INFECTION AND IMMUNITY, July 2007, p. 3633–3640 Vol. 75, No. 7
0019-9567/07/$08.00+0 doi:10.1128/IAI.00256-07
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Babesial Vector Tick Defensin against *Babesia* sp. Parasites▽†

Naotoshi Tsuji,[1] Badgar Battsetseg,[2] Damdinsuren Boldbaatar,[2] Takeharu Miyoshi,[1] Xuenan Xuan,[2] James H. Oliver, Jr.,[3] and Kozo Fujisaki[2,4]*

Laboratory of Parasitic Diseases, National Institute of Animal Health, National Agricultural and Food Research Organization, Tsukuba, Ibaraki 305-0856, Japan[1]; National Research Center for Protozoan Diseases, Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido 080-0834, Japan[2]; Institute of Arthropodology and Parasitology, Georgia Southern University, Statesboro, Georgia[3]; and Department of Emerging Infectious Diseases, School of Veterinary Medicine, Kagoshima University, Korimoto, Kagoshima 890-0065, Japan[4]

Received 15 February 2007/Returned for modification 17 March 2007/Accepted 26 April 2007

Antimicrobial peptides are major components of host innate immunity, a well-conserved, evolutionarily ancient defensive mechanism. Infectious disease-bearing vector ticks are thought to possess specific defense molecules against the transmitted pathogens that have been acquired during their evolution. We found in the tick *Haemaphysalis longicornis* a novel parasiticidal peptide named longicin that may have evolved from a common ancestral peptide resembling spider and scorpion toxins. *H. longicornis* is the primary vector for *Babesia* sp. parasites in Japan. Longicin also displayed bactericidal and fungicidal properties that resemble those of defensin homologues from invertebrates and vertebrates. Longicin showed a remarkable ability to inhibit the proliferation of merozoites, an erythrocyte blood stage of equine *Babesia equi*, by killing the parasites. Longicin was localized at the surface of the *Babesia* sp. parasites, as demonstrated by confocal microscopic analysis. In an in vivo experiment, longicin induced significant reduction of parasitemia in animals infected with the zoonotic and murine *B. microti*. Moreover, RNA interference data demonstrated that endogenous longicin is able to directly kill the canine *B. gibsoni*, thus indicating that it may play a role in regulating the vectorial capacity in the vector tick *H. longicornis*. Theoretically, longicin may serve as a model for the development of chemotherapeutic compounds against tick-borne disease organisms.

Antimicrobial peptides are major defensive molecules of the innate immune system in animals (43). They appear to be well-conserved, evolutionally ancient molecules useful for the survival of vertebrates and invertebrates (23, 24, 46, 50). These peptides respond differently from those of antibiotics and currently used chemotherapeutic drugs. They are less toxic and more effective against multidrug-resistant bacteria, and it is hoped that they might be better choices for control of some bacterial and fungal infectious diseases (30).

Parasite disease-bearing vectors may require an extensive spectrum of innate immunity mechanisms, as evidenced by their diverse protective strategies. The presence of antibacterial and antiparasitic peptides is observed for mosquitoes (12, 49). Expression of defensin-like proteins has also been claimed for several ticks (9, 15, 24, 27, 34, 42, 44); however, their parasiticidal mechanisms in insect and tick vectors still remain unclear. Molecules operating in the innate immune system of numerous vectors (4, 11, 14, 21, 23, 41) may be candidates for the development of a chemotherapeutic effective against arthropod-borne diseases.

Babesiosis is a well-recognized malaria-like disease that occurs in animals and people worldwide and has recently gained increased attention as an emerging zoonosis (25, 47). The suffering and financial cost associated with this disease demand a search for new methods of control. *Babesia* species undergo a complex developmental cycle in the vertebrate host and tick vector somewhat analogous to that of the malaria parasite and mosquito vector (32). The major tick vectors of *Babesia* globally are *Boophilus* species and *Haemaphysalis longicornis* (40). The ixodid tick *H. longicornis*, one of the most important tick species in Asia and Australia, is a natural vector of the pathogens causing babesiosis of humans and domestic animals (16, 28). We hypothesize that *H. longicornis* possesses a specific gene product(s) that mediates partial protective responses against *Babesia*.

Here we report a defensin peptide, longicin, from the tick *H. longicornis* that exerts a babesiacidal effect. Longicin inhibited the growth of *Babesia* in vitro and in vivo. The babesiacidal effect was demonstrated at the merozoite stage, which causes babesiosis and *Babesia*-associated pathology, and was induced by the specific adherence of longicin to the parasite membrane. Interestingly, functional analysis by our validated double-stranded RNA (dsRNA) knockdown procedure revealed that longicin is involved in *Babesia* killing in *H. longicornis*. Our findings suggest that longicin might be useful in designing new chemotherapeutic agents against human and animal babesiosis. Vector ticks possess specific defense molecules probably acquired in the process of their evolution. Elucidation of the relationship between longicin and the *Babesia* parasite may thus help us to better understand the origin of differences between ticks that do and those that do not transmit pathogens.

* Corresponding author. Mailing address: Laboratory of Emerging Infectious Diseases, School of Frontier Veterinary Medicine, Kagoshima University, 1-21-24 Korimoto, Kagoshima 890-0065, Japan. Phone: 81-99-285-3569. Fax: 81-99-285-3570. E-mail: tick@ms.kagoshima-u.ac.jp.

† Supplemental material for this article may be found at http://iai.asm.org/.

▽ Published ahead of print on 7 May 2007.

MATERIALS AND METHODS

Ticks. *H. longicornis* ticks were obtained from the parthenogenetic Okayama strain maintained at the National Research Center for Protozoan Diseases (NRCPD), Obihiro University, Obihiro, Hokkaido, Japan, and were fed on rabbits (16).

Babesia **sp. parasites.** The *Babesia* sp. parasites used in this study were as follows: the horse parasite *Babesia equi* (22), the dog parasite *B. gibsoni* (17), and the mouse parasite *B. microti* (37). The U.S. Department of Agriculture strain of *B. equi* and the NRCPD strain of *B. microti* were maintained in in vitro culture at NRCPD. The NRCPD strain of *B. gibsoni* was maintained in chronically infected dogs at NRCPD.

Animals. All animals used this study were acclimatized to these conditions for 2 week prior to the experiment. Animal experiments at the National Institute of Animal Health (NIAH) were conducted in accordance with the protocols approved by the NIAH Animal Care and Use Committee (approval nos. 441, 508, and 578). Animal experiments at Obihiro University were conducted in accordance with the guiding principles for Care and Use of Research Animals promulgated by Obihiro University (approval nos. 6 to 42, C-2).

Cloning of longicin cDNA. Longicin was identified from expressed sequence tags constructed from the midgut cDNA libraries of *H. longicornis* as described previously (6). The plasmids containing longicin-encoding gene inserts were extracted using a QIAGEN DNA purification kit (QIAGEN, Hilden, Germany). The nucleotide sequences of the cDNAs were determined by the BigDye Terminator method on an ABI PRISM 3100 automated sequencer (Applied Biosystems, Foster City, CA). The GENETYX-WIN DNA analysis software system (Software Inc., Tokyo, Japan) was used to deduce the amino acid sequence of longicin (3) and the BLAST program (2) for alignment was used to compare this sequence with previously reported sequences available in GenBank (5). The putative signal sequence was analyzed using the prediction server SignalP V2.0.b2 (http://www.cbs.dtu.dk/services/SignalP) (36). Analysis of the secondary structure was done using the PSIPRED (http://bioinf.cs.ucl.ac.uk) and *SSThread* (http://www.ddbj.nig.ac.jp) programs.

Recombinant longicin. The entire coding region for longicin except the signal sequence was subcloned into a plasmid expression vector, pTrcHisB (Invitrogen, Carlsbad, CA), as described previously (10). The plasmid was transformed into *Escherichia coli* strain TOP10F′ (Invitrogen) and the purification process was monitored by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) using a T7 Taqâ monoclonal antibody (Takara, Otsu, Japan). The recombinant protein was purified using AKTA equipped with a HiTrap chelating HP column (Amersham Pharmacia Biotech, Piscataway, NJ), and the recombinant longicin band resolved by SDS-PAGE was excised (10). The purified protein was cleaved with EK max (Invitrogen), and the digested proteins corresponding to longicin were purified by SDS-PAGE gels with a zinc stain kit (Bio-Rad Laboratories, Hercules, CA). EK-digested longicin proteins were dialyzed against 20 mM Tris-HCl (pH 7.5), 150 mM NaCl by use of a Slide-A-Lyzer dialysis cassette (Pierce, Rockford, IL). Protein concentrations were measured using a Micro BCA protein assay reagent (Pierce). The results of matrix-assisted laser desorption ionization–time of flight (mass spectrometry) [MALDI-TOF (MS)] analysis of longicin agreed with its expected mass as determined using a Kratos Axima CFR (Shimadzu, Kyoto, Japan).

Synthetic peptides. Peptides were synthesized using a Perkin-Elmer Applied Biosystems 431 A synthesizer by use of prederivatized polyethylene glycol polystyrene arginine resin, FastMoc chemistry, and double coupling for residues. The reduced peptides were purified using reversed-phase high-performance liquid chromatography. The partial peptides were as follows: P1 (residues 23 to 37), P2 (33 to 45), P3 (42 to 57), and P4 (53 to 73). Peptide purity and integrity were assessed by MALDI-TOF (MS) (Kratos Axima CFR).

Production of an antibody against longicin. A mouse polyclonal antibody was generated against a peptide consisting of the N-terminal 20 amino acids of mature longicin. The animals were immunized with 50 μg of bovine serum albumin-conjugated peptide by use of TiterMax Gold (Syntex, Norcross, GA) and were boosted two more times with the bovine serum albumin-conjugated peptide as described previously (10).

Immunoblot analysis. Immunoblot analysis was performed as previously described (51). Adult female ticks were homogenized under liquid nitrogen. Antigens separated by one- or two-dimensional gel electrophoresis were transferred onto nitrocellulose membranes. For detection of endogenous longicin, the membranes were incubated with mouse anti-longicin serum followed by alkaline-phosphatase-conjugated goat anti-mouse immunoglobulin G (ICN Pharmaceutical, Irvine, CA) secondary antibody. The membranes were washed and visualized with the alkaline-phosphatase substrate 5-bromo-4-chloro-3-indolylphosphate and Nitro Blue Tetrazolium (Promega, Madison, WI).

Tick immunohistochemistry. The adult female ticks were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer and processed for immunohistochemistry using a mouse anti-longicin serum as described previously (51). The color was developed by incubation with 3,3′-diaminobenzidine (Sigma, St. Louis, MO) solution containing 0.03% H_2O_2. After dehydration and clearance, the sections were observed under an Axiophot instrument (Carl Zeiss, Jena, Germany). Preimmune mouse serum was used as a negative control.

Bactericidal and fungicidal assay. Bactericidal activity was determined by a CFU assay (19). The following bacteria used in these assays were a gift from the NIAH, Tsukuba, Japan: the human pathogenic bacteria *Escherichia coli* O-157 (ATCC 35150), *Pseudomonas aeruginosa*, and *Staphylococcus aureus*, derived from bovine mastitis, and plasmid-dependent multidrug-resistant *Salmonella enterica* serovar Typhimurium. Overnight bacterial culture was subcultured in 3% trypticase soy broth (TSB) with shaking at 37°C to obtain log-phase bacterial cells. Bacterial cells were then washed and diluted to 1×10^6/ml in 10 mM sodium phosphate buffer, pH 7.4, in 1% TSB. The bacterial cell suspensions (90 μl) were mixed with 10 μl of longicin (0, 10, 50, 100, 200 μmol) or synthetic peptide stock (0, 10, 20, 100 μmol) solutions and incubated at 37°C for 2 h. Samples were then diluted 100-fold in 1% TSB and spread on TSB agar plates with a spiral plater. Plates were incubated for 16 h at 37°C, colonies were counted, and CFU per ml were calculated. Fungicidal activity was also determined by a CFU assay (19). The yeast *Pichia pastoris* (GS115) was obtained from Invitrogen. Fungal cell suspensions (90 μl of 1×10^5/ml) were mixed with 10 μl of longicin or synthetic peptide stock solutions (0, 20, 100, 200, 400 μmol) and incubated in 10 mM sodium phosphate buffer, pH 7.0, at 37°C for 2 h. Samples were then diluted 10-fold in yeast-tryptone broth (YTB) and spread on YTB agar plates.

Hemoparasiticidal assay. Hemoparasiticidal activity was determined against *B. equi* in an in vitro culture system (22). *B. equi* merozoites were grown in horse erythrocytes in vitro and incubated in the presence of longicin or various synthetic peptides at different concentrations. Parasitemia was assessed every day using Giemsa-stained medium smears and microscopic observation (24).

Confocal fluorescence microscopy. The target peptide P4 was labeled with fluorescein isothiocyanate (FITC) according to the manufacturer's protocol (Pierce). The conjugate was purified by reversed-phase high-performance liquid chromatography (Shimadzu CLASS-VP; Shimadzu) and resuspended in distilled water. The labeled peptide was added to culture medium and washed several times with phosphate-buffered saline (PBS). Cells were smeared and fixed in 1% paraformaldehyde. Specimens were imaged using a confocal laser scanning microscope (Leica TCS-NT; Leica Microsystems, Wetzlar, Germany) with excitation of FITC. Images were collected by using Leica confocal software.

In vivo parasiticidal assay. Parasiticidal activity of longicin was examined in a *B. microti*-BALB/c mouse infection system (37). Mice that were intravenously inoculated with 1×10^7 *B. microti*-infected erythrocytes were simultaneously treated with various doses of longicin (0 to 3 mg/kg). Parasitemia was assessed every day by microscopic observation of Giemsa-stained blood smears and the results were expressed as mean values ± standard deviations from five mice per dosage. The survival of the mice was monitored for the next 8 weeks.

RNAi. The RNA interference (RNAi) procedure in ticks was carried out using dsRNA as described previously (33, 35). The coding sequence of mature longicin was cloned into pBluescript II SK+ plasmid, and the inserted sequence was amplified by PCR using the oligonucleotides T7 (5′-GTAATACGACTCACTA TAGGGC-3′) and CMo422 (5′-GCGTAATACGACTCACTATAGGGAACA AAAGCTGGAGCT-3′) to attach T7 promoter recognition sites at both the 5′ and 3′ ends. The PCR products were purified by use of a gel extraction kit (QIAGEN). dsRNA complementary to the DNA insert was synthesized by in vitro transcription using the T7 RNA polymerase (Promega, Madison, WI) according to the manufacturer's protocol. Two micrograms of double-stranded DNA was used as a template, and 50 to 100 μg of dsRNA was synthesized. We injected 1 μg of *longicin* dsRNA in 0.5 μl of PBS into the hemocoel through the fourth coxae of unfed adult *H. longicornis* females fixed on a glass slide with adhesive tape. The injections were carried out by using 50-μl microcapillaries (MICROCAP; Drummond Scientific, Broomall, PA) drawn in to fine-point needles by heating. The needles were connected to an air compressor. Control ticks were injected with 0.5 μl PBS alone. The ticks were allowed to rest for 1 day at 25°C. No mortality resulted from the injection alone, as both control and *longicin* dsRNA-treated ticks survived after injection while being held in an incubator prior to placement on the host.

Infestation by RNAi-treated ticks of dogs infected with canine *Babesia* sp. parasite. The dsRNA-injected ticks were placed on the ears of 8-month-old female beagles preinfected with *B. gibsoni* (18). During attachment, the dogs kept 12.5% intraerythrocytic parasitemia in the peripheral blood. The pattern of the control ticks injected with the buffer alone was comparable to that for

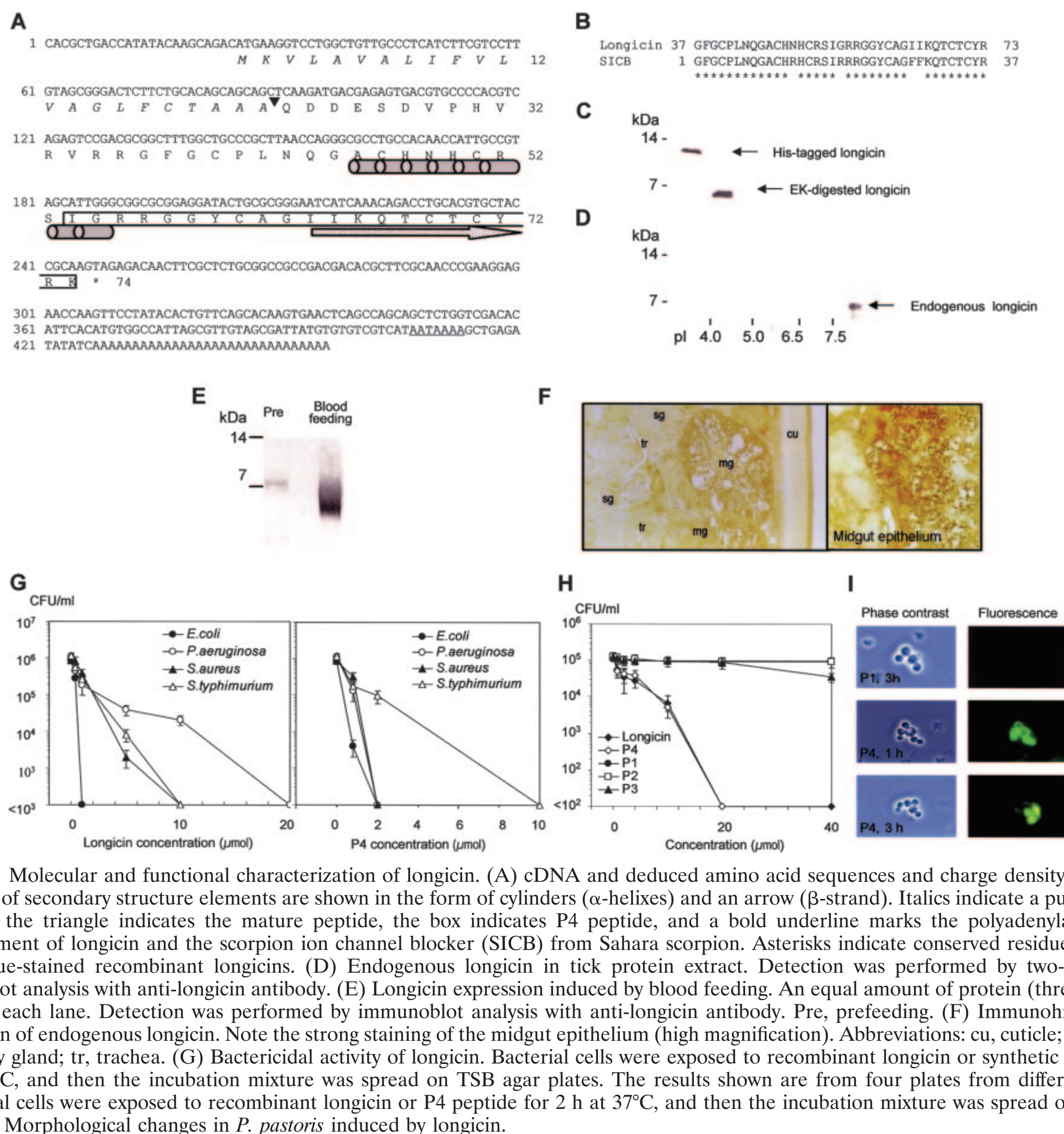

FIG. 1. Molecular and functional characterization of longicin. (A) cDNA and deduced amino acid sequences and charge density of longicin. Locations of secondary structure elements are shown in the form of cylinders (α-helixes) and an arrow (β-strand). Italics indicate a putative signal sequence, the triangle indicates the mature peptide, the box indicates P4 peptide, and a bold underline marks the polyadenylation signal. (B) Alignment of longicin and the scorpion ion channel blocker (SICB) from Sahara scorpion. Asterisks indicate conserved residues. (C) Coomassie blue-stained recombinant longicins. (D) Endogenous longicin in tick protein extract. Detection was performed by two-dimensional immunoblot analysis with anti-longicin antibody. (E) Longicin expression induced by blood feeding. An equal amount of protein (three ticks) was loaded in each lane. Detection was performed by immunoblot analysis with anti-longicin antibody. Pre, prefeeding. (F) Immunohistochemical localization of endogenous longicin. Note the strong staining of the midgut epithelium (high magnification). Abbreviations: cu, cuticle; mg, midgut; sg, salivary gland; tr, trachea. (G) Bactericidal activity of longicin. Bacterial cells were exposed to recombinant longicin or synthetic peptides for 2 h at 37°C, and then the incubation mixture was spread on TSB agar plates. The results shown are from four plates from different batches. (H) Fungal cells were exposed to recombinant longicin or P4 peptide for 2 h at 37°C, and then the incubation mixture was spread on YTB agar plates. (I) Morphological changes in *P. pastoris* induced by longicin.

uninjected ticks infested simultaneously on the same host. On day 6, ticks were recovered from the dogs. After dissection of the ticks, individual organs were removed and the midgut contents were opened under a microscope. To verify gene silencing of *longicin* dsRNA, reverse transcription-PCR was performed as described previously (51). Total mRNA was isolated using a QuickPrep Micro mRNA purification kit (Amersham Pharmacia Biotech) as described in the protocols. cDNA was then synthesized with 30 μg of mRNA using an RNA PCR kit (AVM) Ver.3.0 (Takara) following the manufacturer's instructions. PCR was performed using *longicin*-specific oligonucleotides and β-actin-specific oligonucleotides for *H. longicornis* with 500 ng of cDNA as the template in a final volume of 50 μl. PCR products were resolved by agarose gel electrophoresis.

Immunofluorescence microscopy. We examined endogenous longicin expression and localization of *B. gibsoni* in dissected tick organs. Tick immunofluorescent analysis was performed as described previously (26). Bound mouse anti-longicin or mouse anti-*B. gibsoni* antibodies (17) were detected using anti-mouse immunoglobulin G Alexa 488 (Invitrogen). The sections were mounted in Vectashield (Vector, Burlingame, CA) with 4′,6′-diamino-2-phenylindole (DAPI) and photographed with a fluorescence microscope (Leica) using appropriate filter sets. Images were collected by using Leica FW4000 software.

Real-time PCR assay for quantifying *B. gibsoni* infection. The numbers of *B. gibsoni* organisms and the intensity of *B. gibsoni* infection in the dissected organs were evaluated using a real-time quantitative PCR assay. Initially, we standardized the PCR protocol by use of *B. gibsoni* P18 gene-specific primers (D3, 5′-TCCGTTCCCACAACACCAGC-3′; D4, 5′-TCCTCCTCATCATCCTCATTCG-3′) and purified *B. gibsoni* genomic DNA. *B. gibsoni* P18, encoding a major surface protein, is a well-known gene, and its use as a diagnostic tool for dog *B. gibsoni* infection has been demonstrated (18). PCR was performed using a LightCycler 1.5 (Roche Diagnostics GmbH, Nonnenwald, Germany) and DNA master SYBR green I (Roche) with 4 mM $MgCl_2$. Standard curves used to quantify relative gene concentrations were made from a 10-fold serial dilution (3×10^0 to 3×10^3) of the *B. gibsoni* parasites with the following setting: 95°C for 600 s (denaturing step), 45 cycles of 95°C for 15 s, 55°C for 10 s, and 72°C for 15 s under the fit point method in LightCycler software, version 3.5.3. The protocol was observed to be highly specific for *B. gibsoni* P18, with no amplification of dog, tick, or a range of other *B. gibsoni* DNA. The standard plot is shown elsewhere (see Fig. S1 in the supplemental material). Evaluation of the number of *B. gibsoni* parasites from excised organs was determined on the basis of the standard plot. DNA extraction and concentration were determined as described previously (51).

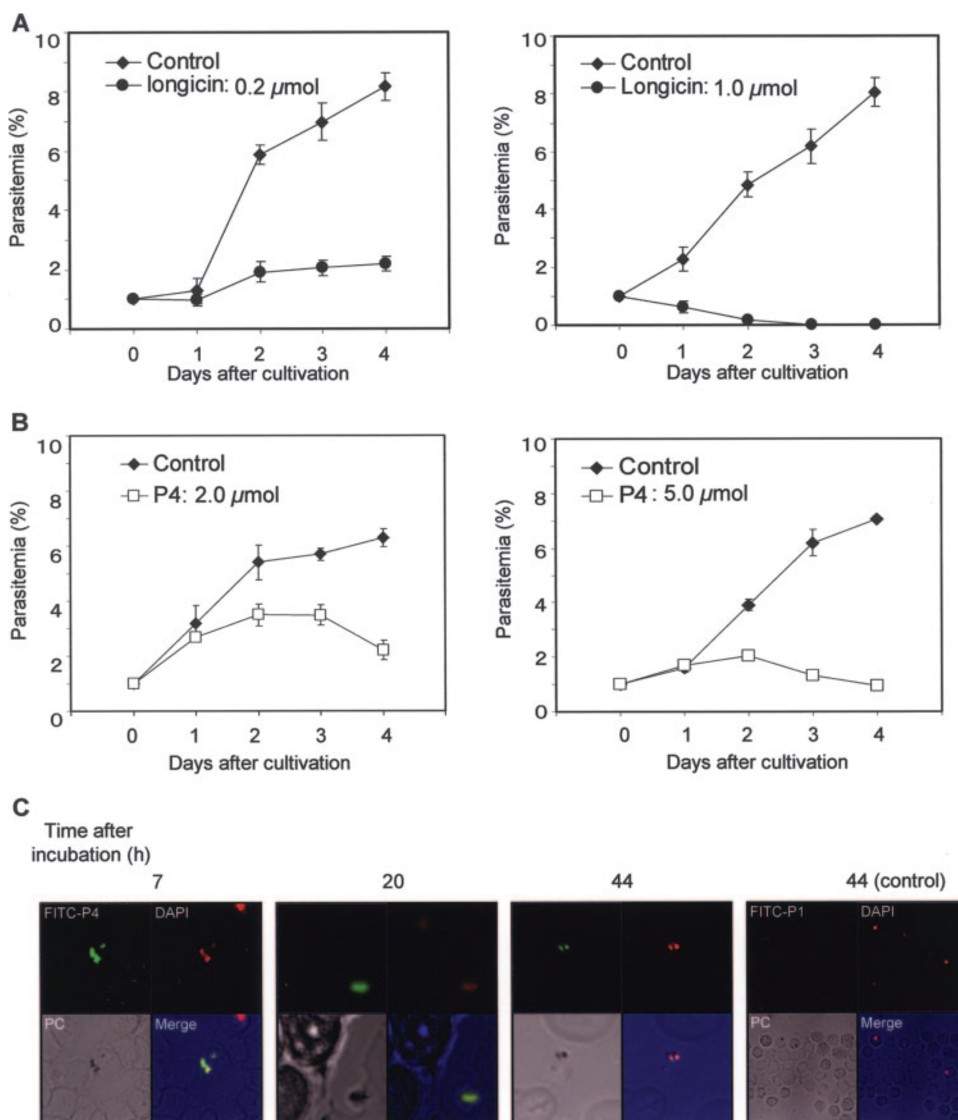

FIG. 2. Babesiacidal activity of longicin. Longicin or synthetic peptides were incubated with *B. equi*-infected erythrocytes (1% parasitemia) in culture medium. Parasite-infected erythrocytes were counted as percentages of total erythrocytes. (A) Parasiticidal effect in the presence of longicin. *Babesia*-infected cells had almost disappeared on day 2 of 1.0 μmol treatment. (B) Parasiticidal effect in the presence of synthetic peptides. The error bars indicate standard errors of the mean (SEM). (C) Detection of longicin at the surface of *B. equi* merozoites. FITC-conjugated longicin P4 synthetic peptide was used for localization by fluorescent confocal microscopy. The control panel shows that FITC-labeled P1 did not bind to any cells. PC, phase contrast.

Statistical analysis. Statistically significant analysis was performed by using Student's *t* test.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper have the DDBJ/EMBL/GenBank accession no. AB105544.

RESULTS

Molecular and functional characterization of longicin. The composite full-length *longicin* cDNA sequence was 456 nucleotides long and contained a single open reading frame of 216 bases. The open reading frame coded for a protein of 74 amino acids, including a signal peptide of 20 residues (Fig. 1A). The putative mature protein has a molecular mass of 5,820 Da and a pI of 8.3, including six cysteine residues. The predicted secondary structure of longicin showed a well-defined β-sheet at the C terminus. The greatest amino acid sequence similarity (86%) found for longicin was with the scorpion ion channel blocker (13) (Sahara scorpion, accession no. P56686; Fig. 1B). Longicin also shares great similarity with defensins from ixodids and argasids (hard and soft ticks) (9, 15, 27, 34, 42, 44). The cDNA corresponding to the deduced premature protein was subcloned into a plasmid expression vector to produce a recombinant fusion longicin (Fig. 1C). To characterize the expression of endogenous longicin, we generated a specific polyclonal antibody. The antibody bound to a single spot in two-dimensional immunoblots (Fig. 1D), indicating that the peptide with a molecular mass of 6.4 kDa and pI of 8.5 from adult *H. longicornis* extract was the endogenous form of longicin. We confirmed by MALDI-TOF (MS) analysis that this

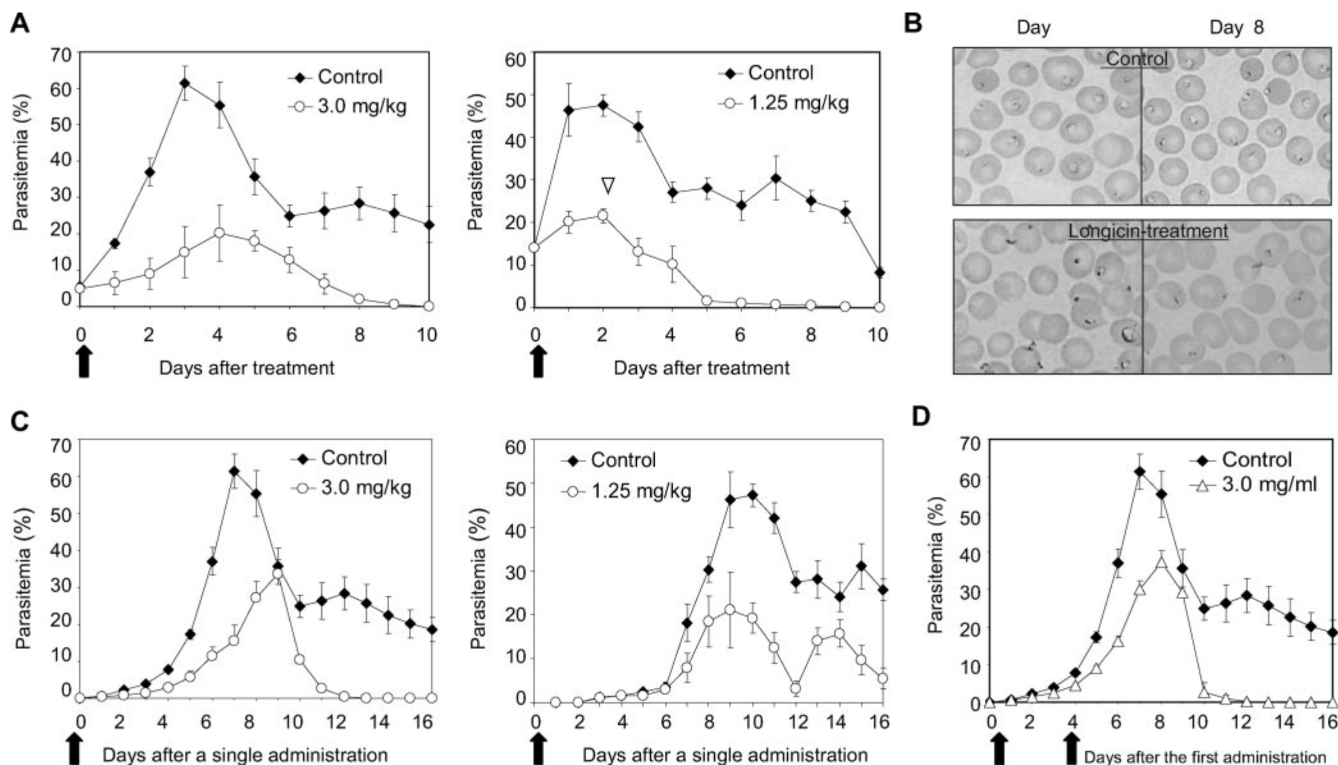

FIG. 3. In vivo babesiacidal activity of longicin. (A) Therapeutic effect of a single treatment dose. Arrows and an arrowhead indicate administration of longicin and 60% reduction of parasitemia, respectively. (B) Representative images of the parasites from mice treated with longicin (3.0 mg/kg). Longicin blocked parasite invasion but not host erythrocyte rupture. (C) Growth inhibition was dependent on the dose of longicin. (D) Growth inhibition by a double treatment with longicin. The error bars indicate SEM.

protein was consistent with the polypeptide predicted from the cDNA (data not shown). Constitutive expression of longicin was detected throughout the normal life cycle, including larval, nymphal, and adult stages, and the level was clearly increased after blood feeding (Fig. 1E). This pattern of endogenous expression resembled that of defensin homologues from other bloodsucking arthropods (11). However, endogenous longicin was produced mainly in the midgut epithelium (Fig. 1F), suggesting that longicin is secreted into the lumen, unlike mosquito defensin (1).

Recent studies indicate that some ixodid tick defensins possess bactericidal activity against several bacterial pathogens (15, 27, 42). To assess the functional properties of longicin, we measured the antimicrobial activity against several human and animal pathogenic bacteria. Recombinant longicin and synthetic peptides consisting of 16 to 22 residues were also prepared based on the deduced amino acid sequences. The bactericidal activity of longicin was tested against a variety of gram-positive and gram-negative bacteria, including multidrug-resistant strains from human and animal patients (Fig. 1G). Although the synthetic peptides P1, P2, and P3 did not exert a bactericidal effect against any of the screened bacterial strains, P4 consistently caused concentration-dependent killing of the four species tested at concentrations of 2.0 to 10.0 μmol (Fig. 1G). It also possessed fungicidal activity. Proliferation of the yeast *P. pastoris* was completely inhibited by P4 at 20.0 μmol (Fig. 1H). When *P. pastoris* was incubated with FITC-labeled P4, the cell membrane was clearly fluorescent (Fig. 1I).

Babesiacidal activity of longicin. To further assess the properties of longicin, we incubated the equine *Babesia* sp. parasite, *B. equi*, in medium supplemented with longicin. Interestingly, recombinant longicin completely inhibited merozoite proliferation at a concentration of 1.0 μmol (Fig. 2A). The inhibitory effects of longicin were concentration dependent. Next, we attempted to identify the babesiacidal active site of longicin against *B. equi* by using the four types of synthetic peptides (P1 to P4) described above. The merozoite-infected erythrocytes were maintained for 4 days in the presence of the peptides at a dose of 0 to 5.0 μmol. Similarly to the full-length longicin, P4 induced complete protection against the infection of new erythrocytes (Fig. 2B), and the parasiticidal effects were concentration dependent. No synergistic effects were induced by treatment with any two of the peptides. Neither longicin nor the four types of partial peptides caused hemolysis at any concentration that exerted a parasiticidal effect.

To explore how longicin interacted with the parasites, we used fluorescence confocal microscopy to examine *B. equi* at the merozoite stage. *B. equi* was incubated with FITC-labeled P4 peptide in culture medium. Fluorescent reactions were seen at the surface of the merozoites, but morphological changes did not occur at 7 h of incubation (Fig. 2C). At 44 h, longicin clearly caused lysis of the parasites. Quantitative assays showed that the residual peptide was decreased in the presence of

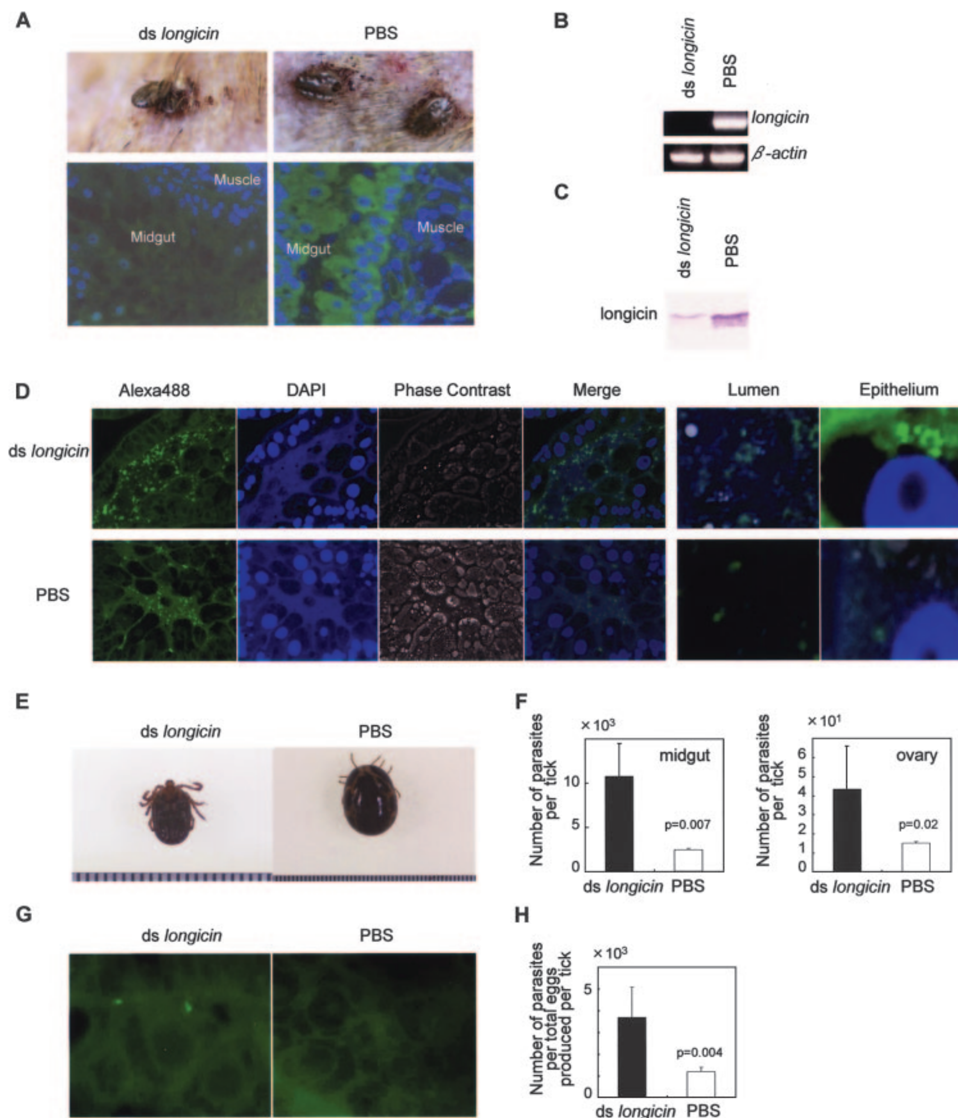

FIG. 4. Knockdown of *longicin* by RNAi facilitates transmission of *Babesia* parasite through the vector ticks. Unfed adult female ticks were injected with *longicin* dsRNA (ds *longicin*) into the hemocoel through the fourth coxae by use of fine-point glass needles. Control ticks were injected with PBS alone. Ticks were collected from the ear of a *Babesia gibsoni*-infected dog on day 6 after attachment. (A) Microinjection-treated ticks at day 3 on the ear of a dog. Injection of *longicin* dsRNA inhibited endogenous longicin expression (green) in the midgut. Figures are representative of three independent RNAi experiments. (B) RT-PCR analysis (day 3). Note the reduced expression of *longicin* mRNA in *longicin* dsRNA-injected ticks. (C) Immunoblot analysis of endogenous longicin expression (day 3). Results showed the absence of longicin expression, indicating that the effective knockdown of *longicin* mRNA was achieved by dsRNA injection. (D) Images of the tick midgut. *B. gibsoni* parasites were visualized by use of mouse anti-*B. gibsoni* antibody (green). The two right panels highlight the lumen and epithelium from the merge section of the midgut. (E) Ticks on day 6. The body weights of ticks with silencing *longicin* were significantly lower than those of control ticks at day 6. Suppression of endogenous longicin expression in *longicin* dsRNA-treated ticks was seen up to engorgement. Preoviposition, oviposition, and egg periods of ticks treated with dsRNA were similar to those of PBS control ticks. Smaller engorged ticks silenced with *longicin* subsequently transmitted larger numbers of *Babesia* sp. parasites than did PBS control ticks. One scale, 1 mm. (F) Prevalence and intensity of *B. gibsoni* infection. The numbers of invaded parasites were evaluated by P18 genes on the *B. gibsoni* genome DNA by use of a real-time quantitative PCR. (G) Representative image of the migrating *Babesia* parasite in the tick ovary. (H) Intensity of *B. gibsoni* infection. Data represent the means ± SEM for three experiments with five ticks. Quantitative results demonstrated that repression of *longicin* enhanced the *B. gibsoni* transmission in the vector tick. The error bars indicate SEM for three independent experiments with three ticks.

merozoites (data not shown), indicating that this difference was the result of subsequent interactions of peptides with parasites.

In vivo babesiacidal activity of longicin. For babesiosis or *Babesia*-associated pathology to occur, the invasive merozoites must recognize, bind, and enter into the circulating erythrocytes. Subsequent replication of the babesial blood-stage parasites causes rupture of the animal erythrocytes (40). The efficacy of the hemoparasiticidal activity of longicin was tested in vivo against a murine *Babesia* parasite, *B. microti*. When infected mice were inoculated with a single dose of 3.0 mg/kg longicin at 5% parasitemia, the parasitemia increased significantly more slowly, and a 72% inhibition was noted at the peak

parasitemia. Infected mice inoculated with 1.25 mg longicin/kg of body weight at 15% parasitemia showed a 60% inhibition of parasitemia (Fig. 3A and B). Next, a challenge infection using longicin-treated mice was initiated. Treatment of mice with 3.0 mg/kg maximally inhibited parasitemia by 40% and cleared it at day 12, whereas 1.25 mg/kg allowed a low level of parasitemia throughout the observation period (Fig. 3C). Mice treated with 3.0 mg/kg followed by a second treatment at the same dose did not show any synergistic effects (Fig. 3D). All mice treated with longicin and P4 were as active and healthy as untreated mice, with normal parameters for liver and kidney function as indicated by blood examination (data not shown).

The role of longicin in *H. longicornis*. We next hypothesized that endogenous longicin may directly affect *Babesia* parasite survival. We took an RNAi approach to knock down *longicin* mRNA by dsRNA in adult *H. longicornis*. We then assessed whether the transmission of *Babesia* sp. parasites was affected by the endogenous longicin. The dsRNA-treated ticks were attached to a dog that was preinfected with dog *Babesia* sp. parasite *B. gibsoni*. Interestingly, the knockdown of *longicin* resulted in a significant reduction in the ability of the ticks to feed and engorge. Longicin depression clearly impaired tick blood feeding at day 3, although the underlying mechanism responsible for the developmental effect is unclear (Fig. 4A). Inhibition of *longicin* mRNA and endogenous longicin was clearly seen for the *longicin* dsRNA-treated ticks (Fig. 4B and C). Immunofluorescent studies indicate increasing ratios of the parasites in the midgut of the *longicin* knockdown ticks (Fig. 4D). A significant difference in body weight at engorgement between the knockdown (mean ± standard deviation, 89.5 ± 32.1 mg; $n = 12$) and control (312.5 ± 48.3 mg; $n = 15$) groups was observed (Fig. 4E). In addition, the knockdown of *longicin* showed a significantly increased number of *B. gibsoni* parasites in the midgut and the ovary (Fig. 4F and G). Longicin deletion in ticks resulted in a twofold increase in the transmission ability of *Babesia* sp. parasites into the eggs (Fig. 4H). Although *longicin* dsRNA-treated ticks were attached to non-*Babesia*-infected dogs, no significant differences were observed in the tick feeding and engorgement abilities on the infected and the noninfected dogs.

DISCUSSION

Babesia sp. parasites must complete a complex developmental cycle in the tick for transmission to occur. Their development depends on a balance between the ability of the tick to establish a defense response against the parasite and the ability of the parasite to escape the tick's immune response. Thus, it may be that *H. longicornis* has some defense molecules against invading *Babesia* sp. parasites. An excessive number of parasites may destroy the midgut epithelium, resulting in the hemolymph flowing into the lumen and causing the death of the tick (20, 45). Longicin expressed by *H. longicornis* may have acquired the parasiticidal action of controlling the number of *Babesia* parasites in addition to its antibacterial and antifungal actions. On the other hand, for babesiosis or *Babesia*-associated pathology to occur, the invasive merozoites must recognize, bind, and enter circulating erythrocytes. Subsequent replication of the babesial blood-stage parasites causes rupture of animal erythrocytes (40).

Current information indicates that longicin is not involved in erythrocyte rupture during the blood phase of *Babesia* but rather has a specific role in the invasion of the host cell by extracellular merozoites (7). Antimicrobial peptides recognize pathogen-associated molecular patterns in microbes and may form ion-permeable transmembrane pores, resulting in the rupture of target microbes (31). Some antimicrobial peptides derived from non-parasite-bearing vectors are known to possess antiparasite activity (8). However, these are very toxic for mammalian cells, since they cause hemolysis. The present results suggest that the bactericidal, fungicidal, and babesiacidal active sites are located in the C-terminal amino acid sequence, which consists of a β-sheet, in contrast to insect defensins, whose recognition site is located in an α-helix (38). Ticks are phylogenetically closer to spiders and scorpions (Arachnida, Chelicerata) than to insects (24, 29). Cationic defensin peptides from both spiders and scorpions act as ion channel blockers, and the ion channel recognition site is located in the β-sheet of their C termini (39). Longicin may have evolved from a common ancestral peptide resembling spider and scorpion toxins. Longicin is the first molecule isolated from a parasite-bearing vector that exerts a hemoparasiticidal effect without any demonstrable toxicity to mammalian host cells.

Our data show that longicin can prevent or retard proliferation of merozoites. Moreover, longicin appears to be stable in vivo and therefore able to act against *Babesia* parasites when exported to them. This may be due to its lack of degradation by murine proteases (39). Surprisingly, present therapeutic test results indicate that the efficacy of longicin is superior to that reported for antibabesial drugs commonly used in clinical practice (48). Our data confirm the efficacy of longicin in inhibiting the growth of *Babesia* sp. parasites both in vitro and in vivo and strongly suggest that it may be useful in designing new chemotherapeutic agents.

After the acquisition of a blood meal by adult *H. longicornis*, the ingested *Babesia* sp. parasites invade the midgut epithelium, move to the ovary, and finally enter into the eggs (32). The present results suggest that longicin-mediated killing of *Babesia* may control the number of parasites as they exit and/or invade the midgut epithelium. Prior studies have shown that the tick transmits only a limited number of *Babesia* sp. parasites during blood feeding, suggesting the existence of a partially successful natural defense mechanism against the parasites (20). Thus, longicin expressed by vector ticks may have acquired parasiticidal action in reducing the number of *Babesia* sp. parasites in addition to its antibacterial and antifungal actions.

ACKNOWLEDGMENTS

We thank H. Kobayashi and T. Sameshima for providing bacterial stocks. We also thank H. Shimada and M. Kobayashi for help in preparing tick paraffin sections.

This work was supported by the 21st Century COE Program (A-1, to K.F.) and grants-in-aid (to N.T. and K.F.) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This work was also supported by a grant (to N.T. and K.F.) for Promotion of Basic Research Activities for Innovative Biosciences from the Bio-oriented Technology Research Advancement Institution.

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Editor: W. A. Petri, Jr.