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***Drosophila* C-type Lectins Enhance Cellular Encapsulation**

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Abstract

C-type lectins are calcium-dependent carbohydrate binding proteins, and animal C-type lectins participate in innate immunity and cell-cell interactions. In the fruit fly *Drosophila melanogaster*, more than 30 genes encode C-type lectin domains. However, functions of *Drosophila* C-type lectins in innate immunity are not well understood. This study is to investigate whether two *Drosophila* C-type lectins, CG33532 and CG33533 (designated as DL2 and DL3, respectively), are involved in innate immune responses. Recombinant DL2 and DL3 were expressed and purified. Both DL2 and DL3 agglutinated Gram-negative *Escherichia coli* in a calcium-dependent manner. Though DL2 and DL3 are predicted to be secreted proteins, they were detected on the surface of *Drosophila* hemocytes, and recombinant DL2 and DL3 also directly bound to hemocytes. Coating of agarose beads with recombinant DL2 and DL3 enhanced their encapsulation and melanization by *Drosophila* hemocytes *in vitro*. However, hemocyte encapsulation was blocked when the lectin-coated beads were pre-incubated with rat polyclonal antibody specific for DL2 or DL3. Our results suggest that DL2 and DL3 may act as pattern recognition receptors to mediate hemocyte encapsulation and melanization by directly recruiting hemocytes to the lectin-coated surface.

Keywords

C-type lectin; encapsulation; melanization; innate immunity; pattern recognition receptor; *Drosophila melanogaster*

1. Introduction

Insects have developed an effective immune system similar to the innate immune system of vertebrates. Insect immunity is composed of cellular and humoral immune responses, such as nodule formation, phagocytosis, encapsulation, synthesis of antimicrobial peptides, and activation of the prophenoloxidase system (Lavine and Strand, 2002; Kanost et al., 2004; Hetru et al., 2003; Cerenius and Soderhall, 2004). These immune responses are triggered by innate immune recognition, which is mediated by pattern recognition receptors (PRRs) (Janeway 1992; Janeway and Medzhitov, 2002). PRRs are germ-line cell encoded receptors that can recognize molecular structural patterns, termed pathogen-associated molecular patterns (PAMPs), present on the surface of microorganisms. Common PAMPs include microbial surface molecules or cell wall components such as bacterial lipopolysaccharide (LPS), lipoteichoic acid (LTA) and peptidoglycan, and fungal β -1, 3-glucan and mannan.

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In the fruit fly *Drosophila melanogaster*, peptidoglycan recognition proteins (PGRPs) are major PRRs involved in the Toll and immune deficiency (IMD) pathways to activate antimicrobial peptide genes (Michel et al., 2001;Gottar et al., 2002;Choe et al., 2002;Gobert et al., 2003;Bischoff et al., 2004;Takehana et al., 2004;Steiner, 2004;Choe et al., 2005;Garver et al., 2006). But PGRPs only recognize bacterial peptidoglycans. Therefore, there must be other PRRs involved in innate immune recognition in *Drosophila*. In the tobacco hornworm *Manduca sexta*, a family of immulectins, which are members of the C-type lectin superfamily, functions as important PRRs to promote hemocyte encapsulation and stimulate prophenoloxidase activation (Yu et al., 1999;Yu and Kanost, 2000;Yu et al., 2002;2005;2006;Ling and Yu, 2006a). C-type lectins are calcium-dependent carbohydrate-binding proteins that can bind terminal sugars on the surface of microorganisms. *Drosophila* genome contains more than 30 genes encoding C-type lectin domains (Dodd and Drickamer, 2001). However, only a few C-type lectin genes have been described. A galactose-specific lectin was purified from *Drosophila* pupal extract (Haq et al., 1996). This lectin, designated as DL1 (*Drosophila* lectin 1), bound to Gram-negative *Escherichia coli* and *Erwinia chrysanthemi*, but not to some other Gram-negative or Gram-positive bacteria (Tanji et al., 2006). CG2958 encodes a protein with a carboxyl-terminal C-type carbohydrate recognition domain (CRD) specific for fucose and mannose, and it is a potential endogenous receptor for neural-specific glycoproteins (Bouyain et al., 2002). CG1500, the furrow gene, is a member of the selectin subgroup, and it is involved in *Drosophila* eye and bristle development (Leshko-Lindsay and Corces, 1997). However, it is not clear whether *Drosophila* lectins can function as PRRs in innate immunity.

CG33532 and CG33533 encode two galactose-specific lectins, designated as DL2 and DL3, respectively. These two genes, together with CG9976 (DL1), are clustered at 37D6 on the *Drosophila* chromosome (Tanji et al., 2006). Each of the three genes encodes a C-type lectin with a single CRD. DL2 shares high similarity in CRD (66% identity) to DL1, but has low similarity to DL3 (30% identity). In the larval stage, DL1 is expressed in most tissues but not in midgut and Malpighian tube, DL2 and DL3 are expressed in cuticle and muscles, midgut and Malpighian tube, and fat body, and DL3 is also expressed in hemocytes (Tanji et al., 2006). It was proposed that DL1 may participate in hemocyte-mediated immune responses, though it does not have an impact on expression of antimicrobial peptide genes, but (Tanji et al., 2006). However, little is known about functions of DL2 and DL3 in *Drosophila* innate immunity.

This study is to investigate whether DL2 and DL3 can act as PRRs in *Drosophila* innate immune recognition. Recombinant DL2 and DL3 were expressed in bacteria and purified. Both recombinant DL2 and DL3 agglutinated *E. coli* in a calcium-dependent manner, but they did not agglutinate *Staphylococcus aureus* or yeast (*Saccharomyces cerevisiae*). DL2 and DL3 are predicted to be secreted lectins, but they were detected on the surface of *Drosophila* hemocytes, and recombinant DL2 and DL3 directly bound to hemocytes. *In vitro* encapsulation assay showed that coating of recombinant DL2 and DL3 to agarose beads enhanced their encapsulation and melanization by *Drosophila* hemocytes. Pre-incubation of the lectin-coated beads with rat polyclonal antibody specific for DL2 or DL3 blocked hemocyte encapsulation. These results suggest that DL2 and DL3 may act as PRRs to mediate encapsulation and melanization by directly recruiting hemocytes to the lectin-coated surface.

2. Materials and Methods

2.1. *Drosophila* strain

Drosophila melanogaster (wild-type Canton S strain) were reared on artificial diets. Larvae and adult flies were kindly provided by Dr. Jeffery Price, School of Biological Sciences at University of Missouri-Kansas City. Flies were reared at 25°C.

2.2. Isolation of total RNA

Total RNA from *Drosophila* adult flies was extracted with Trizol Reagent (Invitrogen) according to the manufacturer's protocol. Briefly, about 50–100 *Drosophila* adult flies were frozen in liquid nitrogen and homogenized with 1 mL of Trizol Reagent. The homogenate was incubated at room temperature for 5 min, and then 0.2 mL of chloroform was added and mixed by vigorously shaking. After incubation at room temperature for 3 min, the mixture was centrifuged at 4°C, 12,000×g for 15 min. The upper aqueous phase was transferred to a new tube and mixed with 0.5 mL of isopropyl alcohol to precipitate total RNA. The sample was incubated at room temperature for 10 min and centrifuged at 4°C, 12,000×g for 10 min. After removing the supernatant, the RNA pellet was washed with 70% ice-cold ethanol, air-dried and dissolved in nuclease free double-distilled water. The quality and concentration of total RNA were determined by spectrometry.

2.3. Expression of recombinant DL2 and DL3

Total RNA (1 µg) from *Drosophila* adult flies was used as a template to synthesize the first strand cDNA using the ThermoScript™ reverse transcriptase (Invitrogen) and Oligo(dT) primer. The cDNA was then used as a template for polymerase chain reactions (PCR) to clone DL2 (CG33532) and DL3 (CG33533) genes using the following primers: DL2_N (5'-TCA CCA TGG ACA AGT ACA CCA CAC- 3') and DL2_C (5'-CTA AAG CTT CTA CTT CCA AAC AAC AAT AGA- 3') for DL2 gene, DL3_N (5'-AGT CCA TGG CCT TGG GTA ACC GAT- 3') and DL3_C (5'-CTA AAG CTT CTA GTT AAG CTG GCA AAT G) for DL3 gene. PCR reactions were performed as followings: initial denaturing at 94°C for 2 min, then 35 cycles of denaturing at 94°C for 30 sec, annealing at 55°C for 30 sec, and extension at 72°C for 30 sec, followed by a final extension at 72°C for 10 min. After PCR reactions, the amplified PCR products were recovered using gel clean-up system (Promega) and digested with *Nco*I and *Hind*III. The digested cDNA fragments were inserted into *Nco*I/*Hind*III digested H6pQE-60 expression vector (Lee et al., 1994) and then transformed into *E. coli* XL1-Blue competent cells. Recombinant proteins were expressed in *E. coli* XL1-Blue and purified under denaturing conditions in 8 M urea using nickel-nitrilotriacetic acid (Ni-NTA) resin according to the manufacture's instruction (Qiagen). Purified recombinant proteins (200 µg each) were applied to a preparative SDS-PAGE, and the gel slice containing DL2 or DL3 was cut out and used as an antigen to inject rats for polyclonal antibody production (Cocalico Biologicals, Inc., Reamstown, PA, USA).

Purified DL2 and DL3 were diluted to 0.01–0.05 mg/mL and renatured by three-step dialysis as described previously (Yu et al., 2005). Dialysis was performed for at least 12 h at 4°C. Renatured protein solution was centrifuged at 12,000 rpm for 20 min at 4°C and the supernatant was concentrated with Amicon ultrafiltration membrane (10 kD cut-off, Millipore). Protein concentration was determined by spectrometry and SDS-PAGE (polyacrylamide gel electrophoresis).

2.4. Agglutination of microorganisms by recombinant DL2 and DL3

Tetramethylrhodamine isothiocyanate (TRITC)-labeled *E. coli*, *S. aureus* and *S. cerevisiae* (Molecular Probes, Invitrogen, USA) were suspended at 1.0×10^9 cells/mL in Tris-buffer saline containing calcium (TBS-Ca) (20 mM Tris.HCl, pH 7.5, 500 mM NaCl, 10 mM CaCl_2). Renatured recombinant DL2 or DL3 was added to a final concentration of 20 $\mu\text{g/mL}$. After incubation at room temperature for 1 h, 5 μL of the mixture was removed for observation under fluorescent microscope. Bovine serum albumin (BSA) was used as a control protein at 40 $\mu\text{g/mL}$.

To test whether agglutination was calcium-dependent, TRITC-labeled *E. coli* was incubated with DL2 or DL3 in TBS buffer containing 2 mM EDTA. After 1h incubation, 5 μL of the mixture was removed for observation, and extra calcium was added to a final concentration of 10 mM to the remained mixture. This mixture was incubated for another hour at room temperature. Then cells were observed by fluorescent microscopy (Nikon Eclipse TE2000-U).

2.5. Immunolocalization of DL2 and DL3 on hemocytes

Hemolymph was collected from 20 *Drosophila* larvae and mixed with 50 μL Schneider's *Drosophila* Medium (Invitrogen) containing 50 $\mu\text{g/mL}$ tetracycline and 2 μL saturated 2-phenylthiourea (PTU). The diluted hemolymph was added to each well of a 12-well multi-test slide (ICN Biomedicals) and the hemocytes were allowed to adhere to the slide for 15–20 min at 25°C. Plasma was then removed, 50 μL of paraformaldehyde fixative solution (4% paraformaldehyde, 5% sucrose, 0.1% citric acid-monohydrate, 0.4% sodium citrate, 0.15% EDTA, pH 6.8) was added to each well, and hemocytes were fixed for 20 min. After removing the fixative solution, hemocytes were washed with TBS for 3 times, each for 5 min. Then hemocytes were blocked with 50 μL of 3% BSA in TBS at room temperature for 1 h and washed 3 times with TBS. These hemocytes were then used for the following experiments.

To test direct binding of recombinant DL2 and DL3 to hemocytes, renatured proteins were diluted to 40 $\mu\text{g/mL}$ with TBS (pH 7.5) containing 0.5% BSA. Fifty microliters of the protein solutions were added to each well and incubated with hemocytes for 1 h at room temperature. Then the protein solution was discarded and hemocytes were washed with TBS as described above. Mouse anti-poly-histidine monoclonal antibody (Sigma) diluted in TBS containing 0.5% BSA at 1:1000 was used as the primary antibody. Goat anti-mouse IgG-fluorescein isothiocyanate (FITC) conjugate (Sigma) diluted in TBS containing 0.5% BSA at 1:200 was used as the secondary antibody. Fifty microliters of the primary and secondary antibody solutions were added to each well sequentially and incubated at room temperature for 1 h and 30 min, respectively. Hemocytes were washed 3 times with TBS after each incubation. At the last washing step, 4', 6-Diamidino-2-phenylindole (DAPI) was added to a final concentration of 0.5 $\mu\text{g/mL}$ to stain the nuclei. Recombinant CP36, an *M. sexta* cuticle protein (Suderman et al., 2003), was used as a control protein.

To detect whether endogenous DL2 and DL3 bind to the surface of *Drosophila* hemocytes, rat anti-DL2 or DL3 polyclonal antiserum, or pre-immune rat serum was diluted in TBS containing 0.5% BSA at 1:200, and 50 μL of the antiserum solution was added to each well and incubated with hemocytes for 1 h. After removing the antiserum solution, hemocytes were washed with TBS for 3 times, each for 5 min. Then 50 μL Goat anti-rat IgG-FITC conjugate (Sigma) diluted in TBS containing 0.5% BSA at 1:200 was used as the secondary antibody and incubated with hemocytes for 30 min. Hemocytes were washed again with TBS for 3 times, and at the last washing, the nuclei were stained with DAPI as described above. Hemocytes were observed by fluorescent microscope (Nikon Eclipse TE2000-U).

2.6. In vitro encapsulation and melanization

To coat agarose beads with His-tagged recombinant proteins, Ni-NTA agarose beads (Qiagen) were equilibrated in TBS containing 5 mM CaCl₂. Renatured His-tagged DL2, DL3 or CP36 (as a control protein) was added and incubated with agarose beads in a 1.5 mL tube with shaking at 4°C overnight. Additional proteins may be added until binding to agarose beads was saturated (excess proteins were detected in the remaining supernatant). Generally, 200 µL of agarose beads can bind up to 1 mg of renatured DL2, DL3 or CP36. Protein-coated beads were washed with TBS four times, each for 5 min, and suspended in TBS at 80–100 beads per microliter (about 1 µg protein per microliter beads).

In vitro encapsulation and melanization were performed as described previously (Ling and Yu, 2006a). Briefly, a 48-well cell culture plate (Falcon) was treated with 1% agarose (ISC Bioexpress). Hemolymph collected from 30 *Drosophila* larvae was combined with 200 µL Schneider's *Drosophila* Medium (Invitrogen) containing 50 µg/mL tetracycline and 8 µL saturated PTU. The diluted hemolymph was added to each well of the agarose-coated plate. Hemocytes were allowed to settle down for at least 10 min. Then 1 µL (80–100 beads) of the protein-coated agarose beads was added to each well, and the plate was incubated at 25°C. Encapsulation and melanization of the agarose beads were observed after 6 and 24 h incubation, respectively. For each recombinant protein, assay was performed in three different wells for statistic analysis.

To test whether *in vitro* encapsulation and melanization can be blocked by rat polyclonal antiserum specific for DL2 or DL3, or by galactose, 5 µL protein-coated beads (about 5 µg of recombinant proteins on the surface) were placed in a microcentrifuge tube containing 50 µL of rat anti-DL2 or anti-DL3 antiserum, or 100 mM galactose (final concentration) in a total of 100 µL TBS (pH 7.5), and the mixture was incubated at 4°C overnight with shaking. Then the beads were washed with TBS and resuspended in 5 µL TBS. *In vitro* encapsulation assay was performed the same as described above.

2.7. Immunoblot analysis

Renatured recombinant DL2 and DL3 were analyzed by SDS-PAGE by the method of Laemmli (1970). For immunoblotting analysis, proteins were separated on 15% SDS-PAGE, and then transferred to a nitrocellulose membrane. The membrane was blocked with 5% dry skim milk in TBS and then incubated with rat polyclonal antiserum to DL2 or DL3 (1:1000), or with mouse anti His-tag monoclonal antibody (Sigma) (1:3000). Antibody binding was visualized by a color reaction catalyzed by alkaline phosphatase conjugated to goat anti-rat IgG (Sigma) (1:10,000) (for rat antiserum) or goat anti-mouse IgG (Sigma) (1:10,000) (for anti His-tag).

3. Results

3.1. Expression and purification of recombinant DL2 and DL3

CG33532 and CG33533 encode 186- and 150-residue polypeptides, designated as DL2 and DL3, respectively. Both DL2 and DL3 are predicted to be secreted proteins, each contains a signal peptide of 20 residues. To express recombinant mature DL2 and DL3, a cDNA fragment encoding residues 21–186 of DL2 or 21–150 of DL3 was obtained by PCR, and cloned into a bacterial expression vector, H6pQE-60 (Lee et al., 1994). Recombinant proteins expressed in H6pQE-60 vector contain a 6×His-tag at the amino-terminus, which can be detected by monoclonal antibody to the His-tag. Recombinant plasmids were transformed into competent *E. coli* XL1 blue cells, and protein expression was induced by IPTG. Recombinant DL2 and DL3 were purified by nickel affinity chromatography under denaturing conditions in 8 M urea, renatured by a three-step dialysis (Yu et al., 2005). Analysis of the purified recombinant DL2

and DL3 by SDS-PAGE showed that they had apparent molecular masses of 20 and 15 kDa, respectively (Fig. 1A), which are consistent with the masses calculated from the deduced amino acid sequences (mature DL2 is 19.3 kDa, while DL3 is 14.8 kDa). Both recombinant DL2 and DL3 were recognized by mouse monoclonal anti-His-tag antibody (Fig. 1B), but DL2 or DL3 was recognized only by its specific rat polyclonal antiserum (Fig. 1C and 1D). Faint signals at about 40 and 30 kDa (indicated by asterisks) were also detected in recombinant DL2 and DL3, respectively, by rat antibodies, which correspond to dimers of DL2 and DL3. Specific rat polyclonal antibody did not cross-react with DL2 or DL3 (Fig. 1C and 1D), which is consistent with their low similarity (23% identity) in amino acid sequence.

3.2. Agglutination of microorganisms by DL2 and DL3

To test whether recombinant DL2 and DL3 can agglutinate microorganisms, we performed a hemagglutination assay. When *E. coli* was incubated with renatured DL2 or rDL3 at a protein concentration of 20 µg/mL, large aggregates of *E. coli* were observed (Fig. 2A). No aggregates of *E. coli* were formed when BSA, a control protein, was used. To determine whether agglutination of *E. coli* by DL2 and DL3 is calcium-dependent, hemagglutination assay was performed in the presence of EDTA. No aggregates of *E. coli* were observed when EDTA was added to chelate calcium (Fig. 2B). However, when extra calcium was added to the same mixture to overcome the effect of EDTA, large aggregates of *E. coli* were re-formed (Fig. 2B). These results suggest that agglutination of *E. coli* by recombinant DL2 and DL3 is calcium-dependent. But DL2 and DL3 did not agglutinate *S. aureus* (a Gram-positive bacterium) or *S. cerevisiae* (yeast) (data not shown), indicating that they recognize surface molecules of *E. coli*, but not *S. aureus* or *S. cerevisiae*.

3.3. Localization of endogenous DL2 and DL3 on the surface of *Drosophila* hemocytes

mRNAs of DL2 and DL3 are detected in fat body, but only DL3 transcript is present in hemocytes (Tanji et al., 2006). Both lectins are predicted to be secreted into hemolymph. Lectins secreted into hemolymph can bind to insect hemocytes. For example, *M. sexta* immulectin-2 and *Bombyx mori* lectins bind to the surface of hemocytes (Ling and Yu, 2006b; Ohta et al., 2006). To determine whether DL2 and DL3 also bind to *Drosophila* hemocytes, an immuno-localization assay was performed. *Drosophila* larval hemocytes were fixed in paraformaldehyde, and then incubated with rat polyclonal antiserum specific for DL2 or DL3, or with pre-immune rat serum (as a control). Binding of rat antibody to DL2 or DL3 on hemocyte surface was visualized by FITC-labeled Goat anti-rat IgG (Fig. 3). Green fluorescence was observed on the surface of hemocytes when they were incubated with rat antibody specific to DL2 or DL3 (Fig. 3), but no fluorescence was observed when hemocytes were incubated with rat pre-immune serum (data not shown). These results suggest that both DL2 and DL3 can bind to *Drosophila* hemocytes.

To further confirm direct binding of DL2 and DL3 to hemocytes, recombinant DL2 or DL3, or *M. sexta* cuticle protein CP36 (Suderman et al., 2003) (as a control protein), was incubated with *Drosophila* hemocytes, and binding of recombinant proteins to hemocytes was detected by monoclonal anti-His-tag antibody, because all three recombinant proteins contain a 6×His-tag. Recombinant DL2 and DL3, but not CP36, were detected on the surface of hemocytes (Fig. 4), suggesting that DL2 and DL3 bind to surface molecules on *Drosophila* hemocytes.

3.4. DL2 and DL3 promote hemocyte encapsulation and melanization

Binding of DL2 and DL3 to hemocytes may recruit hemocytes to the lectin coated surface to promote hemocyte encapsulation. To investigate whether DL2 and DL3 can enhance cellular encapsulation and/or melanization, we performed an *in vitro* encapsulation assay using protein-coated agarose beads. Nearly all the beads coated with recombinant DL2 or DL3 were

encapsulated by *Drosophila* hemocytes within 6 h of incubation, while only 7.5% of the beads coated with *M. sexta* CP36 were encapsulated (Fig. 5). After 24h incubation, about 31% and 40% of DL2- and DL3-coated beads were melanized, respectively, but no CP36-coated beads were melanized (Fig. 5B and 5C). DL3-coated beads were encapsulated more quickly and effectively than DL2-coated beads, and initial encapsulation of DL3-coated beads was observed within 1 h after incubation with hemocytes (data not shown).

To test whether enhanced encapsulation by DL2 and DL3 was caused by direct interaction between DL2/DL3 with hemocyte surface molecules, lectin-coated beads were pre-incubated with rat polyclonal antiserum to block the surface, or with 100mM galactose to saturate lectin binding sites, because DL2 and DL3 are specific for galactose. Then *in vitro* encapsulation assay was performed. Pre-incubation of rat antiserum with the lectin-coated beads effectively blocked hemocyte encapsulation (Fig. 6). However, pre-incubation with galactose had no impact on hemocyte encapsulation and melanization (data not shown). This may be because DL2 and DL3 have higher affinity to complex carbohydrates on the surface of hemocytes than to galactose. These results suggest that *Drosophila* DL2 and DL3 can promote hemocyte encapsulation and melanization through direct interactions with carbohydrates on the surface molecules of hemocytes.

4. Discussion

Animal innate immunity is mediated by different classes of pattern recognition receptors (PRRs). In mammals, more than 10 Toll-like receptors (TLRs) have been identified as major PRRs for pathogens (Medzhitov, 2001;Takeda et al., 2003). In addition to TLRs, mammalian C-type lectins also act as PRRs in innate immunity (Weis et al., 1998;Vasta et al., 1999;Holmskov et al., 2003). For example, mammalian mannose-binding lectin (MBL) (also called mannose-binding protein, or MBP), a member of the collectin subgroup, is a major PRR in the innate immune system (Holmskov et al., 2003). It binds high-mannose carbohydrates present on the surface of many microorganisms, including bacteria, fungi and viruses, to directly enhance phagocytosis or activate the complement system through the lectin pathway (Jack et al., 2001;Holmskov et al., 2003;Jack and Turner, 2003).

In *D. melanogaster*, multiple toll receptors have been identified (Tauszig et al., 2000). However, these *Drosophila* toll receptors have not been shown to function directly as PRRs. *Drosophila* Toll actually binds to a ligand called Spatzle, a cytokine-like protein, but not to microbial components, in the Toll pathway to activate antimicrobial peptide genes (Hoffmann, 2003;Beutler, 2004). In contrast, *Drosophila* PGRPs are major PRRs involved in phagocytosis and activation of the Toll and IMD pathways (Michel et al., 2001;Gottar et al., 2002;Choe et al., 2002;Ramet et al., 2002;Gobert et al., 2003;Bischoff et al., 2004;Takehana et al., 2004;Steiner, 2004;Choe et al., 2005;Garver et al., 2006). In *M. sexta*, a family of lectins named immulectins has been identified as PRRs (Yu et al., 1999;2005;2006;Yu and Kanost, 2000). Immulectins have a broad binding ability for microbial components, including bacterial LPS, LTA and peptidoglycan, as well as fungal β -1, 3-glucan (laminarin and curdlan) and mannan (Yu et al., 2005;2006;Yu and Ma, 2006). They act as PRRs to enhance innate immune responses, such as phagocytosis, encapsulation, melanization, and prophenoloxidase activation (Yu et al., 1999;Yu and Kanost, 2000;2003;2004;Ling and Yu, 2006a;2006b). Immulectins have unique domain architecture with dual CRDs, which differs from other insect lectins and most mammalian C-type lectins that contain a single CRD.

In the *Drosophila* genome, more than 30 genes encode C-type lectin domains (Dodd and Drickamer, 2001). All the *Drosophila* lectin genes encode a single C-type CRD, and no dual-CRD lectins have been identified. So far, functions of most *Drosophila* C-type lectins have

not been defined, and little is known about *Drosophila* lectins in innate immunity. We demonstrated that two galactose-specific C-type lectins, DL2 (CG33532) and DL3 (CG33533), may function as PRRs to promote hemocyte encapsulation. Recombinant DL2 and DL3 agglutinated *E. coli* in a calcium-dependent manner (Fig. 2), suggesting that they are typical C-type lectins. Unlike *M. sexta* immulectins that have a broad binding specificity for microbial components, DL2 and DL3 have a more restrict specificity for ligands, because they did not agglutinate a Gram-positive bacterium (*S. aureus*) or yeast (*S. cerevisiae*). This may be because immulectins contain two CRDs, and each CRD may have different ligand specificity, thus immulectins can bind to a broad spectrum of microorganisms (Watanabe et al., 2006).

Both DL2 and DL3 were detected on the surface of hemocytes (Fig. 3), a result similar to that of *M. sexta* immulectin-2 (Ling and Yu, 2006b). Binding of DL2 and DL3 to hemocyte surface may increase hemocyte-hemocyte interactions, thus recruiting hemocytes to the lectin-coated surface to promote hemocyte encapsulation. We showed that coating of agarose beads with recombinant DL2 or DL3 did enhance their encapsulation and melanization by *Drosophila* hemocytes (Fig. 5). Interaction of recombinant DL2 or DL3 with hemocytes was blocked by rat polyclonal antiserum specific for the lectin (Fig. 6), suggesting that DL2 and DL3 directly interact with hemocytes, probably through specific carbohydrates present on the surface of hemocytes. Encapsulation of the lectin-coated beads was not inhibited by galactose at 100 mM (data not shown), indicating that DL2 and DL3 have higher affinity for complex carbohydrates on the surface of hemocytes than for galactose. We also observed that recombinant DL2 and DL3 bound to *M. sexta* hemocytes, and agarose beads coated with DL2 and DL3 were encapsulated by *M. sexta* hemocytes (data not shown). These results suggest that insect hemocyte surface may contain common carbohydrates that can be recognized by C-type lectins.

DL1, DL2 and DL3 are all galactose-specific lectins. DL1 and DL2 are both 186 amino acid residues, and they are highly similar (59% identity) in amino acid sequence. DL1 has a restrict specificity too, only binds to *E. coli* and *E. chrysanthemi*, but not to other Gram-negative and Gram-positive bacteria, or two fungal strains tested (Tanji et al., 2006). Based on the similarity between DL1 and DL2, and the localization of the three genes as a cluster on the genome, we speculate that DL1 may have a function similar to DL2 and DL3. As to other *Drosophila* C-type lectins, they may bind to different microorganisms, thus groups of *Drosophila* C-type lectins with different specificity can serve as a pool of PRRs for a variety of microorganisms. For example, CG9134 encodes a C-type CRD with a predicted specificity for mannose/glucose. It is also predicted to be a secreted lectin in hemolymph. Thus, CG9134 may also act as a PRR in innate immunity but recognize different microorganisms. Future work is to investigate other *Drosophila* C-type lectin genes and elucidate their functions in innate immunity.

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The abbreviations used are

CRD	carbohydrate recognition domain
DAPI	4', 6-diamidino-2-phenylindole
FITC	fluorescein isothiocyanate
IMD	immune deficiency
LPS	lipopolysaccharide
LTA	lipoteichoic acid
Ni-NTA	nickel-nitrilotriacetic acid
PAGE	polyacrylamide gel electrophoresis
PAMP	pathogen-associated molecular pattern
PGRP	peptidoglycan recognition protein
PRR	pattern recognition receptor
PCR	polymerase chain reaction
SDS	sodium dodecyl sulfate
TBS	Tris-buffered saline
TRITC	tetramethylrhodamine isothiocyanate

Fig 1. Analysis of recombinant DL2 and DL3 by SDS-PAGE and immunoblotting

Renatured recombinant DL2 or DL3 (2 μ g each for Coomassie blue staining, 1 μ g each for immunoblotting) were analyzed by 15% SDS-PAGE. Proteins were stained with Coomassie brilliant blue (A), or transferred to nitrocellulose membranes and identified by immunoblotting using mouse monoclonal anti-His-tag antibody (B), or rat polyclonal antiserum to DL2 (C) or DL3 (D). The arrows indicate DL2, while the arrow heads indicate DL3. Asterisks indicate DL2 and DL3 dimers.

Fig 2. Calcium-dependent agglutination of *E. coli* by recombinant DL2 and DL3

Renatured recombinant DL2 or DL3 (20 μ g/mL final concentration), or BSA (40 μ g/mL) was incubated with *E. coli* (1.0×10^9 cells/ml) in TBS containing 10 mM CaCl₂. After incubation for 1h at room temperature, bacterial cells were examined by microscopy (A). DL2 and DL3 (each at 20 μ g/mL) were incubated with *E. coli* (1.0×10^9 cells/ml) in TBS containing 2 mM EDTA for 1h at room temperature. Five microliters of bacterial cells were removed and observed by microscopy. Extra CaCl₂ was then added to 10 mM final concentration to the rest of bacterial cells, and the mixture was incubated for another 1h. Bacterial cells were then observed by microscopy (B).

Fig 3. Localization of endogenous DL2 and DL3 on *Drosophila* hemocytes

Hemolymph was collected from *Drosophila* larvae and mixed with Schneider's *Drosophila* Medium. Diluted hemolymph was added to a 12-well multi-test slide and hemocytes were allowed to adhere for 15–20 min. Hemocytes were fixed with paraformaldehyde and then incubated with rat polyclonal antiserum to DL2 or DL3. Binding of antibody to DL2 or DL3 on the surface of hemocytes was visualized by FITC-labeled secondary antibody (green), and the nuclei of hemocytes were stained with DAPI (blue). Panels (c) and (d) are fluorescent views, while panels (a) and (b) are differential interference contrast (DIC) views of hemocytes.

Fig 4. Binding of recombinant DL2 and DL3 to *Drosophila* hemocytes

Drosophila hemocytes were collected from larvae and treated as described in Figure 3. Renatured recombinant DL2, DL3 or CP36 (as a control protein) was diluted to 40μg/mL and incubated with hemocytes. Binding of recombinant proteins to hemocyte surface was detected using mouse anti-His-tag monoclonal antibody (green), and the nuclei of hemocytes were stained with DAPI (blue). Panels (d), (e) and (f) are fluorescent views, while panels (a), (b) and (c) are differential interference contrast (DIC) views of hemocytes.

Fig 5. Recombinant DL2 and DL3 promote encapsulation and melanization by *Drosophila* hemocytes

Nickel agarose beads (~80–100 beads) coated with renatured recombinant DL2, DL3, or CP36 (as a control protein) were incubated with *Drosophila* larval hemocytes. Encapsulation and melanization of the protein-coated beads were observed by microscopy after 6 and 24h incubation. (A): Protein-coated beads were encapsulated and melanized by *Drosophila* hemocytes. (B) and (C): The numbers of encapsulated (at 6h) and melanized (at 24h) beads. The columns represent the mean of three individual counts $\pm$ SEM.

Fig 6. Antibody specific for DL2 or DL3 blocks hemocyte encapsulation of the lectin-coated beads DL2- or DL3-coated agarose beads (400–500 beads coated with ~5 μ g proteins) were pre-incubated with rat polyclonal antiserum (50 μ L) specific for DL2 or DL3. Excess antiserum was removed, and antibody bound beads (~80–100 beads) were incubated with *Drosophila* hemocytes as described in Figure 5. Encapsulation of the antibody-bound beads was observed by microscopy after 24h incubation.