Baculovirus-mediated expression of human apolipoprotein E in *Manduca sexta* larvae generates particles that bind to the low density lipoprotein receptor

**Daniel G. Gretch**, **Stephen L. Sturley**, **Paul D. Friesen**, **Nancy E. Beckage**, and **Alan D. Attie**

Departments of Biochemistry and Comparative Biosciences, University of Wisconsin-Madison; Madison, WI 53706; Institute of Molecular Virology and Department of Biochemistry, University of Wisconsin-Madison; Madison, WI 53706; and Department of Entomology, University of California-Riverside, Riverside, CA 92521

Communicated by Donald F. Steiner, July 1, 1991 (received for review March 18, 1991)

**ABSTRACT**  Human apolipoprotein E (apoE) is a ligand for the low density lipoprotein (LDL) receptor and mediates the catabolism of several classes of lipoprotein particles. Binding of apoE to the LDL receptor requires association of apoE with lipid in a vesicle or a lipoprotein particle. Because of this requirement, purified apoE or apoE derived directly from bacterial expression systems does not bind to the LDL receptor. To overcome this problem and to facilitate analysis of apoE structure, recombinant baculoviruses containing the human apoE cDNA fused to the polyhedrin promoter of *Autographa californica* nuclear polyhedrosis virus were constructed. The recombinant viruses were used to infect larvae of the tobacco hornworm *Manduca sexta* in vitro. High levels of lipoprotein particles containing human apoE were present in the hemolymph of infected larvae. In contrast to apoE produced by recombinant baculovirus-infected insect cells in vitro, these particles were excellent ligands for the LDL receptor.

Human apolipoprotein E (apoE) is one of two known ligands for the low density lipoprotein (LDL) receptor (1). It provides the specificity required for the receptor-mediated clearance of certain classes of lipoproteins. Genetic variation at the apoE locus that results in defective receptor binding is associated with hyperlipoproteinemia and accelerated coronary heart disease (1).

Although apoE is water-soluble when stripped from its lipid milieu, binding of apoE to the LDL receptor requires association of apoE with lipid in a vesicle or a lipoprotein particle (2). Thus, previous studies relating the structure of apoE to its function have relied upon denaturation followed by in vitro reconstitution of apoE with lipid (3). An alternative approach to the production of biologically active apoE would be to express this protein in a system that facilitates lipid association in vivo.

In vertebrates, neutral lipids are transported in association with lipoprotein particles. This process is conserved in insects such as the tobacco hornworm *Manduca sexta* (4, 5). Insect lipoproteins, like their mammalian counterparts, have a core of nonpolar neutral lipids surrounded by a monolayer of amphiphilic lipids (6). The insect apolipoprotein, apolipophorin III, has an amphipathic α-helical secondary structure (7), a motif suggested to play a role in the lipid binding of mammalian apolipoproteins such as apoE (8).

In insect larvae, the fat body is the site of synthesis of hemolymph lipoproteins (9) and is also a target of infection by baculoviruses (10). Therefore, we hypothesized that this in vivo system could be exploited to produce recombinant lipoprotein particles containing the products of heterologous Apolipoprotein genes. Such recombinant lipoproteins might have similar biological properties to native mammalian lipoproteins. In the present study, recombinant baculoviruses possessing a human apoE cDNA in place of the polyhedrin gene were constructed. After infection of *M. sexta* larvae, high concentrations of human apoE were secreted into the hemolymph in a lipid-associated and biologically active form.

**MATERIALS AND METHODS**

**Production of Recombinant Baculoviruses.** A 1.2-kilobase-pair (kb) EcoRI–HindIII fragment encoding the apoE-3 cDNA from plasmid pHE53 (11) was tagged with BamHI linkers (Promega) and cloned into the BamHI site of pAcYM1 (12), yielding pAcE3. An Aat II–HinfI 1.1-kbp fragment corresponding to the apoE-3V cDNA from PHAEB13 (13) was ligated into the Sma I site of pBluescript M13+ (Stratagene). An 8-bp BamHI linker (Promega) was then inserted at the EcoRV site of this plasmid to produce p52-Bam. The 1.1-kbp BamHI fragment from p52-Bam was ligated into the BamHI site of pAcYM1, yielding pAcE3V. The resulting plasmids were cotransfected with the wild-type baculovirus genome (*Autographa californica* nuclear polyhedrosis virus; AcNPV)) into Spodoptera frugiperda Sf-21 cells. Recombinant viruses encoding apoE-3 and apoE-3V (AcNPV-E3 and AcNPV-E3V, respectively) were selected, plaque-purified, and titered as described (14, 15).

**Infection of Cultured Insect Cells and Insect Larvae.** Viruses were used to infect 4 × 10^6 Sf-21 cells per dish at a multiplicity of infection of 10. SF-900 (GIBCO) medium (2 ml) was harvested from infected cells 42 hr after infection. Fourth-instar larvae (day 2; average mass, 0.5 g; reared as described (16)) were injected with 1 × 10^6 plaque-forming units (pfu) of recombinant virus and administered a booster with 5 × 10^6 pfu the following day. Hemolymph was collected 6 days after infection and diluted 1:2 (vol/vol) with phosphate-buffered saline (PBS; 150 mM NaCl/2.8 mM KCl/1.5 mM KH2PO4/6.5 mM Na2HPO4, pH 7.4) containing 5 mM glutathione, 0.002% phenylmethylsulfonyl fluoride, and 0.3 mg of benzamidone per ml (PBSI). Media and hemolymph samples were subjected to sodium dodecyl sulfate/10% polyacrylamide gel electrophoresis (SDS/PAGE) (17). Immunoblotting (18) was performed with anti-human apoE monoclonal antibodies 1E1 and 13E (19) and alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma).

Abbreviations: apoE, apolipoprotein E; LDL, low density lipoprotein; AcNPV, *Autographa californica* nuclear polyhedrosis virus; Myr2-PtdCho, dimyristoyl phosphatidylcholine.

2To whom reprint requests should be addressed: Department of Biochemistry, University of Wisconsin-Madison, 420 Henry Mall, Madison, WI 53706.
Lipid Reconstitution of apoE from Sf-21 Cell Culture Medium. Dimyristoyl phosphatidylcholine (Myr<sub>p</sub>-PtCho) liposomes were produced by extrusion through a French pressure cell (20). Cell culture medium from infected Sf-21 cells was incubated with Myr<sub>p</sub>-PtCho liposomes at a 1:1 lipid/protein ratio for 3 hr at 37°C. Lipid-free proteins were removed by Sephadex G-50 column chromatography. The concentrations of liposome-associated apoE-3 and apoE-3V were estimated by scanning densitometry (Molecular Dynamics, model 300A) of xylene-clarified immunoblots prepared as described above.

Ultracentrifugation of Media and Hemolymph. Samples (12 ml) of infected Sf-21 culture medium were adjusted to a density of 1.21 g/ml with NaBr and centrifuged at 175,000 × g for 48 hr in an SW-41 rotor. Hemolymph samples (0.5 ml) were diluted to 12 ml with PBS, adjusted with NaBr to a density of 1.21 g/ml, and centrifuged as above. After centrifugation, the top 1 ml of 12 was collected and dialyzed against PBS. Samples were analyzed by SDS/PAGE and immunoblotting as described above.

To assess the distribution of apoE across a buoyant density range, 1 ml of culture medium (42 hr after infection) or 0.5 ml of hemolymph (6 days after infection) was layered beneath a NaBr step gradient (21) (density 1.0178–1.2418 g/ml) and subjected to ultracentrifugation in an SW-41 rotor at 175,000 × g for 48 hr. Twelve 1-ml fractions were collected from the tops of the tubes and dialyzed against PBS, and aliquots were assayed for apoE by radioimmunoassay (22).

Receptor Binding Experiments. Media from infected Sf-21 cells (36 hr after infection) and hemolymph of insect larvae of M. sexta (6 days after infection) were collected and dialyzed against PBS (pH 7.4). CHO cells (TR715-19, provided by J. L. Goldstein) that stably overexpress the human LDL receptor (23, 24) were grown in Ham's nutrient mixture F-12 (GIBCO) containing 20 mM Hepes (pH 7.2), 1% (vol/vol) fetal calf serum, 4% (vol/vol) lipoprotein-deficient serum (LDS), 2 mM glutamine, and 100 units of penicillin and 100 µg of streptomycin per ml. Twelve hours prior to use, confluent cells were switched to a medium containing Ham's F-12, 20 mM Hepes (pH 7.2), 5% LDS, 2 mM glutamine, and 100 units of penicillin, 100 µg of streptomycin, 10 µg of cholesterol, and 0.1 µg of 25-hydroxycholesterol per ml. Each dish received 2 ml of Ham's F-12 medium/20 mM Hepes (pH 7.2), containing 5 mg of bovine serum albumin and 0.5 µg of 125I-labeled LDL per ml and the indicated concentration of apoE-3 or apoE-3V as determined by radioimmunoassay. The cells were incubated for 3 hr at 4°C on a rotary shaker prior to washing and release of bound 125I-labeled LDL by dextran sulfate (25). Radioiodination of LDL was performed as described (26).

RESULTS

In Vitro apoE Expression. Two allelic forms of the human apoE gene, APOE, were cloned into the AcNPV transfer vector pAcYM1. These encode the wild-type protein, apoE-3, and apoE-3V, an apoE-3 derivative with two mutations, Ala-152 → Pro and Ala-99 → Thr (13). Previous studies have shown that particles reconstituted with [Pro<sup>125I</sup>]-apoE-3 bound to the LDL receptor with only 27% of the receptor binding activity of wild-type apoE-3 (3). The chimeric apoE expression vectors were cotransfected with wild-type viral DNA into cultured S. frugiperda (SF-21) cells to obtain recombinant viruses, which were subsequently injected into fourth-instar M. sexta larva.

A protein of 35 kDa, the predicted molecular mass of apoE, that was uniquely immunoreactive with two monoclonal antibodies raised against human apoE was secreted into the medium of SF-21 cultures infected with the virus harboring the human apoE-3 cDNA (Fig. 1). The medium apoE-3 concentration was 4 and 30 µg/ml 36 and 60 hr after infection, respectively, as determined by radioimmunoassay. Cultures infected with the virus carrying the variant allele encoding apoE secreted a protein of identical molecular mass and immunoreactivity (Fig. 1) but at ≈75% of the concentration of cultures infected with the wild-type allele (2 µg/ml 60 hours after infection). This inequality may have resulted from the presence of extraneous DNA sequences between the polyhedrin promoter and the apoE-3V open reading frame, or alternatively, from the apoE-3V-specific mutations.

In Vivo apoE Expression. A much higher level of apoE expression was observed in the hemolymph of infected M. sexta larvae (Fig. 1). The larval hemolymph apoE-3 concentration in pooled samples was 218 µg/ml, while the apoE-3V protein was expressed at 128 µg/ml. The apoE produced in vivo displayed molecular mass heterogeneity. This may be a reflection of differential glycosylation as is observed in mammalian plasma apoE (27).

Lipid Binding of Recombinant apoE. In the bloodstream of mammals and in the hemolymph of insects, apolipoproteins are usually found in association with lipoprotein particles. The lipid association properties of the expressed proteins were assayed by ultracentrifugation in NaBr at a density of 1.21 g/ml (Fig. 2). Less than 1% of apoE expressed from tissue culture was lipid-associated. However, a much greater proportion of apoE isolated from larval hemolymph floated under these conditions. When density gradient ultracentrifugation was performed, approximately one-third of both apoE-3 and apoE-3V produced by M. sexta larvae floated at a density <1.02 g/ml (Table 1). At this density, mammalian lipoproteins are composed of >80% lipid. Since lipoproteins were not detected at this buoyant density in wild-type AcNPV-infected larvae, it appears that heterologous apoE facilitated the formation of distinct lipoprotein particles.

LDL Receptor Binding of Recombinant apoE. To assess the biological activity of the lipoprotein particles containing recombinant apoE, their ability to compete with 125I-labeled LDL for binding to the LDL receptor was tested (Fig. 3). Neither the apoE-3 nor apoE-3V produced by SF-21 cells in vitro was able to effectively compete for binding to the LDL receptor. By contrast, the apoE-3-containing lipoprotein particles in M. sexta hemolymph were excellent ligands for the LDL receptor (50% displacement at =0.2 µg/ml). The apoE-3V produced in M. sexta had little detectable receptor
binding activity, consistent with a previously described receptor binding defect associated with the Ala-152 → Pro change (3). Media and hemolymph derived from infections with wild-type virus did not compete with LDL for receptor binding (data not shown). LDL receptor binding activity of apoE-3 from SF-21 cell media was regenerated upon association with Myr2-PtdCho liposomes in vitro. Conversely, apoE-3V from SF-21 cell media remained a poor ligand for the LDL receptor even after lipid association (Fig. 3 Inset).

**DISCUSSION**

Various hosts have been used for the expression of recombinant apoE. The utility of these systems is limited by the relatively low levels of apoE expression in mammalian cells or its lack of lipid association when expressed in bacterial cells. For studies of LDL receptor binding, formation of *bona fide* lipoprotein particles is a significant advantage because apoE requires a lipid milieu to recognize the LDL receptor. In the present studies, lipid-free apoE expressed in recombinant baculovirus-infected insect tissue culture was not a ligand for the LDL receptor. Consistent with previous studies (2), LDL receptor binding activity of apoE-3 was restored upon *in vitro* lipid association. Conversely, expression of apoE in insect larvae resulted in the production of high concentrations of lipoprotein particles containing apoE, which effectively competed with LDL for binding to the LDL receptor.

Particles containing apoE-3V, which were derived from infected larvae or from *in vitro* Myr2-PtdCho lipid association, were poor ligands for the LDL receptor. This confirms the original observation of the disruptive nature of the Ala-152 → Pro mutation (3) and emphasizes the fidelity of the larval expression system.

The apoE in hemolymph of infected *M. sexta* floated at a density substantially more buoyant than that of the endogenous lipoproteins (lipophorins), suggesting the presence of lipoprotein particles unique to apoE-expressing larvae. The mechanism for lipid association is presently unclear. The apoE could have associated with lipids from preexisting insect hemolymph lipoproteins. Alternatively, apoE might have been assembled *de novo* into a more buoyant lipoprotein particle within the fat body cells. The latter possibility would make *M. sexta* an attractive system for studying lipoprotein assembly.

*M. sexta* larvae are easy to maintain, grow 4000-fold in 20 days, and are of sufficient size to simplify injection of the baculovirus into the hemocoele. The larvae have an open circulatory system from which large quantities of hemolymph containing secreted recombinant protein can easily be collected. Because *M. sexta* is semipermanent for baculovirus

---

**Table 1. Distribution of apoE across a buoyant density range**

<table>
<thead>
<tr>
<th>Density range</th>
<th>apoE-3</th>
<th>apoE-3V</th>
<th>apoE-3</th>
<th>apoE-3V</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.02</td>
<td>&lt;1%*</td>
<td>&lt;1%*</td>
<td>33%</td>
<td>27%</td>
</tr>
<tr>
<td>1.02-1.21</td>
<td>&lt;1%*</td>
<td>&lt;1%*</td>
<td>&lt;1%*</td>
<td>&lt;1%*</td>
</tr>
<tr>
<td>&gt;1.21</td>
<td>&gt;99%</td>
<td>&gt;99%</td>
<td>67%</td>
<td>73%</td>
</tr>
</tbody>
</table>

Percentages represent the proportion of recovered apoE. *<1%* indicates the limits of detection of the radioimmunoassay (<25 ng/ml).
infection (28), rapid mortality does not result from viral infection, allowing the infected insects to sustain relatively stable dietary intake and weight gain while producing recombinant proteins. Thus, baculovirus-directed expression in *M. sexta* is an effective approach for the production of large quantities of a complex, biologically active lipoprotein without *in vitro* reconstitution. *In vivo* virus propagation may be utilized to achieve high levels of expression of other therapeutically important proteins that require association with lipid for full biological activity.

The first two authors contributed equally to this work. We are indebted to David Bishop, John Taylor, Linda Curtiss, and Lois Miller for kindly providing vectors, cDNAs, antibodies, and helpful advice. We thank Robert Aiello and David Nevin for providing 125I-labeled LDL and for help with LDL receptor binding assays and Bruce Kottke and Nina Bren for performing the radioimmunoassays of apoE. We are grateful to Julie Dickson for excellent technical assistance and the members of our laboratories for helpful discussions and critical appraisal of this manuscript. This work was supported by grants from the National Heart, Lung, and Blood Institute, Council for Tobacco Research (to A.D.A.), the National Institute of Allergy and Infectious Diseases (to P.D.F.), and the National Science Foundation (to N.E.B.). D.G.G. was supported by a Biotechnology Training Program, National Research Service Award from the National Institute of General Medical Sciences.