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Evolution of Developmental Control Mechanisms

Segmental pairs of giant insect cells discharge presumptive immune proteins at each larval molt

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ABSTRACT

A pair of massive secretory cells exists within each thoracic and the nine abdominal segments of *Manduca* larvae. Each of these cells is nestled between the dorsal integument and underlying muscles. Contents of large vacuoles in these cells are abruptly discharged at each molt and have always been considered to contribute to shedding and/or formation of cuticle. Peanut agglutinin is a specific lectin label for these secretory vacuoles; vacuoles label intensely immediately before each molt as vacuoles attain their maximal size. Contents of vacuoles are restored after each molt and throughout most of each intermolt. During the molt cycle these cells secrete contents of their vacuoles into the interior hemocoel rather than onto the exterior cuticle. Vacuoles discharge via a distinctive mechanism involving partitioning of contents into numerous vesicles that move to the cell surface. Dermal secretory cells were dissected from larvae before and after the 4th–5th instar molt. Proteins from pre-molt and post-molt secretory cells were separated by two-dimensional electrophoresis to establish which proteins are discharged at the molt. While secreted proteins are novel, all have presumptive roles in immune responses. Dermal secretory cells may represent a new, unsuspected component of the innate immune system that release their proteins during the vulnerable molting period of an insect's life.

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1. Introduction

Despite their imposing sizes, dermal secretory cells of larval dermal glands have remained cells with uncertain functions. Each dermal gland consists of three cells – duct cell, saccule cell, and secretory cell. Secretory cells are present throughout larval life and grow in size without any cell divisions. The size of each secretory cell and its vacuoles is closely coupled to each molt cycle and hormonal titers (Horwath and Riddiford, 1988; Lane et al., 1986); at each molt cells attain their maximum size for the preceding instar. At each molt, the contents are abruptly expelled, and secretory cells shrink dramatically in size. Growth of the secretory cell increases throughout the subsequent intermolt and continues until the next molt. At the larval-pupal molt, dermal glands undergo programmed cell death.

Since their discovery (Verson, 1890), these giant cells of the dermal glands have been assumed to function in secreting a substance at the time of the molt – such as molting fluid (Barbier, 1970) or a cement layer (Horwath and Riddiford, 1988; Wigglesworth, 1947) – that is discharged onto the surface of the cuticle. Secretory cells of dermal glands had therefore always been assumed to discharge the contents of their large vacuoles through the associated saccule and duct cells.

Lai-Fook (1973) observed that while the smaller saccule cell of the dermal gland clearly releases granules to the cuticular surface through the channel of its contiguous duct cell, the larger secretory cell has no evident structure resembling a secretory apparatus. Her detailed observations of larval secretion and discharge are not consistent with the dermal secretory cells having a function in molting as was suggested by Wigglesworth (1947), Way (1950) and Barbier (1970) and as subsequently assumed by Horwath and Riddiford (1988).

Despite all these earlier claims about functions for these large cells, Delhanty and Locke (1990) cautiously observed that "the function of the secretion is still uncertain". New information on the

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sequences of proteins that are secreted at the molt by *Manduca* dermal secretory cells provides clues about the function of the secretion and reinforces the claim that secretion from the vacuoles of the large secretory cell is discharged into the surrounding hemocoel and internal larval environment rather that to the external environment and surface of the integument.

2. Materials and methods

2.1. Rearing of larval Manduca sexta

All developmental stages of this insect were fed an artificial diet and maintained in an incubator at constant temperature (26 °C) and constant photoperiod (16L:8D).

2.2. Preparation of whole mounts for lectin and antibody labeling

For dissections, anesthesized larvae were placed in petri dishes in which black Sylgard (Dow Corning) had been added as a substrate. To this silicone surface, whole first and second instar larvae were pinned ventral surface up with stainless steel minutien pins (0.1 mm diameter) and dissected in sterile Grace's insect culture medium (Invitrogen, pH adjusted to 6.5). Each cylindrical larva was cut along its ventral midline with iridectomy scissors from head capsule to anus. The cut edges of the larval integument were spread and pinned down, with two pins at the anterior end and two pins at the posterior end. Converting the initially cylindrical integument to a rectangular planar integument exposes the internal alimentary canal and ventral nerve cord. After excising the entire gut and nerve cord, the dermal cells, muscles, dorsal vessel and fat body that line the inner surface of the dorsal integument are exposed for viewing. After dissection, tissues were either processed for (1) sectioning or for (2) preparation of whole mounts. Upon addition of fixative, the pinned tissue retained its planar configuration. Either whole larvae were labeled as whole mounts or specific regions were excised for sectioning or labeling.

Tissues were fixed for 30 min with 4% paraformaldehyde that had been dissolved in phosphate-buffered saline (PBS, pH 7.4). After three rinses in PBS, tissues were permeabilized for at least 30 min by the addition of blocking buffer (PBS + 10% normal goat serum + 0.1%Triton X-100). After an overnight incubation with PNA lectin coupled to either rhodamine or fluorescein (10 μg/ml, Vector Laboratories) or with primary antibody (mouse anti-lacunin or mouse anti-neuroglian, 1:2000) dissolved in blocking buffer at 4° C, tissues were rinsed at least three times with blocking buffer. After labeling with primary mouse antibodies, rinsed tissues were incubated overnight in the cold with 7.5 mg/ml of a secondary fluorescein isothiocyanate (FITC) coupled goat anti-mouse antibody (Vector). The two primary antibodies are mouse monoclonals prepared against the *Manduca* proteins neuroglian and lacunin (Nardi et al., 1999; Nardi, 1994). In addition, a specific marker for DNA was sometimes used to label nuclei of cells; its labeling concentration was 1 μg/ml. This specific DNA marker is a blue fluorescent compound known as 4′,6-diamidino-2-phenylindole or DAPI. Following three more rinses with blocking buffer, labeled tissues were mounted in 70% glycerin (v/v) in 0.1 M Tris (pH 9.0). Fluorescently labeled specimens were imaged with a Nikon E600.

2.3. Preparation of tissues for high-resolution microscopy

For high resolution imaging of the internal structures, whole dermal secretory cells or abdominal segments or hemisegments containing one or more dermal secretory cells were fixed in the cold for three hours with a mixture of 0.5% paraformaldehyde and 2.5% glutaraldehyde in a rinse buffer (0.1 M cacodylate buffer (pH 7.4) containing 0.18 mM $CaCl_2$ and 0. 58 mM sucrose). After this initial fixation, tissues were washed three times with rinse buffer before being post-fixed for three – four additional hours in the cold with rinse buffer containing 2% OsO_4. Three additional washes with rinse buffer followed the post-fixation. To enhance membrane contrast, tissues were placed in filtered, saturated uranyl acetate for 30 min immediately prior to being passed through a graded series of ethanol concentrations (10–100%).

From absolute ethanol, tissues for sectioning were transferred to propylene oxide and infiltrated with mixtures of propylene oxide and resin before being embedded in pure LX112 resin. Resin was polymerized at 60 °C for three days followed by an additional overnight treatment in an 80 °C oven.

Embedded tissues were sectioned with a diamond knife either at 0.5 μm for light microscopy or at ~0.09 μm for electron microscopy. Sections for light microscopy were mounted on glass slides and stained with a solution of 0.5% toluidine blue in 1% borax. Thin sections of those regions chosen for ultrastructural examination were mounted on copper grids and stained briefly with saturated aqueous uranyl acetate and Luft's lead citrate to enhance contrast. Images were taken with a Hitachi 600 transmission electron microscope operating at 75 kV.

2.4. Processing of dermal secretory cells for electrophoresis

Two samples of dermal secretory cells were mailed to Kendrick Laboratories in Madison, Wisconsin for two-dimensional (2D) electrophoresis of deglycosylated proteins. One sample contained 61 pre-molt cells; the second sample contained 66 post-molt cells. To each sample was added 50 μl of SDS Boiling Buffer without reducing agents in addition to 50 μl of Osmotic Lysis Buffer with 10 × nuclease stock, phosphatase inhibitor I and II stocks, and protease inhibitor stock. These samples were vigorously vortexed and heated at 100 °C for 5 min. Concentrations of proteins were measured with the BCA (BiCinchoninic Acid) assay (Pierce Chemical Co., Rockford, IL). From each sample, an aliquot of 200 μg was deglycosylated with Enzymatic DeGlycoMix Kit (QA Bio). The procedure was carried out at 37 °C for 3 h. The two lyophilized samples were finally dissolved in 1:1 diluted SDS Boiling Buffer: Urea Sample Buffer containing reducing agents prior to loading 200 μg/50 μl of each on gels.

A third sample of 20 dermal secretory cells was mailed to Kendrick Laboratories for lectin (PNA) blotting following 2D electrophoresis of glycosylated proteins. This sample was prepared for electrophoresis as described in the preceding paragraph but without the 3-h deglycosylation procedure.

2.5. Two-dimensional electrophoresis

Two-dimensional electrophoresis was performed according to the carrier ampholine method of isoelectric focusing (IEF) (Burgess-Cassler et al., 1990). Focusing was carried out in a glass tube with an inner diameter of 2.3 mm using 2% pH 3–10 Isodalt Servalytes (Serva, Heidelberg, Germany) for 9600 V-hours. An internal standard of tropomyosin added to each sample migrated as a doublet with lower polypeptide spot of MW 33,000 and pI 5.2. After equilibration in the following buffer (10% glycerol, 50 mM dithiothreitol, 2.3% SDS, and 0.0625 M Tris, pH 6.8), each tube gel was sealed to the top of a stacking gel overlying a 10% acrylamide slab gel (0.75 mm thick). Protein separation in the slab gel occurred over a 4-h period at 15 mA/gel. Six molecular weight standards ranging between 220,000 kDa and 14,000 kDa marked the basic edge of each slab gel.

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