Participation of a galactose-specific C-type lectin in *Drosophila* immunity

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A galactose-specific C-type lectin has been purified from a pupal extract of *Drosophila melanogaster*. This lectin gene, named *DL1* (*Drosophila* lectin 1), is part of a gene cluster with the other two galactose-specific C-type lectin genes, named *DL2* (*Drosophila* lectin 2) and *DL3* (*Drosophila* lectin 3). These three genes are expressed differentially in fruit fly, but show similar haemagglutinating activities. The present study characterized the biochemical and biological properties of the DL1 protein. The recombinant DL1 protein bound to *Escherichia coli* and *Erwinia chrysanthemi*, but not to other Gram-negative or any other kinds of microbial strains that have been investigated. In addition, DL1 agglutinated *E. coli* and markedly intensified the association of a *Drosophila* haemocytes-derived cell line with *E. coli*. For *in vivo* genetic analysis of the lectin genes, we also established a null-mutant *Drosophila*. The induction of inducible antibacterial peptide genes was not impaired in the *DL1* mutant, suggesting that the galactose-specific C-type lectin does not participate in the induction of antibacterial peptides, but possibly participates in the immune response via the haemocyte-mediated mechanism.

Key words: C-type lectin, *Drosophila*, galactose-specific lectin, immunity, phagocytosis.

INTRODUCTION

Insects have an effective defence system against microbial infection that shares many characteristics with the innate immune system of vertebrates [1,2]. These systems discriminate among various classes of infecting micro-organisms and mount a proper immune response. In the case of *Drosophila melanogaster*, the infection of Gram-negative bacteria engenders the expression of antibacterial peptide genes via the IMD (immune deficiency) pathway; on the other hand, the infection of fungi engenders the expression of antifungal peptide genes via the Toll pathway [1–5]. These selective responses are based on molecules that specifically recognize pathogens. These proteins, called pattern-recognition receptors, bind to microbial components such as LPS (lipopolysaccharide) or peptidoglycans, which trigger the activation of corresponding immune responses.

In addition to such recent progress in the investigation of immune-regulation pathways, many lectins have been purified from various insect species such as flesh fly and cockroach [6,7]. Some of them putatively participate in self-defence by recognizing polysaccharide chains on the surface of pathogens. Some insect lectins are believed to bind to microbial components such as LPS, activate prophenoloxidase, and to enhance cellular immune responses by modulating haemocytes.

Insect immune-system-activation mechanisms have been uncovered using *D. melanogaster* as a model organism by genetic and molecular studies [1–5]. For that reason, further analysis of lectins in *Drosophila* will elucidate their biological role in immune systems. *Drosophila* is estimated to have more than 30 C-type lectin genes [8]. However, little is known about the participation of lectin in *Drosophila* immunity [9,10].

Previously, we purified a galactose-specific C-type lectin from a pupal extract of *Drosophila*, which agglutinated, trypsinized and glutaraldehyde-fixed bovine red blood cells [9]. This lectin is partly glycosylated and is believed to form a trimer. In addition, cDNA cloning of the lectin gene revealed that the 20 N-terminal residues out of 186 residues were a leader peptide and that most of the other residues corresponded to the carbohydrate-recognition domain of C-type lectin. Expression of the lectin gene in larvae was enhanced by body injury, suggesting that this lectin is a defence molecule.

Genomic analysis of this lectin gene suggested that the lectin gene consists of a gene cluster with the other C-type lectin genes [11]. This result implied that the original lectin gene was multiplicated, possibly to diversify or amplify its function. The biological meaning of the multiplication can be clarified through comparative analysis of these lectins and mutant analysis of this gene cluster.

MATERIALS AND METHODS

Drosophila stocks and mutagenesis

D. melanogaster stocks were kept on a standard medium at 25 °C.

The *EP(2)1173* strain was obtained from Exelixis FlyStation. The P element was transposed by crossing with *yw*; *CyO*/*Sp*; *TMS Sb P*{*ry*$^{+}$(Δ*2-3*)}(*99B*)/*TM3* jumpstarter males; the F1 male flies with both the *Sp* and *Sb* markers were crossed with *yw*; *CyO*/*Gla* balancer females. F2 flies with different eye colour from that of their progenitors were collected. Flies with the P element on the second chromosome were selected by crossing with *yw* flies. Positive F3 males were balanced by *CyO*. The transposed lines (250 lines) were screened by genomic PCR using primers for the inverted repeat of the P element and the genomic sequences around the lectin gene locus, which were designed to detect amplification of the PCR products when the P element was inserted near the respective lectin genes. The PCR product derived from a P-transposed line #914 was sequenced on subcloned fragments using a

Abbreviations used: CBB, Coomassie Brilliant Blue; FBS, foetal bovine serum; GFP, green fluorescent protein; IMD, immune deficiency; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; PGRP, peptidoglycan-recognition protein; RACE, rapid amplification of cDNA ends; RT, reverse transcription; UTR, untranslated region.

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The nucleotide sequence data reported will appear in the DDBJ, EMBL, GenBank® and GSDB Nucleotide Sequence Databases under the accession numbers AB190811 and AB190812.

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DNA Sequencing kit (Applied Biosystems). The P insertion site was mapped through comparison with the *Drosophila* genomic sequence. The P element was excised from the chromosome of #914 by crossing with the jumpstarter males again to generate the null-mutant for the lectin gene. The F1 male flies with the *Cy* marker were then crossed with w^{1118}; *CyO*/*Gla* balancer females. F2 flies with white eyes were collected and were used to initiate 79 independent P-excised lines. The P-excised lines were screened by genomic PCR using appropriate primers for genomic sequences. The PCR product derived from a P-excised line E136 was sequenced; the deletion was mapped through comparison with the genomic sequence.

We also established transgenic flies to rescue the expression of the *DL1* gene. The genomic sequence from 0.98 kb 5′-upstream to 0.04 kb 3′-downstream of the *DL1* gene was inserted into pP{CaSpeR-4}. Flies carrying this construct on the third chromosome were obtained by P element-mediated germline transformation into w^{1118} flies.

The GFP (green fluorescent protein)-tagged balancer chromosome *CyO* $P\{w^{+mC} = GAL4\text{-}Kr.C\}^{DC3}$ $P\{w^{+mC} = UAS\text{-}GFP.S65T\}^{DC7}$ [12] was used to discriminate between heterozygous and homozygous larvae for the mutation.

RT (reverse transcription)–PCR analysis

Total RNA from tissues of Canton-S flies was isolated using an RNeasy Mini Kit (Qiagen). RT–PCR was carried out according to standard procedures with gene-specific primers. First-strand cDNA was synthesized from 0.25 μg of total RNA using StrataScript reverse transcriptase (Stratagene).

cDNA and genome analysis

The cDNA for the lectin genes were obtained using PCR from a *Drosophila* adult cDNA library (Stratagene) using the primers for the lectin genes and the vector. The PCR products were sequenced on subcloned fragments as mentioned above. The 5′-region was isolated by a 5′-RACE (rapid amplification of cDNA ends) method using a Marathon cDNA amplification kit (BD Biosciences/Clontech). Genomic PCR was performed using isolated DNA from Canton-S adult flies and appropriate primers.

Northern blot analysis

Total RNA from Canton-S flies was isolated using TRIzol® reagent (Invitrogen). RNA from each sample was separated on 1.2 % agarose/formaldehyde gel and was transferred to a GeneScreen Plus membrane (NEN Life Science Products/PerkinElmer). Hybridization with the probe was performed in ExpressHyb Hybridization Solution (BD Biosciences/Clontech) at 68 °C for 2 h. The following probes [γ-^{32}P]dCTP-labelled with a random primer labelling kit (TaKaRa Holdings) were used for hybridization: *DL1* and *DL2*, 470 bp of the translational region on the second exon; *DL3*, 327 bp of the translational region on the second exon. Probes for *rp49* (ribosomal protein 49), *Cecropin A* and *Defensin* were prepared from the genomic clone. After washing with 0.1 × SSC (15 mM NaCl and 5 mM sodium citrate) containing 0.1 % (w/v) SDS, the membrane was exposed on X-ray film.

Plasmid construction and transfection experiment

Expression vectors were constructed by inserting the cDNA into pAC5.1/V5-His A (Invitrogen), which encoded the protein with V5 epitope and His_6 tags at the C-terminus. A vector inserted with the cDNA including the stop codon was also constructed to obtain the constructs encoding the intact lectins for stable transfection. *Drosophila* Schneider-2 cells (Invitrogen) were cultured at 25 °C in DES® (*Drosophila* Expression System) expression medium (Invitrogen) supplemented with 10 % FBS (foetal bovine serum), 50 units/ml penicillin and 50 μg/ml streptomycin. The cells were transiently transfected with the lectin expression vector or the β-galactosidase expression vector (pAC5.1/V5-His/lacZ; Invitrogen) by calcium phosphate transfection, followed by incubation until expression of the recombinant protein reached the maximum concentration. For stable transfections, the cells were co-transfected with the expression vector and the selection vector pCoHygro (Invitrogen). Hygromycin-B-resistant cells were selected by culture in the medium containing 300 μg/ml hygromycin B. After establishment of the stable cell lines, cells were cultured in Schneider's *Drosophila* medium (Invitrogen) supplemented with 10 % FBS, 50 units/ml penicillin, 50 μg/ml streptomycin and 300 μg/ml hygromycin B.

Detection of the recombinant lectins with anti-(His_6 tag) antibody

The transfected cells were lysed in 50 μl of 50 mM Tris/HCl, pH 7.8, containing 150 mM NaCl, 1 % Nonidet P-40, 1 mM PMSF, 1 μg/ml pepstatin A and 1 μg/ml leupeptin. Protein concentration in the lysate was measured using a protein assay kit (Bio-Rad Laboratories). Then, 10 μl of the culture medium or 36 μg of the cell lysate was separated by SDS/PAGE (15 % gels) and blotted on to an Immobilon transfer membrane (Millipore) that was subsequently incubated at 4 °C for 16 h in blocking solution [1 % (w/v) casein]. The blot was probed with His · Tag monoclonal antibody (Novagen) at room temperature (25 °C) for 1 h. A goat anti-mouse horseradish-peroxidase-conjugated antibody (Promega) was used as the secondary antibody; target protein detection was performed using ImmunoStar reagents (Wako Pure Chemical Industries).

Assay of haemagglutinating activity

The stable cell line was cultured in Schneider's *Drosophila* medium supplemented with 50 units/ml penicillin and 50 μg/ml streptomycin for 4 days. Haemagglutinating activity (titre) was expressed as in [9]. The effect of sugars on the activity was measured as the minimum concentration required for inhibition of two titres.

Detection of the recombinant intact DL1

The medium containing 20 μg of protein was separated by SDS/PAGE (15 % gels), and proteins were detected with CBB (Coomassie Brilliant Blue) stain. For detection of recombinant lectin by Western blot analysis, 10 μl of the culture medium was separated by SDS/PAGE (15 % gels), and proteins were blotted on to an Immobilon transfer membrane. It was then incubated at 4 °C for 16 h in blocking solution [5 % (w/v) dried skimmed milk]. The blot was probed with an affinity-purified rabbit anti-peptide antibody against CVKAEPFTKINDGYYFFGT-NH_2 at room temperature for 1 h. A goat anti-rabbit horseradish-peroxidase-conjugated antibody (Promega) was used as the secondary antibody, and peroxidase activity was detected as mentioned above.

Purification of the recombinant DL1

Harvested stable cells were suspended in Schneider's *Drosophila* medium supplemented with 50 units/ml penicillin and 50 μg/ml streptomycin to adjust the cell density to 6×10^6 cells/ml. The cells were then cultured for 1 week with stirring. The culture medium was filtered through a 0.22 μm pore-size filter, concentrated by ultrafiltration, then dialysed against buffered insect

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saline (10 mM Tris/HCl, pH 7.9, containing 130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$). The medium was applied to an immobilized D-galactose column (Pierce Biotechnology), and the column was then washed with the saline. Thereafter, the galactose-binding proteins were eluted with saline containing 0.2 M galactose. The elution was concentrated using Centricon-10 (Amicon), then gel-filtered with Superose 12HR10/30 using an FPLC system (Amersham Biosciences). The fractions containing the purified recombinant protein were dialysed against the saline. Proteins from each step were separated by SDS/PAGE (15 % gels) and detected by silver stain.

Determination of N-terminal amino acid sequence of the recombinant DL1

The recombinant DL1 (10 μg) was subjected to SDS/PAGE (15 % gels) and then blotted on to Immobilon-P^{SQ} transfer membrane (Millipore). After staining with CBB, the recombinant protein band was excised and analysed using a protein sequencer (PPSQ-21; Shimadzu).

Digestion with N-glycosidase

The recombinant DL1 (2 μg) was treated with *N*-glycosidase F (Roche Diagnostics) in 250 mM Tris/HCl, pH 7.9, containing 0.5 % (w/v) SDS, 50 mM 2-mercaptoethanol, 50 mM PMSF, 7 mg/ml leupeptin and 1 mM EDTA at 37 °C for 18 h. Then the protein was separated by SDS/PAGE (15 % gels) and stained with CBB.

Microbial strains

Escherichia coli K-12 W3110, *Enterobacter cloacae* (A.T.C.C. 13047), *Erwinia carotovora* [CFBP (Collection Française de Bacteries Pathogenes, 194, 1401, 1488, 2140 and 2141], *Erwinia chrysanthemi* (CFBP 1446, 2811 and 3477), *Pseudomonas aeruginosa* (A.T.C.C. 10145), *Salmonella* serotype Minnesota (A.T.C.C. 9700), *Salmonella* serotype Typhimurium [IFO (Institute for Fermentation), Osaka, Japan, 14193), *Serratia marcescens* (A.T.C.C. 13880), *Sphingomonas paucimobilis* (A.T.C.C. 29837), *Bacillus megaterium* (A.T.C.C. 14581), *Bacillus subtilis* (A.T.C.C. 6051), *Enterococcus faecalis* (A.T.C.C. 19433), *Micrococcus luteus* (A.T.C.C. 4698), *Staphylococcus aureus* (Cowan 1 strain), *Beauveria bassiana* (A.T.C.C. 9453) and *Metarhizium anisopliae* (A.T.C.C. 22099) were used. All bacterial strains except *Sphingomonas paucimobilis* were cultured at 30 °C in a medium comprising 1 % (w/v) peptone, 0.3 % (w/v) meat extract and 0.5 % (w/v) NaCl, with the pH adjusted to 7.2. *Sphingomonas paucimobilis* was cultured in brain heart infusion broth (Difco Laboratories). Fungal strains were cultured on potato dextrose agar plates.

Binding assay of DL1 to micro-organisms

Each bacterial strain or spores from fungal strain (300 μg) was washed with buffered insect saline and centrifuged at 20 000 ***g*** at 4 °C for 2 min. The harvested micro-organisms were then suspended in 30 μl of the saline containing 400 ng of recombinant DL1 and incubated at 4 °C for 1 h. After washing with 100 μl of saline three times, the micro-organism-binding proteins were released by suspension in 15 μl of saline containing 50 mM galactose or mannose. Eluted proteins were subjected to Western blot analysis with the anti-peptide antibody, as mentioned above.

Agglutination of *E. coli*

E. coli was transformed with the GFP expression vector pEGFP (BD Biosciences/Clontech). These transformants were cultured at 30 °C in a medium containing 50 μg/ml ampicillin and 0.4 mM IPTG (isopropyl β-D-thiogalactoside). *E. coli* (2×10^7 cells) was mixed with the recombinant DL1 and incubated at 4 °C for 1 h. The bacteria were harvested, then washed with buffered insect saline. The bacteria were fixed with 4 % (w/v) paraformaldehyde, washed with the saline, and then applied to a flow cytometer (EPICS Elite ESP; Beckman Coulter). The mean GFP fluorescence intensity of the 10 000 counts was calculated.

Flow-cytometric analysis of the association of *E. coli* with mbn-2 cells

Drosophila mbn-2 cells were cultured at 25 °C in Schneider's *Drosophila* medium supplemented with 12 % FBS, 100 units/ml penicillin and 100 μg/ml streptomycin. The cell suspension was diluted with fresh medium to adjust the cell density to 8×10^5 cells/ml. Then the culture was continued for 3–4 days. For experiments, 2×10^6 mbn-2 cells were harvested and resuspended in 1.8 ml of Schneider's *Drosophila* medium supplemented with 170 μg/ml chloramphenicol instead of penicillin and streptomycin. The GFP-labelled *E. coli* (2×10^8 cells) were washed with Schneider's *Drosophila* medium, then harvested by centrifugation at 800 ***g*** for 3 min and resuspended in 0.2 ml of medium containing the recombinant DL1 and 50 mM galactose or xylose. Xylose was used instead of mannose as a non-hapten sugar because mannose had inhibited the DL1-independent association of mbn-2 cells with *E. coli* (results not shown). The suspension was incubated at 4 °C for 1 h, mixed with a suspension of mbn-2 cells and then incubated at 27 °C for 10 min with rotating. The harvested mbn-2 cells were washed with buffered insect saline, fixed with 4 % (w/v) paraformaldehyde, washed with the saline and then resuspended in 1 ml of the saline containing 10 μg/ml propidium iodide. Then the suspension was applied to a flow cytometer. The mean GFP fluorescence intensity of the 10 000 propidium-iodide-positive counts was calculated. The *E. coli* cells that were agglutinated, but not associated with mbn-2 cells, were readily discernible from mbn-2 cells by the propidium iodide fluorescence intensity.

Southern blot analysis

Genomic DNA (5 μg) prepared from third instar larvae was digested with 30 units of PstI (TaKaRa Holdings) for 18 h; it was then separated by agarose gel electrophoresis. The DNA was transferred to a GeneScreen Plus membrane. After UV cross-linking, hybridization with the probe was performed in ExpressHyb hybridization solution at 60 °C for 2 h. The same probes as those for Northern blot analysis were used for hybridization. After washing with 0.1 × SSC containing 0.1 % (w/v) SDS, the membrane was exposed on X-ray film.

Infection experiment

Natural infection of third instar larvae with *E. coli* was performed following the method of Basset et al. [13] for phytopathogenic bacteria *Erwinia carotovora*.

RESULTS

A gene cluster of three C-type lectin genes at 37D6 on the genome of *D. melanogaster*

The Berkeley *Drosophila* Genome Project revealed that the *Drosophila* genome has more than 30 C-type lectin-like genes [8]. Among these genes, the *CG33532* and *CG33533* genes, known together as the *CG9978* gene, are localized next to the galactose-specific C-type lectin gene locus examined in [9]. To find out whether the predicted genes were expressed, we performed

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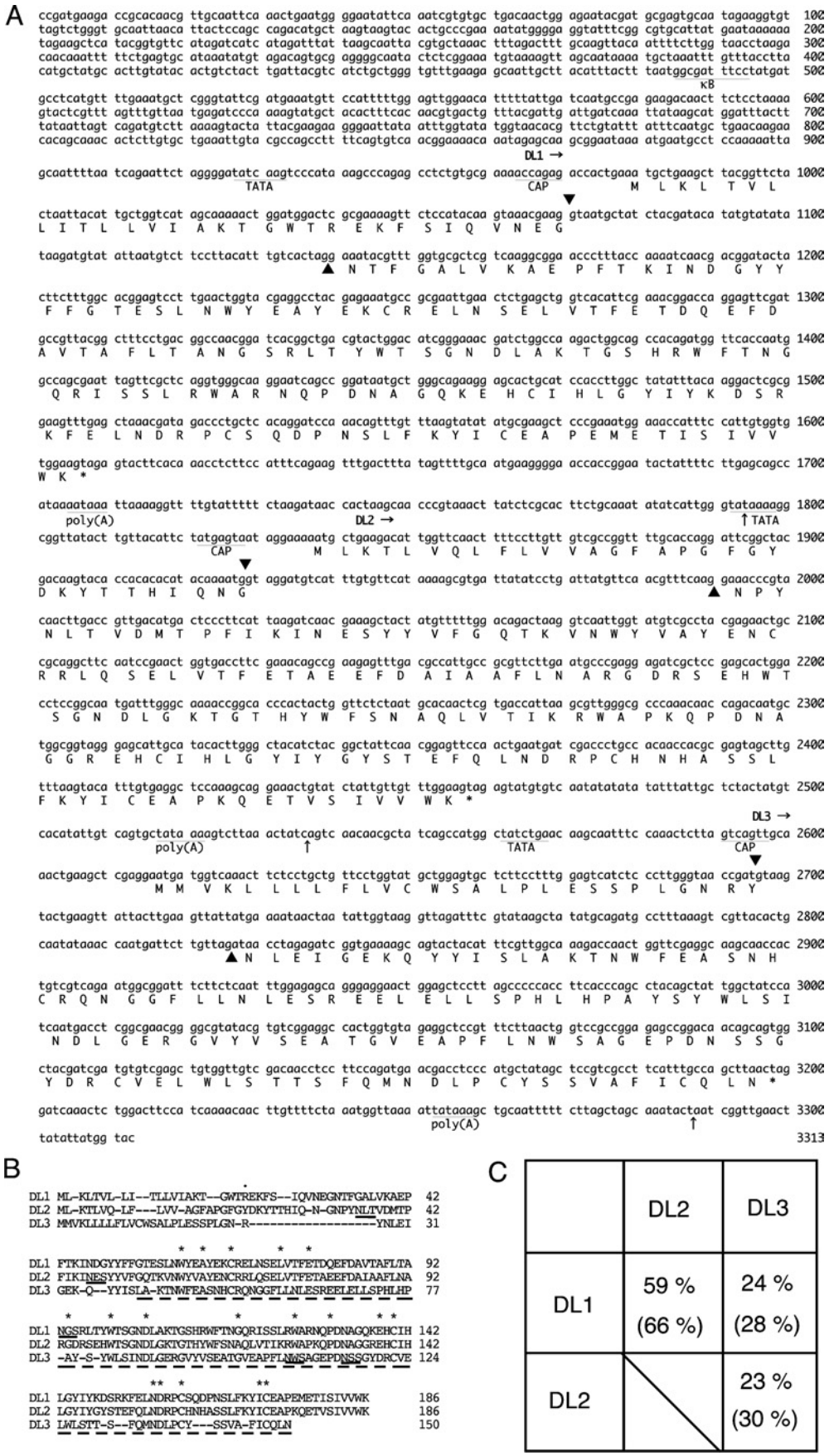

Figure 1 **For legend see facing page**

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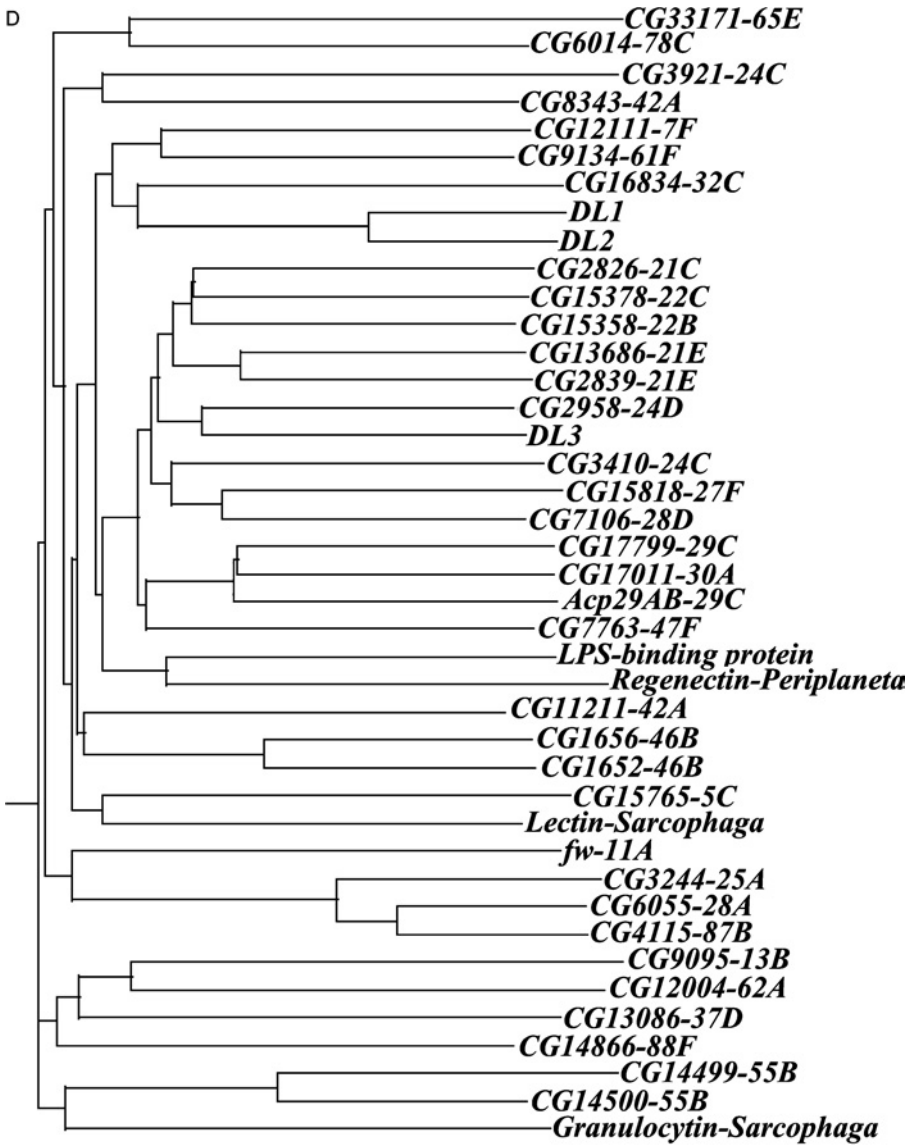

Figure 1 Gene structure of *Drosophila* lectins on 37D

(**A**) Genomic sequence of the lectin genes locus. Intron splice sites are indicated by ▲ for donor and acceptor sites. Possible NF-κB site (κB), and TATA, cap and polyadenylation [poly(A)] signal sequences are dotted underlined. *DL1*, *DL2* and *DL3* are translated using one-letter symbols below their respective exons. The polyadenylation sites are indicated with arrows. (**B** and **C**) Comparison of the amino acid sequences of *DL1*, *DL2* and *DL3*. (**B**) Gaps are introduced to obtain maximum matching. The N-terminal residue of *DL1* is indicated by a dot. Residues corresponding to the carbohydrate-recognition domain are broken-underlined. Asterisks indicate residues conserved among various C-type lectins. Possible N-glycosylation sites are indicated by solid underlining [34]. (**C**) Identities between the lectins at the amino acid level. Identities of the carbohydrate-recognition domains are shown in parentheses. (**D**) Phylogenetic analysis of *Drosophila* C-type lectins. Carbohydrate-recognition domains of these proteins were aligned using the ClustalW program. Based on alignments, a phylogenetic tree was constructed using the dendrogram method. Gene names are shown with cytological locations. For comparison, C-type lectins identified from *Sarcophaga peregrina* [35,36] and *Periplaneta americana* [37,38] were analysed together.

RT–PCR analysis using Canton-S third instar larvae, pupae and adults. Gene-specific amplifications of both carbohydrate-recognition domains were detected during those developmental stages (results not shown).

To elucidate the complete structure of these genes, we obtained their corresponding cDNAs from an adult cDNA library of Canton-S, and the genome fragments from Canton-S adults. We also performed 5′-RACE to get the 5′-terminal region. The intron splice sites and the plural polyadenylation sites were identified through comparison of the cDNA and genome sequences. As shown in Figure 1(A), we named these three lectins on 37D6 as *DL1* (identical with the lectin studied in [9]), *DL2* (*CG33532*) and *DL3* (*CG33533*), according to their order on the genome. Each cDNA consists of the short first exon and the second exon containing the carbohydrate-recognition domain. Each of the three genes has a promoter-like sequence with a TATA box, a cap site at the upstream of the coding region [14], and a polyadenylation signal sequence at the downstream. The gaps between these lectin genes were very short; the putative TATA sequence for the *DL2* genes overlaps the 3′-UTR (untranslated region) of the *DL1* gene, and only 25 bp of nucleotides exist between the polyadenylation site for the *DL2* genes and the putative TATA sequence for the *DL3* gene.

The amino acid sequences of three lectins are compared in Figures 1(B) and 1(C). The number of amino acid residues of DL2 is equal to that of DL1; their identity is 59 % in the

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full-length and 66 % in the carbohydrate-recognition domain. Their homology is extremely high, as shown by phylogenetic analysis of *Drosophila* C-type lectins (Figure 1D). In contrast, DL3 has fewer amino acid residues, and DL3 has relatively low homology with DL1 and DL2. The hydrophobic N-terminal regions of DL2 and DL3, like DL1 [9], suggest that they are also secreted proteins.

Expression analysis of the lectin genes

We analysed expression during development using Northern blotting to compare expression of the *DL1*, *DL2* and *DL3* genes. As reported previously [9], expression of the *DL1* gene was detected from the larval to adult stage (Figure 2A). When probed for the *DL2* gene, 0.8 and 1.6 kb RNA bands were found; both bands were detected from the late pupal stage. Expression of the *DL3* gene was detected only at the adult stage. For all three lectin genes, 0.8 kb bands were consistent with their gene structures, although the structure for the additional minor 1.6 kb bands of the *DL2* gene was unclear. Three lectin genes all showed the highest expression at the adult stage, but the expression at the pupal stage was different between the *DL1* and *DL2* genes: the expression of the *DL1* gene was higher at the early pupal stage than at the late pupal stage, but the expression of the *DL2* gene was higher at the late pupal stage. These results indicate that the expression of these lectin genes was differentially regulated. As *DL1* gene expression has been known to be enhanced on injury at the larval stage [9], we also examined whether *DL2* and *DL3* gene expression was changed on injury or septic injury with *E. coli* at larval and adult stages; no change was detected (results not shown).

We also investigated the expression of these lectin genes in various tissues. During the larval stage, the expression of all three genes was detectable by RT–PCR. Figure 2(B) shows that the expression of the *DL1* gene was detected in all examined tissues except the midgut and Malpighian tubules. On the other hand, the expression of the *DL2* and *DL3* genes was relatively tissue-specific. The expression of these genes was detected in cuticle and muscles, midgut and Malpighian tubules, and fat bodies. Additional expression of the *DL3* gene was also detected in haemocytes. These results indicate that the expression pattern of the *DL1* gene differs greatly from that of the *DL2* and *DL3* genes at the larval stage.

At the adult stage, expression of the *DL1* gene was detected in cuticle and muscles, ovaries, and testes, but not in the midgut or Malpighian tubules (Figure 2C). However, expression of the *DL2* and *DL3* genes was detected in midgut or Malpighian tubules. Differences in *DL2* and *DL3* gene expression were found, especially in reproductive organs; the *DL2* gene was expressed in ovaries, but the *DL3* gene was expressed in testes. These results indicate that the expression patterns of the *DL1*, *DL2* and *DL3* genes are also different in the adult stage.

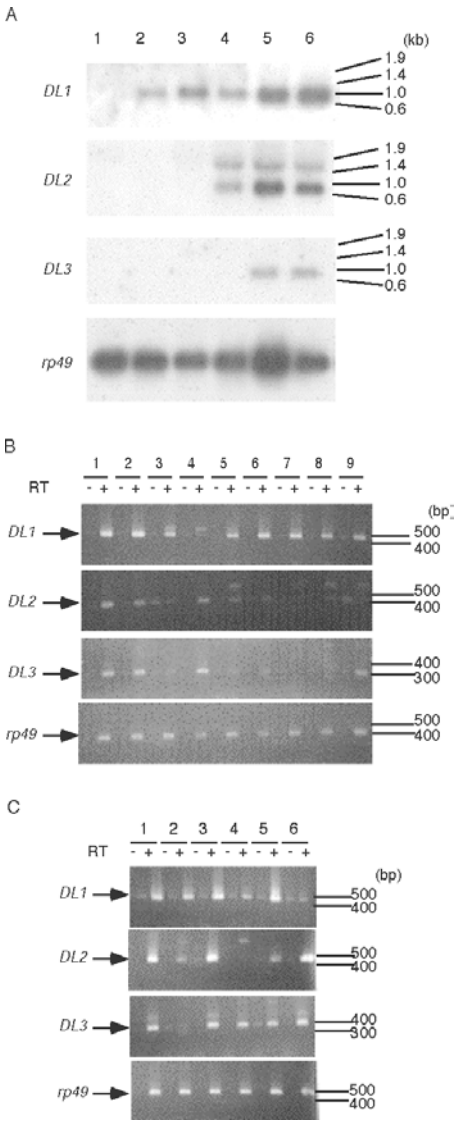

Figure 2 Comparisons of expression profiles of the lectin genes

(**A**) Northern blot analysis of the expression of the lectin genes during development. Total RNA was extracted from *Drosophila* Canton-S at different developmental stages and then subjected to Northern blot hybridization. RNA was extracted from embryos (lane 1), third instar larvae (lane 2), early pupae (lane 3), late pupae (lane 4), female adults (lane 5) and male adults (lane 6). The *Drosophila rp49* gene probe was used as a loading control. Sizes are indicated in kb. (**B** and **C**) RT–PCR analysis of the expression of lectin genes in *Drosophila* tissues. Representative RT–PCR for the lectin genes and the *rp49* gene is shown. Sizes are indicated in bp. (**B**) RT–PCR for larval tissues. Total RNA from the whole body (lane 1), cuticle and muscles (lane 2), salivary glands (lane 3), midgut and Malpighian tubules (lane 4), fat body (lane 5), tracheae (lane 6), brain and eye-antennal discs (lane 7), leg discs and wing discs (lane 8), and haemocytes (lane 9) were analysed. (**C**) RT–PCR for adult tissues. Total RNA from female whole body (lane 1), ovaries (lane 2), male whole body (lane 3), testes (lane 4), cuticle and muscles (lane 5), and midgut and Malpighian tubules (lane 6) were analysed. Arrows indicate positions of gene-specific amplification.

Localization of the recombinant lectins at the transfected cells

The lectins expressed in fat body and haemocytes are expected to be secreted proteins to haemolymph [7]. To determine the secretion of DL1, DL2 and DL3, we transfected *Drosophila* Schneider-2 cells with the expression vector for each lectin and localization of the recombinant protein with the His_6 tag was analysed. Figure 3(A) shows that the recombinant DL1, DL2 and DL3 were all detected mainly in the culture medium on the condition that β-galactosidase, transfected as a control, was detected in the cell lysate as a 120 kDa band. This result indicates that these lectins are all secreted proteins, as suggested by hydropathy profiles of these proteins.

Recombinant proteins of DL1 and DL2 were detected as having a larger molecular size than that of DL3. That result is consistent with the estimated values of the deduced amino acid sequences from their cDNAs. Two bands of DL3 suggest the existence of the N-glycosylated form, which is supported by the existence of possible N-glycosylation sites, as indicated in Figure 1(B).

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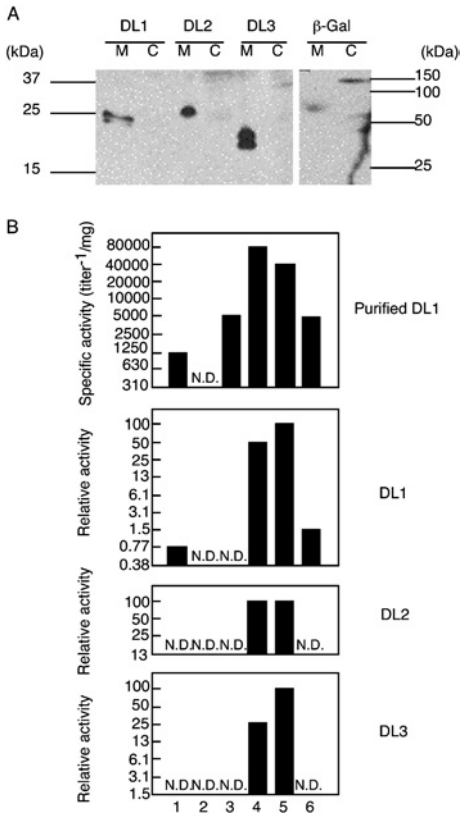

Figure 3 Comparisons of localization and haemagglutinating activity of lectins

(**A**) Expression of recombinant proteins from the transfected Schneider-2 cells. From the culture medium (M) or cell lysate (C) of the transiently transfected cells with the expression vector for each lectin or the β-galactosidase expression vector (β-Gal), recombinant proteins were detected using Western blot analysis using anti-(His_6 tag) antibody. (**B**) Agglutinating activity of the recombinant intact lectins against red blood cells from various species. Culture media from the stably transfected Schneider-2 cells were serially diluted and mixed with bovine (lane 1), chicken (lane 2), horse (lane 3), porcine (lane 4), rabbit (lane 5) or sheep (lane 6) red blood cells. Results of the purified DL1 are also shown (see Figure 4). The highest level of activity of the culture medium in a series was normalized as 100; the representative results are given as relative activity (percentages). N.D. means not detected (<630, <0.77, <25 and <3.1 titre$^{-1} \cdot$ mg^{-1} for the purified *DL1* and the culture medium from the Schneider-2 cells transfected with expression vectors for *DL1*, *DL2* and *DL3* respectively). No haemagglutinating activities were detected when the culture medium from Schneider-2 cells transfected with the mock expression vector was analysed.

Activity of the recombinant lectins

We stably transfected Schneider-2 cells to make them secrete each recombinant intact lectin constitutively. We thereby determined the activity of these lectins. We investigated haemagglutinating activity of the culture medium using bovine, chicken, horse, porcine, rabbit and sheep red blood cells. We clarified that the recombinant DL1 had agglutinating activity against porcine and rabbit red blood cells (Figure 3B). Weaker agglutinating activity, or none at all, was detected when the other red blood cells were used. Activity against bovine red blood cells was not detected because the cells were not treated with trypsin and glutaraldehyde, as in [9]. Similar results were obtained when a culture medium containing recombinant DL2 or DL3 was examined, suggesting that these lectins had binding activity with similar specificity to polysaccharide chains. Furthermore, haemagglutinating activities of these lectins were all inhibited by addition of EDTA, indicating that they were C-type lectins.

Table 1 Competing effects of sugars on agglutinating activity of the recombinant lectins against rabbit red blood cells

The assay was performed with sugars at concentrations of 1, 3, 10, 30 and 100 mM.

	Minimum concentration (mM)			
Sugar	Purified DL1	DL1	DL2	DL3
D-Galactose	3	3	3	3
Raffinose	3	3	3	3
Lactose	10	10	10	3
D-Glucose	100	30	100	30
D-Xylose	100	30	100	30
D-Mannose	>100	>100	>100	>100
Fructose	>100	>100	>100	>100
Sucrose	>100	>100	>100	>100
Trehalose	>100	>100	>100	>100
N-Acetylgalactosamine	>100	>100	>100	>100
N-Acetylglucosamine	>100	>100	>100	>100

Next, we examined the effect of sugars on the haemagglutinating activity of these lectins. Table 1 shows that the activity of the recombinant DL1 was inhibited by galactose or oligosaccharides containing galactose (raffinose and lactose). Similar results were obtained when the culture medium containing the recombinant DL2 or DL3 was examined, suggesting that all of these lectins are galactose-specific.

Preparation of recombinant DL1

DL1, DL2 and DL3 have similar haemagglutinating activity. For that reason, we focused our attention on one lectin, DL1, for further biochemical characterization. We expressed the recombinant protein of DL1 in the stably transfected Schneider-2 cells to obtain a large amount of DL1. From the culture medium, additional 19 kDa and 20 kDa proteins were detected, as shown in Figure 4(A) (lane 2). Both 19 and 20 kDa proteins were detected using Western blot analysis with the anti-peptide antibody for DL1 (Figure 4A, lane 4). As reported previously [9], the native DL1 is partly N-glycosylated, therefore the 20 kDa protein seemed to be the N-glycosylated form of the 19 kDa protein. We purified the recombinant protein by affinity chromatography on a galactose column, followed by gel-filtration; thereby, both 19 and 20 kDa proteins were purified (Figure 4B).

As is evident from Figure 4(C), because of N-glycosidase treatment, the 20 kDa band disappeared and the 19 kDa band was intensified, indicating that the 20 kDa protein is a glycosylated form of the 19 kDa protein. The N-terminal residue of the purified lectin was determined as arginine at position 21 from the first methionine, which was the same as the native DL1 [9].

This recombinant protein had agglutinating activity against porcine and rabbit red blood cells, which was similar to the unpurified culture medium of the transfectant (Figure 3B). Table 1 shows that effects of various sugars on the haemagglutinating activity of the purified recombinant DL1 were identical with that of the native protein purified from *Drosophila*. Therefore this purified recombinant DL1 is available for *in vitro* investigation of the biochemical function of the lectin.

Binding of DL1 to bacteria

We examined the binding activity of DL1 to micro-organisms. Each bacterial strain was mixed with the purified recombinant DL1. Then, the binding lectin was eluted with galactose (a hapten sugar) or mannose, followed by Western blot analysis to detect the lectin eluted from bacteria. Of the nine Gram-negative and five

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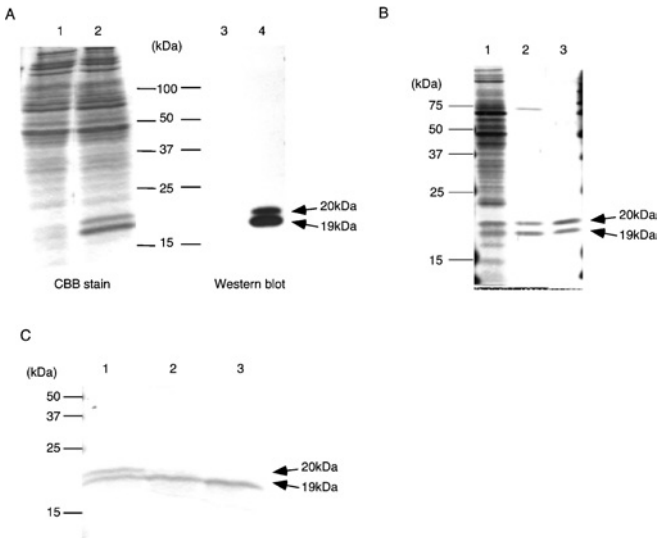

Figure 4 Preparation of recombinant DL1

(**A**) Constitutive expression of the recombinant DL1 from the culture medium of the stable transfectants of Schneider-2 cells. Culture medium of the stable transfectant with mock (lanes 1 and 3) or the expression vector for the intact DL1 (lanes 2 and 4) was separated by SDS/PAGE (15 % gels), followed by CBB staining (lanes 1 and 2) or Western blot analysis using anti-peptide antibody for DL1 (lanes 3 and 4). (**B**) Purification of the recombinant DL1. The culture medium of the stable transfectant of Schneider-2 cells with the expression vector for DL1 (lane 1), proteins purified from the culture medium with galactose-affinity column (lane 2) and proteins subsequently purified using gel-filtration (lane 3) were separated by SDS/PAGE (15 % gels), followed by silver staining. (**C**) Digestion of the purified recombinant DL1 with N-glycosidase. Purified lectin (10 μg) was digested with increasing amounts of N-glycosidase F for 18 h at 37 °C. Then the mixtures were subjected to electrophoresis under denaturing conditions. Amounts of N-glycosidase used were: lane 1, 0 units; lane 2, 0.3 unit; lane 3, 1 unit. Arrows indicate positions of the 19 and 20 kDa proteins. Molecular-mass sizes are indicated in kDa.

Gram-positive bacterial species that we examined, the binding lectin was detected when *E. coli* (K-12 W3110) was used (Table 2 and Figure 5A). Binding with *Erwinia chrysanthemi* strains, which are phytopathogenic bacteria that can infect *Drosophila* naturally [13], was also detected. Nevertheless, the binding was weaker than that with *E. coli*. Furthermore, the binding lectin to *E. coli* was not eluted with mannose, indicating that binding was dependent on the activity as a lectin (Figure 5A). We also tested spores from two fungal strains, but the binding lectin was not detected (Table 2).

The native DL1 forms a homotrimer [9]. Therefore it is likely that the DL1 protein binds bacteria and then agglutinates it. For that reason, we examined whether or not the lectin agglutinated *E. coli*. The GFP-labelled *E. coli* was mixed with the purified recombinant DL1, before measuring the fluorescence intensity using a flow cytometer. Figure 5(B) shows that the mean fluorescence intensity increased by the addition of the lectin in a dose-dependent manner. The total fluorescence intensity of the bacterial suspension was unaffected by the addition of the lectin (results not shown). Consequently, increased fluorescence intensity means an increase in the number of *E. coli* cells per count: the *E. coli* cells were agglutinated. Agglutination of *E. coli* was inhibited completely by galactose, but not by mannose, suggesting that the effect was dependent on the activity of DL1 as a lectin.

Effect of DL1 on the association of mbn-2 cells with *E. coli*

In an innate immune system, bacterial aggregation by lectin often engenders bacterial clearance by scavenger cells [7]. For that reason, we examined whether the binding of DL1 to *E. coli* affected the association of haemocytes, *Drosophila* blood cells, with *E. coli* or not. The GFP-labelled *E. coli* was mixed with and without the purified recombinant DL1, then added to cultured mbn-2 cells, a *Drosophila* haemocyte-derived cell line. The GFP fluorescence associated with mbn-2 cells was measured using a flow cytometer, and the mean fluorescence intensity of 10000 mbn-2 cells was calculated. Figure 6(A) shows that the mean fluorescence intensity associated with mbn-2 cells was increased by the addition of the lectin in a dose-dependent manner. That

Table 2 Detection of the binding of DL1 to microbial strains

+, Binding detected; −, binding not detected.

Strain	Binding
Gram-negative bacteria	
Escherichia coli	+
Enterobacter cloacae	−
Erwinia carotovora	
CFBP194	−
CFBP1401	−
CFBP1488	−
CFBP2140	−
CFBP2141	−
Erwinia chrysanthemi	
CFBP1446	+
CFBP2811	+
CFBP3477	+
Pseudomonas aeruginosa	−
Salmonella typhimurium	−
Salmonella minnesota	−
Serratia marcescens	−
Sphingomonas paucimobilis	−
Gram-positive bacteria	
Bacillus megaterium	−
Bacillus subtilis	−
Enterococcus faecalis	−
Micrococcus luteus	−
Staphylococcus aureus	−
Fungi	
Beauveria bassiana	−
Metarhizium anisopliae	−

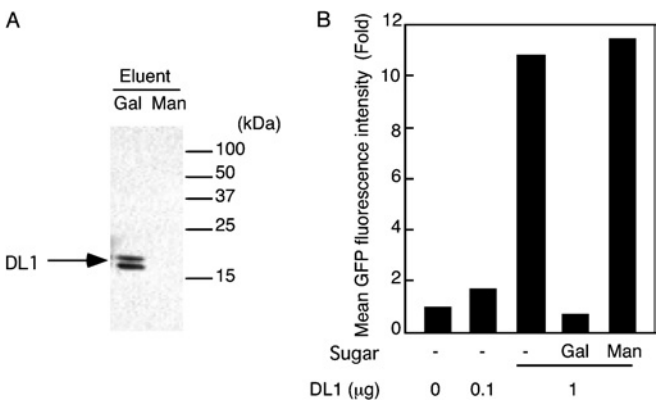

Figure 5 Binding of DL1 to *E. coli*

(**A**) Detection of the bound DL1 to *E. coli*. The purified recombinant DL1 (400 ng) was mixed with 300 μg of bacteria. Binding lectin was then eluted with 50 mM sugars. The eluted lectin was detected using Western blot analysis. An arrow indicates the position of DL1. Gal, eluted with galactose; Man, eluted with mannose. Sizes are indicated in kDa. (**B**) Agglutination of *E. coli* with DL1. Increasing amounts of the purified recombinant DL1 were mixed with 2×10^7 cells of the GFP-labelled *E. coli*; the mean GFP fluorescence intensity from the representative experiment was then measured using a flow cytometer. Inhibitory effects by 50 mM sugars are also shown. Gal, galactose; Man, mannose.

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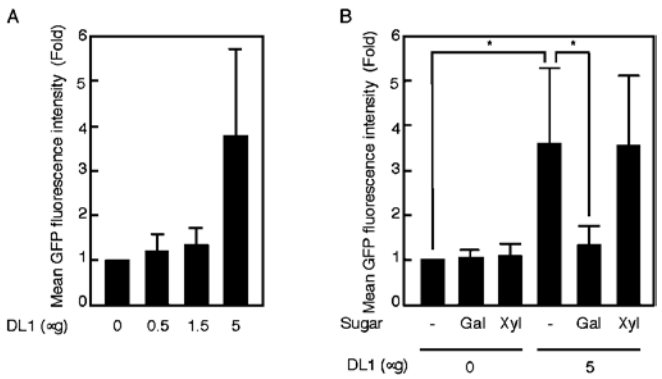

Figure 6 Effect of DL1 on the association of mbn-2 cells with *E. coli*

(**A**) Increasing amounts of the purified recombinant DL1 was mixed with 2×10^8 cells of the GFP-labelled *E. coli*; then the mixture was mixed with 2×10^6 mbn-2 cells. The mean GFP fluorescence intensity associated with mbn-2 cells was measured using a flow cytometer. Results are means ± S.D. ($n = 3$). (**B**) Inhibition of the effects of 50 mM sugars on the association of DL1 with *E. coli*. Gal, galactose; Xyl, xylose. Results are means ± S.D. ($n = 4$); $^*P < 0.05$.

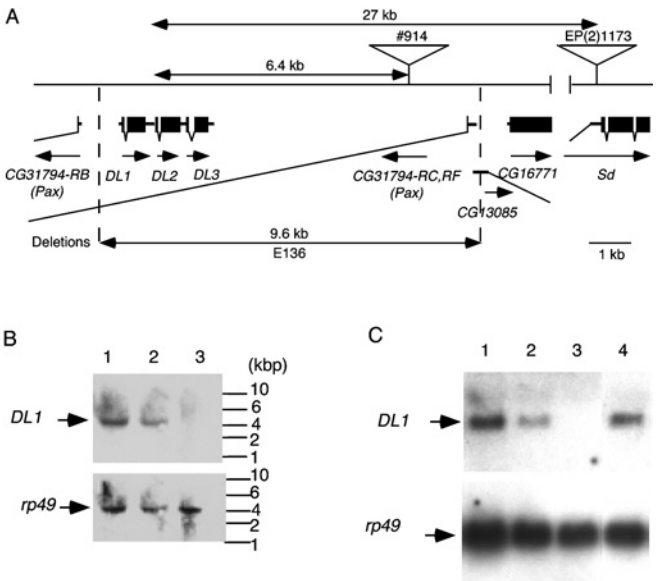

Figure 7 Generation of the deletion mutant of the lectin gene locus

(**A**) Genomic map of 37D, the mutant and the progenitors. The #914 line was generated by local transposition of the P element from *EP(2)1173*, followed by generation of E136 by imprecise excision. Transcripts of the *DL1* gene and the neighbouring genes are shown. Coding parts of the transcripts are shown as closed boxes; UTRs are shown as bars. Southern (**B**) and Northern (**C**) blot analysis of the *DL1* gene. In the upper panel, the filter was probed for *DL1* expression. In the lower panel, the same filter was reprobed with the *rp49* probe as a loading control. Lane 1, w^{1118}; lane 2, w^{1118}; $DL1^{E136}/CyO$; lane 3, w^{1118}; $DL1^{E136}/DL1^{E136}$; lane 4, w^{1118}; $DL1^{E136}/DL1^{E136}$; P{*DL1*}/P{*DL1*}. (**B**) Southern blot analysis of the *DL1* gene. Sizes are indicated in kb. (**C**) Northern blot analysis of the *DL1* gene. Total RNA isolated from the third instar larvae was analysed.

effect was inhibited markedly by galactose, but not by xylose, suggesting that the effect was dependent on the activity as a lectin, which was the same as the binding of the lectin to the bacteria (Figure 6B). That result strongly suggests that the binding of DL1 to *E. coli* enhanced the association of haemocytes with the bacteria.

Generation of the lectin locus deletion mutant

The lectin genes *DL1*, *DL2* and *DL3* localize at 37D on the second chromosome. From a database, we found the P-insertion line *EP(2)1173* in which the P element was inserted 27 kb 3′-downstream of the lectin genes (Figure 7A). The P-insertion site was on the 5′-UTR of the *Sd* gene. We used the $P\{ry^+(\Delta 2\text{-}3)\}(99B)$ jumpstarter element to mobilize the inserted P element and screened transposed lines for novel insertion sites by PCR analysis to generate the mutant [15]. From 250 transposed lines on the second chromosome, one line, named #914, carried the P element 6.4 kb 3′-downstream of the *DL1* gene (Figure 7A).

Homozygous flies for the #914 chromosome reached adulthood, but died soon after emerging from pupae. This phenotype reverted to wild-type after the precise excision of the transposed P element. This phenotype was mapped genetically to the cytological area containing the P-insertion site by deficiency mapping. These results indicate that the P-insertion at the site caused the phenotype.

We mobilized the inserted P element from the #914 line and used PCR analysis to screen the excision lines for deletions, thereby generating the null-mutant for the lectin genes. From 79 excised lines, one line, named E136, carried approx. 9.6 kb of deletions that covered the *DL1*, *DL2* and *DL3* genes (Figure 7A). The deletion site was determined precisely by sequencing the PCR product obtained from the analysis. This line also lacked the first exon of the *CG31974-RC* and *-RF* genes (*Paxillin*) and the part of the first exon of the *CG13085* gene, a predicted gene whose expression and function are unknown.

Deletion of the *DL1* gene from the E136 fly genome was confirmed using Southern blot analysis (Figure 7B). Figure 7(C) shows that the expression of the lectin gene in the mutant larvae was not detected by Northern blot analysis. These results indicate that the E136 line is the null-mutant for the *DL1* gene.

Homozygous flies for the E136 chromosome, just as with the #914 line, reached adulthood, but the adults died soon after emerging from pupae. This phenotype was mapped to the cytological area containing the P-excised site by deficiency mapping, suggesting that the P-insertion and the following excision caused the phenotype. In addition, the phenotype was not rescued by the transgene of the *DL1* gene. For that reason, the lethality was not likely to be the result of *DL1* mutation; rather, it was caused by the other neighbouring gene's mutation.

Persistence of *E. coli* in the E136 larvae

We examined whether the E136 homozygous larvae had some defect in their defence against bacteria. As shown in Figure 5(A), DL1 recombinant protein strongly bound to an *E. coli* strain, so we considered the possibility that the lectin participated in eliminating the infecting bacteria. The E136 homozygous adult flies die soon after emerging from pupae, so we focused our attention on the immune response during the larval stage. *Drosophila* larvae are known to induce expression of various antibacterial peptide genes in response to infection with Gram-negative bacteria [1–5]. Expression was induced by IMD pathway activation, which is indispensable for defence against infection by Gram-negative bacteria [16]. We examined the expression of antibacterial peptide genes after infection with *E. coli*.

Merely pricking the body wall of *Drosophila* larvae induces the immune response [17]; we could not detect a difference in the induction of antimicrobial peptide genes by septic injury between the E136 larvae and wild-type (results not shown). Therefore we examined whether we could infect *Drosophila* larvae with the *E. coli* strain naturally, as do phytopathogenic bacteria *Erwinia carotovora*, to avoid pricking [13]. Expression of the gene for an antibacterial peptide Cecropin A1 was detected in fat body by the coexistence of larvae with the bacteria, indicating

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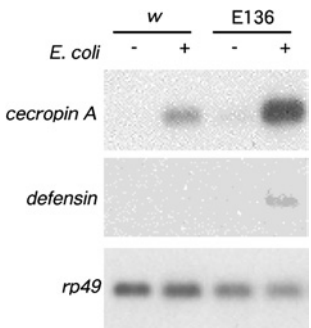

Figure 8 Expression of the antibacterial peptide genes after natural infection with *E. coli* in the lectin deletion mutant larvae

Total RNA was isolated from the third instar larvae of w^{1118} (*w*) or the *DL1* mutant E136 3 h after infection with (+) or without (−) bacteria. Probes: *Cecropin A* (top panel), *Defensin* (middle panel) and *rp49* as a loading control (bottom panel).

the natural infection of the *Drosophila* larvae with the *E. coli* strain and the consequent systemic immune response (results not shown).

At that time, we infected the E136 larvae naturally with *E. coli*, and examined the expression of antibacterial peptide genes. Figure 8 shows that expression of the *Cecropin A* gene was detected in wild-type larvae 3 h after infection. The *Cecropin A* gene expression in the E136 larvae was more intensified than in the wild-type larvae. Such enhanced expression in the E136 larvae was also detected in another antibacterial peptide gene: *Defensin*. These results indicate that DL1 does not participate in the immune response by induction of the antibacterial peptides.

DISCUSSION

We analysed the expression and activity of three lectins arranged as a gene cluster at 37D on the genome of *D. melanogaster*. The genome structure and similarity of the sequences of these three genes implies that an original ancestral gene might be duplicated to create the *DL3* gene and another gene, and that the latter gene was duplicated again to produce the *DL1* and *DL2* genes later on.

We showed that these three lectins are secreted galactose-specific C-type lectins and have similar binding specificities to the carbohydrate chains on red blood cells. The result for DL2 is exactly that expected from the high homology with DL1. However, the result for DL3 is not expected because DL3 has, at most, 30 % identity in the carbohydrate-recognition domain with DL1 and DL2. Regarding *Sarcophaga* lectin, its carbohydrate-recognition domain had 30 % identity with that of DL1, but *Sarcophaga* lectin showed high agglutinating activity against sheep red blood cells [18], which is different from that of DL1. Apparently, the multiplication of three genes at the locus does not produce variation in activity as haemagglutinin.

In spite of their similar activity, the expression patterns of these lectin genes differed markedly. First, expression of the *DL1* gene was higher in the early pupal stage than in the late pupal stage, whereas the expression of the *DL2* gene was higher at the late pupal stage. Secondly, in the midgut and Malpighian tubules at the larval stage, the *DL1* gene was not expressed at detectable levels, but the *DL2* and *DL3* genes were. In contrast, in some other tissues where the *DL1* gene was highly expressed, the *DL2* and *DL3* genes were not expressed. Finally, the expression of these genes in the reproductive organs differed from each other. These results are surprising because these lectin genes are adjacent to each other on the genome, and the specific regulation of these genes using the sequences on the gaps was thought to be improbable. Each gene's expression mechanism is yet to be elucidated, but the upstream and downstream sequences of the gene cluster are thought to act as *cis*-elements. Generation of lectins with different expression patterns presents the possibility of extending the original lectin function. It is an attractive question how the variation of the expression pattern was obtained following lectin gene multiplication.

Regulation of insect C-type lectin genes is not yet well characterized. It is noteworthy that a typical NF-κB (nuclear factor κB)-binding site exists upstream of the *DL1* gene up-regulated by body injury; it is a common regulatory element among various insect immune genes [1–5,19,20]. Previously, we reported that the *Sarcophaga* lectin promoter was activated in various digestive tissues and fat body of transgenic *Drosophila* [21]. Common features of the regulation of insect lectin genes may be revealed in further investigations.

The *DL1*, *DL2* and *DL3* genes were expressed in various larval tissues including fat body and haemocytes, which are known as tissues that secrete various proteins to haemolymph. These lectins are secreted proteins, suggesting the localization of these lectins in haemolymph, which is similar to those of many lectins found in insects [6,7]. In haemocoel, the systemic immune system functions along with both humoral and cellular immune responses against invading pathogens. These results suggest that these lectins participate in immune responses.

By virtue of the *D. melanogaster* genome project, the *Drosophila* genome is predicted to encode more than 30 C-type lectins [8]. The predicted lectin genes are distributed to all chromosomes except the fourth and Y chromosomes, and many members of this family also arrange as gene clusters [11]. The possibility exists that they have a common ancestor until a recent stage in evolution. Comparative analysis of these *Drosophila* C-type lectin genes will shed light on the elucidation of the evolution of C-type lectins.

We demonstrated that DL1 bound to an *E. coli* and some *Erwinia chrysanthemi* strains. The most abundant polysaccharide chains on the surface of Gram-negative bacteria were those of LPS. However, haemagglutinating activity of DL1 against rabbit red blood cells was not inhibited by addition of commercially available LPS (results not shown), suggesting that DL1 did not bind to them via LPS, but via other polysaccharide chains on their surfaces.

In some other insect species, multiple lectins have been identified. In American cockroach, *Periplaneta americana*, a C-type lectin named LPS-binding protein binds to LPS from *E. coli*, but not to LPS from *Salmonella* serotype Minnesota [22]. On the other hand, another C-type lectin named *Periplaneta* lectin binds to both of them when their 2-oxo-3-deoxyoctonate residues are exposed [23]. Both lectins have been suggested to function as opsonins upon infection [23,24]. In another cockroach, *Blaberus discoidalis*, three C-type lectins with different sugar specificities and a β-1,3-glucan-specific lectin have been identified [25,26]. They promote the activation of the prophenoloxidase system and phagocytosis against different spectra of micro-organisms [27,28]. Therefore, although DL1 binds to the restricted strains of bacteria, other C-type lectins could recognize other microbial components in the same way. The C-type lectin family in *Drosophila* may recognize many strains of pathogens collectively.

Furthermore, DL1 increased the association of the *Drosophila* haemocyte-derived cell line with *E. coli*. Probably by agglutinating *E. coli* in haemolymph of *Drosophila*, a larger number of *E. coli* can be trapped by haemocytes via the receptor for *E. coli* on the cell surface, such as dSR-C1 [29]. The present paper makes the first biochemical suggestion of lectin participation in immunity in *D. melanogaster*.

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We also established the null-mutant for the *DL1/DL2/DL3* gene locus. However, expression of antibacterial peptide genes was not impaired in the E136 larvae, suggesting that DL1 does not participate in the elimination of bacteria by the induction of antibacterial peptide genes. Enhancement of the expression of the antibacterial peptide genes in the E136 larvae is likely to result from the increased number of bacteria that persist in the larvae, rather than the loss of the inhibitory effect of the *DL1* gene on expression, since more infected bacteria were detected in the mutant larvae at the early stage of infection (results not shown). Although we could not have shown in the present study the function of DL1 in humoral immune response *in vivo*, cellular immune responses such as phagocytosis might be affected by the *DL1* gene mutation, possibly through the decreased number of associated bacteria with haemocytes as shown in Figure 6. Further analyses using the mutant that we established might lead to the elucidation of the function of lectins in the *Drosophila* cellular immune response.

Recently in *Drosophila*, proteins of the PGRP (peptidoglycan-recognition protein) family have been found to participate in the recognition of infecting bacteria [30,31]. They discriminate the different structures of the peptidoglycans between Gram-positive and Gram-negative bacteria, and activate appropriate immune responses, i.e. the IMD pathway or the Toll pathway. However, in Gram-negative bacteria, peptidoglycans are enveloped in thick cell-wall layers. As a result, the existence of the other kind of recognition proteins that recognize the cell wall components of Gram-negative bacteria, preceding recognition by PGRPs, will facilitate the efficient initiation of the immune response. Some C-type lectins will be involved in *Drosophila* immunity as such pattern-recognition receptors. In addition, although we could not detect the change in *DL2* and *DL3* gene expression by septic injury with *E. coli*, a recent study using microarray analysis has revealed that expression of at least one of the *DL2* and *DL3* genes was up-regulated by infection with a protozoan parasite, *Octosporea muscaedomesticae* [32]. Little has been elucidated about the recognition mechanism of non-bacterial pathogens in *Drosophila*, although there are some reports of the interaction of lectins with them in other insect species [26,33]. These results support the possibility that some C-type lectins also participate in immune response against non-bacterial pathogens.

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REFERENCES

1 Hultmark, D. (2003) *Drosophila* immunity: paths and patterns. Curr. Opin. Immunol. **15**, 12–19
2 Hoffmann, J. A. (2003) The immune response of *Drosophila*. Nature (London) **426**, 33–38
3 Brennan, C. A. and Anderson, K. V. (2004) *Drosophila*: the genetics of innate immune recognition and response. Annu. Rev. Immunol. **22**, 457–483
4 Leclerc, V. and Reichhart, J. M. (2004) The immune response of *Drosophila melanogaster*. Immunol. Rev. **198**, 59–71
5 Naitza, S. and Ligoxygakis, P. (2004) Antimicrobial defences in *Drosophila*: the story so far. Mol. Immunol. **40**, 887–896
6 Natori, S. (2001) Insect lectins and innate immunity. Adv. Exp. Med. Biol. **484**, 223–228
7 Franc, N. C. and White, K. (2000) Innate recognition systems in insect immunity and development: new approaches in *Drosophila*. Microbes Infect. **2**, 243–250
8 Dodd, R. B. and Drickamer, K. (2001) Lectin-like proteins in model organisms: implications for evolution of carbohydrate-binding activity. Glycobiology **11**, 71R–79R
9 Haq, S., Kubo, T., Kurata, S., Kobayashi, A. and Natori, S. (1996) Purification, characterization, and cDNA cloning of a galactose-specific C-type lectin from *Drosophila melanogaster*. J. Biol. Chem. **271**, 20213–20218
10 Pace, K. E., Lebestky, T., Hummel, T., Arnoux, P., Kwan, K. and Baum, L. G. (2002) Characterization of a novel *Drosophila melanogaster* galectin: expression in developing immune, neural, and muscle tissues. J. Biol. Chem. **277**, 13091–13098
11 Theopold, U., Rissler, M., Fabbri, M., Schmidt, O. and Natori, S. (1999) Insect glycobiology: a lectin multigene family in *Drosophila melanogaster*. Biochem. Biophys. Res. Commun. **261**, 923–927
12 Casso, D., Ramírez-Weber, F. A. and Kornberg, T. B. (2000) GFP-tagged balancer chromosomes for *Drosophila melanogaster*. Mech. Dev. **91**, 451–454
13 Basset, A., Khush, R. S., Braun, A., Gardan, L., Boccard, F., Hoffmann, J. A. and Lemaitre, B. (2000) The phytopathogenic bacteria *Erwinia carotovora* infects *Drosophila* and activates an immune response. Proc. Natl. Acad. Sci. U.S.A. **97**, 3376–3381
14 Hultmark, D., Klemenz, R. and Gehring, W. J. (1986) Translational and transcriptional control elements in the untranslated leader of the heat-shock gene hsp22. Cell **44**, 429–438
15 Tower, J., Karpen, G. H., Craig, N. and Spradling, A. C. (1993) Preferential transposition of *Drosophila* P elements to nearby chromosomal sites. Genetics **133**, 347–359
16 Tzou, P., Reichhart, J. M. and Lemaitre, B. (2002) Constitutive expression of a single antimicrobial peptide can restore wild-type resistance to infection in immunodeficient *Drosophila* mutants. Proc. Natl. Acad. Sci. U.S.A. **99**, 2152–2157
17 Bettencourt, R., Tanji, T., Yagi, Y. and Ip, Y. T. (2004) Toll and Toll-9 in *Drosophila* innate immune response. J. Endotoxin Res. **10**, 261–268
18 Komano, H., Mizuno, D. and Natori, S. (1980) Purification of lectin induced in the hemolymph of *Sarcophaga peregrina* larvae on injury. J. Biol. Chem. **255**, 2919–2924
19 Sun, S. C. and Faye, I. (1992) *Cecropia* immunoresponsive factor, an insect immunoresponsive factor with DNA-binding properties similar to nuclear-factor κB. Eur. J. Biochem. **204**, 885–892
20 Kobayashi, A., Matsui, M., Kubo, T. and Natori, S. (1993) Purification and characterization of a 59 kilodalton protein that specifically binds to NF-κB-binding motifs of the defense protein genes of *Sarcophaga peregrina* (the flesh fly). Mol. Cell. Biol. **13**, 4049–4056
21 Tanji, T., Kobayashi, A. and Natori, S. (2002) Activation of the *Sarcophaga* lectin gene promoter in transgenic *Drosophila*. Arch. Insect Biochem. Physiol. **50**, 131–138
22 Jomori, T., Kubo, T. and Natori, S. (1990) Purification and characterization of lipopolysaccharide-binding protein from hemolymph of American cockroach *Periplaneta americana*. Eur. J. Biochem. **190**, 201–206
23 Kawasaki, K., Kubo, T. and Natori, S. (1993) A novel role of *Periplaneta* lectin as an opsonin to recognize 2-keto-3-deoxy octonate residues of bacterial lipopolysaccharides. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. **106**, 675–680
24 Jomori, T. and Natori, S. (1992) Function of the lipopolysaccharide-binding protein of *Periplaneta americana* as an opsonin. FEBS Lett. **296**, 283–286
25 Chen, C., Ratcliffe, N. A. and Rowley, A. F. (1993) Detection, isolation and characterization of multiple lectins from the haemolymph of the cockroach *Blaberus discoidalis*. Biochem. J. **294**, 181–190
26 Chen, C., Rowley, A. F., Newton, R. P. and Ratcliffe, N. A. (1999) Identification, purification and properties of a β-1,3-glucan-specific lectin from the serum of the cockroach, *Blaberus discoidalis* which is implicated in immune defence reactions. Comp. Biochem. Physiol. Part B Biochem. Mol. Biol. **122**, 309–319
27 Chen, C., Durrant, H. J., Newton, R. P. and Ratcliffe, N. A. (1995) A study of novel lectins and their involvement in the activation of the prophenoloxidase system in *Blaberus discoidalis*. Biochem. J. **310**, 23–31
28 Wilson, R., Chen, C. and Ratcliffe, N. A. (1999) Innate immunity in insects: the role of multiple, endogenous serum lectins in the recognition of foreign invaders in the cockroach, *Blaberus discoidalis*. J. Immunol. **162**, 1590–1596
29 Rämet, M., Pearson, A., Manfruelli, P., Li, X., Koziel, H., Göbel, V., Chung, E., Krieger, M. and Ezekowitz, R. A. (2001) *Drosophila* scavenger receptor CI is a pattern recognition receptor for bacteria. Immunity **15**, 1027–1038
30 Dziarski, R. (2004) Peptidoglycan recognition proteins (PGRPs). Mol. Immunol. **40**, 877–886
31 Steiner, H. (2004) Peptidoglycan recognition proteins: on and off switches for innate immunity. Immunol. Rev. **198**, 83–96
32 Roxström-Lindquist, K., Terenius, O. and Faye, I. (2004) Parasite-specific immune response in adult *Drosophila melanogaster*: a genomic study. EMBO Rep. **5**, 207–212
33 Mello, C. B., Nigam, Y., Garcia, E. S., Azambuja, P., Newton, R. P. and Ratcliffe, N. A. (1999) Studies on a haemolymph lectin isolated from *Rhodnius prolixu*s and its interaction with *Trypanosoma rangeli*. Exp. Parasitol. **91**, 289–296

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34 Marshall, R. D. (1974) The nature and metabolism of the carbohydrate-peptide linkages of glycoproteins. Biochem. Soc. Symp. **40**, 17–26
35 Takahashi, H., Komano, H., Kawaguchi, N., Kitamura, N., Nakanishi, S. and Natori, S. (1985) Cloning and sequencing of cDNA of *Sarcophaga peregrina* humoral lectin induced on injury of the body wall. J. Biol. Chem. **260**, 12228–12233
36 Fujita, Y., Kurata, S., Homma, K. and Natori, S. (1998) A novel lectin from *Sarcophaga*: its purification, characterization, and cDNA cloning. J. Biol. Chem. **273**, 9667–9672
37 Jomori, T. and Natori, S. (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
38 Arai, T., Kawasaki, K., Kubo, T. and Natori, S. (1998) Cloning of cDNA for regenectin, a humoral C type lectin of *Periplaneta americana*, and expression of the regenectin gene during leg regeneration. Insect Biochem. Mol. Biol. **28**, 987–994

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