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Cloning and expression of the VHDL receptor from fat body of the corn ear worm, *Helicoverpa zea*

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Abstract

In Noctuids, storage proteins are taken up into fat body by receptor-mediated endocytosis. These include arylphorin and a second, structurally unrelated very high-density lipoprotein (VHDL). Previously, we have isolated a single storage protein receptor from the corn earworm, *Helicoverpa zea*, which binds both VHDL and arylphorin. The receptor protein is a basic, N-terminally blocked, ~80 kDa protein that is associated with fat body membranes. Microsequencing of proteolytic fragments of the isolated receptor protein revealed internal sequences that were used to clone the complete cDNA of the VHDL receptor by 3' and 5' RACE techniques. The receptor protein, when expressed in vitro via a suitable insect expression vector, reacted with antibodies against the native VHDL receptor and bound strongly to its ligand VHDL, thus confirming that the cloned cDNA represents indeed the previously purified VHDL receptor. The receptor protein and a second, similar protein also found associated with the fat body membrane show considerable homology to putative basic juvenile hormone suppressible proteins cloned previously from other Noctuid species. Sequence analysis revealed that the receptor is likely a peripheral membrane protein that may mediate the selective uptake of VHDL.

Keywords: Storage protein receptor, receptor mediated endocytosis, arylphorin, very high density lipoprotein, basic juvenile hormone suppressible protein, hexamerin, insect metamorphosis

Abbreviation:

BJHSP	basic juvenile hormone suppressible protein
FITC	fluorescein isothiocyanate
HRP	horseradish peroxidase
RACE	rapid amplification of cDNA ends
VHDL	very high density lipoprotein

Introduction

Metamorphosis, that is the transition from the larval to the adult stadium of holometabolous insects, involves the breakdown of various structures and the formation of numerous new proteins and tissues (Sehnal, 1985). The synthesis of most of these proteins takes place during the pupal stadium in the fat body, which acquires the biosynthetic precursors from the hemolymph prior to pupation (Dean et al., 1985). Amino acids are provided by storage proteins, large larval serum proteins that are synthesized by fat body tissue of larvae and released into the hemolymph, where they accumulate in high concentration. Most, but not all storage proteins belong to a conserved family of hexameric proteins (hexamerins) related to hemocyanin (Beintema et al., 1994). All insects investigated utilize arylphorin, a storage protein rich in aromatic amino acids that are

needed for the formation of cuticular proteins. Various other storage proteins have been found, including proteins that are rich in methionine, or basic amino acids, but their specific functions remain unresolved (Haunerland, 1996). Similarly, it is not clear why certain lepidopteran species, like the corn earworm, *Helicoverpa zea*, and other Noctuids, possess an additional, structurally unrelated storage protein that contains considerable amounts of lipids and a blue chromophore (Haunerland and Bowers, 1986; Jones et al., 1988). Like arylphorin, this blue very high density lipoprotein (VHDL, density 1.26 g/ml) accumulates in the hemolymph of last instar larvae, and is sequestered by fat body prior to pupation (Haunerland et al., 1990). Due to its color, it is possible to visually trace the fate of this protein. Only perivisceral fat body, which appears in the second half of the last larval stage, sequesters VHDL, which gives

this tissue its blue color. Its synthesis takes place at the beginning of the last larval stage in another region of the fat body (peripheral fat body) which cannot sequester any storage proteins and remains white (Haunerland and Shirik, 1995).

The uptake of storage proteins in *H. zea* is selective and reminiscent of receptor-mediated endocytosis. After fat body of pre-pupae had been incubated with colloidal gold-labeled VHDL or arylphorin, electron micrographs revealed that both proteins are concentrated in membrane pits, vesicles, and endosomes. In pupae, both VHDL and arylphorin were found in protein granules, but only arylphorin was seen in the crystalline areas of these granules (Wang and Haunerland, 1994a). Fat body cells from perivisceral fat body bound radio-iodinated VHDL or arylphorin in a saturable manner (binding constant $\sim 8 \times 10^{-8}$), while cells from the peripheral fat body did not bind either protein. The putative VHDL receptor was isolated from membrane fractions of perivisceral fat body by a complex purification scheme (Wang and Haunerland, 1993). It is a basic, glycosylated protein with a subunit molecular weight of 80 kDa, which in the presence of calcium strongly binds both VHDL and arylphorin at pH values between 6 and 8.5. Antibodies detected the receptor only in the latter half of the last larval instar, but in large amounts. The VHDL receptor mostly lines the plasma membrane of the perivisceral fat body, but is also visible within protein granules, suggesting that the protein is sequestered with its bound ligand and is not recycled (Wang and Haunerland, 1993).

Although the receptor has been purified more than a decade ago, no information is yet available on its sequence. Because the receptor protein is N-terminally blocked, it was not possible to obtain sequence information useful for cloning its cDNA, and the complex purification method did not yield sufficient amounts for internal sequence analysis. Various attempts to screen cDNA libraries with antibodies carried out in our laboratory were not successful. Recently, we developed a novel affinity purification method that yielded larger quantities of the VHDL receptor sufficient for proteolytic digestion and microsequencing of its fragments (Persaud et al., 2003). The present paper describes the cloning and sequencing of the VHDL receptor, and its functional characterization.

Materials and Methods

Insects

H. zea colonies were reared in a controlled environment. The temperature was maintained at 26 °C. Eggs (AgriPest, Zebulon, NC) were hatched on paper towels enclosed in plastic bags. Once hatched, the larvae were immediately placed into plastic containers containing an artificial diet (Corn Earworm Diet, Southland Products, Lake Village, AR). After the larvae had reached the 3rd instar, they were placed into individual 2 oz cups filled with 2 ml of diet. The cups were sealed with a perforated lid to allow for the exchange of gases. Larvae pupated after approximately one week, and adult ecdysis occurred 10 to 14 days afterwards.

Preparation of solubilized membrane proteins

Perivisceral fat body tissues were isolated from 5-8 day old 5th instar larvae. Hemolymph was first removed by bleeding the insect through an excised proleg. The larvae were dissected and the remaining hemolymph washed away with protein extraction

buffer. The fat bodies from 10 larvae were excised and placed immediately in 6 ml protein extraction buffer at 4 °C, (50 mM Tris-base, 150 mM NaCl, 1 % Nonident P-40, 1 mM phenylmethylsulfonyl-fluorophosphate, pH 7.8). Following disintegration with a Polytron homogenizer, the homogenate was centrifuged at 800 x g for 1 h at 4 °C. The supernatant was centrifuged again at 30,000 x g for 1 h at 4 °C. The pellet was washed twice with protein extraction buffer, treated overnight with 200 μ l of a 2 % solution of Triton X-100, and re-centrifuged at 100,000 x g for 1 h at 4 °C to remove insoluble components.

Labeling of VHDL

Protein biotinylation was done using the ECL protein biotinylation system (Amersham Pharmacia Biotech, www.apbiotech.com), following the manufacturer's instructions. Briefly, 100 μ l of N-hydroxysuccinimide-biotin ester was added to 2.5 ml of a VHDL solution (1 mg/ml in 0.1 M bicarbonate buffer, pH 9.0) and incubated for 1 h under constant agitation. The labeled protein was separated by chromatography on a Sephadex G25 column (1 cm x 10 cm), and stored at 4 °C.

Fluorescein isothiocyanate-labeled VHDL (VHDL-FITC) was prepared with the FluoroTag™ FITC Conjugation Kit (Sigma, www.sigmaaldrich.com). Lyophilized and dialyzed VHDL was dissolved in 0.1 M bicarbonate buffer, pH 9.0 to make a 1.0 mg/ml protein solution. The conjugation of FITC to 1 mg of VHDL was carried out according to manufacturer's protocol using a molar ratio FITC:VHDL of 10:1, and labeled protein was separated by gel filtration on Sephadex G25.

Immobilization of VHDL-biotin on streptavidin-coated Dynabeads

Streptavidin-coated M-280 Dynabeads (Dyna, www.dynal.no) were used according to manufacturer's protocols, except that ligand binding buffer (20 mM Tris-HCl, 0.15 M NaCl, 4 mM CaCl₂, 0.1% TritonX-100, pH to 7.0) was used during all washing steps. Streptavidin-coated Dynabeads (100 μ l) were incubated with biotinylated VHDL (100 μ g/100 μ l binding buffer) for 90 min at room temperature with gentle agitation. The beads were washed twice with ligand binding buffer, using a Dynal MPC magnetic holder. The VHDL-biotin-streptavidin-Dynabeads complex was stored at 4 °C until needed.

Affinity purification of VHDL-binding proteins

Paramagnetic beads were used for the efficient affinity purification of the VHDL receptor, as described (Persaud et al., 2003). Briefly, the lyophilized, desalted 30,000 x g supernatant was dissolved in 100 μ l ligand binding buffer, and incubated for 60 min at room temperature with the VHDL-biotin-streptavidin-Dynabeads complex. The Dynabeads were washed three times for 5 minutes each with ligand binding buffer, each washing being for 5 min duration, and then treated with elution buffer (20 mM Tris-HCl, 0.15 M NaCl, 0.17 μ M PMSF, 0.1% TritonX-100, pH 9.5) to break the interactions between the immobilized VHDL and its receptor.

Western and ligand blotting

Proteins were separated on an 8 x 10 cm SDS-polyacrylamide gel (separating gel: 12 %T, 5 % C, 0.1 % SDS, 0.375 M Tris/HCl, pH 8.9; stacking gel: 4 % T, 20 % C, 0.1 %

SDS, 0.125 M Tris/HCl, pH 6.8; loading buffer: 2 % SDS, 60 mM Tris/HCl, pH 6.8; 10 % glycerol, 0.025 % bromophenol blue), and blotted onto a PVDF membrane, using a semi-dry blotting device (transfer buffer: 25 mM Tris/glycine, pH 8.7, 20 % methanol, 0.04 % SDS). For dot blots, equal amounts of protein were immobilized on a pre-conditioned PVDF membrane in a Bio-Dot Microfiltration Apparatus (BioRad Laboratories, www.bio-rad.com). Western blots were carried out with the ECL system (Pharmacia Amersham Biosciences, www.Pharmacia.com). The concentrations of primary antiserum (rabbit anti-VHDL, Haunerland and Bowers, 1986) and secondary antiserum (goat anti rabbit-IgG-HRP conjugate) were 1:3000 and 1:5000, respectively, except for the analysis of the recombinant receptor, where dilutions of 1:6,000 and 1:10,000 were used.

For ligand blots, SDS polyacrylamide gels were run in the normal fashion, but to preserve binding activity the protein sample was not boiled in loading buffer prior to electrophoresis, but incubated for 4 h at room temperature. Since membrane proteins that contain biotin as a co-factor are present in the solubilized membrane fractions, ligand blots were carried out with fluorescein isothiocyanate labeled VHDL (VHDL-FITC), at a dilution of 1:5000. Anti-FITC-HRP conjugate (1:5000) was used for enzymatic detection. In the analysis of recombinant proteins, the dilutions of used were 1:8000 (VHDL-FITC) and 1:10,000 (anti-FITC-HRP conjugate antibody).

Amino acid sequencing

The affinity purified VHDL receptor fraction (3 µg) was further resolved on a 15 % T, 5 % C SDS-PAGE gel (12x10 cm). In order to obtain complete separation of the two proteins contained in this fraction, electrophoresis was continued for 1 hour after the tracking dye had left the gel. Subsequently, the proteins were blotted onto a PVDF membrane. The membrane was lightly stained with Coomassie Brilliant Blue R-250 (0.025% in a methanol:water 40:60), and destained with methanol:water (50:50). Individual bands were excised and sent for N-terminal sequence determination by automated Edman degradation to the Biotechnology Laboratory at the University of British Columbia.

For internal sequencing larger amounts of proteins were needed. A total of 50 µg of protein was loaded into sixteen wells of a 15 % SDS-PAGE gel. Following electrophoresis, the gel was stained and destained as described above, and individual gel bands were cut out and digested with endoprotease Lys-C *in situ*. The resulting peptides were separated using a ABI 173A Microblotter Capillary HPLC (Perkin-Elmer www.perkinelmer.com) and then collected onto a PVDF membrane for subsequent Edman Sequencing.

Rapid amplification of cDNA ends (RACE)

Degenerate oligonucleotide primers were constructed from internal peptide sequences, relying on published codon preferences for Noctuid species to reduce degeneracy (Nakamura et al., 2000). Both, 5' - and 3' RACE libraries were constructed with the Clontech SMART™ RACE cDNA Amplification kit (Clontech Laboratories, Inc., www.clontech.com), following the manufacturer's instructions. Total RNA was extracted from 5-7 day-old last instar larvae by the method of Chomczynski and Sacchi (1987). For 5'

RACE, first-strand synthesis was carried out in the presence of 5'CDS primer (5'-T₂₅VX-3') and the SMART II primer (5'-AAG CAG TGG TAA CAA CGC AGA GTA CGC GGG-3'), while the 3'-CDS primer (5'-AAG CAG TGG TAA CAA CGC AGA G TACT₂₅VX-3') was used for 3' RACE. PCR amplification of the 3' part of the 80 kDa VHDL receptor cDNA was carried out with the following primers: gene-specific forward primer F1 (5'-AAA AGA TTA AAC CAC CAG CCA T-3') and universal primer NUP (5'- AAG CAG TGG TAA CAA CGC AGA GT-3'). The PCR product was diluted 1:100, re-amplified with the same primers, and sequenced. Two nested reverse primers R4 (5'-ACA GCA TAC AGT CCATA CTC CAA CCG AA-3') and R3 (5'-GAT AGT ATT CT T GCC AGT TGT CAG TTT G-3') were constructed from the cDNA sequence of the 3' RACE, and used for the amplification of the 5'-RACE library: universal primer UPM (5'-CTA ATA CGA CTC ACT ATA GGG CAA GCA GTG GTA ACA ACG CAG AGT-3') and gene-specific lower primer R4, followed by re-amplification of the 1:100 diluted PCR product with the nested primers NUP and R3.

The primers for the 3'-RACE PCR amplification of the 78 kDa protein were the gene-specific primer UPM and F2-1 (5'-AGA TAC TGG TAC CGT CCT CAT AGG CAA GGA-3'), followed by re-amplification of the diluted PCR product with the same primers.

TOPO cloning reaction

Full-length cDNA of the VHDL receptor was obtained by RT-PCR from total RNA with the primers F5 (5'-GTT GAC TCC ACG ATG AGG GCT GTC CTA CTG-3') and R5 (5'-CAT GTC GGT CAG GTT AAT GTT GCG GTC CAT-3'). The PCR product (6 ng) was immediately cloned into a TOPO cloning vector, following the manufacturer's instructions (Invitrogen, www.invitrogen.com). One Shot Top10 cells were transformed and grown overnight on LB-ampicillin plates. Individual colonies were excised and cultured overnight in 5 ml LB medium containing 50 µg/ml ampicillin. Plasmid removal was carried out using S.N.A.P. MiniPrep Kit (Invitrogen). Five clones with the correct insert size were sequenced with OpiE2 forward (5'-CCG CAA CGA TCT GGT AAA CA-3') and reverse (5'-GAC AAT ACA AAC TAA GAT TTA GTC AG-3') vector primers.

Recombinant protein expression

The VHDL receptor was expressed in *Spodoptera Sf9* cells with the InsectSelect expression system (Invitrogen), following the manufacturer's instructions. For preparation of each transfection mixture, 1 ml of Grace's Sf9 insect medium, 1 µl of pIB/V5-His plasmid construct (1 µg/µl in TE, pH 8), and 20 µl of Insectin-Plus™ Liposomes (Invitrogen) were incubated at room temperature for 15 minutes. The transfection mixture was added to a 60 mm culture plate containing Sf9 cells in TNM-FH medium at 30 % confluency, and incubated at room temperature for 4 h. After addition of 2 ml complete TNM-FH medium, the plate was kept for 4 days at 27 °C. Subsequently, the cell medium was removed and concentrated by lyophilization. Cell Lysis Buffer (100 µl 50 mM Tris-base, 150 mM NaCl, 1 % Nonidet P-40, 1 mM PMSF, pH 7.8) was added to the plate and the cells were scraped off into a microcentrifuge tube. After vortexing the mixture, nuclei and cell debris were pelleted by centrifugation at 10,000 x g for 2 min. For SDS-PAGE, each fraction was resuspended in cell lysis buffer to a final protein concentration

of 0.1 µg/µl. Protein separation was carried out on a 10 % T, 5 % C SDS-PAGE gel with a stacking gel of 4% T, 20 % C. Aliquots of each fraction (30 µl, 0.1 µg/µl) were mixed with 10 µl of SDS-PAGE sample buffer and boiled for 5 min. Electrophoresis was carried out at 30 mA.

Results

The recently developed affinity purification method (Persaud et al., 2003) yielded sufficient amounts of protein for sequence analysis. The protein appeared to be homogenous and behaved in SDS gels, Western blots, and ligand blots just as the previously isolated receptor (Figure 1A). As earlier results had shown that the VHDL receptor is N-terminally blocked (Wang and Haunerland, 1993), no sequence was expected from Edman degradation of the isolated protein. However, when large amounts (150 pmol) of the protein were subjected to Edman degradation, weak signals were obtained, indicating that the VHDL receptor was not homogenous, but contaminated with another protein that was not N-terminally blocked. Indeed, in SDS PAGE gels occasionally a minor second band with a slightly lower molecular weight was observed. To separate the contaminating protein from the receptor, electrophoresis conditions were modified as to allow a complete separation of the two proteins (Figure 1 B). Two bands of 80 kDa and 78 kDa, respectively, were easily identifiable, with the upper band of greater intensity being the VHDL-receptor.

For the preparative separation of the proteins, all 16 lanes of a gel were used to separate approx. 50 µg of the affinity-purified receptor. The proteins were blotted onto a PVDF membrane, from which the individual bands were excised, pooled, and submitted to sequence analysis. The 78 kDa protein was not blocked and revealed 29 amino-terminal residues similar to those seen previously in the affinity purified receptor fraction: NH₂-SVEKDTGTVLIGKDNMVNMDIKMELCLIK-COOH. The 80 kDa VHDL-receptor, however, was N-terminally blocked and did not yield a signal. Following digestion of the blocked 80 kDa protein in situ with endoprotease Lys-C, the resulting peptides were separated by HPLC and individually sequenced by Edman degradation, yielding the following two internal peptide sequences that could be used for the design of PCR primers for RACE, peptide 1 (NH₂-KXRLNHQPF-COOH), and peptide 2 (NH₂-KVILYDFRSTVLL-COOH).

cDNA sequence of the VHDL receptor

To obtain the cDNA sequence of the VHDL receptor, degenerate primers were prepared from each of the internal sequences, and used to screen 5' and 3' RACE libraries. The 3' RACE PCR product migrated as a fairly sharp band that could be easily re-amplified and sequenced (Figure 2A). From the cDNA sequence thus obtained two new primers were constructed (R4 and R3) and used as nested primers to amplify the 5' end of the VHDL receptor message from the 5' RACE library. The resulting 1600 bp PCR product was sufficient for cloning and DNA sequencing (Figure 2A). The complete full-length cDNA sequence of the VHDL receptor is shown in Figure 3. It codes for a basic protein (calculated pI 8.9) with a molecular weight of approx. 89 kDa. Only one potential transmembrane region was found, near the amino-terminus, which has the hallmarks of an ER-targeting signal

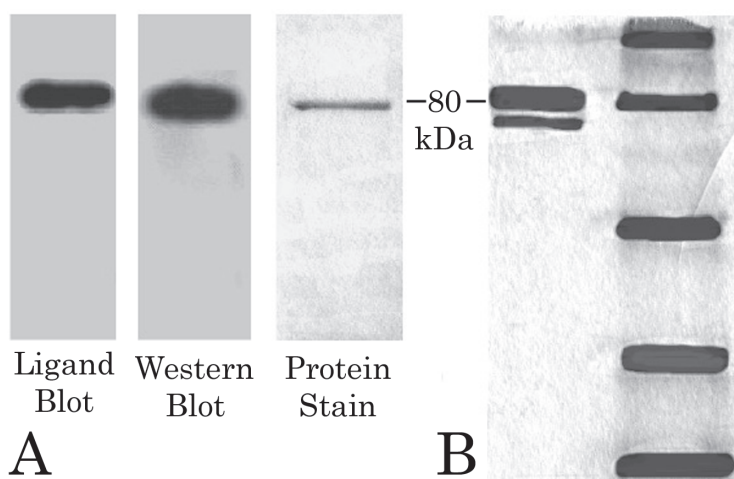


Figure 1. Purification of the VHDL receptor
 Panel A: Affinity-purified VHDL receptor (1 µg) appeared homogenous in a 12 % SDS gel. The ligand blot was carried out with FITC-labeled VHDL, and the Western blot with anti-VHDL-receptor antiserum, as described in Materials and Methods.
 Panel B: The affinity-purified fraction could be further separated into two proteins on a larger 15 % SDS gel: the 80 kDa band is the receptor and less prominent 78 kDa protein. Molecular weight markers (175, 83, 62, 48, 33 kDa) are shown on the far right lane.

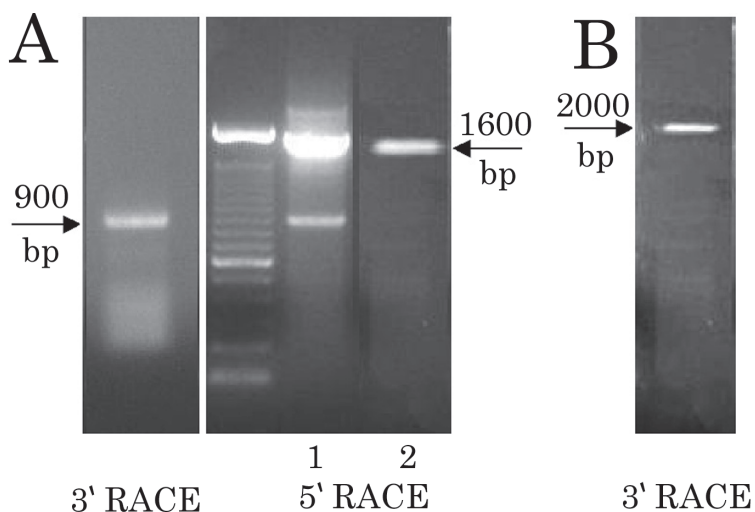


Figure 2. RACE-amplification of the VHDL receptor and the 78 kDa protein.
 Panel A: RACE of the 80 kDa receptor. PCR of the 3'-RACE-library was carried out with primers R1 and the universal primer NUP, while the 5'-RACE was first amplified with primers R4 and UPM (1), followed by re-amplification of the 1600 bp band with nested primers R3 and NUP (2).
 Panel B: 3'-RACE of the 78 kDa protein. PCR was carried out with primers F2-1 and UPM.

sequence. The protein also contains one potential N-glycosylation site each, adjacent to its amino- and carboxy-terminus (Figure 3, consensus sequence N-X-S/T).

Partial cDNA sequence of the 78 kDa protein

In order to determine whether the 78 kDa protein was a modified form of the VHDL receptor (e.g., a breakdown product),

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-----F5----->
GACCGTTGAACTCTCGCTGAGTTGACTCCACGATGAGGGCTGTCTACTGATACTCGCAAGCCTGGCCGCGCTGGCCATGGCTAGGCCTGAACTCGACGA 100
      M R  A V L L I L A S L A A V A M A R P E L D D
CAACACTAGTATGGGGAACATGGACATCAAACACCGGCAACTAGTCATCCTGAAATTGCTGAACCACATCACGGAGCCGTTGATGTACAAAGATCTGGAG 200
  N T S M G N M D I K H R Q L V I L K L L N H I T E P L M Y K D L E
GATTGGGGCAAGAACTTCAAATTAAGACAACATGGAATTATCTACTAAAACCGATGTTGTAAAGCACTTCATCAAATGATCAAGACCGGAGTTCTGC 300
  D W G K N F K I E D N M E L F T K T D V V K H F I K M I K T G V L
CGCGCGGGGAGATCTTCACTCTGCACATTGACCGCCAGCTCAAGGAAGTTGTCACCATGTTCCACATGCTGTACTACGCCAAAGACTTCAACACCTTCAT 400
  P R G E I F T L H I D R Q L K E V V T M F H M L Y Y A K D F N T F I
CAAGACCGCCTGCTGGATGCGCCTTCACTCAACGAGGGTATGTTGCTATACGCTCTCACCGTGGCAGTCAGACACCGTGAGGACTGCAAGGGAATCATC 500
  K T A C W M R L H L N E G M F V Y A L T V A V R H R E D C K G I I
TTGCCTCCTCCCTATGAAATCTACCCATACTACTTCGTACGTGCCGATGTTATCCAGAAAGCTTACTTATTGAAGATGAAGAAGGGAGATGTGGATCTTA 600
  L P P P Y E I Y P Y Y F V R A D V I Q K A Y L L K M K K G D V D L
AACTGTGTGATTTCTATGGAATCAAGAAGACCGACAAGATGTTTTCATAATCGACGAGAATGTGTTGACAAACGTGTACATCTCTCCGACGAAGACAA 700
  K L C D F Y G I K K T D K D V F I I D E N V F D K R V H L S D E D K
ACTCCGCTACTTCACTCAGGATATTCATCTCAATACCTACTATTACTACTTCCACGTTCACTATCCATTCTGGATGAAGGACACCGTAATGGATAAGAAC 800
  L R Y F T Q D I H L N T Y Y Y Y F H V H Y P F W M K D T V M D K N
TTGAAGACTAGGCGTTTGTAGCTTACAGTGTACATGTACCAACAGATCCTTGCCTAGATACTACTTGGAGCGTTTGTCCAACAGGATGGGCATGATCAAGG 900
  L K T R R F E L T V Y M Y Q Q I L A R Y Y L E R L S N R M G M I K
AGTTCCTTGGCACAAAACCATTAAGAAGGGATACTGGCCGTGGTTGAAAACGAGCAATGGTATTGAATTCCTGTAAAGTTCAACAACCTACGTCATTGC 1000
  E F S W H K T I K K G Y W P W L K T S N G I E F P V R F N N Y V I A
ACACGATTACAACCGTGACGTCATCCGCTGTGCGAGGAGTATGAGAGGATTATCCGGGAGGCTATCATCAAAGGATTCATCGAAATTAACGGCATGAGA 1100
  H D Y N R D V I R L C E E Y E R I I R E A I I K G F I E I N G M R
CTGGAACCTACCAAGACTGAGGACATGGAGGTTCTTGGAAAACGATCTACGGTAAAACGACAAGCTTGATCTTGACAGGACCGTGGTTGACTCATAACC 1200
  L E L T K T E D M E V L G K L I Y G K I D K L D L D R T V V D S Y
GCTACCTGCTCATCGTCATGAAGGCTGCTCTTGGTCTTAACACTCTCCACTCCGACAAGTACTTCGTTGTTCCCTTCTGTCTGGACCAATACCAGACAGC 1300
  R Y L L I V M K A A L G L N T L H S D K Y F V V P S V L D Q Y Q T A
TCTTCGTGACCCAGTATTCTACATGCTGCAGAAACGCATCCTGGATTTGGTATTCTTGTTCAGCTGCGTTTGGCCCTGTTACACCAAAGAGGACCTGTAC 1400
  L R D P V F Y M L Q K R I L D L V F L F K L R L P C Y T K E D L Y
TTCCCGGTGTGAAGTGCACAACGTTAACGTTGACAAGCTCGTTACTTACTTCGATGACTATCTTATGGACATGACTAACGCTGTCTTCTTGACGGAAG 1500
  F P G V K V D N V N V D K L V T Y F D D Y L M D M T N A V F L T E
-----F1----->
AAGAGATGAAGAAGACAAAGTCAGACATGAAATTCATGGTACGCAAGCGCCGCTCAATCACCAGCCATTCAAGGTCCTTGGACATATTATCTGACAA 1600
  E E M K K T K S D M K F M V R  K R R L N H Q P F K V T L D I L S D K
GGCGTTGACTGTGTGTAAGAATATTCCTTGGACCGAAGAAAGATCACATGGATCGCCTCATGACATCAACATTAATCGCCTTAACTTCGTCGAATTA 1700
  A V D C V V R I F L G P K K D H M D R L I D I N I N R L N F V E L
<-----R3----->
GATACTTTCCTTTTCAAACCTGACAACCTGGCAAGAATACTATCGTCAGAAAACCTCTCATGACATGCACAACATTGTTACGACCGCATGTTTACCCGTGACT 1800
  D T F L F K L T T G K N T I V R N S H D M H N I V H D R M F T R D
TGATGAAGAAGGTTGAATCTATCACCGACATGAGGGACTTATTGATCAAGGACTTGAGGAACACCACACTGGCTTCCCACCAGGCTACTTCTTCTCTAG 1900
  L M K K V E S I T D M R D L L I K D L R N Y H T G F P T R L L L P R
<-----R4----->
GGGCTTCGTTGGAGGTATGGACTGTATGCTGTACGTTATTGTGACACCACTGAGGCTGGTTCGACAACGTCGATATGAACGTTGGATATCTACCGTAAG 2000
  G F V G G M D C M L Y V I V T P L R L V D N V D M N V L D I Y R  K
GACTTAGTGCGGACTTCAGATCGACTGTCTTCTCGACAAAATGCCTCTTGGCTTCCCCTTGTATCGCCGAATTGATGTTGGAAACTTCTTACGCCAA 2100
  D L V R D F R S T V L L D K M P L G F P F D R R I D V G N F F T P
ACATGAAGTTCATTGATGTAAGATCTTCCACAAGAAGATGACATGTGATATGAAGACCAGGTGGAACCGATGGGTGCTGAGGGACTACAACATGGTGGA 2200
  N M K F I D V K I F H K K M T C D M K T R W N R W V L R D Y N M V D
<-----R5----->
CAGGACAACCATCGATCTTGACACCTACTTTCGTGGATACCGACCTGAACATGAAATGGACCGCAACATTAACCTGACCGACATGTGAATTCATCACGGA 2300
  R T T I D S D T Y F V D T D L N M K M D R N I  N L T D M *
CGACTAAACTGAAAATACTGACTGTGTGCTAGTAATATACGCATAAAAAAAAAAAAAAAAAAAAAA 2370

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Figure 3. Sequence of the VHDL receptor (Genbank Accession AY422205)
The 2.4 kb cDNA contains a single open reading frame of 741 amino acids. The signal sequence is shaded in yellow, and the two peptide fragments obtained from internal sequencing are shown in red. The location of the primers is indicated above the nucleotide sequence. Potential N-glycosylation sites are shaded green.

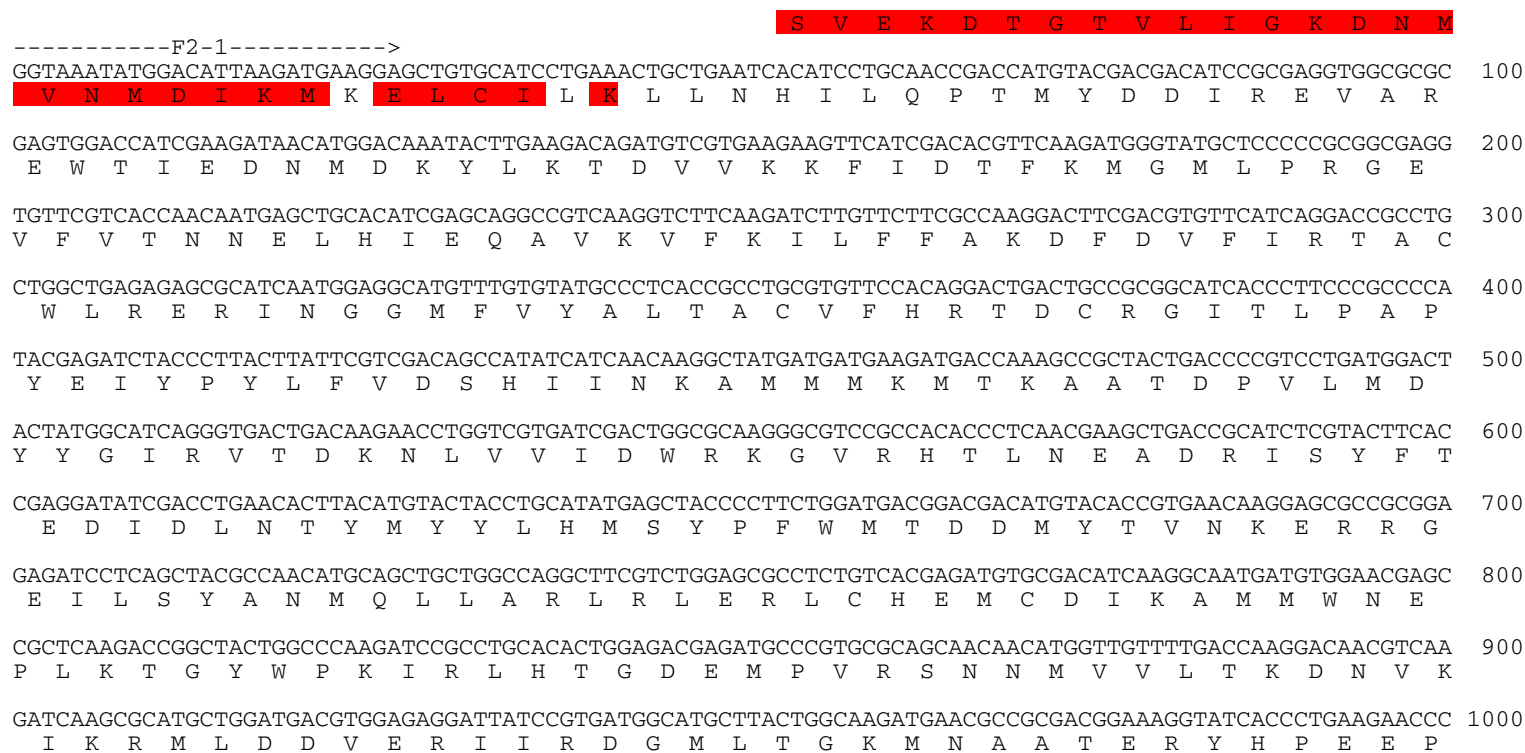


Figure 4. Partial sequence of the 78 kDa protein (Genbank Accession AY422206) Shown is the amino-terminal 346 amino acids deduced from the N-terminal sequence (red shading) and the amplified cDNA. The location of the gene specific PCR primer is shown above the nucleotide sequence..

part of its cDNA was amplified from the 3' RACE library and sequenced (Figure 2B). This resulting 2 kb product coded for a protein that is clearly different from the VHDL receptor (Figure 4).

Sequence comparison

Sequence alignment between these proteins and the closed matches from Genbank revealed that the VHDL receptor is virtually identical to the basic juvenile hormone suppressible protein 2 (BJHSP-2) from *Trichoplusia ni* (Jones et al., 1993), while the 78 kDa protein has higher similarity to the BJHSP-1 from the same species (Figure 5).

Expression of the VHDL receptor and functional analysis

To assess whether the 80 kDa protein sequenced here is indeed the VHDL receptor, its cDNA was cloned into an expression vector and expressed in *Spodoptera Sf 9* cells. The presence of the expressed protein in transfected cells was confirmed by Western dot blots (Figure 6A). No immuno-reactivity with anti-receptor antibodies was seen in the lysate of non-transfected control cells, or the growth medium of transfected or control cells. Strong reactivity, however, was observed in the lysate of the transfected cells. Ligand blots confirmed that the in vitro expressed VHDL receptor has the same apparent molecular weight as the wild-type proteins, and also can bind its natural ligand VHDL (Figure 6B).

Discussion

With the improved isolation procedure, it was finally possible to obtain internal sequences of the VHDL receptor, and use these to clone the entire protein. The theoretical properties of the protein are similar to the biochemical data obtained by Wang and Haunerland (1993): the receptor is a basic protein of approx. 80 kDa, which is likely to be glycosylated. While its high sequence homology to a basic juvenile hormone-suppressible protein of *T. ni* is surprising, it is noteworthy that the latter protein shares some properties with the receptor. Both proteins are expressed later in insect life than most storage proteins. While arylphorin and VHDL are expressed strongly throughout the feeding stage of last instar larvae (day 1-5), the receptor is not seen until shortly before the beginning of the wandering stage, and remains high until just before pupation (Wang and Haunerland, 1994b). Similarly, the strong expression of BJHSP-2 in *T. ni* commences when the arylphorin message declines, in the second half of the final instar (Jones et al., 1993). While the BJHSP-2 mRNA remains high until shortly before pupation, the protein disappears from the hemolymph towards the end of the larval stadium and shows up in the fat body fraction. The VHDL-receptor from *H. zea*, on the other hand, was not detected in the hemolymph. Given that the VHDL-receptor is most likely located at the extracellular side of the fat body membrane, it is possible that

BHSP-2	1	MRAVLLFVVSLLAALRMRPEIDD-TTTLVTMDIKQRQLVILKLLNHVVPEPLMYKDLLEELGKNFKIEENTDLEFTKTDVLLKDFIKMRKVCFLPRGEIFTL	96
VHDL-R	1	MRAVLLILASLAAVAMARPELDDNTSMGNMDIKHRQLVILKLLNHITTEPLMYKDLLEDWGNFKIEEDNMELEFTKTDVVKHFVKMIKTGVLPRGEIFTL	97
P78	1	SVEKDTGTVIGKDNVNMIDIKMKEICILKLLNHILQFTMYDDIREVAREWTEEDNMDKYLKTDVVKKFIIDTFKMGMLPRGEVFT	70
BHSP-1	1	MRVVLVLSLGLRGSVVKDDTTVVTGKDNVTMTDVKMKEICILKLLNHILQFTMYDDIREVAREWTEENMDKYLKTDVVKKFIIDTFKMGMLPRGEVFT	100
BHSP-2	97	HVDRQLKEVVTMFHMLYYAKDFNTFTVKTACWMLYLNIEGMFVYALTVAVRHREDCKGITLPPPYEYIYPYFVRADVIQKAYLLKMKKGLLDDLKLCDFYGI	196
VHDL-R	98	HIDRQLKEVVTMFHMLYYAKDFNTFTVKTACWMLYLNIEGMFVYALTVAVRHREDCKGITLPPPYEYIYPYFVRADVIQKAYLLKMKKGLLDDLKLCDFYGI	197
P78	71	NNELHIEQAKVFKILFFAKDFDVFIRTACWLRERINGEMFVYALTVAVRHREDCKGITLPPPYEYIYPYFVRADVIQKAYLLKMKKGLLDDLKLCDFYGI	170
BHSP-1	101	TNELHIEQAKVFKILFFAKDFDVFIRTACWLRERINGEMFVYALTVAVRHREDCKGITLPPPYEYIYPYFVRADVIQKAYLLKMKKGLLDDLKLCDFYGI	200
BHSP-2	197	KKTDKDFIIDENVYDKRVHLNKEKLRYPTEEDLNTYYFYFHVDPFPMKDKFMDK-MKMRRFELTYIYQOILARYILERLSNGMGMKDLSDWHKTI	295
VHDL-R	198	KKTDKDFIIDENVYDKRVHLNKEKLRYPTEEDLNTYYFYFHVDPFPMKDKFMDK-MKMRRFELTYIYQOILARYILERLSNGMGMKDLSDWHKTI	297
P78	171	RVTDKNLVVIDWRKG-VRHTLNEADRISYFTEEDLNTYYFYFHVDPFPMKDKFMDK-MKMRRFELTYIYQOILARYILERLSNGMGMKDLSDWHKTI	268
BHSP-1	201	KVTDKNLVVIDWRKG-VRHTLNEADRISYFTEEDLNTYYFYFHVDPFPMKDKFMDK-MKMRRFELTYIYQOILARYILERLSNGMGMKDLSDWHKTI	297
BHSP-2	296	KKGYWPMKLLHNGVHIVPVRFDNYIVRDRNRDVIRLCEYERIRIDATIKGFIEIN-GMRLELTKTDDIETLGKLIIFGKIDRVLDLDRTRVDSYRYLLIVM	394
VHDL-R	298	KKGYWPMKLLHNGVHIVPVRFDNYIVRDRNRDVIRLCEYERIRIDATIKGFIEIN-GMRLELTKTDDIETLGKLIIFGKIDRVLDLDRTRVDSYRYLLIVM	396
P78	269	KTGYWPKIRLHTGDEMPVRSNNMIVTKDVKIKRMLDDVERIIRDGMLTKMNDKRRGELLSANMQLARLRERLERSHEMCDIKSIMNEPL	322
BHSP-1	298	KTGYWPKIRLHTGDEMPVRSNNMIVTKDVKIKRMLDDVERIIRDGMLTKMNDKRRGELLSANMQLARLRERLERSHEMCDIKSIMNEPL	393
BHSP-2	395	KAALGLNTHSDKYFVVPVSLDQYQALRDPVFYMLQKRIIDLHFLFKLRPSYTKEDLYFPGVKIDNVVVDKLVTVFDDYLDMDMTNAVFLTEDEIKKTK	494
VHDL-R	397	KAALGLNTHSDKYFVVPVSLDQYQALRDPVFYMLQKRIIDLHFLFKLRPCYTKEDLYFPGVKVDNVVVDKLVTVFDDYLDMDMTNAVFLTEDEIKKTK	496
BHSP-1	394	KRLSYNVVNFDKYTVVPTALDLSTCLRDPEVWRIMKRVTDTFFLFKKMLPKYTRREDFEPGVKLEKFTTDLKTFIFIDEMDITNAMFDVDDVEMKCKR	493
BHSP-2	495	SDMFMVRRRLNHQPFKVTLDILSDKSVDCVVRVFLGPKKDLNLRILIDINRNLNFVELDTFLYKILNTGKNTIVRNSYDMHNLVDRMTRDLMKKVES	594
VHDL-R	497	SDMFMVRRRLNHQPFKVTLDILSDKAVDCVVRVFLGPKKDHMDRLIDINRNLNFVELDTFLYKILNTGKNTIVRNSYDMHNLVDRMTRDLMKKVES	596
BHSP-1	494	SDMTMVARMAALNHHFFKVTLDVTSKTVDCVVRVIFGPKYVCLGRMLMSVNDKMDMIDMTFLYKLEFGKNTIVRNSLEMHGVIEQSPWTRRILNLMIG	593
BHSP-2	595	--ITDMRDLMIKDLRNLPHWFPTRLLLPKGFVGGMMLLYVIVTPLRLVDNVDINILDLNRRKDLMRDFRSTVLLDKMPLGFPFDRRI DVGNF FTPNMKPF	692
VHDL-R	597	--ITDMRDLMIKDLRNLPHWFPTRLLLPKGFVGGMMLLYVIVTPLRLVDNVDNMLVDIYRKLVDLDFRSTVLLDKMPLGFPFDRRI DVGNF FTPNMKPF	694
BHSP-1	594	TVGKISKTVDESWYKRRHLPHRMLLPLRRCGMPMQMVFIVTIVKTNLLENLDMNIMKE--RKTACAGASVSTRCSGFPFDRKIDMTHEFTTRMKKFT	691
BHSP-2	693	EVTIFHKRMTCDMKTRWRNWLKDYDMVDRTRIESLSYFVDTDLDMKVNRNVLIDV	749
VHDL-R	695	DVKIFHKRMTCDMKTRWRNWLKDYDMVDRTRIESLSYFVDTDLDMKVNRNVLIDV	751
BHSP-1	692	DVMLEFRKDLSLSNTIKDVIDSDMMMKKDLIYLSMLVRSYKAVMMSKDDMMRM	748

Figure 5. Aligned sequences of VHDL binding proteins from *H. zea* and the basic juvenile hormone suppressible proteins from *T. ni*. Amino acids that are identical with those of the VHDL-receptor are shaded red, and conservative substitutions are shaded grey. BHSP-2: basic juvenile hormone suppressible protein 2 (accession Q06343); BHSP-1: basic juvenile hormone suppressible protein 1 (accession Q06342); VHDL-R, 80 kDa VHDL receptor (accession AY422205); P78, 78 kDa protein from *H. zea* (accession AY422206).

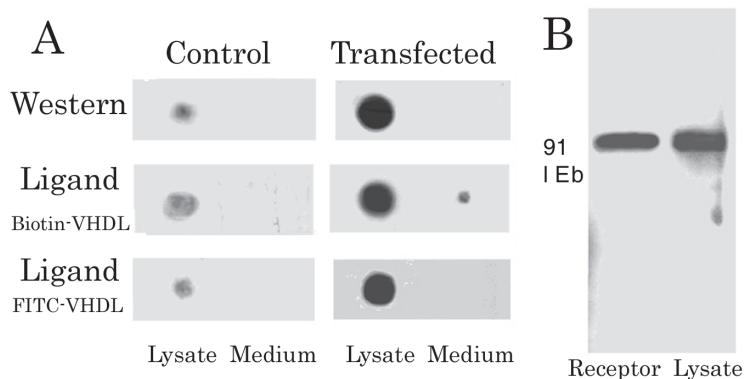


Figure 6. Western and ligand blots of the recombinant VHDL receptor. Sf9 cells were transfected with a VHDL-receptor cDNA expression vector, as described in Materials and Methods. Transfected and wild-type cells (control) were lysed, and the presence of VHDL receptor analysed in the growth medium and the cell lysate.

Panel A: Dot blots of 3 μ g of cell lysate (lysate) or concentrated growth medium (medium) were spotted onto PVDF membrane, and processed as described in Materials and Methods.

The Western blot was probed with anti-VHDL-receptor antiserum (1:6,000, secondary antibody 1:10,000; Western). Ligand blots were carried out with biotinylated VHDL (1:8,000, Ligand Biotin-VHDL) or FITC-labeled VHDL (1:8000, Ligand FITC-VHDL)

temporal and/or inter-species differences in the hemolymph composition are responsible for these different observations. For example, it may be possible that at lower pH values the interactions of a basic protein with the negatively charged membrane are weaker, so that at least some of the protein can dissociate from the membrane. Thus, it is conceivable that BHSP-2, too, may serve as receptor for VHDL, a protein that is also expressed in *T. ni*.

Since the N-terminus of the *H. zea* receptor is blocked, we do not know whether the endoplasmic reticulum-targeting signal sequence (von Heijne, 1994) is still present in the mature receptor, or cleaved as one would normally expect. If indeed this hydrophobic helix were still attached to the protein, it could serve as membrane anchor to keep the VHDL-receptor bound to the membrane, and prevent its release into the hemolymph. While it has been shown that the signal sequence has been removed from the hemolymph form of BHSP-2, it may well be possible that part of the BHSP-2 protein remains uncleaved and resides in the membrane.

In any case, even if anchored via a membrane spanning helix, the VHDL receptor is unlike any known endocytotic receptor that can trigger protein uptake by receptor-mediated endocytosis (see Figure 7 a). The endoplasmic reticulum-targeting membrane spanning helix (residues 3-17, flanked by positively charged residues)

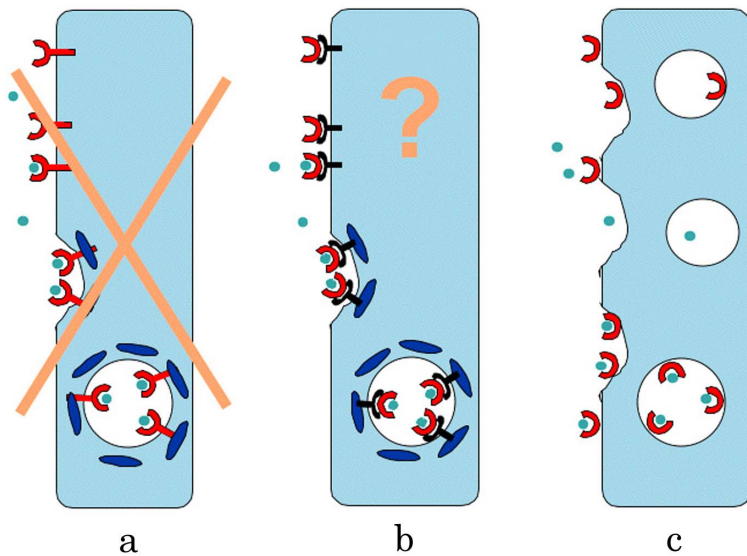


Figure 7. Potential models for the action of the VHDL receptor.

Panel a: From sequence analysis it is apparent that the VHDL receptor (red) is unlike a classical receptor of receptor mediated endocytosis, which bind its ligand VHDL (green) but also intracellular components like clathrin (blue).

Panel b: It is possible, but less likely, that the VHDL receptor acts as an intermediary link between storage proteins (green circle) and a different, yet unknown receptor (black).

Panel c: Since massive phagocytosis takes place in insect fat body prior to pupation, selective uptake can be achieved without intracellular contacts. As long as the receptor remains bound to the plasma membrane, its ligands will become enriched at the extracellular membrane surface and sequestered more efficiently than unbound hemolymph proteins.

binds co-translationally to a signal receptor at the endoplasmic reticulum membrane and directs the nascent polypeptide chain into the endoplasmic reticulum lumen. Therefore, almost the entire protein will end up facing the extracellular space, and only 3 residues will remain at the cytosolic side; these are unlikely to interact with clathrin or adaptor proteins that are known to lead to the formation of coated pits. Indeed, sequence analysis revealed that the receptor does not contain the tyrosine-based endocytic sorting signal which is recognized by adaptin in receptor mediated endocytosis (Kirchhausen, 2002).

Yet, from biochemical and ultrastructural studies it is clear that the 80 kDa protein cloned here functions as a receptor and is responsible for the selective uptake of storage proteins. In electron micrographs with gold-labeled proteins, the VHDL-receptor colocalized with its ligands VHDL and arylphorin at the plasma membrane, in coated pits and coated vesicles, and in the multivesicular vesicles that give rise to protein storage granules (Wang and Haunerland, 1994a). Only fat body that contains the receptor is capable of selectively sequestering storage proteins. When perivisceral fat body was incubated with equimolar amount of storage proteins and non-target proteins, both VHDL and arylphorin were highly enriched in the fat body. In contrast, only small amounts of immunoglobulin G entered the tissue, together with other hemolymph components enclosed in the lumen of the endocytotic

vesicle (Wang and Haunerland, 1994a). The receptor itself can bind both VHDL and arylphorin with high affinity, and the binding of either protein can be out-competed with an excess of the other one. The binding is specific, saturable, and pH- and calcium dependent (Wang and Haunerland, 1994b). In short, these properties resemble those of other receptor proteins, but the intracellular contacts that initiate endocytosis appear to be absent.

While the receptor apparently cannot trigger the uptake, it could fulfill its function of mediating the selective endocytosis of certain proteins by virtue of being a peripheral membrane protein, as long as endocytosis is induced by other means. It is well known that in the prepupal stadium, massive endocytosis takes place in fat body cells. Pinocytosis occurs at all fat body plasma membrane surfaces, but prior to pupation fat body cells switch rapidly to massive heterophagy (Dean et al., 1985). Abundant microvesicles form through invaginations of the cell membrane, which enter the fat body cells and fuse to multivesicular bodies that eventually form storage granules (Locke, 1998). It has been calculated that the entire larval fat body membrane is replaced at metamorphosis (Locke, 2003). If one assumes that this process takes place independent of an external trigger, the presence of a storage protein-binding protein like the VHDL-receptor would achieve an enrichment of VHDL and arylphorin at the plasma membrane surface, and lead to the selective uptake of these proteins. Receptor and ligands would be endocytosed together with the plasma membrane. Indeed, our earlier studies suggested that the VHDL receptor is probably not recycled, but is digested in multivesicular bodies prior to the formation of protein granules. A mechanism as proposed in Fig 7c is therefore sufficient to explain the selective uptake of storage proteins. Alternatively, the VHDL-receptor sequenced here could bind to yet another, integral membrane protein that is a true endocytotic receptor, thus acting as an intermediary of endocytosis (Figure 7b). While possible, we consider this explanation as less likely to be correct.

Numerous papers have studied storage protein uptake in various species (Haunerland, 1996), but very little is known about storage protein receptors in Lepidoptera other than *H. zea*. KiranKumar et al. (1997) demonstrated selective uptake of hexamerins in the rice moth, *Corcyra cephalonica*, and revealed that a 130 kDa protein is involved in the uptake process. These authors recently showed that the hexamerin receptor is activated through phosphorylation by a tyrosine kinase, which in turn appears to be activated by ecdysteroids (Arif et al., 2003). Similar non-transcriptional activation by ecdysone was observed by Burmester and Scheller for the hexamerin receptor from *Calliphora vicina* (Burmester and Scheller, 1997). The dipteran hexamerin receptor, which has been cloned and sequenced, is itself a member of the hexamerin gene family (Burmester and Scheller, 1995). It lacks the usual features of endocytotic receptors, such as an adaptin-binding sequence motif or membrane spanning helices. Moreover, the 130 kDa receptor requires a complex series of proteolytic cleavage steps before it can bind its ligands (Burmester and Scheller, 1997, 1999). The full-length receptor-precursor is comprised of a 17 amino acid signal peptide at its N-terminus, followed by the 65 kDa active receptor and two additional cleavage products of 30 and 45 kDa, respectively. Thus, at least the N-terminal part of the active receptor should be localized at the outside of the cell, and since the protein

lacks a hydrophobic helix other than the signal peptide, it remains unclear how this protein is attached to the membrane as well. Using a yeast two-hybrid screen, Hansen et al. (2003) recently characterized the molecular interactions between *Calliphora* arylphorin and its receptor. It appears that the ligand-binding domain is located within the N-terminal 24 amino acids of the protein; this region does not show any similarity to other receptors, or the VHDL receptor of *H. zea* described here. While the details of the storage protein uptake in *Calliphora* and the physical characteristics of the receptor are certainly distinct from the VHDL-receptor from *H. zea*, it is interesting that both proteins are members of the hexamerin superfamily. This finding lends credence to the belief that the receptors arose from mutations in this abundantly expressed gene family (Burmester and Scheller, 1999).

At present, we have no indication that the 78 kDa protein that we have isolated together with the VHDL receptor is also involved in storage protein uptake. It is not a breakdown product of the receptor, but resembles the BJHSP-1 from *T. ni* (Jones et al., 1993). Given its abundance of positive charges it is certainly possible that it also interacts with the plasma membrane, perhaps less strongly since the mature protein lacks the hydrophobic signal sequence that may still be present at the receptor. It is also possible that this protein is a ligand for the receptor, like VHDL and arylphorin, and remains bound under the conditions of the affinity purification. It has been shown in Diptera that the hexamerin receptor binds various storage proteins as well; the receptor-binding domain was mapped to a freely accessible domain within the C-terminal region of the arylphorin subunit, which appears to be conserved between many members of this gene family (Hansen et al., 2003). Whether or not similar interactions are present in Lepidoptera remains to be seen. Certainly, arylphorin and BJHSP-1 show high sequence homology in this area, but it is unknown whether such common sequence motifs are present in VHDL as well. The structure elucidation of this unique storage protein is therefore of great interest.

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