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Nuclear translocation of immulectin-3 stimulates hemocyte proliferation

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Abstract

Immulectin-3 (IML-3) is a C-type lectin from the tobacco hornworm *Manduca sexta* that contains a motif (NWGV) similar to the BH1 motif (NWGR) of the mammalian galectin-3. IML-3 is synthesized in fat body and secreted into hemolymph, but can be translocated into hemocytes. In this study, we showed that IML-3 was predominantly localized to the nucleus of hemocytes and some metaphase, anaphase and telophase hemocytes from *M. sexta* larvae injected with bacterial lipopolysaccharide (LPS). IML-3 was detected in the membrane and soluble extracts of hemocytes, suggesting that it may be translocated into hemocytes via receptor-mediated endocytosis. To investigate the role of IML-3 translocation to the nucleus, we expressed recombinant wild-type IML-3 and a deletion mutant IML-3 that has the NWGV motif deleted in *Drosophila* S2 cells. We found that recombinant wild-type IML-3, but not IML-3, was localized to the nucleus of some S2 cells and also detected in the nuclear extract. Expression of recombinant wild-type IML-3, but not IML-3 or GFP, increased the number of proliferating S2 cells. Our results suggest that nuclear translocation of IML-3 may stimulate hemocyte proliferation.

Keywords

Immulectin-3; C-type lectin; Nucleus; Hemocyte; Translocation; S2 cells; Proliferation; *Manduca sexta*

1. Introduction

Insects respond to microbial infections with both humoral and cellular immune responses (Lavine and Strand, 2002; Kanost et al., 2004), and cellular immunity is central to the host defense in the fruit fly *Drosophila melanogaster* (Matova and Anderson, 2006). In insect cellular immune responses, circulating hemocytes (blood cells) participate in phagocytosis, nodule formation and encapsulation (Lavine and Strand, 2002; Kanost et al., 2004). Hemocytes released from the hematopoietic organs are the main source of circulating hemocytes in insects (Akai and Sato, 1971). Proliferation of hemocytes and the number of hemocytes in circulation can be affected by injury, infections by microorganisms and parasites, and hormonal factors (Shapiro, 1968; Carton and Kitano, 1979; Hazarilca and

Gupta, 1997; Ling et al., 2003). In some host insects that are parasitized by wasps, the number of circulating hemocytes decreases dramatically due to breakdown of the circulating hemocytes and the hematopoietic organs (Teramoto and Tanaka, 2004; Ibrahim and Kim, 2006). Hemocyte density also reduces significantly in larvae of the great wax moth *Galleria mellonella* inoculated with pathogenic isolates of yeast (Bergin et al., 2003). Decrease in the number of circulating hemocytes may result in killing of insects by a variety of microorganisms or parasites. For example, decrease in circulating hemocytes of the mosquito *Aedes aegypti* correlates with age-associated mortality in immune challenged mosquitoes due to reduced overall phagocytic capacity of mosquitoes (Hillyer et al., 2005). Therefore, insects must increase hemocyte density by proliferation to keep up the immune capacity.

In the silkworm, *Bombyx mori*, *in vitro* culture of hematopoietic organs shows that the plasma contains hematopoietic factor(s) (Nakahara et al., 2003). In *Drosophila*, a platelet-derived growth factor (PDGF)/vascular endothelial growth factor (VEGF)-like factor (PVF2) can induce a dramatic increase in circulating hemocytes in larvae (Munier et al., 2002). Research in *Drosophila* also suggests that the Toll/Cactus pathway, Rel/NF- κ B proteins (Dorsal and Dif), receptor tyrosine kinases, Ras/mitogen-activated protein kinase pathway as well as JAK/STAT pathway may regulate hemocyte proliferation and hemocyte density (Qiu et al., 1998; Agaisse and Perrimon, 2004; Zettervall et al., 2004; Matova and Anderson, 2006).

Lectins are carbohydrate binding proteins that play an important role in many biological processes. Galectins, originally called S-type lectins (Drickamer, 1988), are galactoside-specific animal lectins involved in development, differentiation, cell-cell adhesion, cell-matrix interaction, growth regulation, apoptosis and tumor metastasis (Perillo et al., 1998; Danguy et al., 2002; Liu et al., 2002; Yang and Liu, 2003; Takenaka et al., 2004). Galectin-1 can promote or inhibit cell proliferation, and promote apoptosis (Yang and Liu, 2003; Scott and Weinberg, 2004). Galectin-3 can modulate T-cell growth and apoptosis, and it is the only member in the galectin family that has anti-apoptotic activity (Yang et al., 1996; Akahani et al., 1997). Also, a C-type (calcium-dependent) lectin from the tunicate, *Styela plicata*, can stimulate tunicate and mammalian cell proliferation (Nair et al., 2001).

Previously, we reported that immulectin-3 (IML-3), a C-type lectin from the tobacco hornworm *Manduca sexta*, is synthesized in fat body (an equivalent to mammalian liver) and secreted into hemolymph, but can be translocated into hemocytes (Yu et al., 2005). The carboxyl-terminal carbohydrate-recognition domain (CRD) of IML-3 contains an NWGV motif similar to the Bcl-2 homology domain 1 (BH1) motif (NWGR) of the mammalian galectin-3 (Yu et al., 2005). The BH1 motif in galectin-3 plays a crucial role in modulation of cell growth and apoptosis (Akahani et al., 1997), and this motif is highly conserved in the Bcl-2 family proteins. In this study, we found that IML-3 was predominantly localized to the nucleus of hemocytes and some metaphase, anaphase and telophase hemocytes from *M. sexta* larvae injected with bacterial lipopolysaccharide (LPS). IML-3 mRNA was not expressed in hemocytes, but its protein was detected in the membrane and soluble extracts of hemocytes, suggesting that IML-3 may be translocated into hemocytes via receptor-mediated endocytosis. To investigate the role of IML-3 translocation to the nucleus, we

expressed recombinant wild-type IML-3 and a deletion mutant IML-3 that has the NWGV motif deleted in *Drosophila* Schneider 2 (S2) cells. We found that recombinant wild-type IML-3, but not IML-3, was localized to the nucleus of some S2 cells and also detected in the nuclear extract. Expression of recombinant wild-type IML-3, but not IML-3 or green fluorescent protein (GFP), increased the number of proliferating S2 cells. Our results suggest that nuclear translocation of IML-3 may stimulate hemocyte proliferation.

2. Materials and methods

2.1. Insects

M. sexta eggs were kindly provided by Dr. Michael Kanost, Department of Biochemistry at Kansas State University. Larvae were reared on an artificial diet at 25 °C (Dunn and Drake, 1983). The fifth instar larvae were used for the experiments.

2.2. Preparation of soluble and membrane extracts from hemocytes

Membrane and soluble extracts from hemocytes were prepared using The Mem-PER Eukaryotic Membrane Protein Extraction Reagent Kit (PIERCE, Cat. No. 89826) according to the manufacturer's instruction with slight modifications. Briefly, 1 ml hemolymph (about 1.4×10^7 hemocytes) from day 2 fifth instar naïve larvae or larvae injected with saline (control) or LPS (*Escherichia coli* 0111:B4, 20 µg per larva) at 12 h post-injection was mixed with 1 ml phosphate-buffered saline (PBS) (25 mM sodium phosphate buffer, 137 mM NaCl and 3 mM KCl, pH 6.8) containing a few crystal of 2-phenyl thiourea (PTU) and 1 mM EDTA. Hemocytes were collected by centrifugation at $3300 \times g$ for 5 min at 4 °C, washed twice with PBS, and re-suspended in Reagent A (450 µl) by pipetting. The hemocyte lysates were incubated for 20 min at room temperature with occasionally vortexing and placed on ice. Then Reagent C (900 µl) and Reagent B (450 µl) were added to the lysed cells, vortexed and incubated on ice for 30 min with vortexing at every 5 min. Cell lysates were centrifuged at $10,000 \times g$ for 3 min at 4 °C, and the supernatants were transferred to new tubes and incubated for 20 min at 37 °C. Then the hydrophobic fraction was separated from the hydrophilic fraction by centrifugation at room temperature for 2 min at $10,000 \times g$. The hydrophilic phase (top layer) was quickly and carefully removed from the hydrophobic protein phase (bottom layer), saved in new tubes and both fractions were placed on ice. Most membrane proteins are in the lower hydrophobic fraction, while soluble proteins are in the hydrophilic phase. Proteins were finally dialyzed in PBS containing 1 mM EDTA and 0.5% Tween-20 (for membrane proteins) or 1 mM EDTA (for soluble proteins) at 4 °C, with four changes of the dialysis buffer. The dialyzed proteins were centrifuged at $10,000 \times g$ for 15 min at 4 °C, the supernatants were saved and protein concentrations were determined by the Bradford method (Bradford, 1976). Both membrane and soluble proteins were stored at -80 °C until use.

2.3. Construction of recombinant plasmids for expression of IML-3 and IML-3 in *Drosophila* Schneider 2 (S2) cells

The plasmid pBluescript KS, which contains the full length IML-3 cDNA sequence with the complete open reading frame, was used as a template for polymerase chain reaction (PCR). A pair of sequence-specific primers Des3F (5'-GGA AGA TCT GTG TTT CGT GCC

GAC-3', containing a *Bgl* II restriction site) and Des3R (5'-CAA GGG CCC GTT GTT TGG GAC TGG-3', containing an *Apa* I site) was used to clone wild-type IML-3. To clone IML-3, which has the BH1-like motif (NWGV) deleted, Des3F, Des3R and another pair of primers IML3dF (5'-TAT GCG ACC CAG CCG GAC AGC-3') and IML3dR (5'-CTG GGT CGC ATA GCC AGC TTC-3') were used for PCR reactions. Two separate PCR reactions were performed using pBluescript KS plasmid DNA as a template, Des3F/IML3dR and IML3dF/Des3R as primers, respectively. PCR products were purified using Wizard® SV Gel and PCR Clean-Up System (Promega). Then the two PCR products were equally mixed as a template for IML-3 cDNA using Des3F/Des3R primers. All PCR reactions were performed in 50- μ l reactions: 10–100 ng template (plasmid DNA or PCR products), 5 μ l 10 $\times$ PCR buffer, 0.5 μ l 50 mM dNTPs, 1 μ l (100–200 ng/ μ l) each of the forward and reverse primers, 1 μ l *Taq* polymerase (1 unit/ μ l), and sterilized water to a final volume of 50 μ l. The conditions for PCR reactions were: initial denaturing at 94 °C for 2 min, 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 45 s, and extension at 72 °C for 60 s, followed by a final extension at 72 °C for 10 min. PCR products were purified and recovered using the gel clean-up system, and cloned into plasmid vector pGEMR-T (Promega). Plasmid DNA containing the insert was prepared, and the nucleotide sequence of the insert was determined. Plasmid DNA from the positive clones was purified and digested with *Bgl* II and *Apa* I enzymes. The digested IML-3 or IML-3 fragment was recovered and ligated to *Bgl* II/*Apa* I digested expression vector, pMT/BiP/V5-His A (Invitrogen). The ligated product was then used to transform competent *Escherichia coli* strain DH5 α cells, and plasmid DNA containing the interest gene was purified and used to transfect *Drosophila* Schneider 2 cells. Green fluorescent protein in pMT/BiP/V5-His A (Invitrogen) was used as a control plasmid.

2.4. Expression of recombinant IML-3, IML-3 and GFP proteins in S2 cells

Drosophila S2 cells (Invitrogen) were transfected with recombinant plasmid DNAs using CELLFECTIN (Invitrogen). Briefly, fetal bovine serum (FBS)-free medium (250 μ l) was placed in each of the two 1.7-ml sterilized Eppendorf tubes. In Tube 1, plasmid DNA (10 μ g) was mixed with 250 μ l FBS-free medium by pipetting. For establishing stable cell lines, 0.5 μ g plasmid DNA containing a blasticidin-resistant gene (pCoBlast) (Invitrogen) was also included. In Tube 2, CELLFECTIN (40 μ l) was mixed with 250 μ l FBS-free medium by pipetting. Then, the DNA solution (Tube 1) was mixed with the CELLFECTIN solution (Tube 2) and the mixture was incubated at room temperature for 20 min. S2 cells (about 1–2 $\times 10^6$ cells) were washed with FBS-free medium 1–2 times and re-suspended in 0.8 ml FBS-free medium in a 15-ml conical tube. The DNA/CELLFECTIN solution was added to the cell suspension by gently tapping, and the cells were transferred to a culture plate. The cells were incubated for 3 h at 25 °C and the medium was removed by centrifugation at 500 $\times g$ for 1 min. Cells were re-suspended in medium containing 10% FBS and incubated at 25 °C. To generate stable transfected S2 cell lines, blasticidin (Blasticidin S Hydrochloride, Waken Inc.) was added to the transfected cells on day 2 at 25 μ g/ml. Cells were re-plated in fresh medium every 4 days over the selection period. After 2–3 weeks of selection, resistant cells containing the interest gene grew well. Stocks of stable S2 cell lines were prepared in 80% FBS and 10% dimethyl sulfoxide and kept frozen in liquid N₂. For protein expression, CuSO₄ was added to the transfected cells at 1 mM to induce protein expression. Expression

of recombinant proteins was detected by immunoblotting, because recombinant proteins contain a V5-His-tag at the carboxyl-terminus. About 10^6 S2 cells were collected by centrifugation and cell culture medium was removed. S2 cells were lysed in 100 μ l of lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% Nonidet P-40, pH 7.8) containing 1% sodium dodecyl sulfate (SDS) and 0.1 M DTT, and whole cell lysates were collected. Aliquots of whole cell lysates were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

2.5. Preparation of nuclear proteins from hemocytes and S2 cells

Nuclear proteins from hemocytes of day 2 fifth instar *M. sexta* larvae injected with saline (control) or LPS (*E. coli* 0111:B4, 20 μ g per larva) at 12 h post-injection were extracted using the Nuclear Extract Kit (Active Motif, Cat No. 40010 & 40410). Briefly, hemolymph (2 ml from at least four larvae) was mixed with ice-cold PBS (2 ml) containing 1 mM EDTA, 50 μ l phosphatase inhibitors and a few crystals of PTU (pH 6.8) on ice. Hemocytes were collected by centrifugation at $3300 \times g$ for 5 min at 4 °C, washed with ice-cold PBS three times, and gently re-suspended in 1.5 ml ice-cold $1 \times$ hypotonic buffer by pipetting up and down several times. Hemocytes were then incubated for 15 min on ice, and detergent (75 μ l) was added and vortexed for 10 s at highest setting. The suspension was centrifuged at $14,000 \times g$ for 30 s at 4 °C, and the supernatant (cytoplasmic fraction, 1.5 ml) was transferred into a pre-chilled microcentrifuge tube. The pellet that contains nuclei was re-suspended in 150 μ l complete lysis buffer (15 μ l 10 mM DTT, 133.5 μ l Lysis Buffer AM1 and 1.5 μ l Protease Inhibitor Cocktail) by pipetting up and down, vortexed for 10 s at highest setting and incubated for 30 min on ice on a rocking platform at 150 rpm. The suspension was fully vortexed for 30 s at highest setting and centrifuged at $14,000 \times g$ for 10 min at 4 °C. The supernatant (nuclear fraction) was transferred into a pre-chilled microcentrifuge tube. Nuclear and cytoplasmic extracts from S2 cells and stable S2 cells expressing recombinant IML-3, IML-3 or GFP (after induced expression of recombinant proteins for 24 h) were prepared the same as described above. Protein concentrations were determined by the Bradford method (Bradford, 1976). Both nuclear and cytoplasmic proteins were aliquoted and stored at -80 °C.

2.6. Western blot analysis

Plasma samples or protein fractions were analyzed on SDS-PAGE by the method of Laemmli (1970), and IML-3 was identified by immunoblotting. Samples were separated on 12% SDS-PAGE, and proteins were transferred to nitrocellulose membrane. The membrane was blocked with 5% dry skim milk and then incubated with rabbit polyclonal antibody to IML-3 (1:2000). Antibody binding was visualized by a color reaction catalyzed by alkaline phosphatase conjugated to goat anti-rabbit IgG (1:3000) (Bio-Rad). For each group, plasma from four individual larvae was mixed and analyzed. The IML-3 band intensities were measured using Kodak Digital Science 1D gel analysis software, and relative amount of IML-3 in each protein sample was calculated based on the band intensity.

2.7. Localization of native IML-3 in *M. sexta* larval hemocytes and recombinant IML-3 in S2 cells

Hemocytes were collected from day 2 fifth instar *M. sexta* naïve larvae or larvae injected with LPS (*E. coli* 0111:B4, 20 µg per larva) at 12 h post-injection. Larvae were surface-sterilized with alcohol, and 10 µl of hemolymph was collected directly into 50 µl of the Grace's tissue culture medium (ICN Biomedicals) on wells of a 12-well slide. Hemocytes were allowed to adhere to the slide for at least 5 min. Plasma was then removed and hemocytes were washed with PBS (pH 6.8) and fixed in 4% paraformaldehyde for 15 min at room temperature. After washing with PBS (pH 6.8) three times (each for 5 min), hemocytes were incubated with rabbit polyclonal antibody to IML-3 for 30 min at room temperature. Hemocytes were washed with PBS (pH 6.8) as the above and then incubated with goat anti-rabbit IgG conjugated with fluorescein isothiocyanate (FITC) for 30 min. All antibodies were diluted 1:200 in PBS containing 0.1% BSA. Hemocytes were washed again and stained with 4',6-diamidino-2-phenylindole (DAPI) (20 µl of 1 mg/ml) for 30 min. Finally, after washing, a drop of 2% 1,4-diazabicyclo [2,2,2] octane (DABCO) was added to the slide and hemocytes were observed by fluorescence microscopy (Nikon Eclipse TE2000-U).

To detect recombinant IML-3 in S2 cells, stable S2 cells were cultured in the medium containing 1 mM CuSO₄ for a period of time and the S2 cells were detached. Then the S2 cells were re-suspended in fresh medium and seeded in wells of a 12-well slide. Cells were allowed to adhere to the slide for 10–20 min and fixed in 4% paraformaldehyde for 15 min. Cells were then incubated with mouse monoclonal antibody to the His-tag (1:200 in PBS containing 0.1% BSA) for 30 min, followed by incubation with goat anti-mouse IgG conjugated with fluorescein isothiocyanate (1:200 in PBS containing 0.1% BSA) for 30 min. Cells were washed, stained with DAPI, and then observed by fluorescence microscopy.

2.8. Proliferation of S2 cell

Proliferation of S2 cells was determined using the 5-bromo-2'-deoxy-uridine (BrdU) Labeling and Detection Kit III (Roche) with a slightly modified procedure. Briefly, CuSO₄ was added to about 10⁶ S2 cells or stable S2 cells containing recombinant wild-type IML-3, IML-3 or GFP gene in 1 ml medium to induce protein expression. Cells were cultured for the indicated times and then detached. BrdU stock solution (1000×) (1 µl) was added to the cells and fully mixed (final concentration of BrdU was 10 µM). Aliquots of cells (50 µl) were mixed with equal volumes (50 µl) of medium containing 10 µM BrdU, and 50 µl of these cells were seeded to each well of a 12-well slide. A parallel well of cells without BrdU was used as a negative control. Cells were incubated for 3 h at 22 °C and washed with PBS for three times (each for 5 min). Cells were then fixed in a cold fixative (70% ethanol and 0.5 M HCl) for 15 min and washed with PBS for three times. Anti-BrdU-POD solution (1:100) was prepared in PBS containing 0.1 mg/ml BSA, and an aliquot of anti-BrdU-POD solution (50 µl) was added to each well and cells were incubated at room temperature for 30 min. Anti-BrdU-POD solution was discarded and cells were washed with PBS for three times. POD original substrate solution (50 µl) was then added to each well and cells were incubated at room temperature for 5 min for color development. Cells with blue color were BrdU-positive. Then S2 cells were washed again in PBS and mounted for counting BrdU-positive cells under microscopy. At least 1000 total cells were counted from each well (at

least three wells for each group). This experiment was repeated two times. The p value was calculated using a t -test program.

3. Results

3.1. Translocation of IML-3 may be mediated by endocytosis upon LPS injection

We previously showed that IML-3 is synthesized in fat body and secreted into hemolymph, and it can be translocated from hemolymph into hemocytes (Yu et al., 2005). Using real-time PCR analysis, we have further confirmed that IML-3 mRNA is not expressed in hemocytes and expression of IML-3 transcript in fat body was significantly induced (increased more than fourfold) by LPS injection (data not shown).

To understand how IML-3 is translocated into hemocytes, we prepared membrane and soluble extracts from hemocytes of fifth instar *M. sexta* larvae injected with saline (control) or LPS at 12 h post-injection. We found that IML-3 was present in both membrane and soluble extracts of hemocytes by Western blot analysis (Fig. 1A). About 75% more IML-3 was detected in the membrane extract of LPS-injected hemocytes than in the control hemocytes (Fig. 1B), while almost same amounts of IML-3 were detected in the soluble extract of LPS-injected and control hemocytes (Fig. 1B). However, the concentration of IML-3 in plasma remained almost unchanged in the LPS-injected larvae compared to the control larvae (Fig. 1A and B), a result consistent with our previous one (Yu et al., 2005). These results suggest that translocation of IML-3 to hemocytes may be mediated by receptor-mediated endocytosis upon injection of LPS. We also found that the protein level of IML-3 in hemocytes relative to that in plasma after LPS injection was increased about one-fold at 36 h post-injection (data not shown), which is consistent with our previous result that injection of LPS increases the number of IML-3-positive hemocytes (Yu et al., 2005).

3.2. IML-3 is localized to the nucleus of larval hemocytes

We previously showed that IML-3 is located in larval hemocytes (Yu et al., 2005). To further localize IML-3 in hemocytes of naïve larvae, we performed an immunolocalization assay. IML-3 in hemocytes was detected by rabbit antibody to IML-3, and the nucleus of hemocytes was stained with DAPI. We found that IML-3 was predominantly co-stained with DAPI in the nucleus of day 2 naïve larval hemocytes (Fig. 2A, the arrows). We then performed a Western blot to determine whether IML-3 is present in the nuclear extract of larval hemocytes. We found that IML-3 was present in the nuclear extracts (in 50 µg total proteins) of hemocytes from both saline- (control) and LPS-injected larvae (Fig. 2B). However, almost twice as much IML-3 was detected in the nuclear extract in LPS-injected hemocytes than in the control hemocytes (Fig. 2C), suggesting that translocation of IML-3 to the nucleus of hemocytes is enhanced by LPS injection. Furthermore, we found that IML-3 also accumulated in the nucleus of some hemocytes that are proliferating (Fig. 3). IML-3 was co-stained with DAPI in the nucleus of some metaphase, anaphase and telophase hemocytes (Fig. 3, the arrows), suggesting that translocation of IML-3 to the nucleus of hemocytes may play a role in hemocyte proliferation.

3.3. Expression of recombinant wild-type IML-3, IML-3 and GFP in S2 cells

IML-3 is the only member of the immunectin family that contains an NWGV motif (residues 262–265 starting from the first Met), which is similar to the BH1 motif (NWGR) of the mammalian galectin-3 (Yang et al., 1996; Akahani et al., 1997; Yu et al., 2005). The BH1 motif is critical for the apoptosis-suppressing activity of galectin-3 (Yang et al., 1996) and it is highly conserved in the Bcl-2 family proteins. To investigate the role of the BH1-like motif (NWGV) of IML-3 in hemocyte proliferation, we constructed expression vectors for recombinant wild-type IML-3, a deletion mutant IML-3 that has the NWGV motif deleted, and green fluorescent protein (a control protein). Recombinant proteins were expressed in *Drosophila* S2 cells.

Analysis of recombinant protein expression by immunoblotting showed that recombinant wild-type IML-3 and IML-3 were detected in whole S2 cell lysates by rabbit polyclonal antibody to IML-3 (Fig. 4A). Recombinant IML-3 and IML-3 appeared larger in the gel than native IML-3 from hemolymph (Fig. 4A, lane HL) because of addition of the V5-His-tag (~2.8 kDa) to the carboxyl-terminus (Fig. 4A). Recombinant IML-3, IML-3 and GFP were all detected in S2 cells by mouse monoclonal antibody to the His-tag (Fig. 4B), since all three recombinant proteins contain a V5-His-tag at the carboxyl-terminus. No recombinant protein was detected in normal S2 cells that do not contain a foreign gene by either IML-3 or His-tag antibody. These results indicate that recombinant IML-3, IML-3 and GFP were successfully expressed in S2 cells.

3.4. Recombinant wild-type IML-3 but not IML-3 is localized to the nucleus of some S2 cells

Plasmid vector pMT/BiP/V5-His is used to express secreted proteins, thus recombinant IML-3 proteins should be expressed in S2 cells and then secreted into cell culture medium. However, we observed that wild-type IML-3 was co-localized to the nucleus of some S2 cells (Fig. 5A, the arrows), while IML-3 was localized in the cytoplasm of S2 cells (Fig. 5A). Analysis of the nuclear extracts by immunoblotting showed that recombinant wild-type IML-3, but not IML-3 or GFP, was detected in the nuclear extract of S2 cells (Fig. 5B), further confirming that wild-type IML-3, but not IML-3, can be targeted to the nucleus of some S2 cells.

3.5. Expression of recombinant wild-type IML-3 but not IML-3 increases the number of proliferating S2 cells

To investigate whether translocation of recombinant wild-type IML-3 to the nucleus of S2 cells plays a role in cell proliferation, we performed an assay to label proliferating cells with 5-bromo-2'-deoxy-uridine (BrdU). S2 cells or stable S2 cells containing wild-type IML-3, IML-3 or GFP gene were induced to express recombinant proteins for 0, 3, 12 and 24 h, and the cells were then collected and labeled with BrdU. BrdU-positive cells were the cells that are proliferating. We found that expression of recombinant wild-type IML-3 for 3 h significantly ($p = 0.0048$) increased the number of proliferating S2 cells compared to 0 h when no protein was expressed (Fig. 6A). The number of proliferating cells at 3 h after expression of recombinant wild-type IML-3 was also significantly higher than that of normal S2 cells ($p = 0.0061$). Expression of wild-type IML-3 for 12 and 24 h also greatly

increased the numbers of proliferating S2 cells (Fig. 6A). Recombinant IML-3 expression for 3 h slightly increased the number of proliferating S2 cells but not significantly ($p = 0.0706$). However, expression of IML-3 for 12 and 24 h did not change the numbers of proliferating S2 cells. Expression of GFP (a control protein) for 3, 12 and 24 h also had no effect on the number of proliferating S2 cells (Fig. 6A). In all these stable S2 cells, recombinant wild-type IML-3, IML-3 and GFP were all expressed (Fig. 6B). These results suggest that the BH1-like motif (NWGV) of IML-3 may play a role in cell proliferation.

4. Discussion

In this study, we showed that *M. sexta* IML-3, a member of insect C-type lectins with a BH1-like motif (NWGV), was translocated into hemocytes and accumulated in the nucleus of hemocytes, and it may play a role in cell proliferation. This is, to our knowledge, the first report of an insect C-type lectin that may promote hemocyte proliferation. IML-3 shares many properties with the mammalian galectin-3, which can modulate cell growth and apoptosis (Yang et al., 1996; Akahani et al., 1997). For example, IML-3 is the only member of the immulectin family that contains an NWGV motif similar to the BH1 motif (NWGR) of galectin-3. Galectins are mainly localized in the cytoplasm and nucleus of cells (Wang et al., 2004), while IML-3, though secreted into hemolymph, can be translocated into hemocytes and accumulated in the nucleus (Figs. 2 and 3). More IML-3 was detected in hemocytes after larvae were injected with bacterial LPS (data not shown) and more IML-3 was also detected in the nuclear extract in the LPS-injected hemocytes than in the control hemocytes (Fig. 2). These results suggest that translocation of IML-3 into hemocytes and accumulation of IML-3 to the nucleus may stimulate hemocyte proliferation during infection.

When we expressed recombinant proteins in *Drosophila* S2 cells, we intended to target the recombinant proteins for secretion into the cell culture medium. Recombinant wild-type IML-3 and IML-3, as well as GFP, were detected in the cell culture medium (data not shown), however, only wild-type IML-3, but not IML-3 or GFP, was detected in the nuclear extract (Fig. 5B). These results suggest that IML-3 can indeed be translocated to the nucleus, and the BH1-like motif (NWGV) may play a role in nuclear translocation of IML-3. In mammalian galectins, galectin-3 can be translocated to the perinuclear membrane after stimulation by apoptotic stimuli (Yu et al., 2002), and it can interact with transcription factors such as TTF-1 that regulates gene transcription in thyroid cells (Hsu and Liu, 2004).

We think that IML-3 may not have a direct activity in hemocyte proliferation. Instead, it may facilitate nuclear translocation of some nuclear factors that play a central role in hemocyte proliferation. In *Drosophila*, the Toll pathway, Cactus and Rel/NF- κ B factors (Dif and Dorsal) are implicated in regulation of hemocyte proliferation (Qiu et al., 1998; Matova and Anderson, 2006). In the absence of both Dif and Dorsal, the number of circulating hemocytes in *Drosophila* larvae decreases dramatically due to apoptosis of hemocytes triggered by infection, and hemocytes cannot ingest microbes (Matova and Anderson, 2006). Expression of Dorsal or Dif in hemocytes of Dif/Dorsal double mutants can increase the number of circulating hemocytes and restore the ability of hemocytes to clear microbes (Matova and Anderson, 2006). Dif and Dorsal also play an important role in promoting

hemocyte proliferation. The *Drosophila* Cactus (an I κ B) mutants have an overabundance of hemocytes that can cause melanotic capsules, and the lethality can be rescued by the introduction of mutations in Toll, tube or pellet (key components in the Toll pathway) (Qiu et al., 1998). Over-expression of either Dif or Dorsal in hemocytes increases the number of circulating hemocytes in *Drosophila* larvae, and Dorsal primarily promotes plasmatocyte proliferation, while Dif controls production of both plasmatocytes and lamellocytes (Huang et al., 2005; Matova and Anderson, 2006). These results suggest that Rel/NF- κ B factors like Dif and Dorsal play an essential role in regulation of hemocyte proliferation. We found that IML-3 directly interacted with some nuclear proteins from *Drosophila* S2 cells by far Western blot analysis (E. Ling and X.-Q. Yu, unpublished results). It is possible that IML-3 interacts with Dif or Dorsal and facilitates nuclear localization of Dif/Dorsal to stimulate hemocyte proliferation. Identification of *Drosophila* nuclear proteins that can interact with IML-3 is underway and our future work is to elucidate how IML-3 stimulates hemocyte proliferation.

Acknowledgments

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Abbreviations

BH1	Bcl-2 homology domain 1
BrdU	5-bromo-2'-deoxy-uridine
DAPI	4',6-diamidino-2-phenylindole
FBS	fetal bovine serum
FITC	fluorescein isothiocyanate
GFP	green fluorescent protein
IML-3	immulectin-3
LPS	lipopolysaccharide
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PTU	2-phenyl thiourea
SDS	sodium dodecyl sulfate

References

- Agaisse H, Perrimon N. The roles of JAK/STAT signaling in *Drosophila* immune responses. *Immunol Rev.* 2004; 198:72–82. [PubMed: 15199955]
- Akahani S, Nangia-Makker P, Inohara H, Kim HR, Raz A. Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. *Cancer Res.* 1997; 57:5272–5276. [PubMed: 9393748]

- Akai H, Sato S. An ultrastructural study of the haemopoietic organs of the silkworm, *Bombyx mori*. *J Insect Physiol.* 1971; 17:1665–1676.
- Bergin D, Brennan M, Kavanagh K. Fluctuations in haemocyte density and microbial load may be used as indicators of fungal pathogenicity in larvae of *Galleria mellonella*. *Microbes Infect.* 2003; 5:1389–1395. [PubMed: 14670452]
- Bradford M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem.* 1976; 72:248–254. [PubMed: 942051]
- Carton Y, Kitano H. Changes in the hemocyte population of *Drosophila* larvae after single and multiple parasitization by *Cothonaspis* (Parasitic cynipidae). *J Invertebr Pathol.* 1979; 34:88–89.
- Danguy A, Camby I, Kiss R. Galectins and cancer. *Biochim Biophys Acta.* 2002; 1572:285–293. [PubMed: 12223276]
- Drickamer K. Two distinct classes of carbohydrate-recognition domains in animal lectins. *J Biol Chem.* 1988; 263:9557–9560. [PubMed: 3290208]
- Dunn PE, Drake D. Fate of bacteria injected into naive and immunized larvae of the tobacco hornworm, *Manduca sexta*. *J Invertebr Pathol.* 1983; 41:77–85.
- Hazarilca LK, Gupta AP. Effect of juvenile hormone on haemocyte counts of *Blattella germanica*. *Pesticide Res J.* 1997; 9:252–255.
- Hillyer JF, Schmidt SL, Fuchs JF, Boyle JP, Christensen BM. Age-associated mortality in immune challenged mosquitoes (*Aedes aegypti*) correlates with a decrease in haemocyte numbers. *Cell Microbiol.* 2005; 7:39–51. [PubMed: 15617522]
- Hsu DK, Liu FT. Regulation of cellular homeostasis by galectins. *Glycoconj J.* 2004; 19:507–515. [PubMed: 14758074]
- Huang L, Ohsako S, Tanda S. The lesswright mutation activates Rel-related proteins, leading to overproduction of larval hemocytes in *Drosophila melanogaster*. *Dev Biol.* 2005; 280:407–420. [PubMed: 15882582]
- Ibrahim AM, Kim Y. Parasitism by *Cotesia plutellae* alters the hemocyte population and immunological function of the diamondback moth, *Plutella xylostella*. *J Insect Physiol.* 2006; 52:943–950. [PubMed: 16872627]
- Kanost MR, Jiang H, Yu XQ. Innate immune responses of a lepidopteran insect, *Manduca sexta*. *Immunol Rev.* 2004; 198:97–105. [PubMed: 15199957]
- Laemmli MK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature (Lond).* 1970; 227:680–685. [PubMed: 5432063]
- Lavine MD, Strand MR. Insect hemocytes and their role in immunity. *Insect Biochem Mol Biol.* 2002; 32:1295–1309. [PubMed: 12225920]
- Ling E, Shirai K, Kanekatsu R, Kobayashi Y, Tu Z, Funayama T, Watanabe H, Kiguchi K. Why does hemocyte density rise during the wandering stage of the silkworm, *Bombyx mori*? *J Insect Biotechnol Sericol.* 2003; 72:101–109.
- Liu FT, Patterson RJ, Wang JL. Intracellular functions of galectins. *Biochim Biophys Acta.* 2002; 1572:263–273. [PubMed: 12223274]
- Matova N, Anderson KV. Rel/NF-kappaB double mutants reveal that cellular immunity is central to *Drosophila* host defense. *Proc Natl Acad Sci USA.* 2006; 103:16424–16429. [PubMed: 17060622]
- Munier AI, Doucet D, Perrodou E, Zachary D, Meister M, Hoffmann JA, Janeway CA Jr, Lagueux M. PVF2, a PDGF/VEGF-like growth factor, induces hemocyte proliferation in *Drosophila* larvae. *EMBO Rep.* 2002; 3:1195–1200. [PubMed: 12446570]
- Nakahara Y, Kanamori Y, Kiuchi M, Kamimura M. *In vitro* studies of hematopoiesis in the silkworm: cell proliferation in and hemocyte discharge from the hematopoietic organ. *J Insect Physiol.* 2003; 49:907–916. [PubMed: 14511823]
- Nair SV, Burandt M, Hutchinson A, Raison RL, Raftos DA. A C-type lectin from the tunicate, *Styela plicata*, that modulates cellular activity. *Comp Biochem Physiol C Toxicol Pharmacol.* 2001; 129:11–24. [PubMed: 11369297]
- Perillo NL, Marcus ME, Baum LG. Galectins: versatile modulators of cell adhesion, cell proliferation, and cell death. *J Mol Med.* 1998; 76:402–412. [PubMed: 9625297]

- Qiu P, Pan PC, Govind S. A role for the *Drosophila* Toll/Cactus pathway in larval hematopoiesis. *Development*. 1998; 125:1909–1920. [PubMed: 9550723]
- Scott K, Weinberg C. Galectin-1: a bifunctional regulator of cellular proliferation. *Glycoconj J*. 2004; 19:467–477. [PubMed: 14758070]
- Shapiro M. Changes in the haemocyte population of the wax moth, *Galleria mellonella*, during wound healing. *J Insect Physiol*. 1968; 14:1725–1733.
- Takenaka Y, Fukumori T, Raz A. Galectin-3 and metastasis. *Glycoconj J*. 2004; 19:543–549. [PubMed: 14758078]
- Teramoto T, Tanaka T. Mechanism of reduction in the number of the circulating hemocytes in the *Pseudaletia separata* host parasitized by *Cotesia kariyai*. *J Insect Physiol*. 2004; 50:1103–1111. [PubMed: 15670857]
- Wang JL, Gray RM, Haudek KC, Patterson RJ. Nucleocytoplasmic lectins. *Biochim Biophys Acta*. 2004; 1673:75–93. [PubMed: 15238251]
- Yang RY, Hsu DK, Liu FT. Expression of galectin-3 modulates T-cell growth and apoptosis. *Proc Natl Acad Sci USA*. 1996; 93:6737–6742. [PubMed: 8692888]
- Yang RY, Liu FT. Galectins in cell growth and apoptosis. *Cell Mol Life Sci*. 2003; 60:267–276. [PubMed: 12678492]
- Yu F, Finley RL Jr, Raz A, Kim HR. Galectin-3 translocates to the perinuclear membranes and inhibits cytochrome c release from the mitochondria. A role for synexin in galectin-3 translocation. *J Biol Chem*. 2002; 277:15819–15827. [PubMed: 11839755]
- Yu XQ, Tracy ME, Ling E, Scholz FR, Trenczek T. A novel C-type immulectin-3 from *Manduca sexta* is translocated from the hemolymph into the cytoplasm of hemocytes. *Insect Biochem Mol Biol*. 2005; 35:285–295. [PubMed: 15763465]
- Zettervall CJ, Anderl I, Williams MJ, Palmer R, Kurucz E, Ando I, Hultmark D. A directed screen for genes involved in *Drosophila* blood cell activation. *Proc Natl Acad Sci USA*. 2004; 101:14192–14197. [PubMed: 15381778]

Fig. 1.

IML-3 is present in the membrane and soluble extracts of hemocytes. Membrane and soluble extracts were prepared from hemocytes of day 2 fifth instar larvae injected with saline (control) or LPS (*E. coli* 0111:B4, 20 μ g per larva) at 12 h post-injection. Aliquots of membrane and soluble extracts (50 μ g total proteins from each sample), or cell-free plasma (2 μ l) were analyzed by SDS-PAGE, and IML-3 was identified by immunoblotting using rabbit polyclonal antibody to IML-3. Each protein sample was prepared from hemocytes of at least four larvae, and the plasma was a mixture from the same larvae. The IML-3 band intensity in each lane was measured using Kodak Digital Science 1D gel analysis software. IML-3 in the samples of control larvae was arbitrarily set as 100% and relative amount of IML-3 in each protein sample was then calculated (panel B).

Fig. 2.

IML-3 is localized to the nucleus of larval hemocytes. (A) IML-3 is co-localized to the nucleus of naïve larval hemocytes. Hemocytes were collected from day 2 fifth instar naïve larvae and fixed in 4% paraformaldehyde. IML-3 in hemocytes was detected by rabbit antibody to IML-3 (green), and nuclei of hemocytes were stained with DAPI (blue). (a) Light phase images of hemocytes; (b and c) fluorescent images; (d) merged image of (b and c); Green = IML-3, blue = nuclei. The arrows indicate co-localization of IML-3 with nuclei. (B) IML-3 is detected in the nuclear extract of hemocytes. Nuclear proteins were prepared from hemocytes of day 2 fifth instar larvae injected with saline (control) or LPS (*E. coli* 0111:B4, 20 µg per larva) at 12 h post-injection. Aliquots of nuclear proteins (50 µg total proteins) were analyzed on SDS-PAGE, and IML-3 was identified by immunoblotting. (C) Relative amount of IML-3 in the nuclear extracts of hemocytes calculated based on the band intensity.

Fig. 3.

IML-3 is co-localized to the nucleus of metaphase, anaphase and telophase hemocytes.

Hemocytes were collected from day 2 fifth instar *M. sexta* larvae injected with LPS (*E. coli* 0111:B4, 20 µg per larva) at 12 h post-injection and fixed in 4% paraformaldehyde. IML-3 in hemocytes was detected by rabbit polyclonal antibody to IML-3 (green), and the nucleus of hemocytes was stained by DAPI (blue). (a–c) Light phase images of hemocytes; (d–l) fluorescent images; (j–l) merged images of IML-3 (green) and nucleus (blue) staining.

Green = IML-3, blue = nucleus. The arrows indicate a hemocyte in the metaphase, anaphase or telophase.

Fig. 4.

Expression of recombinant wild-type IML-3, Δ IML-3 and GFP in S2 cells. Whole cell lysates (10 μ g total proteins each) from normal S2 cells, stable S2 cells expressing recombinant wild-type IML-3, Δ IML-3 or GFP (after 24 h protein expression), or cell-free plasma (HL) (2 μ l) from day 2 fifth instar *M. sexta* naïve larvae (as a control sample) were separated on 15% SDS-PAGE, and proteins were identified by immunoblotting using rabbit polyclonal antibody to IML-3 (A) or mouse monoclonal antibody to the His-tag (B).

Fig. 5.

Recombinant Wild-type IML-3, but not Δ IML-3, is co-localized to the nucleus of some S2 cells. S2 cells and stable S2 cells expressing recombinant wild-type IML-3 or Δ IML-3 were induced to express proteins for 24 h and then fixed in 4% paraformaldehyde. Recombinant wild-type IML-3 or Δ IML-3 in S2 cells was detected by mouse monoclonal antibody to the His-tag (green), and the nucleus of S2 cells was stained by DAPI (blue) (A). (a–c) Light phase images of S2 cells; (d–i) fluorescent images; (g–i) merged images of IML-3 (green) and nucleus (blue) staining. Green = IML-3, blue = nucleus. The arrows indicate co-localization of recombinant wild-type IML-3 to the nuclei. Nuclear proteins were prepared from S2 cells, stable S2 cells expressing recombinant wild-type IML-3, Δ IML-3 or GFP (after 24 h protein expression). Aliquots of nuclear proteins (50 μ g total proteins) were analyzed by SDS-PAGE, and recombinant proteins were identified by immunoblotting using mouse monoclonal antibody to the His-tag (B).

Fig. 6.

Expression of recombinant wild-type IML-3, but not Δ IML-3, increases the number of proliferating S2 cells. S2 cells and stable S2 cells expressing recombinant wild-type IML-3, IML-3 or GFP were induced to express proteins for 0, 3, 12 and 24 h. Cells were then labeled with 5-bromo-2'-deoxy-uridine (BrdU) as described in the Section 2. Over 1000 total cells were counted from each well (three wells for each group). The percentage of BrdU-positive cells was calculated by the number of positive cells over the number of total cells counted. The columns represent the means of three individual counts $\pm$ S.E.M. Significance of difference was calculated using the Student's *t*-test (A). Recombinant proteins in S2 cells (1/10 of whole cell lysates from about 10^6 cells) were also detected by immunoblotting using mouse monoclonal antibody to the His-tag (B).