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A novel granulocyte-specific α integrin is essential for cellular immunity in the silkworm *Bombyx mori*

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ABSTRACT

Haemocytes play crucial roles in immune responses and survival in insects. Specific cell markers have proven effective in clarifying the function and haematopoiesis of haemocytes. The silkworm *Bombyx mori* is a good model for studying insect haemocytes; however, little is known about haemocyte-specific markers or their functions in silkworm. In this study, we identified the α subunit of integrin, *BmintegrinαPS3*, as being specifically and highly expressed in silkworm haemocytes. Immunofluorescence analysis validated the specificity of BmintegrinαPS3 in larval granulocytes. Further analyses indicated that haemocytes dispersed from haematopoietic organs (HPOs) into the circulating haemolymph could differentiate into granulocytes. In addition, the processes of encapsulation and phagocytosis were controlled by larval granulocytes. Our work demonstrated that BmintegrinαPS3 could be used as a specific marker for granulocytes and could be applied to future molecular cell biology studies.

1. Introduction

Insects defences against exogenous pathogens mainly depend on the innate immune system (Lavine and Strand, 2002; Rodrigues et al., 2010), which includes humoral and cellular defence responses. Great progress has been made in the study of humoral defence, including the identification of antimicrobial peptides, as well as the signalling pathways that regulate their synthesis. However, less is known about cellular defence responses, such as phagocytosis, nodulation and encapsulation, for which granulocytes and plasmatocytes are responsible (Nardi, 2004; Schmidt et al., 2001).

Many cell adhesion molecules, including immunoglobulin, cadherin and integrin, which are located on the surface of haemocytes (Hynes et al., 2002), are involved in cellular immune responses. Integrins, which are found in many animal species ranging from invertebrates to vertebrates, are transmembrane receptors comprised of heterodimers between two distinct subunits. In mammals, the integrin family contains 18α and 8β subunits, which are capable of forming at least 24 distinct heterodimers that regulate cell growth, survival, phagocytosis, migration, and signal transduction across the plasma membrane (Huhtala et al., 2005; Shimaoka and Springer, 2003; Takada et al., 2007). In *Drosophila*, the 5α and 3β integrin subunits can form 3 different heterodimers through the combination of αPS1, αPS2, αPS3 and βPS, whereas heterodimers containing αPS4, αPS5, and another two β subunits have yet to be found. These integrins were shown to be involved in embryonic development, epithelial remodelling, muscle attachment, tracheal terminal branching, and the luminal organisation of tracheae (Brown, 2000; Bunch et al., 1998; Levin et al., 2005b; Schotman et al., 2008). Integrins regulate insect development (Surakasi et al., 2011b) and play important roles in immune defence responses, especially in phagocytosis (Mamali et al., 2009; Moita et al., 2006) and encapsulation (Hu et al., 2010; Lavine and Strand, 2003; Levin et al., 2005b; Pech and Strand, 1995; Xu et al., 2012) in insects. Integrin β1 is upregulated in haemocytes in response to various microbes in *Spodoptera exigua* (Surakasi et al., 2011a). When *Ostrinia furnacalis* integrin β1 is knocked down by RNA interference, encapsulation by haemocytes is significantly inhibited (Xu et al., 2012; Zhang et al., 2012). Integrin β also plays crucial roles in proPO activation, phagocytosis, and the antioxidant system for immunomodulation in white shrimp, which suggests that integrins may play wide roles in innate immune defence responses, even in the humoral response in invertebrates. In mammals, some integrins, such as α4β1, αLβ2, and αMβ2, are mainly found on haematopoietic cells, such

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as lymphocytes and monocytes, to regulate immune functions (Krieger et al., 2004). In insects, cell immune responses, such as nodulation and encapsulation, participate in the transformation of resting, non-adherent and/or adherent haemocytes. Additionally, these immune responses require a number of integrins that are specifically expressed in haemocytes, especially those that are only found in certain types of haemocytes (Gillespie and et al., 1997; Lavine and Strand, 2002).

The silkworm *Bombyx mori* has five types of haemocytes: prohaemocytes, plasmatocytes, granulocytes, spherulocytes, and oenocytoids (Tan et al., 2013). Our previous work identified the potential haematopoietic stem cell or progenitor, which may contribute to larval haematopoiesis in the silkworm (Tan et al., 2013). Silkworm larval haemocytes in haematopoietic organs (HPOs) were divided into two major lineages: a granulocyte lineage and a plasmatocyte–oenocytoid lineage (Nakahara et al., 2010a). However, the identification of different types of haemocytes currently depends only on cell morphology, and the exact function of the five types of haemocytes remains unknown (Yamashita and Iwabuchi, 2001a).

In this study, a specific integrin α subunit was identified from the haemocytes of the silkworm *B. mori*. Phylogenetic analysis indicated that this protein belongs to the αPS3 family; thus, we named it *BmintegrinαPS3*. qRT-PCR analyses and immunofluorescence assays showed that *BmintegrinαPS3* is a specific marker of circulating granulocytes and is located on the cell membrane. These characteristics of *BmintegrinαPS3* expression might be a promising tool to help researchers understand the origin and function of granulocytes.

2. Materials and methods

2.1. Biological materials

Dazao (p50) from the Gene Resource Library of Domesticated Silkworm of Southwest University, China, were reared with mulberry leaves as described previously (Tan et al., 2013). Haemocytes were collected from silkworm larvae by cutting a leg. Other tissues, including wing disc, epidermis, fat body, head, midgut, Malpighian tubules, silk gland, testis, and ovary, were dissected and stored at −80 °C. Eggs were collected on different days during whole embryo stages and were stored as above. The larval period was designated as the number of larvae (L) and day (D). For example, silkworms at the 3rd day of 5th larvae and the moulting stage of 4th larvae were L5D3 and L4M, respectively.

2.2. Cloning of BmintegrinαPS3

Total RNA was isolated from silkworm haemocytes using an SV Total RNA Isolation System kit (Promega, USA) according to the manufacturer's instructions. After the RNA was treated with RNase-free DNaseI, first-strand cDNA was synthesised using M-MLV reverse transcriptase (Promega, USA). The sequences of the primers used in this experiment were as follows: int-CDS-F, 5′-ATGTTTGGCCTAAGAACATCA-3′ and int-CDS-R, 5′-ATCTTCCAACGATGTTACAACTT-3′. PCR was performed with HiFi Taq DNA polymerase (TransGen Biotech, China) under the following conditions: 94 °C for 2–4 min, followed by 30 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 2 min, and a final extension at 72 °C for 10 min. The PCR product was cloned into a pEASY™-T5 Zero Cloning Vector (TransGen Biotech, China) and sequenced at Invitrogen (Shanghai, China).

2.3. Alignment and comparison of integrin amino acid sequences

The full-length *BmintegrinαPS3* cDNA was acquired using PCR amplification and rapid amplification of cDNA ends (RACE) in our previous study (Zhang et al., 2014). The amino acid sequences of integrin α subunits from *Apis mellifera* (Am), *Tribolium castaneum* (Tc), *Anopheles gambiae* (Ag), *Danaus plexippus* (Dp), *Drosophila melanogaster* (Dm), *Manduca sexta* (Ms), *Pseudoplusia includens* (Pi), *Caenorhabditis elegans* (Ce), and *Homo sapiens* (Hs) were downloaded from the National Center for Biotechnology Information (NCBI); the sequences used for this analysis were shown in Supplementary data Table S1, and the alignment was performed using ClustalW. The phylogenetic tree was constructed using the neighbour-joining method (Tamura et al., 2007).

2.4. Quantitative Real-Time PCR (qRT-PCR)

Total RNA from each tissue was isolated using TRIzol regent (Invitrogen) and subjected to cDNA synthesis using reverse transcriptase AMV (Promega). *BmintegrinαPS3* mRNA expression was determined by qRT-PCR using the StepOne Plus™ Real-Time PCR system (Applied Biosystems). *GAPDH* was used as an internal control. The sequences of the primers for qRT-PCR were as follows: int-qRT-F, 5′-CGGAGAACTATTGTGGGCGT-3′; int-qRT-R, 5′-CTCAGTGAAAGCCGAAAGCAT-3′; GAPDH-F, 5′-CATTCCGCGTCCCTGTTGCTAAT-3′; and GAPDH-R, 5′-GCTGCCTCCTTGACCTTTTGC-3′. All reactions were performed in triplicate in a total volume of 20 μL. The conditions for PCR were 95 °C for 30 s following by 40 cycles of 95 °C for 5 s and 60 °C for 30 s. Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta CT}$ method (Bustin et al., 2009; Livak and Schmittgen, 2001). The online t-test software GraphPad Software (http://www.graphpad.com/quickcalcs/ttest1.cfm) was used to evaluate statistical significance ($P < 0.05$).

2.5. Expression of recombinant BmintegrinαPS3 protein and antibody preparation

The truncated coding sequence of BmintegrinαPS3 was amplified by PCR from the haemocyte cDNA template. The sequences of the primers were as follows: int-F, 5′-TGTAACCCAGTTGACATTGACG-3′ and int-R, 5′-CAGTGAAAGCCGAAAGCATC-3′. The PCR product was digested with BamHI and XhoI (Takara, Japan) and ligated into PET28a. The recombinant plasmid (PET28a-His-BmintegrinαPS3-His) was transformed into competent *Escherichia coli* BL21 (DE3) cells, and the expression of recombinant BmintegrinαPS3 was induced by 0.6 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) for 4 h at 37 °C. Recombinant protein was purified using the HIS*BIND PURIFICATION KIT (Merck, Germany) according to the manufacturer's instructions. The purified protein was mixed and homogenised with an equal volume of complete Freund's adjuvant (Sigma) for the initial injection and with an equal volume of incomplete Freund's adjuvant (Sigma) for the subsequent three injections to immunise New Zealand rabbits according to standard procedures (Harlow and Lane, 1988). Antiserum against integrinαPS3 in rabbits was then prepared.

2.6. Immunofluorescence assay

Collected haemocytes were cultured on coverslips for 30 min at room temperature as described previously (Tan et al., 2013). The cells were fixed in 4% (w/v) paraformaldehyde (PFA) for 15 min. After being washed three times with PBS, the cells were blocked with 10% goat serum in PBS for 1 h at room temperature. Cells were stained with anti-integrinαPS3 serum (1:1000) and an anti-tubulin mouse monoclonal antibody (1:1000, Sigma). Then, the cells were stained with the following secondary antibodies: Alexa Fluor® 595 goat anti-rabbit IgG (H+L)(1:1000, Invitrogen), Alexa Fluor® 645 goat anti-rabbit IgG (H+L)(1:1000, Invitrogen), and Alexa Fluor® 488 goat anti-mouse IgG (H+L)(1:1000, Invitrogen). Nuclei were counterstained by adding 300 μL of DAPI. The stained samples