Multiple interactions between insect lipoproteins and fat body cells: extracellular trapping and endocytic trafficking

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Abstract **The binding and internalization of a circulating insect lipoprotein, high density lipophorin (HDLp), by insect fat body cells was studied at the electron-microscopic level using ultrasmall gold-labeled HDLp and DiI-labeled HDLp, which were visualized by silver enhancement and diaminobenzidine photoconversion, respectively. Internalization of HDLp seems to conflict with the selective process by which the lipids are transported between HDLp and fat body cells. The pathway followed by the internalized lipoproteins was investigated. In addition, the localizations of HDLp in fat body cells of young and older adult locusts were compared because of the previously reported age-related differences in distribution of cell-associated and internalized HDLp. In the present study, internalized labeled HDLp was observed in early endosomes, late endosomes, and putative lysosomes. In older adults, these labeled structures were much less abundant than in young adults. Moreover, in these animals, the labeled endosomal/lysosomal vesicles were located close to the plasma membranes. A more intense labeling was observed in the extracellular matrix in older adults compared to young adults. In both developmental stages, an apparent accumulation of labeled HDLp was found in extracellular spaces. We propose that this entrapment of HDLp may be essential for selective lipid transport between HDLp and fat body cells.—**Dantuma, N. P., M. A. P. Pijnenburg, J. H. B. Diederen, and D. J. Van der Horst. **Multiple interactions between insect lipoproteins and fat body cells: extracellular trapping and endocytic trafficking. *J. Lipid Res.* 1998.** 39: **1877–1888.**

Supplementary key words selective lipid transport • receptor-mediated endocytosis • lipophorin • locust • DiI • ultrasmall gold • electron microscopy • DAB photoconversion

In the circulatory compartment of insects a single lipoprotein class is abundantly present, termed lipophorin, and accomplishes transport of diacylglycerol (DAG) (1). DAG is the principle form in which fatty acids are transported in insects. Lipophorins contain two integral apolipoproteins, apolipophorin I and II, which are derived from a common precursor by proteolytic splicing (2). The recently reported homology between this precursor and the mammalian apolipoprotein B (3–5) suggests an evolutionary relationship between lipophorins and apolipoprotein B-containing lipoprotein. Nevertheless, the way in which these lipid vehicles are involved in lipid transport differs at major points (1, 5, 6).

A characteristic feature of lipid transport in insects is the selective mechanism by which the lipophorin shuttles its lipid cargo between cells without concomitant degradation of the protein matrix of the lipophorin particle (7). After selective lipid delivery to recipient cells, the resulting lipophorin can be reloaded with lipids. This recycling mechanism of lipophorin reflects its capacity to transport large amounts of lipids without the need to synthesize new lipoproteins. The efficient lipid transport accomplished by lipophorins is crucial during flight activity of migratory insects, when transport of massive amounts of DAG to the flight muscle cells is achieved by additional loading of DAG upon circulating lipophorins (8).

The fat body tissue plays a key role in the insect lipid metabolism as this is the site of both lipid storage and mobilization (1, 7, 8). This tissue is composed of two cell layers and is surrounded by the circulating hemolymph. Consequently, all fat body cells encounter at their superficial plasma membrane the high concentration of circulating lipophorins in the hemolymph. Despite the fact that selective lipid transport in insects is well established, little is known about the molecular mechanisms underlying lipid uptake and lipid mobilization by fat body cells. Previously, we focused on the interaction between high density lipophorins (HDLp) and fat body cells in migratory locusts (9). It appeared that although the majority of the lipid cargo was exchanged between HDLp and fat body cells by a selective mechanism, the fat body cells nevertheless internalized HDLp by means of receptor-mediated endocytosis. In addition, only limited degradation of the apolipophorins was observed. We postulated, therefore, that the pathway followed by HDLp may branch off from the classical endosomal/lysosomal pathway. Another in-

Abbreviations: DAB, diaminobenzidine; DAG, diacylglycerol; DiI, 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate; HDLp, high density lipophorin

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teresting observation was a strong developmental down-regulation of receptor-mediated uptake of HDLp during the adult stage. Coincidental with this down-regulation a dramatic increase in cell surface-associated HDLp occurred, which corresponds well with the point of time in development at which the insects gain the ability to fly (10, 11), and to facilitate the flight-specific loading of DAG upon HDLp (12).

In the present study, we extend these observations by studying the localization of labeled HDLp in fat body tissue at the electron-microscopic level in order to gain insight in the pathway followed by the internalized lipoprotein as well as to study the developmental shift to mainly cell surface-bound HDLp.

MATERIALS AND METHODS

Animals and fat body tissue

Migratory locusts, *Locusta migratoria*, were reared under crowded conditions as described previously (13). Fat body tissue was dissected from adult male locusts, 4 and 15 days after the imaginal ecdysis.

Isolation and labeling of lipoproteins

HDLp was isolated from hemolymph of adult locusts 15–20 days after the imaginal ecdysis, by density gradient ultracentrifugation (14). Lipoproteins were labeled with the fluorescent lipid 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine perchlorate (DiI; Molecular Probes), essentially according to Pitas et al. (15), with some minor modifications that we have described previously (9). Custom labeling of the HDLp with ultrasmall colloidal gold (diameter 1 nm) was performed by Aurion (Wageningen, The Netherlands). Protein concentrations of HDLp samples were determined according to Schacterle and Pollack (16).

Silver enhancement of ultrasmall gold-labeled HDLp

Freshly dissected fat body tissue was rinsed in buffer A (10 mm HEPES, 150 mm NaCl, 10 mm KCl, 4 mm $CaCl_2$, 2 mm $MgCl_2$, pH 7.0). The tissue was incubated in buffer A + 0.05 mg/ml ultrasmall gold-labeled HDLp for 90 min at 30°C. Afterwards, the tissue was rinsed 3 × 10 min in buffer B (0.1 m NaH_2PO_4/Na_2HPO_4, pH 7.4) at 0°C, fixed for 3 days in 2% paraformaldehyde and 0.5% glutaraldehyde in buffer B, and rinsed again 3 × 10 min in buffer B, both at 4°C. The samples were post-fixed for 1 h at 4°C in buffer B + 1% OsO_4. After sequential dehydration in a graded ethanol series and propylene oxide, the tissue samples were embedded in an epoxy resin (glycide ether 100; Merck). Ultrathin sections were cut, silver-enhanced according to Danscher (17), and stained with uranylacetate and lead citrate. Ultrathin sections were examined with transmission electron microscopy (EM10A, Zeiss).

Diaminobenzidine photoconversion of DiI-labeled HDLp

The modified diaminobenzidine (DAB) photoconversion technique that was used for the visualization of DiI-labeled lipoproteins in fat body cells will be presented in detail elsewhere (18). The initial steps before the fixation were identical to those for the tissue samples incubated with ultrasmall gold-labeled HDLp except that these samples had been incubated with 0.3 mg/ml DiI-labeled HDLp and fixed overnight. The tissue was embedded in 7.5% agar and fixed overnight in 2% paraformaldehyde. Fifty-μm vibratome slices of the agar-embedded tissue were subjected to two subsequent preincubations: *1*) in buffer C [buffer B + 20 mm 3-amino-1,2,4-triazole (Sigma) + 0.001% H_2O_2] for 1 h at room temperature, and *2*) in buffer C containing 1.5 mg/ml 3,3′-diaminobenzidine tetrahydrochloride (DAB; Sigma) for 1.5 h at 0°C. The sections were illuminated in the same buffer using a conventional fluorescence microscope (Axioskop, Zeiss) with a rhodamine filter setting (BP 520–560, FT 580, LP 590; Zeiss). Upon rinsing 3 × 10 min in buffer B at 0°C, the samples were post-fixed for 30 min in buffer B + 1% OsO_4 at 4°C, and rinsed again for 3 × 10 min in distilled water. The samples were sequentially dehydrated in a graded ethanol series and propylene oxide and embedded in an epoxy resin (glycide ether 100; Merck). Ultrathin sections were examined with transmission electron microscopy.

Pulse-chase experiment with ultrasmall gold-labeled HDLp

Freshly dissected fat body tissue of young adults, 4 days after the imaginal ecdysis, was rinsed in buffer A for 6 × 10 min at 4°C and, subsequently, incubated with 0.05 mg/ml ultrasmall gold-labeled HDLp in buffer A for 3 h at 4°C to establish binding of the lipoproteins. After this incubation, the fat body tissue was rinsed 3 × 10 min in buffer A at 4°C. Subsequently, the tissue was incubated for various time intervals with 0.05 mg/ml unlabeled HDLp at 30°C. From this point the fat body tissue samples were prepared for electron-microscopic examination as described above (Silver enhancement of ultrasmall gold-labeled HDLp). A control was included consisting of fat body tissue that had been incubated with 0.05 mg/ml ultrasmall gold-labeled HDLp in the presence of a 100-fold excess of unlabeled HDLp during the initial incubation and chased for 60 min with 0.05 mg/ml unlabeled HDLp (the longest time interval examined).

RESULTS

Interaction between HDLp and fat body tissue of young adult locusts

Two approaches were used to monitor the extra- and intracellular distribution of HDLp upon interaction with the insect fat body by electron microscopy. Fat body tissue was incubated *1*) with DiI-labeled HDLp, which was subsequently visualized by DAB photoconversion, and *2*) with ultrasmall gold-labeled HDLp, which was detected by silver enhancement. We preferred the use of these lipoprotein tracers for several reasons. The photoconversion technique allowed us to directly relate our electron micrographs to the previously reported fluorescence micrographs of fat body cells that had been incubated with DiI-labeled HDLp (9). Ultrasmall gold-labeled lipoproteins have previously been shown to be appropriate probes for tracing endocytosis of lipoproteins (19). Compared to the classical probes used for studying lipoprotein internalization, that consist of several lipoproteins attached to a single large colloidal gold particle of 20–40 nm (20), ultrasmall gold hardly influences the size of the lipoprotein. The main reason for using ultrasmall colloidal gold instead of this classical labeling is that we aimed to minimize the amount of non-specifically internalized lipoprotein. Large colloidal gold–lipoprotein conjugates generated with the classical labeling method could result in artificial uptake of these conjugates which would than be transported to the lysosomal compartment.

Fat body tissue dissected from young adults, 4 days after the imaginal ecdysis, was incubated with DiI-labeled or ultrasmall gold-labeled HDLp for 90 min at 30°C. Fat body tissue from the same individual incubated with labeled HDLp in the presence of an excess of unlabeled HDLp was included as a control, in order to determine whether the appearance of labeled HDLp in the fat body tissue was receptor mediated. Tissue incubated with free ultrasmall colloidal gold (coated with bovine serum albumin) was used to determine possible non-specific binding and internalization caused by the colloidal gold label.

We chose to determine the endosomal and lysosomal structures containing the labeled lipoproteins by means of their morphology and labeling pattern. The reason for this is that a satisfactory preservation of the ultrastructure of the lipid-rich fat body tissue, in combination with the in vitro incubations and the visualization of the labeled HDLp, required an embedding method (epoxy resin) that appeared not to be compatible with immunocytochemical methods to characterize the different endosomal/lysosomal compartments. The following criteria were applied for the morphological characterization. Spherical vesicles were termed early endosomes when the labeling was restricted to the perimeter of these vesicles (see for example Fig. 2B). The vesicles that we classified as late endosomes had in common an irregular shape, the presence of a limited number of large intralumenal vesicles, and the label being distributed throughout their lumina (see for example Fig. 2C). Vesicles with slightly electron-dense lumina (this criterion could only be used for tissue incubated with ultrasmall gold-labeled HDLp) and homogeneous distribution of label were classified as lysosomes (see for example Fig. 1E). As it is difficult to distinguish late endosomes from lysosomes, we will refer to structures that are in accordance with the latter criteria, as putative lysosomes. We emphasize that the morphological characterization of endosomal/lysosomal organelles applied by us should be considered as indicative and cannot be conclusive.

A 6-fold higher concentration of DiI-labeled HDLp, compared to ultrasmall gold-labeled HDLp, was required to produce a clear staining. (It is noteworthy that with some of the DiI-labeled HDLp preparations, the DAB photoconversion experiments resulted in moderately blurred images. We do not know the reason but it can be caused, for example, by differences in the number of DiI molecules per lipoprotein.) Despite the different concentrations of DiI-labeled and ultrasmall gold-labeled HDLp used, the distribution of label in the fat body tissue was similar. However, only with ultrasmall gold-labeled HDLp, we occasionally observed a modest labeling of the lumina of large structures (**Fig. 1A**) that was unaffected by an excess of unlabeled HDLp. The same labeling was also present in fat body tissue incubated with free ultrasmall gold (not shown) but not in fat body tissue that had been subjected to silver enhancement, but from which the ultrasmall gold had been omitted. The latter excludes the possibility that this staining was due to silver enhancement of an endogenous compound. Together these results suggest that this signal reflects either fluid-phase uptake or non-specific adsorptive uptake due to binding of gold particles to the cell surface. The specific labeling of the characteristic endosomal/lysosomal structures could be easily distinguished from these low abundant non-specifically labeled structures described above.

With both types of labeled HDLp, internalized lipoproteins were found in a large number of endocytic vesicles (**Fig. 2A**). Labeled invaginations were demonstrated at the superficial plasma membrane (Fig. 1B) and at the plasma membrane bordering the intercellular spaces (not shown). Early endosomal structures, with label situated in a rim at the perimeter membrane but otherwise free of label, were also present (Figs. 1C and 2B). Although most of these early endosomes were smoothly rounded, occasionally tubular extensions were observed (Fig. 2B) that are characteristic for this endocytic compartment (21).

Because we aimed to identify whether HDLp is transported to the lysosomes, we focused on labeling of late endosomal structures as the accessibility of the internalized lipoproteins to be returned to the plasma membrane and to escape from lysosomal hydrolysis decreases with their localization in more mature endosomes (22). DiI-labeled HDLp also appeared to be present in the lumen of large endosomes with a limited number of large intraluminal vesicles, typical for late endocytic structures (Fig. 2C). Late endosomes that contained ultrasmall gold-labeled HDLp revealed the morphology of these structures in more detail, confirming the identification of these structures as late endosomal or prelysosomal structures, with characteristic membranous whorls in their lumen (Fig. 1D). An advantage of the gold-labeling method is that the inherent colloidal gold accumulates in the lysosomes, thereby reflecting the total amount of gold-labeled HDLp transported to this compartment during the incubation. We observed putative lysosomal structures in fat body tissue that contained gold label (Fig. 1E). These vesicles were completely absent in control incubations in which tissue was incubated with ultrasmall gold-labeled HDLp in the presence of an excess of unlabeled HDLp, indicating that this lysosomal labeling is the result of receptor-mediated endocytosis rather than a non-specific uptake process.

In order to study whether the 90-min period was sufficient to label the entire pathway followed by internalized HDLp, an additional experiment was performed in which fat body cells were incubated for 3 h with DiI-labeled HDLp. Examination of photoconverted fat body tissue of this experiment did not reveal labeled intracellular structures that were morphologically different from those found after 90 min.

Interaction between HDLp and fat body cells of older adult locusts

At the electron-microscopic level, the striking differences in the distributions of HDLp in young and older adult locusts were similar to those shown previously using fluorescence microscopy (9). Much more cell surface-associated ultrasmall gold-labeled HDLp was observed in fat body tissue of older adults (**Fig. 3A–C**) compared to that of young adults (Fig. 1B). The extracellular labeling

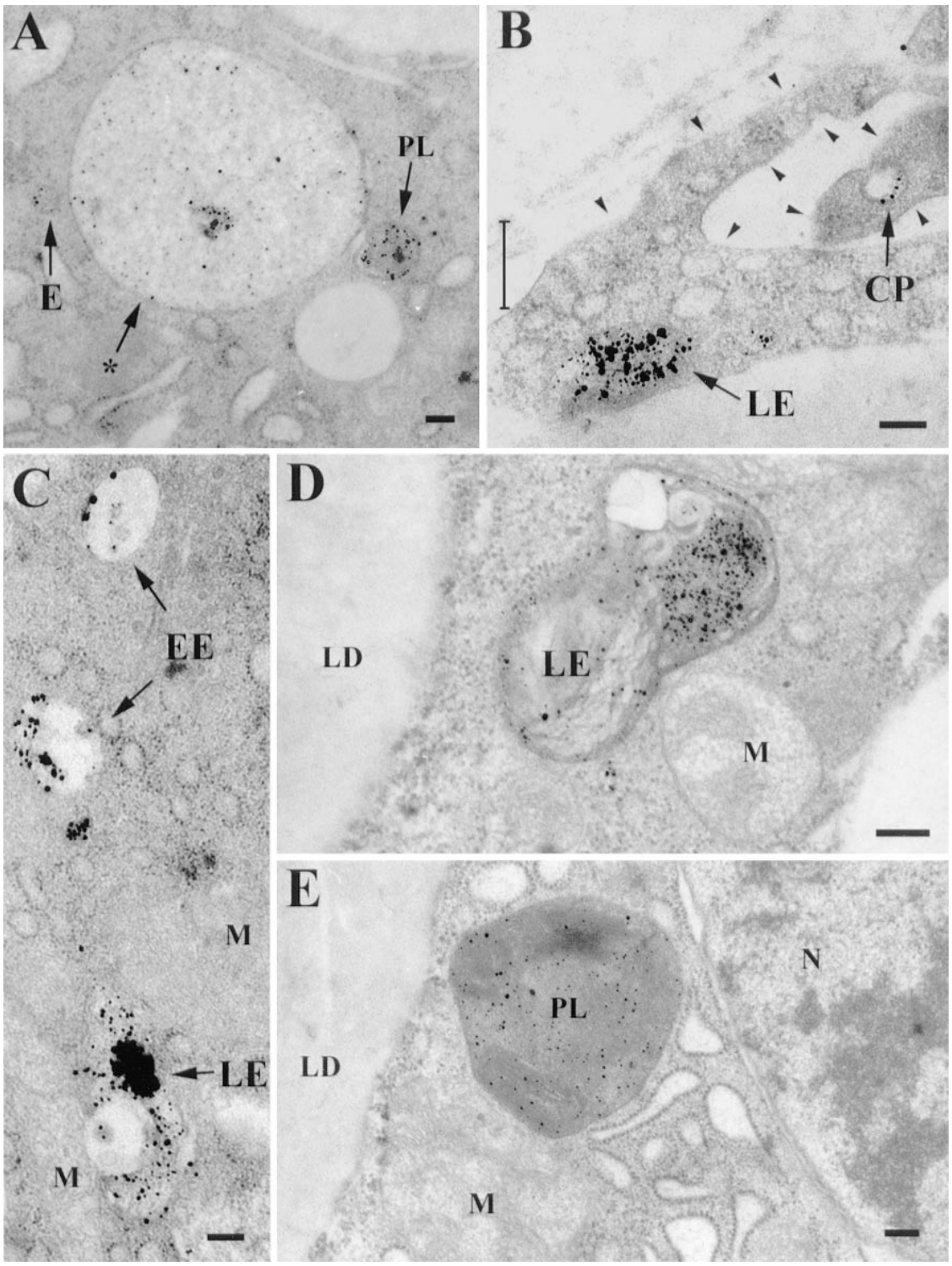

Fig. 1. Localization of ultrasmall gold-labeled HDLp in fat body tissue of young adult locusts. Electron micrographs of fat body tissue of young adults that had been incubated with 0.05 mg/ml ultrasmall gold-labeled HDLp for 90 min at 30°C. Ultrasmall gold was visualized by silver enhancement. A) Label was present in large vesicles (asterisk) that were also labeled in control tissue incubated with an excess of unlabeled lipoproteins or with free ultrasmall gold. For comparison, a labeled endosome (E) and a labeled putative lysosome (PL) are indicated in the same micrograph. B) Labeled coated pit (CP) located in the intercellular space and labeled late endosome (LE). The plasma membrane (arrowheads) as well as the size of the extracellular matrix (vertical bar) are indicated. C) Labeled early endosomes (EE) and late endosome (LE). D) Labeled late endosome (LE) containing large vesicles and membrane whorls. E) Label that has been accumulated in a putative lysosome (PL) located in close proximity to the nucleus (N). LD, lipid droplet; M, mitochondrion. Scale bars are 200 nm.

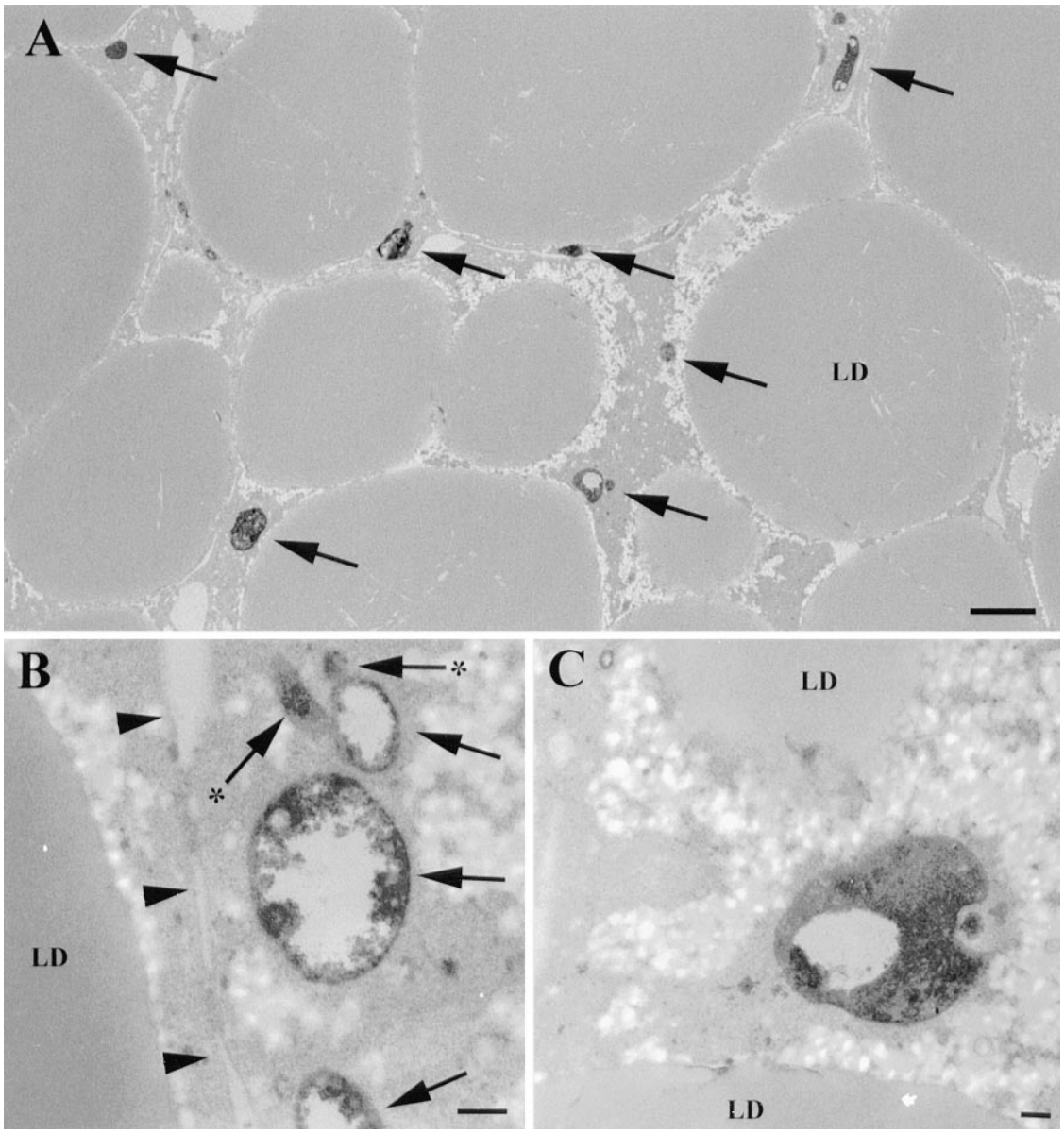

Fig. 2. Localization of DiI-labeled HDLp in fat body tissue of young adult locusts. Electron micrographs of fat body tissue of young adults that had been incubated with 0.3 mg/ml DiI-labeled HDLp for 90 min at 30°C. DiI label was visualized by DAB photoconversion. A) Overview of labeled structures (arrows) in fat body tissue. B) Labeled early endosomes (arrows) located just near the intercellular space (arrowheads). Tubular extensions (asterisks) of an early endosome are indicated. C) Labeled late endosome (LE). LD, lipid droplet. Scale bars are 500 nm (A) and 200 nm (B and C).

was mainly located at the plasma membrane (Fig 3A). With 0.3 mg/ml DiI-labeled lipoproteins, however, additional labeling of the extracellular matrix was observed (see next paragraph). In older adults, the endosomal and lysosomal structures containing ultrasmall gold-labeled HDLp (Fig. 3B and C) or DiI-labeled HDLp (not shown) were located in the periphery of the fat body tissue close to the superficial plasma membranes. Labeled early endosomes, late endosomes, and putative lysosomes were less abundant than similar endocytic structures present in fat body cells of young adults. In the latter, these structures were also much more dispersed throughout the cytoplasm. The early endosomes (Fig. 3B) and late endosomes (Fig. 3C) found in fat body cells of older adults were distinct from those present in young adults (Fig. 1C and D), as the structures in the older adults were larger and more strongly labeled.

The labeling observed at the plasma membrane and in the endosomal/lysosomal compartment of fat body tissue of older adults was almost abolished when the tissue was incubated with labeled HDLp in the presence of an excess of unlabeled HDLp (not shown).

Pulse-chase study of HDLp internalization

In order to get a better insight into the intracellular pathway followed by the HDLp once internalized, an additional study was performed in which the localization of the lipoproteins was monitored in time. In this study,

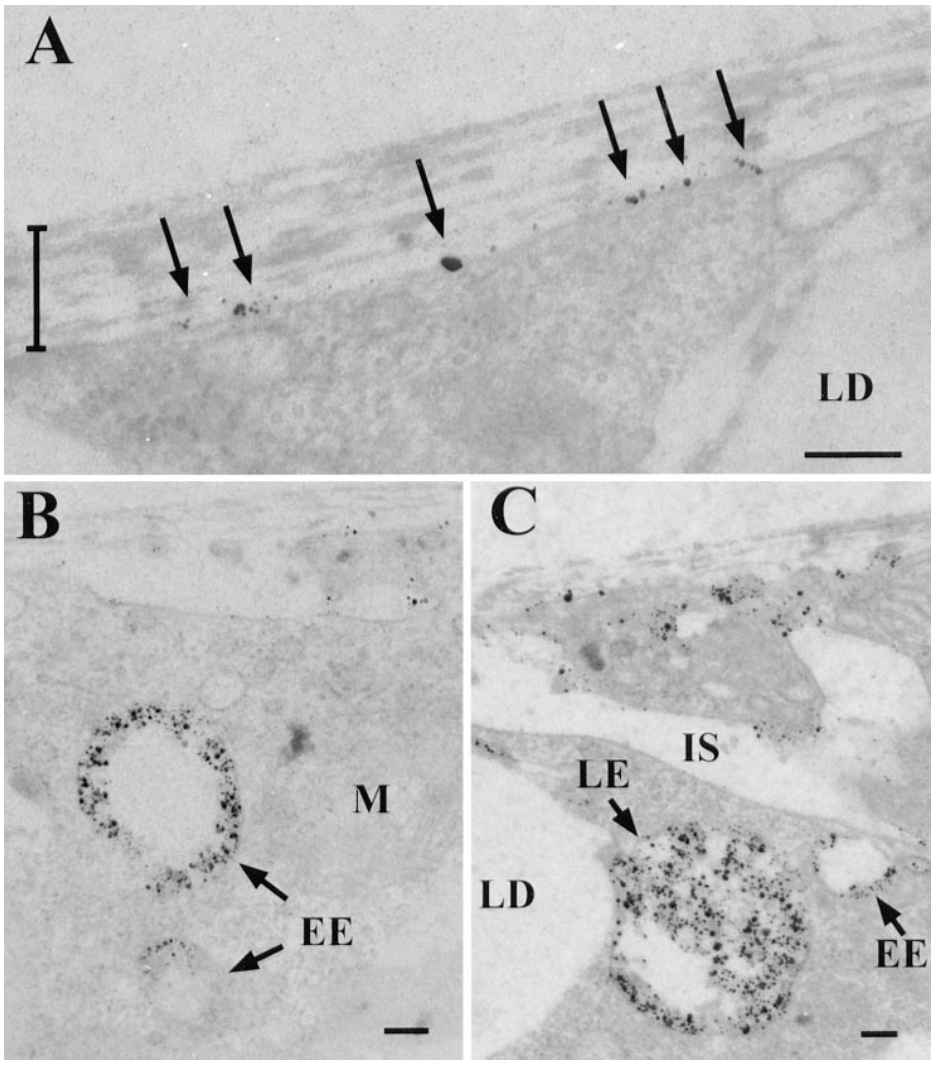

Fig. 3. Localization of ultrasmall gold-labeled HDLp in fat body tissue of older adult locusts. Electron micrographs of fat body tissue of older adults that had been incubated with 0.05 mg/ml ultrasmall gold-labeled HDLp for 90 min at 30°C. The gold label was visualized by silver enhancement. A) Labeling was observed in close association with the superficial plasma membrane (arrows), whereas the extracellular matrix was relatively devoid of label (vertical bar indicates the size of the extracellular matrix). B) Labeled early endosomes (EE) with the label located in a rim at the perimeter membrane. C) Labeled early endosome (EE) and labeled late endosome (LE). In micrographs B and C, labeling can also be observed at the plasma membrane and in the intercellular space (IS). LD, lipid droplet; M, mitochondrion. Scale bars are 200 nm.

binding of ultrasmall gold-labeled HDLp to fat body tissue of young adults, 4 days after the imaginal ecdysis, was allowed to proceed by incubating the tissue with labeled HDLp. Subsequently, the samples were incubated with unlabeled HDLp for 0, 2, 10, 30, 45, and 60 min. These experiments were performed only with 0.05 mg/ml ultrasmall gold-labeled HDLp as a preliminary study using 0.3 mg/ml DiI-labeled HDLp and subsequent photoconversion of the tissue revealed the signal as being too weak for studying the distribution accurately.

We quantified the two parameters in the chased samples: *1*) the number of labeled endocytic structures classified according to our, above-mentioned, morphological criteria; and *2*) the ratio between labeling at the superficial cell surface and labeling in the intercellular space (**Table 1**).

After the binding incubation (0 min chase), HDLp was situated close to the superficial plasma membrane and in the intercellular space (**Fig. 4A**). In the intercellular space, the labeling was mainly present in the superficial part; the signal strongly declined from the periphery to the center of the fat body tissue. Throughout the complete chase, the majority of extracellular labeling was observed in the intercellular space (Table 1). In addition, the extracellular matrix was also weakly labeled. In general, the silver-enhanced colloidal gold label was much smaller when compared to the labeling observed later during the chase. A few labeled vesicles, classified as early endosomes, were observed in the center of the fat body, which suggests that the endocytic uptake was not completely inhibited despite a preincubation for 1 h at 4°C (Table 1).

TABLE 1. Semi-quantitative analysis of the localization of ultrasmall gold-labeled HDLp in fat body tissue after various chase intervals

Chase Time	Early Endosomes	Late Endosomes	Lysosomes	Number of Particles at the Superficial Cell Surface	Number of Particles in the Intercellular Space	Ratio Particles Intercellular Space / Superficial Cell Surface
min		*vesicles/section*			*particles/section*	
0	5.7 ± 2.9	0	0	95 ± 7	260 ± 51	2.7
2	8.3 ± 5.3	2.0 ± 2.8	0	161 ± 72	409 ± 275	2.5
10	12.3 ± 3.8	6.7 ± 0.5	0	46 ± 17	166 ± 79	3.6
30	5.0 ± 1.4	21.0 ± 2.2	1.0 ± 0.8	88 ± 23	414 ± 67	4.7
45	4.7 ± 1.2	18.7 ± 3.1	5.0 ± 1.4	67 ± 37	315 ± 104	4.7
60	2.3 ± 0.5	9.7 ± 2.9	1.7 ± 0.5	57 ± 33	273 ± 169	4.8

Three sections of each time interval of the pulse-chase study (see legend Fig. 4 for technical details) were used for further semi-quantitative analysis. Labeled endocytic structures were classified as early endosomes, late endosomes, and lysosomes according to our above-mentioned morphological criteria. The total number of these three types of labeled endocytic structures per section is listed. In addition, the absolute number of particles at the superficial cell surface and in the intercellular space was quantified for each time point. Only the intercellular space that was close to the superficial border of the tissue was evaluated for this quantification (this area is a similar to that shown in Fig. 4A and F). These data were used to calculate for each section the ratio between the labeling intensities at these two locations. Values given as mean ± SD. Note that the distribution of the labeling among the different compartments and at the cell surface is most informative. As the area of analyzed tissue was not quantified, the labeling at different timepoints cannot be compared directly.

After 2 min of chase, a large number of endocytic structures were observed in the periphery of the fat body cells (Fig. 4B and Table 1). The majority of these structures were characterized as early endosomes, as the label was situated at the perimeter membrane of the vesicles. In addition, we occasionally observed late endosomes that were clearly distinguishable from the early endosomes because the label was distributed throughout the lumen of these vesicles. These vesicles were mainly located close to the cell surface, similar to the early endosomes. The labeling at the superficial membrane was more apparent at this time point compared to the labeling present directly after the binding incubation (not shown).

The intracellular label was predominantly present in late endocytic structures from 30 min up to 60 min (Fig. 4C). A decrease in the number of early endocytic structures coincided with an increasing number of late endocytic structures (Table 1). However, throughout the period studied, label was continuously found at the plasma membrane, in the intercellular space, and in early endosomes.

At 30 min the first structures with a lysosomal appearance were labeled (Fig. 4D and Table 1). No obvious accumulation of the label in these putative lysosomes was observed. In general, the labeling of these structures was rather constant during the period studied (compare Figs. 4D and E).

The labeling of the intercellular spaces at different time points was intriguing. Even after a chase incubation of 60 min, labeling was still observed in the intercellular space close to the superficial border of the tissue (Fig. 4F). There was a shift in the distribution of extracellular labeling which is illustrated by the increase in the ratio between the intercellular space labeling and the superficial cell surface labeling (Table 1; compare also Fig. 4A and F).

No labeling was observed in control samples that had been incubated with 0.05 mg/ml ultrasmall gold-labeled HDLp in the presence of an excess unlabeled HDLp and chased for 60 min (not shown), indicating that the above-described labeling represents specific internalization of HDLp.

Accumulation of HDLp in the intercellular space and at the superficial cell surface

A striking observation was the accumulation of HDLp in the large intercellular spaces between adjacent fat body cells, which was most clearly visible after photoconversion of DiI-labeled HDLp (**Fig. 5A**). These spaces appeared to be filled with lipoprotein particles according to the stained spherical shapes with diameters of 15–20 nm. The plasma membranes bordering the intercellular space often contained invaginations resembling coated pits (Fig. 5A) that were occasionally labeled, suggesting that this part of the plasma membrane contributed to the endocytic uptake of HDLp by the fat body cells. Also, with ultrasmall gold-labeled HDLp, labeling throughout the intercellular space was observed (see Fig. 4F).

The region of the intercellular space located close to the superficial border of the fat body tissue was heavily stained when fat body tissue was incubated for a prolonged period (3 h) with 1 mg/ml concentration of DiI-labeled HDLp (Fig. 5B). The HDLp was also present in intercellular spaces located more to the center of the fat body tissue, though less abundant than the region close to the superficial border of the tissue (not shown). Under those conditions, an apparent labeling of the extracellular matrix was observed in the fat body tissue of both young (Fig. 5B) and older adults (not shown). Although less prominent, labeling of the extracellular matrix could be achieved with a low (0.3 mg/ml) DiI-labeled HDLp concentration (not shown). Moreover, a developmental difference in staining of the extracellular matrix was found as labeling of the ex-

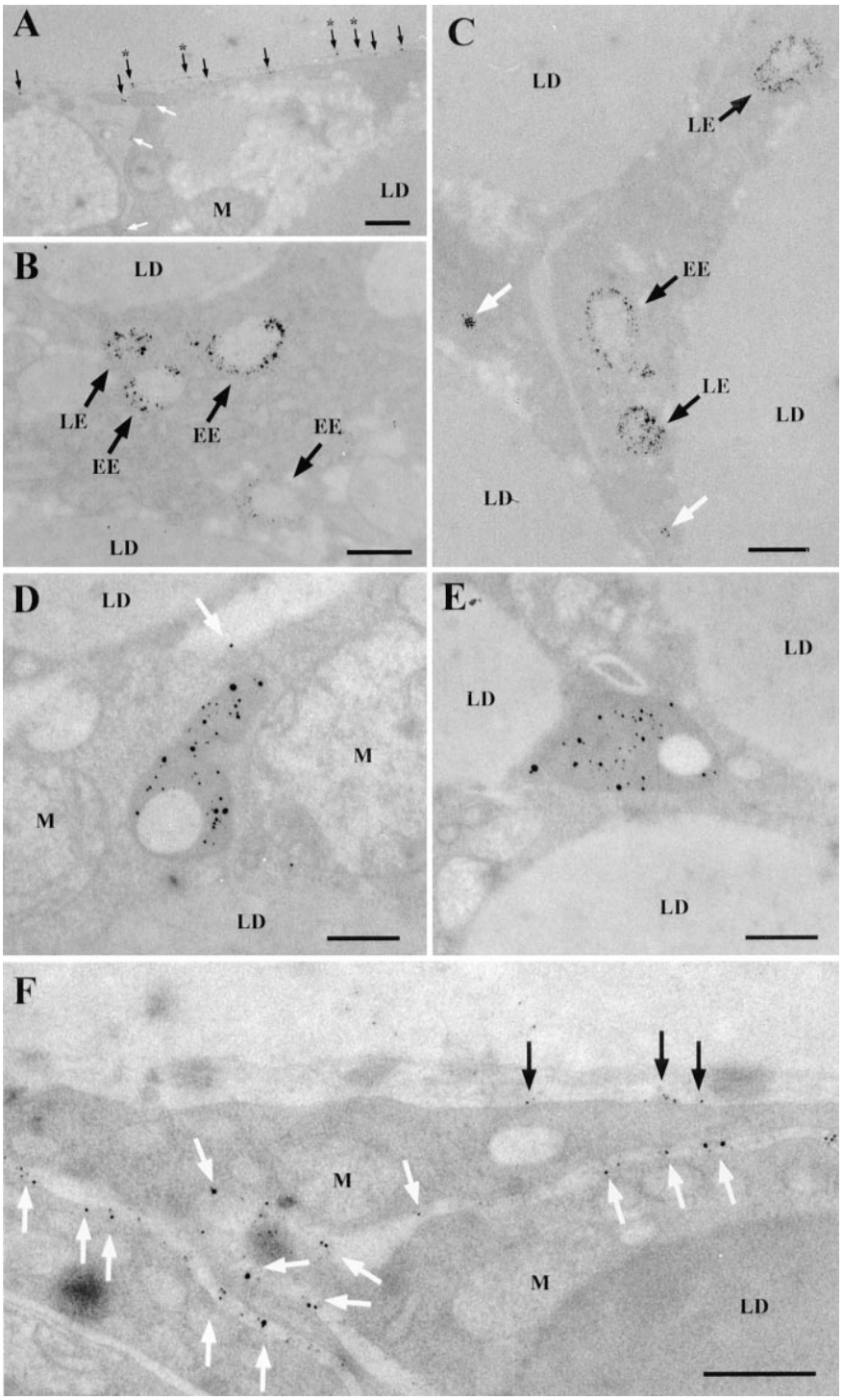

Fig. 4. Pulse–chase study of uptake and trafficking of HDLp in fat body tissue of young adult locusts. Electron micrographs of fat body tissue of young adults that had been incubated with 0.05 mg/ml ultrasmall gold-labeled HDLp and chased for various times with 0.05 mg/ml unlabeled HDLp. The gold label was visualized by silver enhancement. A) Directly after binding, the labeling was present in the extracellular matrix (arrows with asterisks), in close proximity to the superficial plasma membrane (black arrows) and in the intercellular space (white arrows). Note that the silver-enhanced gold label was very small at this time point. B) Early (EE) and late endosomes (LE) labeled after 2-min chase. C) When the chase incubation had proceeded for 20 min, the label was mainly present in late endosomes (LE) but labeled early endosomes were still observed (EE). White arrows indicate small endocytic structures. D) Labeled putative lysosome and label in the intercellular space (arrow) after 30 min. E) Labeled putative lysosome after 60 min. F) After 60 min, the intercellular space close to the superficial border of the tissue was still strongly labeled (white arrows) whereas the superficial cell surface was only modestly labeled (black arrows). IS, intercellular space; LD, lipid droplet; M, mitochondrion. Scale bars are 500 nm.

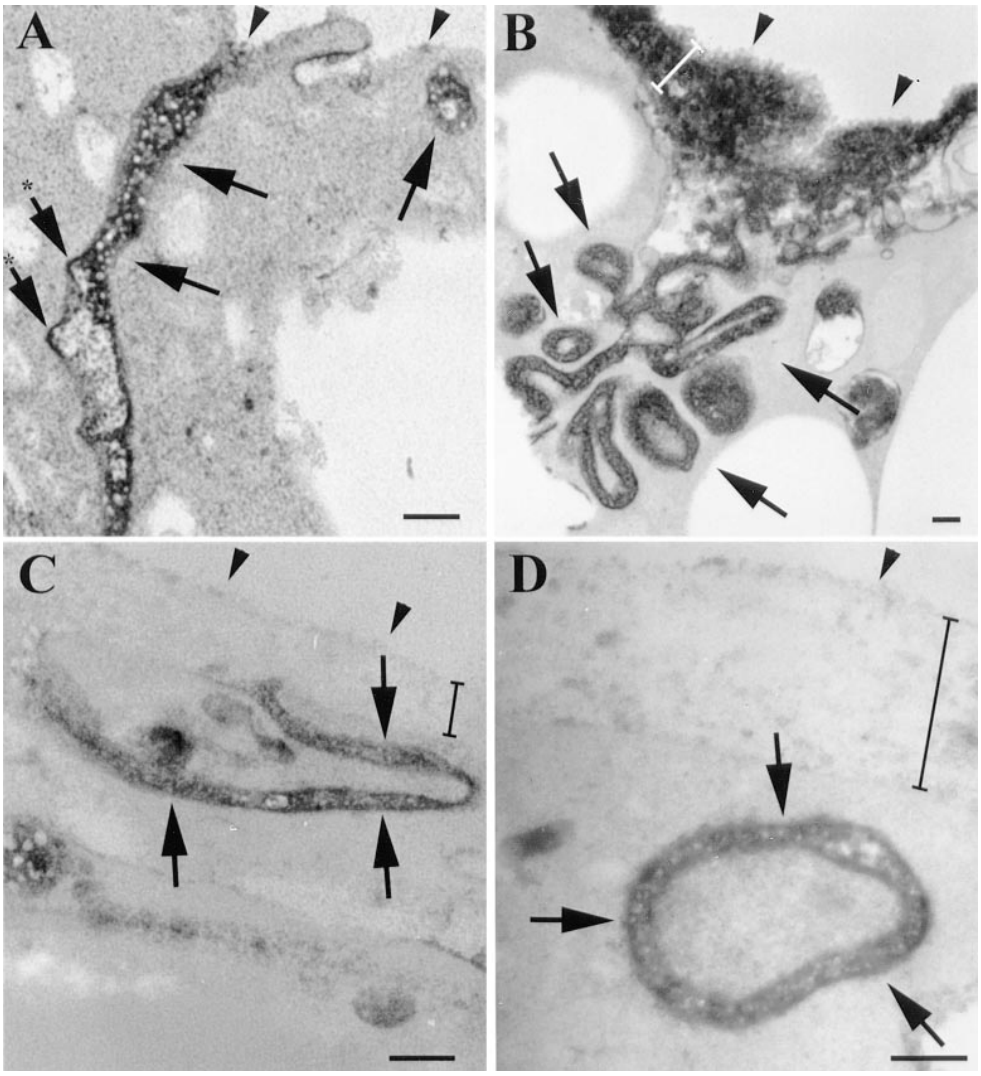

Fig. 5. Accumulation of DiI-labeled HDLp at the superficial border of the fat body and in the intercellular space. Electron micrographs of fat body tissue of young adults that had been incubated for 90 min with 0.3 mg/ml (A, C, D) or for 3 h with 1 mg/ml (B) DiI-labeled HDLp at 30°C. DiI-labeled HDLp was visualized by DAB photoconversion. Due to negative staining, the shapes of individual lipoproteins can be observed in the intercellular space in these micrographs. A) HDLp accumulated in the intercellular space (arrows). At some places the membrane bordering the intercellular space formed coated pit-like structures (asterisks). The superficial border of the tissue is indicated by arrowheads. B) Prolonged incubation with a higher concentration of DiI-labeled HDLp resulted in a strong labeling of the intercellular space, illustrating the folded appearance of this space close to the superficial border of the tissue. HDLp particles were also covering the entire extracellular matrix under these conditions (arrowheads). C) A pilus-like structure that is folded backwards, thereby penetrating the cell surface of the fat body cell. The created space near the pilus is filled with HDLp (arrows). D) Presumably a different section of similar structure as shown in C. Arrowheads, superficial cell surface; vertical bars, size of the extracellular matrix. Scale bars are 200 nm.

tracellular matrix of fat body cells of older adults was more intense compared to that of younger adults.

At the superficial border of the tissue, we observed HDLp to be present in small spaces between different parts of the superficial plasma membrane. Most common were pilus-like extensions that were folded backwards, penetrating the fat body cell and creating a small space between the membranes in which labeled HDLp was accumulated (Fig. 5C). The circular labeled profiles in which the labeled HDLp is situated between two circular membranes most likely represent different sections of similar pilus-like structures (Fig. 5D).

DISCUSSION

As lipids are selectively transported into the fat body cells without substantial concomitant apolipophorin degradation, it has been postulated that transport of lipids into the fat body cells is not established by the classical endosomal/lysosomal pathway (23). Previously, we confirmed the existence of selective lipid transport for the fat body cells of migratory locusts (9). Nevertheless, we demonstrated that HDLp is internalized by means of receptor-mediated endocytosis in fat body tissue of young adult lo-

custs. Endocytic uptake of lipophorins by fat body has also been reported for larvae of the dragon fly (24), suggesting that lipophorin internalization by fat body cells is a common mechanism in insects.

The present electron-microscopic study revealed that ultrasmall gold-labeled and DiI-labeled HDLp were present throughout the endosomal/lysosomal compartment of fat body cells, both in young and older adults. In fat body cells of older adults, however, the labeling was much less abundant. Typical early endocytic and late endocytic structures contained labeled HDLp. Despite the fact that the bulk of the lipids is transported into the fat body cells by a selective mechanism without substantial degradation of the apolipophorins (9, 23), labeled lipoproteins were also present in putative lysosomes.

The reliability of the determination of the different endocytic structures according to morphological characteristics was strengthened by the pulse–chase study where the vesicles, that we classified as early endosomes, late endosomes, and putative lysosomes, appeared to be labeled in the expected sequence. An increase in the size of the silver-enhanced label at the plasma membrane early during the chase was observed. This is not surprising as the size of the particle after silver enhancement is related to the amount of gold label and, therefore, this increase in the size of the label may reflect clustering of ligand–receptor complexes at the cell surface. Interestingly, the ratio between the amount of label in the intercellular space and the superficial cell surface increased during the 60-min period studied. There are three explanations for this finding. First, this may be due to the fact that the plasma membrane bordering the intercellular space is devoid of endocytic receptors and therefore does not contribute to internalization of the lipoproteins. However, we observed invaginations at these sites which were occasionally labeled. Second, it is possible that there is a delay in the uptake from HDLp that is entrapped in the intercellular space. This would result in a constitutive flow of HDLp to endocytic receptors during the chase, giving rise to newly bound and internalized lipoproteins during the 60-min incubation. Retroendocytic trafficking of HDLp is the third possibility, as such an intracellular pathway would result in resecretion of the HDLp at the plasma membrane after intracellular trafficking. The absence of substantial accumulation of label in the putative lysosomes favors the latter possibility.

Previously, we observed by means of fluorescence microscopy that fat body tissue of older adults incubated with DiI-labeled HDLp showed a surface staining (9), that resembled the staining of the extracellular matrix as observed for mammalian lipoprotein (25). In the present study, we confirm that the lipoproteins bind to the extracellular matrix of fat body tissue of older adults. When fat body tissue of young adults was used, this surface staining was only observed upon incubation with higher concentrations of DiI-labeled HDLp. Thus, indications are provided for two types of binding sites at the surface of fat body cells: a high abundant binding site with a relatively low affinity in the extracellular matrix and a low abundant high affinity binding site at the plasma membrane.

By studying the saturation kinetics of the interaction between fat body cells and radiolabeled HDLp, we previously identified an HDLp binding site that is up-regulated during development (14). In retrospect, it is likely that this binding site, according to its affinity for HDLp and its abundance, represents the binding site in the extracellular matrix. An explanation for the fact that the putative second binding site located at the plasma membrane was not noticed in these binding studies might be that this binding site is less abundant compared to the binding site at the extracellular matrix.

Binding of the lipophorins to the extracellular matrix is strongly increased during development. This may be established by developmental changes in the composition of the extracellular matrix itself. In mammals, extracellular matrix components have been found that contain domains related to the ligand-binding domain of the low density lipoprotein receptor gene superfamily (26, 27). Developmental changes in such matrix components might lead to an increased binding of HDLp to the extracellular matrix of locust fat body cells. Alternatively, the expression of a matrix-associated protein with affinity for lipoproteins, like lipoprotein lipase (28), might be up-regulated. However, there are no data available on the presence of such extracellular matrix components or lipoprotein lipases in insect fat body tissue.

We have recently identified a lipophorin receptor belonging to the low density lipoprotein receptor gene superfamily that is expressed by fat body cells of young adult locusts and mediates internalization of HDLp (N. P. Dantuma, unpublished results). Although this endocytic receptor explains the endosomal/lysosomal staining pattern in fat body cells of young adults, it is most likely not responsible for the binding at the fat body plasma membrane in older adults as the expression of this receptor is strongly down-regulated in this developmental stage. Additional experiments are required to identify the binding site present at the plasma membrane of fat body cells of older adults.

There is an interesting similarity between the flight-specific selective mobilization of DAG in insects and the reverse cholesterol transport in mammals: both include selective loading of lipids upon pre-existing lipoprotein (29). Based on this analogy, we postulated previously that if a receptor is involved in the lipid mobilization established by fat body cells in older locusts, it might be related to the mammalian scavenger receptor class B (14). This receptor plays a key role in reverse cholesterol transport (30), as it facilitates both the cholesterol extraction from (31) and loading upon (32) circulating lipoproteins in mammals. However, no scavenger receptor class B homologs were found upon screening of a fat body cDNA library of older adults with a murine scavenger receptor B probe (N. P. Dantuma, unpublished results). In addition, locust HDLp does not compete with natural ligands of the mammalian scavenger receptor class B for binding to this receptor (33). As these experiments do not fully exclude the possibility that fat body cells express an HDLp receptor that is related to scavenger receptor class B, an insect

homolog of this mammalian receptor should still be considered as a candidate for the above-mentioned cell-surface binding site of fat body cells of older adults.

We observed that HDLp particles accumulated in the intercellular space and near folded pilus-like structures at the superficial border of fat body tissue. The accumulation of HDLp in these extracellular cavities resembles a similar phenomenon observed by Reaven and co-workers for different mammalian steroid hormone-producing tissues (34, 35). This is especially interesting as these tissues extract cholesterol from circulating lipoproteins without internalizing and degrading the lipoproteins (36), similar to the selective DAG uptake from HDLp by fat body cells. Reaven and co-workers (34, 35) argue that the extracellular entrapment of lipoprotein is likely to be required for accelerating selective cholesterol transport into the recipient cells. The accumulation of HDLp in the intercellular spaces may serve a similar function in selective lipid transport into locust fat body cells as the DAG and cholesterol cargo can also be easily exchanged between HDLp and fat body cells when endosomal uptake is inhibited (9).

Consequently, there may be three distinct mechanisms involved in the transport of lipids between lipophorins and fat body cells. First, we have demonstrated that HDLp is internalized by a receptor-mediated mechanism and transported through the endocytic pathway, especially in young adults where lipid storage dominates. It needs to be evaluated whether all of the internalized lipophorins are targeted to the lysosomes or whether a part is re-secreted after intracellular trafficking and eventually unloading of their lipid cargo. Second, selective transport of lipids from lipophorins into the fat body cells may be accelerated by the entrapment of lipophorins in the intercellular spaces and in the spaces at the superficial cell surface created by pilus-like structures, thereby increasing the concentration of lipophorins at the cell surface. Third, the increase in the amount of HDLp associated with the low affinity binding sites in the extracellular matrix and with binding sites located at the plasma membrane coincides with the ability of insects to mobilize massive amounts of DAG during flight activity, suggesting that these interactions are likely candidates for establishing DAG loading upon HDLp.

We thank Dr. Jan Leunissen for helpful comments regarding the silver enhancement of ultrasmall gold label, Dr. Paul van Bergen en Henegouwen for support, and Dr. Willem Stoorvogel for critical reading of the manuscript. The investigations were supported by the Life Science Foundation (SLW), which is subsidized by the Netherlands Organization for Scientific Research (NWO) (SLW-805.27.062).

Manuscript received 25 June 1997, in revised form 17 February 1998, and in re-revised form 20 May 1998.

REFERENCES

1. Ryan, R. O. 1990. Dynamics of insect lipophorin metabolism. *J. Lipid Res.* **31:** 1725–1739.
2. Weers, P. M. M., W. J. A. Van Marrewijk, A. M. T. Beenakkers, and D. J. Van der Horst. 1993. Biosynthesis of locust lipophorins. Apolipophorins I and II originate from a common precursor. *J. Biol. Chem.* **268:** 4300–4303.
3. Kutty, R. K., G. Kutty, R. Kambadur., T. Duncan, E. V. Koonin, I. R. Rodriguez, W. F. Odenwald, and B. Wiggert. 1996. Molecular characterization and developmental expression of a retinoid- and fatty acid-binding glycoprotein from *Drosophila*. A putative lipophorin. *J. Biol. Chem.* **271:** 20641–20649.
4. Sundermeyer, K., J. K. Hendricks, S. V. Prasad, and M. A. Wells. 1996. The precursor protein of the structural apolipoproteins of lipophorin: cDNA and deduced amino acid sequence. *Insect Biochem. Mol. Biol.* 26: 735–738.
5. Van der Horst, D. J., P. M. M. Weers, and W. J. A. Van Marrewijk. 1993. Lipoproteins and lipid transport. *In* Insect Lipids: Chemistry, Biochemistry and Biology. D. W. Stanley-Samuelson and D. R. Nelson, editors. University of Nebraska Press, Lincoln, NE. 1–24.
6. Goldstein, J. L., M. S. Brown, R. G. W. Anderson, D. W. Russel, and W. J. Schneider. 1985. Receptor-mediated endocytosis: concepts emerging from the LDL receptor system. *Annu. Rev. Cell Biol.* **1:** 1–39.
7. Law, J. H., and M. A. Wells. 1989. Insects as biochemical models. *J. Biol. Chem.* **264:** 16335–16338.
8. Van der Horst, D. J. 1990. Lipid transport function of lipoproteins in flying insects. *Biochim. Biophys. Acta.* **1047:** 195–211.
9. Dantuma, N. P., M. A. P. Pijnenburg, J. H. B. Diederen and D. J. Van der Horst. 1997. Developmental down-regulation of receptor-mediated endocytosis of an insect lipoprotein. *J. Lipid Res.* **38:** 254–265.
10. Beenakkers, A. M. T., A. T. M. Van den Broek, and T. J. A. De Ronde. 1975. Development of catabolic pathways in insect flight muscles. A comparative study. *J. Insect Physiol.* **21:** 849–859.
11. Haunerland, N. H., P. Andolfatto, J. M. Chisholm, Z. Wang, and X. Chen. 1992. Fatty-acid-binding protein in locust flight muscle. Developmental changes of expression, concentration and intracellular distribution. *Eur. J. Biochem.* **210:** 1045–1051.
12. Van der Horst, D. J., A. M. T. Beenakkers, J. M. Van Doorn, K. Gerritse, and T. K. F. Schulz. 1987. Adipokinetic hormone-induced lipid mobilization and lipophorin interconversions in fifth larval instar locusts. *Insect Biochem.* **17:** 799–808.
13. Van der Horst, D. J., A. M. C. Baljet, A. M. T. Beenakkers, and E. Van Handel. 1978. Turnover of locust haemolymph diglycerides during flight and rest. *Insect Biochem.* **8:** 369–373.
14. Dantuma, N. P., W. J. A. Van Marrewijk, H. J. Wynne, and D. J. Van der Horst. 1996. Interaction of an insect lipoprotein with its binding site at the fat body. *J. Lipid Res.* **37:** 1345–1355.
15. Pitas, R. E., T. L. Innerarity, J. N. Weinstein, and R. W. Mahley. 1981. Acetoacetylated lipoproteins used to distinguish fibroblasts from macrophages in vitro by fluorescence microscopy. *Arteriosclerosis.* **1:** 177–185.
16. Schacterle, G. R., and R. L. Pollack. 1973. A simplified method for the quantitative assay of small amounts of protein in biological material. *Anal. Biochem.* **51:** 654–655.
17. Danscher, G. 1981. Localization of gold in biological tissue. A photochemical method for light and electron microscopy. *Histochemistry.* **71:** 81–88.
18. Dantuma, N. P., M. A. P. Pijnenburg, J. H. B. Diederen, and D. J. Van der Horst. 1998. Electron microscopic visualization of receptor-mediated endocytosis of DiI-labeled lipoproteins by diaminobenzidine photoconversion. *J. Histochem. Cytochem.* **46:** 1085–1089.
19. Esbach, S., M. F. Stins, A. Brouwer, P. J. M. Roholl, T. J. C. Van Berkel, and D. L. Knook. 1994. Morphological characterization of scavenger receptor-mediated processing of modified lipoproteins by rat liver endothelial cells. *Exp. Cell Res.* **210:** 62–70.
20. Handley, D. A., C. M. Arbeeny, L. D. Witte, and S. Chien. 1981. Colloidal gold–low density lipoprotein conjugates as membrane receptor probes. *Proc. Natl. Acad. Sci. USA.* **78:** 368–371.
21. Geuze, H. J., J. W. Slot, and G. J. A. M. Strous 1983. Intracellular site of asialoglycoprotein receptor-ligand uncoupling: double-label immunoelectron micrscopy during receptor-mediated endocytosis. *Cell.* **32:** 277–287.
22. Stoorvogel, W., G. J. Strous, H. J. Geuze, V. Oorschot, and A. L. Schwartz. 1991. Late endosomes derive from early endosomes by maturation. *Cell.* **65:** 417–427.
23. Tsuchida, K., and M. A. Wells. 1988. Digestion, absorption, transport and storage of fat during the last larval stadium of *Manduca sexta*. Changes in the role of lipophorin in the delivery of dietary lipid to the fat body. *Insect Biochem.* **18:** 263–268.
24. Bauerfeind, R., and H. Komnick. 1992. Immunocytochemical localization of lipophorin in the fat body of dragonfly larvae (*Aeshna cyanea*). *J. Insect Physiol.* **38:** 185–198.

25. Hamilton, R. L., J. S. Wong, L. S. S. Guo, S. Krisans, and R. J. Havel. 1990. Apolipoprotein E localization in rat hepatocytes by immunogold labeling of cryothin sections. *J. Lipid Res.* **31:** 1589–1603.
26. Noonan, D. M., A. Fulle, P. Valente, S. Cai, E. Horigan, M. Sasaki, Y. Yamada, and J. R. Hassel. 1991. The complete sequence of perlecan, a basement membrane heparan sulfate proteoglycan, reveals extensive similarity with laminin A chain, low density lipoprotein-receptor, and the neural cell adhesion molecule. *J. Biol. Chem.* 266: 22939–22947.
27. Kallunki, P., and K. Tryggvason. 1992. Human basement membrane heparan sulfate proteoglycan core protein: a 467-kD protein containing multiple domains resembling elements of the low density lipoprotein receptor, laminin, neural cell adhesion molecules, and epidermal growth factor. *J. Cell Biol.* 116: 559–571.
28. Edwards, I. J., I. J. Goldberg, J. S. Parks, H. Xu, and W. D. Wagner. 1993. Lipoprotein lipase enhances the interaction of low density lipoproteins with artery-derived extracellular matrix proteoglycans. *J. Lipid Res.* **34:** 1155–1163.
29. Johnson, W. J., F. H. Mahlberg, G. H. Rothblat, and M. C. Phillips. 1991. Cholesterol transport between cells and high-density lipoproteins. *Biochim. Biophys. Acta.* **1085:** 273–298.
30. Rigotti, A., B. L. Trigatti, M. Penman, H. Rayburn, J. Herz, and M. Krieger. 1997. A targeted mutation in the murine gene encoding the high density lipoprotein (HDL) receptor scavenger receptor class B type I reveals its key role in HDL metabolism. *Proc. Natl. Acad. Sci. USA.* **94:** 12610–12615.
31. Acton, S., A. Rigotti, K. T. Landschulz, S. Xu, H. H. Hobbs, and M. Krieger. 1996. Identification of scavenger receptor SR-BI as a high density lipoprotein receptor. *Science.* **271:** 518–520.
32. Ji, Y., B. Jian, N. Wang, Y. Sun, M. de la Llera Moya, M. C. Phillips, G. H. Rothblat, J. B. Swaney, and A. R. Tall. 1997. Scavenger receptor BI promotes high density lipoprotein-mediated cellular cholesterol efflux. *J. Biol. Chem.* **272:** 20982–20985.
33. Xu, S., M. Laccotripe, X. Huang, A. Rigotti, V. I. Zannis, and M. Krieger. 1997. Apolipoproteins of HDL can directly mediate binding to the scavenger receptor SR-BI, an HDL receptor that mediates selective lipid uptake. *J. Lipid Res.* **38:** 1289–1298.
34. Reaven, E., M. Spicher, and S. Azhar. 1989. Microvillar channels: a unique plasma membrane compartment for concentrating lipoproteins on the surface of rat adrenal cortical cells. *J. Lipid Res.* 30: 1551–1560.
35. Reaven, E., J. Boyles, M. Spicher, and S. Azhar. 1988. Evidence for surface entrapment of cholesterol-rich lipoproteins in luteinized ovary. *Arteriosclerosis.* **8:** 298–309.
36. Glass, C., R. C. Pittman, M. Civen, and D. Steinberg. 1985. Uptake of high-density lipoprotein-associated apoprotein A-I and cholesterol esters by 16 tissues of the rat in vivo and by adrenal cells and hepatocytes in vitro. *J. Biol. Chem.* **260:** 744–750.