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ORIGINAL PAPER

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

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Abstract Scavenger receptors (*SRs*) are cell-surface proteins and exhibit distinctive ligand-binding properties, recognizing a wide range of ligands that include microbial surface constituents and intact microbes. The class B scavenger receptor CD36 (*SRB*) is predominantly expressed by macrophages and is considered important in innate immunity. We here show the identification and characterization of *SRB* from the hard ixodid tick, *Haemaphysalis longicornis* (HlSRB). The full-length cDNA was 2,908 bp, including an ORF encoding of 1,518 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 *Escherichia 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 from egg and adult tick but showed a weak reaction with lysates of larva and nymph. In an indirect immunofluorescent antibody test, 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. It is noteworthy that more than a dozen *SRB* orthologs have been identified in the genomes of insect species with functions related to pheromone signaling, innate immunity, phagocytic clearance of apoptotic cells, and various aspects of the fatty acid metabolism. This is the first report of the identification and characterization of the *SRB* homologue in Chelicerata, including ticks, horseshoe crabs, scorpions, spiders, and mites.

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Introduction

Ticks are blood-sucking arthropods that can transmit various pathogens, such as protozoa, rickettsiae, spirochaetes, and viruses, to mammalian hosts. Approximately 10% of the known 867 tick species act as vectors of a broad range of pathogens of domestic animals and humans (Jongejan and Uilenberg 2004). The hard ixodid tick, *Haemaphysalis longicornis*, is distributed mainly in East Asia and Australia, where it transmits a wide range of pathogens, including bovine theileriosis (*Theileria* spp.), bovine babesiosis (*Babesia ovata*), canine babesiosis (*Babesia gibsoni*), and human rickettsiosis (*Rickettsia*

japonica) (Fujisaki et al. 1994; Jongejan and Uilenberg 2004). 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 and cloning of key tick molecules and the characterization of their roles in arthropod physiology (Mulenga et al. 2000).

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 (Krieger and Herz 1994). 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 (Krieger and Herz 1994). The scavenger receptors known as "pattern-recognition receptors" identify the conserved structure of the pathogen ligands and mediate the binding and uptake of microorganism antigens (Gordon 2002). 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* (Pearson 1996). 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 (Elomaa et al. 1995; Ramprasad et al. 1995). The SR class A, expressed on macrophages are involved in innate immunity by facilitating phagocytic activity especially the uptake and killing of bacteria (Gough and Gordon 2000). Other SRs belonging to class B are known to bind and internalize senescent neutrophils (Krieger 1997; Savill et al. 1992) and to be involved in cell adhesion, aggregation (Yamada et al. 1998), and signal transduction (Ockenhouse et al. 1989; Huang et al. 1991). The SRB is involved in the first line of body defense and plays a pivotal role in innate immunity (Haworth et al. 1997; Febbraio et al. 2001), clearance of apoptotic cells, lipid transportation (Ockenhouse and Chulay 1988; Oquendo et al. 1989; Endemann et al. 1993; Moore et al. 2002; Medeiros et al. 2004), macrophage foam cell formation, and the development of atherosclerosis (Oz et al. 2009).

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 (Pearson et al. 1995). However, at least two types of phagocytic cells in ticks, plasmatocytes and granulocytes, have been reported (Dolp 1970; Fujisaki et al. 1975; Kuhn and Haug 1994; Zhioua et al. 1996; Inoue et al. 2001); here, we show the mRNA expression of the *HlSRB* gene in hemocytes, a finding that would contribute to future study of the functional conservation on innate immunity in *H. longicornis* using hemocytes.

A prominent member of SRB is a membrane glycoprotein present on platelets, mononuclear phagocytes, adipocytes, myocytes, and some epithelia. The SRB has been identified in most orders of insects (Diptera, Hymenoptera, Coleoptera, and Lepidoptera) (Hart and Wilcox 1993) as well as nematodes, sponges, and slime mold (Muller et al. 2004). 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 (Acton et al. 1996; Nichols and Vogt 2008). 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 (Silverstein and Febbraio 2009). 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 characterization and identification of the SRB from blood-sucking ticks remain to be finalized. In this study, we report the gene identification, isolation, sequence analysis, endogenous localization, and gene and protein expression pattern in developmental stages and different tissues of this class B scavenger receptor CD36 from *H. longicornis*. The present study is the first report on the identification and characterization of the *SRB* gene in ticks and which will be very helpful for further functional analysis of this gene in ticks.

Materials and methods

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 Agricul-

ture, Kagoshima University, Kagoshima, Japan (Fujisaki 1978; Fujisaki et al. 1994).

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.

Identification and characterization of the cDNA encoding scavenger receptor

The full-length cDNA library was made using the vector capping method as previously reported (Kato et al., 2005). 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) and subsequently subjected to an automated sequencer (ABI PRISM 3100 Genetic Analyzer) using plasmid-specific primers of pGCAP1 vector. 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. 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).

RNA isolation and reverse transcriptase-polymerase chain reaction

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 (Fujisaki et al. 1975; Inoue et al. 2001) containing 100 μl of phosphate-buffered saline (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 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 (ScR Exp-F and ScR Exp-R, listed in Table 1). Control amplification was carried out using the actin-specific primers (Actin-F and Actin-R, listed in Table 1) designed from *H. longicornis β-actin* gene (accession no. AY254898). A series of reverse transcriptase-polymerase chain reaction (RT-PCR) was performed in 50 μl of a mixture containing 0.5 μg of cDNA, a 10× 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

Table 1 Gene-specific primers used for RT-PCR and dsRNA synthesis

Name	Sequence (5′–3′)
ScR Exp-F	TGTGCCCATATACCGGAAGT
ScR Exp-R	TGGTTGCAACTAGTGGGTCA
ScR *Xho*I-F	AC*CTCGAG*ATGGCTGTGAGTCGCGT
ScR *Eco*RI-R	AC*GAATTC*TGCTGTAGAACATGGGGTT
ScR T7-F	ACGGATCCTAATACGACTCACTATAGGATGGCTGTGAGTCGCGT
ScRT7-R	ACGGATCCTAATACGACTCACTATAGGTGGTTGCAACTAGTGGGTCA
Luc T7-F	GTAATACGACTCACTATAGGGCTTCCATCTTCCAGGGATACG
Luc T7-R	GTAATACGACTCACTATAGGCGTCCACAAACACAACTCCTCC
Actin-F	CCAACAGGGAGAAGATGACG
Actin-R	ACAGGTCCTTACGGATGTCC

The T7 promoters are underlined. Restriction enzyme sites are shown in italics

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.

Expression and purification of the recombinant protein

The *HlSRB* ORF was amplified by PCR using a forward primer (ScR *Xho*I-F, listed in Table 1) containing a recognition site for *Xho*I and a reverse primer (ScR *Eco*RI-R, listed in Table 1) containing a recognition site for *Eco*RI. 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 *Escherichia 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 0.1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the culture was grown for an additional 4 h 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.

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.

Western blot analysis

To determine native SRB, 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 (Boldbaatar et al. 2006). 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 (1:2,000 dilution; GE Healthcare, Little Chalfont, UK), a signal was detected using 0.5 mg/ml diaminobenzidine tetrahydrochlodide.

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 (ScR T7-F and ScR T7-R, listed in Table 1). The cDNA of the firefly *luciferase* (*luc*) (Promega, Madison, WI, USA) gene was amplified by PCR using oligonucleotides, including the T7 forward and T7 reverse primers (Luc T7-F and Luc T7-R, listed in Table 1). 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 1 μl of the reaction products in a 1.5% agarose gel.

The dsRNA injection was followed as described previously (Boldbaatar et al. 2006). Briefly, 1 μg of the *HlSRB* dsRNA and *luc* dsRNA in 0.5 μl of an injection buffer (10 mM Tris, 1 mM EDTA, pH 7.4) was injected into 50 unfed ticks in the experimental or control groups, through the fourth coxae into the hemocoel; 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.

Indirect immunofluorescent antibody test

For endogenous localization of HlSRB, female *H. longicornis* adults were fed on the ears of rabbits (Fujisaki 1978) and recovered 4 days post-infestation. The midguts, salivary glands, and ovaries from partially fed ticks were immediately dissected out under the microscope (Fujisaki 1978). 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 min 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 three times with PBS, Alexa 488-conjugated goat anti-mouse immunoglobulin (1:1,000; Invitrogen) was applied as second antibody at 37°C for 1 h. 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).

Results

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 (Rasmussen et al. 1998) (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 (Krieger 1999) (Fig. 2). A phylogenetic tree using amino acid

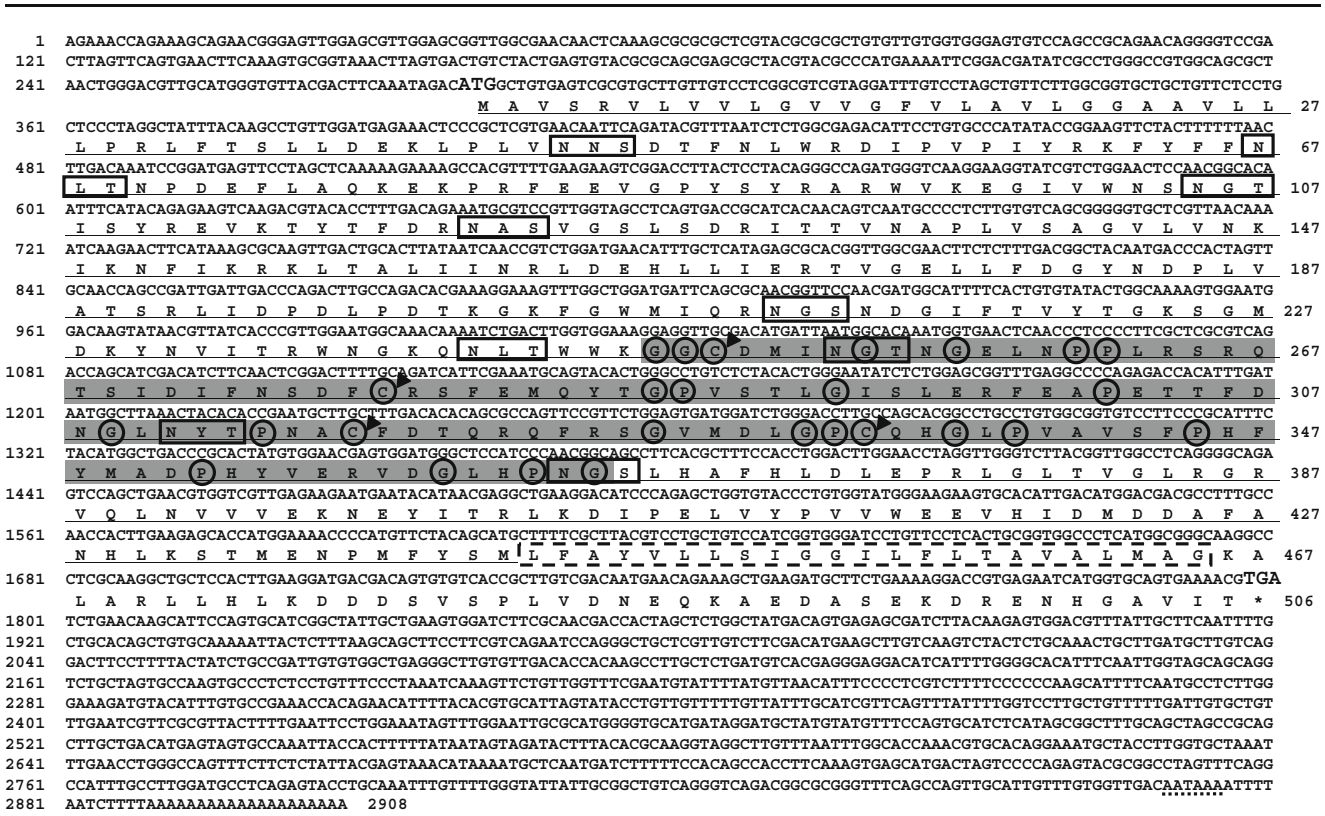

Fig. 1 Nucleotide and deduced amino acid sequence of class B scavenger receptor CD36 (*SRB*) gene from *H. longicornis* tick. Blastx analysis revealed an *SRB* domain (aa 1-442, *underline* showed), which included a highly conserved proline, glycine, and cysteine region (aa 247-365; cysteine, glycine, and proline residues are indicated in *grey*, *underlined*, and *circled*, respectively). The *box with a dotted line* shows a transmembrane segment. The start codon ATG and stop codon TGA are shown by *bolded letter*. The *dotted underline* shows the putative polyadenylation signal (AATAAAA). Nine asparagine residues are shown in *boxes* as potential *N*-linked glycosylation sites. Four cysteine clustered residues were recognized as potential sites for intrachain disulfide linkage (aa 249-334) and are indicated by *triangles*. Amino acids are numbered on the *right*

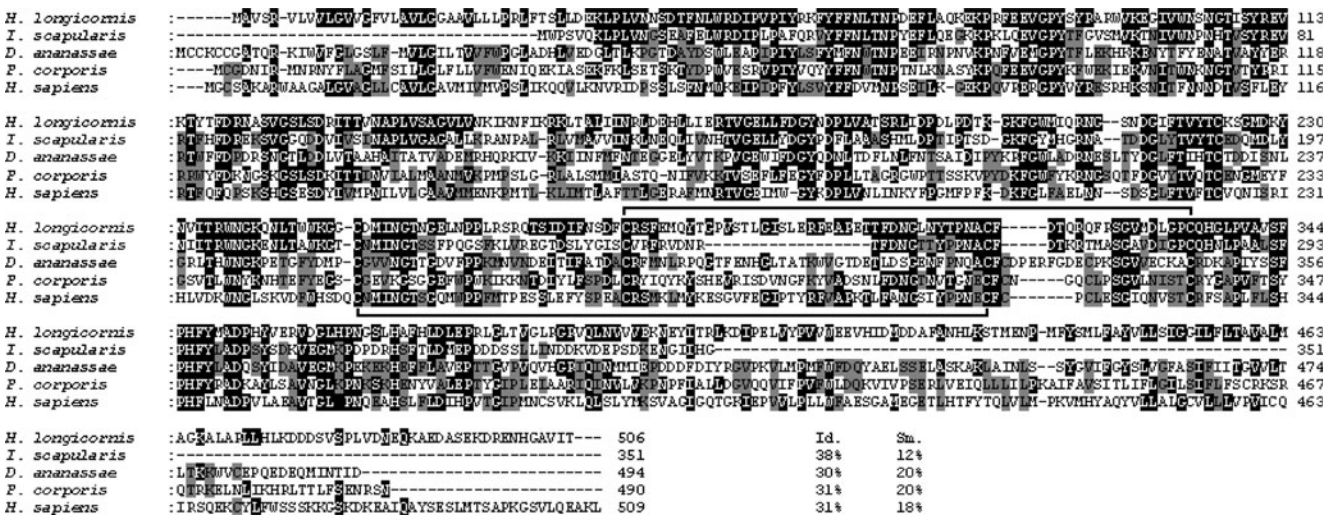

Fig. 2 The alignment of the amino acid sequence of class B scavenger receptor CD36 gene was compared with those of the class B scavenger receptor CD36 transcription factor of the ixodid tick *Ixodes scapularis* (XP 002409323), the flies *Drosophila ananassae* (XP 001965367), the lice *Pediculus humanus 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*

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, *I. scapularis* (Fig. 3).

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 engorgement) 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. We found that the transcript of the *HlSRB* was highly expressed in the midgut, fat body, salivary gland, and hemocytes (Fig. 4b).

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

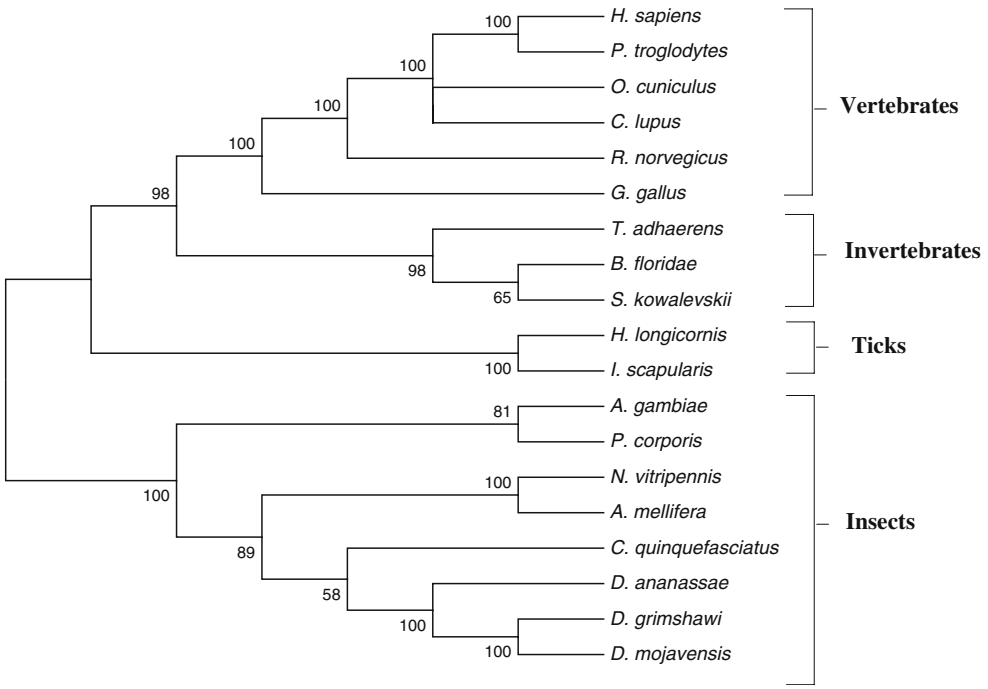

Fig. 3 Phylogenetic tree of the protein sequences of *HlSRB* genes. The *HlSRB* amino acid sequences were used the bee *Apis mellifera* (GenBank accession no. XP392321); the wasps *Nasonia vitripennis* (XP001604561); the mosquitoes *Culus quinquefasciatus* (XP001844488) and *Anopheles gambiae* (XP314281); 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)

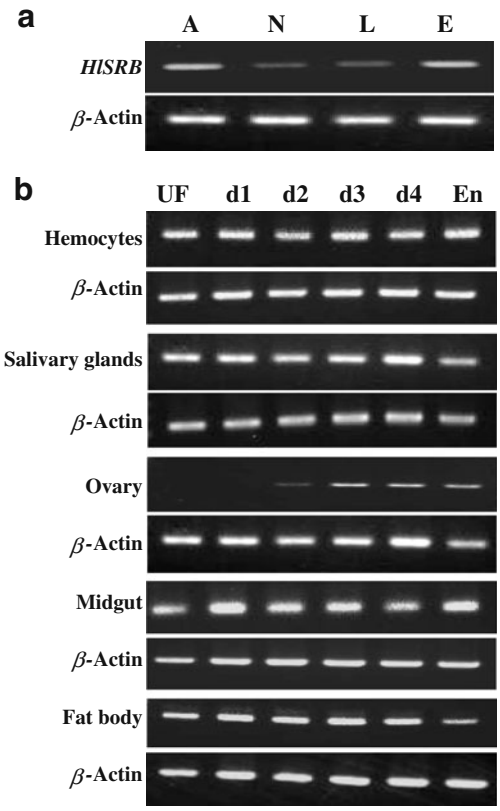

Fig. 4 Transcription profile of *HlSRB* in immature developmental stages and in different tissues of adult females. Total RNA was isolated from partially fed adults, larvae, nymphs, and eggs. Analysis of *HlSRB* gene expression in immature developmental stages (**a**). *A* adult females, *N* nymphs, *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* 1-day-fed ticks, *d2* 2-day-fed ticks, *d3* 3-day-fed ticks, *d4* 4-day-fed ticks, *En* engorged ticks. The transcription profiles of *β-actin* were used as an internal control

columns (Fig. 5a, lane 3). Purified recombinant protein was used in the immunization of mice for the production of antiserum.

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.

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 (ScR Exp-F and ScR Exp-R, listed in Table 1) were used for RT-PCR, and a parallel RNA sample with *β*-actin-specific primers (Actin-F and Actin-R, listed in Table 1) were also amplified as a positive control. We found a considerable

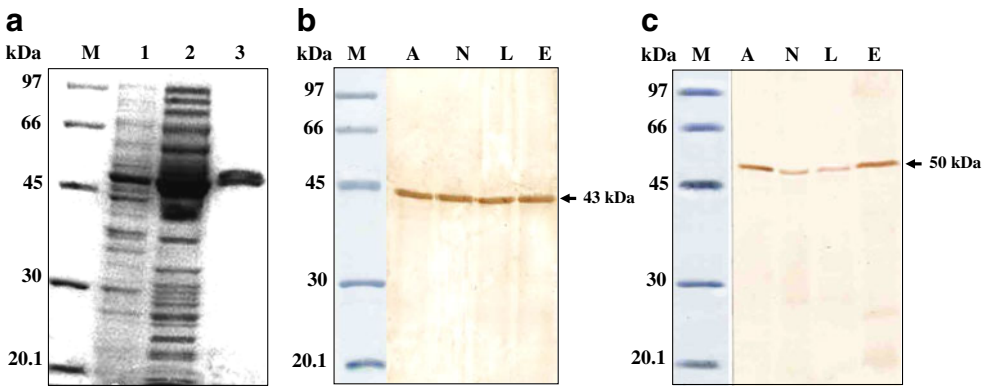

Fig. 5 Expression and purification of rHlSRB in *E. coli*. **a** Recombinant proteins or bacterial lysates were electrophoresed on a 12% SDS-polyacrylamide gel and then stained with Coomassie brilliant blue. *M* molecular weight marker, *lane 1* crude lysate of *E. coli* before induction, *lane 2* IPTG-induced *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

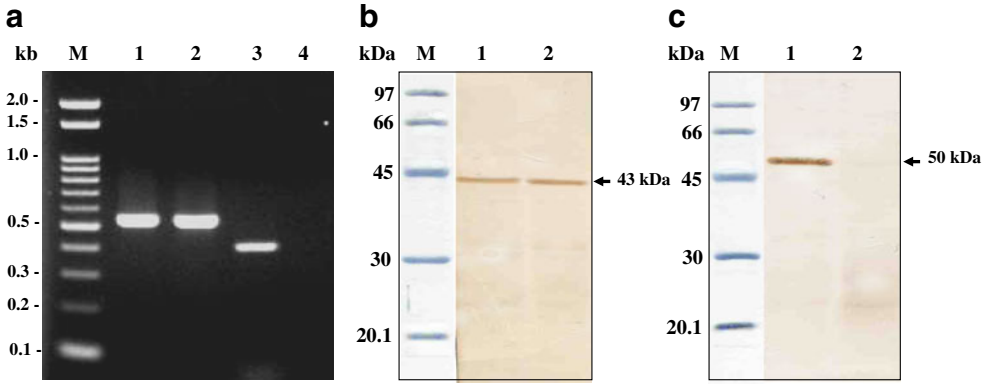

Fig. 6 Effect of dsRNA treatment on *HlSRB* gene disruption. dsRNA complementary to *HlSRB* was injected into *H. longicornis* adult females. The injected ticks (50 individuals for each group) were allowed to feed, and ticks were recovered from the *luciferase* (control) and *HlSRB* dsRNA-treated group, 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

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.

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 2. 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, no 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.

Indirect immunofluorescent antibody test

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

Table 2 Effects of HlSRB RNA interference

Groups	No.	Engorged body weight (mg)[a,c]	Average egg weight (mg)[a,c]	Ratio of body weight/egg 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

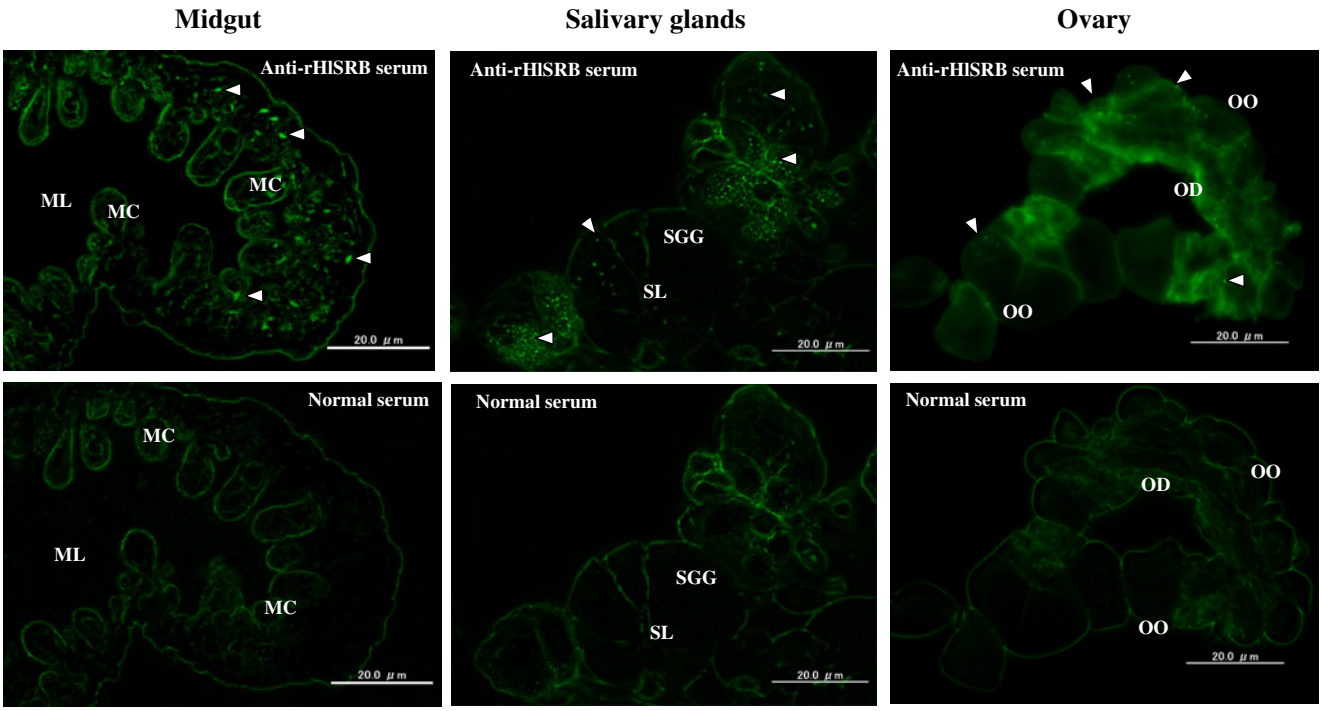

Fig. 7 Immunohistochemical localization of endogenous HlSRB in the midgut, salivary gland, and ovary of partially fed adult *H. longicornis* by IFAT. Staining pattern of anti-HlSRB serum and normal mouse serum were used as primary antibodies with a fluorescence microscopy. The mouse anti-IgG conjugated 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 20 μm

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 paper, we 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 aa with 30% identity with CD36, a class B scavenger receptor protein found in vertebrates and invertebrates (Greenwalt et al. 1992). As a member of the SRB superfamily, the HlSRB possessed several shared structural characteristics (Greenwalt et al. 1992) 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 (Rasmussen et al. 1998). 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 (Gruarin et al. 1997). Overall, based on these critical structural similarities, we 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 (Oquendo et al. 1989), which differed from the value of 88 kDa reported in other studies (Martin et al. 2007; Sun et al. 2007). 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 (Xu et al. 2010). In this study, we 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 (Ji et al. 1997; Jian et al. 1998). A similar case was also observed in *Drosophila*: SRB is expressed in the embryonic stage (Kiefer et al. 2002) 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 (Voolstra et al. 2006).

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 (Abrams et al. 1992; Krieger et al. 1993; Cociancich et al. 1994). 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 (Krieger 1997; Svensson et al. 1999). 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 (Pearson et al. 1995). 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 engorgement) by RT-PCR (Fig. 4b); moreover, endogenous HlSRB protein was also detected in several tissues of ticks including ovary, by indirect immunofluorescent antibody test (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). Our result suggests that HlSRB appears to be involved in the functional activities of SRB, providing to further study 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 SNMP from mammalian and insect SRBs (Rasmussen et al. 1998). Here, we 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 (Arenas et al. 2004). In our experiment, we 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 (Aljamali et al. 2003; Narasimhan et al. 2004; Miyoshi et al. 2004; Boldbaatar et al. 2006; Liao et al. 2008). In this paper, we also applied RNAi with one part of the fragment of the *HlSRB* gene to *H. longicornis*. We 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, c). These findings suggest that post-transcriptional 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 *Schistosoma 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 (Dinguirard and Yoshino 2006). Similar to what has been reported in *Drosophila*, a reduction in

infection was observed 3 days after dsRNA treatment for mycobacrerial infection (Philip et al. 2005).

In summary, we 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 further study, we will focus on the details of the functional analysis of the class B scavenger receptor CD36 gene from *H. longicornis*.

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