Human Apolipoprotein E Mediates Processive Buoyant Lipoprotein Formation in Insect Larvae†

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ABSTRACT: The expression of human apolipoprotein E in tobacco hornworm larvae causes a dramatic change in the buoyant density of the insect's endogenous lipoproteins. Larvae without apoE have lipoproteins that are found exclusively in the high-density range. Baculovirus-mediated apoE expression results in the conversion of approximately one-fourth of the endogenous lipoproteins to low-density species. This density conversion is progressive and parallels a similar change in apoE density distribution. ApoE is secreted from the lipoprotein producing fat body tissue in a lipid-poor form, but readily associates with circulating insect lipoproteins in the hemolymph where the density conversion takes place. Analysis of the buoyant lipoprotein particles indicates that they contain apoE and insect apolipophorins I and II with few or no other proteins present. Immunoprecipitation of apolipophorins I and II results in coprecipitation of apoE. This association is disrupted by detergent, consistent with the three proteins sharing the same lipoprotein particles. The ability of apoE to influence buoyant lipoprotein formation in an invertebrate system leads us to suggest that small apolipoproteins such as apoE may play a role in buoyant lipoprotein production in mammals.

Plasma lipoproteins have diameters ranging from 100 to 1000 Å. The stability of these particles is maintained by their complement of apolipoproteins. The largest lipoprotein particles, such as very low density and low-density lipoproteins (VLDL[1] and LDL, respectively), contain apolipoprotein B100 (apoB100), a high molecular weight nonexchangeable apolipoprotein (Young, 1990).

VLDL particles approach 800 Å in diameter and are synthesized and secreted by the liver (Vance & Vance, 1990). The protein moiety of VLDL consists of apoB100, apoE, apoCI, apoCII, apoCIII, apoAIV, and traces of other proteins (Davis et al., 1979); Hamilton et al., 1991). All of the non-apoB apolipoproteins have molecular weights less than 10% of apoB100 and can exchange between lipoprotein particles. Postsecretory lipolysis of VLDL causes a decrease in lipid content and a concomitant loss of the smaller apolipoproteins. This results in an LDL particle which has a diameter of approximately 230 Å and a protein moiety that consists almost entirely of apoB100. Since newly-synthesized VLDL is never devoid of the exchangeable apolipoproteins, it is possible that these smaller apolipoproteins are involved in the assembly and structure of large lipoprotein particles.

A clear role for an exchangeable apolipoprotein in determining lipoprotein size has been demonstrated in invertebrates (Ryan, 1990). Apolipophorin III (apoLp-III), an apolipoprotein of molecular weight 18 000−20 000, is essential for dramatic transformations in the size and lipid content of adult insect lipoprotein particles (Wells et al., 1987). The basic matrix of circulating insect lipoproteins consists primarily of apolipophorin I (apoLp-I) and apolipophorin II (apoLp-II), two nonexchangeable apolipoproteins analogous to mammalian apoB. In the circulation of adult insects, a relatively small, high-density lipoprotein particle accepts diglycerides released from fat body tissue. The growth of this particle to a low-density lipoprotein is dependent upon its stabilization by several apoLp-III molecules. *In vitro* reconstitution experiments have demonstrated an absolute requirement for apoLp-III in this extracellular event (Chino et al., 1989). A parallel role for exchangeable mammalian apolipoproteins has been more difficult to demonstrate because the mammalian lipoprotein assembly process is primarily an intracellular event.

Larval insect lipoproteins do not normally undergo the dramatic size changes that occur in adult insects (Tsuchida & Wells, 1988). At this developmental stage, insect lipids are transported exclusively on high-density particles that lack apoLp-III. The absence of larger lipoproteins has allowed us to test whether expression of an exchangeable mammalian apolipoprotein can affect particle size in insect larvae. In previous studies, we showed that human apoE expressed in insect larvae is associated with lipoproteins and is able to function as a ligand for the human LDL receptor (Gretch et al., 1991).

In the present study, we further characterize the apoE-containing lipoproteins and explore the role of apoE in the formation of buoyant particles. When human apoE is

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[1] Abbreviations: VLDL, very low density lipoprotein; LDL, low-density lipoprotein; apoB, apolipoprotein B; apoE, apolipoprotein E; apoLp, apolipophorin; PBSI, phosphate-buffered saline with inhibitors; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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produced in the tobacco hornworm, a surprising lipoprotein profile results. The apoE is associated with particles that are much larger than those normally found in the insect larvae. Here we show that human apoE is able to associate with insect lipoproteins in the hemolymph and facilitate the processive formation of large, buoyant lipoprotein particles. ApoE may be exerting its effect by stabilizing these lipoproteins as they accumulate lipid, or by influencing the transfer of lipids into or out of the lipoproteins so that more buoyant species are formed. This demonstrates the ability of a small mammalian apolipoprotein to influence the amount of lipid packaged into lipoprotein particles.

EXPERIMENTAL PROCEDURES

Animals Used in This Study. Manduca sexta (tobacco hornworm) eggs and hornworm diet were obtained from Carolina Biological Supply Co. Insects were hatched and reared individually at room temperature with constant diet.

Infection of Insect Larvae and Lipoprotein Isolation. Recombinant baculoviruses encoding human apoE3 or nonrecombinant viruses were used to infect tobacco hornworm larvae. Four or six days following infection, hemolymph was collected and processed as described (Gretch et al., 1991). Samples (0.5 mL) were subjected to NaBr density gradient ultracentrifugation in an SW-41 rotor at 175000*g* for 30−32 h. The resulting gradient range was 1.018−1.246 g/mL. Twelve 1-mL fractions were collected from the top of each tube and were dialyzed at 4 °C against PBSI [150 mM NaCl, 2.8 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM $NaHPO_4$, 0.001% phenylmethanesulfonyl fluoride (PMSF), and 0.015% benzamidine, pH 7.4] with 5 mM ethylenediaminetetraacetic acid (EDTA).

Analysis of ApoE, ApoLp-I, and ApoLp-II Densities. Dialyzed gradient fractions were subjected to sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE). 7.5% gels were used for the analysis of apoLp-I and -II while 12% gels were used for the analysis of apoE. Immunoblotting (Burnette, 1981) was performed for apoE with anti-apoE monoclonal antibodies 1E and 13E (Takagi et al., 1988) and alkaline phosphatase-conjugated goat anti-mouse IgG (Sigma). ApoLp-I and -II were detected by immunoblot analysis with anti-lipophorin polyclonal antibodies and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma). The distribution of apoLp-I, apoLp-II, and apoE across the gradient was assessed by scanning densitometry of xylene-clarified immunoblots using an LKB Ultroscan XL enhanced laser densitometer. All 12 fractions from 1 tube were analyzed on the same blot, and the volumes of the fractions were adjusted so that band densities were analyzed within the linear range of detection.

Analysis of ApoE Produced by the Fat Body. Fat body tissue was dissected 4 or 6 days post-infection from larvae infected with apoE recombinant or nonrecombinant viruses and was washed with PBSI, pH 6.2. Tissue (0.15 g wet weight) from each animal was then incubated with rocking for 1 h in 1 mL of methionine-free Grace's media (GIBCO) containing 100 μCi of [^{35}S]methionine (Du Pont Express Protein Labeling Mix). Following the incubation, fat body was removed, and medium (1×10^5 trichloroacetic acid precipitable counts) from either apoE recombinant infected or nonrecombinant infected fat body was immunoprecipitated in immuno-buffer A (PBSI with 5 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 0.2 mg/mL ovalbumin) using the anti-apoE polyclonal antibody 265-2. The samples and antibody were incubated together for 4 h at 4 °C after which time protein A−Sepharose in immuno-buffer A was added. This was allowed to rock slowly for 1 h at room temperature. The immunocomplexes were washed twice with immuno-buffer A, and again with immuno-buffer A without ovalbumin. The samples were then subjected to SDS−PAGE in 12% gels. Following electrophoresis, the gels were treated with Enhance (ICN) according to the manufacturer's recommendations, and dried. Fluorography of the dried gels was carried out at −70 °C.

To assess the density of the apoE secreted by the fat body, density gradient ultracentrifugation was performed on 1.5×10^6 cpm of the [^{35}S]methionine-labeled samples as described above. Twelve 1-mL fractions from the gradients were dialyzed against PBSI, pH 7.4, and each fraction was subjected to immunoprecipitation and fluorography using the above conditions.

Association of Lipid-Free ApoE with Hemolymph Lipoproteins. Spodoptera frugiperda Sf-21 cells were infected with apoE recombinant viruses or wild-type nonrecombinant viruses as described (Gretch et al., 1991). At 27 h post-infection, the medium from each dish was replaced with 2 mL of methionine-free Grace's medium containing 200 μCi of [^{35}S]methionine. Five hours later, the media from the apoE-expressing and wild-type virus-infected cells were harvested and dialyzed against PBSI, pH 6.2. A portion (1×10^5 cpm) of each dialyzed sample was spun through a density gradient, fractionated, and subjected to SDS−PAGE and fluorography. The same amount (1×10^5 cpm) of dialyzed sample from apoE-expressing or wild-type virus-infected cells was injected into the hemolymph of insect larvae that had been infected with wild-type or recombinant virus 6 days earlier. After 4 h at 25 °C, the hemolymph was collected and subjected to gradient ultracentrifugation, fractionation, SDS−PAGE, and fluorography as described above.

Characterization of Buoyant Lipoproteins Containing ApoE. To determine the protein composition of the buoyant lipoprotein particles formed during apoE expression, particles from the *d* 1.02 g/mL fraction of the gradient spins were subjected to SDS−PAGE under reducing conditions on 5−12% polyacrylamide gradient gels. Following electrophoresis, the gels were stained with silver to detect the resolved proteins.

Larval lipoprotein particles from the *d* 1.02 g/mL fraction, endogenous particles from the *d* 1.13 g/mL fraction, and human LDL were subjected to nondenaturing and nonreducing electrophoresis in 2−16% native polyacrylamide gels as described (Nichols et al., 1986). Following electrophoresis, the lipoproteins were analyzed by immunoblot using anti-apoLp-I and -II polyclonal antibodies or anti-apoE monoclonal antibodies as described above, except that the immunoblot transfer buffer consisted of 25 mM Tris, 192 mM glycine, 20% v/v methanol, and 2% v/v Triton X-100, pH 8.3.

Coimmunoprecipitation of ApoLp-I and -II and ApoE. Protein A−Sepharose was coupled to anti-lipophorin (anti-Lp-I and -II) polyclonal antibodies or to antibodies from irrelevant rabbit serum in the presence of immuno-buffer B (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM EDTA, 0.02% NaN_3, 0.2% ovalbumin, 0.001% PMSF, and 0.015% ben-

zamidine) with rocking for 1 h at 4 °C. The antibody−protein A complexes were washed extensively with immuno-buffer B. Hemolymph from larvae infected with apoE recombinant or wild-type virus was collected 6 days post-infection as described and was dialyzed against immuno-buffer B without ovalbumin. Following dialysis, samples containing 33 μg of total protein each (roughly 20 μL) were combined with immuno-buffer B in the presence or absence of 0.01% Triton X-100. Each 20 μL of sample from apoE-expressing larvae has approximately 2 μg of apoE (Gretch et al., 1991). As a control for nonspecific binding of apoE in the absence of detergent, purified apoE was substituted for hemolymph, and 4 μg was used in each immunoprecipitation reaction. Protein A−antibody complexes were added to the samples which were then incubated for 2 h at 4 °C with rocking. Extensive washes were then performed with immuno-buffer B in the presence or absence of 0.01% Triton X-100. The immunoprecipitated proteins were then subjected to SDS−PAGE and immunoblot analysis as described above.

Analysis of the Lipid Content of the ApoE-Containing Lipoproteins. Lipoproteins (*d* 1.02 g/mL) from apoE-expressing larvae and *d* 1.13 g/mL endogenous lipoproteins from wild-type virus-infected animals were isolated 6 days post-infection, and their lipids were extracted (Folch et al., 1957). Samples were subjected to thin-layer chromatography using Silica Gel H plates (Analtech) and a hexane/ether/acetic acid (80:20:2) solvent system. Following visualization with iodine vapors, species that comigrated with triglyceride, diglyceride, and phospholipid standards were collected and quantitated by charring with concentrated sulfuric acid (Marsh & Weinstein, 1966). Standard curves were generated using triolein, diolein, and phosphatidylcholine.

RESULTS

Human ApoE Produced in Insect Larvae Dramatically Alters the Profile of Insect Lipoproteins. Human apolipoprotein E was expressed in insect larvae to assess its potential influence on buoyant lipoprotein formation. Fourth instar *Manduca sexta* larvae were injected with either recombinant baculoviruses harboring the human apoE3 cDNA or nonrecombinant (wild-type) baculoviruses. Hemolymph was collected 4 and 6 days post-infection, and the lipoprotein profiles were assessed following density gradient ultracentrifugation. The apoE, apoLp-I, and apoLp-II distributions in the gradient fractions were determined by quantitative immunoblot analysis.

Expression of human apoE caused a striking change in the insect's lipoprotein profile. The endogenous apoLp-I and -II of control larvae infected with wild-type virus were found predominantly in the *d* 1.10−1.15 g/mL range, with none of the lipoproteins floating at a density less than 1.05 g/mL (Figure 1, top panel). However, in larvae infected with virus encoding human apoE, approximately one-fourth of the endogenous apolipoproteins were at $d < 1.05$ g/mL. Commensurate with the appearance of these buoyant lipoprotein particles was a striking change in the apoE density distribution. While at the fourth day of infection about one-fourth of the apoE was in the $d < 1.05$ g/mL density fractions (Figure 1, bottom panel), by the sixth day of infection >90% of the apoE floated in this range.

Therefore, as a result of apoE expression, endogenous larval lipoproteins became considerably more buoyant. This

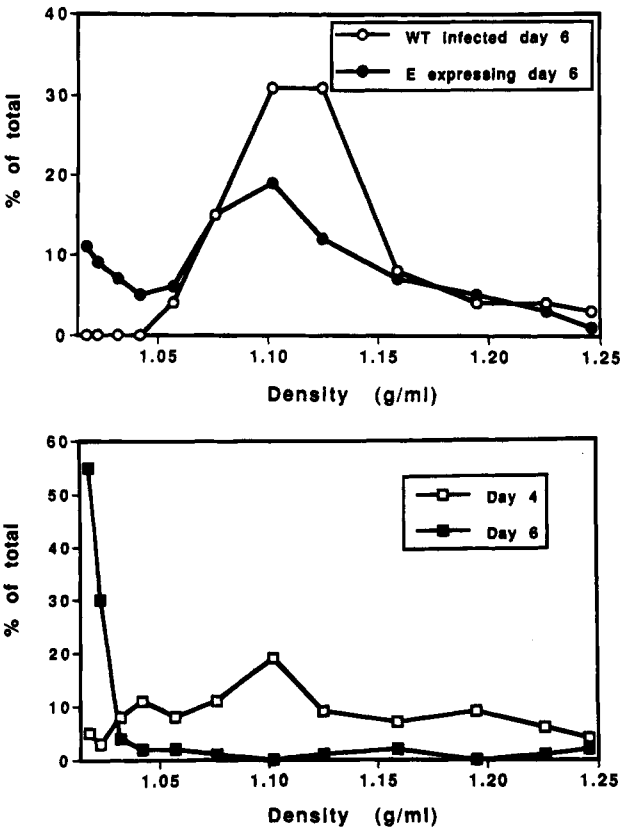

FIGURE 1: Buoyant density distribution of apolipophorins I and II and apolipoprotein E. Hemolymph samples from larvae infected with wild-type or apoE-expressing viruses were subjected to density gradient ultracentrifugation followed by quantitative immunoblot analysis of apoLp-I and -II as well as apoE. The top panel represents the density distribution of apoLp-I and -II 6 days after infection with wild-type (control) virus (○) or with apoE-expressing virus (●). The bottom panel represents the density distribution of apoE 4 (□) and 6 (■) days after infection with the apoE-expressing virus.

change in density paralleled a similar, progressive change in apoE density distribution, suggesting that apoE may interact with the insect lipoproteins and enable them to assemble more lipid and form larger lipoprotein particles.

Lipoprotein Association by ApoE Is a Postsecretory Event. The insect fat body tissue is the site of synthesis and secretion of apoLp-I and apoLp-II (Prasad et al., 1986). Since the fat body is a target for baculovirus infection (Keddie et al., 1989), infected cells were likely to be synthesizing apoE as well as the endogenous apolipoproteins. Thus, it was conceivable that all three apolipoproteins were coassembled into lipoprotein particles during their passage through the secretory pathway. To test for fat body synthesis of apoE, and to ascertain whether apoE was causing low-density lipoprotein formation before or after secretion, infected fat body tissue was isolated at 4 or 6 days post-infection and incubated with [^{35}S]methionine-containing media *in vitro*. Immunoprecipitation with apoE-specific antibodies indicated that apoE was being synthesized and secreted by the fat body tissue of insects infected with apoE encoding virus (Figure 2, top panel). While apoE was secreted by this tissue, density gradient ultracentrifugation showed that at 4 and 6 days post-infection its buoyant density was >1.20 g/mL, consistent with very high density lipoproteins or with a lipid-free form of the protein (Figure 2, bottom panel). Therefore, apoE was not stimulating low-density lipoprotein formation prior to secretion.

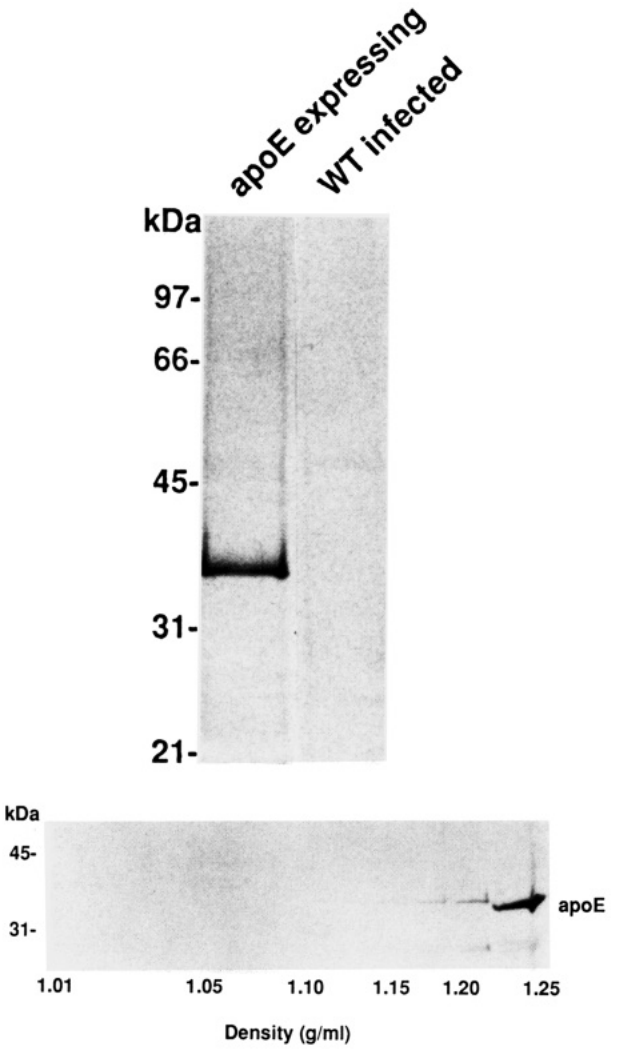

FIGURE 2: Analysis of apolipoprotein E produced by the fat body tissue. Fat body tissue was removed from larvae infected with wild-type (control) virus or with virus encoding apoE. Following [^{35}S]-methionine labeling *in vitro*, proteins secreted by the fat body were immunoprecipitated using an apoE-specific polyclonal antibody. The top panel shows the specific immunoprecipitation of apoE that is secreted by fat body infected with the apoE-encoding virus. The bottom panel represents immunoprecipitation of fat body produced apoE following density gradient ultracentrifugation. Similar results were observed from fat body dissected from larvae either 4 or 6 days after infection.

Since apoE did not stimulate low-density lipoprotein synthesis prior to secretion, its effect would have to be postsecretory. We therefore tested its ability to interact with insect lipoproteins after secretion. To test whether apoE could associate with larval lipoproteins in the hemolymph, ^{35}S-labeled proteins from cultured insect cells expressing apoE were injected into the hemolymph of *M. sexta* larvae. The larval hemolymph was then collected and subjected to density gradient ultracentrifugation. Prior to injection, no detectable protein from the apoE-expressing cells floated in the density gradient. Injection of the labeled proteins from apoE-expressing cells into the hemolymph of wild-type virus-infected larvae resulted in a labeled protein with the size of apoE floating in the high-density lipoprotein range (Figure 3). This is consistent with the apoE associating with existing lipoproteins that are found in that density range. By contrast, in hemolymph from the recombinant virus-infected insects, the exogenous apoE showed a bimodal distribution; a substantial proportion was in the $d < 1.05$ g/mL fraction,

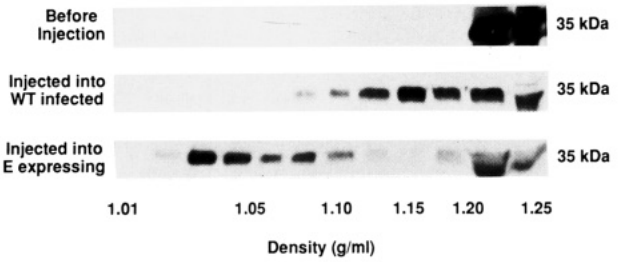

FIGURE 3: Association of lipid-free apolipoprotein E with larval lipoproteins. Insect cells expressing apoE were labeled with [^{35}S]-methionine, and the secreted proteins were injected into the hemolymph of insect larvae. Prior to, and following, injection, the proteins were analyzed by density gradient ultracentrifugation, SDS−PAGE, and fluorography. The top row represents the density of 35 kDa proteins prior to injection. The second row shows the density of the same sample after injection into wild-type virus-infected larvae. The bottom row represents the density of the sample after injection into larvae infected with apoE-expressing virus. No proteins from control insect cells floated in the gradient prior to or after injection (not shown).

and some was in the $d > 1.20$ g/mL fraction. This is consistent with the exogenous apoE associating with the recombinant low-density lipoproteins found in these larvae. Parallel experiments showed that no proteins float in the gradient when labeled proteins from insect cells not expressing apoE are injected (data not shown). The association of the exogenous apoE with hemolymph lipoproteins does not require host tissues since this association can occur in hemolymph that has been removed from the animal (Gretch and Attie, unpublished observations).

ApoE Shares Lipoprotein Particles with ApoLp-I and ApoLp-II. The apoE expression experiments did not distinguish between apoE forming an autonomous lipoprotein particle that overlaps in density with endogenous lipoproteins or apoE interacting directly with endogenous lipoproteins to facilitate buoyant recombinant lipoprotein formation. To assess the protein content of particles isolated from the *d* 1.02 g/mL density fraction of apoE-expressing larvae, SDS−PAGE was performed on these samples followed by silver staining of the resolved proteins (Figure 4, left panel). This analysis confirmed that the particles in this fraction contained proteins with mobilities consistent with those of apoLp-I, apoLp-II, and apoE. This is in agreement with the immunological detection of these proteins in the *d* 1.02 g/mL fraction (Figure 1). This result also suggests that this fraction contains few or no other protein components. Immunoblot analysis following nondenaturing gel electrophoresis of *d* 1.02 g/mL samples from apoE-expressing larvae showed that particles containing apoE migrated in the same position as particles containing apoLp-I and apoLp-II and had a mobility comparable to human LDL (Figure 4, right panel). This supports the hypothesis that these apolipoproteins are transported in the same lipoprotein particles. In contrast, these particles had a decreased mobility relative to the endogenous *d* 1.13 g/mL particles. This is consistent with the *d* 1.02 g/mL particles being larger than the endogenous particles.

To directly determine whether all three apolipoproteins were on the same lipoprotein particles, hemolymph from apoE-expressing and nonexpressing larvae was immunoprecipitated with polyclonal antibodies to apoLp-I and apoLp-II. The precipitated proteins were then analyzed by immunoblot analysis (Figure 5). All three apolipoproteins were

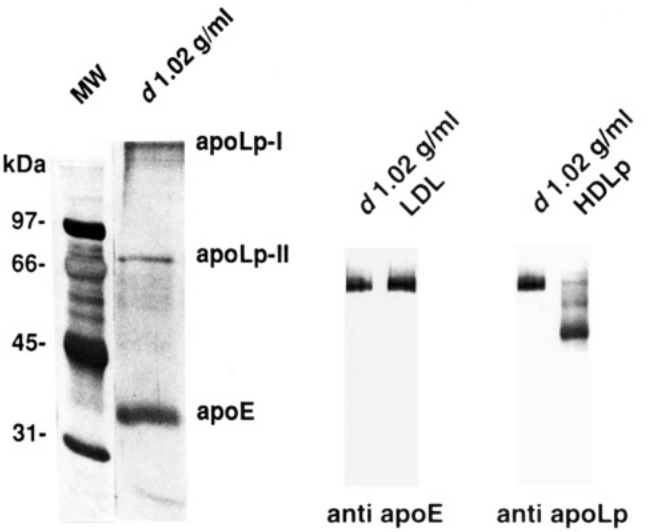

FIGURE 4: Characterization of *d* 1.02 g/mL lipoproteins from apoE-expressing larvae. The left panel shows the protein composition of the *d* 1.02 g/mL lipoprotein particles after gradient ultracentrifugation of hemolymph from apoE-expressing larvae. Proteins were resolved on 5−12% SDS−PAGE gradient gels under reducing conditions and were stained with silver. The right panel represents immunoblot analysis of the same particles after native gel electrophoresis using 2−16% polyacrylamide gels. For mobility comparisons, the particles were electrophoresed on the same gel as human LDL and endogenous larval high-density lipophorin. The samples were probed with anti-apoE or anti-apoLp-I and -II antibodies.

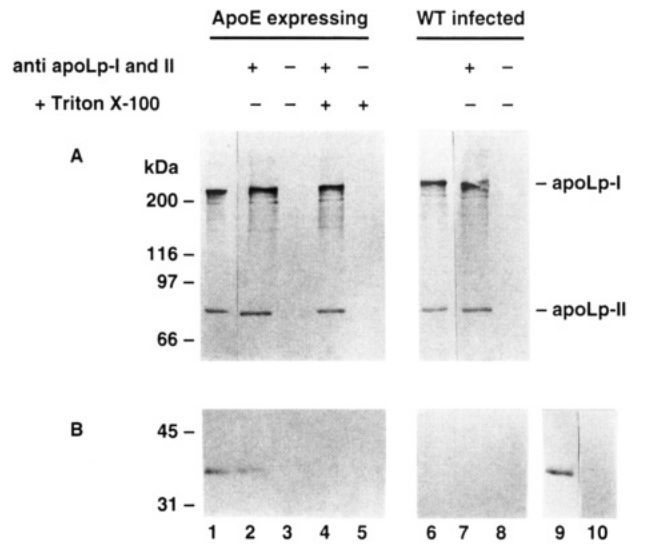

FIGURE 5: Coimmunoprecipitation of apoE with apoLp-I and -II. Hemolymph proteins from larvae infected with wild-type (control) virus or apoE-expressing virus were immunoprecipitated with anti-apoLp-I and -II antibodies or with irrelevant antibodies in the presence or absence of Triton X-100. Panel A shows an immunoblot of precipitated proteins probed with anti-apoLp-I and -II antibodies. Panel B shows an immunoblot of the same samples probed with anti-apoE antibodies. Lanes 1 and 6 contain hemolymph proteins prior to immunoprecipitation. Lanes 9 and 10 show the results of immunoprecipitation of purified apoE using anti-apoLp-I and -II in the absence of Triton X-100. Lane 9 shows apoE remaining in the supernatant while lane 10 shows the lack of precipitated apoE protein.

found in the immunoprecipitate of the apoE-producing larvae (lane 2), but only apoLp-I and -II were precipitated from the nonexpressing hemolymph (lane 7). To ensure that apoE was not cross-reacting with the anti-apoLp-I/apoLp-II antibodies during the immunoprecipitation, apoE alone was tested and was shown to remain in the supernatant (lane 9)

Table 1: Mass of Lipids in *d* 1.13 g/mL Endogenous Lipoproteins and *d* 1.02 g/mL Lipoproteins from ApoE-Expressing Larvae[a]

	mass of lipid (μg/mg of protein)	
lipid class	endogenous lipoproteins	*d* 1.02 g/mL lipoproteins
phospholipids[b]	269 ± 23[c]	184 ± 14[c]
diglycerides	258 ± 10[c]	1287 ± 21[c]
triglycerides	15 ± 4	17 ± 4

[a] Values represent means ± standard deviations ($n = 3$). [b] Includes all lipids remaining at the origin following chromatography. [c] $p < 0.05$ endogenous lipoproteins *versus* *d* 1.02 g/mL lipoproteins using the Student's *t*-test.

and not to be precipitated by the antibodies (lane 10). The nonionic detergent Triton X-100 is known to disrupt the integrity of lipoprotein particles (Mahley & Holcombe, 1977). When the hemolymph from apoE-expressing larvae was mixed with Triton X-100, the antibodies failed to coimmunoprecipitate apoE along with apoLp-I and -II (lane 4). This detergent-sensitive association is consistent with apoE sharing the same lipoprotein particles with the endogenous apolipoproteins.

The anti-apoLp-I and -II antibodies reacted with some minor proteins with mobilities between those of apoLp-I and the 116 kDa marker. It is likely that they are products of limited apoLp-I degradation that may have occurred during sample manipulation.

ApoE-Containing Lipoproteins Are Enriched in Diacylglycerol. To assess the effect of apoE expression on the lipid composition of the buoyant lipoprotein particles, the lipids were extracted, separated by thin-layer chromatography, and quantitated. The *d* 1.02 g/mL particles from apoE-expressing larvae showed a 5-fold increase in diacylglycerol relative to *d* 1.13 g/mL particles from wild-type virus-infected controls (Table 1). This is consistent with apoE promoting the transfer of diacylglycerol into lipoprotein particles in the hemolymph. This resembles the diacylglycerol loading that occurs in adult insects. The phospholipid-to-protein ratio is lower in the apoE-containing particles, presumably because of the acquisition of protein (apoE) with little or no phospholipid enrichment.

DISCUSSION

The assembly of lipid-rich VLDL in mammals involves the association of a variety of lipids with several apolipoprotein comonents within the secretory pathway of hepatocytes. Apolipoprotein B100 synthesis is absolutely required for VLDL assembly. While apoB100 plays a critical role in the assembly of lipoproteins, the role of smaller apolipoproteins in stabilizing the lipid−protein complex is less well-defined.

Small apolipoproteins such as apoE are able to exchange between lipoprotein particles and move from a lipid-free state onto existing lipoproteins (Weisgraber et al., 1990). It has therefore been difficult to assess the potential for these proteins to participate in the lipoprotein assembly process. The presence of small apolipoproteins on VLDL may simply be a reflection of their affinity for an exposed lipid surface rather than an indication of their role in assembling these particles.

Like mammals, many adult insects are able to assemble low-density lipoproteins. However, unlike mammals, the

protein−lipid association that results in insect low-density lipoprotein formation is largely an extracellular event (Ryan, 1990). Insect lipoproteins contain a nonexchangeable protein component comprised of two proteins: apoLp-I (M_r ~240 000) and apoLp-II (M_r ~78 000). These two proteins are derived from a common precursor and are secreted together from the fat body tissue in a nascent very high density particle (Weers et al., 1993). Once in the circulation, these particles can interact with donor tissues and receive lipid. In adult insects, the lipid efflux comes primarily from the fat body tissue. As they accumulate lipid, the recipient lipoproteins bind several molecules of circulating apoLp-III. The apoLp-III facilitates the formation and stabilization of low-density particles.

While clear differences exist between mammalian and invertebrate lipoprotein assembly, some common themes have emerged. Recently, assembly of apoB containing mammalian lipoproteins has been shown to be dependent upon a protein complex, termed the microsomal triglyceride transfer protein (MTP) (Wetterau et al., 1992; Sharp et al., 1993). This soluble complex exists within the lumen of microsomes isolated from lipoprotein-producing tissues of mammals. *In vitro* studies have shown that this complex can transfer various lipids (preferentially triglycerides) between membranes (Wetterau & Zilversmit, 1984). Subjects with dysfunctional MTP are unable to assemble and secrete apoB-containing lipoproteins.

Extracellular insect lipoprotein assembly seems to be facilitated by a similar type of protein complex. The lipid transfer particle (LTP) of insects is a soluble protein complex that appears to direct the transfer of lipids into insect lipoproteins (Blacklock & Ryan, 1993). *In vitro* studies have shown that like MTP, LTP can transfer a variety of lipids between membranes. This activity includes the ability to transfer triglycerides and phospholipids into human LDL (Ando et al., 1990). Interestingly, the similar activities of these complexes are paralleled by similar subunit compositions. LTP is made up of a lipid−protein complex that includes proteins of M_r ~320 000, 85 000−89 000, and 55 000−68 000. MTP has a subunit composition that includes proteins of M_r 88 000 and 58 000.

Here we have shown that expression of human apoE in insect larvae causes a dramatic density change in the host's lipoprotein profile. In the absence of apoE expression, the *M. sexta* larvae contain lipoproteins that exist only in the high-density range. The expression of apoE greatly alters this density profile. In the presence of apoE, a significant fraction of the insect apolipoproteins are found in the low-density lipoprotein range. This suggests that apoE enables the insect lipoproteins to accumulate more lipid and form larger particles. The observed density conversion is more pronounced after 6 days of expression than after 4 days. Therefore, the conversion to low-density lipoproteins in the presence of apoE appears to be processive in nature.

Results from experiments using infected fat body tissue suggest that newly secreted apoE is lipid-poor. However, after 4 days of expression, the hemolymph apoE appears to be evenly distributed across the density gradient. The presence of one-fourth of the apoE in the low-density range suggests that by this time apoE not only has bound to the endogenous lipoproteins but also has stimulated the conversion of some of these lipoproteins to low-density species. By 6 days post-infection, greater than 90% of the hemolymph apoE is at a density less than 1.05 g/mL, and roughly one-fourth of the insect apolipoproteins have been converted to this low-density range.

Injection of exogenous lipid-free apoE into insects or *in vitro* incubation of apoE with larval hemolymph results in a change in apoE's density characteristics. The resulting density distribution suggests that the apoE is associating with the hemolymph lipoproteins. Analysis of the buoyant particles that result from apoE expression shows that apoE is in association with the same lipoprotein particles as the endogenous insect apolipoproteins (Figure 5) and that these particles are larger than the endogenous lipoproteins (Figure 4, right panel). This is consistent with the hypothesis that apoE is able to bind to the endogenous lipoproteins in the hemolymph and enable these particles to accommodate additional lipid, thus increasing in size and decreasing in density.

Comparison of the lipid content of the *d* 1.02 g/mL apoE-containing lipoproteins with the lipids found in the endogenous *d* 1.13 g/mL lipoproteins showed that the apoE-containing particles are enriched in diacylglycerol (Table 1). Therefore, apoE expression has mediated the acquisition of lipid by these particles, thus promoting buoyant lipoprotein formation. This is similar to the diglyceride enrichment that occurs in adult lipoproteins that are stabilized by apoLp-III (Ryan et al., 1986) and supports the idea that apoE is functioning in a similar way. The mass of phospholipids in the *d* 1.02 g/mL particles relative to protein is lower than in the high-density particles. This observation is consistent with the *d* 1.02 g/mL particles having acquired protein (apoE) and diglyceride but relatively little phospholipid. The enlargement of these particles in the absence of significant phospholipid acquisition suggests that apoE binding alone is able to stabilize the particle surface, or that diacylglycerol may be partitioning into both the core and outer leaflet of the expanded particles.

The ability of apoE to mediate buoyant lipoprotein formation in insect larvae may provide some additional insights into the apolipoprotein structural elements that are required for efficient density conversions. While apoE is able to facilitate buoyant lipoprotein formation in insect larvae, apoLp-III is unable to do so at this developmental stage despite circulating at a concentration of ~500 μg/mL (Kawooya et al., 1984) compared to ~200 μg/mL for apoE (Gretch et al., 1991). The three-dimensional structures for both apoLp-III and the amino-terminal two-thirds of apoE have been solved (Breiter et al., 1991; Wilson et al., 1991). These structures are remarkably similar, consisting of elongated up-and-down helical bundles, with apoLp-III containing five such helices and the apoE fragment containing four. However, the apoE sequences that mediate optimal lipoprotein association are found in the carboxy-terminal region of the protein (Westerlund & Weisgraber, 1993) and are absent from the solved structure. These sequences may contribute to the ability of apoE to mediate the lipoprotein density conversions that do not occur in the presence of aopLp-III alone. ApoLp-III is able to mediate density conversions in adult insects (Wells et al., 1987). This process requires adipokinetic hormone stimulation of fat body tissue to mobilize lipid. Therefore, apoLp-III may play a more passive role in density conversions (stabilizing lipoproteins after hormone-stimulated lipid mobilization), whereas apoE may have the capacity to play a more active role (perhaps

due to its carboxy-terminal region) and mediate density conversions without hormonal stimulation.

The putative role of small apolipoproteins in buoyant lipoprotein formation is supported by transgenic mice overexpressing the human apoCIII gene (Aalto et al., 1992). These mice contain larger, triglyceride-rich VLDL particles with 2.5 times the normal amount of apoCIII when compared to control animals. Hepatocytes cultured from these animals secrete twice the amount of triglyceride as control hepatocytes when stimulated with oleic acid but have no elevation in apoB100 secretion. *In vivo* experiments with these animals support these results. ApoCIII-expressing transgenic animals treated with Triton WR 1339 to block VLDL clearance showed an increased incorporation rate of [^{3}H]-glycerol into VLDL triglycerides compared to controls but showed no increase in [^{35}S]methionine incorporation into VLDL apoB. These data support the hypothesis that small mammalian apolipoproteins can influence the amount of lipid packaged into a lipoprotein particle and the resulting particle size.

Analysis of mammalian lipoproteins resulting from subjects with apolipoprotein deficiencies and from transgenic animals with null mutations in apolipoprotein genes can provide insights into the potential role of small apolipoproteins in mammalian lipoprotein assembly. Human patients with apoE deficiencies are able to synthesize VLDL that is similar in density to that of normal subjects (Schaefer et al., 1986). These particles do, however, have an increased amount of apoAIV which seems to replace the missing apoE. This suggests that if the small apolipoproteins do influence lipoprotein assembly, they may be somewhat interchangeable, with one substituting in another's absence. Similarly, transgenic animals with null mutations in their apoE genes have VLDL particles with normal densities but which are enriched with apoAI and apoAIV (Zhang et al., 1992; Plump et al., 1992).

The ability of LDL to accept exogenous lipids and increase in diameter has been analyzed *in vitro* (Singh et al., 1992). These studies show that LDL with apoB100 alone has a finite capacity to accept neutral lipid without becoming destabilized and aggregating. However, non-apoB apolipoproteins and apoLp-III can bind to LDL and increase in capacity to accept lipid. The resulting particles are stable and have a significantly increased diameter compared to LDL. This is consistent with the potential role of exchangeable apolipoproteins in stabilizing large lipoproteins such as VLDL during their formation.

Additional studies have demonstrated that apoE is able to promote lipid accumulation by high-density lipoproteins *in vitro* (Koo et al., 1985). In the presence of apoE, canine HDL_3 particles were able to accept cholesterol from cholesterol-loaded macrophages and form larger HDL_1/HDL_c particles. During this size conversion, the particles acquired apoE as they accepted cholesterol from the macrophages. In the absence of apoE, no HDL_1/HDL_c particles were formed from HDL_3 even in the presence of continued macrophage cholesterol efflux. Therefore, apoE is required for the efficient formation of these cholesterol-enriched, large HDL particles. These observations further demonstrate the potential role of small, exchangeable apolipoproteins in the modulation of lipoprotein size and density.

Clearly, the synthesis of large, nonexchangeable apolipoproteins such as apoB and apoLp-I and -II and also the availability of appropriate lipids are major determinants for buoyant lipoprotein formation. However, *in vitro* studies, work with adult insects and transgenic animals (e.g., the apoCIII overproducers), as well as the data presented here suggest that small, exchangeable apolipoproteins can influence the amount of lipid assembled into a lipoprotein and thus affect the size and buoyant density of the resulting particle.

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