The lipopolysaccharide-binding protein participating in hemocyte nodule formation in the silkworm *Bombyx mori* is a novel member of the C-type lectin superfamily with two different tandem carbohydrate-recognition domains

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Received 26 October 1998; received in revised form 30 November 1998

Abstract **We recently isolated and characterized the lipopolysaccharide (LPS)-binding protein, BmLBP, from the larval hemolymph of the silkworm *Bombyx mori*. BmLBP is a pattern recognition molecule that recognizes the lipid A portion of LPS and participates in a cellular defense reaction. This paper describes the cDNA cloning of BmLBP. The deduced amino acid sequence of BmLBP revealed that BmLBP is a novel member of the C-type lectin superfamily with a unique structural feature that consists of two different carbohydrate-recognition domains in tandem, a short and a long form.**

Key words: Lipopolysaccharide-binding protein; C-type lectin; Carbohydrate-recognition domain; Pattern recognition; Insect; Immunity

1. Introduction

Innate immunity is a primary defense mechanism in the animal kingdom and innate immune recognition is based on pattern recognition. Pattern recognition is a recognition system that recognizes structures common among invading pathogens known as pathogen-associated molecular patterns (PAMPs) [1,2]. Molecules that recognize PAMPs are called pattern recognition molecules. C-type lectins are pattern recognition molecules that play a central role in pattern recognition [2]. C-type lectins likely function in both mammal and insect innate immunity [3–6].

Lipopolysaccharide (LPS) is a PAMP of Gram-negative bacteria. Previously, we isolated and characterized the LPS-binding protein, BmLBP, from the larval hemolymph of the silkworm *Bombyx mori* [7]. BmLBP is a pattern recognition molecule that recognizes the lipid A portion of LPS and participates in a cellular defense reaction, hemocyte nodule formation ([7], Koizumi et al., submitted).

This paper describes the cDNA cloning of BmLBP. The deduced amino acid sequence revealed that BmLBP is a novel member of the C-type lectin superfamily, with a unique structural feature that consists of two different carbohydrate-recognition domains (CRDs) in tandem, a short and a long form [8]. Furthermore, a database search suggests that proteins with this structural feature may participate in pattern recognition in lepidopteran insects generally.

2. Materials and methods

2.1. Animals and collection of hemocytes

Silkworms, *B. mori* (Kinshu×Showa), were reared on an artificial diet (Nihonnosanko) at 25°C. Hemolymph from a fifth-instar 3- or 4-day larva was collected directly into a petri dish containing ice-cold insect physiological saline (IPS) (150 mM NaCl, 5 mM KCl), mixed with 10 mM benzamidine (final conc.), by puncturing the prolegs, which were directly immersed in the solution. Hemocytes were collected by centrifuging the hemolymph solution at 200×*g* for 10 min at 4°C.

2.2. Construction and cloning of cDNA library

Total RNA was extracted from the hemocytes by AGPC method [9], and poly(A)$^+$ RNA was separated using mRNA Purification Kit (Pharmacia). An oligo(dT)-primed cDNA library was constructed in a λMOS*Elox* vector using a complete rapid cloning system (Amersham) according to the supplier's instruction. About 3×10^5 clones of the cDNA library were screened with anti-BmLBP serum and peroxidase-conjugated secondary antibody. A positive clone (BmL2) was selected, plaque-purified, and a plasmid (pMOS*Elox*) containing insert cDNA was autoexcised according to the supplier's instruction. Sequencing was performed by the dideoxynucleotide chain termination method using the AutoRead Sequencing Kit (Pharmacia). BmL2 was subjected to further characterization.

2.3. 5′-Rapid amplification of cDNA ends (5′-RACE)

The 5′-terminal cDNA ends was amplified using 5′-Full RACE Core Set (Takara) according to the supplier's instruction. The antisense strand synthesis was directed with 1 μg of total RNA and primed with 5′-(P)TCGCATGGATGCCGG-3′. PCR was conducted using 5′-CCTCTGGACGATGCGTTGAA-3′ (first forward primer), 5′-TCCCGCATGTACGTGTAGTC-3′ (first reverse primer), 5′-GAGCGGCATGCTATCTCTAA-3′ (second forward primer) and 5′-GCTGACCATCCAGATAAGCG-3′ (second reverse primer). The PCR product was cloned into a p123T vector (Mo Bi Tec) for sequencing.

2.4. Deglycosylation of BmLBP

Purified BmLBP was denatured by heating it at 100°C for 3 min in

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Abbreviations: LPS, lipopolysaccharide; CRD, carbohydrate-recognition domain; RACE, rapid amplification of cDNA ends; LBP, LPS-binding protein

The sequence reported in this paper has been deposited in the EMBL database (accession number AJ011573).

PII: S0014-5793(98)01701-3

20 mM phosphate buffer, pH 7.2, containing 1% SDS and 2% (v/v) 2-mercaptoethanol. The denatured protein (0.5 μg) was incubated with 1 unit of *N*-glycosidase F (Boehringer Mannheim) in 50 μl of 50 mM phosphate buffer, pH 7.2, containing 0.1% SDS, 0.5% (v/v) Nonidet-P 40 and 0.5% (v/v) 2-mercaptoethanol for 24 h at 37°C. The resulting digest was analyzed by SDS-PAGE under denaturing condition [10].

2.5. Southern blot analysis of the BmLBP gene

Genomic DNA was extracted from the posterior silk glands of fifth-instar 5-day larvae [11]. 20 μg of genomic DNA was digested with restriction enzymes, electrophoresed on a 0.9% agarose gel and transferred to a nylon membrane (Hybond-N^+, Amersham). An insert cDNA of the BmL2 clone was fluorescein-labeled using ECL random prime labelling and detection systems (Amersham) and used as a probe. The membrane was hybridized and detected according to the supplier's instruction.

2.6. Gene expression in hemocytes

RT-PCR was carried out to examine whether the expression of the BmLBP gene in hemocytes was up-regulated by bacterial challenge.

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AGTTGTTTTACAGAAGCGTAAACATGAAAGCGGCACTGGCCAGCCTCGTTTTTGTCCTGA  60
                      M  K  A  A  L  A  S  L  V  F  V  L  T  13

CCATCGCTTATCTGGATGGTCAGCAGTTTCGTTATGACTACACGTACATGCGGGACATAA 120
  I  A  Y  L  D  G  Q  Q  F  R  Y  D  Y  T  Y  M  R  D  I  N  33

ATGGGTGGTTGAAGCTTCAAGAGATCCCAGCTACCTGGCAGGAGGCTCGATTGAGATGTC 180
  G  W  L  K  L  Q  E  I  P  A  T  W  Q  E  A  R  L  R  C  H  53

ATCTTGAAGGGTCCCTTTTGGCATCTCCTCTGGACGATGCGTTGAAGAGCGGCATGCTAT 240
  L  E  G  S  L  L  A  S  P  L  D  D  A  L  K  S  G  M  L  S  73

CTCTAATAAAGAACAAGAAGACTTCATGTGGTATATTCACCGGCATCCATGCGACATTTT 300
  L  I  K  N  K  K  T  S  C  G  I  F  T  G  I  H  A  T  F  S  93

CTAAAGGAGACTACCGTTCTGTTGAAGGGGTCCCATTGGCGAAAATTCCTCACGATTGGG 360
  K  G  D  Y  R  S  V  E  G  V  P  L  A  K  I  P  H  D  W  A  113

CCGATTATGAACCAGATAATGCTGGAGGTGATGAAAACTGTATCCTCATGAACCCTGATG 420
  D  Y  E  P  D  N  A  G  G  D  E  N  C  I  L  M  N  P  D  G  133

GAAACTTCGCCGATGTTAATTGCACTGAAACGTTCCAATACGTTTGCTATAAGAAAAAGA 480
  N  F  A  D  V  N  C  T  E  T  F  Q  Y  V  C  Y  K  K  K  T  153

CTGCAACTCTTGCAATGGCTTCTTGTGGCAGTGTCGATAGCGAATATGTTCTCAGCAAAG 540
  A  T  L  A  M  A  S  C  G  S  V  D  S  E  Y  V  L  S  K  D  173

ACACTGGTAATTGTTACAAGTTCCACAAAGTTCCCCGTACCTGGTCGCGGGCCTACATGG 600
  T  G  N  C  Y  K  F  H  K  V  P  R  T  W  S  R  A  Y  M  A  193

CTTGTTCAGCTGAAGGCGGATACCTCACCATTATTAACAACGAGAAGGAGGCCACGTTCC 660
  C  S  A  E  G  G  Y  L  T  I  I  N  N  E  K  E  A  T  F  L  213

TAAGGGACCTTTTCGCGAAGAACCCTGCCGGTCAAATGATCGGAAGTTTCTGGAAAGACG 720
  R  D  L  F  A  K  N  P  A  G  Q  M  I  G  S  F  W  K  D  V  233

TTGCCTTCATTGGCTTCCACGACTGGAATGAGCGCGGGGAATGGTTAACGATCAATGGTG 780
  A  F  I  G  F  H  D  W  N  E  R  G  E  W  L  T  I  N  G  E  253

AGAAATTACAAGAGGCCGGTTACGAGAAATGGTCTGGCGGCGAGCCTAGTAACGCGACGA 840
  K  L  Q  E  A  G  Y  E  K  W  S  G  G  E  P  S  N  A  T  T  273

CTGGTGAATATTGCGGCTCTATCTACCGCTCGGCTCTACTCAACGACCTTTGGTGCGAAA 900
  G  E  Y  C  G  S  I  Y  R  S  A  L  L  N  D  L  W  C  E  K  293

AGCCGGCACCGTTTATCTGCGAGAAGGAACCTCGCAGCTTACTCCGAGAGCACGACGACA 960
  P  A  P  F  I  C  E  K  E  P  R  S  L  L  R  E  H  D  D  K  313

AATGAATGATACTAATTCCGAGTTAGCATTTCGAGTTAGTTGCAAAGATCAAAATAACGA 1020
  *
AATAATGATATTTATTGACGACAAATTAAATGTATTTAATATCAAAAAAAAAAAAAAAA  1079
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Fig. 1. Nucleotide sequence of cDNA for BmLBP. Nucleotide numbers are shown on the right of the nucleotide sequence, and the deduced amino acid sequence (one-letter symbols) is shown below the nucleotide sequence. The numbers of the amino acid residues, starting from the first Met, are given to the right of each line. The partial amino acid sequences determined are underlined, possible *N*-glycosylation sites are boxed, the putative amino-terminal residue is indicated by an arrow, and the asterisk and double underlining denote the termination codon and poly(A) addition signal, respectively. Putative RNA instability signals are indicated by boldface type.

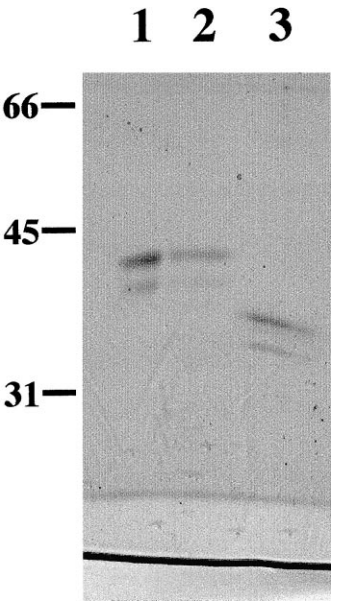

Fig. 2. Deglycosylation of BmLBP by the treatment with *N*-glycosidase F. Purified BmLBP (0.5 μg) was incubated with *N*-glycosidase F, and the resulting product (0.2 μg) was subjected to SDS-PAGE. Lane 1, purified BmLBP (1 μg); lane 2, without *N*-glycosidase F; lane 3; with *N*-glycosidase F. Protein molecular mass standards are shown on the left in kDa.

Fifth-instar 5-day larvae were immunized with *Escherichia coli* W3110. Hemocytes were collected 12 h after immunization, and total RNA was prepared as described above. As a control, total RNA from hemocytes were extracted from non-immunized larvae. Reverse transcription was performed with 1 μg of total RNA, MMLV-RTase (10 units), 0.2 μmol of oligo(dT) as a primer. Then, PCR was performed (25, 30, 35 cycles; 94°C (1 min), 56°C (1 min), 72°C (1 min)) with the following primers: 5′-CAGCAGTTTCGTTATGACTACACGTAC-3′ (forward) and 5′-TTTGTCGTCGTGCTCTCGGA-3′ (reverse). As an internal marker, primers for the *B. mori* actin gene [12] and cecropin A gene [13] were used. The PCR products were analyzed by agarose gel electrophoresis.

3. Results

3.1. cDNA cloning and nucleotide sequencing

We first determined which tissues synthesize BmLBP. Almost all known insect defense molecules are produced by the fat body or hemocytes. Since BmLBP is a defense molecule, we expected it to be synthesized by the fat body or hemocytes, so Western blotting against these tissue proteins was performed. It turned out that BmLBP was produced by hemocytes (data not shown). A cDNA library from the mRNA of hemocytes was constructed, and screened with anti-BmLBP serum. A single clone (BmL2) remained positive after a second screening. Sequence analysis of BmL2 revealed that the protein encoded in this cDNA contained five peptide fragments, obtained by digesting purified BmLBP with lysyl endopeptidase: (1) LQEIPANWQEARLRXHLEGSVLASP, (2) GDYRSVEGVPLANIPXDXADY, (3) EAAFLRDLFAK, (4) NPAGQMIGXFWK, (5) DVAFIG. The unidentified amino acid residues in peptide fragments 1, 2, and 4 were assigned as Cys, His, Trp, and Ser, respectively. Therefore, we concluded that this is cDNA for BmLBP. However, there are some differences between the amino acid residues predicted by the cDNA and the amino acid residues in the purified BmLBP (Asn-Thr in peptide fragment 1, Val-Leu in peptide

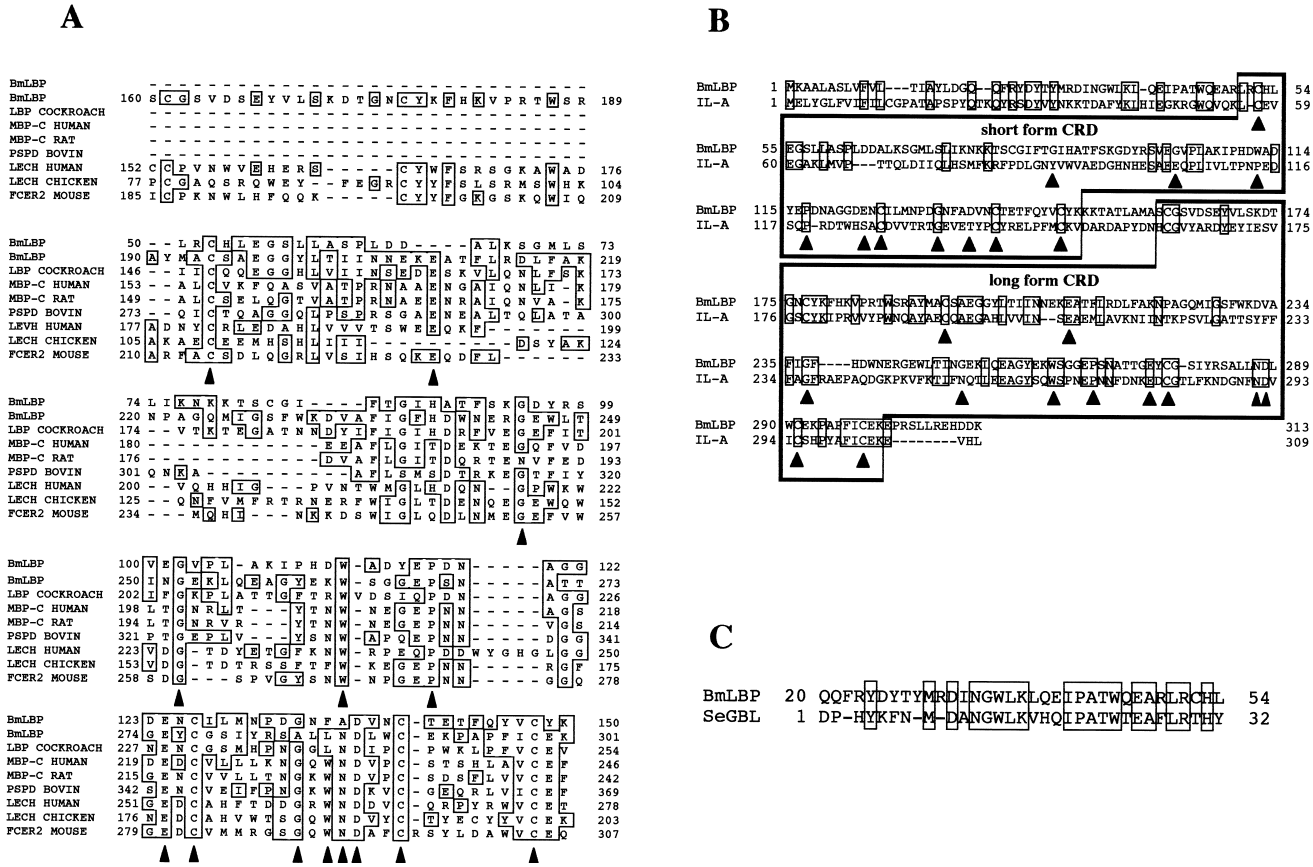

Fig. 3. Comparison of BmLBP and other proteins. A: Alignment of CRD in BmLBP and seven C-type lectins; LBP, LBP of *P. americana* [4]; MBP-C, mannose-binding protein C of human and rat [15,16]; PSPD, pulmonary surfactant-associated protein D of bovine [17]; LECH, hepatic lectin of human and chicken [18,19]; FCER2, low affinity IgE Fc receptor of mouse [20]. Sequence identities between BmLBP and the other lectins are indicated by boxes. The numbering is based on the sequence of each protein. Triangles indicate residues conserved in C-type lectins. B: Comparison of amino acid sequence between BmLBP and immunolectin-A (IL-A); Identical amino acids are indicated by boxes and triangles indicate residues conserved in C-type lectins. The numbering is based on the sequence of each protein. C: Comparison of amino acid sequence between BmLBP and galactose-binding lectin of S. exigua (SeGBL); Identical amino acids are indicated by boxes and the numbering is based on the sequence of each protein.

fragment 1, Asn-Lys in peptide fragment 2 and Ala-Thr in peptide fragment 3). These differences may come from another allele because the larvae used in this study are the F_1 of hybrid strains. Sequence analysis of BmL2 also revealed that it did not extend to the 5′-end of the mRNA. Next, we performed 5′-RACE, and the cDNAs obtained from 5′-RACE were thought to contain the complete protein-encoding region as expected, since every cDNA extended and stopped at the same site. Thus, we concluded that we had determined the complete nucleotide sequence (1079 bp) of the cDNA for BmLBP.

The nucleotide and deduced amino acid sequences encoded in this cDNA are shown in Fig. 1. We could not determine the amino-terminal residue of mature BmLBP because it seemed to be blocked in some way. It was estimated to be Gln at position 20 by the method of Von Heijne [14]. Therefore, this cDNA encodes BmLBP consisting of 294 residues (ca. 33 224 Da) and its leader peptide consisting of 19 residues. BmLBP consists of two highly similar protein components with molecular masses of 43 and 40 kDa [7]. Two possible *N*-glycosylation sites were found in this cDNA. The purified BmLBP was deglycosylated by treating it with *N*-glycosidase F and the digests were subjected to SDS-PAGE to determine molecular masses. The two bands of BmLBP shifted to 37 and 34 kDa, respectively (Fig. 2). The latter coincides with the deduced molecular weight of the protein encoded by this cDNA, revealing that this cDNA encodes 40 kDa BmLBP.

3.2. Sequence comparison with other proteins

A search for homology between BmLBP and proteins listed in the SwissProt and PIR databases revealed that BmLBP contains two CRDs of animal C-type lectin (Fig. 3A) [8]. Therefore, BmLBP is a novel member of the C-type lectin superfamily with the unique structural feature of two different CRDs in tandem, a short and a long form (Fig. 3A,B). The two CRDs are approximately 20% homologous. A further database search showed that this unique structural feature is also found in immunolectin-A from the tobacco hornworm *Manduca sexta* (accession number AF053131) (Fig. 3B). Moreover, there was homology between the N-terminal sequence of BmLBP and galactose-binding lectin from the beet armyworm *Spodoptera exigua* [21]. Although BmLBP is a novel member of the C-type lectin superfamily, none of the monosaccharides tested so far is a hapten that specifically inhibits BmLBP binding to LPS (data not shown). It is also unclear whether BmLBP is dependent on Ca^{2+} for LPS binding. Thus, it remains unclear whether BmLBP is a C-type lectin per se.

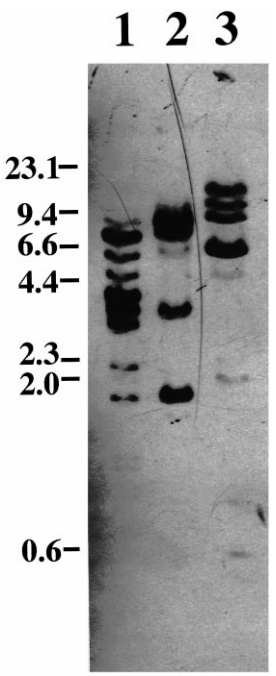

Fig. 4. Southern blot analysis of the BmLBP gene. 20 μg of genomic DNA was digested by *Eco*RI, *Eco*RV, and *Hin*dIII. Digested samples were electrophoresed on a 0.9% agarose gel, transferred and hybridized with BmLBP cDNA as a probe. Lane 1, *Eco*RI; lane 2, *Eco*RV; lane 3; *Hin*dIII. Positions of size markers are presented on the left in kb.

3.3. Southern blot analysis of the BmLBP gene

Genomic DNA from the silk glands was digested with *Eco*RI, *Eco*RV, and *Hin*dIII, and analyzed by Southern blot hybridization using the insert cDNA of the BmL2 clone as a probe. As shown in Fig. 4, several positive bands were detected in each digest. This suggests the existence of many genes similar to BmLBP in the *B. mori* genome.

3.4. Gene expression in hemocytes

RT-PCR was carried out to examine whether the expression of the BmLBP gene in hemocytes was up-regulated by bacterial challenge. The BmLBP gene was expressed constitutively in hemocytes and was not up-regulated by bacterial challenge (Fig. 5).

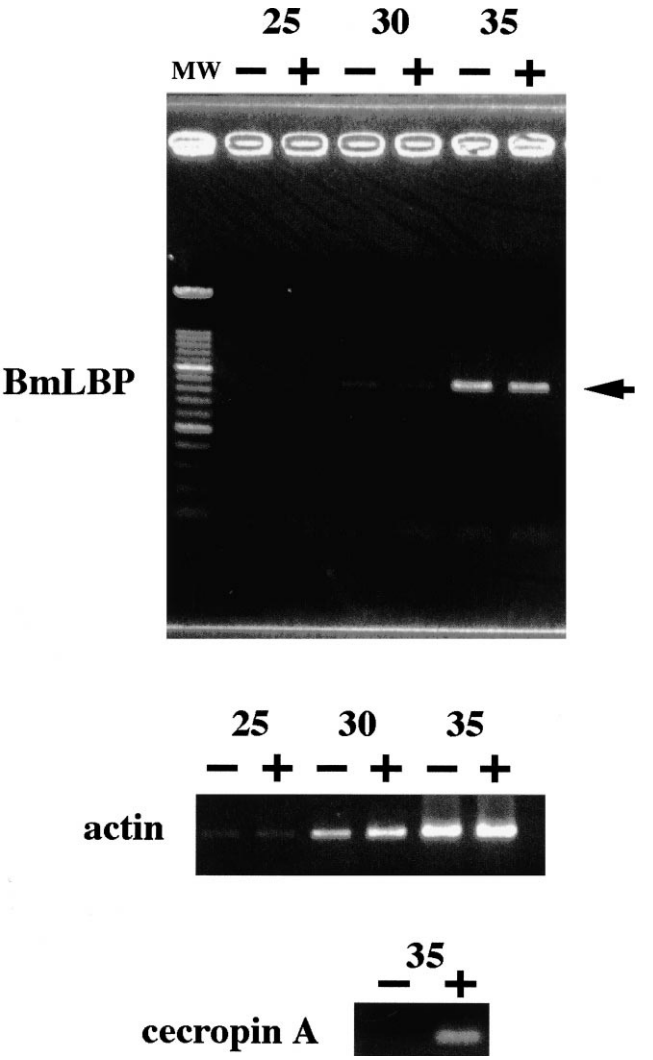

Fig. 5. BmLBP gene expression in hemocyte. mRNA samples were extracted from hemocytes of immunized and non-immunized larvae. RT-PCR products were analyzed by agarose gel electrophoresis. Numbers indicate the PCR cycle, and + and − indicate results with and without bacterial injection. As internal markers, actin and cecropin A primers were used. MW, 100 bp ladder molecular weight marker.

4. Discussion

In this study, we cloned the cDNA of an LPS-binding protein of *B. mori*, BmLBP. The cDNA clone is believed to encode the 40 kDa BmLBP (Figs. 1 and 2). BmLBP consists of two proteins with molecular masses of 43 kDa and 40 kDa and peptide mapping using two enzymes and partial amino acid sequences indicates that these are very similar proteins [7]. Moreover, the internal amino acid sequences described in Section 3.1 were obtained from both of the proteins. Fig. 5 shows that only a single DNA fragment was amplified by RT-PCR using primers that were designed to amplify the fragment containing all of the amino acid fragments described in Section 3.1. This suggests that the 43 kDa BmLBP protein is constructed by adding amino acid residues to the N- or C-terminus of the 40 kDa protein or carbohydrate chains that are not cut by *N*-glycosidase F to the 40 kDa protein.

The deduced amino acid sequence of BmLBP revealed that it is a novel member of the C-type lectin superfamily [8] and has a unique structural feature consisting of two different CRDs in tandem, a short and a long form (Figs. 1 and 3). The two CRDs are approximately 20% homologous. As far as we know, this is the first report of a LPS-binding protein with this structural feature. The function of each CRD of BmLBP is not clear. We speculate that the overall three-dimensional structure of BmLBP consisting of two CRDs might be required to recognize the lipid A portion of LPS, or that each CRD might have a different binding specificity, where each CRD recognizes a different part of LPS or LPS and another carbohydrate on *E. coli* or different microorganisms. Furthermore, one CRD might recognize LPS and the other a ligand on the hemocyte membrane, because BmLBP interacts with hemocytes. To test these hypotheses, we prepared each recombinant CRD, and the binding specificity of each CRD is now being investigated.

In insects, it is reported that the American cockroach, *Periplaneta americana*, has two LPS-binding C-type lectins, LBP and *Periplaneta* lectin [4–6,22]. These C-type lectins function as an opsonin in pattern recognition. However, these structures and the binding sites on LPS differ from those of BmLBP. In this study, overall structural similarity between BmLBP and immunolectin-A from *M. sexta* was demonstrated (Fig. 3B). BmLBP and galactose-binding lectin from *S. exigua* have similar N-terminal sequences (Fig. 3C) and molecular weights [21]. This suggests that there are two CRDs in the galactose-binding lectin. Furthermore, it was reported that galactose-binding lectin binds to an entomopathogenic hyphomycete *Paecilomyces farinosus* and functions as an opsonin [23]. BmLBP binds to the LPS of Gram-negative bacteria and participates in hemocyte nodule formation ([7], Koizumi et al., submitted), indicating a functional similarity between BmLBP and galactose-binding lectin. This suggests that proteins with two different tandem CRDs exist in insects generally, or at least in lepidopteran species, and function as a pattern recognition molecule in their innate immunity.

The existence of C-type lectin family proteins in *P. americana* was demonstrated [24]. The result of Southern blot analysis of the BmLBP gene (Fig. 4) suggests that there are many copies of the BmLBP gene in the *B. mori* genome or that there are C-type lectin family genes in *B. mori* as well as *P. americana*. We have already isolated two other C-type lectins with molecular masses similar to that of BmLBP (unpublished results). In short, we suggest that a repertoire of C-type lectins with BmLBP-like structure and various carbohydrate binding specificities plays a central role in innate immune recognition in *B. mori*.

5. Note added in proof

The citation which appears as (Koizumi et al., submitted) has now been accepted for publication [25].

Acknowledgements: We are grateful to Dr. M. Ashida for amino acid sequencing. We thank A. Saito for her technical assistance. This work was supported by a Grant-in-Aid for Scientific Research (10680601) from the Ministry of Education, Science, Sports and Culture of Japan.

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