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 M. luteus cells and S. cerevisiae were 1.23 × 10^{-8} and 1.00 × 10^{-11} M, respectively. rBmMBP triggered the aggregation of hemocytes from B. mori larvae in vitro and microorganisms recognized by BmMBP were surrounded by aggregated hemocytes in vivo, forming a nodule, which is the typical cellular reaction in insect immune responses. These observations suggest that BmMBP functions as a trigger for the nodule reaction and that the multirecognition characteristic of BmMBP plays an important role in the early stages of infection by a variety of microorganisms. The Journal of Immunology, 2006, 177: 4594–4604.

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2 Address correspondence and reprint requests to Dr. Ryoichi Sato, Graduate School of Bio-Applications and Systems Engineering, Tokyo University of Agriculture and Technology, Tokyo, Japan. Received for publication January 13, 2006. Accepted for publication July 5, 2006.
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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-acetylgalacosamine (GlcNAc), galactose, sucrose, 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 MP Biomedicals, and trehalose was purchased from Hayashibara Biochemical Laboratories.

Animals

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

Preparation of bacterial and yeast cells

The bacteria 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 0.1% Triton X-100 in IPS. The cells were resuspended in yeast physiological saline (areas) and centrifuged at 1800 × g for 30 min at 4°C. Yeasts were cultured in YM broth (10 g of glucose, 5 g of yeast extract, 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 ice-cold IPS mixed with benzamidine (10 mM final conc.) and centrifuged at 1800 × g for 10 min at 4°C. Then, the supernatant was stored at −80°C. The plasma was mixed with 80 µl of the precipitate of fixed M. luteus or S. cerevisiae cells and incubated at 4°C for 1 h using 10 µl of IPS. 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). Antiserum against M. luteus-binding protein or S. cerevisiae-binding protein was raised by injecting 15 µg of the respective HPLC-purified binding protein into a female mouse with CFA and giving a booster injection containing the same amount of protein without the adjuvant 2 wk later.

For immunoblotting, the proteins were separated using SDS-PAGE and transferred to a nitrocellulose membrane. After blocking with 2% BSA, the membrane was incubated in 15,000-fold diluted mouse anti-M. luteus-binding protein antiserum for 1.5 h. After washing, the membrane was incubated in 15,000-fold diluted peroxidase-conjugated rabbit anti-mouse IgG (Wako Pure Chemical) for 1.5 h, and then the proteins were detected using the ECL Western blotting detection system (Amersham Biosciences). For peptide mapping, M. luteus- or S. cerevisiae-binding protein was subjected to SDS-PAGE. The gel was stained with CBB and the stained bands were cut from the gel. The gel fragments were subjected to Tricine SDS-PAGE and the proteins were digested with V8 protease at an enzyme:protein ratio of 1:25 in the stacking gel at 25°C for 1 h according to Cleveland (20). The digested proteins were separated using Tricine SDS-PAGE and stained.

Internal amino acid sequencing

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 491LC; Applied Biosystems).

Computer-assisted homology search and alignment

We searched the B. mori expressed sequence tag (EST) database ((http://papilio.ab.a.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 e040h0121. 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 (http://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(12–18 mix)T primer: 5′-GTGTTTCCACGTCAGCAAGCAGGTCTCAGATGTT-3′) from the M. luteus-binding protein (later renamed BmMBP) cDNA using Rever Tra Ace (Toyobo) as the reverse transcriptase and then polyadenylated with TdT (Amersham Biosciences). Double-stranded cDNA fragments were amplified by PCR using the following primer sets: 1) sense-1 (5′-GCAHTHTGTTTGYACNCNNYT-3′) or sense-2 (5′-AYYCAR ATHTCAARTYCC-3′) and M13M4 (5′-GTGTTTCCACGTCAGCAAGCAGGTCTCAGATGTT-3′) for 3′-RACE; 2) M13M4-oligo d(12–18 mix)T and anti-2 (5′-GGTG CAGGGACATGT GTT-3′) for 5′-RACE; and 3) M13M4 and anti-3 (5′-ATTGCAGCTGAGGGGAT-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-overhang vector p123T (MoBiTec). The cDNA was sequenced using a Long Read Tower DNA sequence (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 23–118) using primer-1 (5′-TTCATGGATC CGGGAATGAAAGTGTTTTCCT-3′) and primer-4 (5′-ACACAAACAT GCGTTGCTATTGAGCAC-3′); a cDNA fragment encoding the CRD1 region (residues 23–158) was amplified using primer-1 and primer-2 (TTTCTTGATATAAAATGGGAAAGATGTCG-3′); and a cDNA fragment encoding the CRD2 region (residues 159–318) was amplified using primers-1 and primer-3 (5′-TATCGCAAGTCACATCGAGAC-3′) and primer-4. Each cDNA fragment was subcloned into plasmid vector pGEX-4T-3 (Amersham Biosciences) and transfected into E. coli BL21. An overnight culture of transformed BL21 cells was diluted with fresh 1-broth
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 urea were reanimated 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, glycerol, 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 × 10^10^ 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% glycerol) and 24 μl of M. luteus-binding protein, and 24 μl of the individual CRDs were detected by immunoblottig.

### 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 hemolymph 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, 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 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 exposed for 4 h. The dark nodules were observed under a stereomicroscope. 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 × 10^8^ fluorescence-labeled E. coli cells and the hemocytes were exposed 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 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 exposed for 4 h. The dark nodules were observed under a stereomicroscope. 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 × 10^8^ fluorescence-labeled E. coli cells and the hemocytes were exposed 4 h postinjection. The melanized nodules were then put on a slide glass, squashed with a cover glass, and observed under a fluorescence microscope.

### Immunoblotting 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. The cuvette was then equilibrated using sodium acetate buffer containing 20 mM NaCl, 2 mM reduced glutathione, 0.2 mM oxidized glutathione, glycerol, 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.

### 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 1 h. Then the plates were blocked with the BSA solution. Recombinant M. luteus-binding protein (CRD1, CRD2, and CRD1 + 2) was 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 microinor. Mouse anti-M. luteus-binding protein protein 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 rinsed out in a similar manner to that described above, replaced with 100 μl/well of peroxidase-conjugated rabbit anti-mouse IgG diluted 3,000-fold with TBS containing 20 mg/ml BSA, and then 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-H₃PO₄ 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 exposed for 4 h. The dark nodules were observed under a stereomicroscope. 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 × 10^8^ fluorescence-labeled E. coli cells and the hemocytes were exposed 4 h postinjection. The melanized nodules were then put on a slide glass, squashed with a cover glass, and observed under a fluorescence 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 digests of the two proteins, indicated by arrows in Fig. 1A; in both cases, the sequence MEGATFFY was obtained using an amino acid sequencer. Furthermore, S. cerevisiae-binding protein was detected on Western blots using antisera directed against M. luteus-binding protein (Fig. 1Ba) and M. luteus-binding protein similarly cross-reacted with an antisera directed against S. cerevisiae-binding protein (Fig. 1Bc). In contrast, the M. luteus- and S. cerevisiae-binding proteins were not detected using anti-BmLBP antisera (Fig. 1Bb). These results indicate that the M. luteus- and S. cerevisiae-binding proteins were 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 (AATTAAA) 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 (MNKLFPILFFLLTLLPSELIHGFLLTLLPSELIH), with a cleavage site between Gly22 and Arg318. The predicted mature M. luteus-binding protein consisted of 296 aa, from Gln23 to Arg60 (Fig. 2). The MEGATFFY sequence obtained from direct amino acid sequencing of the 17-kDa fragment was present from Met46 to Tyr68. A recombinant protein expressed from a CDNA spanning the entire encoding region was detected by the anti-M. luteus-binding protein antiserum.
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 (AF023275); BmLBP (AJ011573); BmIML (AY297159)).

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 analyzed by multiple alignments 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 *M. luteus*-binding protein, which bind GalNAc and glucose,
clustered 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 a Lasys 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 $1 \times 10^8$ 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.
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 $1 \times 10^9$ 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 antiserum 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 CRD1/2 in microplate wells for 1 h at 25°C. Hemocyte aggregations 20- to 100-μm long were observed (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/H110012 and hemocytes (data not shown). Four other microorganisms (A. globiformis, E. coli W3110, S. ludwigii, or C. albicans) aggregated when mixed with recombinant CRD1/H110012 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 immulectin-1, -2, -3, and -4 from M. sexta (7), a lectin from H. cunea (9), BmLBP (10), and BmIML (12) from B. mori. Immulectin-1 and, -2, H. cunea lectin and BmLBP all bind to LPS and to Gram-negative bacteria (7, 9, 10, 15). Immulectin-3 specifically binds to LPS and lipoteichoic acid from bacteria, and to laminarin, a β-1,3-glucan (13) and immulectin-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); 2.03 × 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 × 10⁻¹¹ (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, mannos, 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 mannan 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. ludwigi*, *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 is thus 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.

References
1. Janeway, Jr., C. A. 1989. Approaching the asymptote: evolution and revolution in Immunology. Cold Spring Harbor Symp. Quant. Biol. 54: 1–13.
2. Medzhitov, R., and C. A. Janeway, Jr. 1997. Innate immunity: impact on the adaptive immune response. Curr. Opin. Immunol. 9: 4–9.
3. Weis, W. I., M. E. Taylor, and K. Drickamer. 1998. The C-type lectin superfamily in the immune system. Immunol. Rev. 163: 19–34.
4. Jomori, T., and S. Natori. 1991. Molecular cloning of cDNA for lipopolysaccharide-binding protein from the hemolymph of the American cockroach, Periplaneta americana: similarity of the protein with animal lectins and its acute phase expression. J. Biol. Chem. 266: 13318–13323.
5. Jomori, T., and S. Natori. 1992. Function of the lipopolysaccharide-binding protein of Periplaneta americana as an opsonin. FEBS Lett. 296: 283–286.
6. Kawasaki, K., T. Kubo, and S. Natori. 1993. A novel role of Periplaneta lectin as an opsonin to recognize 2-keto-3-deoxy octonate residues of bacterial lipopolysaccharides. Comp. Biochem. Physiol. 106: 675–680.
7. Yu, X. Q., Gan, Hong, and M. R. Kanost. 1999. Immunelectin, an inducible C-type lectin from an insect, Manduca sexta, stimulates activation of plasma prophenol oxidase. Insect Biochem. Mol. Biol. 29: 585–597.
8. Shin, S. W., S. S. Park, D. S. Park, M. G. Kim, S. C. Kim, P. T. Bray, and H. Y. Park. 1998. Isolation and characterization of immune-related genes from the fall webworm, Hyphantria cunea, using PCR-based differential display and subtractive cloning. Insect Biochem. Mol. Biol. 28: 827–827.
9. Shin, S. W., D. S. Park, S. C. Kim, and H. Y. Park. 2000. Two carbohydrate recognition domains of Hyphantria cunea lectin bind to bacterial lipopolysaccharides through O-specific chain. FEBS Lett. 467: 70–74.
10. Koizumi, N., A. Morozumi, M. Imamura, E. Tanaka, H. Iwahana, and R. Sato. 1997. Lipopolysaccharide-binding proteins and their involvement in the bacterial clearance from the hemolymph of the silkworm Bombyx mori. Eur. J. Biochem. 248: 217–224.
11. Koizumi, N., M. Imamura, T. Kadotani, K. Yaoi, H. Iwahana, and R. Sato. 1999. 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. FEBS Lett. 443: 139–143.
12. Kim, S. R., K. S. Lee, I. Kim, S. W. Kang, S. K. Nho, H. D. Sohn, and B. R. Jin. 2003. cDNA sequence of a novel immunecom homologue from the silkworm, Bombyx mori. J. Insect Physiol. 49: 583–591.
13. Yu, X. Q., M. E. Tracy, E. Ling, F. R. Scholz, and T. Turecek. 2005. A novel C-type lectin molecule from Manduca sexta is translocated from hemolymph into the cytoplasm of hemocytes. Insect Biochem. Mol. Biol. 35: 285–295.
14. Yu, X. Q., Y. F. Zhu, C. Ma, J. A. Fabricck, and M. R. Kanost. 2002. Pattern recognition proteins in Manduca sexta plasma. Insect Biochem. Mol. Biol. 32: 1287–1293.
15. Yu, X. Q., and M. R. Kanost. 2000. Immulectin-2, a lipopolysaccharide-specific lectin from an insect, Manduca sexta, is induced in response to Gram-negative bacterial. J. Biol. Chem. 275: 37373–37381.
16. Yu, X. Q., and M. R. Kanost. 2003. Manduca sexta lipopolysaccharide-specific immulectin-2 protects larvae from bacterial infection. Dev. Comp. Immunol. 27: 189–196.
17. Yu, X. Q., and M. R. Kanost. 2004. Immulectin-2, a pattern recognition receptor that stimulates hemocyte encapsulation and melanization in the tobacco horn-worm, Manduca sexta. Dev. Comp. Immunol. 28: 891–900.
18. Koizumi, N., Y. Imai, A. Morozumi, M. Imamura, T. Kadotani, K. Yaoi, H. Iwahana, and R. Sato. 1999. Lipopolysaccharide-binding protein of Bombyx mori participates in a hemocyte-mediated defense reaction against Gram-negative bacteria. J. Insect Physiol. 45: 853–859.
19. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680–685.
20. Cleveland, D. W., S. G. Fischer, M. W. Kirschner, and U. K. Laemmli. 1977. Peptide mapping by limited proteolysis in sodium dodecyl sulfate and analysis by gel electrophoresis. J. Biol. Chem. 252: 1102–1106.
21. Imamura, M., J. D. Stoler, J. H. Plesch, S. L. Imai, J. Jeanmougin, and D. G. Higgins. 1997. The CLUSTAL-X windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. Nucleic Acids Res. 25: 4876–4882.
22. Mizuno, Y., Y. Kozutsumi, T. Kawasaki, and I. Yamashina. 1981. Isolation and characterization of a mannose-binding protein form rat liver. J. Biol. Chem. 256: 4247–4252.
23. Tinari, N., I. Kuwabara, M. E. Hullete, P. F. Shen, S. Iacobelli, and F. T. Liu. 2001. Glycoprotein 90kMAC-2BP interacts with galectin-1 and mediates galectin-1-induced cell aggregation. Int. J. Cancer 91: 167–172.
24. Kawabata, S., and R. Tsuda. 2002. Molecular basis of non-self recognition by the horseshoe crab tachylectins. Biochim. Biophys. Acta 1572: 414–421.
25. Ichyoshi, T., M. Zhou, and P. Casali. 1995. A human anti-insulin IgG autoantibody apparently arises through clonal selection from an insulin-specific “Germ-Line” natural antibody template. J. Immunol. 154: 226–238.
26. Hartshorn, K. L., M. R. White, and E. C. Crouch. 2002. Contributions of the N- and C-terminal domains of surfactant protein D to the binding, aggregation, and phagocytic uptake of bacteria. Infect. Immun. 70: 6129–6139.
27. Ferreira, V., C. Valck, G. Sanchez, A. Gingrich, S. Tzima, M. C. Molina, R. Sim, W. Schwaeble, and A. Ferreira. 2004. The classical activation pathway of the human complement system is specifically inhibited by calreticulin form Trypanosoma cruzi. J. Immunol. 172: 3042–3050.
28. Uemura, K., M. Saka, T. Nakagawa, N. Kawasaki, S. Thiel, J. C. Jensenius, and T. Kawasaki. 2002. L-MBP is expressed in epithelial cells of mouse small intestine. J. Immunol. 169: 6945–6950.
29. Tumer, M. W. 2003. The role of mannose-binding lectin in health and disease. Immunology 40: 423–429.
30. Ohba, M., N. Kido, T. Hasegawa, H. Ito, Y. Fujii, Y. Arakawa, T. Komatsu, and N. Kato. 1986. Contribution of the mannose O side-chains to the adjuvant action of lipopolysaccharides. Immunology 60: 503–507.