A nuclear juvenile hormone-binding protein from larvae of Manduca sexta: a putative receptor for the metamorphic action of juvenile hormone

Subba R. Palli, University of Washington
Kazushige Touhara
Jean-Philippe Charles, University of Washington
Bryony C. Bonning, University of California, Davis
A nuclear juvenile hormone-binding protein from larvae of *Manduca sexta*: A putative receptor for the metamorphic action of juvenile hormone

(photosaffinity labeling/baculovirus expression/metamorphosis)

**SUBBA R. PALLI**, *KAZUSHIGE TOUHARA, JEAN-PHILIPPE CHARLES, BRYONY C. BONNING, JEFFREY K. ATKINSON, STEPHEN C. TROWELL, KIYOSHI HIRUMA, WALTER G. GOODMAN, THEMIS KRYIAKIDES, GLENN D. PRESTWICH, BRUCE D. HAMMOCK, AND LYNN M. RIDDIFORD*

*Department of Zoology, University of Washington, Seattle, WA 98195; Department of Chemistry, State University of New York, Stony Brook, NY 11794-3400; Departments of Entomology and Environmental Toxicology, University of California, Davis, CA 95616; and Department of Entomology, University of Wisconsin, Madison, WI 53706*

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**ABSTRACT** A 29-kDa nuclear juvenile hormone (JH)-binding protein from the epidermis of *Manduca sexta* larvae was purified by using the photosaffinity analog for JH II ([3H]epoxyhomofarnesyl diazocacete) and partially sequenced. A 1.1-kb cDNA was isolated by using degenerate oligonucleotide primers for PCR based on these sequences. The cDNA encoded a 262-amino acid protein that showed no similarity with other known proteins, except for short stretches of the interphotoreceptor retinoid-binding protein, rhodopsin, and human nuclear protein p68. Recombinant baculovirus containing this cDNA made a 29-kDa protein that was covalently modified by [7H]epoxyhomofarnesyl diazocacete and specifically bound the natural enantiomer of JH I (Kᵢ = 10.7 nM). This binding was inhibited by the natural JHs but not by methoprene. Immunocytochemical analysis showed localization of this 29-kDa protein to epidermal nuclei. Both mRNA and protein are present during the intermolt periods; during the larval molt, the mRNA disappears but the protein persists. Later when cells become pupally committed, both the mRNA and protein disappear with a transient reappearance near pupal ecdysis. The properties of this protein are consistent with its being the receptor necessary for the antimetamorphic effects of JH.

Juvenile hormone (JH), a sesqui-terpenoid, regulates both metamorphosis and reproduction in insects (1). In the larva, JH is present throughout the growth phase, and its presence at the time of the ecdyseroid-induced molt is critical to prevent metamorphosis. The primary morphogenetic effect of JH is to guide ecdyseroid action so as to prevent its activation of new programs resulting in a change of form. In the adult, by contrast, JH apparently acts alone to stimulate vitellogenin synthesis in the fat body or vitellogenin uptake into the oocyte. The fat body has high-affinity cytosolic and nuclear-binding proteins for JH, whereas in facilitating yolk uptake, JH apparently acts via a membrane receptor on the follicle cells for review, refs. 1 and 2.

In the epidermis of tobacco hornworm (*Manduca sexta*), one-third of the JH that enters the cell is retained in the nucleus by two proteins (Kᵢ = 7 and 88 nM) (3). Palli et al. (4), using tritiated photosaffinity analogs of JH, identified a 29-kDa nuclear protein and a 38-kDa cytosolic protein that specifically bound JH. The 29-kDa protein was present in the epidermis during the fourth- and fifth-instar growth phases but declined during the molt and disappeared at the time of pupal commitment of the epidermis (5). Its reappearance in both the larval and pupal epidermis after ecdysis depended on the presence of JH during the molt. Our paper reports the purification and subsequent cloning of the cDNA for this protein.‡

**MATERIALS AND METHODS**

Experimental Animals, Hormones, and Binding Studies. *M. sexta* larvae were reared and staged according to published morphological markers (6). (10R, 11S)-JH I (>95% EE) (7), (10R, 11S)-JH II (>95% EE) (7), racemic JH II (SciTech, Czech Republic), 10R-JH III (8), methyl farnesolate (E/Z, 7:3), and 7S-methoprene (Sandoz) were used as competitors. [3H]Epoxyhomofarnesyl diazocacete (EHDA; 47 Ci/mmol; 1 Ci = 37 GBq) (9) and [3H]epoxybisonhomofarnesyl diazocacete (EBDA) (38 Ci/mmol) (4) were used for photosaffinity labeling. Hormones were checked periodically by thin-layer chromatography and repurified as necessary. Enantiomerically pure [7H]JH I (84 Ci/mmol) was prepared enzymatically as described (10). Purified JH I acid (11) was incubated with *Hyalophora cecropia* male accessory gland extract and 5-[methyl-3H]adenosyl-L-methionine (84.3 Ci/mmol, NEN) at 27°C for 3 hr. The radiolabeled JH I was extracted with ethyl acetate, then redissolved in hexane, and purified through a Sep-Pak silica cartridge (Millipore) by elution with hexane/ethyl acetate, 4:1.

All glassware used for the JH solutions was coated with 1–2% polyethylene glycol 20,000. For photoaffinity labeling, hormone was dissolved in buffer C (20 mM Tris-HCl, pH 7.9/5 mM magnesium acetate/1 mM EDTA/1 mM dithiothreitol/1 mM phenylmethylsulfonyl fluoride). Photoaffinity labeling and subsequent SDS/PAGE followed by fluorography were as described (4). For binding assays, DEAEPurified baculovirus recombinant protein (see below) was incubated with [7H]JH I in 20 mM Tris, pH 8.0/1 mM dithiothreitol with or without excess unlabeled hormone for 1 hr at room temperature. Binding parameters were determined by a modified dextran-coated charcoal assay (12).

Abbreviations: EBDA, epoxybisonhomofarnesyl diazocacete; EHDA, epoxyhomofarnesyl diazocacete; JH, juvenile hormone.

‡To whom reprint requests should be addressed.

The sequence reported in this paper has been deposited in the GenBank data base (accession number U50270).

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Purification and Analysis of the 29-kDa JH-Binding Nuclear Protein. Nuclei were isolated from the dorsal epidermis of ~200 day 0 and day 3 fifth-instar larvae, according to Palli et al. (4), and then extracted for 4–12 hr in 0.5 M KCl. The supernatant (13,000 × g, 10 min) was dialyzed to 10 mM Tris-HCl, pH 8.6/10 mM NaCl/1 mM EDTA. Both this extract and buffer C washes of the nuclei (4) contained a 29-kDa protein that bound EHDA.

The protein thus prepared was photolabeled with 75 nM [3H]EHDA. Excess unbound ligand was removed with de-Exun-coated charcoal, 3% amphotolys (pH 3–10) (Bio-Rad) were added, and then the protein was focused in a Bio-Rad Rotofor cell (13). The radioactive fractions were combined, refocused, and then pooled; 1 M NaCl was added, and the mixture was dialyzed against 20 mM Tris-HCl, pH 8.0/1 mM EDTA for 6 hr followed by concentration on an Amicon YM-5 membrane.

After preparative gel electrophoresis on 1.5-mm SDS/12.5% PAGE, the 29-kDa band was visualized by Coomasie blue R-250; electroeluted and dialyzed against [14]pH 7.5, 0.1 M sodium phosphate, pH 8.0/14 mM EDTA (Fig. 1C). The protein was electroeluted by [15]Bio-Rad Systems model 475A.

Analysis and Determination of the cDNA Encoding the 29-kDa Protein. Degenerate oligonucleotides based on the peptide sequences (see Results) were used in 37-cycle PCR (17) using Ampli-Taq (Perkin–Elmer/Cetus) with cDNA made from day 1 fifth-instar epidermal poly(A)+ RNA using Superscript and random primers (GIBCO/BRL). The PCR product was cloned into Bluescript pBSK– (Stratagene) and sequenced. Methods for RNA extraction, poly(A)+ selection, RNA hybridization, DNA labeling, cDNA library screening, and sequencing were as described (6).

Fusion Protein, Antibody Production, and Immunoanalysis. A fusion protein was made by ligating the 786-nt coding region of the 29-kDa JH-binding protein cDNA into the pGEX-3X expression vector (Pharmacia) (18). The resultant 55-kDa protein was isolated from the cell pellet by SDS/PAGE. The excised, minced band was used for the initial antibody production (Pocono Rabbit Farm, Canadensis, PA) with subsequent injections of 50 μg of electroeluted protein. The rabbit polyclonal antiserum was purified by 40% ammonium sulfate precipitation. For immunocytochemistry the antisera was further purified with two affinity columns [cyanogen bromide-activated Sepharose 4B (Pharmacia)] (19): column I, induced Escherichia coli cell lysate containing the pGEX-3X vector alone, and column II, electroeluted 29-kDa glutathione S-transferase fusion protein. Flow-through from column I was applied to II; then the bound antibodies were eluted with low pH.

For immunoblotting, epidermal extracts in phosphate-buffered saline (PBS: 0.015 M sodium phosphate, pH 7.4/0.15 M NaCl) were electrophoresed on SDS/12% PAGE and transferred to nitrocellulose. After incubation with 1:1000 purified antiserum, the blots were developed according to ref. 20.

Immunocytochemistry was done on whole mounts of dorsal abdominal epidermis fixed in 4% paraformaldehyde/PBS overnight at 4°C, incubated in 1:1000 affinity-purified antiserum overnight, and immunostained as described (21).

Production of the 29-kDa Protein by Recombinant Baculovirus. A 870-nt cDNA fragment encoding the 29-kDa protein cDNA was inserted into the transfer vector for Autographa californica nuclear polyhedrosis virus (pAcUW21) (12). Cotransfection with linearized polyhedrin-negative Autographa californica nuclear polyhedrosis virus DNA (AcPR6-SC) (22) and screening for recombinant viruses were as described (12).

For production of the 29-kDa protein, SF21, or Tr5 B1-4 (Invitrogen) cells cultured in ExCell 401 medium (JRH Biosciences)/3% fetal calf serum were infected at 5 plaque-forming units per cell (5 × 105 cells per ml) with the recombinant virus. Cells and medium were harvested 3 days later, quick-frozen in ethanol/dry ice, and stored at −80°C. Before use the cell pellet was extracted in 5 vol of buffer C. Further purification was by DEAE chromatography (DE52, Whatman), using gradient elution of 0–0.5 M NaCl in buffer C monitored by gel electrophoresis.

RESULTS

Purification and Sequencing of the 29-kDa JH-Binding Protein. Purification of the EHDA-binding nuclear protein from Manduca fifth-instar larval epidermis yielded a 29-kDa protein (Fig. 1A). Fig. 1C shows the N-terminal sequences of three peptides from the Lys-C digestion of this protein.

Isolation and Sequencing of the Full-Length cDNA. On the basis of the sequences in Fig. 1C, six degenerate oligonucleotides were synthesized: 1, AAR TCR AAR CTC ATY TTC ATY TT and 2, ATG AAR ATG AGY TTY GAY TT for peptide 1; 3, TTC CAR TGY TOR TTC ANY GYTT and 4, GYN ATG AAY CAR CAY TGG AA, for peptide 3; 5, ARR ATR TCI ACR TTR AAY TT, and 6, TTY GAY TTY AAR CCI TTY AAR GTI GAR CAR CG, for peptide 2. Amplification by PCR using different primer combinations yielded a 191-bp fragment from the day 1 fifth-instar cDNA only with primers 2 and 3 (Fig. 1B, gel). Moreover, this fragment hybridized to primer 5 (Fig. 1B, blot), indicating that it included all three peptide fragments.

The deduced amino acid sequence of the PCR fragment (arrows in Fig. 2) showed 100% identity with the amino acid sequence of the recombinant protein. The deduced amino acid sequence of the PCR fragment (arrows in Fig. 2) showed 100% identity with the amino acid sequence of the recombinant protein.
sequence of all three peptide fragments (underlined in Fig. 2), except for two of the three C-terminal amino acids of peptide 3 in Fig. 1C.

Using the PCR fragment as a probe, we isolated 20 clones from a ZapiI (Stratagene) cDNA library made to day 1 fifth-instar epidermal RNA. Two of the longest clones had the same 982-bp sequence (Fig. 2). The longest open reading frame was 262 amino acids translating into an ~29-kDa protein.

Comparison of the encoded protein sequences in the GenBank data base by FASTA (24) and by the BLOCKS search motif (25) showed no proteins with significant similarity. A stretch of 21 amino acids (box 1, Fig. 2) was 43% identical with the human nuclear protein p68, an RNA helicase (26). Four small domains of 8–12 amino acids in the C-terminal third of the protein have 60–75% identity with the bovine interphotoreceptor retinoid-binding protein (27) (boxes 2 and 4) and human rhodopsin (28) (boxes 3 and 5) (Fig. 2). The similarities to interphotoreceptor retinoid-binding protein are in its conserved repeat sequences that may be involved in binding fatty acids (27), suggesting that this region could be involved in JH binding.

Binding of JH and JH Analogs by Baculovirus-Produced 29-kDa Protein. When SF21 cells were infected with baculovirus containing the 29-kDa protein cDNA, the cell extracts contained a 29-kDa protein as detected both by immunoblotting and by [3H]-JH3 labeling (Fig. 3A). A 29-kDa protein was not found in cells infected with control wild-type virus or in uninfected cells. The presence of 500-fold excess (10R, 11S)-JH II somewhat reduced EHDa binding to this 29-kDa protein and eliminated binding to a 50-kDa protein present in cells infected with either control or recombinant virus. After DEAE purification, the binding of [3H]-JH3 to the 29-kDa recombinant protein was nearly eliminated by 250-fold excess JH I (Fig. 3A).

DEAE purification resolved the expressed protein from Ts5 cells into two immunoreactive forms, the ratio of which varied. Neither form was glycosylated as detected by the DIG glycan detection kit (Boehringer Mannheim). The smaller form bound significantly more JH I than the larger form. This binding was specific and saturable (Fig. 3B). Scatchard analysis showed a Kd of 10.7 nM for JH I (Inset, Fig. 3B) and of 6.9 nM for JH II (data not shown). Fig. 3C shows that excess (10R, 11S)-JH I, racemic JH II, and (10R)-JH III competed effectively for binding of [3H]-JH3 to an effective 10-fold less effective. Methoprene was ineffective because 1000- and 5000-fold excess only reduced binding of 10 nM [3H]-JH I by 29 and 54 ± 3%, respectively (n = 3 each).

Developmental Expression of the RNA for the 29-kDa Protein. Northern hybridization analysis of larval epidermal RNA using the 191-bp PCR fragment showed that a 1.1-kb RNA was present during the intermolt periods of both the fourth and the fifth instars (Fig. 4A). This mRNA disappeared during the larval molt and at the time of pupal commitment of the epimemis on day 3 of the fifth instar. Trace amounts reappeared transiently just before pupal ecdysis.

The polyconal antisera recognized only a 29-kDa protein present in larval epidermal cell extracts (Fig. 4B). This protein was present in the epimeris throughout the intermolt and through most of the larval molting phase until about 6 hr before ecdysis. In the final instar the protein again was present until the wandering stage. No 29-kDa protein was detectable at the time of pupal ecdysis or up to 48 hr thereafter.

Immunocytochemical Localization of the 29-kDa Protein. Immunocytochemical analysis shows that the 29-kDa protein is confined to the nucleus of the epidermal cells of day 1 fifth-instar larvae (Fig. 5A). Moreover, an uneven distribution within the nucleus is found throughout both the fourth and the fifth instars. In wandering-stage epimemis, many nuclei had lost their immunoactivity, although some showed small punctate staining (arrow, Fig. 5B). Preincubation of the antiserum with purified baculovirus-produced protein eliminated this staining.

DISCUSSION
Previous studies identified 29-kDa nuclear proteins in epidermis of Manduca larvae which specifically bound photoaffinity analogs of JH I (EBDA), JH II (EHDA), and meth-
oprene (methoprene diazomethyl ketone) (4). In the present study, the EHDA-binding protein was purified to homogeneity, and its cDNA was isolated. Protein produced by this cDNA in a baculovirus expression system was covalently modified by both EHDA and EBDA and specifically bound JH I and II with \( K_d \) values (10.7, 6.9 nM) of the same order of magnitude as the high-affinity binder found in whole nuclei (6.6 nM) (3). This binding was saturable and inhibited by the natural JHs but not by the JH analog methoprene, as was also reported (3) with isolated epidermal nuclei. The biological activities of JH III and methyl farnesoate are 300- to 1000- and 30,000-fold less.

**Fig. 3.** Binding of JH and EHDA to the 29-kDa protein produced in a baculovirus expression system. (A) (Left) Immunoblot of 30 \( \mu \)g of protein from SF-21 cells infected with recombinant (lane 1), wild-type (control) (lane 2), or no (lane 3) virus. (Right) Binding of 20 nM \(^{3}H\)EHDA to 30 \( \mu \)g of protein from SF-21 cells infected with recombinant (lanes 1, 2), control (lane 3), or no (lane 4) virus and of 200 nM \(^{3}H\)EBDA to 20 \( \mu \)g of DEAE-purified protein from recombinant virus-infected Tn5 cells (lanes 5, 6). Lanes 2 and 6 had excess unlabeled (10 R, 11S)-JH II (500x) and (10 R, 11S)-JH I (250x), respectively. For lanes 5 and 6, the photolabeled protein was not lyophilized before electrophoresis as in lanes 1–4. (B) Binding of 30 \( \mu \)g of DEAE-purified protein from the Tn5 cell extract to \(^{3}H\)(10 R, 11S)-JH I in the absence (o) or absence (L) of 100-fold excess unlabeled (10 R, 11S)-JH I. Specific binding (o) is the difference between total (c) and nonspecific (L) binding. (Inset) Scatchard analysis of specific binding data. (C) Competition for binding of 2.5 nM \(^{3}H\)(10 R, 11S)-JH I to 20 \( \mu \)g of purified protein as in B by (10 R, 11 S)-JH II (o), racemic JH II (c), (10 R)-JH III (c), and methyl farnesoate (m). One hundred percent total binding was determined in the absence of competitor (\( n = 3 \)).

**Fig. 4.** (A) Developmental RNA blot of 29-kDa JH-binding protein mRNA in the epidermis during the fourth- and fifth-larval stages using 10 \( \mu \)g of total RNA hybridized with a \(^{32}P\)-labeled 191-bp PCR fragment. Reprobing with the full-length cDNA showed similar expression. Ethidium bromide staining showed equal loading (data not shown). The TMB + 13-hr lane was exposed three times longer; dot blots indicate 30–60 times less mRNA than in day 1 fifth instar (data not shown). (B) Developmental immunoblot of 30 \( \mu \)g of epidermal protein from various larval stages, 1–3. Days of each stage: HCS, head capsule slippage; YM, yellow mandibles; and AF, air-filled head capsule (29, 10, and 6 hr before ecdysis, respectively); FE, freshly ecdysed; W0–W2, 0–2 days after onset of wandering; TMB, tanned metathoracic bars, 19 hr before pupal ecdysis; P1, 1-day-old pupa.

JH I. The nature of the two forms produced by the baculovirus in Tn5 cells is still unknown because there is no apparent glycosylation, and preliminary sequence analysis indicates identical N-termini for both forms.

Under reversible binding conditions, DEAE-purified recombinant protein specifically bound both JH I and II with \( K_d \) values (10.7, 6.9 nM) of the same order of magnitude as the high-affinity binder found in whole nuclei (6.6 nM) (3). This binding was saturable and inhibited by the natural JHs but not by the JH analog methoprene, as was also reported (3) with isolated epidermal nuclei. The biological activities of JH III and methyl farnesoate are 300- to 1000- and 30,000-fold less.

**Fig. 5.** Immunocytochemical staining of Manduca larval epidermis. (A) Day 1 fifth instar. (Inset) Higher (×2.5) magnification. (B) Wandering stage. Arrow points to punctate immunostaining within nucleus. (Bar = 50 \( \mu \)m for A and B.)
respectively, than JH I and II in the Manduca black larval assay (refs. 29 and 30; J.-P.C., unpublished work). Yet JH III is nearly as good a competitor as JH II, whereas the structurally similar methyl farnesoate is much less effective. Apparently other factors such as metabolism and rate of uptake are critical in the determination of biological activity.

Our immunochemical studies on the epidermis showed that the encoded 29-kDa protein is localized to the nucleus. Moreover, the developmental patterns of both mRNA abundance and immunoreactive protein were similar to those of the nuclear 29-kDa JH-binding protein (4, 5) and of nuclear JH binding (30). Interestingly, during the larval molt, the mRNA declines early during the ec dysdrost stage, whereas the protein remains for at least 20 more hr. The mRNA reappears just before ecdysis (data not shown) and is present until day 3 of the last larval instar. The mRNA then disappears followed by the protein in response to the ec dysdrost rise in absence of JH (5), which causes pupal commitment of the epidermis (31). The low levels of RNA seen just before pupal ecdysis apparently result in little protein because the latter was undetectable by immunoblotting.

These studies thus show that the 29-kDa protein is nuclear and specifically binds JH I with an affinity (Kd ~ 10 nM) that corresponds closely to the JH concentration in the hemolymph at the time of the larval molt (~ 20 nM) (29) or in the early fifth instar (29, 32). Moreover, this protein is present in the epidermis during the larval intermolt periods and the larval molts when JH is high and exerting its morphogenetic effects. Its disappearance from the abdominal epidermis at the time of pupal commitment occurs after the loss of JH (32) and correlates with the loss of JH sensitivity of this tissue (31). Thus, the nuclear 29-kDa JH-binding protein has the characteristics expected for a hormone receptor—high affinity, specific binding, and developmental expression that correlates with hormone presence and action. These data are consistent with its being responsible for the "status quo" action of JH.

Although this putative receptor for the lipophilic JH molecule is nuclear, the predicted protein contains no known DNA-binding motifs. It may represent another class of hormone receptors whose action within the nucleus somehow modulates the outcome of hormone-induced cascade of transcription factors, as in the ec dysdrost model (33).

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