Interaction of an insect lipoprotein with its binding site at the fat body

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Abstract A single type of high density lipophorin (HDLp) binding sites is present at intact fat body tissue and in fat body membranes of larval and adult locusts. HDLp is bound with high affinity ($K_d \approx 10^{-7}$ M). This interaction does not require divalent cations and is heat-labile because heat-treatment of fat body membranes results in a substantial reduction of the maximal binding capacity. In addition to unlabeled HDLp and low density lipophorin (LDLp), human low density lipoprotein also seems to compete with radiolabeled HDLp for this binding site, suggesting a relaxed specificity. Induction of lipid mobilization with adipokinetic hormone did not change the binding characteristics of the fat body. An increase in the binding capacity of intact fat body tissue in the adult stage suggests that the number of cell surface binding sites is upregulated during development. However, the total number of HDLp binding sites appears to be constant, because larval and adult fat body membranes have similar binding capacities.—**Dantuma, N. P., W. J. A. Van Marrewijk, H. J. Wynne, and D. J. Van der Horst.** Interaction of an insect lipoprotein with its binding site at the fat body. *J. Lipid Res.* 1996. **37:** 1345–1355.

Supplementary key words locust • lipophorin • receptor • development • lipid mobilization

It is well established that lipoproteins are of major importance in transporting hydrophobic molecules in the circulatory compartment of organisms. Although many different lipoproteins and tissues are involved, the majority of interactions between lipoproteins and target cells display common biochemical characteristics. In essence, lipoproteins are synthesized and secreted by donor cells and subsequently transported to acceptor cells, which internalize the lipoproteins by receptor-mediated endocytosis, followed by their breakdown in lysosomes (1).

During physical activity, lipids are transported in insects not in the form of fatty acids, which is the principal form in vertebrates, but as diacylglycerol (DAG), which requires lipoprotein carriers for transport in the circulation (2). Consequently, several adaptations are required to transport large amounts of this lipid in an energetically favorable manner. The major difference, compared to the common vertebrate lipoprotein transport mechanism, is that insects reuse the lipoprotein matrix (3, 4). For example, during sustained flight activity of gregarious locusts, DAG is loaded from the storage organ, the fat body, onto a lipoprotein, the high density lipophorin (HDLp), which contains two non-exchangeable apolipoproteins, apolipophorin (apoLp) I and II. In addition, several molecules of an exchangeable apolipoprotein, apoLp-III, associate with the expanding lipoprotein to stabilize the resulting lipid-rich low density lipophorin (LDLp). LDLp is transported to the muscles, where the DAG is hydrolyzed. As the lipid content of the lipoprotein particle diminishes, HDLp and free apoLp-III are regenerated and reused (2, 5, 6). This shuttle mechanism, which is initiated by the adipokinetic hormones (AKHs) (7), allows substrate delivery to the muscle without requiring new synthesis of apolipoproteins.

Although insect lipoproteins have been studied in detail, only limited attention has been paid to the interaction between insect lipoproteins and their target tissues. Because this shuttle mechanism is a result of selective transport of lipids between these lipoproteins and cells, understanding of the binding events involved is essential to elucidating the molecular basis for the way insect lipoproteins act as reusable shuttles. Differences between the classical endosomal-lysosomal delivery pathway and the shuttle mechanism of insect lipoproteins

Abbreviations: AKH, adipokinetic hormone; apoLp, apolipophorin; B_{max}, maximal binding capacity; BSA, bovine serum albumin; DAG, diacylglycerol; HBS, HEPES-buffered saline; HDLp, high density lipophorin; K_d, dissociation constant; K_n, nonspecific association constant; LDLp, low density lipophorin.

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may be reflected in the binding sites involved. In this study, we characterized the interaction between HDLp and the fat body of adult locusts. In addition, we examined HDLp binding to the fat body at different stages of development and after AKH-induced lipid mobilization.

MATERIALS AND METHODS

Animals

Gregarious locusts, *Locusta migratoria,* were reared under crowded conditions as described previously (8). For isolation of lipoproteins from the hemolymph, both male and female locusts were used. All other experiments were performed with male locusts only to exclude possible interaction with vitellogenic processes occurring in females (9).

Isolation and labeling of lipoproteins

Hemolymph was collected from adults (15–20 days after imaginal ecdysis) by puncturing with a Hamilton microsyringe, and was directly diluted 2-fold in ice-cold insect saline buffer (130 mM NaCl, 5 mM KCl, 1.9 mM NaH_2PO_4, 1.7 mM K_2HPO_4, 10 mM EDTA, pH 7.5). Hemocytes were removed by centrifugation (14,000 *g,* 5 min). HDLp and LDLp, with densities of ≈1.11 and ≈1.04 g/ml, respectively, were isolated from the hemolymph by KBr gradient ultracentrifugation in a vertical rotor (Sorvall TV 850) at 200,000 *g* for 4 h using a Sorvall OTD-2 ultracentrifuge. HDLp was isolated from untreated locusts and LDLp from hemolymph of locusts that had been injected, 90 min prior to collection of the hemolymph, with 10 pmol AKH-I (Peninsula Laboratories), which gives a complete conversion of HDLp into LDLp.

Lipoproteins were labeled in vitro in the protein moiety with [^{3}H]succinimidyl-propionate (Amersham) according to Bolton and Hunter (10), resulting in specific activities in the range of 5000–30,000 dpm/μg protein.

Isolation of membrane fraction

Fat body tissue was dissected and rinsed by gently shaking in 10 ml/fat body of HEPES-buffered saline (HBS: 10 mM HEPES, 150 mM NaCl, 10 mM KCl, pH 7.0) for 1 h at 4°C. After rinsing, the tissue was homogenized by 5 strokes of a motor-driven glass-Teflon homogenizer in sucrose buffer (250 mM sucrose, 2 mM KH_2PO_4, 8 mM K_2HPO_4, pH 7.4) including protease inhibitors (1 mM EDTA, 25 μg/ml PMSF). Large debris was removed by centrifugation at 1000 *g,* 0°C for 10 min. The supernatant was centrifuged at 100,000 *g,* 0°C for 1 h. The pellet, which contained insoluble proteins, was dissolved in HBS by mild sonification. The protein concentration of the membrane preparations was determined according to Schacterle and Pollack (11).

Tissue binding assay

Tissue binding assays were performed according to a modified version of the method described previously (12). Fat body tissue was dissected and rinsed by gently shaking in medium A (HBS + 4 mM $CaCl_2$ + 2 mM $MgCl_2$) and divided in equal parts. For adult locusts, each binding assay was performed with 1/8 fat body per incubation. For fourth and fifth larval instars, 1/8 fat bodies from two individuals were pooled to obtain similar amounts of tissue. Consequently, determination of bound labeled HDLp was limited to a maximum of eight different ligand concentrations per individual for adults or per two pooled individuals for larvae. Each sample was incubated with radiolabeled lipoproteins in 300 μl medium A under gentle mixing for 3 h at 4°C. Afterwards, the tissue was rinsed 6 times by floating each tissue sample for 5 min in 3 ml medium A. In Ca^{2+}/Mg^{2+}-dependency experiments, HBS without $CaCl_2$ and/or $MgCl_2$ was used for incubations and rinsing. Fat body tissue was homogenized in 200 μl distilled water by sonification. The amount of tissue was standardized by quantifying total fat body proteins. As large amounts of lipids in the fat body homogenate interfere with the protein assay, lipids were removed from 100 μl of homogenate with 450 μl chloroform–methanol 1:2 (v/v). After sonification, 300 μl chloroform–distilled water 1:1 (v/v) was added and the sample was centrifuged (14,000 *g,* 10 min). Protein content was determined in the infranatant, which had been dissolved in 0.5 M KOH by sonification and an incubation for 1 h at 55°C (11). Radioactivity was determined in Emulsifier Safe scintillation fluid (Packard) using a Tri-Carb 4550 liquid scintillation counter (Packard). From the radioactivity and protein content, the amount of bound HDLp per total fat body protein was calculated.

Fat body from AKH-I-treated animals was dissected 1 h after injection. For competition experiments, fat body tissue was incubated with tritiated HDLp in the presence of HDLp, LDLp, human low density lipoprotein (Sigma), and bovine serum albumin (BSA; Fraction V, Merck). The following molecular weights were used for the protein moieties of the lipoproteins: 289,000 for HDLp, 545,000 for LDLp (12) and 533,000 for human low density lipoprotein (13).

Solid phase binding assay

A modified version of the previously described enzyme-linked immunosorbent assay for monitoring lipoprotein binding was used (14). Plates (96-wells, Costar) were coated with fat body membrane preparations (0.5 μg membrane protein/well) for 16 h at 4°C in HBS. All

following incubations were performed in medium A + 1% BSA for 1 h at 37°C, unless otherwise stated. After each incubation, plates were washed 5 times with medium A. After coating, plates were blocked with 150 µl medium A + 1% BSA and subsequently incubated with 100 µl HDLp in medium A. The amount of bound HDLp was quantified by an enzyme-linked immunosorbent assay: wells were incubated with 100 µl of a 1:5000 dilution of rabbit anti-HDLp polyclonal antibodies (14), followed by an incubation with 100 µl of a 1:5000 dilution of a goat anti-rabbit peroxidase complex (Sigma). After staining the wells with 75 µl *o*-phenylenediamine (Baker Chemicals, 4 mg in 10 ml 0.25 M citrate, 51.4 mM Na_2HPO_4, 5 µl 30% H_2O_2) for 10 min, the reaction was terminated by addition of 75 µl 1 M H_2SO_4. Extinction was recorded at 490 nm, using a microplate reader (Bio-Rad model 450). Each plate contained a standard incubation in order to allow comparison of different plates. Temperature sensitivity of the binding site was determined by preincubation of the membrane preparation for 15 min at different temperatures. Binding of HDLp in the presence of increasing amounts of NaCl was determined in HBS containing 30 mM HEPES instead of 10 mM.

Statistical analysis

Tissue binding assays were evaluated by unweighted least squares regression, simultaneously fitting the measurements from five experiments (each experiment represents one individual for adults or two pooled individuals for larvae) to the ligand binding model $B = B_{max}F/(K_d + F) + K_nF$, where B is the observed binding, F is the free HDLp concentration, K_d is the dissociation constant of HDLp, and K_n is the nonspecific association constant (15–17). The maximum binding capacity is represented in the model by $B_{max} = B_1 + B_2D_2 + B_3D_3 + B_4D_4 + B_5D_5$, where the dummy-variable $D_i = 1$ for the i-th experiment and zero for all other experiments, and where B_1, B_2, B_3, B_4, and B_5 are model parameters to be estimated. Thus, B_1 is the binding capacity in the first experiment and $B_1 + B_2$ is the binding capacity in the second experiment etc. The model allows for a different value of the binding capacity in each single experiment due to one (or two pooled) individual(s). The data obtained with the solid phase binding assay were fitted to the ligand binding model without the dummy-variable. Analyses with different models were tested for significant differences by a partial *F*-test. Parameter estimates are expressed as fitted value ± asymptotic standard error.

Differences between group means were tested for overall statistical significance by one-way analysis of variance followed, where appropriate, by a Student-Newman-Keuls procedure to test all possible differences. Otherwise a Student's *t*-test was used to compare group means.

Data are expressed as mean ± standard error of the mean (SEM). A level of statistical significance of $P = 0.05$ was adopted throughout this paper.

RESULTS

Interaction between HDLp and intact fat body tissue

Binding studies were performed using intact fat body tissue in a medium containing radiolabeled lipoprotein. This resembles the in vivo situation, in which the fat body is surrounded by hemolymph due to the open circulatory system of insects. Furthermore, the fat body tissue is composed of lobes that are only two cell layers thick and therefore all cells are in direct contact with the incubation medium (18). In order to inhibit physiological activity of the fat body, which might result in processing of bound lipoproteins, binding studies were performed at 4°C. Time course experiments with fat body tissue from adult locusts, 15 days after imaginal ecdysis, revealed that a substantial amount of radiolabeled HDLp was bound to the fat body tissue (**Fig. 1**). Binding of radiolabeled HDLp in the presence of a 50-fold excess of unlabeled ligand resulted in a strong reduction of the amount of bound radiolabeled HDLp, indicative of the presence of saturable HDLp binding sites at the fat body. Although binding of HDLp occurs mainly during the first 2 h, complete equilibrium was not established within 4 h. Because long incubations may induce cell

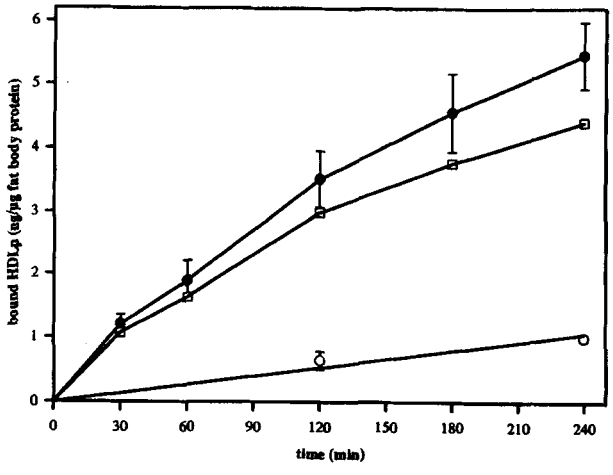

Fig. 1. Time course of total (●), specific (□), and nonspecific (○) binding of labeled HDLp (0.1 mg/ml) to fat body tissue of adult locusts (15 days after imaginal ecdysis). Nonspecific binding was determined as the amount of bound tritiated HDLp in the presence of a 50-fold excess of unlabeled HDLp. Specific binding was calculated by subtracting the estimated value along the linear regression line for nonspecific binding from the total binding. Error bars represent the SEM (n = 9 for total binding, n = 5 for nonspecific binding).

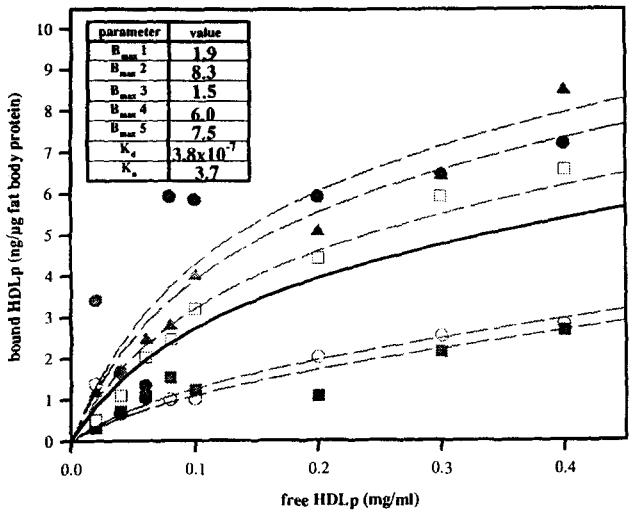

Fig. 2. Saturation kinetics of total binding of labeled HDLp to fat body tissue of adult locusts (15 days after imaginal ecdysis). Each dashed curve represents a curve obtained for fat body tissue of one individual. The solid curve represents the mean of the individual curves. The data were fitted as described in Materials and Methods. K_d, B_{max}, and K_n are expressed as M, ng bound HDLp/µg fat body protein, and (ng × ml)/(mg × mg), respectively.

damage, we preferred to use a 3-h incubation period for our experiments. Consequently, values determined for the B_{max}, K_d, and K_n should be considered as approximate.

Simultaneous fitting of independent binding assays is appropriate in cases where notable heterogeneity exists for B_{max}, K_d, or K_n (15–17). The large interindividual variation in our data appears to be due to differences in B_{max} values, because simultaneous fitting with different B_{max} values for the individuals proved to be significant against the alternative simple model with an undifferentiated B_{max} (partial F-test, $P < 0.05$). Moreover, fitting either the raw data or the means of the binding measurements to the simple model gives statistically nonsignificant values for B_{max}, K_d, and K_n, whereas fitting the raw data to the extended model gives statistically significant results. Consequently, we used the extended model which allows for a different B_{max} value for each individual, and supposes K_d and K_n to be identical for all individuals. Fat body tissue from adult locusts appears to contain a single type of HDLp binding sites, as testing a model for two binding sites against the above-mentioned one-site model yielded no statistically significant result (partial F-test, $P > 0.05$). This binding site binds HDLp with an approximate K_d of 3.8 (± 1.8) × 10^{-7} M (**Fig. 2**). The average B_{max} of these five adult fat bodies is 5.0 ng HDLp/µg fat body protein. The high variation between the B_{max} values of the various individuals is

TABLE 1. Effect of Ca^{2+}/Mg^{2+} on total HDLp binding to intact fat body tissue

$CaCl_2$	$MgCl_2$	EDTA	bound HDLp
			ng/µg fat body protein
4 mM	2 mM	–	3.82 ± 0.66
4 mM	–	–	4.49 ± 0.90
–	2 mM	–	4.47 ± 0.85
–	–	–	4.41 ± 1.11
–	–	2 mM	4.70 ± 0.37

Comparison of total binding of labeled HDLp (0.06 mg/ml) to adult fat body (15 days after imaginal ecdysis) in the presence and absence of 4 mM $CaCl_2$, 2 mM $MgCl_2$, and 2 mM EDTA. Data are expressed as mean ± SEM (n = 5). There are no significant differences (paired t-test, $P > 0.05$).

demonstrated by its standard deviation of 3.2 ng HDLp/µg fat body protein.

For many lipoprotein-receptor interactions, in both invertebrates (19–21) and vertebrates (22, 23), ligand binding has been found to be Ca^{2+}-dependent. However, binding of HDLp to the locust fat body does not require extracellular Ca^{2+} or Mg^{2+}, as we could not detect any significant difference between HDLp binding in the presence or absence of these divalent cations (paired t-test, $P > 0.05$; **Table 1**).

Another binding characteristic is the ionic strength at which the interaction between the ligand and the binding site is optimal. Increasing the NaCl concentration results in a significant reduction of HDLp binding (paired t-test, $P < 0.05$; **Table 2**), suggesting that HDLp binding is optimal at low NaCl concentrations.

Competition experiments revealed that unlabeled HDLp and LDLp compete with radiolabeled HDLp for its binding site at the fat body (paired t-test, $P < 0.05$; **Table 3**). Examination of the competitive ability of human low density lipoprotein showed that this lipoprotein may compete with HDLp to a limited extent, though our sample sizes did not permit demonstration of this effect statistically significant (20-fold molar excess, paired t-test, $P = 0.052$). An unrelated protein, BSA, gave no reduction of HDLp binding at a 20-fold molar excess; instead a significant increase was observed in the presence of BSA (paired t-test, $P < 0.05$). The reason for this

TABLE 2. Effect of NaCl concentration on total HDLp binding to intact fat body tissue

[NaCl]	bound HDLp
	ng/µg fat body protein
150 mM	4.56 ± 0.43
300 mM	2.30 ± 0.55[a]

Fat body tissue was incubated with 0.1 mg/ml labeled HDLp in incubation medium containing 150 and 300 mM NaCl. Data are expressed as means ± SEM (n = 5).

[a]Significantly different from control (paired t-test, $P < 0.05$).

TABLE 3. Competition between labeled HDLp and unlabeled HDLp, LDLp, human low density lipoprotein, and BSA for binding to intact fat body tissue

competitor	concentration	bound HDLp
	μM	*ng/μg fat body protein*
–	–	1.47 ± 0.19
HDLp	0.70	0.78 ± 0.05[a]
LDLp	0.70	0.93 ± 0.07[a]
Human low density lipoprotein	0.70	1.19 ± 0.13
	7.0	0.99 ± 0.07
BSA	7.0	2.06 ± 0.18[a]

Fat body tissue from adult locusts (15 days after imaginal ecdysis) was incubated with 0.35 μM labeled HDLp in the presence of unlabeled HDLp, LDLp, human low density lipoprotein, or BSA. Data are expressed as means ± SEM (n = 5).

[a]Significantly different from control (paired *t*-test, $P < 0.05$).

is not yet clear. In the binding studies with the immobilized membranes, which are discussed below, where BSA was used as blocking protein, we did not observe substantial specific HDLp binding to BSA, so this cannot be the explanation.

Administration of AKH evokes the mobilization of DAG and this, in turn, results in the conversion of HDLp into LDLp (7). To examine HDLp binding to lipid-mobilizing fat bodies, adult locusts were injected with 10 pmol AKH-I and fat body tissue was dissected after 1 h. The binding characteristics of the fat bodies from AKH-treated locusts did not differ from those of untreated locusts (**Fig. 3A**). The K_d, 5.7 (± 2.3) × 10^{-7} M, as well as the average B_{max}, 6.4 ng HDLp/μg fat body protein, is similar to the K_d and B_{max} values of untreated animals.

Lipid metabolism in adult insects differs in many respects from that in larvae. For instance, the ability to produce substantial amounts of LDLp is restricted to the adult stage of locust development (24). This might be due to different binding characteristics of larval and adult fat bodies. Therefore, binding characteristics of fat bodies of fifth instar larvae, 4 days before imaginal ecdysis, were studied (Fig. 3B). It was demonstrated that fat bodies of larvae bind HDLp with a K_d of 5.1 (± 2.1) × 10^{-7} M, which is similar to the K_d of adult fat bodies. However, significantly less HDLp is bound to fat bodies of fifth instar larvae (unpaired *t*-test, $P < 0.05$), as evidenced by the average B_{max} of larval fat body: 1.4 ng HDLp/μg fat body protein, which is only 28% of the average B_{max} of adult fat body.

To determine the developmental period in which the HDLp binding capacity of the fat body increases, we quantified the amount of bound HDLp during different developmental stages from fourth instar larvae up to 14 days after the imaginal ecdysis. This experiment was performed at a low HDLp concentration, at which only a minor amount of the total bound HDLp is attributable to nonspecific binding. The binding capacity appeared to remain constant during the fourth and fifth larval stages and the first 7 days of the adult stage (**Fig. 4**). A significant increase in HDLp binding was observed from day 7 to day 11 (Student-Newman-Keuls test, $P < 0.05$), after which the binding capacity remained relatively constant.

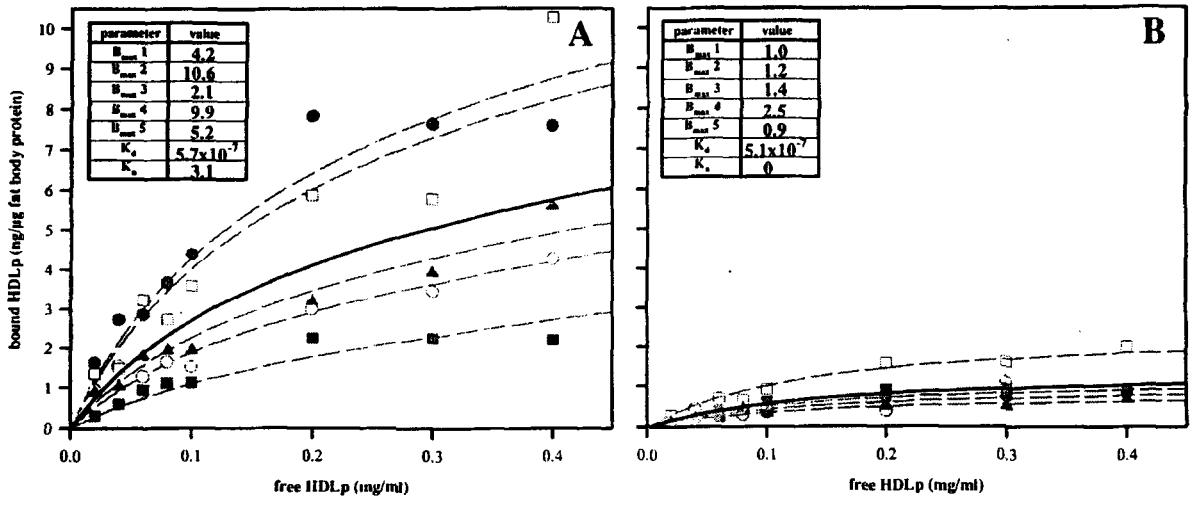

Fig. 3. Saturation kinetics of total binding of labeled HDLp to fat body tissue of AKH-treated adult locusts (15 days after imaginal ecdysis) (A), and larval locusts (fifth instar, 4 days before imaginal ecdysis) (B). Each dashed curve represents a curve obtained for fat body tissue of one individual (adults) or two pooled individuals (larvae). The solid curve represents the mean of the individual curves. The data were fitted as described in Materials and Methods. K_d, B_{max}, and K_n are expressed as M, ng bound HDLp/μg fat body protein, and (ng × ml)/(mg × mg), respectively.

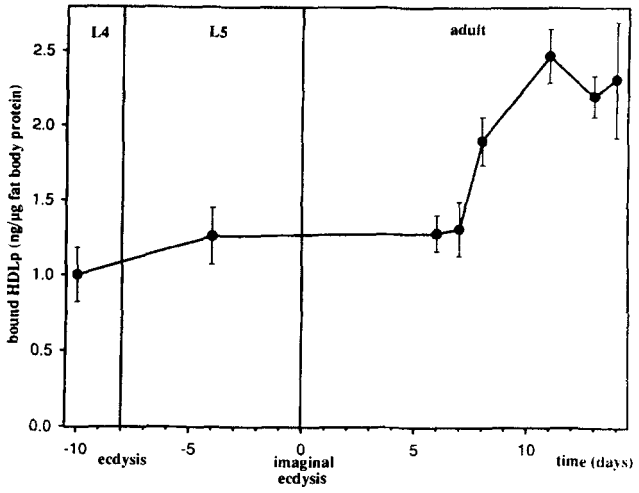

Fig. 4. Total binding of HDLp to fat body tissue during development. Fat bodies of locusts of different developmental stages were incubated with 0.07 mg/ml labeled HDLp. Error bars represent the SEM (n = 5). L4, fourth larval instar; L5, fifth larval instar.

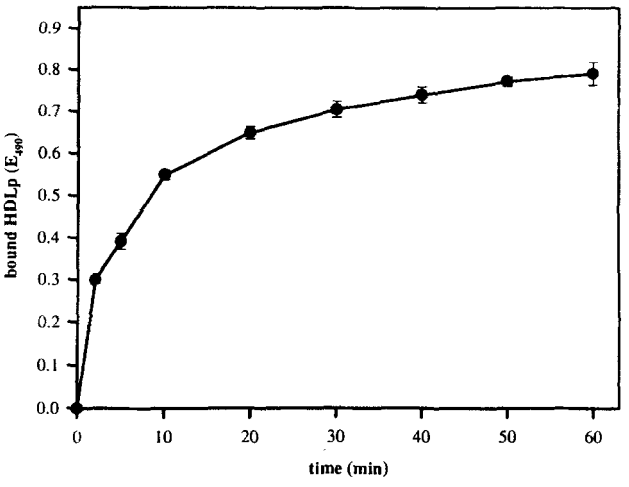

Fig. 5. Time course of total binding of HDLp (0.1 mg/ml) to immobilized fat body membranes from adult locusts (15 days after imaginal ecdysis). Error bars represent SEM (n = 5).

Interaction between HDLp and fat body membranes

An additional binding assay was used that enabled us to study the interaction between HDLp and immobilized fat body membranes. Bound HDLp was measured with an enzyme-linked immunosorbent assay as described previously (14). Two important modifications were introduced: *1)* isolated membranes were used instead of total fat body proteins, and *2)* we used untreated membrane preparations in the present study instead of solubilized precipitated proteins. A dose–response relationship between the amount of immobilized fat body membranes and the amount of bound HDLp indicated that HDLp interacts specifically with the immobilized membranes (not shown). In each experiment, a control lacking HDLp was included to allow subtraction of background signal in the membrane preparations. Background appeared to be caused by endogenous HDLp, as could be demonstrated by Western blotting (not shown). The presence of HDLp in the fat body membrane preparations is not surprising, as HDLp is synthesized by fat body cells (25).

Time course experiments with immobilized fat body membranes indicated that binding approaches equilibrium after approximately 30 min at 37°C (**Fig. 5**). All following experiments were performed using an incubation time of 1 h. The HDLp binding site, observed with intact fat body tissue, appears also to be present in membrane preparations of adult fat body tissue, as shown by saturation kinetics (see Figs. 6 and 8). The saturation kinetics most likely fit a ligand binding model for a single binding site with a linear nonspecific component, as addition of a second binding site to this model did not result in a significant improvement of the fit (partial *F*-test, $P > 0.05$). The average K_d obtained for adult locusts, 15 days after imaginal ecdysis (13 independent experiments with five different membrane preparations), is 1.18 (± 0.18) × 10^{-7} M.

Heat-treatment of membrane preparations results in a strong temperature-dependent reduction of the B_{max} (**Fig. 6**). After preincubation of a membrane preparation for 15 min at 100°C, the B_{max} is only 27% of the B_{max} of an untreated control.

With the immobilized membranes we could confirm that this binding site does not require divalent cations

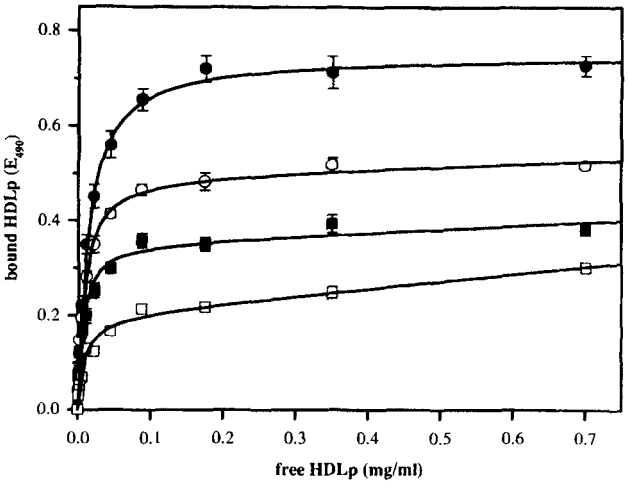

Fig. 6. Saturation kinetics of total HDLp binding to untreated immobilized fat body membranes of adult locusts (15 days after imaginal ecdysis) (●) and membranes that had been incubated for 15 min at 60°C (○), 80°C (■), and 100°C (□). Error bars represent the SEM (n = 5).

for HDLp binding as observed with intact fat body tissue; binding in the absence or presence of divalent cations gave similar saturation curves (not shown).

As demonstrated above with intact fat body tissue, binding to immobilized membranes was also maximal at low NaCl. Initially, HDLp binding decreased almost linearly with increasing NaCl concentrations, and was reduced to 50% at 0.2 M NaCl (**Fig. 7**). Binding was reduced even further at NaCl concentrations exceeding 0.2 M.

Fat body membranes from fifth instar larvae, 4 days before imaginal ecdysis, bind HDLp with a K_d of 1.20 (± 0.02) × 10^{-7} M (three independent experiments with two different membrane preparations), which is similar to the affinity for HDLp of adult fat body membranes (**Fig. 8**). In contrast to the significant difference in the B_{max} values of intact fat body tissue of larvae and adults, no difference could be demonstrated between the B_{max} values of the membrane preparations of fat bodies of both developmental stages. The identical binding capacities cannot be due to coating with an excess of fat body membranes, as the protein concentrations of larval and adult fat body membrane preparations used for coating were similar for larvae and adults and below the saturable concentration for coating.

DISCUSSION

Fat bodies from larval and adult migratory locusts were shown to contain high-affinity HDLp binding sites. The estimated K_d values are 5.1 (± 2.1) × 10^{-7} M and 3.8 (± 1.8) × 10^{-7} M for larval and adult fat bodies, respectively, indicating the presence of a single type of HDLp binding sites during development. Although these values should be regarded as approximate because the binding experiments were performed under not fully equilibrated conditions, the K_d values are similar to the K_d of 3.1 × 10^{-7} M reported previously (12). The latter value had been obtained at a physiological temperature under equilibrated conditions, suggesting an acceptable viability of our experiments. In morphological studies, we found recently that HDLp is not only located at the superficial plasma membranes, which are directly exposed to the hemolymph, but also at the plasma membranes bordering the intercellular spaces (N. P. Dantuma, M. A. P. Pijnenburg, J. H. B. Diederen, and D. J. Van der Horst, unpublished results). These spaces may be less accessible to HDLp. Therefore, the fact that no equilibrium is obtained within 4 h might be due to limited transport or diffusion of HDLp into the intercellular spaces. This could explain why the average B_{max} of 5.0 ng HDLp/μg fat body protein, observed at low temperature, is lower than the 9.8 ng HDLp/μg fat body protein determined at physiological temperature (12), as transport or diffusion of HDLp to the intercellular space may occur at a lower rate at low temperature. Moreover, internalization of HDLp, as discussed below, could also give rise to higher B_{max} values at physiological temperature.

Fitting of the data was strongly improved when simultaneous fitting with different B_{max} values for the different individuals was performed. In general, simultaneous fitting increases the statistical power of analysis and explains the heterogeneity as a real phenomenon of biological variation among individuals (15–17). This

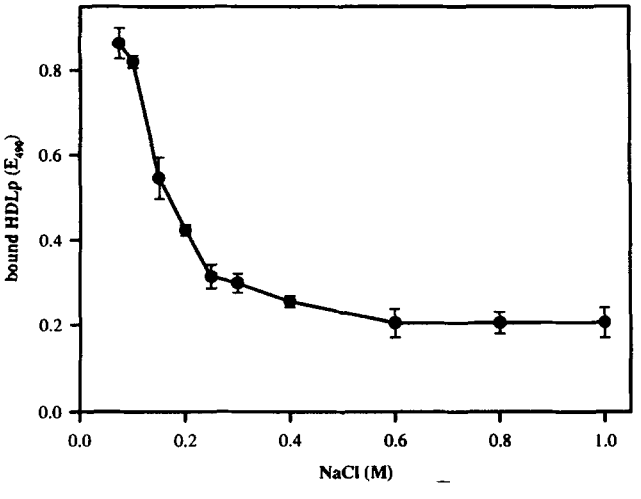

Fig. 7. Effect of NaCl concentration on total binding of HDLp (0.1 mg/ml) to immobilized fat body membranes of adult locusts. Error bars represent the SEM (n = 4).

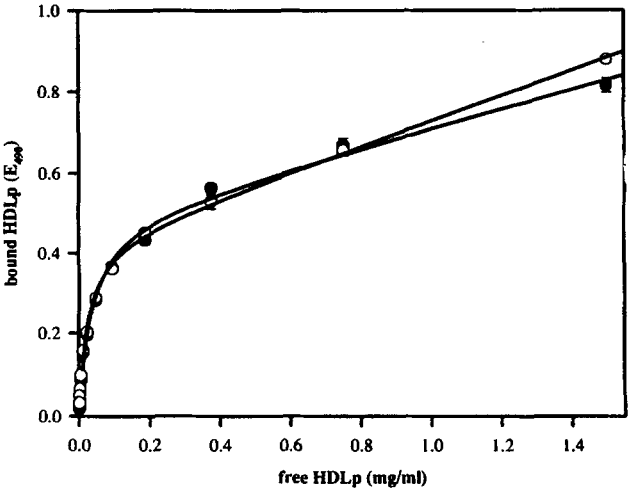

Fig. 8. Saturation kinetics of total binding of HDLp to immobilized fat body membranes of adults (15 days after imaginal ecdysis) (●) and larvae (fifth instar, 4 days before imaginal ecdysis) (○). Error bars represent the SEM (n = 4).

indicates that there is a substantial variation among the B_{max} values of the fat bodies, which can, for example, be due to differences in lipid metabolism.

Membrane preparations of fat bodies from larval and adult locusts bind HDLp with equal affinities (larval: K_d = 1.20 (± 0.02) × 10^{-7} M, adult: K_d = 1.18 (± 0.18) × 10^{-7} M), which are comparable with the K_d determined with intact fat body tissue at physiological temperature (12) and at low temperature, as found in this study. Previously, we have found that immobilized fat body proteins bind HDLp with a higher affinity than fat body tissue at physiological temperature (14). This discrepancy may be due to the experimental procedure used; in the previous study precipitated proteins from fat body homogenates were solubilized with an ionic detergent.

Many lipoprotein receptors, including members of the low density lipoprotein receptor gene superfamily, require Ca^{2+} ions for ligand binding (19–23). At variance with these receptors, the interaction between HDLp and its binding site is Ca^{2+}/Mg^{2+}-independent, because omission of these cations from the incubation medium was without effect.

The HDLp binding site may have a relaxed specificity, as not only unlabeled HDLp and LDLp compete with radiolabeled HDLp for the HDLp binding site, but also human low density lipoprotein, although to a limited extent. Competitive ability of human low density lipoprotein may be due to related structures in the apolipoproteins of these two lipoproteins, as suggested by the homology we found recently between the cDNA of the common apoLp-I/II precursor and human apoB-100, a major apolipoprotein in low density lipoproteins (5). On the other hand, relaxed specificity of lipoprotein receptors for apolipoproteins has been reported previously, mainly for the mammalian high density lipoprotein (26–28). It has been postulated that binding epitopes on lipoproteins may be class A amphipathic helical structures, which are typical for apolipoproteins (29), and this could explain the relaxed specificities of some lipoprotein receptors for apolipoproteins (30, 31).

Two additional binding characteristics were examined. First, the strong reduction in B_{max} for HDLp binding after incubation of the fat body membranes at high temperatures indicates that the HDLp binding site is heat-labile. Second, we found that the amount of bound HDLp is maximal at low ionic strength; increasing the NaCl concentration in the incubation medium strongly reduces HDLp binding.

No differences between the binding characteristics of fat bodies from AKH-treated and control adult locusts were observed with intact fat body tissue. It thus appears that if LDLp formation is mediated by this HDLp binding site, the abundance of these sites at adult fat body is sufficiently high to cope with the enhanced need for lipid mobilization during sustained flight activity. As yet, however, no conclusive evidence for the involvement of HDLp binding sites in LDLp formation is available, although both HDLp binding and LDLp formation are inhibited by monoclonal antibodies directed only against apoLp-II and not by those directed against apoLp-I (14, 32).

Compared with adults, larvae have similar but fewer HDLp binding sites at the fat body cell surface. The increase in the number of HDLp binding sites at intact fat body tissue was shown to occur between day 7 and day 11 after the imaginal ecdysis. This may reflect an important role in an adult-specific process such as flight activity, as this increase in binding sites coincides with several changes in flight muscle metabolism that are required for flight activity (33, 34). Interestingly, fat bodies from larvae demonstrate only limited conversion of HDLp into LDLp, even in the presence of high concentrations of apoLp-III (24). One of the limitations in larvae might be the number of cell surface HDLp binding sites at the fat body.

Surprisingly, the B_{max} values of the membrane preparations isolated from larval and adult fat bodies did not differ, suggesting equal amounts of HDLp binding sites in the fat body membranes of the two developmental stages. The membrane preparations used in this study, 100,000 *g* pellets of fat body homogenates, do not exclusively contain plasma membranes, but membranes of cell organelles will be present as well. Thus, the observed increase of the B_{max} of fat body tissue implies an increase in cell surface binding sites, whereas experiments with membrane preparations suggest a constant number of HDLp binding sites per fat body protein during development. A possible explanation for this observation is that internalization of HDLp by receptor-mediated endocytosis may give rise to intracellularly localized HDLp binding sites, which are inaccessible to the lipoproteins when intact fat body tissue is used. Down-regulation of endocytosis of HDLp binding sites during development could be responsible for an increase in cell surface binding sites. Thus, according to this hypothesis, larval fat bodies internalize more HDLp by receptor-mediated endocytosis than adult fat bodies.

Receptor-mediated endocytosis of lipoproteins is a common internalization pathway, reported for all lipoprotein receptors belonging to the low density lipoprotein receptor gene superfamily (1, 22, 35), and demonstrated for several insect lipoproteins as well (36–38). It has been shown that HDLp is internalized by the fat body of larval *Aeshna cyanea* (36). However, in *Manduca sexta* it has been demonstrated that DAG is selectively taken up from HDLp by fat body cells, which suggests that HDLp is not transported through the classical endosomal–lysosomal pathway (39). Two other tissues

involved in lipoprotein metabolism, the flight muscles of *L. migratoria* (40) and the gut of *A. cyanea* (41), do not internalize HDLp, implying that endocytosis of HDLp does not occur in all target tissues. Consequently, there are two possible mechanisms for lipid transport between HDLp and target cells: *1)* a surface-mediated transport mechanism, which includes binding of HDLp to the membrane, followed by transport of the lipids from or into the cell and *2)* a retroendocytotic transport mechanism, including endocytosis of HDLp, which accepts or donates lipids during the intracellular trafficking and resecretion of the modified lipoprotein in the circulation. The indications for intracellular localized binding sites, presented in this study, are in favor of intracellular trafficking of HDLp binding sites. Whether (retro)endocytosis of HDLp by fat body cells occurs is currently under investigation.

Specific binding sites for HDLp have been identified for the larval fat body and the gut of *M. sexta* (20, 42). The affinity of the HDLp binding site at the larval fat body of *M. sexta* (20) is much higher than the corresponding value determined in this study for *L. migratoria*. Ca^{2+}-dependency and suramin-sensitivity of the *M. sexta* fat body binding site are similar to the characteristics observed for classical lipoprotein receptors (19–23) but different from the *L. migratoria* HDLp binding site, which was shown to bind HDLp Ca^{2+}-independently. However, the HDLp binding site at gut cells of *M. sexta* shows a similar Ca^{2+}-independency and a similar affinity ($K_d \approx 10^{-7}$ M) (42) as the fat body HDLp binding site of *L. migratoria*.

The interaction between HDLp and the fat body shows several similarities with the interaction between high density lipoprotein and target cells in mammals. First, the binding sites of these lipoproteins have similar binding characteristics, which are different from those of the classical lipoprotein receptors: *1)* a relatively low affinity ($K_d \approx 10^{-7}$ M); *2)* the binding does not require divalent cations; *3)* a relaxed specificity for lipoproteins; and *4)* inhibition at high NaCl concentrations (26–28, 43). Second, in contrast to other mammalian lipoproteins, high density lipoprotein transports cholesterol between cells without being degraded (44), analogous to the transport of DAG by insect lipoproteins. And third, high density lipoprotein is the only lipoprotein for which retroendocytosis has been demonstrated to be the major intracellular pathway (45–47). Therefore, it is tempting to speculate that the insect HDLp binding site and the mammalian high density lipoprotein binding site, as well as the mechanisms in which both are involved, may be evolutionarily related. Recently, a candidate for the mammalian high density lipoprotein binding site was identified as the class B scavenger receptor (48). Further research will reveal whether the insect HDLp binding site is related to this receptor.

Better understanding of the nature of the HDLp binding site is not only of major importance for elucidation of the lipophorin shuttle mechanism at the molecular level, but it may also be relevant to understanding how, in general, selective transport of lipids between lipoproteins and cells is established.

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