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An insect homolog of the vertebrate very low density lipoprotein receptor mediates endocytosis of lipophorins

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Abstract A novel member of the low density lipoprotein (LDL) receptor family was identified, which is expressed in locust oocytes, fat body, brain, and midgut. This receptor appeared to be a homolog of the mammalian very low density lipoprotein receptor as it contains eight cysteine-rich repeats in its putative ligand-binding domain. When transiently expressed in COS-7 or stably expressed in LDL receptor-deficient CHO cells, the receptor mediates endocytic uptake of high density lipophorin (HDLp), an abundant lipoprotein in the circulatory compartment of insects. Moreover, in the latter cell line, we demonstrated that an excess of unlabeled HDLp competed with fluorescent labeled HDLp for uptake whereas an excess of human LDL did not affect uptake. Expression of the receptor mRNA in fat body cells is down-regulated during adult development, which is consistent with the previously reported down-regulation of receptor-mediated endocytosis of lipophorins in fat body tissue (Dantuma, N. P., M.A.P. Pijnenburg, J. H. B. Diederen, and D. J. Van der Horst. **1997. *J. Lipid Res.* 38: 254–265). The expression of this receptor in various tissues that internalize circulating lipophorins and its capability to mediate endocytosis of HDLp indicate that this novel member of the LDL receptor family may function as an endocytic lipophorin receptor in vivo.**—Dantuma, N. P., M. Potters, M. P. J. De Winther, C. P. Tensen, F. P. Kooiman, J. Bogerd, and D. J. Van der Horst. **An insect homolog of the vertebrate very low density lipoprotein receptor mediates endocytosis of lipophorins. *J. Lipid Res.* 1999.** 40: **973–978.**

Supplementary key words LDL receptor family • receptor-mediated endocytosis • vitellogenin • high density lipophorin • development • locust

We recently reported that insect fat body cells demonstrate endocytic uptake of circulating high density lipophorin (HDLp) which, in contrast to vertebrate lipoproteins, carry diacylglycerol as their major neutral lipid cargo (1). Receptor-mediated endocytosis of lipoproteins has been studied in detail in vertebrate cells (2). Cloning of different receptors that accomplish lipoprotein uptake has resulted in the identification of the low density lipoprotein (LDL) receptor family, the members of which share structural and functional features (3–8). These receptors appear to originate from an ancient receptor in view of the identification of a similarly composed cell surface molecule in *Caenorhabditis elegans* (9) and two insect vitellogenin receptors belonging to this gene superfamily (10, 11).

We expected the endocytic uptake of HDLp by the insect fat body cells to be mediated by a member of the LDL receptor family. The cloning of a related transcript from locust fat body cells is presented. This novel member of the LDL receptor family is a homolog of the vertebrate very low density lipoprotein (VLDL) receptor. Moreover, we show that this receptor can mediate internalization of HDLp.

MATERIALS AND METHODS

Construction and screening of fat body cDNA library

Poly(A^+) RNA was isolated from total fat body RNA, that had been purified by cesium chloride gradient ultracentrifugation (12) from fat body tissue of adult migratory locusts (gregarious phase), *Locusta migratoria*, and was used as template for cDNA synthesis with a random unidirectional primer harboring a *Xho*I restriction site at the 5′-end. The double-stranded cDNA fragments were unidirectionally cloned in the ZAP Express vector (Stratagene). Hybridization screening was performed using random primed ^{32}P-labeled probes (12).

Abbreviations: CHO, Chinese hamster ovary; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; EGF, epidermal growth factor; HDLp, high density lipophorin; LDL, low density lipoprotein; ldlA, LDL receptor-deficient CHO cell line; ldlA(iLR), iLR expressing ldlA cell line; VLDL, very low density lipoprotein.

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PCR amplification

Fat body cDNA, derived from total RNA of adult locust fat body, was used as template in PCR amplifications using two degenerated primers. The sense primer was: 5′-CGCGAATTC GA(C/T) TG(C/T) III GA(C/T) GG(A/C) TC(C/G) GA(C/T) GA-3′ (I = deoxyinosine), and the antisense primer: 5′-CGCAAGCTT (A/G)CA GAT GTG (C/G)GA ACA (T/G)CC (A/G)CC ATT-3′. The PCR products were isolated, digested with *Eco*RI and *Hind*III (underlined), and subcloned in pBluescript (Stratagene).

For 3′-RACE (13), cDNA was synthesized from poly(A^+) fat body RNA using the Marathon cDNA amplification kit (Clontech). This cDNA was amplified with an adaptor primer and a gene specific primer: 5′-TA CTG CAC TGG ATT TCG TAT TCC GG-3′ using Expand polymerase (Boehringer-Mannheim). Products were re-amplified using a nested adaptor primer and a nested gene specific primer: 5′-C TGG ATT TCG TAT TCC GGA CTG GG-3′ using the same PCR cycling parameters. The T/A cloning vector pCR2.1 (Invitrogen) was used for subcloning PCR products of three independent amplifications (piLR-3′-A, B and C).

Sequencing methods

All sequence data were obtained by double-stranded sequencing of plasmid DNA using the dideoxy chain termination method (14) with Dye Terminator Cycle Sequencing Ready Reactions on a 373 DNA Sequencer (both Applied Biosystems).

Functional expression in mammalian cells

An expression construct, piLR-e, was generated by subcloning in pcDNA3 (Invitrogen) the resulting full-length cDNA (piLR-5′ linked to piLR-3′-A, which did not contain any PCR-derived mutations resulting in amino acid substitutions).

African green monkey COS-7 kidney cells were transiently transfected with this expression construct. COS-7 cells were grown in DMEM containing 5% fetal calf serum and 2 mm glutamine and transfected using the DEAE-dextran method (15). Transfection efficiencies were checked by co-transfecting 100 ng pCMV-β-galactosidase (Stratagene) with 400 ng of piLR-e or pcDNA3 and staining the cells for β-galactosidase. Forty-eight h post-transfection, the transfected COS cells were incubated for 5 h with culture medium containing 50 μg/ml HDLp labeled with the fluorescent lipid 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI) (1). After incubation with the DiI-labeled HDLp, the cells were rinsed with 0.1 m NaH_2PO_4/Na_2HPO_4 (pH 7.4) for 3 × 5 min, fixed with 4% paraformaldehyde in the same buffer at room temperature for 10 min, and examined with a fluorescence microscope (Axioskop, Zeiss).

An LDL-receptor-deficient Chinese hamster ovary (CHO) cell line (ldlA; 16), that was kindly provided by Dr. Monty Krieger (Massachusetts Institute of Technology, MA), was used for making stable transfectants. LdlA cells were transfected with iLR-e using lipofectin (Life Technologies). Clonal, stably transfected cell lines were created by limited dilution and continuous selection with the neomycin analog G418 (400 μg/ml) in HAMF10 medium supplemented with 10% fetal calf serum. Clones were screened by fluorescence microscopic examination upon incubation with DiI-labeled HDLp.

For fluorimetric analysis of DiI-HDLp cell association, triplicate wells with confluent ldlA(iLR) cells were incubated for 3 h at 37°C in HAMF10 medium containing 1% bovine serum albumin with DiI-labeled HDLp (10 μg/ml) either with an excess (200 μg/ml) of unlabeled HDLp or unlabeled human LDL, or without unlabeled lipoproteins. Three wells were incubated with medium alone to determine background fluorescence. After this period the cells were washed twice with HAMF10 medium containing 10% fetal calf serum and twice in PBS. The fluorescence taken up by the cells was measured with a Cytofluor II plate-reader (Perceptive Biosystems) and expressed as relative fluorescence per mg cell protein; differences were tested by Student's *t*-test.

Northern blot analysis

Digoxygenin (DIG, Roche Diagnostics)-labeled anti-sense RNA probes were synthesized from piLR-5′ linearized with *PstI* creating a 750 bp RNA run off probe using T7 RNA polymerase. Total RNA was isolated from various insect tissues using RNAzol B (Tel-Test). Agarose gel electrophoresis, Northern blotting, and probe hybridization were performed using standard procedures (17). Subsequently the membrane was incubated with 1:40000 anti-DIG-alkaline phosphatase in blocking reagent and washed. After washing in staining buffer (100 mm NaCl, 100 mm Tris-HCl, pH 9.5), the blots were incubated with CDP-Star (Roche) and exposed to ECL-Hyper films (Amersham).

RESULTS

Cloning of a novel member of the LDL receptor family from insect fat body

In the present study we considered the possibility that receptor-mediated uptake of HDLp by insect fat body cells (1) is mediated by a member of the LDL receptor family. In order to identify this novel receptor, we attempted to clone transcripts encoding the characteristic clusters of this family: a ligand binding domain, consisting of complement type cysteine-rich repeats, directly linked to a domain homologous to the EGF precursor. For this purpose, fat body cDNA was PCR amplified with a set of degenerated primers based on conserved sequences in these domains: a 5′-primer corresponding with the consensus amino acid sequence, DCxDGSDE, present in the majority of individual cysteine-rich repeats in the ligand binding domain, and a 3′-primer corresponding with the consensus amino acid sequence, NGGCSHIC, located at the most 5′ part of the EGF precursor. This amplification resulted in several products. Sequence analysis revealed that three products, of approximately 1, 0.6, and 0.3 kb, were derived from a single transcript that shared sequence similarities with LDL receptor family members. The differences in length were caused by annealing of the 5′-primer within different cysteine-rich repeats of the putative ligand binding domain.

Hybridization screening of a fat body cDNA library (2.8×10^5 primary clones) with the 1 kb product resulted in the identification of a single positive clone. This clone, piLR-5′, was sequenced revealing a 1.8 kb insert that contained a 5′ untranslated region of 127 bp and an incomplete open reading frame encoding a member of the LDL receptor family. A product of 2.6 kb was obtained with a 3′-RACE PCR amplification (13). Three 2.6 kb products of independent PCR amplifications were cloned and sequenced in order to be able to identify mutations generated by the thermostable polymerase during the amplification. These three products, designated piLR-3′-A, B, and C, encoded the 3′-part of the open reading frame followed by a 3′ untranslated region of 1426 bp. Only piLR-3′-A was devoid of PCR-derived mutations resulting in amino acid substitutions. This clone was used for the construction of the full-length cDNA (see below).

According to the deduced amino acid sequence, the

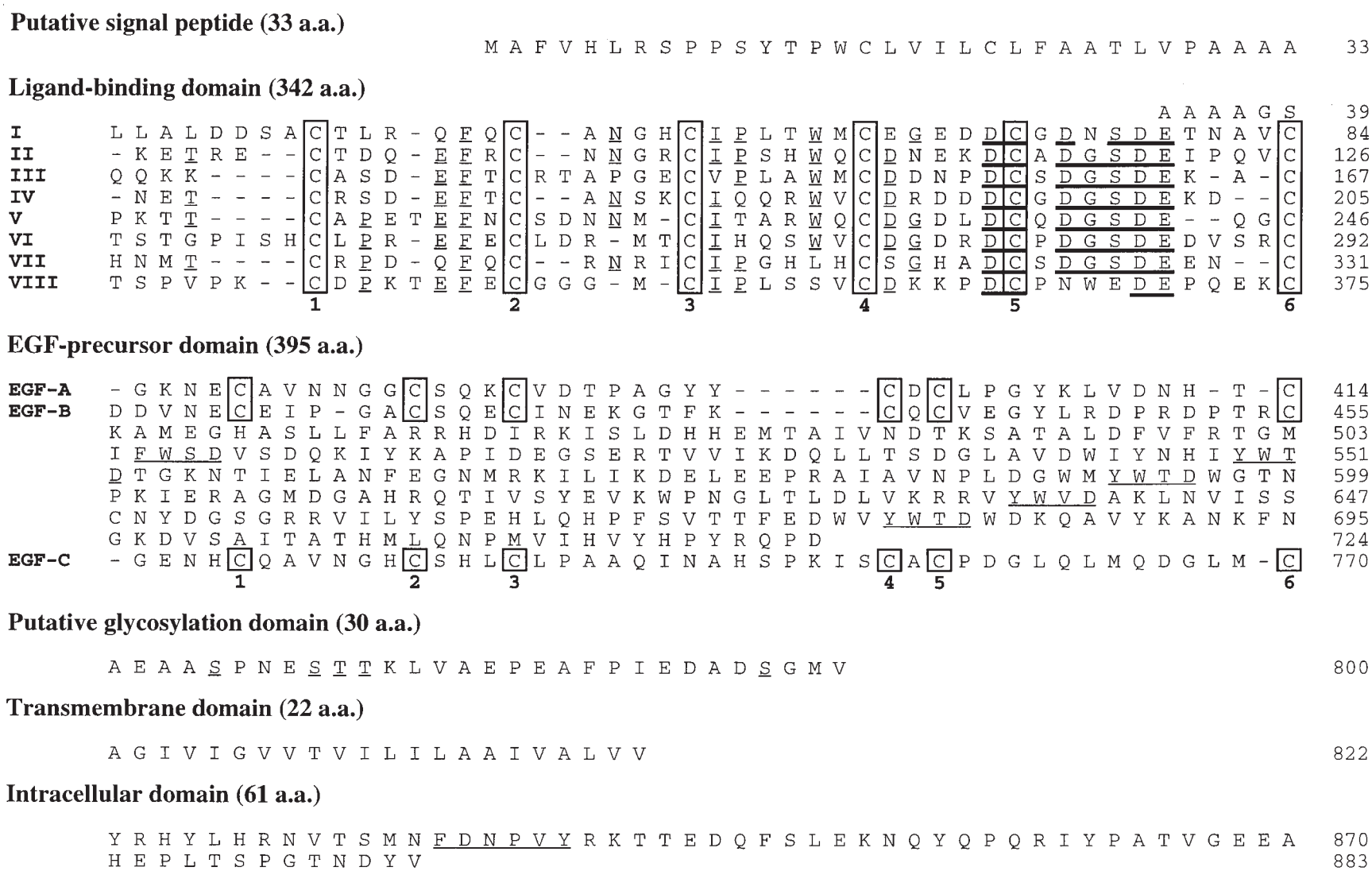

Fig. 1. Deduced amino acid sequence (883 residues) of the insect lipophorin receptor, which is divided into six domains: 1) putative signal peptide, 2) ligand-binding domain, 3) EGF-precursor domain, 4) putative glycosylation domain, 5) transmembrane domain, and 6) intracellular domain. The eight imperfect cysteine-rich repeats (I–VIII), that compose the ligand-binding domain, are aligned. The cysteine residues (1–6), other conserved residues (underlined), and the DCxDGSDE sequence (double underlined) are indicated. The EGF-precursor A, B and C repeats are aligned. The cysteine residues (1–6) in the EGF-precursor repeats and the F/YWxD sequences (underlined), located between EGF-precursor repeat B and C, are indicated. In the putative glycosylation domain, threonine and serine residues (underlined), that can be O-linked glycosylated, are present. The cytoplasmic tail contains the conserved internalization signal FDNPVY (underlined).

protein encoded by the full-length cDNA is a novel member of the LDL receptor family. Six domains were discriminated in the deduced amino acid sequence of the putative receptor (**Fig. 1**). *I*) According to Von Heijne's rules governing signal cleavage (18), the first 33 amino acids might function as a signal peptide. *II*) The second domain encodes the putative ligand-binding domain consisting of eight imperfect cysteine-rich repeats. *III*) The EGF-precursor domain, being the third domain of this receptor, contains three EGF-precursor repeats. In addition, this domain contains five copies of the characteristic F/YWxD sequence, spaced by approximately 50 amino acid intervals. *IV*) The fourth domain is a stretch of 30 amino acid residues in between the EGF-precursor domain and the transmembrane domain. There are only five threonine and serine residues present in this domain that, in several other members of the LDL receptor family, is enriched with these residues and known to be O-linked glycosylated at these positions. *V*) The fifth domain is a hydrophobic stretch of 22 amino acids that most likely functions as a single membrane spanning domain. *VI*) The putative cytoplasmic tail of the receptor, which is the sixth domain, contains the highly conserved internalization signal (amino acid 836–841, FDNPVY) required for internalization in clathrin-coated pits. Interestingly, this receptor contains the crucial tyrosine residue in the internalization signal, which was found to be absent in the recently identified insect vitellogenin receptors (10, 11).

Functional expression of the putative lipophorin receptor in mammalian cells

The expression vector piLR-e (piLR-5′ linked to piLR-3′-A) that contained the full-length cDNA was transiently expressed in COS-7 cells. Fluorescence microscopic examination of transfected cells, that had been incubated with 50 μg/ml DiI-labeled HDLp for 5 h, revealed that the piLR-e transfected cells demonstrated an apparent punctate staining (**Fig. 2A**), which is indicative for receptor-mediated uptake of HDLp. Such a staining was absent from mock transfected cells (Fig. 2B).

Fluorescence microscopic examination of clones of an LDL receptor-deficient CHO cell line that was stably transfected with iLR-e after incubation with DiI-labeled HDLp revealed a punctate staining similar to that observed with transiently transfected COS-7 cells, whereas such a staining was not observed with the untransfected ldlA cell line. One of these clones, ldlA(iLR), and the parental cell line, ldlA, were incubated with 10 μg/ml DiI-labeled HDLp for 5 h at 37°C and subsequently the amount of cell-associated fluorescent label was quantified (**Table 1**). Significantly more label was associated to the ldlA(iLR) cells when compared to the ldlA cells (unpaired t-test, $P < 0.05$). Unfortunately, the ldlA cells also bound substantial amounts of label. Inclusion of a 20-fold excess of unlabeled HDLp strongly reduced the amount of label associated with ldlA(iLR) and resulted in equal amounts of the label associated with the parental and iLR-expressing cell line. An excess of unlabeled HDLp reduced the amount of label associated with ldlA cells, which indicates that the parental cell line contains a saturable HDLp binding site. In contrast to the effect observed with an excess of unlabeled HDLp, a 20-fold excess of human LDL did not affect the amount of cell-associated label.

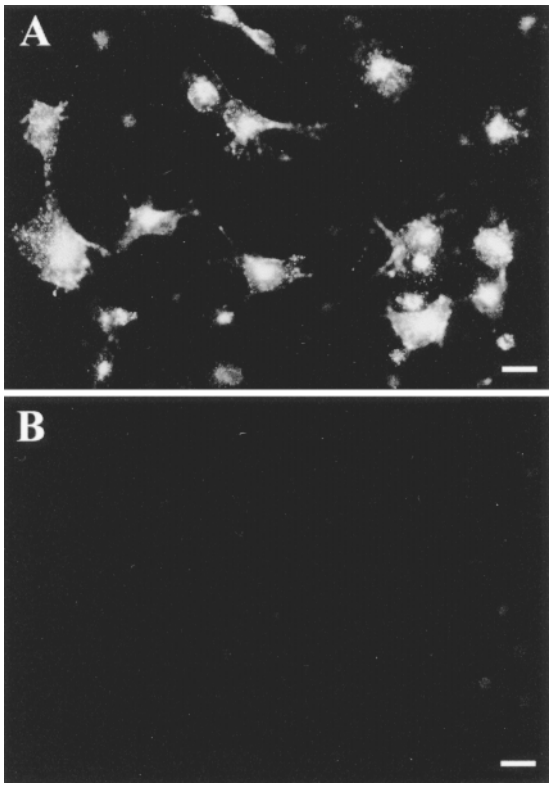

Fig. 2. Functional expression of the insect lipophorin receptor in COS-7 cells which were transiently transfected with expression vector piLR-e or control empty expression vector. Transfected cells, that had been incubated with 50 μg/ml DiI-labeled HDLp for 5 h, were examined using fluorescence microscopy. Approximately 20% of the piLR-e transfected cells demonstrated a punctate fluorescent staining (A), which was absent in mock transfected cells (B). Scale bar = 20 μm.

Expression of lipophorin receptor in fat body cells

Northern blot analysis using total RNA revealed one transcript of approximately 4.8 kb (**Fig. 3**) which corre-

TABLE 1. Association of DiI-labeled HDLp with ldlA(iLR) cells

	No Competitor	Excess HDLp	Excess Human LDL
	AU/total cellular protein		
ldlA	4268 ± 667	2465 ± 216[b]	3807 ± 201
ldlA(iLR)	5584 ± 449[a]	2919 ± 243[b]	5565 ± 48[a]

LdlA(iLR), an LDL receptor-deficient CHO cell line that was stably transfected with iLR-e, and the parental ldlA cell line were incubated for 5 h at 37°C with 10 μg/ml DiI-labeled HDLp in the absence or presence of 200 μg/ml unlabeled HDLp or human LDL. The amount of cell-associated fluorescence per total cellular protein was quantified by fluorimetry. Results given as mean ± SD, n = 3.

[a] Significantly different from parental ldlA cell line (paired *t*-test, $P < 0.05$).

[b] Significantly different from incubation without competitor (paired *t*-test, $P < 0.05$).

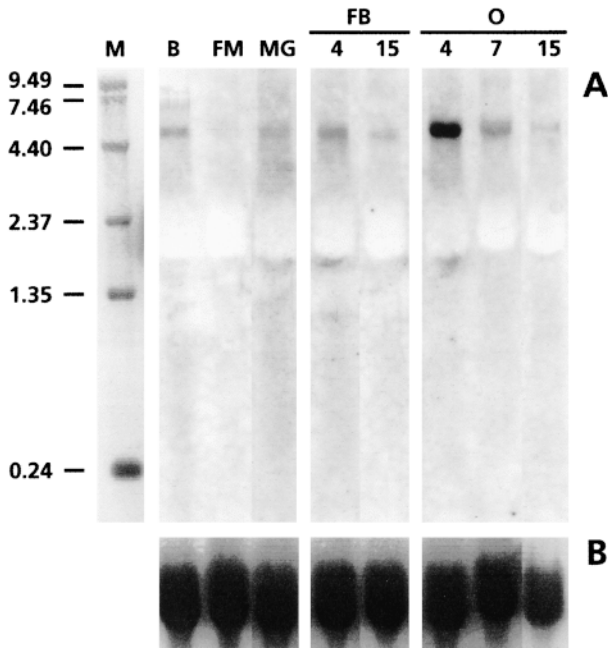

Fig. 3. Expression of the insect lipophorin receptor. Northern blot analysis of 30 μg total RNA per lane isolated from tissues from young (4 days after the imaginal ecdysis) and older adult locusts (10 or 15 days after the imaginal ecdysis) using a DIG-labeled insect lipophorin receptor probe (A). Ethidium bromide staining of the 28S RNA signal from the agarose gel is shown in (B). M, RNA size marker; B, brain; FM, flight muscles; MG, midgut; FB, fat body from 4- and 15-day-old adult locusts, respectively; O, oocytes from 4-, 7-, and 15-day-old adult locusts, respectively.

sponds fairly well with our full-length clone of 4.2 kb. As the latter, however, did not contain an apparent consensus polyadenylation signal, a small part the 3′ untranslated region might be missing. The transcript was predominantly expressed in oocytes and fat body tissue of young adults. Transcription in these two tissues was strongly down-regulated during the adult stage. Expression was also found in the brain and the midgut but the transcript was absent from flight muscles (Fig. 3).

DISCUSSION

In this paper we present the molecular cloning of an insect lipoprotein receptor that is homologous to the vertebrate VLDL receptor. This is the first identification of an invertebrate LDL receptor family member with an extracellular domain composed of a single ligand-binding domain and EGF-precursor domain, a type of lipoprotein receptor that has been found in many vertebrates. Our data suggest involvement of this novel member of the LDL receptor family in endocytic uptake of insect lipophorins. For this reason, the receptor is designated 'the insect lipophorin receptor'.

There are several points that argue in favor of a key role of the insect lipophorin receptor in insect lipid metabolism. First, the ability of this receptor to mediate endocytic uptake of HDLp in transiently transfected COS-7 cells and stable transfected ldlA cells, demonstrating that this receptor can function as a classic endocytic lipoprotein receptor. Second, association of fluorescent label with ldlA(iLR) cells upon incubation with DiI-labeled HDLp is reduced when an excess unlabeled HDLp is included but an excess of human LDL did not affect cell association, which indicates that the insect lipophorin receptor is specific for HDLp. Third, because the insect fat body tissue is in direct contact with the open circulatory system, the insect lipophorin receptor is exposed to the high HDLp concentration in the circulation. Fourth, the receptor is predominantly expressed in two tissues, oocytes and the young adult fat body, which have been shown to internalize HDLp (1, 19). It is unknown whether locust oocytes express a homolog of the two-cluster vitellogenin receptor that has been cloned from the fruit fly and the mosquito (10, 11). Fifth, the developmental down-regulation of expression of the insect lipophorin receptor in adult fat body cells is accompanied by a strong decrease in HDLp endocytosis (1). This also indicates that the cell surface localization observed in fat body tissue of older adults is not due to a redistribution of this endocytic receptor, as we previously postulated (1), but rather to the appearance of a different cell surface binding site. Moreover, the down-regulation observed in the oocytes is very similar to the reported developmental down-regulation of the avian and piscine vitellogenin receptors (7, 8). We consider this novel member of the LDL receptor family a likely candidate for the receptor that mediates HDLp endocytosis by fat body cells and oocytes.

In vertebrates, many different LDL receptor family members have been identified. These can be divided in receptors with a single ligand-binding domain and EGF-precursor domain, such as the classic LDL receptor (3), the apoE receptor 2 (6), and the VLDL receptor (5, 7, 8), and receptors containing multiple clusters of these two domains, such as the LDL receptor-related protein (4). Until now, only the latter receptor type had been found in invertebrates: a nematode homolog of the LDL receptor-related protein containing five clusters (9) and two insect vitellogenin receptors containing two clusters (10, 11). Sequence alignments indicate that the subfamilies with a single cluster and those with multiple clusters evolved independently from a common ancestor with a single cluster of ligand-binding and EGF-precursor domains (19, 20). The identification of the first insect LDL receptor family member belonging to the subfamily of receptors with a single cluster demonstrates that both types of lipoprotein receptors are represented in invertebrates.

The ligand specificity of the different lipoprotein receptors is intriguing. The mammalian VLDL receptor has been shown to bind apoE specifically, whereas apoB, a ligand of the LDL receptor, does not interact with this receptor (5). Yet, the apolipoproteins of HDLp, apolipophorin-I and -II, are not related to the mammalian apoE but share sequence homology with the mammalian apoB (21–23). New insights regarding ligand specificity may be derived by identifying the domains in the different lipo-

proteins that bind to the vertebrate VLDL receptors or their insect homolog: the mammalian apoE, avian apoB, and insect apolipophorin-I and -II.

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