A Novel Protein That Binds Juvenile Hormone Esterase in Fat Body Tissue and Pericardial Cells of the Tobacco Hornworm *Manduca sexta* L.

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Juvenile hormone esterase degrades juvenile hormone, which acts in conjunction with ecdysteroids to control gene expression in insects. Circulating juvenile hormone esterase is removed from insect blood by pericardial cells and degraded in lysosomes. In experiments designed to characterize proteins involved in the degradation of juvenile hormone esterase, a pericardial cell cDNA phage display library derived from the tobacco hornworm moth *Manduca sexta* L. was constructed and screened for proteins that bind juvenile hormone esterase. A 732-base pair cDNA encoding a novel 29-kDa protein (P29) was isolated. Western and Northern analyses indicated that P29 is present in both pericardial cell and fat body tissues and is expressed in each larval instar. In immunoprecipitation experiments, P29 bound injected recombinant juvenile hormone esterase taken up by pericardial cells and native *M. sexta* juvenile hormone esterase in fat body tissue, where the enzyme is synthesized. Binding assays showed that P29 bound juvenile hormone esterase more strongly than it did a mutant form of the enzyme with mutations that perturb lysosomal targeting. Based on these data, we propose that P29 functions in pericardial cells to facilitate lysosomal degradation of juvenile hormone esterase.

Juvenile hormone esterase (JHE); EC 3.1.1.1) is critical to insect development through its action on JH, which regulates gene expression. In many insects, JHE is the predominant anti-JH enzyme found in the hemolymph (blood); JHE hydrolyzes JH to produce JH acid and thereby regulates the titer of circulating JH (1). The importance of precisely regulated JH and JHE in insect development has been demonstrated by topical application of JH analogs or the JHE inhibitor 3-n-octylthio-1,1,1-trifluoro-2-propanone to *Lepidoptera* (butterflies and moths), which can result in production of giant larvae (2). Conversely, larval development is impeded by removal of the corpora allata, which synthesizes JH.

During development of lepidopteran larvae, the titers of JHE and JH are inversely regulated; hemolymph JH titers are high when JHE titers are low and vice versa. The titer of circulating JHE is regulated in part by differential transcription rates in fat body tissue (3). JHE is also cleared from the hemolymph by pericardial cells (4, 5) via receptor-mediated endocytosis and is degraded in lysosomes (6–9). The molecular processes involved in the processing and degradation of JHE in pericardial cells are unknown.

In earlier work, immunoelectron micrographs showed that targeting of JHE to lysosomes in pericardial cells was perturbed when two lysine residues of JHE (Lys29 and Lys524) were mutated to arginines (10). The present study was undertaken to identify proteins that differentially bind JHE and the mutant enzyme JHE K29R/K524R and that may be involved in endocytosis, sorting, and trafficking to lysosomes. Here, the results of screening a *Manduca sexta* pericardial cell cDNA phage display library for proteins that bind JHE are described.

**EXPERIMENTAL PROCEDURES**

**General Methods**—Total RNA and mRNA were isolated using a guanidium-based method (11) and the Micro Poly(A) Pure mRNA purification kit (Ambion Inc.), respectively. All proteins blotted from SDS-polyacrylamide gel for Western analysis were transferred to Hybond-P membrane (Amersham Pharmacia Biotech), and the secondary antibody (HRP-conjugated IgG) was detected using one-step 3,3′,5,5′-tetramethylbenzidine (Pierce).

**Baculovirus Expression and Purification of Juvenile Hormone Esterase**—Recombinant JHE and mutants JHE K29R, JHE K524R, and JHE K29R/K524R were produced by infection of *Spodoptera frugiperda* cells (12) with recombinant baculoviruses (10, 13) in serum-free medium (14). Recombinant enzymes were purified by loading JHE-containing medium (300 ml) onto Q-Sepharose columns (25-ml column volume; Amersham Pharmacia Biotech) and eluting in 10-ml fractions with a sodium chloride step gradient (85–90 mM in 50 mM Tris-HCl, 2 mM EDTA, and 0.02% sodium azide, pH 7.5). Fractions containing JHE activity, identified using 3H-labeled JH-III as described (15, 16), were concentrated using Centricon 30 filters (Amicon, Inc.) and subjected to SDS-PAGE. Purity was assessed by Coomassie Blue and silver staining of the SDS-polyacrylamide gels.

**Construction of the cDNA Phage Display Vector** pBjuFo—Plasmid pBjuFo is shown in Fig. 1. A DNA fragment encoding a Jun leucine zipper domain fused to fd phage coat protein gene III was cloned into pCR2.1TOPO (Invitrogen). The resulting insert was isolated by digestion with EcoRV and NotI sites were added to the 5′- and 3′-ends, respectively, by PCR using the primers JF5′-RV (5′-GGGATATCTTCTTATCCAGGACAGCTCATAG-3′) and JF3′-Not (5′-CCGCGCGCCGACCCGCAAACCACGGTGTCGCCG-3′) prior to cloning into pCR2.1TOPO (Invitrogen). The resulting insert was isolated by digestion with EcoRV and NotI and cloned into pcDNA2.1 (Invitrogen), which had previously been digested with *EcoR* blunted-ended by end filling with Klenow, and digested with *Not*I. The sequence encoding the gene III leader was constructed using overlapping oligonucleotides and inserted 5′ to the *jun* leucine zipper region at the HindIII site. This step replaced the pelB leader sequence that was present in the original fragment with the gene III leader sequence. Next, a V5 epitope tag with a small 3′-multiple...
cloning site was constructed using the same technique and inserted downstream from the fos leucine zipper sequence into the NotI site to produce pBJuFo (see Fig. 1). All constructs were confirmed by sequencing.

**Construction and Enrichment of the Phage Display Library—**Pericardial cell complexes (pericardial cells and associated dorsal aorta) were dissected from 50 *M. sexta* larvae at the fifth instar (day 2 or day 3). Total RNA and mRNA were extracted (see “General Methods”), and cDNA was synthesized (Smart PCR cDNA synthesis kit, CLONTECH). First-strand synthesis was conducted using reverse transcriptase (Promega) with the oligo(T) NotI primer (Invitrogen). Second-strand synthesis was conducted using the Capwith Primer (pCR 2.1-TOPO) synthesis of full-length cDNAs and the Advantage PCR kit (CLONTECH). The PCR products were treated with T7 DNA polymerase, ligated to BstXI adaptors (Invitrogen); size-selected for >400 bp (on Size-Sep400 spin columns, Amersham Pharmacia Biotech); digested with NotI; and ligated into the phage display vector pBcJbFo, which had previously been restricted with BstXI and NotI. *Escherichia coli* strain XL-1 Blue (Stratagene) was transformed with the ligation mixture. An aliquot of the recombinant *E. coli* cells was plated on Luria broth/ampicillin plates for overnight incubation. Recombinant plasmids were isolated and restricted with EcoRI and NotI to show the range of cDNA insert sizes in pBJuFo. The recombinant *E. coli* cells were then infected with the helper phage vector cloning system M13 (Stratagene) to generate a large-scale recombinant phage expression library, which was stored at -70 °C.

The phage display library was enriched by biopanning as described (18). One well of a poly styrene 24-well microtiter plate (Falcon) was coated with JHE (3 μg in 300 μl of 0.1 M sodium bicarbonate, pH 8.6), and recombinant phage (~2.5 × 10^10) plaque-forming units in 250 μl) were added. After binding of phage and removal of unbound phage by washing with TBST (25 mM Tris, 0.5 M NaCl, 0.1% Tween 20, pH 7.4), bound phage were eluted. For the first three rounds of screening, phage were eluted in acidic buffer (300 μl of 50 mM HCl/glycerine, pH 2.2, per well). For the fourth round of screening, phage were eluted with JHE (7.5 μg of JHE in 150 μl of PBS for 15 min). Fifty μl of recombinant phage eluted after the fourth round of enrichment were used to infect *E. coli* cells. After overnight incubation on Luria broth/ampicillin plates, individual colonies were picked to test binding of specific recombinant phage to JHE on 96-well plates by enzyme-linked immunosorbent assay.

**Screening of the Phage Display Library—**JHE (1 μg in 100 μl of 0.1 M sodium bicarbonate, pH 8.6 per well) was adsorbed to the solid phase of alternate rows on 96-well microtiter plates. Recombinant phage isolated from individual *E. coli* colonies (see “General Methods”). Pools of clones (1 μg/well) were added to adjacent wells with or without recombinant JHE and incubated (3-4 h). Unbound phage were removed by washing in TBST, and bound phage were detected by enzyme-linked immunosorbent assay using anti-M13 anti- serum (Amersham Pharmacia Biotech) conjugated to HRP. HRP activity on the substrate ABTS (2.2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt; Amersham Pharmacia Biotech) was quantified by a colorimetric reaction (19). For negative controls, wells were treated with skimmed milk, and scored as positive if bound phage were detected using streptavidin-HRP with the ECL chemiluminescence detection system (Ayerst). Data were analyzed by one-way ANOVA.

**Expression and Purification of Recombinant Juvenile Hormone Esterase-binding Protein—**The insert from a selected clone (pBjuFo.56) was restricted with BstXI and NotI, directionally cloned into the T7 polymerase vector pBluescript (Stratagene) and transformed into E. coli BL21(DE3). Transformants were induced for 3 h (0.5 mM isopropyl-β-D-thiogalactopyranoside), harvested, and lysed, and the recombinant protein was bound to a nickel column. Protein was then eluted in 50-200 mM imidazole according to the manufacturer’s directions (Invitrogen). The purified recombinant binding protein (P29) was separated by SDS-PAGE, electroblotted onto membrane, and detected with anti-polyhistidine primary antibody (anti-Xpress, Invitrogen; see “General Methods”). Purified P29 was used for production of polyclonal antisera in mice as described (20).

**Analysis of JHE Binding by P29 in Vitro—**Purified JHE and P29 were labeled with biotin (biotin labeling kit, Roche Molecular Biochemicals). The biotin-ligated recombinant JHE and recombinant protein, and quantified (Bio-Rad protein assay). Biotinylated proteins were separated by SDS-PAGE, transferred to Hybond-P membrane, and examined using streptavidin-HRP conjugate and the ECL chemiluminescence substrate luminal (Amersham Pharmacia Biotech). Fluorescence was detected by film exposure (Eastman Kodak Co.). For immunoprecipitation experiments, biotin-labeled JHE (50 μl, 3.3 μg) and biotin-labeled P29 (50 μl, 1.4 μg) were mixed and incubated at 37°C for 2 h. Anti-JHE or anti-Xpress antisera (2 μl) was added; the reaction was incubated on ice for 2 h; and Affi-Gel-protein A (200 μl; Bio-Rad) was added to precipitate immune complexes. The immune complexes were washed (2 ml of PBS); pelleted by centrifugation at 10,600 × g for 10 min; and then treated with 0.1 mM sodium citrate, pH 3.0, to release proteins from the affinity gel. Samples were pelleted at 10,600 × g for 5 min, and proteins in the supernatant were separated by SDS-PAGE (12% gel) and transferred to Hybond-P membrane. Biotinylated proteins were detected as described above. For positive controls, purified JHE was immunoprecipitated with anti-JHE antisera, and P29 was immunoprecipitated with anti-Xpress antisera. In negative control reactions, immunoprecipitation reactions contained JHE with anti-Xpress antisera or P29 with anti-JHE antisera.

**Analysis of Expression and JHE Binding of P29 in Vivo—**Pericardial cell and fat body proteins were separated by SDS-PAGE, transferred to membrane, and probed with primary antisera raised against P29 (see “General Methods”). The first 100 μg of enzyme/ml of stock per well) were mixed and incubated at 37°C for 2 h. Anti-JHE or anti-Xpress antisera (2 μl) was added; the reaction was incubated on ice for 2 h; and Affi-Gel-protein A (200 μl; Bio-Rad) was added to precipitate immune complexes. The immune complexes were washed (2 ml of PBS); pelleted by centrifugation at 10,600 × g for 10 min; and then treated with 0.1 mM sodium citrate, pH 3.0, to release proteins from the affinity gel. Samples were pelleted at 10,600 × g for 5 min, and proteins in the supernatant were separated by SDS-PAGE (12% gel) and transferred to Hybond-P membrane. Biotinylated proteins were detected as described above. For positive controls, purified JHE was immunoprecipitated with anti-JHE antisera, and P29 was immunoprecipitated with anti-Xpress antisera. In negative control reactions, immunoprecipitation reactions contained JHE with anti-Xpress antisera or P29 with anti-JHE antisera.
amplification, the size of the phage display library was used to produce cDNA for the phage display library. After production of these proteins was discontinued.

Biotinylated JHE or mutant JHE (200 ng) in PBS, pH 7.4, was added. Bound enzyme was detected using streptavidin-HRP with ABTS at 412 nm. Five replicate assays were carried out, and data were analyzed by one-way ANOVA and Tukey’s test for pairwise comparisons.

RESULTS

Isolation of Juvenile Hormone Esterase-binding Proteins from the Phage Display Library—The phage display vector pBJuFo (Fig. 1) was constructed for expression of pericardial cell-derived proteins as recombinant proteins fused to phage coat protein gene III and displayed on the surface of recombinant phage as a result of the interaction of Fos and Jun. Total RNA (50 μg) extracted from 50 pericardial cell complexes was used to produce cDNA for the phage display library. After amplification, the size of the phage display library was ~108 plaque-forming units/ml (5 ml). Purified recombinant JHE was used to enrich the pericardial cell cDNA phage display library for proteins that bind JHE. After five rounds of enrichment, 287 individual clones were screened by enzyme-linked immunosorbent assay for JHE binding. Of these, 46 clones (16%) were positive for apparent JHE binding and did not bind to wells that were blocked with skimmed milk in the absence of JHE.

Plasmids isolated from the 46 clones were subjected to restriction analysis. The pBJuFo cDNA inserts were from 400 to 1000 bp in size. Nine clones with dissimilar restriction patterns were sequenced. One clone, pBJuFo.56, contained an 830-bp insert (data not shown). Purified recombinant JHE was used to enrich the pericardial cell cDNA phage display library for proteins that bind JHE. After five rounds of enrichment, 287 individual clones were screened by enzyme-linked immunosorbent assay for JHE binding. Of these, 46 clones (16%) were positive for apparent JHE binding and did not bind to wells that were blocked with skimmed milk in the absence of JHE.

Of the eight remaining clones, the cDNA sequences of three clones shared homology with known M. sexta genes (glutathione S-transferase (22), cytochrome oxidase (23), and 16S RNA). The sequences of three other clones contained no poly(A) sequences. The DNA sequences of the two remaining clones did not share homology with previously published sequences and contained no poly(A) sequences. The DNA sequences of the two remaining clones did not contain open reading frames. Therefore, characterization of these proteins was discontinued.

Purification and Binding Characteristics of P29—Recombinant His-tagged P29 migrated at 29 kDa and was purified from transformed E. coli on a nickel column with elution at 150 mM imidazole (Fig. 3A, lanes 2 and 3). Antisera raised against recombinant P29 detected the recombinant 29-kDa protein and showed low background cross-reactivity to other E. coli proteins (Fig. 3C, lanes 1–3). A 29-kDa protein was detected in both pericardial cell and fat body tissues by Western blot analysis using anti-P29 antisera (Fig. 3C, lanes 4 and 5).

Biotinylated JHE bound both crude and purified recombinant P29 (Fig. 3B, lanes 2 and 3). Of greater biological importance, biotinylated JHE bound to a 29-kDa protein in pericardial cell extracts (Fig. 3A, lane 4) as well as in fat body tissue (data not shown). Biotinylated JHE also bound to pericardial cell proteins of 75, 125, and 240 kDa (Fig. 3B, lane 4). The 29-kDa protein was detected by ligand blotting in all five instars of M. sexta (data not shown).

Northern blot analysis of RNA derived from pericardial cells showed a P29 mRNA of 1.1 kilobases. This P29 mRNA was present in M. sexta pericardial cells during the third, fourth, and fifth instars (Fig. 4). No signal was detected for the control
In the efficiency of biotinylation (Fig. 6). There were no significant differences between enzymes K524R, and JHE K29R/K524R were purified and biotinylated (above background levels for the assay (15)).

Dial cell tissue of bovine serum albumin-injected larvae was not precipitated from pericardial cells (Fig. 5 B). Proteins with lower relative molecular mass were precipitated from pericardial cells (Fig. 5 A). In the control reactions, P29 was not immunoprecipitated by anti-JHE antiserum, and JHE was not immunoprecipitated by anti-Xpress antiserum (data not shown).

Following injection of biotinylated JHE into M. sexta larvae and immunoprecipitation with anti-P29 antiserum, biotinylated JHE and several proteins of lower relative molecular mass were precipitated from pericardial cells (Fig. 5 B, lane 3), but not from fat body tissue (lane 1). The proteins with lower relative molecular mass are presumed to be fragments of JHE produced by degradation in lysosomes. Native M. sexta JHE was immunoprecipitated from fat body tissue following injection with bovine serum albumin and detected by radiochemical assay (15). Total activity detected in the immunoprecipitates from fat body tissue was $3.97 \pm 2.6$ nM JH hydrolyzed per min ($n = 3$). JHE activity in the immunoprecipitates from pericardial cell tissue of bovine serum albumin-injected larvae was not above background levels for the assay (15).

Binding of P29 to JHE Mutants—Mutants JHE K29R, JHE K524R, and JHE K29R/K524R were purified and biotinylated (Fig. 6). There were no significant differences between enzymes in the efficiency of biotinylation ($p > 0.05$; one-way ANOVA). P29 was attached at different concentrations to the wells of a microtiter plate, and biotin-labeled JHE or mutant JHE was added (Fig. 7). Analysis of the binding of biotin-labeled enzymes to P29 showed that binding of JHE K29R/K524R was significantly less than that of JHE at 1.5 and 3 \mu{g} of P29 added per well ($p < 0.05$; one-way ANOVA and Tukey’s pairwise comparisons). There were no significant differences between the binding of JHE and mutants JHE K29R and JHE K524R ($p > 0.05$; one-way ANOVA and Tukey’s pairwise comparisons).

The complex of JHE and P29 was immunoprecipitated in vitro using both anti-JHE and anti-Xpress antisera. Both proteins were biotinylated for detection purposes. Lane 1, JHE immunoprecipitated with anti-JHE antiserum; lane 2, JHE and P29 immunoprecipitated with anti-JHE antiserum; lane 3, JHE and P29 immunoprecipitated with anti-Xpress antiserum; lane 4, P29 immunoprecipitated with anti-Xpress antiserum. JHE (3.3 \mu{g}) and P29 (1.4 \mu{g}) were used in these experiments. The positions of molecular mass standards are shown in kilodaltons. Precipitated proteins were separated on a 12% SDS gel and transferred to Hybond-P membrane. Biotin-labeled proteins were detected using streptavidin-HRP and the ECL chemiluminescence substrate. B, binding of P29 and JHE in vitro. M. sexta larvae were injected with biotinylated JHE, and fat body and pericardial cell tissues were removed 1 h after injection. Tissues were homogenized, and immunoprecipitation was carried out with anti-P29 antiserum. Precipitated proteins were separated by SDS-PAGE and transferred to Hybond-P membrane. Biotinylated JHE was detected as described for A. Lane 1, fat body; lane 2, biotinylated JHE immunoprecipitated with anti-JHE antiserum (3 \mu{g}); lane 3, pericardial cell tissue. The positions of molecular mass markers and the presumed degradation products of JHE (arrows) are shown.
binding proteins were selectively enriched in the phage display library by interaction of the gene products with JHE. This is the first time that phage display has been used successfully for screening of a tissue-derived library for specific binding proteins. The results highlight the importance of eliminating false positives, which may result from frameshifting (27) or from production of artificial peptides. Proteins detected for clones with no open reading frame in the cDNA insert are likely to have resulted from the insert being out of frame or from an incomplete coding sequence. The phage display technique limited the clone insert size to ~1 kilobase. There are clearly larger proteins in the pericardial cell complex that bind to JHE and that were not isolated using this technique (Fig. 3B). We are currently using alternative means to isolate and to characterize these proteins.

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