands (C) and fat body (D) tissues were stained with anti-rHlSRB antibodies followed by Alexa 488-conjugated mouse anti-IgG. Arrowheads indicate the native HlSRB expressed in the tissues of salivary glands and fat body tissues. SL, salivary gland lumen; SGG, salivary gland granular acini; FB, fatbody; TR, trachea. The *scale bar* represents 20 μm.

Table 2 Female tick groups injected with a single and a combination of dsRNA(s)

Tick groups	dsRNA used for the first injection	dsRNA used for the second injection
HlSRB	*HlSRB*	–
HlVgR	*HlVgR*	–
HlVg-1	*HlVg-1*	–
HlSRB/HlVgR	*HlSRB*	*HlVgR*
HlSRB/HlVg-1	*HlSRB*	*HlVg-1*
HlSRB/luc	*HlSRB*	*luc*
HlVg-1/HlVgR	*HlVg-1*	*HlVgR*
HlVgR/HlSRB	*HlVgR*	*HlSRB*
HlVg-1/HlSRB	*HlVg-1*	*HlSRB*
luc/HlSRB	*luc*	*HlSRB*
HlVgR/HlVg-1	*HlVgR*	*HlVg-1*
luc	*luc*	–

The first and second dsRNA injections were carried out at 96-hours interval.

Table 3 Phenotypic changes of ticks injected with a single or a combination of different dsRNA(s)

dsRNA groups	Number of ticks attached 24h after infestation[a]	Average engorged body weight (mg)[b]	Mortality rate (%)[c]	Egg weight/ body weight (%)[b]	Hatching rate (%)[d]
HlSRB	40	142.10±30.30*	7.5	41.95±11.25**	83.7
HlVgR	40	172.18±19.92*	5	11.62±12.41**	0
HlVg-1	40	81.56±18.16*	27.5	8.16±30.45**	13.7
HlSRB/HlVgR	38	143.61±14.38*	7.8	41.25±15.08**	82.8
HlSRB/HlVg-1	39	141.57±45.39*	7.6	42.10±16.32**	83.3
HlSRB/luc	39	142.81±17.12*	7.6	41.75±11.21**	83.3
HlVg-1/HlVgR	37	76.91±24.37*	86.4	0	0
HlVgR/HlSRB	38	150.29±11.32*	60.5	0	0
HlVg-1/HlSRB	38	85.81±64.14*	71	0	0
luc/HlSRB	38	143.15±32.63*	7.8	42.87±24.13**	82.8
HlVgR/HlVg-1	37	98.45±68.28*	91.8	0	0
luc	40	245.75±37.35	0	50.70±21.31	100

[a] Sixteen ticks were collected from the host for the subsequent experiments at 4 days after attachment.

[b] These ratios show the fecundity of engorged females. Values are the means of ± SD.

[c] These mortality rates show the percentages of number of dead ticks 20 days after drop-off to the total number of engorged ticks per treatment.

[d] Hatchings from eggs to larvae were examined at 25 °C in an incubator for 60 days.

[*]$P<0.05$, *luc* dsRNA-injected group vs. *HlSRB-*, *HlVgR-*, *HlVg-1-*, *HlSRB/HlVgR-*, *HlSRB/HlVg-1-*, *HlSRB/luc-*, *HlVg-1/HlVgR-*, *HlVgR/HlSRB-*, *HlVg-1/HlSRB-*, *luc/HlSRB-*, and *HlVgR/HlVg-1* dsRNA-injected groups.

[**]$P<0.05$, *luc* dsRNA-injected group vs. *HlSRB-*, *HlVgR-*, *HlVg-1-*, *HlSRB/HlVgR-*, *HlSRB/HlVg-1-*, *HlSRB/luc-*, and *luc/HlSRB* dsRNA-injected groups.

Chapter 3:

HlSRB, a class B scavenger receptor, is key to the granulocyte-mediated microbial phagocytosis in ticks

1. Introduction

Phagocytosis refers to the recognition, engulfment, and intracellular destruction of invading pathogens and apoptotic cells by individual hemocytes (47). In arthropods, such as insects (50) and ticks (35,82), phagocytosis is achieved mainly by the circulating plasmatocytes and/or granulocytes, in the hemolymph. The SRB, the cell surface glycoprotein, is present on a variety of cell types, including insect hemocytes (48, 60). Recent findings provide evidence for the essential role of SRB as a pattern-recognition receptor mediating innate immune responses of the mammalian (83) and insect hosts (50) to a range of exogenous pathogens. In this chapter, I show that HlSRB is the first scavenger receptor molecule contributed to hemocyte-mediated phagocytosis against exogenous

bacteria, isolated and characterized from hematophagous arthropods.

2. Materials and methods

2.1. Preparation of unfed (UF), partially fed (PF), and HlSRB dsRNA-injected female (RNAi-tick) ticks and microinjection of E. coli into these tick groups

Three groups of ticks, i.e., unfed female (UF) 270 ticks, partially fed female (PF) 270 ticks, and *HlSRB* dsRNA-injected female (RNAi-tick) 270 ticks, were used in this experiment. UF ticks were maintained at 15 °C in an incubator. To obtain PF ticks, UF ticks were fed on Japanese white rabbits, and, 3 days after attachment, ticks were collected as PF ticks. For RNAi-ticks, the *HlSRB* dsRNA was injected into UF ticks (total 0.5 µl; 1 µg/tick). The dsRNA injection was followed as described previously (Chapter-1). The UF, PF, and RNAi-ticks were left at 25 °C in an incubator for subsequent experiments. The injection of heat-killed *E. coli* (72 °C for 1.5 mins), *E. coli*, or *HlSRB* dsRNA into ticks and construction of *HlSRB* dsRNA were performed as described previously (Chapter-1).

An *E. coli* (pathogenic strain O157) was grown in a Luria-Bertani broth medium (BD, Sparks, MD, USA) at 37 °C. When the optical density at 600 nm reached 0.5 (OD_{600} = 0.5), *E. coli* cells were induced with 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) and incubated for another 4 hours. An *E. coli* suspension was respectively injected (0.5 µl/tick) to UF, PF, and RNAi-ticks groups through the fourth coxae into the haemocoel.

Control ticks were injected with an equal volume of heat-killed *E. coli* (0.5 μl/tick). The injected ticks were left to rest at 25 °C in an incubator for 24 hours, and hemolymph collection was then performed as described below.

2.2. Preparation of hemolymph and hemocyte samples

Hemolymph samples from UF, PF, and RNAi-ticks injected with or without *E. coli* or heat-killed *E. coli* were collected by amputating the forelegs of female ticks at the coxal-trochanteral joint, drawn into heparinized capillary tubes containing 100 μl of PBS (20, 35, 39) and then loaded to Shandon EZ Double Cytofunnel (Thermo Electron Corp., Milford, MA, USA). Hemocyte smears were obtained from these hemolymph samples by using a Cytospin 4 cytocentrifuge machine (Thermo Electron) at 100 *g* for 5 minutes, and smears on the micro-glass slides were then air-dried and fixed in methanol or cold acetone for 10 minutes. Smears fixed in methanol were immediately stained with a 3% Giemsa solution for 30 minutes for the light microscopic examination of hemocyte morphology and counting of granulocyte population, and other smears fixed in cold acetone were kept at - 80 °C until use for IFAT.

2.3. Indirect immunofluorescent antibody test (IFAT)

To examine endogenous HlSRB localization in hemocytes from UF, PF, and RNAi ticks, hemocyte smears on the micro-glass slides described above were blocked with 5%

skim milk in PBS overnight at 4 °C and incubated for 30 minutes at 37 °C with 1:100 dilution of an anti-rHlSRB (rHlSRB) mouse serum (Chapter-1) as a primary antibody. After washing three times with PBS, Alexa 488-conjugated goat anti-mouse immunoglobulin (1:1000; Invitrogen) was applied as secondary antibody at 37 °C for 1 hour. After washing three times with PBS, hemocyte smears were mounted in a mounting medium with DAPI (Vectashield, Vector Laboratories, Burlingame, CA, USA) and then covered with a cover glass. The images were photographed and recorded using a fluorescence microscope (Olympus, Tokyo, Japan).

2.4. Tick survival monitoring

The changes with time in the survival rates of UF, PF, and RNAi-ticks after injection with heat-killed *E. coli* or *E. coli* were monitored using different tick groups from the ticks for hemocyte examinations. Preparation of *E. coli* incubation and injection were followed as described above. Female ticks injected with heat-killed *E. coli* or *E. coli* were left at 25 °C in an incubator and their survival was checked every 6 hours for 2 days after injection. This survival monitoring was performed at least in triplicate.

2.5. Examination of population of phagocytic hemocytes after E. coli *injection*

To examine the populational changes of phagocytic hemocytes after *E. coli* injection, hemolymph samples (5 μl/tick) of Pf ticks 3, 12, 24, and 48 hours after injection with heat-killed *E. coli* or an *E. coli* suspension were prepared for hemocyte smears, as

described above. Plasmatocytes and granulocytes were counted in a 3% Giemsa solution (5 ticks/group), as described above, using a light microscope (Olympus).

2.6. Reverse transcriptase-polymerase chain reaction (RT-PCR)

To investigate the expression pattern of the *HlSRB* gene after *E. coli* injection, hemolymph samples of PF ticks 3, 12, 24, and 48 hours after injection with heat-killed *E. coli* or an *E. coli* suspension were subjected to total RNA extraction using the TRIzol reagent (Invitrogen, CA, USA). The RT-PCR analysis was performed using a one-step RNA PCR kit (Takara, Otsu, Japan) with the primer sets of the *HlSRB* (Chapter-1) gene. Control amplification was carried out using the *H. longicornis β-actin*-specific primers (accession no. AY254898). The PCR products were subjected to electrophoresis in a 1.5% agarose gel in a TAE buffer; the DNA was visualized by ethidium bromide staining and analyzed using Quantity One 1-D Analysis Software (Quantity One Version 4.5, Bio-Rad Laboratories, Milan, Italy).

2.7. Protein expression analysis by Western blotting

Hemolymph samples of PF ticks 3, 12, 24, and 48 hours after injection with heat-killed *E. coli* or *E. coli* suspension were separated by SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride membrane (Millipore, Bedford, MA, USA). The membrane was blocked with 5% skim milk in PBS-T (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, 0.05% Tween-20, pH 7.4) and

then incubated with 1:100 dilution of anti-rHlSRB or 1:200 dilution of anti-actin serum (49) as a primary antibody. After the incubation of peroxide-conjugated sheep anti-mouse IgG as a secondary antibody (1:2000 dilution; GE Healthcare, Little Chalfont, UK), the specific protein bands were detected using 0.5 mg/ml 3, 3'-diaminobenzidine tetrahydrochloride.

2.8. Construction of recombinant E. coli *expressing green fluorescent protein (EGFP)*

A 759-bp DNA fragment containing an open reading frame of the enhanced green fluorescent protein (EGFP) gene was isolated from pEGFP (Clontech, Palo Alto, CA, USA) and inserted into the *Sal* I-*Not* I sites of the *E. coli* expression vector, pRSET-B (Invitrogen) as recommended by the manufacturer. The resulting plasmid was designated as pRSET-B/EGFP.

An *E. coli*, DH5α strain-competent cells (Invitrogen) colony transformed with pRSET-B/EGFP was cultured in an LB broth medium (BD) supplemented with 50 μg/ml of ampicillin. When the optical density at 600 nm reached 0.5, *E. coli* cells were induced to express the recombinant EGFP by the addition of 1 mM IPTG and incubation for another 4 hours.

2.9. Injection and culture of EGFP-expressing E. coli

EGFP-expressing *E. coli* (E-*E. coli*), readily detectable by microscopy [18], was injected as a suspension (0.5 μl) into UF, PF, and RNAi-ticks, respectively. The injected ticks were left for 24 hours at 25 °C in an incubator, and hemolymph samples were then

collected by amputating the forelegs of female ticks at the coxal-trochanteral joints. One drop of hemolymph was placed on a glass slide and covered with a cover glass, and the hemocyte images were photographed using a fluorescence microscope (Olympus).

Five microliters of hemolymph from UF, PF, or RNAi-ticks 24 hours after E-*E. coli* injection was applied to an LB agar medium including 1 μg/ml of ampicillin and 100 mM/plate of IPTG. All emerged colonies were counted within 24 hours using FluorChem FC2 (Cell Biosciences, California, USA). In addition, the emerged colonies were observed under UV light in order to confirm whether they were E-*E. coli* or not (52).

3. Results

3.1. Plasmatocytes and granulocytes of female ticks

The current observations were performed on plasmatocytes and granulocytes of female *H. longicornis* ticks (Fig. 12A), since they are generally recognized as a predominant class of phagocytes circulating in hemolymph of ticks (9, 31, 35, 53). Plasmatocytes of ticks have a round or irregular shape with processes such as filopodia, and they have few granules in the cytoplasm. The granulocytes of ticks show a spherical form and are filled with many large granules (46, 53, 82).

An immunohistochemical examination using an indirect fluorescent antibody test (IFAT) was conducted to illustrate the localization of the endogenous HlSRB protein in the

phagocytic plasmatocytes and granulocytes of PF ticks without heat-killed *E. coli* or *E. coli* injection (Fig. 12B). IFAT was performed using anti-rHlSRB mouse serum followed by Alexa 488-conjugated anti-mouse immunoglobulin. As shown in Fig. 12B, the dotted localization of native HlSRB protein was detected only on the surface of granulocytes, while no localization was observed in plasmatocytes (Fig. 12B), suggesting that endogenous HlSRB is expressed predominantly in granulocytes of *H. longicornis*.

3.2. Morphological changes of plasmatocytes and granulocytes of female ticks after E. coli *injection*

We examined the morphological changes of two types of phagocytic hemocytes, plasmatocytes and granulocytes, in UF, PF, and RNAi-tick groups 24 hours after heat-killed *E. coli* or *E. coli* injection (Fig. 13). Based on our observation of Giemsa-stained smears, no changes were observed in the shape of plasmatocytes and granulocytes from UF, PF, and RNAi-ticks 24 hours after heat-killed *E. coli* injection (Fig. 13A). Similarly, there were no remarkable morphological changes in plasmatocytes from UF, PF, and RNAi ticks 24 hours after *E. coli* injection (Fig. 13B, panel a, b, and c). Interestingly, large lobopodia-like structures (46) were observed in granulocytes of the Giemsa-stained smears from UF and PF ticks 24 hours after *E. coli* injection (Fig. 13B, panel d, and e, arrows), and many *E. coli* bacteria were found around the top of lobopodia-like structures (Fig. 13B, panel g, h, and i). However, no lobopodia-like structures were detected in granulocytes from RNAi ticks after *E. coli* injection (Fig. 13B, panel f). According to our results of Fig. 2A and B, the different

amount of extracellular heat-killed *E. coli* or *E. coli* was found in the hemolymph because heat-killed *E. coli* could not grow and *E. coli* could grow after 24 hours injection at 25 °C.

3.3. Localization of endogenous HlSRB proteins expressed in granulocytes of female ticks after E. coli *injection*

We observed the immunohistological localization of endogenous HlSRB proteins in granulocytes 24 hours after heat-killed *E. coli* or *E. coli* injection. As shown in Fig. 14A, there were a few dots showing HlSRB localization, around 8 in number, on the surface of granulocytes from UF and PF ticks 24 hours after heat-killed *E. coli* injection (panel a, and b), while no positive fluorescence was observed on the surface of granulocytes from RNAi-ticks injected with *E. coli* (panel c) by IFAT. In addition, no morphological changes were observed in the granulocytes from UF, PF, and RNAi-ticks injected with heat-killed *E. coli* (Fig. 14A).

A significantly large number of fluorescent dots showing localization of native HlSRB protein were observed on almost all the surface of granulocytes from UF and PF ticks 24 hours after *E. coli* injection (Fig. 14B, panel a, and b), and they were 60 to 90 in number and uniformly distributed throughout the surface of granulocytes including lobopodia-like structures. However, no positive fluorescence was detected on the surface of granulocytes from RNAi-ticks injected with *E. coli* (Fig. 14B, panel c). These data suggest that granulocytes of *H. longicornis* ticks might morphologically respond to *E. coli* injection and up-regulate the expression of cell surface HlSRB, but HlSRB-silenced granulocytes might have failed to respond properly to *E. coli* invasion in hemolymph.

3.4. Survival rates of female ticks after E. coli *injection*

High survival rates were consistently observed in UF, PF, and RNAi-ticks after heat-killed *E. coli* injection (Fig. 15). The survival rates of UF, PF, and RNAi-ticks 48 hours after heat-killed *E. coli* injection were 82.1%, 92.1%, and 78.2%, respectively (Fig. 15), indicating that changes with time in survival rates were only modest in ticks injected with heat-killed *E. coli* and no significant differences were obvious among UF, PF, and RNAi-ticks. However, the survival rates of female ticks injected with *E. coli* were quite different between UF and PF ticks and RNAi-ticks. The survival rates of UF and PF ticks 30 and 48 hours after *E. coli* injection were 82.5 to 92.5% and 50.8 to 78.3%, respectively (Fig. 15), showing only a slight decrease with time. However, the survival rates of RNAi-ticks 18, 24, and 30 hours after *E. coli* injection were 64.1%, 22.5%, and 0%, respectively (Fig. 15), indicating a marked decrease with time. These results indicated that almost all UF and PF ticks could survive after proper control of invaded *E. coli* but RNAi-ticks had to succumb to *E. coli* burdens mainly due to the recognition failure in pathogen-associated molecular patterns (PAMPs) (25, 50) caused by HlSRB-silenced and infection due to high bacteremia.

3.5. Populational changes of plasmatocytes and granulocytes of female ticks after E. coli *injection*

We examined changes in population of phagocytic hemocytes from PF ticks after *E. coli* injection in different time courses. Based on our observation of Giemsa-stained smears, the population of plasmatocytes and granulocytes increased after *E. coli* injection. In this

study, we focused on the population of granulocytes because HlSRB was expressed only in the granulocytes (Figs. 12B and 14). As shown in Fig. 16A, the population of granulocytes were increased with a different time course not only slightly in ticks after heat-killed *E. coli* injection but also more significantly in ticks injected with *E. coli*. These percentages represent, on average, the results of five ticks from each group. This result suggests that the increased granulocytes population might be the result of granulocyte-mediated phagocytosis for invasion foreign microorganisms and that the slightly increase is related to possible external injuries caused by microinjections in recipient ticks.

3.6. Expression patterns of HlSRB in hemolymph of PF ticks after E. coli *injection*

The expression patterns of HlSRB in hemolymph from PF ticks after *E. coli* injection were examined by RT-PCR and Western blot analysis in different time courses. As shown in Fig. 16B and C, the β-actin gene and protein levels did not change in ticks injected with heat-killed *E. coli* or *E. coli*. However, the gene and protein expressions of HlSRB were up-regulated with time slightly in ticks after heat-killed *E. coli* injection and more significantly in ticks injected with *E. coli* (Fig. 16D and E), suggesting that HlSRB expression in tick hemolymph containing various types of hemocytes and humoral proteins (29, 82) might be up-regulated for participation in both immunological defense against *E. coli* and wound-healing after microinjection.

3.7. *Key roles of HlSRB in granulocyte-mediated phagocytosis to* E. coli

Hemolymph samples were collected from UF, PF, and RNAi-ticks 24 hours after injection with EGFP-expressing *E. coli* (E-*E. coli*) and examined under fluorescence microscopy (Fig. 17A). In UF and PF ticks injected with E-*E. coli*, granulocytes were found to contain many phagocytosed bacteria and appeared to extend a lobopodia-like structure toward a colony of bacteria (panel a, b, d, and e). In RNAi-ticks injected with E-*E. coli*, bacteria accumulated around granulocytes, and no phagocytosed bacteria were observed in the cytoplasm of granulocytes (panel c). These results suggested that HlSRB expression of granulocytes up-regulated in response to *E. coli* invasion (Fig. 14B) might reflect a critical role of HlSRB in hemocyte-mediated phagocytosis to invading *E. coli* and also that lobopodia-like extension of granulocytes might be specialized for bacteria clearance in hemolymph.

3.8. *Culture of hemolymph from ticks injected with EGFP-expressing* E. coli *(E-*E. coli*)*

The colony numbers of E-*E. coli* after overnight cultivation of hemolymph from UF, PF, and RNAi-ticks 24 hours after E-*E. coli* injection were 88, 21, and 971, respectively (Fig. 17B). These numbers are shown to represent in average results of three different experiments and a significantly highest number of colonies was observed in culture of hemolymph from RNAi-ticks. These results suggested that RNAi-ticks induced high bacteremia in hemolymph 24 hours after E-*E. coli* injection due to the failure in combating

bacteria, caused by knockdown of HlSRB, resulting in the emergence of a large number of colonized bacteria.

Discussion

Previous literature on hemocyte identification of arthropods suggests that the most common types of hemocytes are prohemocytes, plasmatocytes, granulocytes, and spherulocytes (16, 20, 39, 47, 48, 82). At least two types of hemocytes, plasmatocytes and granulocytes, are generally recognized as a predominant class of phagocytic hemocytes circulating in hemolymph of insects (23, 47, 48) and ticks (9, 31, 35, 46, 53, 71). Other finding by Ceraul et al. (10) showed that encapsulation/nodulation may be an important component of the immune response in ticks after direct inoculation of *E. coli* bacteria into the hemocoel cavity. In this chapter, I provide evidence that granulocytes of *H. longicornis* ticks after *E. coli* injection show overt populational and morphological changes, such as an increased number of granulocytes and an extension of lobopodia-like structures toward a colony of *E. coli*. I also demonstrated that phagocytosed EGFP-expressing *E. coli* (E-*E. coli*) was found only inside the granulocytes. These results strongly suggest that granulocytes are almost exclusively involved in hemocyte-mediated phagocytosis for *E. coli* in *H. longicornis* ticks.

Ticks must acquire nutrients from the host blood meal and metabolize these nutrients via metabolism (81). After blood feeding, increase in phagocytosis takes place in the

hemolymph of fed ticks compared with their unfed ticks state (82). In *Ornithodoros moubata* soft tick, the population of hemocytes corresponding increase in fed ticks showed increase eosinophilic granulocytes populations and increase phagocytic activity in fed ticks than their unfed ticks (39). According to our results of UF's hemolymph (Fig. 13B, panels a and d; Fig. 17A, panel a), the amount of extracellular *E. coli* or E-*E. coli* was higher than those of PF's hemolymph (Fig. 13B, panels b and e; Fig. 17A, panel b), while the highest amount of extracellular *E. coli* or E-*E. coli* in RNAi-tick's hemolymph (Fig. 13B, panels c and f; Fig. 17A, panel c), suggest that phagocytic activity of PF ticks is higher than those of UF ticks and loss of phagocytic activity in RNAi-ticks.

It was shown in this study that the gene and protein expressions of HlSRB (Chapter-1) are significantly up-regulated in tick hemolymph after *E. coli* injection. In addition, the fluorescent dots showing localization of native HlSRB, detected only on the surface of granulocytes, demonstrated a marked 10-fold increase after *E. coli* injection. These results indicate that granulocytes up-regulate the expression of cell surface HlSRB in response to exposure to *E. coli*, most likely resulting in increased HlSRB in hemolymph.

In vivo gene silencing study revealed that HlSRB-silenced ticks were unable to properly control invaded *E. coli* burdens and had to succumb to high bacteremia. Interestingly, in HlSRB-silenced ticks, no fluorescent dots showing HlSRB localization were detected in granulocytes before and after *E. coli* injection, and lobopodia-like structures and intracellularly phagocytosed E-*E. coli* bacteria were not observed. It was indicated that the mammalian SRB generally implicates as a sensor of microbial products that mediate phagocytosis in response to a broad range of pathogens (5). Therefore, HlSRB

is critically involved in the uptake of *E. coli* bacteria into granulocytes and thus HlSRB silencing resulted in the complete loss of the granulocyte-mediated phagocytosis, giving rise to the mortality of ticks after *E. coli* injection.

My study raises the possibility that the phagocytosis of tick granulocytes is induced when HlSRB is activated by target pathogens. However, phagocytosis of a microbe by a phagocytic cell is an extremely complex and diverse process which requires multiple successive interactions between the phagocyte and the pathogen as well as sequential signal transduction events (47, 48, 50). Cytokine-related molecules such as PDGF-AB, TNF-α and IL-8 in invertebrates are known to provoke conformational changes in mollusk hemocytes and to affect phagocytosis (65, 66). Scavenger receptors expressed by mammalian myeloid cells have been elucidated to alter cell morphology, and their expression is affected by various cytokines (70). We have already shown in *Ornithodoros moubata* ticks that granulocytes have platelet-derived growth factor (PDGF)-AB (53). Therefore, it may be assumed that the participation of PDGF-AB in the elongation of lobopodia-like structures in tick granulocytes after *E. coli* injection is caused in cooperation with HlSRB.

Collectively, HlSRB, a class B scavenger receptor CD36 of ixodid ticks, is found to play a key role in granulocyte-mediated phagocytosis to invading *E. coli* and contribute to the first-line host defense against various pathogens. These findings indicate that HlSRB may be critical for the survival of ixodid ticks. Furthermore, our data suggest that HlSRB may be a novel promising target molecule for the development of vaccine against ticks and tick-borne diseases.

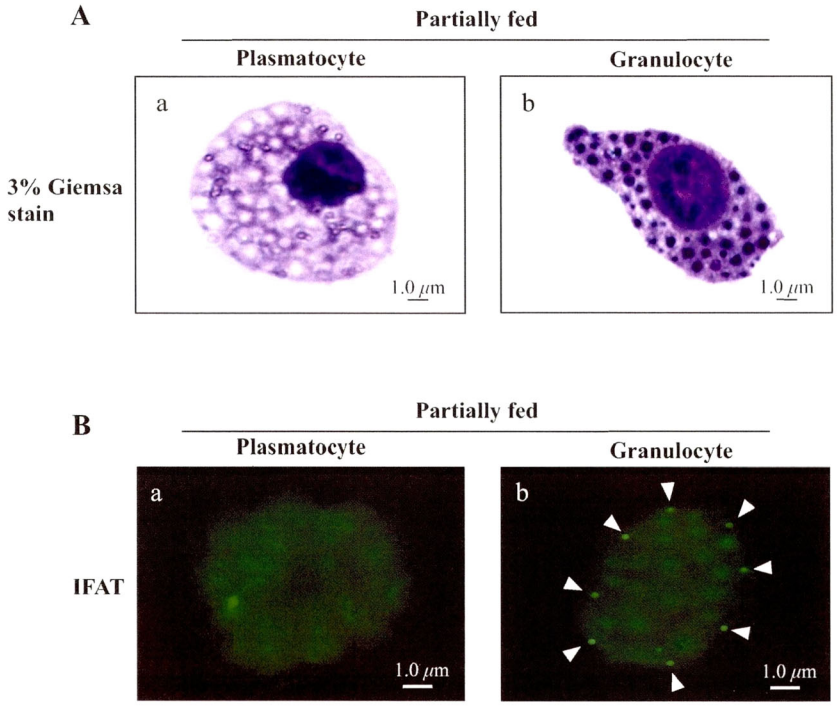


Fig. 12. Giemsa-stained and localization of HlSRB on the plasmatocytes and granulocytes without heat-killed *E. coli* or *E. coli* injection

3% Giemsa-stained plasmatocytes and granulocytes (A) and localization of the endogenous HlSRB on the surface of granulocyte from partially fed female *H. longicornis* adult ticks (PF) by IFAT (B). Hemolymph and hemocyte samples were prepared as indicated in Materials and methods. The hemocytes were stained with anti-rHlSRB antibody followed by Alexa 488-conjugated mouse anti-IgG. Phagocytic plasmatocytes and granulocytes were observed under fluorescence microscopy. Arrowheads indicate the native HlSRB expressed on the surface of granulocytes. Typical plastmatocytes (a) and granulocytes (b) are shown. The *scale bar* represents 1 μm.

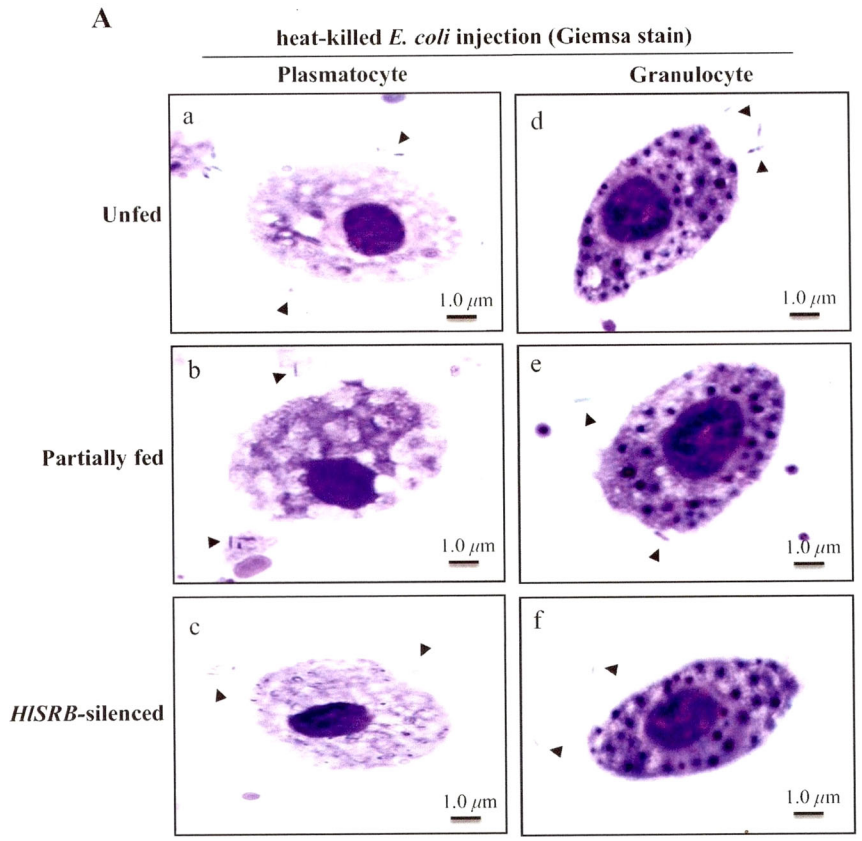

A
heat-killed E. coli injection (Giemsa stain)
Plasmatocyte
Granulocyte
Unfed
Partially fed
HlSRB-silenced
a
b
c
d
e
f
1.0 μm

Fig. 13.

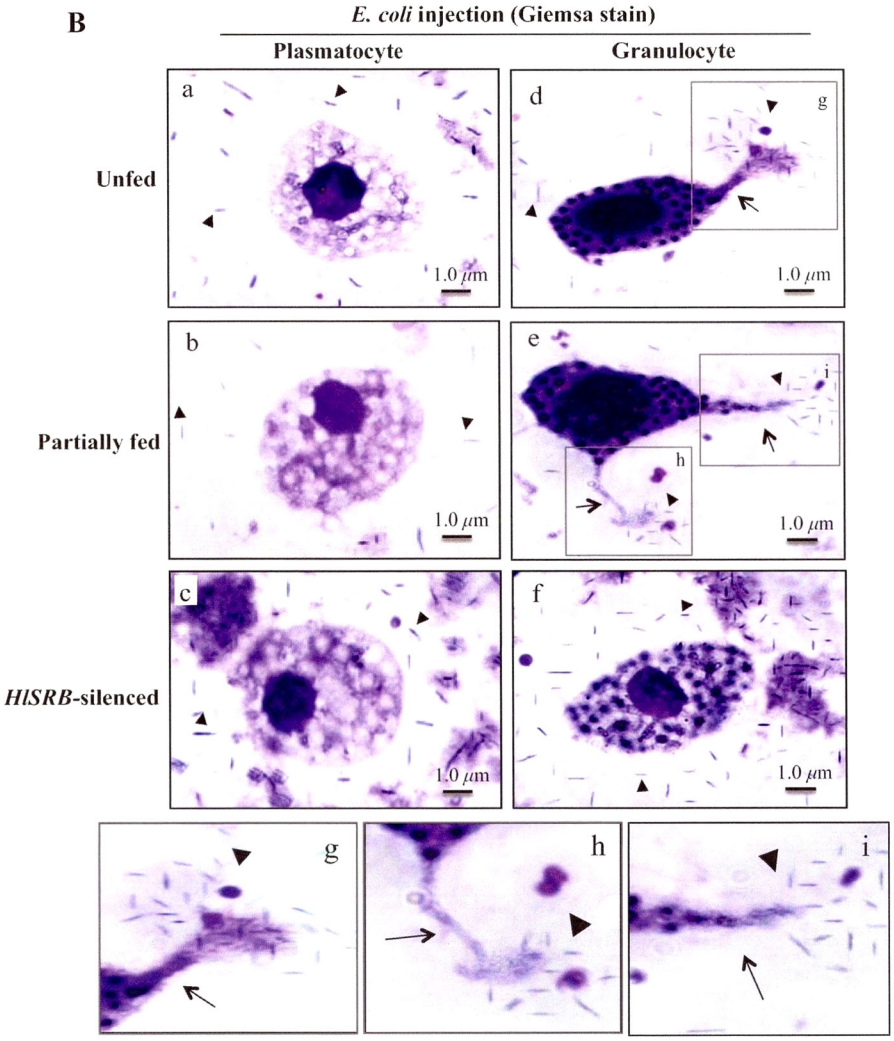


Fig. 13. Giemsa-stained on the plasmatocytes and granulocytes after heat-killed *E. coli* or *E. coli* injection

3% Giemsa-stained plasmatocytes and granulocytes from *H. longicornis* unfed (UF), partially fed (PF), and *HlSRB* dsRNA-injected ticks (RNAi-ticks) 24 hours after the injection with heat-killed *E. coli* (A) or *E. coli* (B). Heat-killed *E. coli* or *E. coli* was percutaneously injected into UF, PF, and RNAi-ticks. The injected ticks were left at 25 °C in an incubator. Twenty-four hours after injections, hemolymph was collected for the examination of hemocytes. Arrowheads indicate *E. coli*, and arrows indicate the lobopodia-like extensions of granulocytes. Plasmatocytes and granulocytes of UF (a and d), PF (b and e), and RNAi-ticks (c and f). Areas marked by squares are shown at higher magnification (g, h, and i). The *scale bar* represents 1 μm.

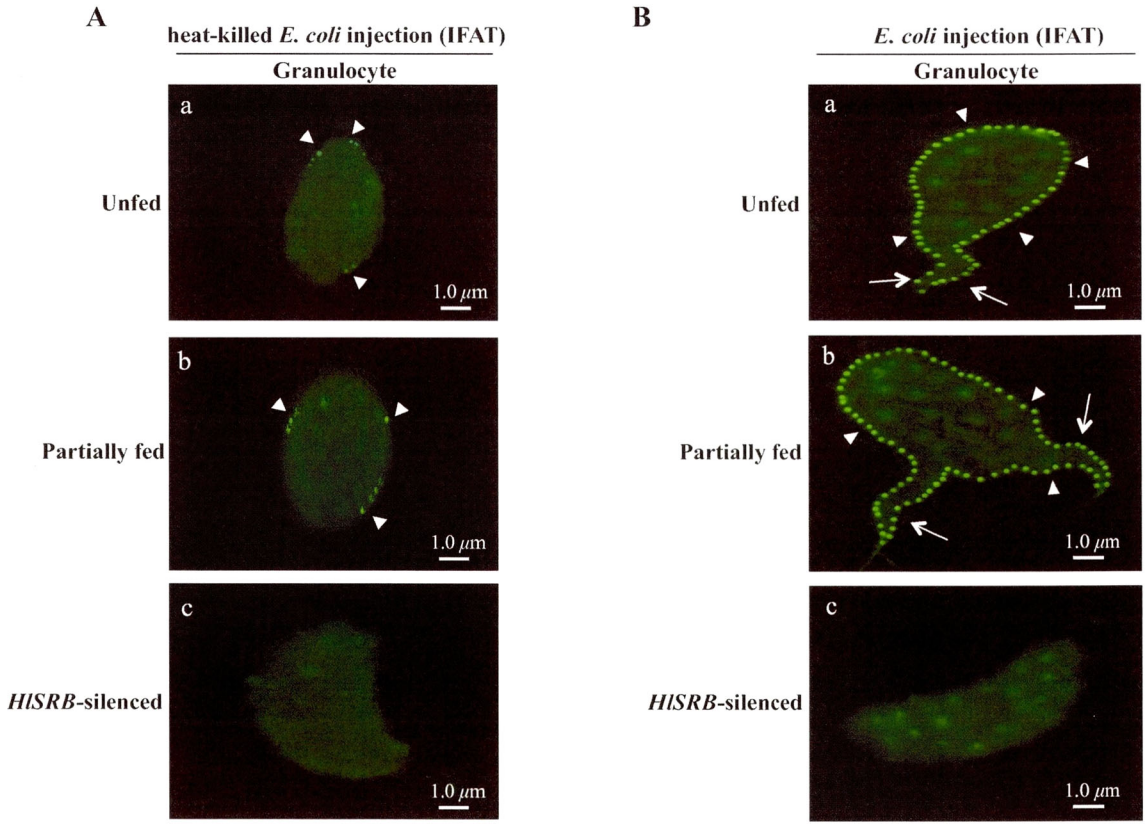


Fig. 14. Immunohistochemical localization of the endogenous HlSRB on the surface of granulocytes from heat-killed *E. coli*- (A) or *E. coli*-injected (B) unfed (UF), partially fed (PF), and *HlSRB* dsRNA-injected ticks (RNAi-ticks) by IFAT

The IFAT experiment was performed as shown in Fig. 1. Arrowheads indicate the native HlSRB expressed on the surface of granulocytes, and arrows indicate the lobopodia-like extensions of granulocytes. Granulocytes of UF (a), PF (b), and RNAi-ticks (c). The *scale bar* represents 1 μm.

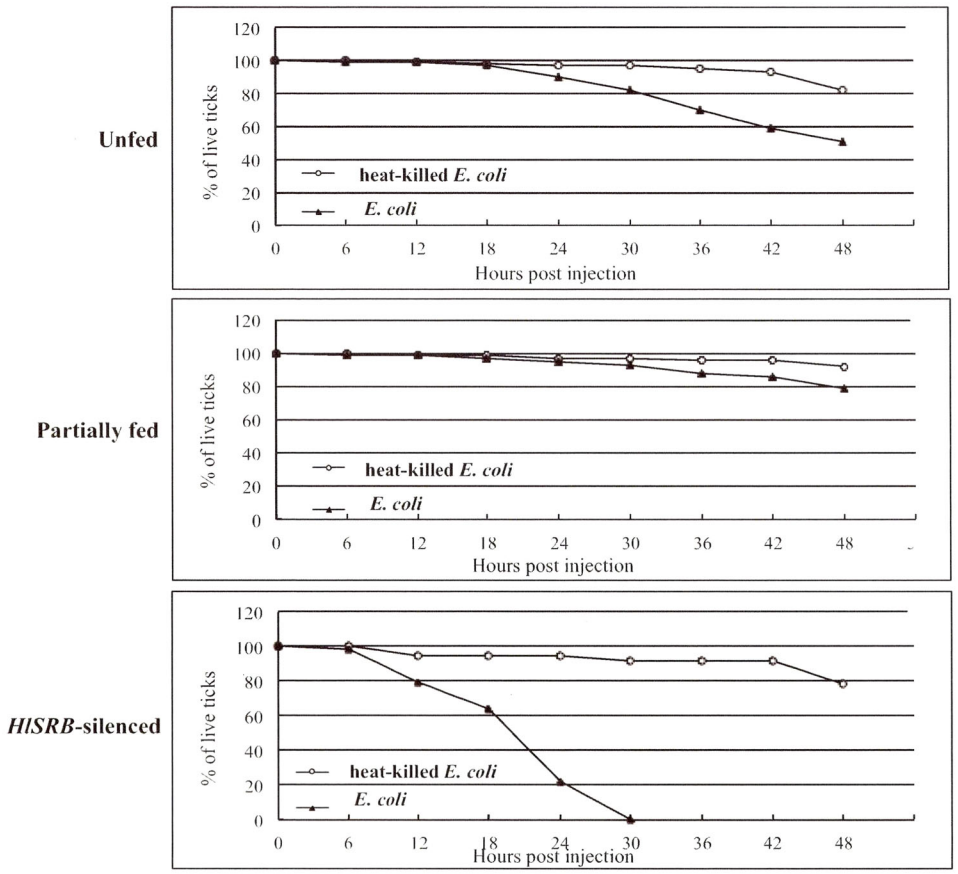
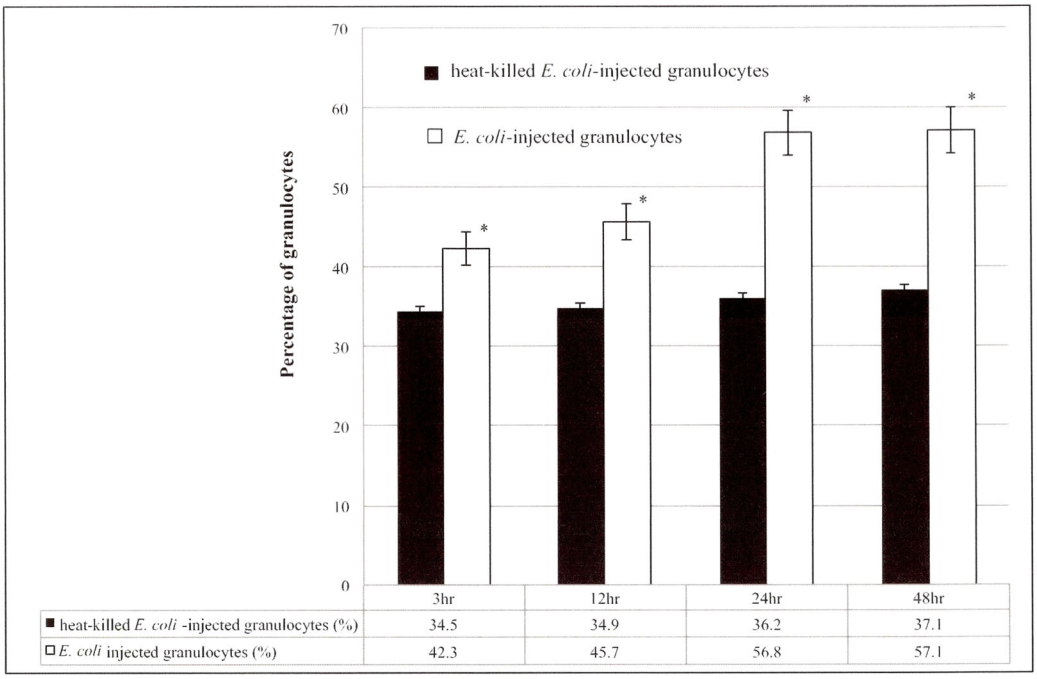


Fig. 15. Survival rate comparisons among unfed (UF), partially fed (PF), and *HlSRB* dsRNA-injected ticks (RNAi-ticks) after heat-killed *E. coli* or *E. coli* injection

Heat-killed *E. coli* or *E. coli* was percutaneously injected into UF, PF, and RNAi-ticks. The injected ticks were allowed to rest at 25 °C in an incubator and then monitored for survival rate. The survival rates were calculated by the percentage of remaining live ticks to the number of ticks used at the beginning of the experiment in different time courses. The moribund ticks were calculated as dead ticks. The figures are shown to represent data in combined results of three different experiments.

A



	3hr	12hr	24hr	48hr
■ heat-killed *E. coli* -injected granulocytes (%)	34.5	34.9	36.2	37.1
□ *E. coli* injected granulocytes (%)	42.3	45.7	56.8	57.1

Fig. 16.

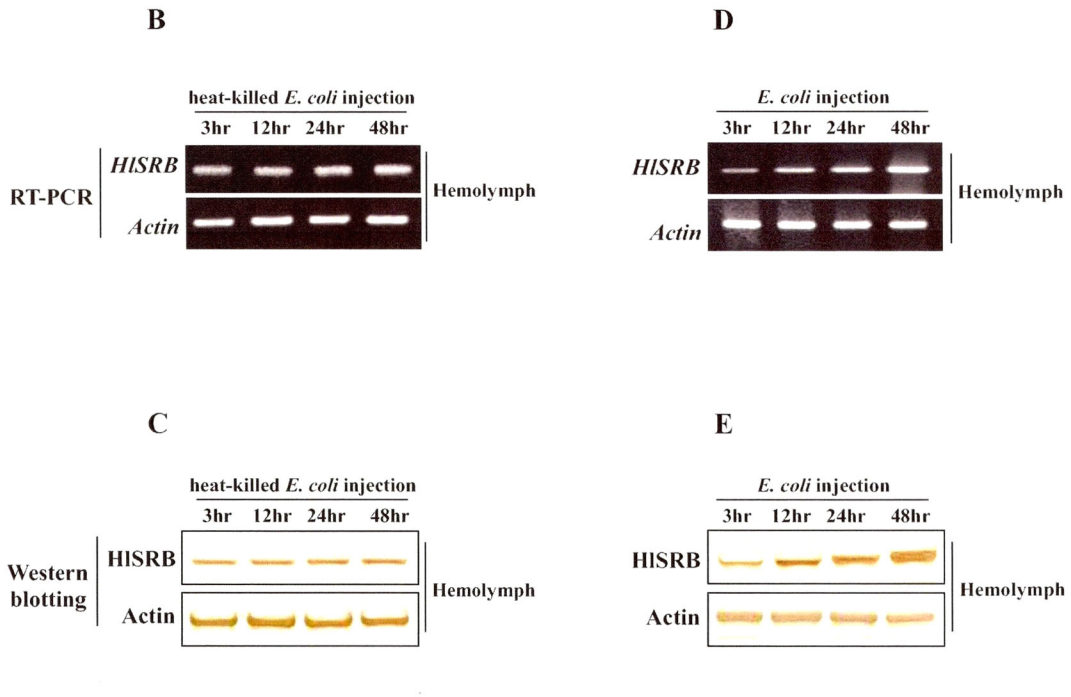


Fig. 16. Populational changes of granulocytes and expression patterns of *HlSRB* gene and endogenous HlSRB protein in the hemolymph from partially fed (PF) ticks injected with either heat-killed *E. coli* or *E. coli*

PF ticks were percutaneously injected with heat-killed *E. coli* or *E. coli*. The injected ticks were left at 25 °C in an incubator. Hemolymph was collected for granulocyte counts, RNA extraction, and preparation of protein lysates in different time courses after heat-killed *E. coli* or *E. coli* injection. Effect of *E. coli* injection on granulocyte population (A). Black and white bars indicate heat-killed *E. coli* and *E. coli* injection, respectively. Values represent the mean ± SD of five ticks. The asterisks indicate a significant difference from the control heat-killed *E. coli* injections ($P<0.05$). RT-PCR analysis (B and D). PCR was performed using cDNA synthesized from the Pf ticks injected with either heat-killed *E. coli* or *E. coli* with primer sets specific to *HlSRB* and *β-actin* gene. Western blot analysis (C and E). Hemolymph samples were subjected to SDS-PAGE under reducing conditions and transferred to a PVDF membrane. The membrane was probed with the mouse anti-rHlSRB or mouse anti-actin serum was used as a control. 3hr, 3 hours after heat-killed *E. coli* or *E. coli* injection; 12hr, 12 hours after heat-killed *E. coli* or *E. coli* injection; 24hr, 24 hours after heat-killed *E. coli* or *E. coli* injection; 48hr, 48 hours after heat-killed *E. coli* or *E. coli* injection.

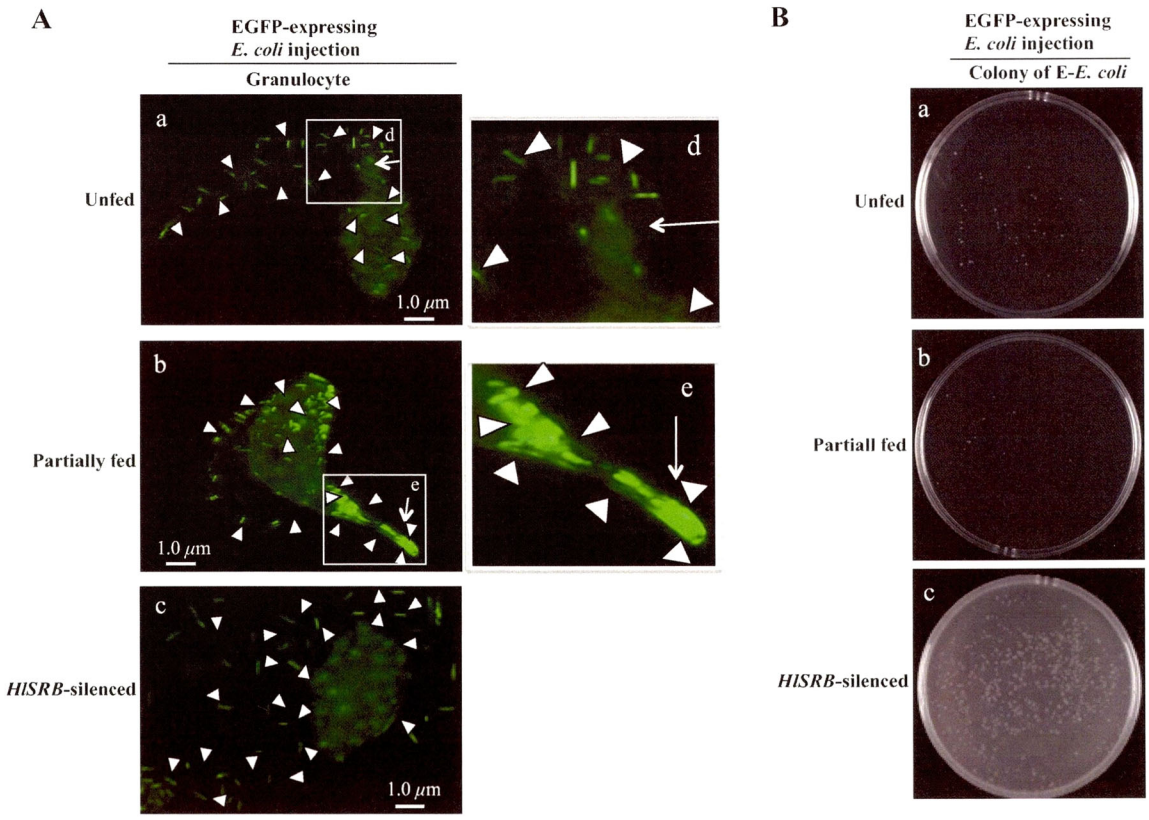


Fig. 17. Fluorescence microscopy showing the fluorescence of EGFP-expressing *E. coli* (E-*E. coli*) in granulocytes (A) and colony number of E-*E. coli* propagated in unfed (UF), partially fed (PF), or *HlSRB* dsRNA-injected ticks (RNAi-ticks) (B)

UF, PF, and RNAi-ticks were injected with E-*E. coli*. Twenty-four hours after the injection, hemolymph was collected from these ticks by amputation of legs. One drop of hemolymph placed on a glass slide was examined under fluorescence microscopy (A). Arrowheads indicate E-*E. coli* and arrows indicate the lobopodia-like extensions of granulocytes of UF (a), PF (b), and RNAi-ticks (c). Areas marked by squares are shown at higher magnification (d and e). The *scale bar* represents 1 μm. Colony numbers of E-*E. coli* propagated in UF, PF, and RNAi-ticks 24 hours after E-*E. coli* injection (B). Hemolymph of these tick groups was applied on an LB agar medium, and the number of emerged colonies of *E. coli* after overnight culture was counted. This experiment was done in triplicate, and similar results were obtained in 3 different experiments.

General Discussion and Conclusions

The ixodid ticks (Arthropoda: Ixodidae), popularly known as hard ticks, serve as a unique vector of various pathogens that cause deadly diseases, such as Lyme disease, tick-borne encephalitis, Rocky Mountain spotted fever, babesiosis, theileriosis, and anaplasmosis, during hematophagy. Approximately 10% of the known 867 tick species act as vectors of a broad range of pathogens of domestic animals and humans (38). The hard ixodid tick, *Haemaphysalis longicornis*, is distributed mainly in East Asia and Australia, where it transmits a wide range of pathogens. Most bacterial and viral diseases can be successfully controlled by vaccination and quarantine procedures. For the tick-borne diseases, a variety of methods, including the application of chemical acaricides have been employed to suppress tick vector population and tick-borne diseases. RNA interference has been proposed to have application possibilities for the autocidal control of tick populations (13) and the characterization of tick-borne pathogens (12, 14). Research in recent years has given new insights into the dsRNA uptake mechanism in the gene silencing of insects. It is of current interest to look at the molecular scenario, in particular, the role of scavenger receptor in hemocyte-mediated phagocytosis, inside vector ticks, which plays an important

role in first-line host defense against invading pathogens. In the present study, I have selected and characterized class B scavenger receptor CD36 gene (*HlSRB*) from the EST data base constructed from ovary cDNA libraries of *H. longicornis* with the aim of exploring their role in the systemic RNA interference and the granulocyte-mediated microbial phagocytosis in ticks.

Scavenger receptors are cell-surface proteins and exhibit distinctive ligand-binding properties, recognizing a wide range of ligands that include microbial surface constituents and intact microbes (46). The class B scavenger receptor CD36 (SRB) is predominantly expressed by macrophages and is considered important in innate immunity. A prominent member of SRB is a membrane glycoprotein present on platelets, mononuclear phagocytes, adipocytes, myocytes, and some epithelia (30). The SRB known as ''pattern recognition receptors'' identifies the conserved structure of the pathogen ligands and mediate the binding and uptake of microorganism antigens (25). It is speculated that exploration of biochemical properties of tick SRB will contribute to our knowledge about tick biology including systemic RNA interference and the granulocytes-mediated phagocytosis and would help in evaluating the SRB as vaccine candidates for the purpose of better control of ticks and tick-borne diseases.

In Chapter-1, I identified a scavenger receptor class B-like protein belonging to the CD36 superfamily in *H. longicornis*, the first to be structurally characterized in ticks. The HlSRB had overall 30% identity to both mammalian and insect SRB membrane proteins. The mRNA transcripts of *HlSRB* were expressed in multiple organs of adult females but with varying levels in the different developmental stages of ticks. The recombinant HlSRB

was expressed in *Escherichia coli* as the His-tagged protein, and anti-mouse recombinant HlSRB serum elucidated the localization of the endogenous protein in the midgut, salivary gland, ovary, fat body, and hemocytes of partially fed *H. longicornis* females. Gene silencing of *HlSRB* in female ticks resulted in a significant reduction of engorged body weights.

Chapter-2 shows for the first time that HlSRB mediated the uptake of exogenous dsRNAs in the induction of the RNAi responses in ticks. Unfed female *H. longicornis* ticks were injected with a single or a combination of *H. longicornis SRB* (*HlSRB*) dsRNA, vitellogenin-1 (*HlVg-1*) dsRNA, and vitellogenin receptor (*HlVgR*) dsRNA. I found that specific and systemic silencing of the *HlSRB*, *HlVg-1*, and *HlVgR* genes was achieved in ticks injected with a single dsRNA of *HlSRB*, *HlVg-1*, and *HlVgR*, repectively. In ticks injected first with *HlVg-1* or *HlVgR* dsRNA followed 96 hours later with *HlSRB* dsRNA (*HlVg-1/HlSRB* or *HlVgR/HlSRB*), gene silencing of *HlSRB* was achieved in addition to first knockdown in *HlVg-1* or *HlVgR*, and prominent phenotypic changes were observed in engorgement, mortality, and hatchability, indicating that a systemic and specific double knockdown of target genes had been simultaneously attained in these ticks. However, in ticks injected with *HlSRB* dsRNA followed 96 hours later with *HlVg-1* or *HlVgR* dsRNAs, silencing of *HlSRB* was achieved, but no subsequent knockdown in *HlVgR* or *HlVg-1* was observed. The Western blotting and immunohistochemical examinations revealed that the endogenous HlSRB protein was fully abolished in midguts of ticks injected with *HlSRB/HlVg-1* dsRNAs but HlVg-1 was normally expressed in midguts, suggesting that *HlVg-1* dsRNA-mediated RNAi was fully inhibited by the first knockdown of *HlSRB*.

Similarly, the abolished localization of HlSRB protein was recognized in ovaries of ticks injected with *HlSRB/HlVgR*, while normal localization of HlVgR was observed in ovaries, suggesting that the failure to knock-down *HlVgR* could be attributed to the first knockdown of *HlSRB*.

Insects have a well-developed innate immune system that allows a general and rapid response to infectious agents. Hemocytes are the primary mediators of cell-mediated immunity in insects, including phagocytosis, nodulation, encapsulation and melanization. Identification of hemocytes is essential to understand hemocyte-mediated immune responses in invertebrates. Chapter-3 shows that HlSRB plays vital roles in granulocyte-mediated phagocytosis to invading *E. coli* and contributes to the first-line host defense against various pathogens. Data clearly revealed that granulocytes that up-regulated the expression of cell surface HlSRB were almost exclusively involved in hemocyte-mediated phagocytosis for *E. coli* in ticks, and post-transcriptional silencing of the HlSRB-specific gene ablated the granulocytes' ability to phagocytose *E. coli* and resulted in the mortality of ticks due to high bacteremia.

In conclusion, the present study demonstrates that the novel gene, *HlSRB*, from the ixodid tick *H. longicornis* belongs to the class B scavenger receptor CD36 family. HlSRB may not only mediate the effective knock-down of gene expression by RNAi but also play essential roles for systemic RNAi of ticks. Additionally, HlSRB is found to play a key role in granulocyte-mediated phagocytosis to invading *E. coli* and contributes to the first-line host defense against various pathogens. Therefore, understanding the mechanisms

underlying class B scavenger CD36-mediated functions in ticks is of paramount importance for the development of vaccine against ticks and tick-borne diseases.

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学 位 論 文 要 旨

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題　　目：Identification and functional characterization of class B scavenger receptor CD36 from the hard tick, *Haemaphysalis longicornis*
(フタトゲチマダニ由来クラスＢスカベンジャー受容体CD36の同定とその機能の解明)

論文要旨：

Ticks are obligate hematophagous ectoparasites of wild and domestic animals and humans. They are considered to be second to mosquitoes as vectors of human diseases and are the most important arthropods transmitting pathogens to domestic animals. The class B scavenger receptor CD36, the cell surface glycoprotein, is present on a variety of cell types, and mediating innate immune responses of the mammalian and insect hosts to a range of exogenous pathogens. However, identification and functional analysis of the class B scavenger receptor CD36 of arthropods, including ticks, are not understood. This study was performed to characterize novel class B scavenger receptor CD36 from the ovary of the disease vector, *Haemaphysalis longicornis*, that has been shown to be linked to the uptake of vitellogenin in oocytes. Also this study was described that HlSRB may not only mediate the uptake of dsRNA but also play essential roles for systemic RNA interference and the granulocyte-mediated microbial phagocytosis in ticks.

In Chapter 1, *HlSRB* gene was cloned from the ovary cDNA library of adult female *H. longicornis*. Cloning and characterization of *HlSRB* gene showed that *HlSRB* cDNA was 2,908 bp, including an ORF encoding of 506 amino acids with a pI value of 5.83. *H. longicornis* SRB contains a hydrophobic SRB domain and four centrally clustered cysteine residues for arrangement of disulfide bridges. Deduced amino acid sequence has an identity of 30-38% with the SRB of other organisms. RT-PCR analysis showed that mRNA transcripts were expressed in multiple organs of adult ticks but with a different transcript level in the developmental stages of *H. longicornis* ticks. His-tagged recombinant HlSRB was expressed in *E. coli* with an expected molecular mass of 50 kDa. In Western blot analysis, mouse anti-rHlSRB serum recognized a strong reaction with a 50 kDa protein band in lysates prepared

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from egg and adult tick but showed a weak reaction with lysates of larva and nymph. In an indirect immunofluorescent antibody test (IFAT), HlSRB antiserum recognized the protein located on the midgut, salivary glands and ovary of partially fed *H. longicornis* females. Silencing of the *HlSRB* gene by RNAi led to a significant reduction in the engorged female body weight.

In chapter 2, *HlSRB* mediated the uptake of exogenous dsRNAs in the induction of the RNAi responses in ticks. Unfed female *Haemaphysalis longicornis* ticks were injected with a single or a combination of *H. longicornis SRB* (*HlSRB*) dsRNA, vitellogenin-1 (*HlVg-1*) dsRNA, and vitellogenin receptor (*HlVgR*) dsRNA. We found that specific and systemic silencing of the *HlSRB*, *HlVg-1*, and *HlVgR* genes was achieved in ticks injected with a single dsRNA of *HlSRB*, *HlVg-1*, and *HlVgR*. In ticks injected first with *HlVg-1* or *HlVgR* dsRNA followed 96 hours later with *HlSRB* dsRNA (*HlVg-1/HlSRB* or *HlVgR/HlSRB*), gene silencing of *HlSRB* was achieved in addition to first knockdown in *HlVg-1* or *HlVgR*, and prominent phenotypic changes were observed in engorgement, mortality, and hatchability, indicating that a systemic and specific double knockdown of target genes had been simultaneously attained in these ticks. However, in ticks injected with *HlSRB* dsRNA followed 96 hours later with *HlVg-1* or *HlVgR* dsRNAs, silencing of *HlSRB* was achieved, but no subsequent knockdown in *HlVgR* or *HlVg-1* was observed. The Westernblot and immunohistochemical examinations revealed that the endogenous HlSRB protein was fully abolished in midguts of ticks injected with *HlSRB/HlVg-1* dsRNAs but HlVg-1 was normally expressed in midguts, suggesting that *HlVg-1* dsRNA-mediated RNAi was fully inhibited by the first knockdown of *HlSRB*. Similarly, the abolished localization of HlSRB protein was recognized in ovaries of ticks injected with *HlSRB/HlVgR*, while normal localization of HlVgR was observed in ovaries, suggesting that the failure to knock-down *HlVgR* could be attributed to the first knockdown of *HlSRB*.

Chapter 3 show that HlSRB plays vital roles in granulocyte-mediated phagocytosis to invading *E. coli* and contributes to the first-line host defense against various pathogens. Data clearly revealed that granulocytes that up-regulated the expression of cell surface HlSRB are almost exclusively involved in hemocyte-mediated phagocytosis for *E. coli* in ticks, and post-transcriptional silencing of the HlSRB-specific gene ablated the granulocytes' ability to phagocytose *E. coli* and resulted in the mortality

of ticks due to high bacteremia. This chapter was performed on plasmatocytes and granulocytes of female *H. longicornis* ticks and the dotted localization of native HlSRB protein was detected only on the surface of granulocytes, while no localization was observed in plasmatocytes, suggesting that endogenous HlSRB is expressed predominantly in granulocytes of *H. longicornis*. Large lobopodia-like structures were observed in granulocytes of the Giemsa-stained smears from unfed and partially fed ticks 24 hours after *E. coli* injection, and many *E. coli* bacteria were found around the top of lobopodia-like structures. However, no lobopodia-like structures were detected in granulocytes from RNAi-ticks after *E. coli* injection. Next, the granulocytes of *H. longicornis* ticks after *E. coli* injection show overt populational and morphological changes, such as an increased number of granulocytes. Phagocytosed EGFP-expressing *E. coli* (E-*E. coli*) was found only inside the granulocytes, strongly suggest that granulocytes are almost exclusively involved in hemocyte-mediated phagocytosis for *E. coli* in *H. longicornis* ticks. It was shown in this chapter that the gene and protein expressions of HlSRB are significantly up-regulated in tick hemolymph after *E. coli* injection. In addition, the fluorescent dots showing localization of native HlSRB, detected only on the surface of granulocytes, demonstrated a marked 10-fold increase after *E. coli* injection. These results indicate that granulocytes up-regulate the expression of cell surface HlSRB in response to exposure to *E. coli*, most likely resulting in increased HlSRB in hemolymph.

In summary, to the best of our knowledge, the identification and characterization of HlSRB is the first class B scavenger receptor CD36 molecule in ixodid ticks and HlSRB may not only mediate the effective knock-down of gene expression by RNAi but also play essential roles for systemic RNAi of ticks. Additionally, HlSRB is found to play a key role in granulocyte-mediated phagocytosis to invading *E. coli* and contribute to the first-line host defense against various pathogens. Collectively, our results provide a comprehensive contribution to studies linked with the development of control measures for ticks and tick-borne diseases.