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Characterization of a Novel C-Type Lectin, *Bombyx mori* Multibinding Protein, from the *B. mori* Hemolymph: Mechanism of Wide-Range Microorganism Recognition and Role in Immunity

Ayako Watanabe, Sousui Miyazawa, Madoka Kitami, Hiroko Tabunoki, Kenjiro Ueda, and Ryoichi Sato

To investigate the system used by insects to recognize invading microorganisms, we examined proteins from the larval hemolymph of *Bombyx mori* that bind to the cell surface of microorganisms. Two hemolymph proteins that bound to the cell surfaces of *Micrococcus luteus* and *Saccharomyces cerevisiae* were shown to be identical. This protein bound to all 11 microorganisms examined—5 Gram-negative bacteria, 3 Gram-positive bacteria, and 3 yeasts—and was consequently designated *B. mori* multibinding protein (BmMBP). The sequence of the cDNA encoding BmMBP revealed that it was a C-type lectin with two dissimilar carbohydrate-recognition domains (CRD1 and CRD2) distantly related to known insect C-type lectins. CRD1 and CRD2 were prepared as recombinant proteins and their binding properties were investigated using inhibition assays. Each domain had wide, dissimilar binding spectra to sugars. These properties enable BmMBP to bind to two sites on a microorganism, facilitating high-affinity binding to many types of microorganisms. The dissociation constants of BmMBP with microorganisms recognized by BmMBP were shown to be identical. This protein bound to all 11 microorganisms examined, whereas recombinant proteins containing the N- and C-terminal halves of BmMBP bound to Gram-negative bacteria via LPS, a Gram-negative PAMP. Immulectin-3 specifically binds to LPS, lipoteichoic acid, and β-1,3-glucan (13). Immulectin-4 binds to N-galactosamine (GalNAc) and glucose (14). Immulectin-1 and -2 participate in the activation of prophenoloxidase (7, 15, 16). Immulectin-2 also participates in hemocyte-mediated encapsulation and bacterial clearance from the hemocoel (16, 17). Immulectin-3 mediates an encapsulation-like hemocyte reaction in vitro (13). BmLBP triggers hemocyte-mediated nodule formation and plays a role in the elimination of invading Gram-negative bacteria from the hemocoel (10, 18). These reports suggest that C-type lectins are important factors in insect defense against invading microorganisms, especially Gram-negative bacteria; however, little is known about their role in defense against Gram-positive bacteria and fungi.

The C-type lectins from lepidopteran insects have two different carbohydrate recognition domains (CRDs) arranged in tandem. Using recombinant proteins, it has been shown that the CRD in the *Caenorhabditis elegans* homologous lectin had differing sugar specificities (9). However, the functional roles of the two CRDs in C-type lectins from lepidopteran insects remain unknown.

Two C-type lectins from *B. mori* have been reported. BmLBP recognizes Gram-negative bacteria, as stated above; however, the recognition target of *B. mori* immulectin (BmIML) is unknown. It was also unknown whether insects had C-type lectins that recognize Gram-positive bacteria and fungi.

We report the characteristics of a novel C-type lectin (*B. mori* multibinding protein [BmMBP]) that can bind to Gram-positive bacteria, Gram-negative bacteria, and yeasts. In addition, we report
the mechanism underlying the recognition by BmMBP of a wide range of microorganisms, as well as the role of BmMBP in the early stages of immune defense in *B. mori* larval hemolymph. We also show that the insect has a unique C-type lectin, which has two CRDs with broad, dissimilar spectra for binding target sugars. It can thus recognize a wide range of microorganisms, which can affect the immune response of the insect.

**Materials and Methods**

**Chemicals**

Mannose, glucose, N-acetylglucosamine (GlcNAc), galactose, succrose, maltose, fucose, GalNAc, benzamidine, and V8 protease were purchased from Wako Pure Chemical. Mannan, peptidoglycan, teichoic acid, laminarin from *Laminaria digitata*, and BSA were obtained from Sigma-Aldrich. LPS (Escherichia coli O26:B6) was acquired from Difco, N-acetylmuramic acid (MurNAc) was purchased from Hayashibara Biochemical Laboratories.

**Animals**

Silkworms, *B. mori* (Kinsyu × Showa), were reared on an artificial diet (Nihonnosanko) at 25°C.

**Preparation of bacterial and yeast cells**

The bacteria listed in Table I were used in this study. The bacteria were cultured in Luria-Bertani medium (10 g of peptone, 5 g of yeast extract, 5 g of NaCl, and 1 g of glucose per liter of distilled water). Cells in the logarithmic growth phase were harvested by centrifugation at 1800 × g for 20 min at 4°C, washed twice with insect physiological saline (IPS, 150 mM NaCl, 5 mM KCl, and 1 mM CaCl2), and fixed with 4% formaldehyde by gentle shaking for 1 h. The fixed cells were harvested by centrifugation at 1800 × g for 20 min at 4°C and washed five times with Clark’s saline (110 mM NaCl, 188 mM KCl, 1 mM CaCl2, 1 mM NaHCO3, 0.07 mM Na2HPO4). Yeasts were cultured in YM broth (10 g of glucose, 5 g of peptone, 3 g of yeast extracts, 3 g of malt extract per liter of distilled water) and fixed as above.

**Purification of Micrococcus luteus- and Saccharomyces cerevisiae-binding proteins**

Fifth-instar day 4 larvae were anesthetized on ice, swabbed with 70% ethanol, and bled by proleg puncture using a sterile needle. A total of 40 ml of hemolymph was collected directly into a 50-ml tube containing 5 ml of an insoluble polysaccharide (IPS) mixed with benzamidine (10 mM final conc.) and centrifuged at 18,000 × g for 10 min at 4°C. Then, the supernatant was stored at −80°C. This plasma was mixed with 40 μl of the precipitate of fixed *M. luteus* or *S. cerevisiae* cells and incubated at 4°C for 1.5 h using a rotator. The cells were centrifuged, washed twice with IPS, and incubated with 1 M GlcNAc at 4°C. After 2 h, the cells were centrifuged and the supernatant was used as *M. luteus*-binding protein or *S. cerevisiae*-binding protein.

**SDS-PAGE, antiserum, immunoblotting, and peptide mapping**

SDS-PAGE was performed using the method of Laemmli (19) and the results were visualized after staining with Coomassie brilliant blue (CBB).

**List of microorganisms used in this study**

Table I.

<table>
<thead>
<tr>
<th>Bacterium/Cell Type</th>
<th>Strain</th>
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<tbody>
<tr>
<td><strong>Gram-negative R</strong></td>
<td></td>
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<tr>
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<td>W3110</td>
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<tr>
<td><em>S. marcescens</em></td>
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<tr>
<td><strong>Smooth strains</strong></td>
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</tr>
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</tr>
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<td><em>A. hydrophila</em></td>
<td>IAM12460</td>
</tr>
<tr>
<td><em>P. vulgaris</em></td>
<td>IAM12542</td>
</tr>
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<td><strong>Gram-positive bacteria</strong></td>
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<td>IAM12243</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>IAM14322</td>
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</tbody>
</table>

**Antiserum against *M. luteus*-binding protein or *S. cerevisiae*-binding protein**

The cleavage products, obtained using the Cleveland method with V8 protease, were separated using Tricine SDS-PAGE and transferred onto a polyvinylidene difluoride membrane. The protein bands were visualized by staining with CBB and washed three times with methanol. These bands were cut out, and their N-terminal sequences were determined by automated Edman degradation using a gas-phase sequencer (model 491C LC; Applied Biosystems).

**Computer-assisted homology search and alignment**

We searched the *B. mori* expressed sequence tag (EST) database (http://papilio.ab.u-tokyo.ac.jp/silkbase/index.html) for the partial amino acid sequence MEGATFFY, described in *Internal amino acid sequencing*, and found one partial sequence, clone e040121A. A computer-assisted homology search was performed using Internet basic local alignment search tool searches of the *B. mori* EST database, at the Bioinformatics Center of Kyoto University (www.genome.ad.jp/Japanese/). The sequences were aligned using ClustalX (21) and DRAWTREE of PHYLIP 3.6 was used to generate an unrooted bootstrap tree.

**Cloning cDNAs and nucleotide sequencing**

Total RNA was extracted from the fifth-instar day 4 larval hemocytes using a QuickPrep Total RNA Extraction kit (Amersham Biosciences). The first strand cDNA was synthesized with 12–18 oligo dT primers associated with M13M4 nucleotide sequences (M13M4 oligo d(12–18mix)T primer: 5′-GTTTTCCCAGTCACGACdT(12–18)-3′; Takara) or an antisense sequence MEGATFFY, described in *Internal amino acid sequencing*, and sequenced using a Long Read Tower DNA sequencer (Amersham Biosciences). Double-stranded cDNA fragments were amplified by PCR using the following primer sets: 1) sense-1 (5′-GCAATHTGTYGAYCCTNYT-3′) or sense-2 (5′-AAYCAR ATHCARTTYCC-3′) and M13M4 (5′-GGTTCAGCTCAGCATGAC-3′) for 3′-RACE; 2) M13M4-oligo d(12–18 mix)T and anti-2 (5′-GTGG CAGGACGATGT-3′) for 5′-RACE; and 3) M13M4 and anti-3 (5′-ATTGCACGTCAGGGATG-3′) for 5′-RACE. The PCR cycling conditions consisted of an initial denaturation at 94°C for 1 min and 40 cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min. The amplified fragment was cloned into T-overnag vector pC123 (MboI/BstEII). The cDNA was sequenced using a Long Read Tower DNA sequencer (Amersham Biosciences).

**Expression of recombinant M. luteus-binding protein and individual CRDs in *E. coli***

The PCR was used to generate a cDNA fragment encoding the CRD1 + 2 region (amino acid residues 23–218) using primer-1 (5′-TTCTATGATCTC GCGGGAATAAGTTCCTTCC-3′) and primer-4 (5′-ACACAAACAT GCTTTGTCATGTGAGGCAC-3′); a cDNA fragment encoding the CRD1 region (residues 23–158) was amplified using primer-1 (5′-TTTCTTGCATATAAATGGGAAAGATTTCGC-3′) and M13M4-oligo d(12–18 mix)T and anti-2 (5′-GTGG CAGGACGATGT-3′) for 5′-RACE; and 3) M13M4 and anti-3 (5′-ATTGCACGTCAGGGATG-3′) for 5′-RACE. The PCR cycling conditions for 5′-RACE consisted of an initial denaturation at 94°C for 1 min and 40 cycles at 94°C for 30 s, 45°C for 30 s, and 72°C for 1 min. The amplified fragment was cloned into T-overnag vector pC123 (MboI/BstEII). The cDNA was sequenced using a Long Read Tower DNA sequencer (Amersham Biosciences).
medium containing 100 μg/ml ampicillin and the cells were grown to mid-log phase at 37°C. Next, 1 mM isopropyl-β-D-thiogalactoside (IPTG) was added to the culture medium to induce the expression of GST fusion proteins. Approximately 4 h after adding the IPTG, three GST fusion proteins were observed microscopically as inclusions. Because the recombinant proteins were insoluble, they were purified using Glutathione Sepharose 4B (Amersham Biosciences) under denaturing conditions in PBS following the manufacturer’s instructions. The purified proteins at 50 μg/ml in 8 μl urca were renatured in two dialysis steps: the first against a buffer containing 20 mM Tris-HCl (pH 7.5), 100 mM NaCl, 2 mM reduced glutathione, 0.2 mM oxidized glutathione, glyceral, and 0.005% Tween 20, and then against the same buffer minus glutathione and Tween 20. Each dialysis step was performed for at least 12 h at 4°C.

**Analysis of the binding spectrum of M. luteus-binding protein**

Formaldehyde-fixed or living 1 × 10^6 bacteria or yeast cells (Table I), silica gel (HisLink Protein Purification Resin; Promega) and Sepharose (His Trap HP; Amersham Biosciences) were mixed and incubated with 1 ml of plasma or a solution of the recombinant proteins (CRD1, CRD2, and CRD1 + 2) described above. The cells were washed and eluted with 30 μl of SDS-PAGE sample buffer (250 mM Tris-Cl (pH 6.8), 5% SDS, 0.25% bromphenol blue, 25% glyceral) and 24 μl of M. luteus-binding protein and 24 μl of the individual CRDs were detected by immunoblotting.

**Analysis of M. luteus-binding protein expression**

To analyze the stage when M. luteus-binding protein was expressed, eggs or first-instar larvae were homogenized using a glass-Teflon homogenizer. The homogenate from day 4 larvae at the second, third, fourth, and fifth instars, prepupa, pupae, and adults was collected directly into a tube by proleg puncture using a sterile needle. To analyze the tissue specificity, the hemocytes, fat body, midgut, silk gland, Malpighian tubule, integument, testis, and ovary were dissected and homogenized in IPS using a glass-Teflon homogenizer. The homogenates were centrifuged at 16,000 × g for 30 min at 4°C and supernatants were obtained. To analyze the inducibility of M. luteus-binding protein, fifth-instar day 4 larvae were surface-sterilized by swabbing with 70% ethanol; 5 μl of IPS or IPS containing 1 × 10^6 M. luteus were injected into the hemocoel and the larvae were kept at 25°C. Hemolymph was collected 0, 0.5, 2, 24, and 72 h postinjection and centrifuged to obtain the plasma fraction. Each sample (25 μg protein) was subjected to SDS-PAGE and the M. luteus-binding protein was detected by immunoblotting.

**Immobilization of bacterial cells on the sensing surface of a biosensor**

Single-well cuvettes with carboxylate coatings were used with the N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide coupling system (Affinity Sensors). The well of each cuvette was coated with an N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide ester chemistry, as described below. The immobilization buffer was 20 mM sodium acetate buffer (pH 6.8). For Ag M. luteus-binding protein coupling, the cuvette was washed with PBS containing 0.05% Tween 20 (pH 7.4) for 10 min before activating the surface using N-hydroxysuccinimide and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide coupling system (Affinity Sensors). The well of each cuvette was coated with activated bacterial cells (M. luteus and S. cerevisiae), which were immobilized on the carboxylate surface via its amino groups using succinimide ester chemistry, as described below. The immobilization buffer was 20 mM sodium acetate buffer (pH 6.8).

**Determination of the dissociation constant of M. luteus-binding protein and M. luteus or S. cerevisiae cells**

After covalent immobilization of the bacterial cells on the carboxylate sensor surface, the compatible binding of M. luteus-binding protein with bacterial cells was determined using an IAsys resonant mirror optical biosensor (Affinity Sensors). To measure the binding of M. luteus-binding protein with individual bacterial cells, each bacterial cell was immobilized on the surface at a concentration of 20–200 μM and the association was observed for 30 min. At the end of this cycle, the PBS in the cuvette was replaced with 20 mM HCl to regenerate the sensor surface. The dissociation constant (Kd) was calculated using a Scatchard plot analysis of the equilibrium values at five concentrations.

**Binding assay for the analysis of the sugar specificity of individual CRDs**

Formalin-killed M. luteus (1 × 10^8 cells/ml in IPS) were used to coat a 96-well plate overnight. The excess binding sites were blocked with 200 μl/well of 2% BSA in TBS (20 mM Tris, 0.15 M NaCl, pH 7.5) at 37°C for 1 h. Then, the plates were blocked with the BSA solution. Recombinant M. luteus-binding proteins (CRD1, CRD2, and CRD1 + 2) were added in a total amount of 0.6 μg/200 μl. Binding of CRD1 + 2, CRD1, or CRD2 was allowed to occur at 37°C for 1 h with several concentrations of sugars. The plate was then rinsed three times with 200 μl/well of TBS (20 mM Tris, 0.15 M NaCl, 0.05% Tween 20 (pH 7.5)) with agitation on a microinertor. Mouse anti-M. luteus-binding protein protein serum diluted 10,000-fold with TBS containing 20 mg/ml BSA was then added at 100 μl/well and incubated at 37°C for 1 h. The antiserum was then removed and replaced with 3,000-fold TBS containing 20 μg/ml BSA, and the plates were incubated at 37°C for 1 h. The rabbit anti-mouse IgG solution was then rinsed out as above. Finally, 100 μl/well of HRP-conjugated rabbit anti-mouse IgG diluted 3,000-fold with TBS containing 20 μg/ml BSA, and the plates were incubated at 37°C for 1 h. The rabbit anti-mouse IgG solution was then rinsed out as above. Finally, 100 μl/well of ABTS solution (0.04% ABTS, 0.01% H2O2, in 100 mM citrate-HNa2PO4 buffer, pH 4.0) was added and allowed to settle until sufficient color had developed. The absorbance of each well at 415 nm was quantified using a microtiter plate reader (Bio-Rad).

**Observation of nodules and fluorescence-labeled E. coli cells**

Fifth-instar day 4 larvae were surface-sterilized by swabbing with 70% ethanol and 5 μl of IPS containing 1 × 10^6 bacteria or yeast cells/ml (Table I) was injected into the hemocoel; the larvae were kept at 25°C. The larvae were anesthetized by chilling on ice, and then the hemocytes were collected directly into a tube by scratching. To label E. coli W3110 cells with a fluorescent tag, Oregon green 488 (Molecular Probes) was dissolved in DMSO at 5 mg/ml, and 100 μl of this solution was added slowly to 1 ml of E. coli solution (1 × 10^10 cells in 50 mM sodium bicarbonate buffer (pH 9.0)) with continuous stirring. The solution was incubated with gentle shaking for 1 h at 4°C. The labeled cells were harvested as above. For fluorescence microscopy observation of nodules, fifth-instar day 4 larvae were injected with 1 × 10^6 fluorescence-labeled E. coli cells and the hemocytes were exposed for 4 h postinjection. The melanized nodules were then put on a slide glass, squashed with a cover glass, and observed under a fluorescence microscope.

**Assay for bacterial clearance from larval plasma**

Fifth-instar day 5 larvae were surface-sterilized by swabbing with 70% ethanol. Then, 5 μl of IPS containing 1 × 10^6 cells of E. coli 12119, M. luteus, and B. cereus were injected into the hemocoel. The bacteria used in this experiment were cultured in LB medium; the cells in logarithmic phase were harvested by centrifugation and washed, as described above. Treated larvae were kept at 25°C, and the hemolymph from five larvae was collected directly into a petri dish containing crystals of phenylthiourea at 1 min, 30 min, and 1, 2, 4, 8, 12, and 24 h postinjection. The hemocytes were removed by centrifugation at 81 × g for 10 min at 4°C, and the number of viable microorganisms in the plasma was determined by plating 30 μl of 10^5 diluted plasma samples on Luria-Bertani agar plates and then incubating them overnight at 37°C.

**In vitro hemocyte aggregation assay**

Hemolymph was collected directly into a petri dish containing 10 ml of ice-cold IPS mixed with 10 mM benzamidine (final concentration) and incubated at 25°C for 4 h. Cells attached to the dish were washed twice with PBS, scraped using a cell scraper, and collected by centrifugation. Recombinant M. luteus-binding proteins (CRD1 + 2, CRD1, and CRD2), hemocytes from fifth-instar day 4 larvae, 1.0 × 10^8 M. luteus cells and anti-M. luteus-binding protein serum or normal mouse serum were mixed in different combinations and incubated with gentle shaking for 1 h at 25°C. The aggregations were observed under a stereoscopic microscope.

**Results**

**Identification and comparison of B. mori proteins that bind M. luteus and S. cerevisiae**

To identify proteins from the larval plasma of B. mori that function in nonspecific recognition of invading microorganisms, we used formalin-fixed M. luteus and S. cerevisiae cells as binding targets. The insect proteins that bound to M. luteus and S. cerevisiae were eluted with GlcNAc and analyzed by SDS-PAGE. A major 43-kDa
protein band was detected in the eluates from both microorganisms (Fig. 1A, lanes 2 and 6). A mixture of these two proteins, designated M. luteus- and S. cerevisiae-binding proteins, also yielded a single 43-kDa band on SDS-PAGE (Fig. 1A, lane 4). Further analysis by digestion of the two proteins with V8 protease and electrophoresis using the Cleveland method (20) revealed that the digestion patterns of the proteins were very similar (Fig. 1A, lanes 3 and 7). Protease digestion of a mixture of the two proteins yielded the same digestion pattern as those of the separate binding proteins (Fig. 1A, lane 5).

We were unable to determine the N-terminal amino acid sequences of the 43-kDa M. luteus- and S. cerevisiae-binding proteins. It revealed that these two proteins were the same protein. Thus, the protein renamed BmMBP apparently because the N-terminal residues of the proteins were blocked. Consequently, we determined the amino acid sequences of the 17-kDa peptide fragments from the V8-protease digestes of the two proteins, indicated by arrows in Fig. 1A; in both cases, the sequence MEGATFFY was found obtained using an amino acid sequencer. Furthermore, S. cerevisiae-binding protein was detected on Western blots using anti-M. luteus-binding protein similarly cross-reacted with an antiserum directed against S. cerevisiae-binding protein (Fig. 1Bc). In contrast, the M. luteus- and S. cerevisiae-binding proteins were not detected using anti-BmLBP antiserum (Fig. 1Bb). These results indicate that the M. luteus- and S. cerevisiae-binding proteins are identical.

cDNA cloning and nucleotide sequencing of M. luteus-binding protein

We searched the B. mori EST database for proteins that shared the MEGATFFY sequence and found one partial sequence, clone e40h0121, that did not include the 5’ and 3’ ends of the coding region. We then conducted 5’- and 3’-RACE and determined the complete nucleotide sequence (1132 bp) of the region encoding M. luteus-binding protein. The encoded protein was 318-aa long, and a polyadenylation signal-like sequence (AATTTAAA) was present 113 bases after the termination codon (TGA; Fig. 2). Analysis of the N-terminal region using the SignalP program (www.cbs.dtu.dk/) predicted a 22-aa signal peptide sequence (MNNLKFPIL), with a cleavage site between Gly22 and Gly23. Thus, the predicted mature M. luteus-binding protein consisted of 296 aa, from Gln23 to Arg319 (Fig. 2). The MEGATFFY sequence obtained from direct amino acid sequencing of the 17-kDa fragment was present from Met4 to Tyr68. A recombinant protein expressed from a cDNA spanning the entire encoding region was detected by the anti-M. luteus-binding protein antiserum.

FIGURE 1. Comparison of M. luteus- and S. cerevisiae-binding proteins by peptide mapping (A) and Western blotting (B). A, The M. luteus- and S. cerevisiae-binding proteins were isolated by SDS-PAGE and digested with V8 protease. The digests were analyzed using Tricine-SDS-PAGE and stained with Coomassie blue. Lane 1, V8 protease; lane 2, M. luteus-binding protein; lane 3, V8 protease-treated M. luteus-binding protein; lane 4, mixture of M. luteus- and S. cerevisiae-binding proteins; lane 5, V8 protease-treated mixture of M. luteus- and S. cerevisiae-binding proteins; lane 6, S. cerevisiae-binding protein; lane 7, V8 protease-treated S. cerevisiae-binding protein. B, M. luteus-binding protein (lane 1), BmLBP (lane 2), and S. cerevisiae-binding protein (lane 3) were separated by SDS-PAGE and immunoblotted being performed using anti-M. luteus-binding protein antiserum (a), anti-BmLBP antiserum (b), or anti-S. cerevisiae-binding protein antiserum (c). The arrow indicates 17-kDa peptide fragments from the V8-protease digests.

FIGURE 2. Nucleotide and amino acid sequences of M. luteus-binding protein. The nucleotide numbers are shown to 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 methionine, are shown to the right of each line. The partial amino acid sequences determined by direct peptide sequencing are underlined. The arrowhead indicates the putative N-terminal residue of the mature protein, underlining indicates the signal peptide sequence, and the asterisk (*) and double underlining denote the termination codon and polyadenylation signal, respectively.
From these results, we concluded that the cDNA that we isolated codes for *M. luteus*-binding protein. The deduced amino acid sequence encoded by the isolated *M. luteus*-binding protein cDNA was aligned with those of four C-type lectins from lepidopteran insects (*M. sexta* immulectin-2, *H. cunea* lectin, *H. cunea* lectin (AF033275); BmLBP (AJ011573); BmIML (AY297159)).

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**Phylogenetic relationship of *M. luteus*-binding protein to other C-type lectins.**

To examine the relationship between *M. luteus*-binding protein and other C-type lectins, the sequences encoding the short- and long-form CRDs from *M. luteus*-binding protein, seven C-type lectins from other lepidopterans, three C-type lectins from the American cockroach, and two C-type lectins from mammals were aligned using ClustalX, and a phylogenetic tree was drawn using PHYLIP. Clusters were formed by all of the short-form CRDs from lepidopteran C-type lectins, five of seven long-form CRDs from lepidopteran C-type lectins, the long-form CRDs from immulectin-1 and BmIML, three C-type lectins from American cockroach, and two C-type lectins from mammals, respectively (Fig. 4). The short-form CRDs from BmLBP, immulectin-1, immulectin-2, and *H. cunea* lectin, which bind LPS (7, 9, 10, 15), clustered in the same group. The short-form CRDs from immulectin-3 and immulectin-4, which bind GalNAc and glucose, respectively (Fig. 8A). From these results, we concluded that the cDNA that we isolated codes for *M. luteus*-binding protein.

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**Phylogenetic relationship of *M. luteus*-binding protein to other C-type lectins.**

To examine the relationship between *M. luteus*-binding protein and other C-type lectins, the sequences encoding the short- and long-form CRDs from *M. luteus*-binding protein, seven C-type lectins from other lepidopterans, three C-type lectins from the American cockroach, and two C-type lectins from mammals were aligned using ClustalX, and a phylogenetic tree was drawn using PHYLIP. Clusters were formed by all of the short-form CRDs from lepidopteran C-type lectins, five of seven long-form CRDs from lepidopteran C-type lectins, the long-form CRDs from immulectin-1 and BmIML, three C-type lectins from American cockroach, and two C-type lectins from mammals, respectively (Fig. 4). The short-form CRDs from BmLBP, immulectin-1, immulectin-2, and *H. cunea* lectin, which bind LPS (7, 9, 10, 15), clustered in the same group. The short-form CRDs from immulectin-3 and immulectin-4, which bind GalNAc and glucose,
clumped together, as did the long-form CRDs from these lectins. However, both short- and long-form CRDs from *M. luteus*-binding protein were not grouped with any of the lectins examined (Fig. 4). Similarly, in a phylogenetic tree based on the complete amino acid sequences of the mature forms of lepidopteran C-type lectins, *M. luteus*-binding protein was placed separate from all other groups (data not shown). These results indicate that *M. luteus*-binding protein is only distantly related to other C-type lectins.

**Stage- and tissue-specificity and inducibility of the expression of *M. luteus*-binding protein**

The expression of *M. luteus*-binding protein was examined by Western blotting of homogenates of *B. mori* eggs and first-instar larvae and plasma from second, third, fourth, and fifth-instar day 4 larvae, and from prepupae, pupae, and adults. *M. luteus*-binding protein was expressed in the larvae from the second instar stage and in the prepupa, pupae, and adults (Fig. 5A). Immunoblotting of tissues collected from fifth-instar day 4 larvae revealed that *M. luteus*-binding protein was expressed only in the fat body and hemocytes (Fig. 5B). To determine whether invading microorganisms can induce the expression of *M. luteus*-binding protein, fifth-instar day 4 larvae were injected with formalin-killed *M. luteus* cells or saline; plasma samples were collected at various times postinjection and examined by immunoblotting. Constitutive expression was confirmed (Fig. 5Cc), and larvae that received *M. luteus* cells also exhibited a little induced expression of *M. luteus*-binding protein 24 h after the injection (Fig. 5Cb).

**Binding targets of *M. luteus*-binding protein**

The initial experiments demonstrated that *M. luteus*-binding protein was identical with the protein that bound to *S. cerevisiae*, indicating that the protein can bind to organisms in at least two different microbial groups. To examine whether the protein can bind to a wide range of microorganisms, formalin-killed or living Gram-negative bacteria, Gram-positive bacteria, and yeasts (Table I) were incubated with plasma from *B. mori* larvae, and the unbound plasma constituents were removed by washing. The bound proteins were eluted with sodium citrate and subjected to immunoblotting with anti-*M. luteus*-binding protein antiserum. *M. luteus*-binding protein was found to bind to all of the formalin-killed and living Gram-negative bacteria, Gram-positive bacteria, and yeasts that we examined (Fig. 6, lanes 1–11). In contrast, *M. luteus*-binding protein did not bind to silica gel and Sepharose as a negative control.

The affinity of binding of *M. luteus*-binding protein to *M. luteus* and *S. cerevisiae* cells was analyzed using an IAsys resonant mirror biosensor (Fig. 7). Scatchard plot analysis demonstrated that the value of the dissociation constant of *M. luteus*-binding protein for *M. luteus* was very low (K_D = 1.23 × 10^{-8}, R^2 = 0.97) and that for *S. cerevisiae* was still lower (K_D = 1.00 × 10^{-11}, R^2 = 0.90; Fig. 7).

**Specificity of binding of CRD1 and CRD2 to microorganisms**

To analyze the function of the short- and long-form CRDs from *M. luteus*-binding protein (CRD1 and CRD2, respectively), we expressed CRD1, CRD2, and both CRD1 and 2 (CRD1 + 2) in *E. coli* cells as GST fusion proteins. The three recombinant proteins were subjected to SDS-PAGE and their identity was confirmed by immunoblotting. The relative molecular masses of the proteins were close to the predicted molecular weights and the purity of the preparations exceeded 90% (Fig. 8A). The ability of the recombinant proteins (CRD1, CRD2, and CRD1 + 2) to bind to Gram-negative bacteria, Gram-positive bacteria, and yeasts was examined (Table I). Although the separate recombinant CRD1 and CRD2 proteins bound to all of the microorganisms tested, recombinant CRD1 + 2 bound to all microorganisms tested except *Corynebacterium glutamicum* and *Bacillus cereus* (Fig. 8B), which was identical with the binding specificity exhibited by native *M. luteus*-binding protein in the larval plasma (Fig. 6).

**Specificity of the binding of CRD1 and CRD2 to sugars**

To investigate the binding mechanism of *M. luteus*-binding proteins to a broad range of microorganisms, the specificities of recombinant CRD1 + 2, CRD1, and CRD2 for various sugars and PAMPs were examined. Seven monosaccharides (mannose, glucose, GlcNAc, galactose, fucose, GalNAc, and MurNAc), three...
disaccharides (sucrose, maltose, and trehalose), and five polysaccharides (mannan, teichoic acid, peptidoglycan, LPS, and laminarin) were tested for their ability to inhibit the binding of recombinant CRD1 + 2, CRD1, or CRD2 to formalin-killed *M. luteus* cells. CRD1 + 2 binding to *M. luteus* cells was significantly inhibited by 0.51 mg/ml teichoic acid and 25 mg/ml mannan (see Fig. 10, CRD1 + 2).

CRD1 binding to *M. luteus* cells was significantly inhibited by 1 M glucose or GlcNAc (Fig. 9, CRD1). The binding of CRD1 was incompletely inhibited by 10 mM MurNAc, which was the maximum concentration tested due to the low solubility of this compound. CRD1 binding was also completely inhibited by 25 mg/ml teichoic acid and partially inhibited by 250 mg/ml mannan (Fig. 10, CRD1).

CRD2 binding to *M. luteus* cells was markedly inhibited by 1 M sucrose, maltose, or galactose, and partially inhibited by 10 mM MurNAc (Fig. 9, CRD2). The binding was also significantly inhibited by 25 mg/ml teichoic acid, 1.0 mg/ml peptidoglycan, and 25 mg/ml mannan (Fig. 10, CRD2).

The binding of recombinant CRD1 + 2, CRD1, and CRD2 to *M. luteus* cells was not inhibited by <3 mg/ml LPS (Fig. 10).

**Role of nodule formation in the elimination of invading microorganisms**

To determine the physiological function of *M. luteus*-binding protein, the role of nodule formation in the elimination of invading microorganisms from the hemocoel of the larva was investigated. Fifth-instar day 4 larvae were injected with 1 × 10⁶ cells of each of the 11 formalin-killed microorganisms used in the previous experiment (Table I), dissected after various periods of time to expose the larval hemocoels, and observed under a stereomicroscope. In larvae injected with the 9 microorganisms that were shown to bind *M. luteus*-binding protein (Figs. 6 and 8), melanized nodules of various sizes and shapes were observed binding to the fat body, midgut, Malpighian tubule, and dorsal vessel at 4 h after injection (Fig. 11B; data shown only for *S. cerevisiae*). Larvae injected with *S. cerevisiae* developed hundreds of nodules that were attached along the dorsal vessel (data not shown). In contrast, injection of *C. glutamicum* and *B. cereus*, to which *M. luteus*-binding protein was shown not to bind (Figs. 6 and 8), did not induce nodule formation.

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**FIGURE 6.** Specificity of binding of *M. luteus*-binding protein to formaldehyde-fixed microorganisms (A) or living microorganisms (B). Formdehyde-fixed or living cells of 11 microorganisms were incubated with the plasma fraction from *B. mori* larvae, and the bound proteins were eluted with sodium citrate. Aliquots of the elutes (10⁶) were subjected to 10% SDS-PAGE under reducing conditions and Western blotting was performed using anti-*M. luteus*-binding protein antiserum. Lane 1, *E. coli* W3110; lane 2, *S. marcescens*; lane 3, *E. coli* 12119; lane 4, *A. hydrophila*; lane 5, *Proteus vulgaris*; lane 6, *S. cerevisiae*; lane 7, *C. albicans*; lane 8, *S. ludwigii*; lane 9, *M. luteus*; lane 10, *C. glutamicum*; lane 11, *B. cereus*; lane 12, silica gel; lane 13, Sepharose.

**FIGURE 7.** Scatchard plot analysis of binding of *M. luteus*-binding protein to *M. luteus* and *S. cerevisiae* cells. *M. luteus* or *S. cerevisiae* cells were fixed to the cuvette of a resonant mirror biosensor and the association profile of *M. luteus*-binding protein with each microorganism was determined for Scatchard plot analysis. A and C, Association profiles of *M. luteus*-binding protein to *M. luteus* and *S. cerevisiae* cells, respectively. B and D, Scatchard plots analyses of A or C, respectively. Conc., concentration of *M. luteus*-binding protein (nanomoles).

**FIGURE 8.** Binding of recombinant CRD1, CRD2, or CRD1 + 2 from *M. luteus*-binding protein to various microorganisms. A, SDS-PAGE and immunoblot analysis of recombinant CRDs from *M. luteus*-binding protein. Recombinant CRD1, CRD2, and CRD1 + 2 proteins were separated by SDS-PAGE, stained with Coomassie blue (lanes 1, 3, and 5, respectively), and subjected to immunoblotting using mouse anti-*M. luteus*-binding protein antiserum (lanes 2, 4, and 6, respectively). B, Binding of recombinant CRD1, CRD2, and CRD1 + 2 proteins to eight bacterial and three yeast species was examined by incubation of the proteins with the microorganisms, elution of the bound proteins, and detection of the recombinant proteins by immunoblotting. Yeast: *a*, *S. cerevisiae*; *b*, *S. ludwigii*; *c*, *C. albicans* Gram-positive bacteria: *d*, *M. luteus*; *e*, *C. glutamicum*; *f*, *B. cereus*. Gram-negative bacteria: *g*, *E. coli* W3110; *h*, *E. coli* 12119; *i*, *A. hydrophila*; *j*, *Serratia marcescens*; *k*, *P. vulgaris*.
formation (Fig. 10, C and D) or other changes as compared with the saline-injected controls (Fig. 11A).

To examine the fate of the injected microorganisms using fluorescence microscopy, fifth-instar day 4 larvae were injected with 1 × 10^8 fluorescently labeled E. coli W3110 cells and the hemocoels were exposed at 2 h postinjection. Melanized nodules were collected onto a glass slide and flattened with a cover glass. Fluorescence microscopy showed that thousands of E. coli cells were trapped in the nodules (Fig. 12B) composed of hemocytes. E. coli cells were located within the clusters or, sporadically, in the matrix of hemocytes (Fig. 12). These observations suggested that all nine microorganisms that bind to M. luteus-binding protein are eliminated from the hemocoel by nodule formation, although other lectins may also be involved.

**In vitro hemocyte aggregation assay**

More than 90% of the injected Gram-positive and Gram-negative bacteria and yeasts that were used in the previous experiment were removed from the larval plasma within 30 min postinjection, and melanized nodules began to appear 30–60 min after injection (data not shown). The same phenomenon was observed in a previous study of BmLBP, in which it was further shown that clearance of E. coli cells by nodule formation was inhibited by antisera to BmLBP (18). From these observations, we hypothesized that nodule formation is the first and most important immune response against invading microorganisms in the larval hemocoel. Thus, we examined whether M. luteus-binding protein directly enhances nodule formation by binding to the microorganisms. Formalin-killed M. luteus cells were incubated with hemocytes from B. mori fifth instar larvae and with either larval plasma containing M. luteus-binding protein or recombinant CRD1 (Fig. 13, A and B). In contrast, no aggregation was observed when any component of the mixture was omitted (Fig. 13, C–E). These results confirmed that all three components were necessary for nodule-like hemocyte aggregation.

Next, we examined the role of the two CRD domains in the formation of the nodule-like hemocyte aggregates, using the same system. Incubation of recombinant CRD1, CRD2, or a mixture of recombinant CRD1 and CRD2 with M. luteus and B. mori hemocytes did not result in nodule formation with any combination (Fig. 13, F–H). This may indicate that the complete protein, including both the short- and long-form CRDs in a single amino acid chain, is required for the aggregation-promoting activity of M. luteus-binding protein.

We also examined whether M. luteus-binding protein promotes hemocyte aggregation in the presence of Arthrobacter globiformis,
E. coli W3110, Saccharomyces ludwigii, or Candida albicans, instead of M. luteus. We observed that four microorganisms that bind to M. luteus-binding protein were capable of promoting aggregate formation when combined with recombinant CRD1/H11001 and hemocytes (data not shown). Four other microorganisms (A. globiformis, E. coli W3110, S. ludwigii, or C. albicans) aggregated when mixed with recombinant CRD1/H11001 alone, and were thus not included in the hemocyte aggregation assay. These results suggest that M. luteus-binding protein is a triggering factor for nodule formation in vivo.

**Discussion**

M. luteus-binding protein exhibits a wide range of recognition and high-affinity binding to microorganisms

Seven lectins from lepidopteran insects have been reported, including immurectin-1, -2, -3, and -4 from M. sexta (7), a lectin from H. cunea (9), BmLBP (10), and BmIML (12) from B. mori. Immurectin-1 and, -2, H. cunea lectin and BmLBP all bind to LPS and to Gram-negative bacteria (7, 9, 10, 15). Immurectin-3 specifically binds to LPS and lipoteichoic acid from bacteria, and to laminarin, a β-1,3-glucan (13) and immurectin-4 binds to GalNAc.
and glucose (14); however, it is not yet known what types of microorganisms are really recognized by these two lectins. The recognition ranges of insect lectins for microorganisms, the mechanisms underlying recognition of PAMPs by insect lectins, and their roles in the insect defense system have not been elucidated in detail.

The *M. luteus* - and *S. cerevisiae*-binding proteins were isolated separately from the plasma fraction of *B. mori* larvae incubated with *M. luteus* and *S. cerevisiae*, respectively. We showed that the two proteins had identical digestion patterns (Fig. 1A), identical partial amino acid sequences (Fig. 2), and reciprocal cross-reactivity to antiserum (Fig. 1B). *M. luteus*-binding protein also bound to *S. cerevisiae* (Figs. 6 and 8B), Gram-positive bacteria, and both rough and smooth strains of Gram-negative bacteria (Fig. 6). In light of these findings, we renamed the protein BmMBP.

The dissociation constants of known pattern-recognition lectins and their binding targets are: 1.62 × 10⁻⁸ for mannose-binding protein from humans binding to mannan (22), 1.10 × 10⁻⁷ for galectin-1 binding to glycoprotein 90K (23); and 1.78 × 10⁻⁹ for tachylectin-5A binding to GlcNAc (24). The dissociation constants of Abs and Ags are reported to range from 1.83 × 10⁻⁷ to 2.60 × 10⁻¹⁰ (25). In this study, the Scatchard plot analysis demonstrated that the dissociation constants of BmMBP with *M. luteus* and *S. cerevisiae* were 1.23 × 10⁻⁸ and 1.00 × 10⁻¹¹, respectively (Fig. 6), indicating that the binding affinity of BmMBP to microorganisms is very high. These results suggest that insect lectin BmMBP has a wide recognition range and high-binding affinity for microorganisms.

Phylogenetic trees generated from either the entire amino acid sequences of mature insect C-type lectins or the two CRDs segregated BmMBP separate from all other clusters (Fig. 4), indicating that BmMBP is only distantly related to the known C-type lectins.

**Mechanism producing the wide range of microorganism recognition by BmMBP**

Human mannose-binding protein binds to a wide range of microorganisms, including bacteria, fungi, and protozoa (26, 27), as well as to cancer cells (28), and it binds specifically to GlcNAc, mannose, N-acetyl-mannosamine, fucose, and glucose (29). It is likely that the wide range of recognition of microorganisms by mannose-binding protein is attributable, at least in part, to its broad-spectrum sugar-binding activity.

As BmMBP belongs to the C-type lectin family and has two CRDs in each molecule (Figs. 3 and 4), we hypothesized that BmMBP binds to microbial cell surface carbohydrates via those domains. Using recombinant CRD1 and CRD2 proteins, we showed that CRD1 bound to teichoic acid and mannann and CRD2 bound to teichoic acid, peptidoglycan, and mannan, PAMPs of Gram-positive bacteria and yeasts (Fig. 10, CRD1 and CRD2). The observed apparent high-affinity binding of BmMBP to *M. luteus* (Fig. 7, A and B) may be attributable to two-point binding to teichoic acid or peptidoglycan by CRD1 and CRD2 arranged in tandem within a single molecule. In addition, high-affinity binding or BmMBP to *S. cerevisiae* (Fig. 7, C and D) may result from intramolecular two-point binding by CRD1 and CRD2 to mannan.

LPS, a Gram-negative bacteria PAMP, did not bind to CRD1 + 2, CRD1, or CRD2 (Fig. 10) at concentrations up to 3 mg/ml. The molecular mass of the LPS from *E. coli* O26:B6 used in this experiment was ~3000 Da; therefore, it is clear that LPS did not bind to recombinant CRD1 + 2, CRD1, or CRD2 at high molecular concentrations (Fig. 10).

The LPS O Ag of some Gram-negative bacteria consists of mannan (30). Therefore, BmMBP might bind to Gram-negative bacteria by recognizing the mannan moiety of the O Ag (Fig. 5). CRD1 + 2 did not bind to any of the 10 sugars tested in our experiments (Fig. 9, CRD1 + 2) because CRD1 and CRD2 have different sugar specificities, and each binds to different sugars.

These findings demonstrate that CRD1 and CRD2 have wide, but different, spectra of sugar specificity.

Although CRD2 bound to a monosaccharide, the protein did not bind to the components of the monosaccharide (Figs. 9 and 10).

Therefore, CRD2 may preferentially recognize higher structures found in disaccharides. It is also plausible that BmMBP acquired its capacity to bind multiple microorganisms by virtue of having two CRDs, each of which has a wide and different range of sugar specificity.

Tachylectin, a lectin from horseshoe crabs, exists as a polymer in the blood and its multipoint high-affinity binding to GlcNAc has a dissociation constant of 10⁻¹⁰ M (24). BmLBP, a C-type lectin that recognizes LPS, is also reported to exist as a polymer in the blood of *B. mori* (10). Therefore, it is possible that the high-affinity binding of BmMBP to microorganisms is dependent not only on the tandem arrangement of the two CRDs in each amino acid chain, but also on polymerization of the protein molecules.

**The smallest unit required for triggering of hemocyte aggregation**

In this study, only recombinant CRD1 + 2 was shown to trigger hemocyte aggregation in vitro, whereas recombinant CRD1 and CRD2 separately and a mixture of recombinant CRD1 and CRD2 did not (Fig. 13). However, each recombinant protein was able to bind a broad range of microorganisms (Fig. 8). Thus, it is possible that the capacity of BmMBP to trigger hemocyte aggregation is dependent on the presence of both CRDs in a single amino acid chain. However, the recombinant proteins were produced as GST fusions and it is possible that the GST moieties inhibited the normal function of CRD1 or CRD2. A recombinant protein containing the C-terminal half of immulectin-2, and thus only a single CRD, was shown to enhance melanization and encapsulation in *M. sexta* (14, 17).

**The role of C-type lectins in the defense systems of lepidopteran insects**

BmLBP binds to Gram-negative bacteria, enhancing nodule formation by hemocytes and resulting in the elimination of bacteria from the hemocoel of *B. mori* larvae (10, 18). Similarly, in *M. sexta*, immulectin-2 was reported to act as an enhancing factor for melanization and encapsulation (7, 15, 16, 17).

Microorganisms to which BmMBP binds with high affinity were easily trapped within nodules when they were injected into the hemocoel of larvae (Figs. 11 and 12). Similarly, recombinant CRD1 + 2 triggered hemocyte aggregation in vitro in combination with *M. luteus*, *C. albicans*, *A. globiformis*, or *E. coli* W3110 cells (Fig. 13). Together, these findings indicate that C-type lectins are probably important recognition proteins in the early stages of microbial infection.

Immulectin-1, -2, and -3, *H. cunea* lectin, and BmLBP have been shown to contribute to defense against Gram-negative bacteria in lepidopteran insects through binding to LPS (8, 10, 13, 14). Immulectin-2 also binds to unidentified surface molecules of *C. elegans* (17). In addition, immulectin-3 specifically has been shown to bind to LPS, lipoteichoic acid, and β-1,3-glucan, but its target microorganisms in vivo remain uncertain (13). The microorganisms that invade insects are not limited to Gram-negative
bacteria and nematodes. The role of insect C-type lectins in defense against the entire spectrum of invading microorganisms has not been fully elucidated and the importance of their role as recognition proteins in the defense system of lepidopteran insects has been questioned. However, the identification of BmMBP in this study provides further evidence that C-type lectins are important molecules for the elimination of not only Gram-negative bacteria but also Gram-positive bacteria and yeasts from insects (Figs. 6, 8, and 12). No ortholog of BmMBP was found among the known lectins from other insects, but one may be found in the near future.

When we searched the B. mori genome sequence database using the C-type lectin sequence, several new lectin-like molecules were identified as matches. It thus is plausible that insects have many kinds of C-type lectin in the blood and that they constitute a recognition network against almost all invading microorganisms.

Disclosures
The authors have no financial conflict of interest.

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