LOCALIZATION AND EXPRESSION PROFILES OF Bombyx mori Ecdysone Receptor-B1 IN THE FAT BODY CELLS UNDERGOING PROGRAMMED CELL DEATH

Mohamed Elmogy
Department of Entomology, Faculty of Science, Cairo University, Giza, Egypt

ABSTRACT

BmEcR-B1 isoform localization, distribution patterns, and expression profile are important for understanding the molecular pathways of the PCD in the fat body cells of Bombyx mori. Different Immuno-histochemical and biochemical localization techniques of BmEcR-B1 show that this protein is clearly indicated and is restricted in the cell nuclei and not translocated to cytosol or cell membrane during the prepupal period. The specific emerging timing of BmEcR-B1 in fat body cells of G1 of the prepupal period demarks the initiation of degeneration of the fat body cells. The expression analyses revealed that changes in BmEcR-B1 RNA levels coincides on that of its protein in the fat body cells during the prepupal period. The obtained data are discussed, and another evidence for the dual functions of ecdysteroids to act through membrane receptor in addition to the conventional nuclear receptor during prepupal period in the fat body cells of B. mori are suggested.

Keywords: Apoptosis; Bombyx; immunolocalization; expression; membrane/nuclear receptors; metamorphosis.

INTRODUCTION

The insect hormone 20E induces PCD of larval specific tissues at larval-pupal metamorphosis (Buszczak and Segraves 2000; Baehrecke, 2000). Genetic regulation of 20E-induced PCD has been extensively studied in Drosophila (Truman et al., 1992; Robinow et al., 1997; Igaki et al., 2000; Baehrecke, 2000; Bangs et al., 2000; Quinn et al., 2000; Martin and Baehrecke, 2003) and in Bombyx anterior silk glands (Kakei et al., 2005; Sekimoto et al., 2006; Iga et al., 2007).

Like other steroid hormones, 20E exerts its effect through genomic pathway (Takaki and Sakurai, 2003) in which the contribution of EcR and its heterodimeric partner USP have been characterized (Baker et al., 1987; Takaki and Sakurai, 2003). Three isoforms of ecdysone receptor, i.e. EcR-A, EcR-B1 and EcR-B2, were first identified in Drosophila (Truman et al., 1992). Subsequently, homologs of Drosophila EcR-A and EcR-B1 isoforms were characterized in Manduca sexta, B. mori, Chironomus tentans, and Aedes aegypti (Imhof et al., 1993; Cho et al., 1995; Fujiwara et al., 1995). During the prepupal period, 20E triggers PCD and up-regulates EcR-B1 in ASGs of B. mori (Kaneko et al., 2006). The temporal expression of Bombyx EcR-A and EcR-B1 are synchronous during the fifth instar in the ASGs, while EcR-B1 is the predominant isoform in wing discs, epidermis, mid- gut, and fat body (Kamimura et al., 1999). However, the developmental profile of BmEcR-B1 expression on the transcriptional and protein levels in Bombyx fat body are not studied yet.

On the other hand, recent studies suggest that steroid hormones use receptors on cellular membranes (nongenomic pathway), both to gain access to the intracelluar compartment and to modulate cellular function (Watson and Gametchu, 1999; Manaboon et al., 2008). The molecular mechanisms that stimulate the 20E nongenomic pathway are largely unknown, but in Drosophila, stimulation of this pathway is known to involve the catecholamine receptor (Dopamine/ecdysone receptor; DmDopEcR) (Srivastava et al., 2005). In addition, previous studies (Elmogy et al., 2004, 2006, 2007a) provided several biochemical and topological evidence for a putative BmEcR located in the plasma

Abbreviations: BmEcR-B1, Bombyx nuclear ecdysone receptor-B1; mEcR, membrane ecdysone receptor; PCD, programmed cell death; 20E, 20-hydroxyecdysone; ASGs, anterior silk glands; USP, ultraspiracle; G0,G1, G2, G3, days 0, 1, 2, 3 after gut purge; PO, pupation day.

*E-mail: elmoogy@yahoo.com
membrane of the ASGs of *B. mori*. Later on, we have recognized and characterized membrane ecdysone binding sites in the plasma membrane of the fat body cells of *B. mori* (Elmogy et al., 2007b). However, considerable controversies exist over the mechanisms underlying these nongenomic effects (Losel et al., 2003). Some may be attributable to the exposure of conventional nuclear steroid receptor proteins to extracellular steroid signals at the plasma membrane so that they interact with G-protein-coupled pathways (Losel and Wehling, 2003). Therefore, the histochemical and biochemical immunolocalization of *BmEcR*-B1, presented in this study are important to eliminate these controversies and to understand the molecular pathways of the PCD in the fat body cells of *B. mori*.

Hence, the aim of the present work was the analysis of distribution and expression patterns of *BmEcR*-B1 and the assessment of the relationships between receptor status and the previously recognized ecdysone binding sites (mEcR) in the plasma membrane of the *Bombyx* fat body cells during PCD.

**MATERIALS AND METHODS**

**Insects**

Larvae of the silkworm, *Bombyx mori* (Kinshu X Showa F1 hybrid) were reared on an artificial diet (Silkmate 2M, Nihon Nosan Kogyo, Yokohama, Japan) at 25°C under a 12 h light: 12 h dark cycle, as described previously (Elmogy et al., 2006). Since the 4th ecdysis to the 5th instar occurred during the scotophase, the newly molted 5th (last) instar were segregated at the beginning of the following photophase and immediately fed. Day 0 was designated as the day consisting of a scotophase [during which larvae underwent gut purge] (Truman and Riddiford, 1974), and the following photophase. Fat bodies of larvae were dissected during the photophase of days 0, 1, 2, 3, and day 4, *i.e.* pupation day (phases: G0, G1, G2, G3 and P0).

**Histochemistry**

Fat bodies collected at G0 to P0 were washed three times by cold insect Ringer (130 mM NaCl, 5 mM KCl, 0.1 mM CaCl2 and 1 mM PMSF) and fixed overnight at 4°C in Bouin solution. Standard histochemical techniques were employed to prepare tissue sections (5 µm) in paraffin that were dewaxed and rehydrated as described previously (Shao et al., 2006). Observations of positive control nuclei were conducted with sections that were stained with 0.001% of Acridine orange (AO) (Sigma, St. Louis, MO), as described previously (Solomon and Wolfe, 1997).

**Immunohistochemistry**

Rehydrated sections were rinsed in Tris-buffered saline containing 0.3 Tween 20 (TBS; 0.05 M Tris-HCl, pH 7.6) and blocked with 5 % normal goat serum in TBS for 30 min at room temperature (RT), followed by incubation with the primary antibody for *BmEcR*-B1 (a gift from H. Fujiwara, University of Tokyo, Japan), 1:500, overnight, at 4°C. Bound IgG was detected with rabbit IgG Vectastain Elite ABC Kit (Vector Laboratories, Burlingame, CA) as described previously (Shao et al., 2008). The activity of the horse radish peroxidase (HRP) marker of the secondary antibody was visualized with 0.005 % hydrogen peroxide and 0.25 mM 3, 3’-diaminobenzidine tetrahydrochloride (DAB) solution (0.1 M Tris-HCl, pH 7.5). In the negative control experiments, the primary antibody was replaced with preimmune rabbit serum.

**Immunofluorescence**

Similar procedures were followed for immunofluorescence detection but, TBS was replaced by phosphate buffered saline (PBS; 145 mM NaCl, 8.5 mM Na2HPO4, 1.45 mM NaH2PO4, pH 7.5) suplimented with 0.3% Tween 20 (PBST). Briefly, after blocking, primary antibody incubation and rinsing with PBST (3x10 min) at RT, the sections were incubated with goat anti-rabbit IgG-Cy3 conjugated secondary antibody (Jackson Immuno Research, West Grove, PA), diluted at 1:500 in blocking serum for 1 h at RT. The sections were then rinsed again with PBST (3x10 min). Stained sections were dehydrated and mounted in Bioleit medium (Kouken Rika, Osaka, Japan). Staining was visualized using
a BX50 microscope (Olympus, Tokyo, Japan) equipped with the Nomarski (differential interference contrast), Epifluorescence optics and a CCD camera (Olympus).

Northern blot hybridization analysis

Total RNA from fat bodies during prepupal period (G0 of larval gut purge to P0) was purified using Sepasol RNA I Super (Nacalai tesque, Kyoto, Japan). Aliquots of 20 µg of total RNA from fat bodies were separated on 1% agarose formaldehyde gel. The resolved RNAs were transferred to Hybond N+ membrane (Amersham-Pharmacia, Buckinghamshire, UK) through capillary transfer. The membrane was prehybridized in ExpressHyb™ Hybridization solution (Clontech, Palo Alto, CA) for 30 min at 58°C, and hybridized for 1 h at 60°C in the same buffer with \( \text{BmEcR-B1} \) antisense oligonucleotide probe 5'- CGGTGTTGTGGGAGGCATTGGTA-3', according to Kamimura et al., 1996, which was 5' end labeled with \( \gamma^{32}\text{P} - \text{ATP} \) (MP Biomedical, Ohaio, USA), using The DNA 5' End-labeling System (Promega, Madison, WI). After hybridization, the membrane was washed twice in wash solution I (2x SSC, 0.05% SDS) for 30 min each at room temperature and twice in wash solution II (0.1x SSC, 0.1 SDS) at 60°C for 40 min each. For signal detection, the membrane was exposed to Hyperfilm-MP (Amersham).

Gel electrophoresis and Western blotting

SDS-PAGE was performed according to the method of Laemmli (1970). Briefly, the fat body at the appropriate developmental stages was rapidly dissected in cold insect Ringer solution, homogenized on ice in 1 ml of homogenization buffer (10 mM Tris-HCl pH 7.4, 1 mM EDTA, 1 mM DTT and 1 mM PMSF), supplemented with protease inhibitors cocktail (Complete, Roche Diagnosis GmbH, Mannheim, Germany), and filtered through 3 layers of gauze to remove the lipid layer. The filtered homogenate was used as total lysate.

The membrane fractions were prepared from G2 fat body as previously described (Elmogy et al., 2007b). Briefly, the filtered homogenate was centrifuged at 1000 xg for 10 min. Supernatant was recovered and centrifuged at 105,000 xg for 60 min. The supernatant was used as cytosolic fractions and the resulting pellet was resuspended into homogenization buffer supplemented with 1M NaCl for 1h at 4°C with gentle stirring. Finally, the mixture was centrifuged at 105,000 xg for 60 min at 4°C to obtain membrane fractions as pellet.

Nuclear extract was also prepared from G2 fat body as previously described (Elmogy et al., 2007b). Briefly, dissected fat body from G2 stage was washed once in 100 mM phosphate buffer (pH 7.9) with 100 mM NaCl and homogenized on ice with 2 vol of 10 mM HEPES (pH 7.9) with 10 mM KCl, 0.3 M sucrose, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM 2-mercaptoethanol, and protease inhibitors cocktail, by 12 strokes in a Downcie tissue grinder (1 ml, Wheaton, Millville, NJ, USA). The suspension was centrifuged for 8 min at 1,600 xg at 4°C. The resulting pellet was resuspended in 2 vol of 10 mM HEPES (pH 7.9) containing 0.4 M NaCl, 5% glycerol, 1.5 mM MgCl₂, 0.1 mM EDTA, 0.5 mM DTT and 0.5 mM PMSF, and 5 M NaCl was added to yield a final concentration of 0.4 M NaCl. The suspension was incubated at 4°C under gentle shaking for 30 min and subsequently centrifuged at 4°C at 105,000 xg for 60 min. The resulting supernatant was dialysed for 4 h using a dialysis bag with 14,000 Da cut off-size (Wako Pure Chemical Industries, Osaka, Japan) against 1000 volumes of 20 mM HEPES (pH 7.9) containing 20 mM NaCl, 20% glycerol, 1 mM EDTA and 0.5 mM 2-mercaptoethanol, and protease inhibitor cocktail. The nuclear extracts were clarified by 10 min centrifugation at 10,000 xg at 4°C.

Protein content was measured using the DC protein assay kit (Bio-Rad, Hercules, CA, USA) according to the instruction of the supplier and Bovine serum albumin (BSA; Sigma, St. Louis, MO) as a standard.

Equal amounts of fat body extracts (20 µg protein equivalent) were separated on 10% SDS-polyacrylamide gels, and either transferred to 0.45 µm Immobilon-P membranes (Millipore), or duplicate gels of identical protein extracts were assessed for integrity by Coomassie blue (CBB) staining (Bio-Rad). Blots were blocked in 5% non-fat milk in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) for 2h and then parallel blots were incubated with the primary antibody (1:1000) raised against \( \text{BmEcR-B1} \), at 4°C overnight. After washing with TBST, the blots were incubated with
HRP-conjugated protein A in the fresh TBST containing 1% BSA (Jackson ImmunoResearch, West Grove, PA) diluted (1: 10,000) for 2h at room temperature (RT). Visualization of immunoblots were carried out using ImmobilonTM-Western Chemiluminescent HRP Substrate Kit (Millipore), according to the manufacturer's instructions, exposed to x-ray film (Fuji Photo Film Co., LTD., Tokyo, Japan) and scanned by using the GS-710 Calibrated Imagina Densitometer (BioRad).

RESULTS AND DISCUSSION

Similar to other larval organs, fat body undergoes a developmental remodeling process during the period of insect metamorphosis, with the massive destruction of obsolete larval tissues by PCD and the simultaneous growth and differentiation of adult tissues from small clusters of progenitor cells (Baehrecke, 2003). The techniques used in the present study are to reveal the signaling pathways regulating the mechanism of PCD in the fat body cells of B. mori.

Immunohistochemical localization of BmEcR-B1

In the present study, the antibody raised against BmEcR-B1 isoform was used for immunolocalization experiments because EcR-B1 is the predominant EcR isoform in the Bombyx fat body tissues (kamimura et al., 1999).

In order to localize BmEcR-B1 protein during the prepupal period of fat body cells, we used immunohistochemistry to localize the binding of anti-BmEcR-B1 antibody. The results shown in figure 1 are the immunohistochemical localization of BmEcR-B1 protein assessed with DAB staining (Fig. 1IA-F), confirmed by immunofluorescence (Fig. 1IIa-f), and compared with the positive control sections stained with the nuclear staining, Acridine orange (AO) (Fig. 1III). In all cases, BmEcR-B1 immunolabeling was clearly corresponding to cell nuclei, whereas no immunosignals were detected in the cytoplasm or on cell membranes. At G0, the cells almost lacked EcR-B1 expression (Fig. 1IA, IIa) as compared with the negative control sections (Fig. 1IF, IIf). The intensity of immunolabeled nuclei increased from G1 to G2. At G1, the intensity was low (Fig. 1IB, IIb) and became comparatively higher by G2 (Fig. 1IC, IIc). At G3, a relatively few nuclei were moderately labeled for EcR-B1, and the majority of cells at this point showed weak immunolabeling (Fig. 1ID, IId). Cyclically, very weak immunolabeled nuclei were observed again at P0. These results indicate that EcR-B1 is restricted to the fat body cell nuclei and is not translocated to cytoplasm or cell membranes during the prepupal period.

Expression pattern and developmental characteristics of BmEcR-B1 transcript and protein

The expression of EcR-B1 transcript was previously detected in the Bombyx fat body (Kamimura et al., 1999), however, nothing is known about its developmental profile in this tissue. In the present study, the estimation of the developmental profile of BmEcR-B1 gene transcript was performed by Northern blot analysis with total RNA extracted from the fat body during different indicated periods (Fig. 2). The BmEcR-B1 transcript was detected as a single hybridization signal of ~ 6.2 kb. The same molecular weight of EcR-B1 transcript was obtained for Bombyx ASGs, epidermis, wing disk, and mid gut (Kamimura et al., 1999). The obtained developmental profile of BmEcR-B1 gene transcript shows that the transcript level started to increase rapidly in fat body after G0, and increased steeply from G1, reaching maximum level at G2, then decreased following to G3 and P0. The obtained developmental profile for EcR-B1 is synchronized with that of the hemolymph ecdysone titer during these developmental periods (Takaki and Sakurai, 2003). Therefore, the presented results suggest that EcR-B1 isoform in the fat body is up regulated by the hemolymph ecdysone titer during these periods.
Fig. 1: Immunohistochemical localization of BmEcR-B1 in the fat body cells of B. mori during the prepupal period. G0 (A, a); G1 (B, b); G2 (C, c); G3 (D, d); PO (C, c). A-E, are DAB immunostained for BmEcR-B1 (I); a-e, BmEcR-B1 immunolocalization was confirmed by fluorescent chromogen Cy3 (II); I-F and II-f are the controls immunostained with preimmune rabbit serum. The positive control nuclei for each stage stained with acridine orange (III). Scale bar = 100 µm for I, II and 75 µm for III. (n = 3).
The estimation of the BmEcR-B1 protein developmental profile (Fig. 3) was performed by Western blot analysis. The results clearly indicate that BmEcR-B1 protein is present in the fat body cells during the prepupal period and is in detectable amounts at G1 reaching its maximum level at G2, then decreased to G3 to P0. Accordingly, the special emerging timing of EcR-B1 from G1 of prepupal period in fat body cells (Fig. 3) demarcates the beginning of degeneration of the fat body cells. This indicates that these cells may respond early to ecdysone via EcR-B1 for PCD process. Similarly, in B. mori, the sequence of PCD begins at G1 for posterior silk glands (Kaneko et al., 2006). However, it begins at G2 for anterior and middle silk glands (Terashima et al., 2000; Manaboon et al., 2008; Goncu and Parlak, 2008).

Fig. 2: Developmental profile of BmEcR-B1 transcript. (A) Northern blot analysis of BmEcR-B1 RNA in fat body during the prepupal period. Total RNA (20 μg / lane) isolated from fat bodies were denatured, electrophoresed, and blotted. The blots were hybridized with a 32P labeled antisense BmEcR-B1 oligonucleotide probe. (B) Ribosomal RNAs are shown as internal controls. (n = 3).

Fig. 3: Detection of BmEcR-B1 developmental protein profile during the prepupal period of fat body of B. mori. (A) Protein extracts (20 μg of each per lane) prepared from fat body at G0, G1, G2, G3 and P0 were separated by 10% SDS-PAGE; (B) blotted and probed with anti-BmEcR-B1. The molecular weight detected for BmEcR-B1 (arrow) is 56 kDa. (C) The average intensities of each band for BmEcR-B1. (n = 3).
Biochemical localization of BmEcR-B1

In order to confirm biochemically that BmEcR-B1 protein is restricted to nuclei as shown immunohistochemically (Fig. 1) and not to cytoplasm or plasma membrane during the prepupal period, Western blot analysis was carried out. In this experiment, BmEcR-B1 antibody was used against G2 fat body [which show the maximum expression of BmEcR-B1 (Fig. 2, 3)] total lysate, plasma membrane, and nuclear and cytosolic extracts (Fig. 4). In the total lysate and nuclear extract, a single immunoreactive signal band of 56 kDa (an approximate molecular mass of Bombyx EcRs) was detected. No immunoreactive signal was found in the membrane or cytosolic extracts. These results indicate biochemically that BmEcR-B1 expression is restricted to the cell nuclei of the Bombyx fat body during the prepupal period. Also, EcR-B1 restriction to the cell nuclei was observed in Bombyx ASGs (Gone and Parlak, 2008). However, in Rhodnius prolixus (Schlattner et al. 2006) and Bombyx brain neurosecretory cells (Hossain et al., 2006) EcR-B1 was localized in the cytoplasm beneath the plasma membrane.

In this context, several signaling pathways have been shown to regulate mechanisms of PCD in vertebrates (Cruz et al., 2006). For example, the signaling pathway for estrogen in mammals (Zivadinovic et al., 2005) is mediated through membrane receptor and/or by an alternative pathway through cytoplasmic receptor. On the other hand, the signaling pathway for estrogen in the sea trout (Zhu et al., 2003) is only through membrane receptor.

**Fig. 4:** Biochemical localization for BmEcR-B1 in G2 fat body. Western blotting using the antibody raised against B. mori nuclear ecdysone receptor B1 (anti-BmEcR-B1) (1:1000) as a primary antibody and horseradish peroxidase-conjugated protein A (1:10,000) as a secondary antibody. Lane 1, G2 fat body total lysate; Lane 2, G2 fat body membrane fraction; Lane 3, G2 fat body nuclear extract; Lane 4, G2 fat body cytosol. The arrow indicates the immunoreactive signal band of 56 kDa. (n = 3).

In conclusion, the present results showed that the previously recognized ecdysone binding sites in the Bombyx fat body cell membranes (Elmogy et al., 2007b) are not due to the conventional nuclear EcR receptor translocation to cytoplasm or cell membranes. Accordingly, this work confirm the dual function of ecdysone through two different receptors, i.e. membrane ecdysone receptor, mEcR (Elmogy et al., 2004, 2006, 2007a and b), in addition to the conventional nuclear receptor, EcR-B1, during larval pupal development in the fat body cells of B. mori.

**ACKNOWLEDGMENTS**

The author is indebted to Dr H. Fujiwara of the University of Tokyo for the gift of Bombyx EcR-B1 antibody, and to Professor Dr. Makio Takeda of the University of Kobe, for giving me the chance to carry experiments in his laboratory.
REFERENCES


