papers and notes on methodology

High level secretion of wild-type and mutant forms of human proapoA-I using baculovirus-mediated Sf-9 cell expression

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Abstract To facilitate the investigation of apoA-I structure:function relationships as they relate to LCAT activation and lipid binding, we have developed an apoA-I baculoviral expression and purification system that yields milligram quantities of wild-type or mutant proapoA-I. Baculovirus-infected Sf-9 cells, grown in suspension, were found to secrete high levels of human wild-type (40–50 mg/l) or mutant apoA-I protein (1–38 mg/l), which was determined to be > 95% pure following a two-step purification procedure. In the case of wild-type apoA-I, ELISA showed that approximately 13–18% of the total protein secreted into the culture medium was apoA-I. To isolate pure protein from culture medium, 72 h post-infection medium was subjected to preparative reverse phase high performance liquid chromatography (HPLC), followed by DEAE ion-exchange chromatography. Purity and molecular size determination of wild-type proapoA-I protein was verified by SDS polyacrylamide gel electrophoresis, electrospray mass spectrometry, and N-terminal sequencing. In addition, recombinant discoidal apoA-I:phospholipid complexes prepared from wild-type or plasma apoA-I showed similar particle size and LCAT activation properties. To fully characterize the utility of this expression system, the expression levels of various mutant apoA-I proteins were compared to wild-type. Despite a lower production level seen with selected apoA-I mutants, milligram quantities of these purified mutant proteins were also obtained. In summary, we show that baculovirus-derived wild-type proapoA-I shows properties similar to plasma apoA-I relative to recombinant HDL formation, LCAT reactivity, and α-helical content. In addition, we show that a variety of mutant forms of human proapoA-I can be expressed and purified in abundant quantity from baculoviral-infected Sf-9 cells.–**Sorci-Thomas, M. G., J. S. Parks, M. W. Kearns, G. N. Pate, C. Zhang, and M. J. Thomas.** High level secretion of wild-type and mutant forms of human proapoA-I using baculovirus-mediated Sf-9 cell expression. *J. Lipid Res.* 1996. **37:** 673–683.

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Apolipoprotein (apo) A-I is the major protein constituent of high density lipoprotein (HDL) and mediates cholesterol transport within the vascular compartment through its role as a co-activator of the plasma enzyme, lecithin:cholesterol acyltransferase (LCAT). LCAT facilitates the directional movement of cholesterol via the conversion of HDL cholesterol to cholesteryl ester, driving cholesterol movement from peripheral tissues to the liver for removal. Thus, apoA-I functions as both an excellent lipid binding protein for peripheral cholesterol efflux as well as a co-activator of LCAT. The inter-dependency of these two functional roles has been the subject of considerable investigation (for review see refs. 1, 2).

Most studies concerning apoA-I structure:function have focused on elucidating the domains within apoA-I that execute these important functions in cholesterol metabolism. ApoA-I is known to be a highly amphipathic α-helical protein (for review see ref. 3) and has been studied using a variety of approaches including the use of natural occurring mutations (4, 5), proteolytic fragments (6, 7), synthetic apolipoprotein mimetic peptides (8, 9), monoclonal epitope mapping (10, 11), ^{13}C NMR (12, 13), physical studies of defined recombinant apoA-I:phospholipid particles (14, 15), and by deletion/substitution mutagenesis (16–18). One theme common to each of these studies is that one or more specific do-

Abbreviations: apo, apolipoprotein; HDL, high density lipoprotein; LCAT, lecithin:cholesterol acyltransferase; HPLC, high performance liquid chromatography; MOI, multiplicity of infection; SDS, sodium dodecyl sulfate; ELISA, enzyme-linked immunosorbent assay.

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main(s) within apoA-I are believed to be directly responsible for orienting the phospholipid substrate to an accessible conformation that "activates" the substrate for catalysis by LCAT (2).

The high content of amphipathic α-helical conformation within apoA-I has been suggested to account for both its lipid binding and LCAT activation properties. The structural basis for the amphipathic α-helix are 22 and 11 amino acid repeats each possessing a distinctive amphipathic character (3). The 8–22 and 2–11 mers within apoA-I, comprise approximately 80% of the mature protein and are believed to be arranged in an antiparallel amphipathic α-helical manner, interrupted by β-turns punctuated by proline or gylcine. A precise model of apoA-I's three-dimensional structure (for review see 19) has not been possible but hypothetical models have included those based on prediction algorithms (20), monoclonal antibody mapping (21), by comparison to insect apoproteins with solved X-ray crystallographic data, such as apoLp-III (22), or by comparison to three-dimensional structural determinations made on truncated forms of apoE (for review see ref. 23). Each of these above-mentioned approaches offers specific information regarding the possible structural motifs present either in the lipid-free or lipid-bound state of apoA-I. Thus, the use of apoA-I structural mutagenesis should complement and extend the information acquired from other methods, allowing us to test hypothetical models of apoA-I conformation in both the lipid-free and -bound state.

In this report we describe a baculoviral expression system and a two-step purification scheme that yields high levels of wild-type and mutant human proapoA-I protein. These studies extend our previously published work on apoA-I structure:function that utilized a Chinese hamster ovary cell expression system to study the LCAT activation properties of a series of 22-mer deletion mutations (17). In our previous report, we sequentially deleted each of the 8–22 or 2–11-mer repeats found within apoA-I. These studies indicated that the removal of most of the 22 mer domains impaired apoA-I's ability to activate LCAT. However, the region corresponding to residues #143 to 186 was found to be absolutely essential for LCAT activation. These studies were limited in that only μg amounts of protein could be generated. Therefore, we have sought to develop a system that would generate greater quantities of wild-type and mutant protein for use in detailed biophysical studies of apoA-I. To achieve this, the full-length human wild-type apoA-I cDNA was cloned into pBluebac III and co-transfected with genomic DNA of *Autographa californica* nuclear polyhedrosis virus (AcMNPV) into Sf-9 cells. Recombinant baculovirus containing wild-type apoA-I cDNA was purified and then used to infect Sf-9 cells. Baculoviral-infected Sf-9 cells grown in serum-free medium are shown to secrete high levels of biologically active human wild-type proapoA-I in suspension culture. To demonstrate biological activity of the baculoviral-derived apoA-I, recombinant discoidal apoA-I:phospholipid complexes were prepared from wild-type and plasma-derived apoA-I and then compared with respect to their size heterogeneity and LCAT reactivity. Additionally, a variety of mutant forms of apoA-I were also characterized and compared to the wild-type apoA-I expression level. In summary, we show that baculovirus-derived wild-type proapoA-I shows properties similar to plasma apoA-I relative to recombinant HDL formation, LCAT reactivity, and α-helical content. In addition, we show that a variety of mutant forms of human proapoA-I can be expressed and purified in abundant quantity from baculoviral-infected Sf-9 cells.

MATERIALS AND METHODS

Anti-human apoA-I polyclonal antibodies and a human apoA-I protein standard were purchased from Chemicon Inc. Sodium cholate was purchased from Calbiochem. HPLC solvents were purchased from Fisher Scientific. Ultrapure guanidine hydrochloride was purchased from Gibco/BRL. DEAE Fast-Sepharose was from Pharmacia Inc. Cholesterol >99% was from Nu-Chek Prep. Egg phosphatidylcholine in chloroform was from Serdary Research.

Preparation of wild-type and mutant pBlueBac III:apoA-I constructs

The full-length human apoA-I cDNA (kindly provided by Dr. Larry Chan) was amplified by PCR using; 5′ gcGGATCCCCCACGGCCCTT 3′ and 5′ cctgcagccCACTTTGGAAACG 3′ for the 5′ and 3′ primers, respectively (with lower case sequences indicating mismatches to the human apoA-I cDNA sequence). These primers contain embedded BamHI and PstI restriction endonuclease sites at the 5′- and 3′-most ends to allow for directional cloning into the expression vector. The resulting 887 bp apoA-I cDNA fragment generated using these PCR primers extends 21 bp from the upstream ATG start-site to 55 bp downstream from the TGA stop codon. After restriction digest, the full-length apoA-I cDNA was cloned into the BamHI/PstI site of pBlueBac III (Invitrogen, Co., San Diego, CA) as shown in Fig. 1. One construct containing the entire wild-type apoA-I cDNA under the control of the polyhedrin promoter/enhancer (PPH), pcWtA-I, was fully sequenced (Sequenase Version 2.0) and was identical to previously

published sequences (24).

The mutant apoA-I cDNA constructs, apoA-IΔ6 (deleted residues #143–164) and apoA-IΔ10 (deleted residues #220–241) were prepared using pcWtA-I as the template, a unique mutant primer for each of the two specific deletion mutations, and the 5′ and 3′ terminal primers containing PstI and BamHI restriction sites, described above. The method of mutagenesis used to introduce these mutations, referred to as the megaprimer method of site directed mutagenesis (25), has been described previously for the generation of the apoA-I genomic clones, pMTAIΔ6 and pMTAIΔ10 (17), containing deletion mutations at residues #143–164, and #220–241, respectively. The mutant apoA-IΔ1–43 was constructed using the primer 5′ GTCAAGGAGCTTTAGTTGCTGCCAGAAATG3′ which effectively deletes only residues #1–43 of the mature protein and also utilizes the previously described megaprimer method of mutagenesis (17). The mutant apoA-I 10F6 represents a replacement mutation in which residues #143–164 of apoA-I have been removed and replaced with residues #220–241, with the original #220–241 domain remaining intact. The method used to introduce this mutation was based on the method described by Zhong and Bajaj (26). This method uses four oligonucleotides of which two are external primers. The external primers were those mentioned above containing the PstI and BamHI restriction sites. The other two primers used in this method of mutagenesis flank the replacement DNA fragment. To amplify the 5′-most end of this DNA fragment, the primer 5′CAAGAGAAGCTGAGCCCCGTGCTGGAGAGC 3′ was used and for the 3′-most end of the fragment 5′CTCGTCGCTGTAGGGCTGGGTGTTGAGCTT3′ was used. This method of mutagenesis used three rounds of PCR amplification (26). All mutant cDNAs were verified by complete sequencing. Both the pcWtA-I and each of the mutant apoA-I plasmids were used in conjunction with the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) linear viral DNA for co-transfection into Sf-9 cells. Recombinant wild-type or mutant baculoviral clones were then purified and high titer viral stocks were generated according to Invitrogen protocols.

Preparation and purification of recombinant wild-type and mutant apoA-I baculovirus clones

Transfection of pcWTA-I and linear wild-type AcMNPV was carried out according to protocols provided by Invitrogen Co. Three rounds of plaque purification were performed before pure final viral titers of $1–2 \cdot 10^8$ PFU/ml were obtained. To optimize culture conditions for high level expression of wild-type and mutant apoA-I expression, Sf-9 cells in SF900-II SFM medium were cultured in 30-mm wells and infected with wild-type apoA-I baculovirus at a multiplicity of infection (MOI) of 0.5, 5.0, and 10.0. These infections were carried out for approximately 75 h. At various timed intervals, aliquots of culture medium were taken and stored at -20°C for ELISA (27). Similar experiments were conducted to determine the optimal MOI for expression of each of the mutant apoA-I baculoviral clones described in this report. Analogous results to those obtained with wild-type were obtained for each of the mutant clones.

Based on the results of these studies, standard 1 liter Sf-9 cell suspension cultures were routinely infected at an MOI of 0.5 and harvested between 68–85 h, or until 20–30% of the cells had lysed, as determined by trypan blue exclusion. Lengthening infection time so that greater than 20–25% of the Sf-9 cells had lysed resulted in a greater degree of contamination with cellular protein and an increase in the presence of degradation products.

Purification of recombinant apoA-I from baculovirus-infected Sf-9 cell culture medium

At the time of harvest, Sf-9 cell post-infection culture medium was spun at 4°C, 10,000 rpm for 10 min to remove cellular debris. The culture medium was adjusted to 10% acetonitrile and then loaded onto a C-18 reverse phase (50 cm × 5 cm) HPLC column. The column was washed overnight with 10% acetonitrile, 0.1% TFA using a Beckman System Gold HPLC pump. The next day the apoA-I peak was eluted from the column at a flow rate of 10 ml/min using a linear gradient of 10 to 95% acetonitrile, 0.1% TFA, over a 1-h period (28, 29). The peak containing apoA-I was collected, adjusted to pH 7.4, and dried immediately under vacuum. The pellet was resuspended in 6 M guanidine hydrochloride, dialyzed extensively against 1 mM ammonium bicarbonate, pH 7.4. This mixture was adjusted to 5 mM Tris/8 M urea, pH 8.0, and then applied to a DEAE Fast-Sepharose anionic exchange column (26 cm × 7 cm). The purified apoA-I peak eluted at approximately 9 mM Tris/8 M urea, pH 8.0, using a linear gradient (100 ml each) that went from 5 mM Tris to 150 mM Tris/8 M urea, pH 8.0, and a flow rate of 144 ml/h (30). The apoA-I peak was collected and dialyzed against 1 mM ammonium bicarbonate, pH 7.4, for 72 h with at least five complete changes of buffer.

From 50 ml to 1 liter of infected Sf-900 II culture medium was purified using the columns described. Recovery of apoA-I from the HPLC and DEAE columns was typically about 70–85%, with the overall yield from culture medium to purified product for mutant or wild-type apoA-I protein ranging from approximately 50 to 75%.

Mass spectrometric and protein sequence analyses

Mass spectrometric analysis was carried out on a Quattro II triple quadrupole mass spectrometer equipped with an electrospray interface. Protein samples (0.4 μM) were prepared in acetonitrile–water 1:1 (v:v) containing 0.2% formic acid and then introduced into the electrospray interface at 5 μl/min. Acquisition parameters were adjusted to maximize resolution.

N-terminal sequence analysis was performed directly on protein samples using an Applied Biosystem Model 475A protein sequencer equipped with an on-line Model 120A PTH analyzer.

Quantification of apoA-I and SDS polyacrylamide gel electrophoresis

Whole medium, purified wild-type or mutant apoA-I were analyzed by sodium dodecyl sulfate, 15% polyacrylamide gel electrophoresis followed by either direct staining of the gel with Coomassie Brilliant Blue R-250 or by Western blot analysis using polyclonal antibodies specific to human apoA-I, as previously described (17). Quantification of apoA-I in whole culture medium or after the purification procedure was performed by ELISA (27). Mass of the final purified protein was also verified by the method of Lowry et al. (31) or by measuring the optical density at 280 nm.

Circular dichroism spectroscopy

Circular dichroism spectra were recorded with a Jasco J720 spectropolarimeter at 25°C using a 0.1-cm path length cell. Mean residue ellipticity was measured at 222 nm on protein solutions adjusted to an absorbance of 0.10 at 280 nm. Five scans were recorded and averaged and the background was subtracted. Molar mean residue ellipticity (θ) was expressed as degrees $\cdot$ cm^2 $\cdot$ dmol^{-1}, and calculated as $[\theta] = \theta_{obs} \cdot 115/10 \cdot l \cdot c$, where θ_{obs} is the observed ellipticity at 222 nm in degrees, 115 is the mean residue molecular weight of the protein, l is the optical path length in centimeters, and c the protein concentration in g/ml. The percent α-helicity was calculated from the formula of Chen, Yang, and Martinez (32), $[\theta]_{222} = -30{,}300\, f_h - 2{,}340$.

Preparation of discoidal complexes and LCAT activation determination

A ratio of 100:5:1 phospholipid to cholesterol to apoA-I protein was used for making discoidal complexes following published procedures (33). Briefly, 1.35 mg egg phosphatidylcholine in chloroform (20 mg/ml) was added to 35 μg cholesterol in ethanol (10 mg/ml) and 10 μl of radiolabeled cholesterol [1,2-^{3}H(N)] (50 Ci/mmol) in ethanol. The organic solvent was removed under a stream of argon and then the tubes were placed under vacuum for 30 min. Sodium cholate, 305 μg, was added to the tube and the solution was vortexed for 1 min and then incubated on ice for 30 min, vortexing every 15 min. To this mixture was added 500 μg of plasma or baculoviral-derived wild-type apoA-I and the incubation was continued for an additional 1 h or overnight at 4°C. Sodium cholate was removed using hydrated Biobeads SM-2, 20–50 mesh (Bio-Rad Inc.) as previously described (12). Recombinant discoidal complexes were purified using a Superose 12 (Pharmacia) column. Phospholipid (34), protein (29), and cholesterol (35) assays were performed to determine the final composition and molar ratio of the recombinant discoidal complexes. LCAT reaction with discoidal apoA-I-containing complexes was quantitated, as previously described, using 0.2 μg of cholesterol mass per assay to maintain < 10% cholesterol esterified during the 30-min incubation (17, 32). Percentage of cholesteryl ester formed was converted to nmole cholesteryl ester formed per ml of recombinant LCAT used.

Preparation of stably transfected CHO cells expressing recombinant human LCAT

CHO cells obtained from ATCC were grown in Dulbecco's modified Eagle's medium and Coon's F12 medium, supplemented with 10% fetal bovine serum, and essential amino acids (standard medium) at 37°C in a 5% CO_2 atmosphere. Transfection was performed using a modification of the calcium chloride precipitation method (17). Human wild-type LCAT cDNA driven by the CMV promoter (36) was co-transfected with herring sperm DNA (ratio of 2:1) and with pSV2neo at a ratio of 10 (LCAT cDNA) to 1 (pSV2neo). Briefly, the DNA mixture was precipitated with calcium chloride and allowed to incubate with the cells overnight. Once cells had become confluent, selection of stably expressing CHO cells was carried out by adding G418 at a concentration of 550 μg/ml to the medium and the antibiotic-resistant cells were selected over a 2-week period. Approximately six independent clones were tested for LCAT activity using a discoidal substrate composed of a 100:5:1 ratio of phosphatidylcholine–cholesterol–apoA-I. The cell line showing the highest level of activity, 12.3 nmol cholesteryl ester formed/h per ml, was then used as the source of LCAT for all studies described in this report.

To obtain human LCAT activity from the stably expressing CHO cells, the monolayer was grown to approximately 75% confluence in a 100-mm dish using standard medium, as described above. The cell monolayer was washed with phosphate-buffered saline, and serum-free standard medium was applied. This conditioned medium was removed approximately 72–90 h later and centrifuged at 10,000 rpm for 10 min to

remove cell debris. Aliquots of the culture medium were then frozen at -20°C until used in LCAT assays.

Nondenaturating gradient gel electrophoresis

Discoidal complexes were characterized by nondenaturating gradient gel electrophoresis as previously described (37). Gels were run for 3,000 volt · hours and then fixed in 10% sulfosalicylic acid. After fixation, gels were then stained in Coomassie G-250 overnight, then destained in 7.5% acetic acid for 1–2 days. Gels were scanned using a Zeinch scanning densitometer SL-504-XL.

RESULTS

Expression of recombinant wild-type apoA-I

The construct, pcWtA-I, contains the wild-type apoA-I cDNA cloned into pBlueBac III, as shown in **Fig. 1.** This construct was co-transfected with the *Autographa californica* nuclear polyhedrosis virus, (AcMNPV) into Sf-9 cells. Wild-type apoA-I containing recombinant baculoviral plaques were selected, purified, and used to generate high titer viral stocks for protein expression studies. Optimal culturing conditions for expressing

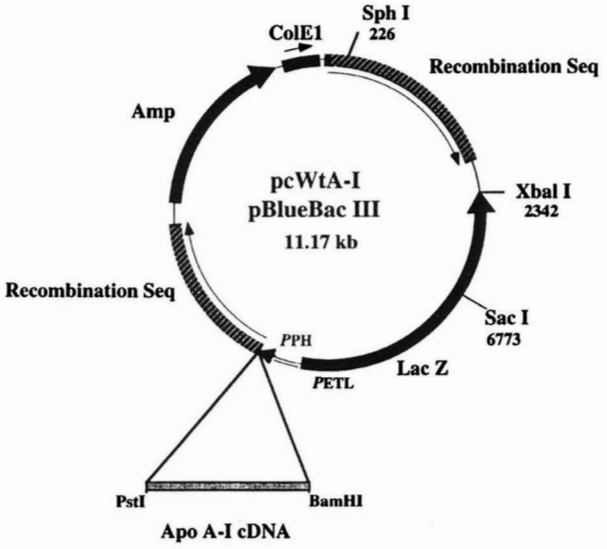

Fig. 1. Shows a map of pcWtA-I, the baculovirus transfer vector used for expression of wild-type apoA-I in Sf-9 cells. The PCR-amplified wild-type apoA-I cDNA was cloned into the PstI/BamHI restriction sites of the pBlueBac III (Invitrogene Co.) to generate pcWtA-I. This expression vector was co-transfected in conjunction with the *Autographa californica* nuclear polyhedrosis virus (AcMNPV) polyhedrin gene and clones derived from homologous recombination were identified by blue/white plaque selection. Recombinant baculoviral clones containing wild-type apoA-I were subjected to three rounds of plaque purification prior to use in these expression studies. All recombinant baculoviral clones containing mutant apoA-I cDNA constructs were prepared in a fashion similar to that described for wild-type apoA-I.

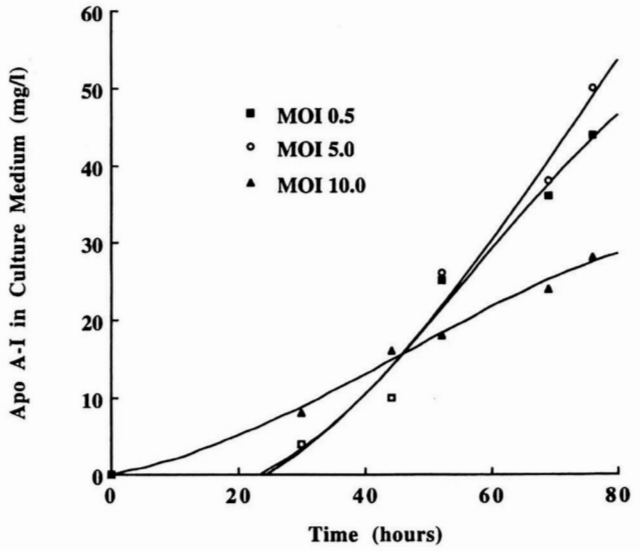

Fig. 2. Effects of increasing the multiplicity of infection (MOI) on wild-type apoA-I expression from baculoviral-infected Sf-9 cells. Sf-9 cells were grown in 30-mm well plates and infected with wild-type apoA-I baculovirus at MOIs of 0.5, filled square; 5.0, open circle; or 10.0, filled triangle. Aliquots of culture medium were taken at various times between 0 and 75 h for ELISA determination of apoA-I protein as described in Materials and Methods. Each point represents the mean (mg/l) obtained from duplicate wells of infected Sf-9 cells for each MOI studied.

wild-type apoA-I protein were examined by measuring protein levels over time and at different multiplicities of infection (MOIs), as shown in **Fig. 2.** Sf-9 cells were infected at MOIs of 0.5, 5.0, and 10.0, using wild-type apoA-I containing baculovirus, as described in Materials and Methods. At given times, aliquots of the culture medium were removed for apoA-I determination by ELISA. Cells infected at an MOI of 10 rapidly expressed apoA-I within 24–30 h after induction; however, this level of expression was not sustained during the entire course of the study (75 h). These results may reflect progressive proteolysis of apoA-I in the culture medium resulting from prolonged cell lysis at higher MOI. In contrast, infections carried out at MOIs of 0.5 and 5.0 had a slower rate of apoA-I expression but reached an overall higher level of expression by 60–70 h than cells infected at MOI of 10.0. Relatively little difference in the apoA-I protein expression level was noted between MOIs of 0.5 and 5.0. Thus, an MOI of 0.5 was routinely used for all large scale wild-type and mutant apoA-I expression studies described in this report.

Purification and characterization of baculoviral-derived wild-type apoA-I

Post-infection Sf-9 culture medium was subjected to a combination of reverse phase HPLC and DEAE chromatography as described in Materials and Methods. After these purification procedures, wild-type apoA-I protein

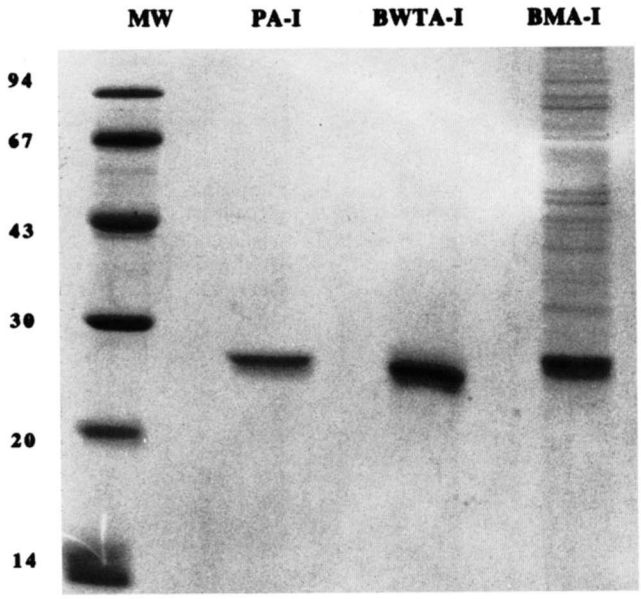

Fig. 3. Coomassie Brilliant Blue-stained 15% SDS polyacrylamide gel of wild-type apoA-I expressed from baculovirus-infected Sf-9 cells. Lane 1, MW, low molecular weight markers, 94,000 (phosphorylase b); 67,000 (albumin); 43,000 (ovalbumin); 30,000 (anhydrase); 20,000 (trypsin inhibitor); ,14,400 (α-lactalbumin); lane 2, PA-I, purified human plasma apoA-I; lane 3, BWTA-I, purified baculoviral-derived wild-type proapoA-I prepared as described in Materials and Methods; lane 4, BMA-I, 72-h post-infection culture medium from wild-type apoA-I baculoviral-infected Sf-9 cells.

was analyzed by SDS PAGE and stained for protein as shown in **Fig. 3.** As seen in this figure, 72 h post-infection wild-type culture medium (lane = BMA-I) showed several bands with the most abundant band co-migrating at the same molecular size with purified human plasma apoA-I (lane = PA-I). Upon completion of the two-step purification, purified baculoviral-derived wild-type proapoA-I (lane = BWTA-I), showed a single distinct band which also co-migrated with plasma apoA-I. This preparation of baculoviral-derived wild-type apoA-I protein was determined to be >95% pure by ELISA and Lowry analyses.

α-Helical content of plasma and baculoviral-derived apoA-I

The results of circular dichroism experiments are listed in **Table 1.** These studies showed 43.0 and 33.7% α-helical content for plasma and baculoviral-derived apoA-I, respectively. These results suggest that the α-helical nature of baculoviral-derived wild-type proapoA-I is similar to human plasma apoA-I, and that these values are within the range of previously reported α-helical contents for plasma-derived apoA-I (7, 38, 39).

Molecular size and purity of baculoviral-derived apoA-I

To further characterize the molecular size and purity of wild-type and mutant proteins produced by the baculoviral Sf-9 cell system, electrospray mass spectrometry was used to determine relative purity and molecular weight. In each case, the protein mass spectra were consistent with the predicted size of the protein and showed no major peak other than the ion corresponding to the mass of wild-type or mutant proapoA-I (data not shown). Protein sequencing confirmed that baculoviral-derived wild-type apoA-I possesses a 6 amino acid (Arg His Phe Trp Gln Gln) n-terminal extension consistent with the sequence of the pro segment for mature apoA-I. Our findings are also consistent with a recently reported baculoviral Sf-21 cell expression system (40).

Preparation and characterization of recombinant discoidal particles containing wild-type apoA-I or plasma apoA-I

The biological activity of baculovirus-derived wild-type proapoA-I was compared to plasma-derived human apoA-I by preparing discoidal recombinant particles at a starting ratio of egg phosphatidylcholine–cholesterol–apoA-I of 100:5:1, as described in Materials and Methods. The size heterogeneity of discoidal particles was compared by running each preparation on a 4–30% nondenaturing gradient gel. **Figure 4** shows the densitometric scan profile from a Coomassie G-stained 4–30% nondenaturing gradient gel. As seen in this figure, the size distribution of particles obtained using baculoviral-derived purified wild-type proapoA-I (panel A) and plasma-derived human apoA-I (panel B) were similar. Each preparation showed a single major peak with a Stokes radius of approximately 5.1 and 5.2 nm, corresponding to a diameter of 10.2 and 10.4 nm for baculoviral-derived wild-type and plasma apoA-I-containing discs, respectively. Compositional analyses of the purified discoidal particle preparation and measurement of their LCAT reactivities are shown in **Table 2.** For wild-type and plasma-derived apoA-I, LCAT reactivity was 8.5 and 9.0 nmol cholesteryl ester formed/h per ml, respectively. These results strongly suggest that similar physical and biological properties exist between apoA-I protein derived from plasma and from the baculoviral expression system.

TABLE 1. Molar ellipticities and calculated α-helical contents

Identification	[θ]222	α-Helix
	deg · cm² · dmol⁻¹	%
Plasma apoA-I	-15,389	43.0
Baculoviral wild-type apoA-I	-12,577	33.7

α-Helical content was calculated from the molar ellipticity at 222 nm using the method of Chen, Yang, and Martinez (32), as described in Materials and Methods.

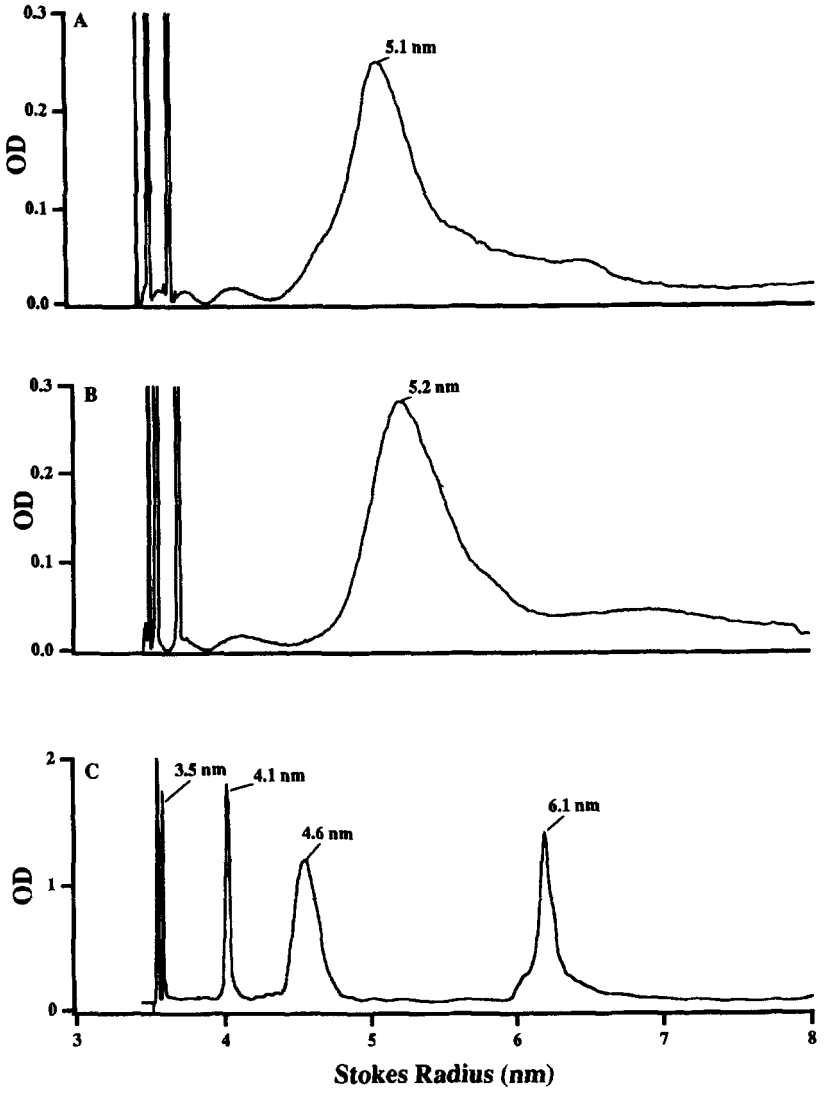

Fig. 4. Densitometric scan of a Coomassie G-250-stained 4–30% nondenaturing gradient gel showing molecular size as Stokes radius (nm). Top panel (A) shows discoidal particles generated using a 100:5:1 ratio of egg phosphatidylcholine–cholesterol–wild-type baculoviral-derived proapoA-I. Center panel (B) shows discoidal particles prepared using a 100:5:1 ratio of egg phosphatidylcholine–cholesterol–plasma-derived apoA-I. Bottom panel (C) shows the calibrating high molecular weight standards and their corresponding Stokes radii: ferritin, 6.1 nm; catalase, 4.6 nm; lactate dehydrogenase 4.1 nm; and albumin, 3.5 nm. Both discoidal particle preparations were made as described in Materials and Methods.

Baculoviral expression of mutant forms of apoA-I

Selected apoA-I mutants were characterized with respect to their protein expression level to determine the utility of expressing these forms of apoA-I from the Sf-9 baculoviral system. As seen in **Fig. 5,** apoA-I wt and apoA-I Δ10 protein expression reported as mg/l in culture medium were similar over time. Whereas, apoA-I Δ6, which lacks amino acids corresponding to #143 to 164 (17), and apoA-I 10F6, in which residues #143–164 are replaced with residues #220–241, displayed a strikingly reduced level of protein expression as a function of time. However, of the various mutants studied, the lowest level of protein expression was noted for apoA-I Δ1-43, which lacks residues #1–43, (where +1 corresponds to the first residue of the processed or mature apoA-I protein). The reduced levels of protein production among deletion mutations as detected by ELISA (Fig. 5) was supported by comparing their relative protein intensity on Coomassie Blue-stained 15% SDS polyacrylamide gels and Western blot analysis of 72-h post-infection culture medium (data not shown). It should, however, be noted that differences may exist among the various apoA-I mutant proteins with regard

TABLE 2. Composition and LCAT reactivity with recombinant discoidal HDL

Identification	Molar Ratio (% Recovery)			LCAT activity
	Protein	Cholesterol	Phospholipid	*nmol CE/h/ml*
Plasma apoA-I	1 (99)	4.2 (84)	87 (87)	8.58
Baculoviral wild-type apoA-I	1 (99)	4.5 (90)	99 (95)	9.01

Recombinant discoidal complexes were made at a starting molar ratio of 100:5:1, phospholipid–cholesterol–apoA-I. Final composition analysis was carried out as described in Materials and Methods. LCAT activity was measured using recombinant discoidal complexes at 0.2 μg of cholesterol per assay.

to their antibody-binding affinity, which may bias ELISA quantification of the mutant apoA-I protein concentrations compared to wild-type apoA-I.

Figure 6 shows the Coomassie Blue-stained SDS PAGE of 72-h post-infection culture medium from apoA-IΔ6 baculovirus-infected Sf-9 cells and after the two-step purification procedure. In the lane designated, BMΔ6A-I, 72-h post-infection apoA-I Δ6 culture medium shows no distinct band co-migrating with plasma apoA-I (lane = PA-I), as was noted when comparing 72-h wild-type culture medium and plasma apoA-I (Fig. 3). After the two-step purification procedure, purified apoA-I Δ6 (lane = BΔ6A-I) shows a single distinct band migrating at a slightly smaller molecular weight than plasma apoA-I. This purified mutant protein product was determined to be >95% pure.

DISCUSSION

In the present report we describe the development of a baculoviral expression system yielding milligram quantities of wild-type or mutant forms of proapolipoprotein A-I. Baculoviral-infected Sf-9 cells grown in serum-free medium produced human wild-type apoA-I at approximately 40–50 mg/l and various mutant apoA-I proteins at levels between 1–38 mg/l. After a simple two-step purification procedure, the overall recovery of purified wild-type or mutant protein from the culture medium ranged from 50–75% of that assayed in the starting medium. ApoA-I degradation products were not detected by Coomassie-stained PAGE (Figs. 3 and 6), Western blot analysis (data not shown), or by electrospray mass spectroscopy. Thus, the use of the baculoviral expression system described represents a highly effec-

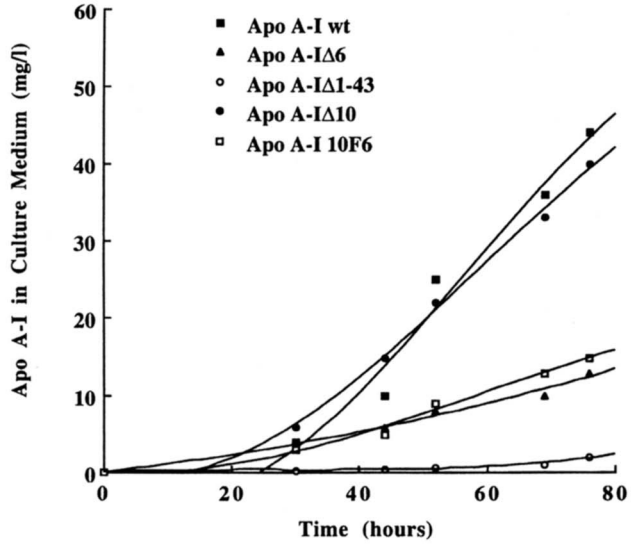

Fig. 5. Time-course expression of wild-type and mutant forms of apoA-I from baculoviral-infected Sf-9 cells. Sf-9 cells grown in suspension as described in Materials and Methods were infected with the indicated baculoviral clone and aliquots of the medium were taken at the indicated times between 0 and 78 h. ApoA-I protein concentrations were determined by ELISA (mg/l) and plotted as a function of time for each individual clone studied. ApoA-I wt, closed square; apoA-IΔ6 (deleted #143–164) closed triangle; apoA-IΔ1-43 (deleted #1–43) open circle; apoA-IΔ10 (deleted #220–241) closed circle; apoA-I 10F6 (replaced #143–164 with #220–241) open square. Each point represents the mean of at least four individual experiments.

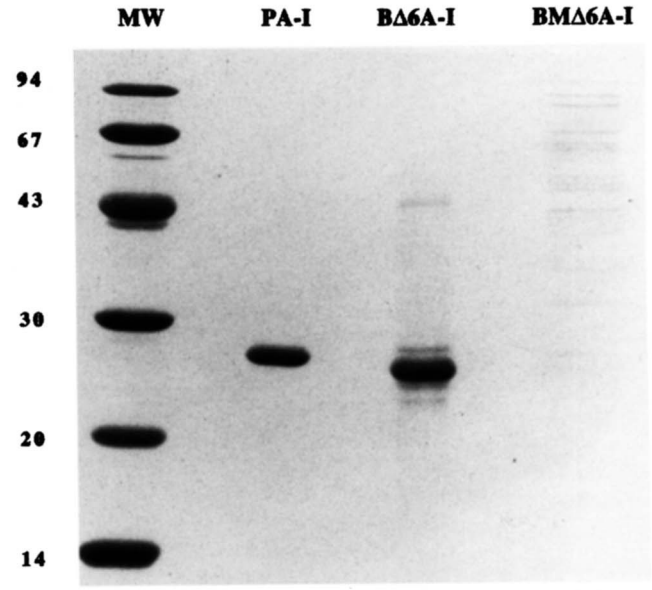

Fig. 6. Coomassie Brilliant Blue-stained 15% SDS polyacrylamide gel of apoA-I Δ6 (deleted residues #143–164) expressed from baculovirus-infected Sf-9 cells. Lane 1, MW, low molecular weight marker, 94,000 (phosphorylase b); 67,000 (albumin); 43,000 (ovalbumin); 30,000 (anhydrase); 20,000 (trypsin inhibitor); 14,400 (α-lactalbumin); lane 2, PA-I, purified human plasma apoA-I; lane 3, BΔ6A-I, purified baculoviral-derived apoA-I Δ6 prepared as described in Materials and Methods; lane 4, BMΔ6A-I, 72-h post-infection culture medium from apoA-I Δ6 baculoviral-infected Sf-9.

tive approach for isolation of biologically active wild-type or mutant forms of apoA-I for use in biochemical and structural studies.

Wild-type and mutant forms of human apoA-I have been isolated from *E. coli* expression systems (41–44) and from several eukaryotic cell expression systems that include African green monkey kidney cells (COS cells) (45), mouse fibroblast cells (C127 cells) (16), Chinese hamster ovary cells (CHO cells) (17, 46), and as a chimeric ubiquitin-proapolipoprotein A-I fusion protein (47) or as proapoA-I (40) from baculovirus-infected insect cells. Many of the earlier *E. coli* systems yielded small amounts of protein as a result of rapid intracellular apoA-I turnover. On the other hand, the utility of most eukaryotic or mammalian cell expression systems has been limited by the need to grow cells in monolayer combined with the modest levels of protein produced, 10–100 μg/ml (16, 17, 46). For these reasons many investigators have continued the development of prokaryotic cell expression systems. Recently, investigators have expressed wild-type and carboxyl-truncated forms of apoA-I as a maltose binding fusion protein in *E. coli* (18). With this system, a high level of protein was produced (30–70 mg/l) but digestion of the fusion protein with factor Xa to release free apoA-I resulted in degradation of the apoA-I protein (18). A recent report describes the expression and purification of large amounts of apoA-I_{Milano} from an *E. coli* expression system (39, 48), in which protein expression was directed into the external medium by use of a specialized expression vector (39). This approach significantly improved the level of apoA-I_{Milano} dimer expression (5 g/l), but required a four-step purification procedure to obtain pure apoA-I_{Milano}.

The baculoviral Sf-9 cell system was chosen for development of large scale apoA-I expression as it displays many of the advantages of both prokaryotic and eukaryotic cell systems. Similar to eukaryotic systems, secreted wild-type apoA-I protein derived from baculoviral-infected Sf-9 cells represents a significant amount of the total protein in the medium. This results in part from a reduction in the synthesis of endogenous cellular proteins during the late stages of infection. Like the prokaryotic cell systems, Sf-9 cells can be grown as suspension cultures with serum-free medium using standard large-scale shaker-incubators.

Baculoviral Sf-9 cell-derived wild-type proapoA-I showed physical properties similar to plasma-derived apoA-I as determined by circular dichroism, mass spectrometric analysis, and SDS PAGE. The baculoviral-derived apoA-I also showed similar biological properties compared to plasma-derived apoA-I as characterized by the formation of recombinant discoidal substrates of similar size, composition, and LCAT reactivity.

The utility of the baculoviral Sf-9 cell expression system described in this report was also demonstrated by the variety of apoA-I mutant proteins expressed in milligram quantities. The expression of mutant forms of biologically active proteins often presents a problem for both prokaryotic and eukaryotic systems. The relative expression level of mutant proteins compared to wild-type protein is often reduced. The basis for the reduction in the amount of mutant protein relative to wild-type protein from the baculoviral-infected Sf-9 cells for apoA-IΔ1-43, apoA-I 10F6, and apoA-IΔ6 is presently unknown, but likely reflects alterations in protein processing, folding, intracellular trafficking and/or secretion. A reduction in the amount of mutant protein produced relative to wild-type was demonstrated by ELISA (Fig. 5) and substantiated by relative comparisons of Coomassie-stained SDS gels of 72-h post-infection culture medium. However, it is also possible that another contributing factor to the reduction in protein production relates to alterations in the global conformation of the protein, which could alter polyclonal antibody recognition of the mutant proteins and thus bias ELISA quantification. Studies are currently in progress to map the tertiary structure of the mutant apoA-I proteins using a panel of monoclonal antibodies to wild-type apoA-I (L. Curtiss, unpublished data).

In summary, we believe that the development of the baculoviral Sf-9 cell expression system will prove to be a useful method for the production of wild-type and mutant forms of apoA-I. The availability of large quantities of mutant apoA-I for structural mutagenesis analysis should allow the elucidation of a more exact model of apoA-I tertiary conformation. From detailed structural and biological analyses of these mutant proteins, new molecular models of apoA-I conformation in the lipid-free and lipid-bound state will advance our current understanding of apoA-I's unique role in cholesterol metabolism.

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