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Mechanisms of black and white stripe pattern formation in the cuticles of insect larvae

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Abstract

Molecular mechanisms that produce pigment patterns in the insect cuticle were studied. Larvae of the armyworm *Pseudaletia separata* have stripe patterns that run longitudinally along the body axis. The pattern in the cuticle became clear by being emphasized by the increasing contrast between the black and white colors of the lines after the last larval molt. We demonstrated that dopa decarboxylase (DDC) mRNA as well as protein are expressed specifically in the epidermal cells under the black stripes. The pigmentation on the stripes was clearly diminished by injection of a DDC inhibitor (*m*-hydroxybenzylhydrazine) to penultimate instar larvae for 1 day before molting, suggesting that DDC contributes to the production of melanin. Further, electron microscopic observation showed that the epidermal cells under the gap cuticle region (white stripe) between the black stripes contain many uric acid granules, which gives a white color. Our findings suggest that the spatially regulated expression of DDC in the epidermal cells produces the black stripes while abundant granules of uric acid in the cells generate the white stripes in the cuticle. Based on these results, we concluded that this heterogeneity in the epidermal cells forms cuticular stripe patterns in the armyworm larvae.

Keywords: Dopa decarboxylase; Cuticle; Pigment pattern; Uric acid; Growth-blocking peptide (GBP)

1. Introduction

To adapt to the environment, animals must have changed their surface pigment patterns under various selective forces such as threats from predators and sexual selection. Changes in the pattern are often the result of mimicry or development. While the significance of the pigment pattern formation is easily recognized by the diversity of the patterns, the molecular mechanisms that generate the pigment patterns are not fully elucidated (Nijhout, 1991).

Wings of insects provide the most conspicuous example of the diversity of the pigment pattern. The molecular mechanisms that generate these wing patterns have been studied in the swallowtail butterfly, *Papilio glaucus*, by analyzing both wild-types and melanic mutants (Koch et al., 1998, 2000). Females of this species can be either the wild type (predominantly yellow with black tiger stripes) or the melanic mutants in which much of the wild-type yellow pattern is replaced with black (Scriber et al., 1996). In this system, the spatially and temporally regulated activity of dopa decarboxylase (DDC), a key enzyme for producing the two major pigments such as papiliochrome (yellow) and melanin (black), produces the yellow/black pattern of the wings (Koch, 1994; Koch and Kaufmann, 1995). Further, in *Drosophila melanogaster*, the regulation and function of the *yellow* and *ebony* genes play crucial roles in the process of the pigment pattern formation: the pattern and levels of Yellow and Ebony expression together determine the pattern and intensity of melanization both in the cuticle and the wing (Wittkopp et al., 2002).

Larvae of the armyworm *Pseudaletia separata* have the stripe pattern, running longitudinally along the body axis,

Abbreviation: DDC, dopa decarboxylase; Dopa, 3, 4-dihydroxy-L-phenylalanine; GBP, growth-blocking peptide; TH, tyrosine hydroxylase

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in the dorsal cuticle throughout all larval stages. However, the pattern becomes clear by being emphasized by the increasing contrast between black and white colors of the lines after the last larval molt. The black line is formed by melanins which belong to one of the diverse classes of polymerized oxidation products of phenolic precursors and are the most widespread pigments in the biological world. In insects, melanins are derived from catecholamine precursors such as 3,4-dihydroxy-L-phenylalanine (Dopa) and dopamine, which are synthesized from tyrosine (Sturm et al., 1998; Barsh, 1996). Some of the enzymatic steps in the melanin synthesis pathway are understood both genetically and biochemically (Wright, 1987). Tyrosine hydroxylase (TH) and DDC convert tyrosine to Dopa and Dopa to dopamine, respectively, which are then processed by a system of phenoloxidases (POs) and co-factors to form melanins during cuticle development. A previous report on the swallowtail butterfly showed that DDC is expressed in patterns coinciding with black color formation (Koch et al., 1998), suggesting that ectopic DDC expression might be involved in the genesis of novel melanin patterns. However, it was later reported that no ectopic pigmentation was seen in the *Drosophila* imaginal discs with strong ectopic DDC expression. In contrast, ubiquitous TH expression caused gradual development of ectopic brown stripes in the intervein areas of the wings during the early adult stage. Further, co-expression of TH and DDC increased the frequency of intense ectopic pigmentation (True et al., 1999). These results were interpreted to mean that DDC activity may be essential to form intense black melanins under the basic expression of TH.

In this study, we focused on the special and temporal patterns of DDC expression in the armyworm larval cuticle to examine the molecular mechanisms that generate pigment stripe patterns which obviously become intense after the final molt. DDC expression was clearly localized in the cells under the black stripe in the dorsal cuticle. Further, the epidermal cells under the white stripe contain a number of granules including uric acid that locally whiten the cuticle. Thus, it is clear that this heterogeneity in the epidermal cells forms cuticular stripe patterns in the armyworm larvae.

2. Materials and methods

2.1. Animals

P. separata larvae were reared on an artificial diet at 25±1 °C in a photoperiod of 16-h light/8-h dark (Hayakawa, 1990). Penultimate instar larvae undergoing ecdysis between 4 and 4.5 h after starting the light period were designated as day 0 last instar larvae.

2.2. Chemicals

Dopamine, Dopa, *m*-hydroxybenzylhydrazine (NSD1015: DDC inhibitor) and 3-iodotyrosine (TH inhibitor) were obtained from Nacalai Tesque Co. (Japan). All other chemicals were reagent grade.

2.3. Dissection of integument

A whole abdominal integument (approximately 1 cm width) between the first and second segments was dissected from the test armyworm larva. Care was taken to remove all the adhering fat body tissue from the integument. The dissected integument was separated into dorsal and ventral parts, and, in some experiments, the black stripes were further isolated from the dorsal part of the dissected integument. After washing with phosphate buffered saline (NaCl/Pi: 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 137 mM NaCl, 2.7 mM KCl, pH7.2), the tissues were lightly blotted with filter paper, weighed and immediately used for experiments.

2.4. Dopa and dopamine quantification

A piece of integument was immediately transferred to a 1.5 ml microtest tube containing 200 µl ice-cold 0.2 N perchloric acid and homogenized using an Altek Sonic Dismembrator (10 pulses at 50 W). The homogenate was centrifuged at 20,000*g* for 10 min at 4 °C, and the supernatant was directly analyzed by HPLC-ECD (electrochemical detection) (Hayakawa et al., 1987).

2.5. DDC assay

Dissected tissue was homogenized in ice-cold 0.1 M phosphate buffer (pH 7.2) containing 0.2 M sucrose and 0.05% phenylthiourea by sonication (10 pulses at 40 W). The supernatant after centrifugation at 20,000*g* for 5 min was assayed for DDC as described previously (Noguchi and Hayakawa, 2001).

To determine whether DDC and TH contribute to produce the black stripes in the dorsal cuticle, larvae were treated with enzyme inhibitors. Each day 2 penultimate instar larva was injected with 0.2 µmol of *m*-hydroxybenzylhydrazine (DDC inhibitor) or 0.04 µmol of 3-iodotyrosine (TH inhibitor) twice at 8 and 14 h after lights on.

2.6. Reverse transcription PCR (RT-PCR)

Total RNA was extracted from larval integument using TRIzol reagent (Invitrogen) according to manufacturer's protocol. The 5 µg RNA was transcribed using ReverTra Ace (TOYOBO) at 42 °C for 60 min. Synthesis of first strand cDNA was initiated by using an oligo(dT) primer. The cDNA was amplified with the DDC-specific primer pair (5′-ATGGAGGCCGGAGATTTCAAAG-3′, +1 to +22 bp; and 5′-ACGGGCTTTAAGTATTTCATCA GGC-3′, +1405 to +1428 bp) and actin primer pair (5′-TTCGAGCAGGAGATGGCCACC-3′ and 5′-GAGATC CACATCTGYTGGAAGGT-3′). The PCR reaction