Lipid transfer particle mediates the delivery of diacylglycerol from lipophorin to fat body in larval *Manduca sexta*

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Abstract **This work analyzed the process of lipid storage in fat body of larval *Manduca sexta*, focusing on the role of lipid transfer particle (LTP). Incubation of fat bodies with [^{3}H]diacylglycerol-labeled lipophorin resulted in a significant accumulation of diacylglycerol (DAG) and triacylglycerol (TAG) in the tissue. Transfer of DAG to fat body and its storage as TAG was significantly inhibited (60%) by preincubating the tissue with anti-LTP antibody. Lipid transfer was restored to control values by adding LTP to fat body. Incubation of fat body with dual-labeled DAG lipophorin or its treatment with ammonium chloride showed that neither a membrane-bound lipoprotein lipase nor lipophorin endocytosis is a relevant pathway to transfer or to storage lipids into fat body, respectively. Treatment of fat body with suramin caused a 50% inhibition in [^{3}H]DAG transfer from lipophorin. Treatment of [^{3}H]DAG-labeled fat body with lipase significantly reduced the amount of [^{3}H]DAG associated with the tissue, suggesting that the lipid is still on the external surface of the membrane. Whether this lipid represents irreversibly adsorbed lipophorin or a DAG lipase-sensitive pool is unknown. Nevertheless, these results indicate that the main pathway for DAG transfer from lipophorin to fat body is via LTP and receptor-mediated processes.**—Canavoso, L. E., H. Kyung Yun, Z. E. Jouni, and M. A. Wells. **Lipid transfer particle mediates the delivery of diacylglycerol from lipophorin to fat body in larval *Manduca sexta*. *J. Lipid Res.* 2004.** 45: **456–465.**

Supplementary key words lipoprotein lipase • developmental stages • triacylglycerol • endocytosis

In insects, the majority of stored lipids are found in the fat body, an organ analogous to vertebrate adipose tissue and liver. In *Manduca sexta*, triacylglycerol (TAG) constitutes more than 90% of the fat body lipids, whereas diacylglycerol (DAG) accounts for less than 2–3%. TAG is derived mainly from dietary fat, which is transferred in the form of DAG by lipophorin from the midgut to the fat body during the feeding stage. A minor source of TAG in the fat body is the de novo lipid synthesis from carbohydrates (1). We have shown recently that the uptake of DAG from midgut to lipophorin takes place by the action of lipid transfer particle (LTP) (2).

LTP is a very high density lipoprotein, first purified from the hemolymph of larval *M. sexta* (3) [see also refs. (4, 5) for recent reviews]. LTP has also been identified in the hemolymph of *Locusta migratoria* (6), *Periplaneta americana* (7), *Musca domestica* (8), and *Bombyx mori* (9). LTP is synthesized in the fat body and secreted into the hemolymph (10). The physiological function of LTP is still not completely understood. The protein catalyzes the transfer of DAG from adult fat body to high density lipophorin (Lp), resulting in the formation of low density lipophorin (LDLp) (11), but not the reverse reaction. LTP catalyzes the transfer of DAG from the larval midgut to Lp, but not the reverse reaction (12), and from Lp to ovarioles (13). LTP also catalyzes the transfer and/or exchange of DAG between Lp or LDLp and vitellogenin (9). The protein also facilitates the transfer of other lipids from Lp to LDLp, including hydrocarbons (7), phospholipids (9), and carotenoids (14), and it catalyzes the exchange and/or transfer of DAG between Lps and human lipoproteins (15).

We have been investigating the role of LTP in DAG transfer in larval *M. sexta* using in vitro assays. In the midgut, LTP is required to export DAG from the midgut to Lp (12). Interestingly, LTP does not catalyze the transfer of DAG from Lp to the midgut. In this paper, we describe the extension of these studies to the larval fat body and show for the first time that LTP is required for the bidirectional transfer of DAG between Lp and fat body.

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MATERIALS AND METHODS

Materials

[9,10-^{3}H]Oleic acid was purchased from NEN (Boston, MA). [1-^{14}C]Oleic acid and [1(3)-^{3}H]glycerol were from Amersham (Arlington Heights, IL). Silica gel plates were obtained from J. T. Baker. 4-(2-Aminoethyl)-benzenesulfonylfluoride (AEBSF) and DEAE-Trisacryl M were from Sigma (St. Louis, MO). Falcon multiwell tissue culture plates and cell strainers were obtained from Becton Dickinson (Franklin Lakes, NJ). Affi-Gel Protein A was from Bio-Rad (Hercules, CA). Centriprep Centrifugal Filter Devices were from Millipore-Amicon (Bedford, MA). *Rhizopus* lipase was purchased from Fluka (Buchs, Switzerland). All other chemicals were analytical grade.

Insects

M. sexta was reared as previously described (16). For the experiments, day 1 or day 2 fifth instar larvae were used unless otherwise indicated.

Lp labeling

Two-day-old fifth instar larvae were fed [^{3}H]oleic acid (5 μCi/animal) on a small piece of artificial diet. One hour later, hemolymph was collected in ice-cold bleeding solution (30 mM KH_2PO_4, pH 6.5, containing 2 mM Na_2EDTA, 10 mM glutathione, and 3 mM NaN_3) by puncturing a proleg and gently pressing the abdomen. Hemolymph was centrifuged for 5 min at 12,000 *g* (4°C) to remove hemocytes and then subjected to two steps of KBr gradient ultracentrifugation as described previously (16, 17). Labeled Lp (density of 1.14 g/ml) was dialyzed against lepidopteran saline (5 mM KH_2PO_4, 100 mM KCl, 4 mM NaCl, 15 mM $MgCl_2$, and 2 mM $CaCl_2$, pH 6.5) and concentrated by ultrafiltration before use (Centriprep YM-50). Under these conditions, more than 95% of the label was recovered in the DAG-Lp moiety, and this material is designated [^{3}H]DAG-Lp.

LTP purification

LTP was isolated from hemolymph of 2-day-old fifth instar *M. sexta* larvae (12, 18). Briefly, 20 ml of hemocyte-free hemolymph was adjusted to a density of 1.31 g/ml with KBr, transferred to a 39 ml Quick-Seal centrifuge tube, and overlaid with 0.15 M NaCl. Centrifugation was carried out for 4 h at 50,000 rpm (4°C) in a Beckman VTi 50 rotor. The LTP fraction (density of 1.23 g/ml) was collected and adjusted to 1.31 g/ml with KBr, and 20 ml fractions of this preparation were overlaid with a 1.21 g/ml KBr solution in 0.15 M NaCl. A second ultracentrifugation was carried out for 16 h, and LTP was recovered from the top 5 ml of the gradient and dialyzed against 20 mM Tris-HCl, pH 8.7, containing 5 mM Na_2EDTA. LTP was purified before each experiment by a double passage through a DEAE-Trisacryl M column (14). The protein was eluted with a linear 0–300 mM NaCl gradient (flow rate of 15 ml/h), and 3 ml fractions were collected. LTP-containing fractions were pooled and stored at 4°C in 3.7 M KBr and 1 mM AEBSF for no longer than 1 week. Before its use, LTP was dialyzed against lepidopteran saline and concentrated by ultrafiltration (Centriprep YM-50).

In vitro transfer of lipid from [^{3}H]DAG-Lp to fat body

Fat bodies from second day fifth instar larvae were dissected on ice-cold lepidopteran saline and then transferred to a 24-well culture plate containing the incubation medium (lepidopteran saline-Grace's insect medium in a 1:1 ratio). After 5 min, each fat body was transferred to 1.5 ml of fresh medium containing [^{3}H]DAG-Lp (1.5 mg/ml; specific activity of 5.5×10^5 dpm/mg Lp). Incubations were performed at room temperature or 4°C (control) with gentle shaking and continuous oxygenation by aeration with 95% O_2-5% CO_2 (19). At different times, fat bodies were removed and washed twice for 15 min each with 1.5 ml of incubation medium. Preliminary data have shown that no significant further reduction in radioactivity was observed when the tissues were subjected to more than two washes. Thus, two washing steps with incubation medium was adopted in these experiments.

Fat bodies were individually homogenized for lipid extraction according to Folch, Lees, and Sloane Stanley (20). Lipids were fractionated by TLC, and spots scraped from the plates were assayed for radioactivity by liquid scintillation counting (2). Cellular viability and tissue integrity were determined by trypan blue exclusion tests.

Effect of LTP on lipid transfer from [^{3}H]DAG-Lp to fat body

Dissected fat bodies were transferred to 1.5 ml of fresh medium and incubated as described above under the following conditions: *a*) for 3 h in the presence of [^{3}H]DAG-Lp (1.5 mg/ml); *b*) for 1 h with anti-LTP antibody (3.5 μg IgG/μl), washed with Grace's medium, and then incubated with [^{3}H]DAG-Lp for 3 h; and *c*) preincubated with anti-LTP antibody (1 h), washed, and then transferred to a medium with both [^{3}H]DAG-Lp (1.5 mg/ml) and LTP (70 μg/ml). After incubation, fat bodies were removed, washed as described above, and processed for lipid analysis.

Transfer of lipid from dual-labeled DAG-Lp to fat body

Insects were fed a mixture of [1-^{14}C]oleic acid (3 μCi/insect) and [1(3)-^{3}H]glycerol (12 μCi/insect) on a small piece of artificial diet 1 h before bleeding. Isolation of doubly labeled DAG-Lp ([^{14}C]/[^{3}H]DAG-Lp) that incorporated the ^{3}H label in glycerol backbone and the ^{14}C label in the fatty acids was performed as described above. Dissected fat bodies were incubated with 1.5 ml of incubation medium containing [^{14}C]/[^{3}H]DAG-Lp (1 mg/ml; ^{14}C/^{3}H ratio of 0.97) with or without unlabeled glycerol (4 μmol/ml). After 1.5 and 3 h of incubation, tissue was removed and washed with unlabeled medium, and lipids were extracted and isolated by TLC. The radioactivity was recorded as ^{14}C/^{3}H ratio in DAG-Lp and fat body DAG and TAG.

Treatment of fat body with lipase

To determine how much residual [^{3}H]DAG remained bound to the fat body after extensive washing, labeled fat bodies were incubated with *Rhizopus* lipase, which is able to hydrolyze only the surface-bound lipids. Briefly, TAG and DAG pools of the fat body were radiolabeled by incubating the tissues with [^{3}H]DAG-Lp (1.5 mg/ml) for 2 h, followed by extensive washing with Grace's medium. The washed fat body was transferred to a tube containing 200 mg of fatty acid-free BSA in the absence (control) or presence of *Rhizopus* lipase (1.2 U) and incubated at 34°C for 30 min. After incubation, the reaction was stopped by adding diisopropyl fluorophosphates (5 mM), PMSF (5 mM), diethyl *p*-nitrophenyl phosphate (Paraoxon; E600) (3 mM), and EDTA (25 mM). Tissues were then removed, washed with Grace's medium, and processed for lipid analysis as described above. In another set of experiments, the fat body was incubated with anti-LTP (4 mg IgG/ml) for 30 min before incubation with [^{3}H]DAG-Lp (1.5 mg/ml) and then with lipase.

Effect of pH and inhibitors of endocytosis

To study the effect of pH on the transfer of lipids from Lp to fat body, [^{3}H]DAG-Lp was dialyzed into a buffer containing 10 mM MES, 10 mM MOPS, 0.5 mM Trizma base, and 0.15 M NaCl of different pH values between 5.0 and 7.5. Transfer studies were

carried out as described above. To study the effect of inhibitors of endocytosis, the fat body was incubated for 2 h in insect Grace's medium containing [^{3}H]DAG-Lp (1.5 mg/ml) containing different concentrations of ammonium chloride or chloroquine. After incubation, the fat body was washed and lipids were analyzed as described above.

Transfer of DAG from [^{3}H]TAG-labeled fat body to Lp

To study the extent of transfer of [^{3}H]DAG from different developmental stages of fat body to Lp, day 1 fifth instar *M. sexta* were fed on a piece of diet labeled with [^{3}H]oleic acid (2 μCi) and then switched to unlabeled diet until the fat body was used. This technique produced [^{3}H]TAG-labeled fat body with the same specific activity in day 4 and 5 of the fifth instar insects and in all wandering stages. At the indicated developmental stages, [^{3}H]TAG-labeled fat body (120 mg) was incubated in Grace's medium containing 1 mg/ml unlabeled larval Lp for 2 h and transfer studies were carried out as described above.

Anti-LTP antibody

Antiserum against purified LTP was obtained from a New Zealand White rabbit as described by Ryan et al. (21). The IgG fraction was then purified using Affi-Gel Protein A and stored at −80°C.

Protein and lipid determination

Protein concentration was determined by the Bradford assay (22) using BSA as the standard. Lipids were extracted according to Folch, Lees, and Sloane Stanley (20) or Bligh and Dyer (23). Lipid classes were separated by TLC on silica gel using hexane-ethyl ether-formic acid (70:30:3, v/v/v) as a solvent system (24).

Statistical analysis

Statistical tests were performed using GraphPad Prism version 3.00 for Windows (GraphPad Software, San Diego, CA). The results are expressed as means ± SEM. $P < 0.05$ was considered a significant difference between means.

RESULTS

DAG transfer from [^{3}H]DAG-Lp to fat body

Figure 1 shows the time course of lipid transfer when labeled Lp was incubated with larval *M. sexta* fat body. Over a 4 h incubation period, a significant amount of the radioactivity recovered in DAG and TAG was transferred from [^{3}H]DAG-Lp to fat body. In contrast, a negligible amount of radioactivity was found in fat body lipids when incubations were performed at 4°C. Trypan blue exclusion tests performed during the incubation time showed that tissue integrity and cellular viability were maintained.

The role of LTP in lipid transfer from [^{3}H]DAG-Lp to fat body

As shown in **Fig. 2**, treatment of fat body with anti-LTP antibody before its incubation with [^{3}H]DAG-Lp resulted in a significant inhibition of the transfer of labeled DAG from Lp to fat body. The decrease of radioactivity in fat body DAG and fat body TAG amounted to ~40% and 60%, respectively, compared with controls. This incomplete inhibition of lipid transfer, in contrast to the results in larval midgut (12), did not change when increasing amounts of anti-LTP antibody up to 6 μg IgG/μl were tested. No inhibition on [^{3}H]DAG transfer from Lp to fat body was observed if the tissue was preincubated with nonimmune rabbit serum (Fig. 2A), showing that the inhibitory effect of anti-LTP antibody was specific. When fat bodies preincubated with anti-LTP antibodies were transferred to a medium containing both [^{3}H]DAG-Lp and LTP, the amount of lipid transferred to the tissue was restored to control values (Fig. 2B). It was also observed that the amount of [^{3}H]DAG transferred to fat body after pretreatment with anti-LTP antibody increased with increasing concentrations of LTP up to 80 μg of LTP per assay, a concentration corresponding to the physiological levels for LTP, which were reported to be between 50 and 80 μg/ml during the fifth instar (10).

Are there other mechanisms for lipid transfer from Lp to fat body?

The presence of a fat body membrane-bound lipase that could hydrolyze DAG-Lp into fatty acids and glycerol, which in turn would be taken up by the tissue and resynthesized into TAG, could account for the fact that anti-LTP antibodies did not completely inhibit DAG transfer to larval fat body. To test this possibility, we prepared dual-labeled DAG-Lp with [^{14}C]fatty acid- and [^{3}H]glycerol-labeled moieties and incubated it with fat body in the presence and absence of unlabeled glycerol. The rationale for this experiment was that if significant hydrolysis of DAG occurred because of the action of a membrane-bound lipase, then the presence of unlabeled glycerol would increase the ^{14}C/^{3}H ratio of the acylglycerols in the fat body. In fact, the presence of unlabeled glycerol had no effect

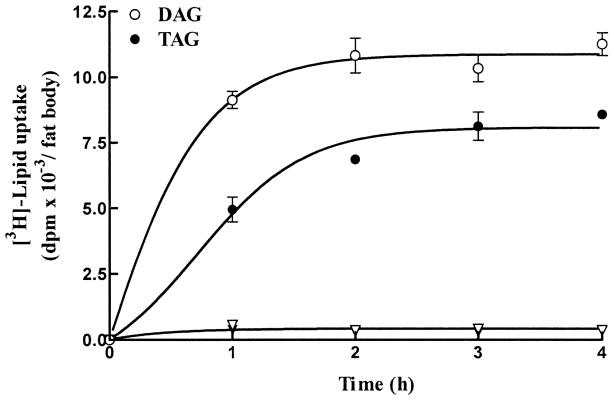

Fig. 1. Time course of [^{3}H]lipid transfer from labeled lipophorin to fat body in vitro. Fat bodies were incubated at room temperature with [^{3}H]diacylglycerol-lipophorin ([^{3}H]DAG-Lp; 1.5 mg/ml incubation medium, final volume of 1.5 ml). At different times, the tissues were removed, washed, and processed for lipid extraction. Extracted lipids were separated by TLC, and the radioactivity was determined by liquid scintillation counting of silica gel scrapings. Results are expressed as total dpm ± SEM (n = 6) found in DAG (open circles) and triacylglycerol (TAG) (closed circles) fat bodies. Total radioactivity in DAG (closed inverted triangles) and TAG (open inverted triangles) fat bodies when incubations were performed at 4°C (control) is also shown.

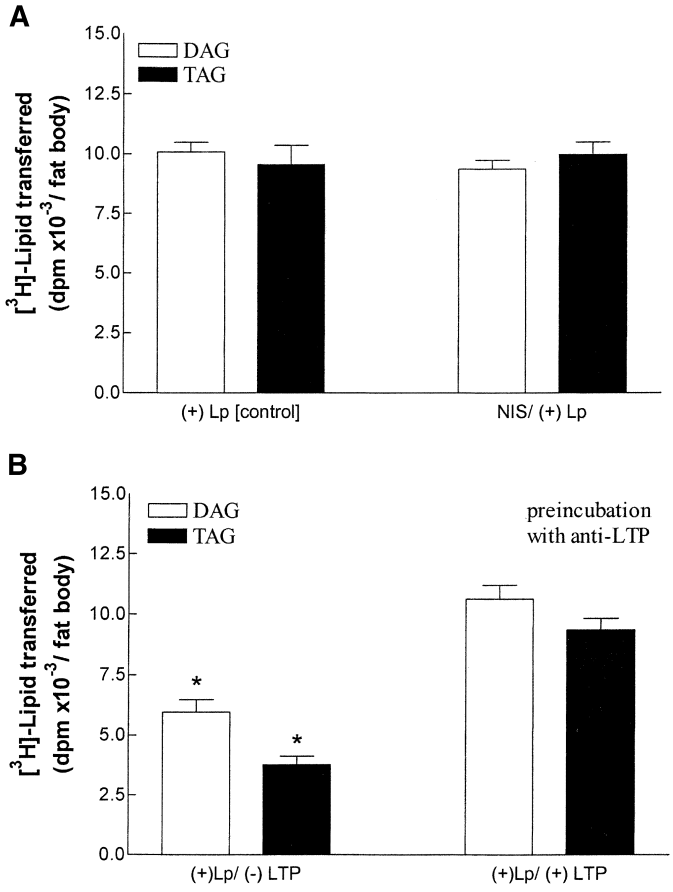

Fig. 2. Effect of lipid transfer particle (LTP) on lipid transfer from Lp to fat body in vitro. Dissected fat bodies were transferred to fresh medium and incubated as described in Materials and Methods under the following conditions: A: for 3 h in the presence of [^{3}H]DAG-Lp (1.5 mg/ml) [control] or preincubated with rabbit nonimmune serum (NIS; 0.3 ml) and then transferred to a medium with [^{3}H]DAG-Lp (1.5 mg/ml) [NIS/(+) Lp]; B: preincubated for 1 h with anti-LTP antibody (3.5 μg IgG/μl) and then transferred to a medium with [^{3}H]DAG-Lp [(+) Lp] or with both [^{3}H]DAG-Lp (1.5 mg/ml) and LTP (70 μg/ml) [(+) Lp/(+) LTP]. Concentrations of Lp and LTP are expressed as milligrams or micrograms per milliliter of incubation medium (1.5 ml final volume). After incubation, fat bodies were removed and processed for lipid extraction. Lipids were fractionated by TLC, and spots were assayed for radioactivity. Results are expressed as total dpm ± SEM (n = 6–8) found in DAG and TAG fractions. * $P <$ 0.01 versus control, NIS/(+) Lp, and (+) Lp/(+) LTP.

on the ^{14}C/^{3}H ratio of acylglycerols in the fat body (**Fig. 3**). These results strongly suggest that DAG-Lp enters the cell without being hydrolyzed.

Another mechanism that could account for the incomplete inhibition of DAG-Lp transfer in the presence of anti-LTP antibody is the possibility that Lp itself is endocytosed. We tested the effect of two endocytic inhibitors, ammonium chloride and chloroquine, on the transfer of lipid from Lp to fat body (**Fig. 4**). Chloroquine had no effect at any concentration tested, and ammonium chloride only inhibited at very high doses (100 mM), which is much higher than usually used in these studies. Thus, neither a lipoprotein lipase nor endocytosis could account for LTP-independent DAG-Lp transfer to the fat body.

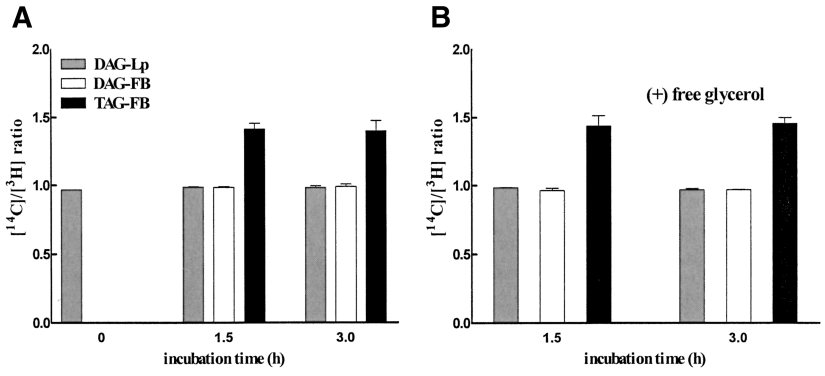

Fig. 3. Lipid transfer from doubly labeled Lp to fat body in vitro. Dissected fat bodies were incubated as previously stated in a medium containing [^{14}C]/[^{3}H]DAG-Lp (1 mg/ml; ^{14}C/^{3}H ratio of 0.97) without (A) or with (B) the addition of unlabeled free glycerol (4 μmol/ml incubation medium, final volume of 1.5 ml). At 1.5 and 3 h of incubation, fat bodies were removed and washed with cold medium, and both tissue and medium were processed for lipid extraction. Lipids were fractionated by TLC, and the spots were assayed for radioactivity by liquid scintillation counting. Results are expressed as means ± SEM (n = 4) of ^{14}C/^{3}H ratio found in DAG-Lp and fat body acylglycerols (DAG-FB and TAG-FB).

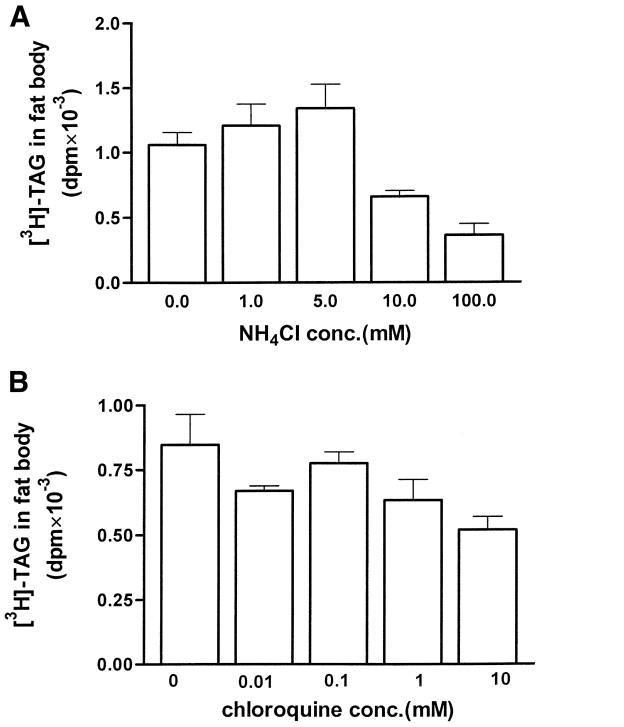

Fig. 4. Effect of endocytic inhibitors of on [^{3}H]DAG transfer in vitro. Labeled Lp was incubated with fat body for 1 h in the presence of the endocytic inhibitors ammonium chloride (A) and chloroquine (B). Results are expressed as total dpm ± SEM (n = 3–4) found in fat body TAG. conc., concentration.

Lipid binding to the external surface of the fat body

To test the possibility that DAG fat body represents a pool attached to the external surface of the fat body, even after Lp was washed away, we treated the fat body with lipase. The rationale for these experiments was that if the lipid remained on the external surface of the fat body, it would be accessible to a lipase added to the medium; on the other hand, if the lipid had been transferred into the tissue, it would not be accessible to lipase. As judged by the production of oleic acid after lipase addition, a significant fraction of the lipids present in the fat body sample after extensive washing, and with or without treatment with anti-LTP antibody, were still on the external surface of the fat body (**Fig. 5**). Our results indicate that added lipase was able to hydrolyze DAG in the anti-LTP antibody-insensitive pool into free fatty acid, which in turn was captured by BSA in the medium. In addition, DAG in this pool was being supplied from the hydrolysis of fat body TAG, which was decreased by 75%. Thus, we concluded that the majority of the 40% of the lipid bound to the fat body after anti-LTP treatment represents lipid present on the external surface of the fat body.

Characterization of the lipid transfer process

Previously, we documented the presence of a Lp receptor on fat body of *M. sexta* (25) and that binding of Lp to its receptor is calcium-dependent. As shown in **Fig. 6**, neither Ca^{2+} nor EDTA in the incubation medium had a significant effect on lipid transfer. The transfer of lipid occurs best below pH 6.0 (**Fig. 7**), although the pH of hemolymph is 6.5. In addition, suramin, which inhibits the binding of Lp to its receptor (25) and binds to Lp, rendering it unavailable for binding (26), also inhibited the transfer of DAG from Lp to fat body (**Fig. 8**). Taken together, these results are consistent with the suggestion that both LTP and a Lp receptor are involved in lipid transfer to the fat body.

Role of LTP in export of lipid during the larval stage

To determine the role of LTP and Lp receptor in the reverse transfer of DAG from the larval and wandering stages to Lp, we incubated [^{3}H]DAG-labeled fat body tissues with larval Lp and determined the amount of [^{3}H]DAG transferred to Lp (**Fig. 9**). The transfer of DAG between Lp and fat body is bidirectional. This occurs with fat body from either feeding-stage larvae or wandering-stage larvae. However, the uptake of DAG from Lp to the fat body was 3-fold higher than in the reverse direction, confirming the function of the fat body as a storage organ. In addition, lipid transfer in both directions is inhibited by anti-LTP antibodies and by suramin, suggesting that the basic process may be the same.

Developmental changes in lipid transfer

Figure 10 depicts the changes in the extent of DAG transfer from and to Lp at different developmental stages. The bidirectional transfer of lipid in this in vitro system

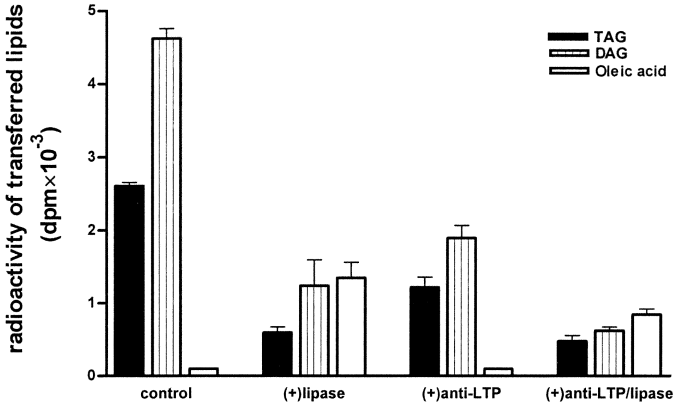

Fig. 5. Lipase susceptibility of fat body surface on [^{3}H]DAG. Fat body was incubated for 2 h in incubation medium containing [^{3}H]DAG-Lp (1.5 mg/ml) followed by extensive washing. The washed fat body was transferred to a tube containing 200 mg of fatty acid-free BSA in the absence (control) or presence of lipase [(+)lipase] and incubated at 34°C for 30 min. Or the fat body was preincubated with anti-LTP antibody for 30 min and then transfer studies were carried out in the absence [(+)anti-LTP] or the presence of lipase [(+)anti-LTP/lipase]. After incubations, fat bodies were washed vigorously with lepidopteran saline and then processed for lipid extraction. Lipids were fractionated by TLC, and spots were assayed for radioactivity by liquid scintillation counting. Results are expressed as total dpm ± SEM (n = 3–4) found in DAG, TAG, and oleic acid fractions.

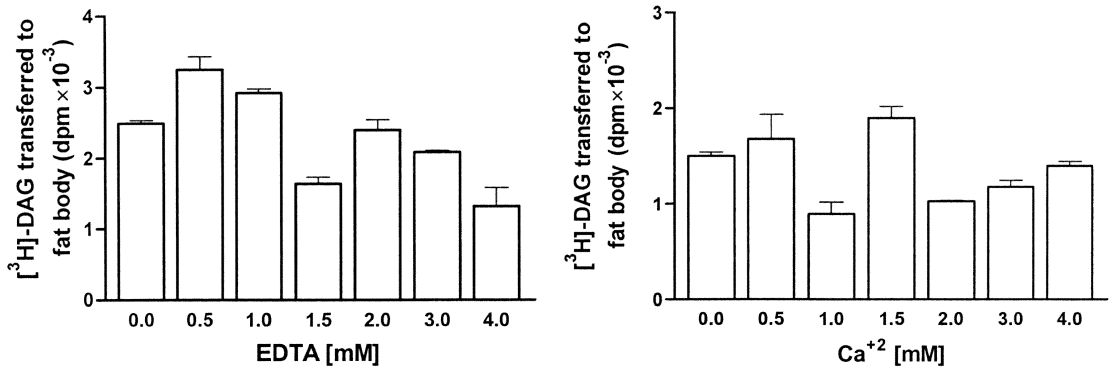

Fig. 6. Effect of EDTA and calcium on [^{3}H]DAG transfer from labeled Lp to fat body. Dissected fat bodies were incubated in Grace's medium containing [^{3}H]DAG-Lp (1.5 mg/ml) and the indicated concentration of EDTA (left panel) or Ca^{2+} (right panel). In the experiments shown at right, all tubes contained 1.5 mM EDTA. Transfer studies were carried out as described in Materials and Methods. Results are expressed as total dpm ± SEM (n = 4–5).

decreased significantly from the feeding larval stage until the second day of wandering. The percentage decrease in the amount of DAG transferred from fat body to Lp and from Lp to fat body ranged from 7% to 60% and from 20% to 50% in the wandering stages compared with day 4 of fifth instar, respectively.

DISCUSSION

Vertebrate systems contain several types of lipoproteins that deliver their neutral lipids to target tissues by a combination of lipoprotein lipase-mediated lipolysis (chylomicrons and VLDL) or endocytosis and degradation of the whole particle (LDL and chylomicron remnants) [reviewed in ref. (2)]. In the more versatile insect system, the same basic Lp particle carries a wide variety of lipids and selectively delivers specific lipids to specific tissues, e.g., cholesterol to oocytes (13, 27) and fat body (27), hydrocarbons to the cuticle (7), and carotenoids to the cuticle or the silk gland, etc. (28). In addition, in insects, lipid delivery to tissues, for the most part, takes place without internalization and degradation of Lp [reviewed in ref. (4)]. This observation led to the idea that Lp functions as a reusable shuttle, removing lipid from sites of absorption or storage to sites of utilization.

In vivo, Lp from feeding *M. sexta* larvae delivers DAG to the fat body, where it is stored as TAG that becomes the most prevalent lipid in fat body, amounting to nearly 30% of its wet weight (5, 29). The appearance of [^{3}H]TAG in the fat body after in vitro incubation indicates that DAG from Lp was taken up by the tissue and converted to TAG. However, the label associated with fat body DAG, which accounted for 30–40% of total lipids, could be the consequence of DAG taken up but not yet converted to TAG, or DAG might still be associated with Lp bound to fat body membranes, which could not be eliminated by washing the tissues. In this case, it might be expected that incubating labeled fat body tissues with unlabeled Lp would displace the remaining [^{3}H]DAG-Lp bound to fat body cells. However, when fat was pre-incubated with [^{3}H]DAG-Lp for 2 h, incubated with unlabeled Lp, high salt (300 mM NaCl), or 2 mM suramin, which has been shown to bind

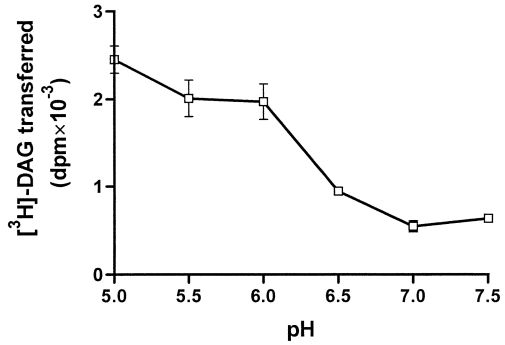

Fig. 7. Effect of pH on [^{3}H]DAG transfer. Labeled Lp was dialyzed into a buffer containing MES (0.01 M), MOPS (0.01 M), Trizma base (0.5 mM), and NaCl (0.15 M) of different pH values between 5.0 and 7.5. Labeled Lp (1.5 mg/ml) was incubated with fat body for 2 h, and transfer studies were carried out as described in Materials and Methods. Results are expressed as total dpm ± SEM (n = 3–4).

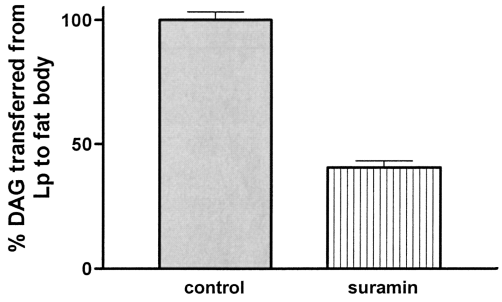

Fig. 8. Effect of suramin on [^{3}H]DAG transfer. Fat body was preincubated in 5 mM suramin for 30 min and then washed extensively with Grace's medium before adding [^{3}H]DAG-Lp (1.5 mg/ml). After incubation for 2 h, the fat body was washed and lipids were analyzed as described for Fig. 1. Results are expressed as percentage DAG transferred ± SEM (n = 3–4).

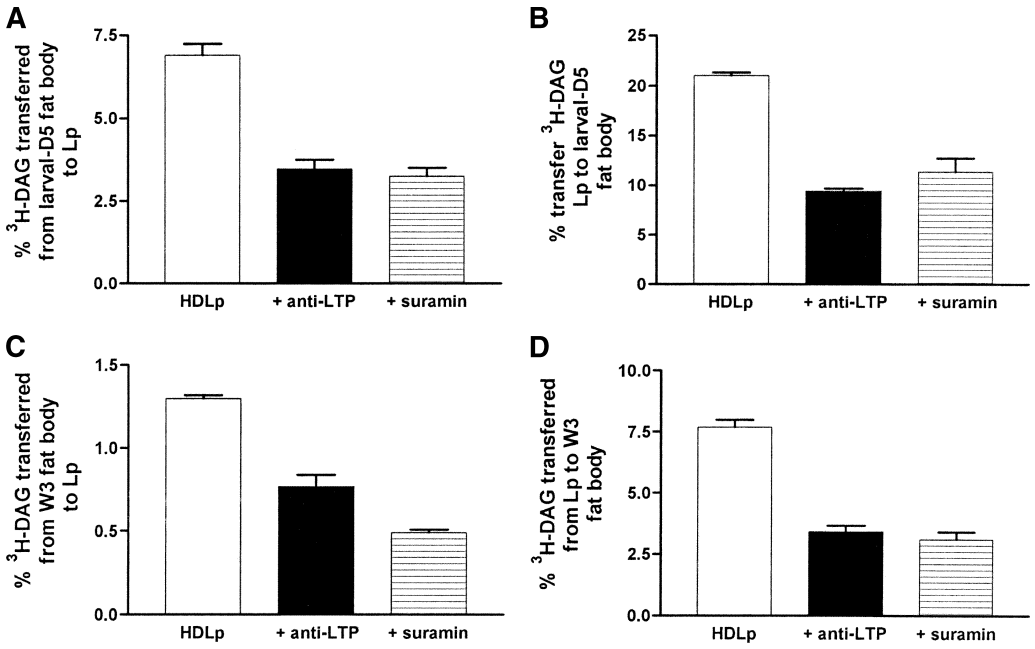

Fig. 9. Lipid transfer in feeding-stage and wandering-stage larvae. To measure lipid transfer from Lp to fat body, [^{3}H]DAG-Lp (1.5 mg/ml) was incubated with fat body (120 mg) from either day 5 fifth instar larvae (A) or day 3 wandering larvae (C). To measure lipid transfer from fat body to Lp, [^{3}H]lipid-labeled fat body from day 5 fifth instar larvae (B) or from day 3 wandering larvae (D) was incubated with unlabeled Lp (1 mg/ml). In each experiment, lipid transfer was measured in control experiments [high density lipophorin (HDLp) alone] and in the presence of either anti-LTP antibodies or suramin. Results are expressed as percentage DAG transferred ± SEM (n = 3–5).

Lp (26), no changes in the amount of DAG-associated fat body cells were observed (data not shown), indicating that [^{3}H]DAG is not associated with Lp that is reversibly bound to fat body. The presence of radioactive DAG in the fat body cells is not limited to our present report. Van Heusden and Law (11) also reported the presence of radioactive DAG when fat body cells from adult *M. sexta* were incubated with [^{3}H]DAG-Lp. However, they did not carry out further experiments to clarify its origin.

Role of LTP in DAG uptake by fat body

The release of labeled DAG from the tissues to Lp was completely inhibited by treating TAG-labeled adult fat body tissues (11) or DAG-labeled larval midgut sacs (12) with anti-LTP antibody. In both systems, the DAG transfer activity was restored to normal by exogenous LTP. Although these results indicate that LTP plays an essential role in promoting DAG transfer from the cells to Lp, the mechanism is unknown. Thus, it was of interest to establish if LTP plays any role in DAG transfer from larval fat body cells to Lp. Treatment of fat body tissues with anti-LTP antibody before incubation with [^{3}H]DAG-Lp resulted in a significant decrease of fat body DAG (35–40%) and fat body TAG (60–65%). The extent of the incomplete inhibition did not change when increasing concentrations of anti-LTP antibody were tested. However, when LTP was exogenously added to tissues that were pretreated with anti-LTP antibody, the amount of lipid transferred to the fat body was restored to control values, indicating that LTP plays an essential role in the transfer of DAG from Lp. Note that lipid transfer from Lp to fat body occurred in the absence of added LTP (Figs. 1, 2A). This observation suggested that the fat body itself contains intrinsic LTP. LTP is synthesized and secreted by the fat body into the hemolymph (10). It is also likely that binding of hemolymph LTP to fat body cells (which cannot be eliminated even with extensive washing) might be sufficient to facilitate lipid transfer. The intrinsic presence of LTP is consistent with reports that although LTP was required for the transfer of DAG from larval *M. sexta* midgut to Lp and from adult fat body to Lp, the addition of an exogenous source of LTP was not (12).

Pathways for DAG accumulation in fat body

DAG is the unique lipid species used by insects to transport dietary fatty acids from midgut to hemolymph and for its storage as TAG in fat body. During the in vitro characterization of lipid transfer from Lp, we encountered the presence of labeled DAG in the fat body. Although the presence of DAG in fat body after in vitro incubation with Lp has been reported previously (11), its localization and origin have never been addressed. Thus, it was of interest to characterize this pool.

DAG-associated fat body was only partially inhibited by anti-LTP antibody. More than one pathway could account for the observation that anti-LTP antibodies reversibly reduced DAG transfer to larval fat body by only

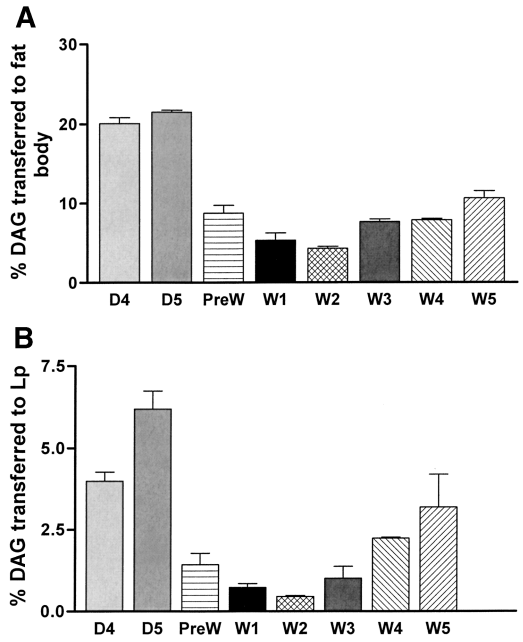

Fig. 10. Lipid transfer in feeding and nonfeeding stages of the fifth larval stadium. The nomenclature used to describe the stages is as follows: D4 and D5 refer to day 4 and day 5 of the feeding fifth instar larvae; PreW is 12 h after day 5 and represents the beginning of the wandering stage; W1–W5 represent 1–5 days after day 5. Transfer studies were carried out by incubating 120 mg of body fat from different stages with Lp (1.5 mg/ml) for 2 h, and transfer studies were carried out as described in Materials and Methods. A: Transfer of DAG from Lp to fat body. B: Transfer of DAG from fat body to Lp. Results are expressed as percentage DAG transferred ± SEM (n = 3–4).

60–65%. One possibility would be the presence of a membrane-bound lipoprotein lipase in fat body. This enzyme would hydrolyze DAG to fatty acids and glycerol, which in turn would be taken up by the tissue and resynthesized into TAG. However, incubating fat body tissues with dual-labeled DAG-Lp in [^{14}C]fatty acid and [^{3}H]glycerol moieties in the presence or absence of glycerol had no effect on the ^{14}C/^{3}H ratio, demonstrating that a lipoprotein lipase does not play a role in this transfer process and that no prerequisite hydrolysis of DAG is required for its transfer from Lp. Although specific membrane-bound Lp lipases have been found in flight muscle (30–32) and developing oocytes (33), such an enzyme does not appear to be relevant for lipid delivery to larval fat body.

Another possibility for the presence of an LTP-independent DAG pool is the endocytosis of Lp, although we have shown previously that this is not a pathway in *M. sexta* (18). Endocytosis of Lp has been reported to occur in *Aeshna cyanea* (34, 37) and *L. migratoria* (35, 36). In *A. cyanea*, the intracellular localization of Lp revealed its presence in the secretory pathway, as would be expected for a secreted protein and in endosomes, consistent with an endocytotic uptake. The authors postulated that endocytosis of Lp may be involved in the removal of old or damaged Lp (37).

In *M. sexta* (25) and *L. migratoria* (38), a Lp receptor has been purified and characterized. In contrast to the *M. sexta* receptor, *L. migratoria* Lp receptor does not require divalent cations, has a broader specificity as it can bind human LDL, and is involved in the endocytosis of Lp (35). Two endocytic inhibitors, ammonium chloride and chloroquine, exhibited no effect on the amount of DAG transferred to larval *M. sexta* fat body even when used at concentrations that were 10-fold and 1,000-fold higher than normally reported, respectively. In contrast to our findings, ammonium chloride and chloroquine completely reduced the amount of intracellularly localized Lp concentrations in adult *L. migratoria*. Furthermore, by using ammonium chloride, Dantuma et al. (35) demonstrated that the inhibition of endocytosis did not significantly affect the rate of exchange of DAG and cholesterol. The authors also postulated that the majority of the endocytosed Lp is not lysosomally degraded and may be resecreted after endocytosis, depleted of its lipid load (35). However, this internalization/secretion cycle cannot readily account for the tissue-specific lipid transfer observed in *M. sexta* as well as the absence of delipidated Lp in hemolymph at all developmental stages.

Lack of Lp endocytosis confirms our earlier report in which larval fat body tissues were incubated with labeled Lp for 1–4 h and then the amount of radioactivity transferred to fat body was determined; in addition, the incubation media were collected, and Lp was repurified and its concentration determined (18). During the 4 h period, about 95% of labeled DAG was taken up, but there was no loss of Lp protein from the incubation medium. In a more recent report, we have shown that a progressive and significant increase in the accumulation of lipids (TAG) in *B. mori* ovaries after incubation with Lp corresponded to the gradual loss of the lipid from Lp without internalization of the protein, as confirmed by immunodiffusion assays (13). Based on these data, we concluded that in larval fat body of *M. sexta*, neither a lipoprotein lipase nor Lp endocytosis could account for the LTP-independent transfer of DAG from Lp to fat body.

Because we could not account for the LTP-independent transfer of DAG from Lp to fat body by any known mechanism, we investigated the possibility that this labeled lipid represented a pool of lipid attached to the external surface of the fat body, even after Lp was washed away. This might represent lipid in transit from Lp into the cell or it might be attributable to irreversible adsorption of Lp to fat body. As described above, we eliminated reversible adsorption of Lp as a cause when we showed that unlabeled Lp did not displace this pool of labeled lipid from the fat body. At present, we do not know whether this lipid represents irreversibly adsorbed Lp. However, in *L. migratoria*, Lp that is endocytosed does not accumulate in the cells, whereas its lipid moiety is able to accumulate intracellularly (35).

Extracellular localization of this DAG pool was studied using exogenous lipase that is able to hydrolyze extracellularly located lipids. The fact that lipase was able to hydrolyze the DAG-associated fat body (although not

completely) and liberate oleic acid into the media demonstrated the presence of this pool extracellularly. Our results also confirmed the continuous communication of this DAG pool, directly or indirectly, with the intracellular pool of TAG. Thus, we concluded that the majority of the 35–40% of DAG-associated fat body after anti-LTP treatment represents lipid present on the external surface of the fat body and that this DAG pool could be an intermediate pool in the lipid transfer process. Given these caveats, we propose that lipid transfer from Lp to fat body in larval *M. sexta* is mediated by a Lp receptor and LTP and that the majority of the transfer is LTP-dependent. Recently, we proposed a mechanism by which LTP facilitates lipid exchange between Lp and tissues (4). One possibility is that LTP plays a role in the formation of the Lp-receptor complex. Alternatively, LTP might act to enhance the function of the tissue-specific lipid transfer factors, which in turn could determine the direction of the lipid transfer. Further experiments are needed to explore these hypotheses.

Developmental changes in lipid transfer

In the midgut of *M. sexta*, DAG is transferred from the tissue to Lp, but the reverse reaction does not occur (12), consistent with the nutrient physiological pathway of absorption from the midgut and its storage in the fat body. In contrast, transfer of DAG between Lp and fat body is bidirectional, emphasizing the function of the fat body as a storage organ that allows mobilization of nutrients in both directions. The mobilization of DAG occurs with fat body from either feeding-stage larvae or wandering-stage larvae. Lipid transfer in both directions is inhibited by anti-LTP antibodies and by suramin, suggesting that the basic process may be the same. The net accumulation of lipid in the larval feeding stage and the mobilization of lipid in the wandering stage (39) must reflect a balance between these two opposing processes. The changes in lipid uptake may be controlled by developmental hormones, resulting in a temporal regulation of lipid mobilization during periods in developmental stages, which will require further analysis.

In summary, Lp in *M. sexta* larvae serves as a reusable shuttle that moves lipids from one tissue to another without itself entering the cell (4, 40, 41). This indicates that lipid transfer from Lp to fat body (and to other tissues) basically occurs at the interface between the hemolymph and the plasma membranes. We recently reported that LTP is required to export DAG from the midgut cells to Lp in feeding *M. sexta* larvae (12). In this work, we demonstrated that LTP plays a critical role in facilitating lipid transfer from Lp to larval fat body. In contrast, lipid transfer from Lp to fat body in adult *M. sexta* was not mediated by LTP (11), but LTP facilitated lipid transfer from fat body to Lp. These findings indicate that LTP plays a role in the physiological direction of lipid metabolism in each stage: lipid loading of Lp at the midgut and the transfer of its lipid cargo at the fat body for feeding larvae, and lipid mobilization from fat body to other tissues in the adult stage.

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