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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 ΔIML-3 that has the NWGV motif deleted in *Drosophila* S2 cells. We found that recombinant wild-type IML-3, but not ΔIML-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 ΔIML-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

Abbreviations: BH1, Bcl-2 homology domain 1; BrdU, 5-bromo-2′-deoxyuridine; 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.

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pathway, Rel/NF-κB 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 galelctin-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 ΔIML-3 that has the NWGV motif deleted in *Drosophila* Schneider 2 (S2) cells. We found that recombinant wild-type IML-3, but not ΔIML-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 ΔIML-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 ΔIML-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 ΔIML-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