

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

(Received for publication, September 27, 1999, and in revised form, November 5, 1999)

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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 (JHE¹; 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 synthesize JH.

* This work was supported by NATO Grant CRG 951318. This is Journal Paper J-18501 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA (Project 3301), supported by the Hatch Act and the State of Iowa. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF153450.

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¹ The abbreviations used are: JHE, juvenile hormone esterase; JH, juvenile hormone; HRP, horseradish peroxidase; PAGE, polyacrylamide gel electrophoresis; PCR, polymerase chain reaction; bp, base pair(s); PBS, phosphate-buffered saline; ANOVA, analysis of variance.

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 (GenBank™/EBI accession number J02448) and a leader sequence fused to the Fos leucine zipper domain was a generous gift from R. Cramer (17, 18). *EcoRV* and *NotI* sites were added to the 5'- and 3'-ends, respectively, by PCR using the primers JF5'RV (5'-GGGATATCTTCTATTCAAGGAGACAGTCATAG-3') and JF3'Not (5'-CCGCGCCGCCACCACCGCAACCACCGTGTGCCGCC-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 *KpnI*, blunt-ended by end filling with Klenow, and digested with *NotI*. 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 aortas) 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 Capswitch primer (CLONTECH) for synthesis of full-length cDNAs and the Advantage PCR kit (CLONTECH). The PCR products were treated with T7 DNA polymerase; ligated to *Bst*XI 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 pBJuFo, which had previously been restricted with *Bst*XI 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 *Eco*RI 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 polystyrene 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 ($\sim 2.5 \times 10^7$ plaque-forming units in 250 μl) were added. After binding of phage and removal of unbound phage by washing with TBST (25 mM Tris, 3 mM KCl, 150 mM NaCl, and 0.01% 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/glycine, 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 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 antiserum (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 at 412 nm. Wells precoated with monoclonal anti-M13 antiserum or skimmed milk were used as positive and negative controls, respectively. Wells with optical density readings of $>2\times$ background levels were considered to be positive, and these clones were screened by enzyme-linked immunosorbent assay a second time. Plasmids were then isolated from positive clones and subjected to restriction analysis with *NotI*, *Eco*RI, and *Hind*III. Clones with dissimilar restriction enzyme fragments were selected for DNA sequencing using Applied Biosystems 377 automated DNA sequencing technology. Sequences were compared with those in GenBank™/EBI Data Bank using BLAST (19). Protein motifs were identified, and the isoelectric point was determined from the deduced amino acid sequence using the program MOTIF (Genome Net). Preparations of pericardial cell protein-gene III fusions were isolated from the periplasm of selected clones, separated by SDS-PAGE, transferred to membrane, and probed with anti-V5 antibody (Invitrogen; see “General Methods”) to confirm the presence of larger proteins (rather than peptides) that bind JHE.

Expression and Purification of Recombinant Juvenile Hormone Esterase-binding Proteins—The insert from a selected clone (pBJuFo.56) was restricted with *Bst*XI and *NotI*, directionally cloned into the T7 polyhistidine expression vector pRSET-JF (Invitrogen) to produce pRSET-JF.56, 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), column-purified on streptavidin to eliminate non-biotinylated 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 luminol (Amersham Pharmacia Biotech). Fluorescence was detected by film exposure (Eastman Kodak Co.).

The binding of JHE to pericardial cell proteins and recombinant P29 was examined by ligand blotting. Pericardial cell complexes were dissected from larvae of *M. sexta* (fifth instar, day 3); homogenized in PBS, pH 7.4, supplemented with 10 mM EDTA and 10 mM phenylmethylsulfonyl fluoride; and centrifuged at $5200 \times g$ for 10 min. The supernatant was used for ligand blot analysis. *E. coli* samples from recombinant BL21(DE3) cells transformed with pRSET-JF.56 were sonicated for 2 min in PBS, pH 7.4, supplemented with 10 mM EDTA and 10 mM phenylmethylsulfonyl fluoride and centrifuged at $5200 \times g$ for 10 min. Protein concentrations were determined (Bio-Rad), and proteins were separated by SDS-PAGE and electroblotted onto Hybond-P membrane. Blots were incubated for 4 h with biotin-labeled JHE (2 $\mu\text{g}/\text{ml}$) in PBS, washed with PBS and 0.1% Tween 20, and then blocked with skimmed milk prior to detection with streptavidin-HRP conjugate and one-step 3,3',5,5'-tetramethylbenzidine.

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 antiserum (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 $10,600 \times g$ for 10 min; and then treated with 0.1 M sodium citrate, pH 3.0, to release proteins from the affinity gel. Samples were pelleted at $10,600 \times g$ 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 antiserum, and P29 was immunoprecipitated with anti-Xpress antiserum. In negative control reactions, immunoprecipitation reactions contained JHE with anti-Xpress antiserum or P29 with anti-JHE antiserum.

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 antiserum raised against P29 (see “General Methods”). Poly(A)⁺ mRNAs (see “General Methods”) from third, fourth, and fifth instar larvae of *M. sexta* were separated on a 2.2 M formaldehyde-containing 1% agarose gel; transferred to nitrocellulose; and probed with a biotinylated P29 coding sequence under high stringency conditions (21). The 1.3-kilobase biotin-labeled probe was prepared from pBJuFo.56 template by PCR with primers flanking the P29 coding sequence (forward primer PhD, 5'-GCGGCACACGGTGGT-TGC-3'; and reverse primer T7, 5'-AATACGACTACTATAG-3'). As a negative control, a second probe was amplified by PCR using the T7 and PhD primers and an irrelevant cDNA insert (including a poly(A) tail) in pBJuFo. Biotinylated probe bound to mRNA on the membrane was detected using streptavidin-HRP with the ECL chemiluminescence substrate.

Larvae of *M. sexta* (fifth instar, day 3) were cooled on ice and injected with 10 μg of biotinylated JHE or 10 μg of bovine serum albumin, and pericardial cell and fat body tissues were dissected 1 h after injection. Tissues were homogenized on ice in 20 mM Tris-HCl, pH 6.8, 150 mM NaCl, 1 mM EDTA, and 10 mM phenylmethylsulfonyl fluoride and centrifuged at $5200 \times g$ for 5 min. Anti-P29 antiserum (1 μl) was added to proteins in the supernatant, followed by immunoprecipitation with Affi-Gel-protein A. Proteins in the immunoprecipitate were separated by SDS-PAGE and transferred to membrane for detection of biotin-labeled JHE. For tissue samples from insects injected with bovine serum albumin ($n = 3$), precipitated native JHE was detected by radiochemical assay (15).

Binding of P29 to JHE Mutants—The degree of biotinylation of JHE K29R, JHE K524R, and JHE K29R/K524R (purified and biotinylated as described above) was quantified by colorimetric assay at 412 nm in a microtiter plate using streptavidin-HRP conjugate with ABTS substrate. All assays (50 μl of 2 μg of enzyme/ml of stock per well) were replicated four times. Data were analyzed by one-way ANOVA.

A competition experiment was conducted to quantify the extent of binding of the JHE mutants to P29. Purified P29 was attached to a microtiter plate at different concentrations (0.75, 1.5, and 3 $\mu\text{g}/\text{well}$).

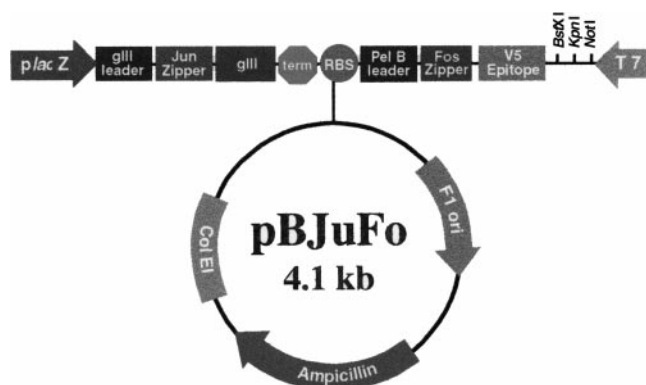


FIG. 1. Plasmid pBJuFo used for construction of the phage display library. Pericardial cell-derived cDNAs were cloned into the *Bst*XI and *Not*I cloning sites. *gIII*, gene III; *RBS*, ribosomal binding site; *ori*, origin; *kb*, kilobases.

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 μ g) 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 $\sim 10^8$ 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 (Fig. 2) that included a 732-bp open reading frame that codes for the protein P29. P29 is predicted to contain 243 residues and to have a mass of 28,450 Da and an isoelectric point of 8.72. This protein has six potential phosphorylation sites and one potential myristoylation site (Fig. 2). Searches of the protein and DNA data bases failed to identify sequences related to P29.

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 16 S 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 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



FIG. 2. cDNA and deduced protein sequences of P29. A, nucleotide sequence of the 830-bp insert in pBJuFo.56 including 732 bp that encode P29. An in-frame start codon and stop codon (TAA) are shown (boxed). The polyadenylation signal is underlined. B, 243-residue deduced amino acid sequence of P29. The sequence includes putative sites for cAMP- and cGMP-dependent protein kinase phosphorylation (dashed underline) and for casein kinase II phosphorylation (double underline), four putative protein kinase C phosphorylation sites (single underline), and an *N*-myristoylation site (thick underline). The asterisk indicates the stop codon.

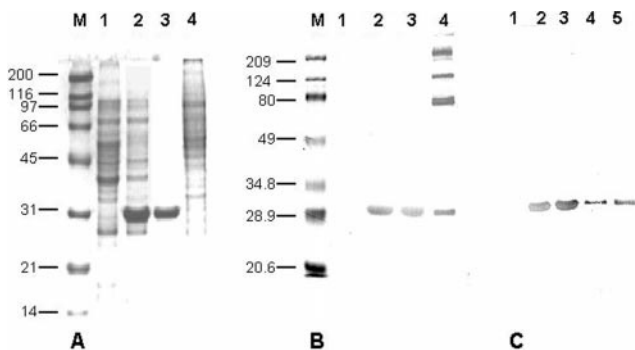


FIG. 3. Purification, detection, and JHE binding of P29. Proteins were separated by SDS-PAGE on a 12% gel. Lanes M, molecular mass markers in kilodaltons; lanes 1, *E. coli* (BL21(DE3)) control; lanes 2, recombinant *E. coli* cells expressing P29; lanes 3, purified P29; lanes 4, *M. sexta* pericardial cell homogenate; lane 5, fat body homogenate. Lanes 1, 2, 4, and 5 were loaded with 7 μ g of protein, and lane 3 was loaded with 2 μ g of protein. A, expression and purification of P29. The SDS gel was stained with Coomassie Blue. B, detection of JHE-binding proteins. Proteins separated by SDS-PAGE and transferred to Hybond-P membrane were probed with biotin-labeled JHE (2 μ g/ml). C, Western blotting of samples with anti-P29 antiserum. Proteins were transferred to Hybond-P membrane and detected using anti-P29 antiserum and anti-mouse IgG conjugated to HRP with one-step 3,3',5,5'-tetramethylbenzidine.

both pericardial cell and fat body tissues by Western blot analysis using anti-P29 antiserum (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. 3B, 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

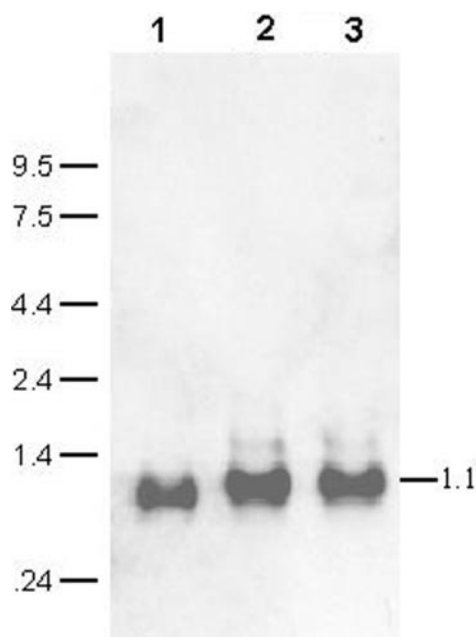


FIG. 4. Northern blot showing P29 mRNA in larvae of *M. sexta*. Poly(A)⁺ RNA was separated on a 1% agarose gel containing 2.2 M formaldehyde, transferred to nitrocellulose, and probed with a biotinylated probe containing the sequence of P29 under high stringency conditions. Lane 1, third instar, day 2; lane 2, fourth instar, day 3; lane 3, fifth instar, day 3. Each lane was loaded with 3 μ g of mRNA. Size markers are shown in kilobases.

probe that was generated from pBJuFo with an irrelevant polyadenylated insert (data not shown).

The binding of P29 to JHE in solution was demonstrated by immunoprecipitation of the two biotinylated proteins using both anti-Xpress and anti-JHE antisera (Fig. 5A). 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. 5B, 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 ± 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 μ 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).

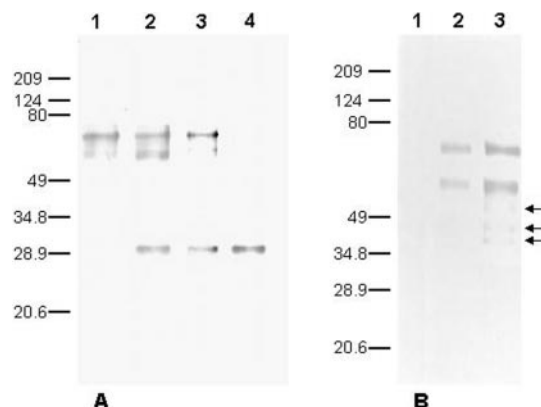


FIG. 5. Binding of P29 to JHE. A, binding of JHE and P29 *in vitro*. The complex of JHE and P29 was immunoprecipitated *in vitro* using both anti-JHE and anti-Xpress antisera. Both proteins were biotinylated for detection purposes. Lane 1, JHE immunoprecipitated with anti-JHE antiserum; lane 2, JHE and 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 μ g) and P29 (1.4 μ 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 vivo*. *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 μ g); lane 3, pericardial cell tissue. The positions of molecular mass markers and the presumed degradation products of JHE (arrows) are shown.



FIG. 6. Analysis of biotin-labeled wild-type and mutant JHEs. Lane 1, wild-type JHE; lane 2, JHE K29R; lane 3, JHE K524R; lane 4, JHE K29R/K524R. Proteins (25 ng/lane) were run on a 12% SDS gel and transferred to Hybond-P membrane. Biotin-labeled proteins were detected with streptavidin-HRP conjugate and luminol (ECL chemiluminescence technique). The positions of molecular mass markers are shown in kilodaltons.

DISCUSSION

Lepidopteran insects regulate titers of JHE to achieve the regulation of JH in the hemolymph that is required for development. Hemolymph JHE titers are regulated in part through clearance via receptor-mediated endocytosis into pericardial cells. The endocytosed enzyme is targeted to lysosomes for degradation. In earlier work, we observed that mutation of two residues in JHE perturbed lysosomal targeting of the enzyme in pericardial cells (10). Furthermore, this perturbed protein targeting was shown to result in toxicity to the insect: a recombinant baculovirus expressing the mutant enzyme JHE K29R/K524R killed the host insect significantly faster than a baculovirus expressing JHE (10, 24). The present study was based

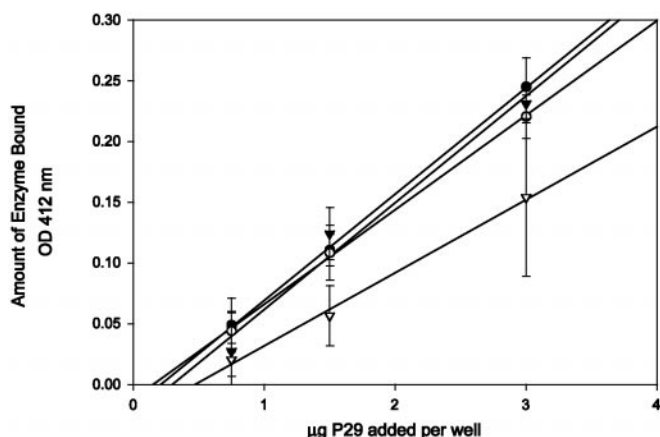


FIG. 7. Binding of JHE and mutant enzymes to P29 attached to the solid phase of a microtiter plate. Biotinylated JHE (●), JHE K29R (○), JHE K524R (▼), or JHE K29R/K524R (▽) (200 ng) was added. Bound enzyme was detected using streptavidin-HRP conjugate with ABTS. Optical density was determined at 412 nm. Four replicate assays were carried out, and means \pm S.D. are shown.

on the hypothesis that disruption of lysosomal targeting of JHE K29R/K524R results from decreased affinity for a binding protein involved in protein sorting in the endocytotic pathway. Here, we report the characterization of P29, which is present in pericardial cell and fat body tissues and shows reduced binding to JHE K29R/K524R relative to JHE.

Previous research showed that targeting of JHE K29R/K524R to lysosomes in pericardial cells was significantly less efficient than targeting of JHE (10). Our data on reduced binding of P29 to JHE K29R/K524R imply a role for P29 in JHE targeting or processing in lysosomes. P29 is also present in the fat body, which is not involved in uptake of JHE, but is involved in endocytosis of other hemolymph proteins (25). The fat body is also a site of synthesis of JHE (3). To deduce the function of P29, immunoelectron microscopy will be used to determine the intracellular location of P29 and the sites of colocalization with JHE in the two tissues.

Because *M. sexta* JHE had not been cloned at the beginning of this study, recombinant JHE derived from the tobacco budworm *Heliothis virescens* (26) was used for enrichment and screening of the *M. sexta* pericardial cell cDNA phage display library. Ligand blotting with *H. virescens*-derived JHE against *H. virescens* pericardial cell and fat body tissues showed the same profiles as blots with *M. sexta* tissues.² *H. virescens* JHE shares 54% identity with *M. sexta* JHE.³ Based on these observations, we expect the processing of the two enzymes to be comparable.

Use of the phage display library enabled the simultaneous screening for JHE-binding proteins and isolation of the cDNAs encoding binding proteins. cDNAs encoding potential JHE-

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 \sim 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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² M. Shanmugavelu and B. C. Bonning, unpublished data.

³ A. C. Hinton and B. D. Hammock, unpublished data.