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Longistatin, a novel EF-hand protein from the ixodid tick *Haemaphysalis longicornis*, is required for acquisition of host blood-meals ☆

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ABSTRACT

Calcium and the EF-hand Ca^{++}-binding proteins have been undisputedly recognised as the key players in almost all aspect of cell functions, starting from the cell's birth, during mitosis to its end with apoptosis. But in a few exceptional cases the EF-hand proteins are secreted from the cells and play their crucial roles extracellularly. Here, to our knowledge for the first time, we have identified and characterised an EF-hand Ca^{++}-binding protein from the salivary glands of the ixodid tick, *Haemaphysalis longicornis*, herein called longistatin. Longistatin possesses two EF-hand domains which conserve canonical structure and bind with Ca^{++}. Both the recombinant and endogenous proteins were stained with Rutheninum red. Reverse-transcription PCR data showed that longistatin-specific transcript was expressed in all life-cycle stages of *H. longicornis* and was up-regulated only in blood-fed ticks. Organ-specific transcription analysis revealed a salivary gland-specific expression of the gene which peaked at 96–120 h of feeding when ticks acquired full blood-meals and become engorged but its expression declined sharply as they detached and dropped off the host. Consistently, endogenous protein was localised in the salivary glands of adult ticks and in the lumen of the functional acini of the salivary glands. Furthermore, longistatin was detected in feeding lesions at the site of attachment of ticks on the host. These results suggest that longistatin is synthesised in, and is secreted from, the salivary glands and may have functional roles in the feeding process of ixodid ticks.

1. Introduction

Ticks are obligate haematophagous ectoparasites and all of their motile life-cycle stages are exclusively dependent on the blood-meals from host animals. Ticks are broadly classified into three families such as Ixodidae (hard ticks), Argasidae (soft ticks) and Nuttalliellidae. *Haemaphysalis longicornis* Neumann 1901, commonly known as Bush Tick or New Zealand Cattle Tick, is a hard tick and is widely distributed in many countries from the Far East to Australia (Fujisaki et al., 1994; Hoogstraal et al., 1968). In addition to direct severe adverse effects on health and productivity of infested animals, *H. longicornis* acts as vector of many bacterial, viral, protozoan and rickettsial diseases (Hoogstraal et al., 1968). Among these diseases, babesiosis, anaplasmosis, theileriosis and Q fever have both veterinary and medical importance. These diseases are associated with human suffering, and are a major constraint to animal production as they may cause morbidity or mortality in affected animals (Fujisaki et al., 1994; Ho et al., 1995).

Control of ticks may be an effective strategy to eradicate tick-borne diseases. But tick control is a herculean task as they have a wide host range. In addition, ticks are able to spend about 95% of their life-time away from the host, especially during the period of starvation (Needham and Teel, 1991). Even in this post-genomic era, various controversial methods are being used to control ticks, including the application of chemical acaricides which are very hazardous for human and animal health as they have direct toxic effects. Due to their residual effects, the acaricides are not environmentally friendly and furthermore, repeated usages of acaricides lead to the development of resistance in ticks (Zaim and Guillet, 2002). These obstacles in controlling ticks necessitate the

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development of sustainable therapeutic interventions. Therefore, identification and characterisation of potential vaccine candidate and drug target bioactive molecules from the blood-feeding ixodid ticks are of considerable interest.

EF-hand proteins have been reported from a variety of sources such as bacteria, protozoa, helminths, arthropods and mammals including humans (Nelson and Chazin, 1998; Kawasaki et al., 1998). Calcium and the EF-hand Ca^{++}-binding proteins have been undisputedly recognised as the key players in almost all aspects of cell functions, starting from the cell's birth, through mitosis to its end with apoptosis (Berridge et al., 1998; Kahl and Means, 2003). EF-hand Ca^{++}-binding proteins modulate various biochemical reactions within the cell and in a few exceptional cases they are secreted from the cells and play their crucial roles extracellularly. For example, Osteonectin, a glycoprotein with EF-hand Ca^{++}-binding domains, is secreted from the cell and exerts versatile functions in the extracellular matrix including, a vital role as a morpho-regulatory element (Pottgiesser et al., 1994). Similarly, acetylcholinesterase, having two EF-hand domains, functions extracellularly in cell surface associations (Tsigelny et al., 2000).

Here, we have identified and cloned a full-length cDNA from the salivary glands of the ixodid tick, *H. longicornis*, which encodes a protein (longistatin) containing two functional EF-hand Ca^{++}-binding domains and is able to bind calcium. We also show that longistatin is secreted from the salivary glands and may function in the feeding process of the blood-meals from host animals by ixodid ticks. To our knowledge, longistatin is the first characterised EF-hand protein isolated from the salivary glands of ticks.

2. Materials and methods

2.1. Ticks

Parthenogenetic Okayama strains of *H. longicornis* were propagated at the Laboratory of Parasitic Diseases, National Institute of Animal Health (NIAH), Tsukuba, Japan, by feeding on the ear of specific pathogen-free (SPF) Japanese White rabbits according to the methods described previously (Alim et al., 2007) to obtain different life-cycle stages of ticks at different feeding intervals. Briefly, the ears of rabbits were cleaned by hair clipping; ticks were attached and given support with ear bags and an Elizabethan collar. Ear bags were changed at 24 h intervals. Ticks were collected after detachment following full engorgement or after the indicated period of attachment. Rabbits used in these experiments were acclimatised to the experimental laboratory conditions for 2 weeks prior to the commencement of the experiment. Animal care was conducted according to the protocols approved by the Animal Care and Use Committee, NIAH (Approval Nos. 441, 508, 578).

2.2. Cloning and sequencing of longistatin cDNA

The gene coding for longistatin was identified from the expressed sequence tags (ESTs) constructed from the salivary gland cDNA libraries of *H. longicornis* following the methods described previously (Boldbaatar et al., 2006). Briefly, the plasmid containing the gene coding for longistatin was extracted using a Qiagen DNA Purification kit (QIAGEN Science, Germantown, MA, USA). The nucleotide sequences of the cDNA were determined using the big dye terminator method on an ABI PRISM 3100 automated sequencer (Applied Biosystem, Foster City, CA, USA). The GENETYX-WIN DNA analysis software system (Software Inc.) was used to deduce the amino acid sequence of longistatin. Sequence similarity searches were performed using the BLAST programme (Altschul et al., 1997). Alignment with the previously reported similar protein sequences, available in GenBank (Benson et al., 2002), were done using CLUSTALW. The putative signal sequence was analysed using the prediction server SignalP V2.0.b2 (http://www.cbs.dtu.dk/services/SignalP) (Nielsen et al., 1997). Theoretical mol. wt and pI were determined using PeptideMass (http://us.expasy.org/tools/peptidemass.html) (Wilkins et al., 1997). Domains were searched using ExPASy-PROSITE (http://au.expasy.org/prosite/). N-linked glycosylation sites were searched using NetNGlyc 1.0 (http://www.cbs.dtu.dk/services/NetNGlyc).

2.3. Expression of longistatin

The open reading frame (ORF) of longistatin was amplified by PCR from PBS/longistatin using a set of primers, 5′ CCG CTC GAG CGG GCA GGC CGG CGA CCA GCA G 3′ and 5′ GGA ATT CCC TAA ATT TGG TTG GTC AGG TC 3′, which contained XhoI and EcoRI restriction sites, respectively. PCR was performed for 3 min at 95 °C followed by 35 cycles of 30 s at 95 °C, for 30 s at 57 °C and 1.5 min at 72 °C with a final elongation at 72 °C for 5 min. Both the PCR product and the vector pTrcHisB (Invitrogen, Carlsbad, CA, USA) were digested by XhoI and EcoRI restriction enzymes. The purified PCR product was inserted into the XhoI and EcoRI sites of the vector pTrcHisB (Invitrogen). The resultant plasmid was transformed into competent cells of *Escherichia coli* Top10F′strain (Invitrogen) following the conventional method. The expression of longistatin in *E. coli* with a Polyhistidine-tag was performed according to the procedure described by Tsuji et al. (2001). Briefly, the transfected cells were allowed to grow in SOB medium (Tryptone 20.0 g, Yeast extract 5.0 g, Sodium chloride 0.5 g, Magnesium sulphate anhydrous 2.4 g and Potassium chloride 0.186 g per litre) containing 50 μg ampicillin/ml at 37 °C under vigorous shaking (200 rpm) until the OD of 1 at 600 nm (OD_{600}) was achieved. To induce recombinant protein expression, isopropyl-β-D-thiogalactopyranoside (IPTG) was added to 1 mM concentration and the culture was grown for an additional 4 h at 37 °C on a shaking incubator. The culture was then centrifuged at 10,000*g* and 4 °C for 20 min and the pellet was resuspended in 10 ml of lysis buffer (20 mM sodium phosphate and 500 mM sodium chloride, pH 7.8). Egg white lysozyme (100 μg/ml) was added to the cell suspension and incubated in ice for 15 min. The suspension was sonicated for 2 min on ice with an ultrasonic processor (VP-15S, TAITEC, Japan) followed by immediate freezing at −80 °C and then thawing at 37 °C for 15 min in each case. After three cycles of sonication, freezing and thawing, the *E. coli* lysate was centrifuged at 23,900*g* and 4 °C for 30 min and supernatant was collected. The recombinant protein was purified using ProBond™ resin (Invitrogen) under native conditions as described by the manufacturer and subsequently eluted with a stepwise gradient of imidazole (25–500 mM). The eluted recombinant protein was concentrated using Centrisart® (Sartorius, Goettingen, Germany) having a mol. wt cut-off of 10 kDa. The concentrated protein was dialysed extensively at 4 °C with successive changes of 20 mM Tris–HCl (pH 7) and a decreasing concentration of NaCl (500–250 mM) using a Slide-A-Lyser Dialysis Cassette (Pierce, Rockford, IL, USA) with a mol. wt cut-off of 10 kDa. Purified recombinant longistatin was electrophoresed on 12.5% SDS–PAGE gel under reducing conditions. The gel was treated with 50% methanol in 10% acetic acid for 10 min at room temperature. The protein was subjected to silver staining (Daiichi Pure Chemicals, Tokyo, Japan) following the manufacturer's instructions. Finally, protein concentration was determined using micro-BCA reagent (Pierce) and stored at −20 °C until further use.

2.4. Western blot analysis

Crude *E. coli* lysate was electrophoresed through 12.5% SDS–PAGE gel under reducing conditions and the proteins were trans-