Lipophorin receptor-mediated lipoprotein endocytosis in insect fat body cells

Dennis Van Hoof,[1] Kees W. Rodenburg, and Dick J. Van der Horst

Department of Biochemical Physiology and Institute of Biomembranes, Utrecht University, Padualaan 8, 3584 CH Utrecht, The Netherlands

Abstract High-density lipophorin (HDLp) in the circulation of insects is able to selectively deliver lipids to target tissues in a nonendocytic manner. In *Locusta migratoria*, a member of the LDL receptor family has been identified and shown to mediate endocytosis of HDLp in mammalian cells transfected with the cDNA of this receptor. This insect lipophorin receptor (iLR) is temporally expressed in fat body tissue of young adult as well as larval locusts, as shown by Western blot analysis. Fluorescence microscopy revealed that fat body cells internalize fluorescently labeled HDLp and human receptor-associated protein only when iLR is expressed. Expression of iLR is down-regulated on Day 4 after an ecdysis. Consequently, HDLp is no longer internalized. By starving adult locusts immediately after ecdysis, we were able to prolong iLR expression. In addition, expression of the receptor was induced by starving adults after down-regulation of iLR. These results suggest that iLR mediates endocytosis of HDLp in fat body cells, and that expression of iLR is regulated by the demand of fat body tissue for lipids.—Van Hoof, D., K. W. Rodenburg, and D. J. Van der Horst. **Lipophorin receptor-mediated lipoprotein endocytosis in insect fat body cells. *J. Lipid Res.* 2003.** 44: **1431–1440.**

Supplementary key words high-density lipophorin • insect lipophorin receptor • low density lipoprotein receptor • very low density lipoprotein receptor • receptor-associated protein • lipid • *Locusta migratoria* • receptor-mediated endocytosis • recycling

Whereas mammals rely on a wide array of lipoproteins with different compositions and functions (1), insects make use of a single type of lipoprotein, high-density lipophorin (HDLp) (2), to effect the transport of lipids through the circulation. HDLp comprises diacylglycerol and phospholipids as major lipid classes. The protein matrix consists of two nonexchangeable apolipoproteins, apolipophorin I (apoLp-I) and apoLp-II, which are derived from a common precursor protein through posttranslational cleavage (3, 4). Sequence and domain structure analyses indicate that this precursor protein is homologous to mammalian apolipoprotein B-100 (apoB-100), the nonexchangeable protein component of VLDL and its resulting LDL, and that both proteins have emerged from an ancestral gene (5–7).

HDLp is secreted by the insect fat body, an organ combining many of the functions of mammalian liver and adipose tissue (8). Similar to mammalian adipose tissue, the fat body retains large intracellular lipid depots that provide the fuel for energy-demanding tissues. Circulatory HDLp is able to take up lipids released from the fat body cells and to selectively unload its lipid cargo at target tissues without endocytosis and lysosomal degradation, and thus functions as a reuseable shuttle [as reviewed in refs. (2, 9–11)].

In spite of the concept of selective lipid transfer mediated by HDLp, a novel member of the LDL receptor (LDLR) family was identified in *Locusta migratoria* (12). The domain structure composition of this insect's lipophorin receptor (iLR) is identical to that of the mammalian VLDL receptor (VLDLR), and both have eight consecutive ligand binding repeats. A similar receptor was found in the mosquito *Aedes aegypti* (13). In a stably transfected Chinese hamster ovary (CHO) cell line, locust iLR was shown to bind and internalize HDLp specifically, but not human LDL (14). In contrast to the lysosomal fate of ligands internalized by mammalian lipoprotein receptors, endocytosed HDLp was observed to escape from degradation after iLR-mediated endocytosis. Both the occurrence of iLR in the insect (12, 13) and its functioning in a mammalian cell line (14) suggested that internalization of HDLp via receptor-mediated endocytosis may be a physiologically relevant process (15).

Using fluorescence microscopy, in this study we demonstrate that fat body tissue of young adult and larval locusts

Abbreviations: apoLp, apolipophorin; CHO, Chinese hamster ovary; HDLp, high-density lipophorin; iLR, insect lipophorin receptor; L_5, fifth instar; LDLR, low density lipoprotein receptor; LPL, lipoprotein lipase; OG, Oregon Green 488; PVDF, polyvinylidene fluoride; RAP, receptor-associated protein; TAG, triacylglycerol; VLDLR, very low density lipoprotein receptor.

[1] To whom correspondence should be addressed.
e-mail: d.vanhoof@bio.uu.nl

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is able to internalize fluorescently labeled HDLp via iLR. In addition, similar to mammalian VLDLR, this receptor appears capable of internalizing human receptor-associated protein (RAP). On Day 4 after the energy-consuming process of ecdysis, expression of iLR drops below detectable levels in young adult and larval locusts. Fat body tissue excised from these insects has lost the ability to endocytose HDLp. Down-regulation of iLR was postponed when adults were starved immediately after ecdysis. In addition, starving adult locusts after down-regulation of iLR induced expression of the receptor. Taken together, these results suggest that iLR mediates endocytosis of HDLp in insect cells, and provide evidence for regulation of iLR expression under specific physiological conditions. The endocytic property of iLR is compared with that of VLDLR. In addition, the proposed lipoprotein recycling function observed in mammalian cells as well as its possible role in lipid storage in insects are discussed.

MATERIALS AND METHODS

Antibodies, reagents, and proteins

Precision protein standards prestained broad range marker (Bio-Rad); alkaline phosphatase-conjugated affinipure goat-anti-rabbit IgG, DiI(C_{18}(3)) (1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine percholate), and Oregon Green 488 (OG) carboxylic acid (Molecular Probes); 4′,6′-diamidino-2′-phenylindole (DAPI) (Roche Diagnostics); leupeptin, aprotinin, and BSA (Sigma); ^{125}I[iodine] (3.9 GBq/ml; Amersham Pharmacia Biochem); Oil Red O (Chroma); Coomassie brilliant blue (Serva); trypsin-EDTA (Invitrogen); and chloramine-T (Merck) were obtained from commercial sources. HDLp was isolated from locust hemolymph by ultracentrifugation (14). Membrane proteins of wild-type CHO and iLR-transfected CHO [CHO(iLR)] cells were isolated as described by Van Hoof, Rodenburg, and Van der Horst (14). Polyclonal rabbit-anti-iLR 9218 antibody was raised against a synthetic peptide representing the unique C-terminal 19 amino acids (865–883) of iLR (14), and polyclonal rabbit-anti-iLR 2189/90 antibody was raised against a synthetic peptide representing the unique N-terminal 20 amino acids (34–53) of the first cysteine-rich repeat of iLR. Human RAP was a generous gift from Dr. Michael Etzerodt (IMSB, Aarhus University, Aarhus, Denmark).

Insects

Insects were reared under crowded conditions in a temperature-controlled environment at 30°C with a relative humidity of 40% and a 12 h light-dark cycle. Immediately after ecdysis, male and female fifth instar (L_5) larvae were transferred to separate cages to obtain synchronized larval fat body. The same procedure was used to obtain synchronized adult male and female locust fat body after the imaginal ecdysis. When starved, individual animals were transferred to separate cages and given access to water to prevent dehydration.

In vitro incubation of fat body tissue with fluorescently labeled ligands

HDLp (1 mg/ml) was fluorescently labeled in PBS with 50 μl/ml DiI in DMSO (3 μg/μl) at 37°C under continuous stirring for 2.5 h. HDLp and RAP (1 mg/ml) were labeled with 20 μl/ml OG dissolved in DMSO (1 μg/μl) at room temperature under continuous stirring for 1 h according to the manufacturer's instructions. DiI and OG-labeled HDLp (DiI-HDLp and OG-HDLp, respectively) were purified with Sephadex G-25 PD-10 columns (Amersham Pharmacia Biotech) to separate fluorescently labeled ligand from free fluorescent label and replace the PBS by incubation medium (10 mM HEPES, 50 mM NaCl, 10 mM KCl, 5 mM $CaCl_2$, and 2 mM $MgSO_4$; pH 7.4). OG-labeled RAP (OG-RAP) was dialyzed against incubation medium using standard cellulose membrane (Medicell International). Fat body tissue was incubated with 10 μg/ml DiI-HDLp, 25 μg/ml OG-HDLp, or 3.6 μg/ml OG-RAP for 30 min at 32°C for endocytic uptake. Tissue was rinsed in incubation medium and immediately fixed in 4% paraformaldehyde diluted in PBS for 30 min at room temperature. Where indicated, prior to fixation, fat body tissue was incubated with 0.05% trypsin in 0.35 mM EDTA for 5 min at room temperature, and washed thoroughly in incubation medium. For cell surface binding, fat body tissue was incubated with fluorescently labeled HDLp for 1 h at 4°C, thoroughly washed in incubation medium, and fixed as described. For endocytosis of surface-bound HDLp, fat body tissue was preincubated with OG-HDLp for 30 min at 4°C, thoroughly washed, and then incubated in medium without fluorescently labeled HDLp for 30 min at 32°C, followed by fixation as described. After fixation, fat body tissue was incubated with 0.25 μg DAPI per ml PBS for 30 min at room temperature to stain the nuclei of the cells.

Microscopy and image processing

Coverslips with fixed tissue were mounted in Mowiol and examined on a light and fluorescence Axioscop microscope (Zeiss) with a Hg HBO-50 lamp and a Plan-Neofluar 100×/1.30 oil lens. Using UV and fluorescein isothiocyanate-tetramethylrhodamine isothiocyanate filters, digital images were recorded with a DXM 1200 digital camera and ACT-1 version 2.00 software (Nikon). Images of centrally localized nuclei and peripherally distributed endocytic vesicles of the same area were obtained sequentially at their respective confocal planes. Corresponding images of nuclei and vesicles were subsequently processed and merged using PaintShop Pro 7.00 (Jasc Software).

Western blot analysis of fat body membrane protein extracts

Fat body tissue from male and female larvae and adult locusts was excised in incubation medium containing protease inhibitors. Fat body tissue of three to five individuals was pooled and fractionated by thoroughly resuspending and vortexing, and kept on ice during the following purification steps. Samples were centrifuged for 10 min at 10°C at 15,000 *g* to separate the fractionated cells from the released lipids. The fat cake was removed with a toothpick and the supernatant was discarded, after which the pellet was resuspended in protease inhibitor-containing incubation medium. The samples were centrifuged again at 15,000 *g* for 10 min at 10°C, after which the supernatant was removed and the remaining lipids were discarded with a tissue. The lipid-depleted pellets were resuspended in 40 μl to 80 μl 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate (CHAPS) buffer (20 mM HEPES, 124 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 2.5 mM Na_2HPO_4, 1.2 mM $MgSO_4$, 1 mM EDTA, 0.1 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml aprotinin, and 1% CHAPS) to resuspend the pellet. The suspension was incubated for 10 min on ice, and spun down at 15,000 *g* for 10 min at 10°C. Supernatant containing 5.0 μg of total membrane protein was transferred to a clean Eppendorf tube and either heated for 5 min at 95°C in Laemmli buffer (16) or directly dissolved in modified Laemmli buffer (containing 0.025% SDS and no disulfide bond-reducing reagents) prior to separation by SDS-PAGE in a 10% polyacrylamide gel. The separated membrane proteins were transferred to polyvinylidene fluoride (PVDF) membrane (Millipore) and incubated with rabbit anti-iLR 9218 (1:2,000, v/v) or

2189/90 (1:100, v/v) antibody for 2 h, followed by 1 h alkaline phosphatase-coupled goat anti-rabbit incubation. Bound second antibody was visualized by incubating the blot in TSM buffer containing 100 mM Tris-HCl, 100 mM NaCl, 10 mM $MgAc_2$, 50 μg/ml p-nitro blue tetrazolium chloride (Boehringer Mannheim), and 25 μg/ml 5-bromo-4-chloro-3-indoyl-phosphate p-toluidine (BCIP; Roche Diagnostics) (pH 9.0).

Ligand blot with ^{125}I-RAP

^{125}I-RAP was prepared using chloramine-T according to Rodenburg et al. (17), resulting in a specific labeling activity of ~45,000 cpm/ng protein. PVDF membrane, containing 20.0 μg per lane of total membrane proteins that were separated by SDS-PAGE under nonreducing conditions, was incubated overnight with 12 nM ^{125}I-RAP in binding buffer [20 mM HEPES (pH 7.4), 50 mM NaCl, 2.5 mM $CaCl_2$, and 0.5% (w/v) BSA], after which the blot was washed several times with binding buffer. RAP binding was detected using a PhosphorImager (Molecular Dynamics), and visualized using MD ImageQuant software version 3.3 (Molecular Dynamics). RAP receptor binding was quantified by determining the radioactivity in those parts of the ligand blots that corresponded to receptor-bound complexes using the data from the PhosphorImager.

RESULTS

In vitro endocytosis of HDLp by fat body tissue of young adult locusts

In contrast to the mechanism by which HDLp selectively unloads lipids at target tissues, endocytosis of HDLp may provide an alternative mechanism for the uptake of lipid components in fat body cells. Therefore, HDLp was fluorescently labeled with DiI to visualize the lipoprotein after incubation of fat body tissue that was excised from young adult male locusts within 24 h of ecdysis. Incubation of fat body tissue with incubation medium containing DiI-HDLp resulted in a punctate staining pattern characteristic for endocytosis (**Fig. 1A**, red dots). To investigate whether, in addition to the lipid, the apolipoprotein component was also internalized, the amine-reactive probe OG was used to label apoLp-I and -II. Analogous incubation conditions with OG-HDLp resulted in a similar endocytic uptake pattern (Fig. 1B, green dots). Scanning vertically through incubated fat body tissue revealed that HDLp-containing vesicles are peripherally localized in the cells (Fig. 1C). Treatment of tissue with trypsin prior to fixation did not alter the punctate staining pattern, verifying that HDLp is encapsulated in membranes (i.e., endocytic vesicles; Fig. 1D).

The trophocyte, or adipocyte, is the main cell type that constitutes the fat body, and is used for storage of lipids and glycogen (8). The size and shape of trophocytes are predominantly determined by the lipid droplets that fill up almost the entire intracellular space (Fig. 1E, F). As a result, cytoplasm is mainly situated peripherally below the cell surface and between the lipid droplets, whereas the nuclei are predominantly localized in the cell center (Fig. 1G). Incubation of young adult female fat body tissue with DiI-HDLp (data not shown) or OG-HDLp (Fig. 1H) resulted in staining patterns identical to those observed with male fat body tissue, which suggests an uptake mechanism that is present in both sexes. In contrast, fat body tissue excised from adults on Day 4 or later after ecdysis remained devoid of fluorescently stained endocytic vesicles when incubated with DiI-HDLp or OG-HDLp (Fig. 1I and J, respectively). These findings suggest a down-regulation of the ability to internalize HDLp via endocytosis that occurs on Day 4 after imaginal ecdysis.

Endocytosis of HDLp by fat body cells is mediated by iLR

To investigate the involvement of a receptor in HDLp endocytosis, fat body tissue was incubated at 4°C, at which receptor-mediated endocytosis is inhibited, whereas cell surface binding still occurs (18). As shown in **Fig. 2A** and B, the internalization of both DiI-HDLp and OG-HDLp was prevented. Transfer of tissue that was preincubated with OG-HDLp at 4°C to medium without HDLp resulted in the formation of OG-HDLp-containing endocytic vesicles when the temperature was raised to 32°C (Fig. 2C). At 32°C, a 100-fold excess of unlabeled HDLp prevented endocytic uptake of DiI- and OG-labeled HDLp (Fig. 2D and E, respectively). These observations imply that endocytosis of HDLp by fat body cells of young adult locusts is mediated by a receptor.

iLR has recently been shown to mediate endocytic uptake of HDLp in CHO cells that were stably transfected with an expression vector harboring iLR cDNA (14). Consequently, iLR was supposed to mediate endocytosis of HDLp in these young adult locusts. The presence of iLR was analyzed using cell membrane extracts from adult locusts at defined time points after ecdysis. Membrane proteins were separated by SDS-PAGE under reducing conditions, and immuno-detected with anti-iLR 9218 antibody raised against the cytoplasmic tail of iLR that is unique for iLRs (14). Under reducing conditions, iLR has a molecular weight of ~140 kDa and is expressed in both males and females (**Fig. 3A** and B, respectively). In addition, the blots show that iLR is expressed during the first 3 days after imaginal ecdysis, which is in agreement with the capability of young adult fat body tissue to endocytose HDLp (Fig. 1A, B). On Day 4, expression of iLR drops below detectable levels (Fig. 3A, B), which coincides with the absence of fluorescently labeled HDLp-containing endocytic vesicles in the fat body tissue of these animals (Fig. 1I, J).

Nonreduced iLR obtained from fat body cells (Fig. 3C, lane 3 and 4) has a higher electrophoretic mobility in SDS-PAGE compared with reduced iLR (Fig. 3D, lanes 3 and 4), indicating the presence of multiple disulfide bonds. The molecular weight of reduced iLR is higher than the theoretical 98 kDa based on the amino acid sequence (12), suggesting that endogenous *L. migratoria* iLR is glycosylated, like all other LDLR family members (19, 20). The iLR isolated from CHO(iLR) cells (Fig. 3C, D, lane 2) has a higher molecular weight compared with that obtained from fat body cells (Fig. 3C, D, lanes 3 and 4), which is most likely the result of a different degree of glycosylation between the two different cell types (21, 22). Immunodetection with anti-iLR 2189/90 antibody raised against the 20 N-terminal amino acids of the first cysteine-rich repeat of iLR gave a similar result with nonreduced membrane extracts of CHO(iLR) and fat body cells as shown in Fig.

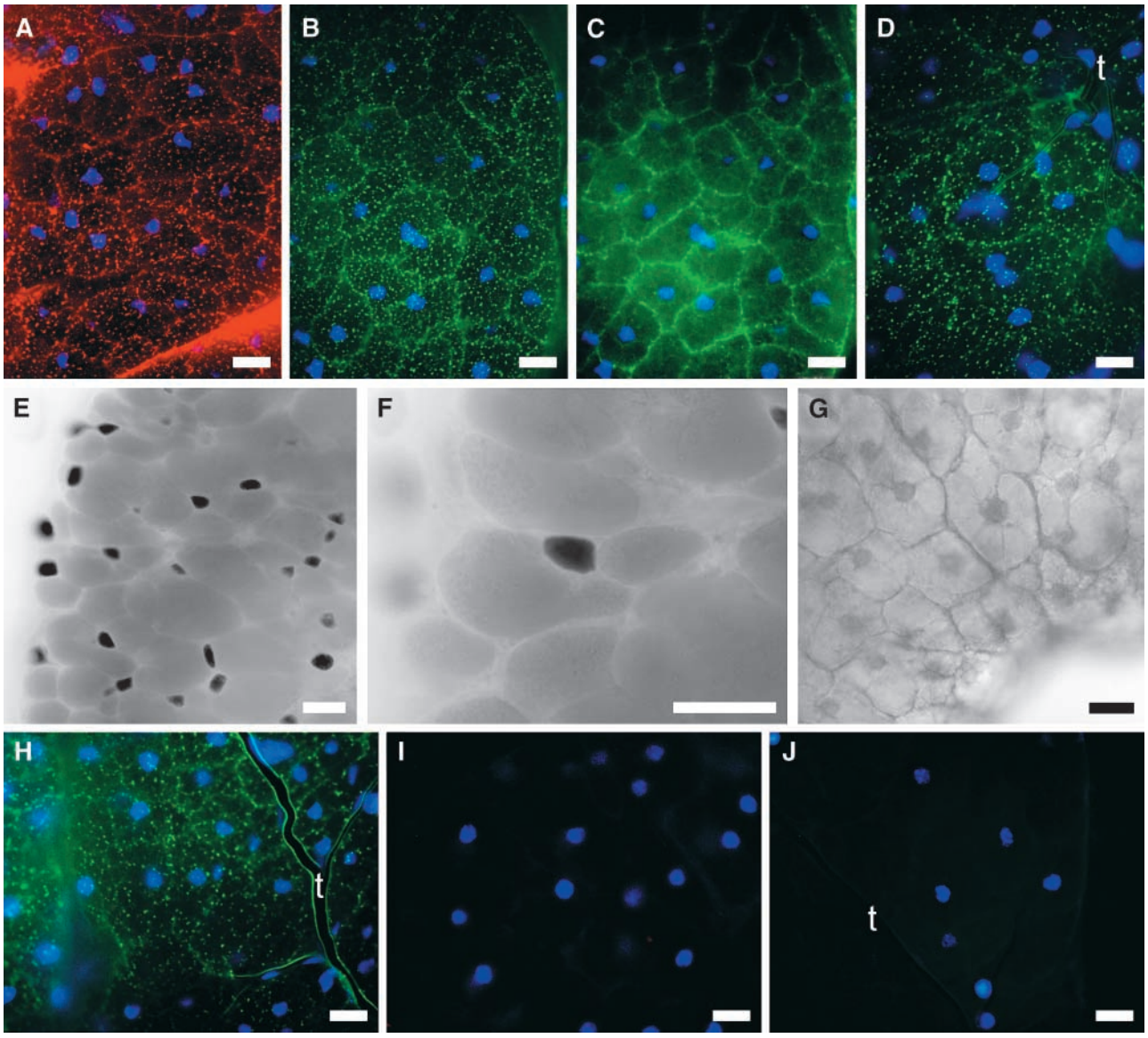

Fig. 1. Multi-color fluorescence microscopic images of locust fat body tissue obtained with fluorescein isothiocyanate (FITC) and tetramethylrhodamine isothiocyanate (TRITC) filters combined with a UV filter. On Day 1 after imaginal ecdysis, fat body tissue was excised from male locusts and incubated for 30 min at 32°C with high-density lipophorin (HDLp) labeled with DiI (DiI-HDLp; A, red) or Oregon Green 488 (OG-HDLp; B–D, green). Two FITC images were captured of the same area of fat body tissue peripherally below the cell surface (B) and at the confocal plane of the nuclei (C). Prior to fixation, fat body tissue was incubated with trypsin and washed thoroughly (D). Light microscopic images were merged with fluorescence microscopic images of fat body tissue excised from adult males, immediately after imaginal ecdysis. E: After fixation, the lipid droplets were stained with Oil Red O (gray) and visualized with light microscopy. Nuclei were stained with 4′,6′-diamidino-2′-phenylindole (DAPI) (black) and visualized with fluorescence microscopy using a UV filter. The colors of the DAPI fluorescence image were inverted and reduced to a gray scale before merging the light and fluorescence images. F: Detailed image of a single nucleus derived from E. Total cell proteins were stained with Coomassie brilliant blue and visualized with light microscopy (G). Images of fat body tissue excised from females on Day 1 (H) or males on Day 4 after imaginal ecdysis (I, J) that was incubated for 30 min at 32°C with OG-HDLp (H and J) or DiI-HDLp (I). Nuclei were stained with DAPI (blue), and the images were merged with those obtained with TRITC and FITC filters (A–D and H–J). Tracheae are indicated with a "t." The scale bars represent 20 μm.

3C (Fig. 3E). These data additionally support that, despite a difference in molecular weight, the recognized membrane proteins from both cell types are iLR.

RAP is a ligand for iLR

RAP serves as a chaperone to assist the folding of LDLR family members and prevents premature binding of ligands in the endoplasmic reticulum (23). It has been shown to inhibit binding of ligands to lipoprotein receptors (24–26), including iLR (14). Fat body tissue of young adult locusts that was incubated with DiI-HDLp or OG-HDLp remained devoid of HDLp-containing vesicles when a 100-fold molar excess ratio of human RAP was added to the incubation medium (**Fig. 4A** and **B**, respectively). Inhibition of HDLp endocytosis by RAP suggests that the protein serves as a ligand for iLR, and thus can also be internal-

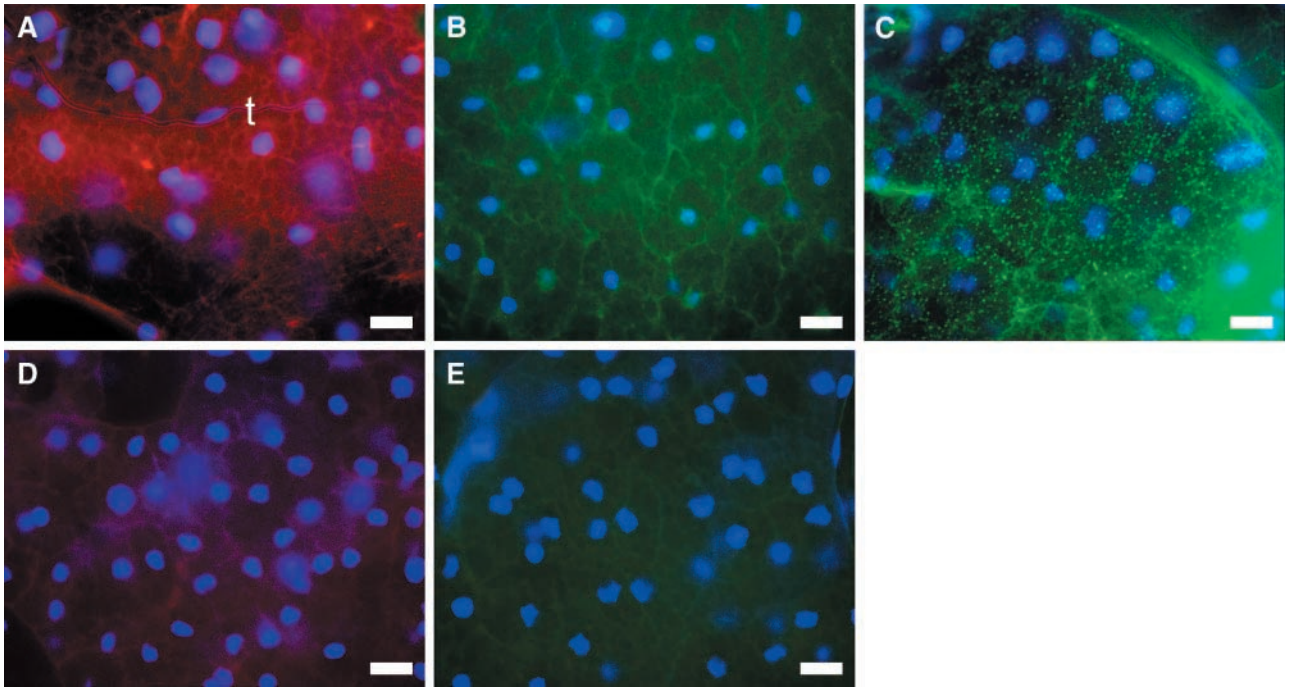

Fig. 2. Fat body tissue was excised from adult male locusts on Day 1 after ecdysis and incubated for 1 h at 4°C with DiI-HDLp (A) or OG-HDLp (B) in incubation medium. After preincubation with OG-HDLp at 4°C, fat body tissue was transferred to medium without fluorescently labeled HDLp and incubated at 32°C (C). Prior to incubation with fluorescently labeled HDLp, fat body tissue excised from males on Day 1 after imaginal ecdysis was preincubated for 30 min at 32°C with 2.5 mg/ml unlabeled HDLp. Subsequently, the preincubation medium was replaced by incubation medium containing the same concentration of unlabeled HDLp supplemented with either 10 μg/ml DiI-HDLp (D) or 25 μg/ml OG-HDLp (E) and incubated for an additional 30 min at 32°C. Digital images were taken from the confocal plane peripherally below the cell surface with TRITC or FITC filters for DiI-HDLp and OG-HDLp, respectively. All TRITC and FITC images were overlaid and merged with images of the nuclei that were stained with DAPI and visualized with a UV filter. Tracheae are indicated with the "t." The scale bars represent 20 μm.

ized by fat body cells. Incubation of young adult fat body tissue with OG-RAP resulted in a particulate pattern identical to that of endocytosed OG-HDLp (compare Figs. 4C and 1B). Endocytosis of OG-RAP was completely inhibited, with a 100-fold molar excess ratio of either unlabeled HDLp (Fig. 4D) or unlabeled RAP (Fig. 4E), suggesting that HDLp and RAP bind to the same fat body receptor.

To confirm that the RAP binding receptor is iLR, fat body cell membrane proteins separated under nonreducing conditions were transferred to PVDF membrane and incubated with ^{125}I-RAP. Immediately after adult ecdysis, fat body membrane extracts contain a single binding protein with a molecular weight of ~110 kDa (Fig. 4F, lanes 1 and 2), which is identical to that of iLR under nonreducing conditions. These results strongly suggest that iLR is the only endocytic lipoprotein receptor expressed in this stage. In Day 4 locust fat body tissue, the RAP binding protein is no longer significantly present (Fig. 4F, lanes 3 and 4). In agreement with this finding, OG-RAP was not endocytosed by fat body tissue derived from these adult locusts (Fig. 4G). Taken together, these findings confirm that, in addition to HDLp, fat body cells are able to internalize RAP via iLR-mediated endocytosis.

iLR mediates HDLp endocytosis in fat body cells of larvae

The finding that iLR is expressed after the imaginal ecdysis raises the question of whether similar up- and down-regulation of the receptor occurs in earlier developmental stages. Similar to adults (**Fig. 5A**, lanes 1 and 2), iLR is highly expressed in L_5 larvae immediately after ecdysis (Fig. 5A, lanes 3 and 4), and is down-regulated on Day 4 (Fig. 5A, lanes 5 and 6). Expression of iLR in L_5 larvae implies that the fat body tissue is capable of internalizing HDLp. On Day 1 after ecdysis to L_5, larval fat body tissue was incubated with OG-HDLp, resulting in a particulate staining pattern (Fig. 5B) similar to that observed for young adults (Fig. 1B). On Day 4, L_5 larvae have lost the ability to internalize OG-HDLp under similar conditions (Fig. 5C) [like Day 4 adults (Fig. 1J)], which coincides with the expression pattern of iLR (Fig. 5A). Collectively, these data on adults and larvae suggest that HDLp is internalized by fat body cells during the first few days after each ecdysis, and that this endocytic uptake is mediated by iLR.

iLR expression is induced by the demand of fat body tissue for lipids

In the first few days after each ecdysis, the fat body rapidly increases in volume due to the storage of a high amount of lipids derived from dietary intake. Combined with the expression pattern of iLR in young adult (Fig. 3A and B) and larval (Fig. 5A) locusts, this observation suggests that iLR mediates the rapid uptake of lipids after the depots in fat body cells have been depleted. Therefore, it

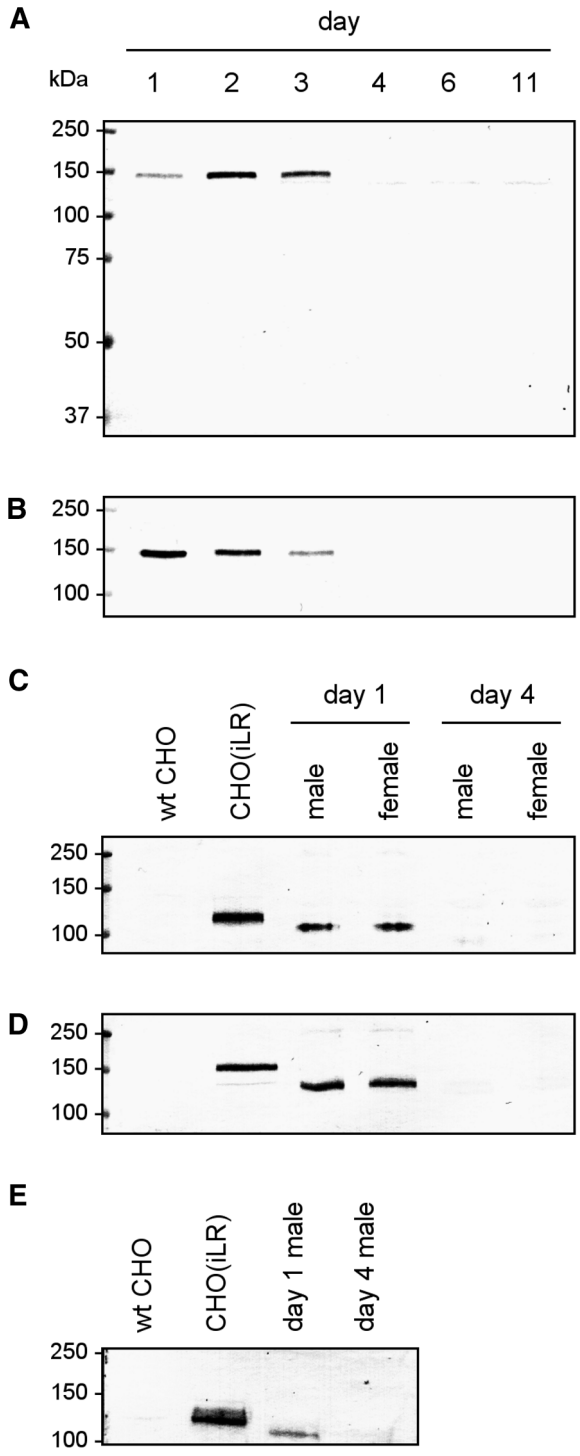

Fig. 3. Fat body tissue was excised from male (A) and female (B) locusts after imaginal ecdysis on the days indicated, and the membrane proteins were separated by SDS-PAGE. C–E: Membrane proteins were obtained from wild-type Chinese hamster ovary (CHO) cells (lane 1) and insect lipophorin receptor (iLR)-transfected CHO [CHO(iLR)] (lane 2) cells harvested from culture flasks, and fat body of Day 1 (C and D, lanes 3 and 4; E, lane 3) or Day 4 (C and D, lanes 5 and 6; E, lane 4; controls) adult locusts. Samples were either dissolved in modified Laemmli buffer and directly subjected to SDS-PAGE under nonreducing conditions (C), or heated for 5 min at 95°C in Laemmli buffer (D, E) and then separated by SDS-PAGE. iLR was detected by immunoblotting using anti-iLR 9218 antibody (A–D) or anti-iLR 2189/90 antibody (E). The molecular weight markers (kDa) are indicated on the left of each panel.

was hypothesized that iLR expression may be regulated by a requirement of fat body cells for lipids. To support this hypothesis, adult locusts were starved to create a physiological condition in which the demand for lipids is increased.

First, we investigated whether the expression of iLR after ecdysis could be prolonged. Young adult locusts were starved immediately after imaginal ecdysis, and membrane proteins were isolated on Day 5; the developmental stage at which iLR expression in fed animals is below detectable levels (Fig. 3A, B). Western blot analysis with a high amount of membrane protein showed that, in contrast to fed animals (**Fig. 6A**, lanes 3 and 4), iLR expression is retained in starved locusts (Fig. 6A, lanes 5 and 6), suggesting that if lipid intake is postponed, the down-regulation of the receptor is delayed. With the high amount of membrane protein used for these Western blots, anti-iLR 9218 antibody also recognized a membrane protein of ~130 kDa, the electrophoretic mobility of which was not altered under nonreducing conditions (data not shown). This protein neither enabled adult fat body tissue of Day 4 and later to visibly internalize HDLp (Fig. 1I and J) or RAP (Fig. 4G), nor bound RAP after ligand blotting under nonreducing conditions (Fig. 4F), and therefore does not seem to function in HDLp endocytosis. However, anti-iLR 2189/90 antibody also recognized this protein (data not shown), suggesting that the protein is most likely structurally related to iLR.

Second, to obtain additional evidence for a regulation of iLR expression by lipid depletion, we investigated whether the expression could be induced after down-regulation had occurred. Adult locusts were starved from Day 4 to 11 after ecdysis, whereafter fat body cell membrane proteins were isolated and analyzed as described above. Whereas iLR is absent in membrane extracts from fed locusts (Fig. 6B, lanes 3 and 4), starved animals show a significant expression of iLR (Fig. 6B, lanes 5 and 6). Although the amount of iLR per total amount of membrane protein appeared to be not as high as that observed in animals immediately after ecdysis (Fig. 6A, B, lanes 1 and 2), these data suggest that iLR expression can be induced by reducing the lipid stores in fat body cells.

DISCUSSION

HDLp has been proposed to selectively unload lipid cargo at the cell surface of target tissues [as reviewed in refs. (9–11)], thus avoiding the lysosomal fate of endocytosed mammalian lipoproteins [as reviewed in refs. (27–30)]. Consequently, the process of lipid storage in fat body cells may be expected to proceed via selective lipid uptake. However, the expression of an insect LDLR family member at the surface of insect fat body cells implies that HDLp can be additionally internalized via receptor-mediated endocytosis. This is also supported by the identification and characterization of a lipophorin receptor that is expressed on the surface of mosquito oocytes and has been shown to bind lipophorin (13).

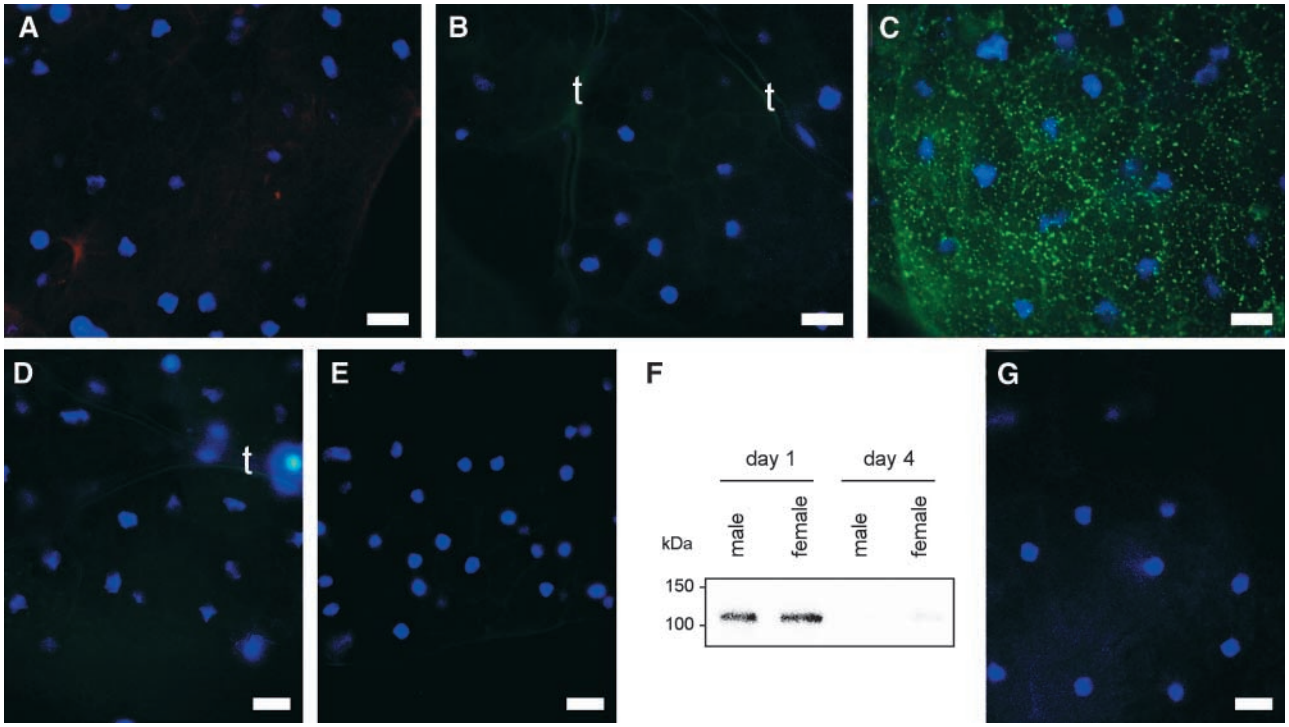

Fig. 4. Fat body tissue, excised from locusts on Day 1 after imaginal ecdysis, was preincubated for 30 min at 32°C with incubation medium containing 0.36 mg/ml human receptor-associated protein (RAP). After preincubation, the medium was removed and the tissue was subsequently incubated with DiI-HDLp (A) or OG-HDLp (B) in the presence of 0.36 mg/ml unlabeled human RAP. C: OG-RAP was used to incubate fat body tissue of young adult locusts immediately after ecdysis. Prior to OG-RAP incubation, fat body tissue of the same developmental stage was preincubated with incubation medium containing either 2.5 mg/ml HDLp (D) or 0.36 mg/ml human RAP (E). After removing the preincubation medium, the tissue was incubated with OG-RAP in the presence of a 100-fold molar excess ratio of unlabeled HDLp (D) or RAP (E). F: Fat body tissue was excised from locusts after imaginal ecdysis on the indicated days, and membrane proteins were dissolved in Laemmli buffer containing 0.025% SDS. The nonreduced proteins were subjected to SDS-PAGE, blotted onto polyvinylidene fluoride membrane, and incubated with ^{125}I-labeled RAP, after which the ligand blot was analyzed with a PhosphorImager. The molecular weight markers (kDa) are indicated on the left of the panel. G: Fat body tissue of Day 4 adult male locusts was incubated with 3.6 μg/ml OG-RAP for 30 min at 32°C. Nuclei of tissues were stained with DAPI after fixation, and the UV images were overlaid and merged with the fluorescence images of DiI- and OG-labeled ligands obtained with TRITC and FITC filters, respectively (A–E, G). Tracheae are indicated with a "t." The scale bars represent 20 μm.

Endocytosis of HDLp has been demonstrated earlier for the dragonfly *Aeshna cyanea* (31) and *L. migratoria* (15). The latter results, however, relate to internalization of HDLp during a period in which iLR is not significantly expressed. The results of the present study provide evidence for an iLR-mediated endocytic uptake mechanism for HDLp that is present during specific periods of development in the locust. Incubation experiments using excised fat body tissue and fluorescently labeled HDLp clearly show that, shortly after ecdysis, fat body cells are able to endocytose the complete particle; both the lipid moiety and the protein matrix of HDLp are internalized (Fig. 1A and B, respectively). The simultaneous expression of iLR (Fig. 3) strongly suggests that this receptor is responsible for HDLp endocytosis. Like the intricate process of ecdysis, the appearance of iLR may be controlled by developmental hormones, resulting in a temporal up-regulation of iLR during critical periods in developmental stages in which endocytosis of HDLp is needed. However, expression of iLR is also triggered by starvation (Fig. 6), which may be a response to rapidly replenish the depleted fat body reserves once lipids from dietary intake are available. Therefore, iLR expression is most likely not restricted to the first few days after an ecdysis, but may be induced after all lipid store-depleting processes (e.g., ecdysis, starvation, and possibly sustained flight).

The simultaneous existence of the two distinct uptake mechanisms in fat body cells cannot be excluded. Whereas HDLp is able to selectively unload its lipid cargo at the cell surface of target tissues, receptor-mediated endocytosis of HDLp may be requisite to internalize components that are unable to diffuse through the cell membrane. In contrast to the selective lipid transfer mechanism, via which degradation of HDLp is circumvented, endocytosis of the lipoprotein would imply that the complete particle is degraded in lysosomes, similar to mammalian LDL [as reviewed in refs (27–30)]. However, experiments involving CHO(iLR) cells demonstrated that endocytosed HDLp is resecreted without substantial degradation (14). In fat body cells, preliminary pulse-chase experiments indicate that the particle may also be resecreted after endocytosis. Extensive incubation of excised tissue in insect growth medium following a pulse of fluorescently labeled HDLp resulted in a significant decrease

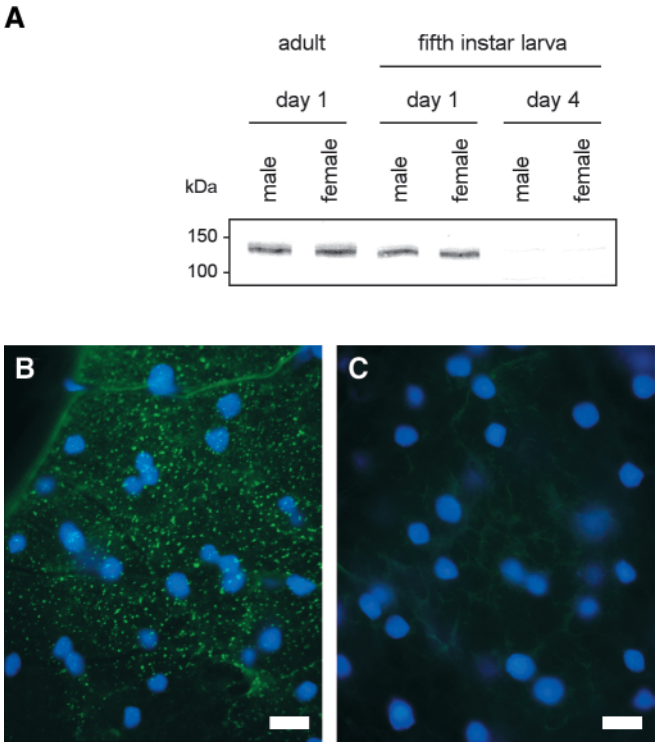

Fig. 5. A: Fat body tissue was excised from adult locusts and fifth instar (L_5) larvae on the days indicated. Membrane proteins were separated by SDS-PAGE under reducing conditions, after which iLR was detected by immunoblotting using anti-iLR 9218 antibody. The molecular mass markers (kDa) are indicated on the left of the panel. Fat body tissue was excised from L_5 larvae on Day 1 (B) and Day 4 (C) after ecdysis, and incubated with OG-HDLp for 30 min at 32°C. Digital images of OG-HDLp and DAPI-stained nuclei were taken with an FITC and UV filter, respectively, after which the images were overlaid and merged. The scale bars represent 20 μm.

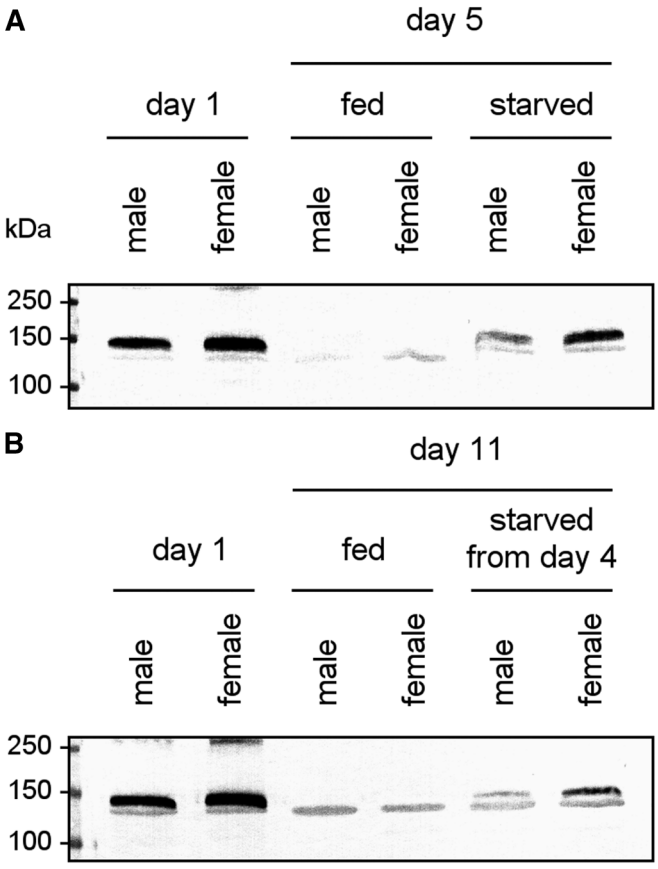

Fig. 6. Twice the standard amount of fat body membrane proteins from male (lanes 1, 3, and 5) and female (lanes 2, 4, and 6) locusts were denatured in Laemmli buffer and subjected to SDS-PAGE, after which iLR was detected by immunoblotting using anti-iLR 9218 antibody. A: Fat body membrane proteins obtained from Day 1 adult locusts (lanes 1 and 2) and Day 5 adult locusts that had either been normally fed (lanes 3 and 4) or starved from the imaginal ecdysis until the day of excision (lanes 5 and 6). B: Lanes 1 and 2 contain fat body membrane proteins of Day 1 adult locusts. Membrane proteins separated in lanes 3 and 4 were obtained from adult locusts that had been normally fed for 11 days. Lanes 5 and 6 contain membrane proteins from locusts that were fed for 4 days after imaginal ecdysis and then starved until the day of isolation. The molecular weight markers (kDa) are indicated on the left of each panel.

in the number of HDLp-containing vesicles (data not shown). However, these remaining vesicles were larger compared with the initial endosomes, which may be due to fusion and maturation of the endosomes into lysosomes (32); thus, degradation of HDLp cannot be excluded. The fragility and typical composition of fat body tissue render it difficult to allow quantitation of HDLp degradation or recycling. We are currently addressing this issue using iLR-transfected insect cell lines (e.g., *Sf*9), which provide a suitable alternative to determine the fate of endocytosed HDLp in insect cells.

iLR and VLDLR share 58.2% amino acid sequence similarity and have an identical domain structure composition. The ligand binding domains of both receptors comprise eight consecutive cysteine-rich repeats, and iLR (Fig. 4F) (14) as well as VLDLR (26, 33) are capable of binding RAP. In spite of their apparent structural homology, iLR and VLDLR seem to function differently. Whereas iLR mediates endocytic uptake of lipoprotein in insect as well as mammalian cells, VLDLR is assumed to function extracellularly. Storage of VLDL-derived triacylglycerol (TAG) is presumed to depend on the extracellular hydrolysis of TAG by lipoprotein lipase (LPL), after which the liberated free fatty acids are taken up by adjacent cells (34). Such a mechanism would require neither internalization of the lipoprotein nor the involvement of an endocytic receptor (i.e., VLDLR). On the other hand, LPL has been reported to bind to LDLR family members, including VLDLR (35, 36). Therefore, it was suggested that LPL and VLDLR together promote retention of circulating VLDL via a process that takes place extracellularly (34). However, the intracellular tail of VLDLR harbors the putative internalization signal sequence NPxY (37), like all other LDLR family members, including iLR. In addition, VLDLR was shown to function as an endocytic receptor for VLDL in CHO cells (35). Moreover, in vivo studies showed that ectopic expression of this receptor in mouse liver results in enhanced internalization of lipoproteins (38, 39). Thus, VLDLR apparently has the ability to function as an endocytic receptor, like all LDLR family members. Possibly,

VLDLR-mediated VLDL endocytosis provides a backup mechanism when extracellular hydrolysis of VLDL-derived TAG is prevented; for instance, LPL-deficient patients were reported to show neither abnormal energy metabolism, nor deprivation of subcutaneous fat (40).

Earlier findings with LDLR and our observations with iLR show that alternative functions for lipoprotein receptors that deviate from the classic lysosomal delivery may depend on the type of cell or developmental stage at which the receptor is expressed (14, 41). Our results indicate that, in spite of the ability of HDLp to selectively unload lipids at target tissues, HDLp can also be taken up by fat body cells via iLR similar to the mammalian LDL uptake system. In addition, evidence is provided for a physiological regulation of iLR expression that is dependent on the requirement of fat body tissue for lipids. Thus, iLR expression is most likely not restricted to the first days after ecdysis, but may also occur by virtue of a need of fat body cells to replenish their storage depots. Further research will elucidate whether fat body cells are able to resecrete HDLp after iLR-mediated endocytosis. Transfection of insect cell lines (e.g., *Sf*9) with iLR cDNA will provide insight into the intracellular transport routes of lipoproteins and other ligands after endocytosis in insect cells. Perhaps more importantly, unravelling the means of lipid storage in the insect as a model may very well contribute to the understanding of lipid storage at the cellular level in general.

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