Lipoprotein Association of Human Apolipoprotein E/A-I Chimeras

EXPRESSION IN TRANSFECTED HEPATOMA CELLS*

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Both apolipoprotein (apo) E and apoA-I are associated with lipoproteins, although with different particle classes. ApoE is associated with very low density lipoprotein (VLDL) and with the larger density lipoprotein (HDL) subclasses, whereas apoA-I associates predominantly with HDL subclasses. The genes encoding these proteins have a similar overall structure with the nucleotide sequences of the third and fourth exons coding for the mature protein. In an effort to understand the difference in lipoprotein association patterns of these two apoproteins, we have constructed and expressed chimeric apoproteins using cDNAs in which the third (n) and fourth (c) exons of human apoE and apoA-I are exchanged. McArdle rat hepatoma cells (MCA-RH7777), which secrete VLDL- and HDL-like particles, were stably transfected with these cDNAs, and the cDNAs for human apoA-I. Single spin NaBr gradient fractions of lipoprotein deficient serum-treated cell medium from transfected MCA-RH7777 cells were analyzed. The distributions of transfected human apoE and apoA-I, and endogenous rat apoE and apoA-I were compared with those of the chimeras. Among HDL subfractions, human apoE expressed by these cells is associated with particles of density 1.108 g/ml. Similarly, chimera apoE/A-Ic (exon 3 of apoA-I and exon 4 of apoE) is found in particles of density 1.111 g/ml. Human apoA-I, however, distributes in a broader range of particles with peak densities of 1.111 g/ml and 1.164 g/ml. The distribution of the complementary chimera, apoE/A-Ic, follows this same pattern, with peak particle densities of 1.098 and 1.137 g/ml. This is in contrast to the narrow distributions of endogenous rat apoE and apoA-I, which were found in particles of density 1.099 and 1.089 g/ml, respectively. When metabolically labeled medium was fractionated via gel filtration column chromatography, apoA-Ic was found to associate with the VLDL fractions; apoE/A-Ic was absent from these same fractions. These results suggest that the fourth exon largely determines the distinctive lipoprotein distribution patterns of these two human apoproteins and that the human apoA-I fourth exon sequence may account for the polydisperse HDL pattern as observed by others in transgenic mice expressing human apoA-I.

The soluble apoproteins appear to have evolved from a common ancestral apoprotein, apoC-I, through whole-gene duplications, intragenic duplications, and intragenic deletions (1). The set of current soluble apoproteins (apoA-I, apoA-II, apoA-IV, apoC-I, apoC-II, apoC-III, and apoE) have maintained similar overall gene structures. The first exon encodes the 5'-untranslated region, the second exon encodes most of the signal peptide, the third exon encodes the rest of the signal peptide and the amino terminus of the mature protein, and the fourth exon encodes the remainder of the mature protein as well as the 3'-untranslated region. In the case of apoA-IV, the first exon fulfills the role of exons one and two in the other six apoproteins. In all cases, the last two exons encode the mature plasma protein. The last exon of all the soluble apoprotein genes codes for a variable number of 11 or 22-amino acid repeats. These repeats are capable of forming amphipathic α-helical secondary structures that in vitro have been shown to be important for binding to phospholipid vesicles (2) and lipoproteins (3). Despite the similar gene structure and the presence of common secondary structure, these apoproteins all have unique roles in lipoprotein metabolism and structure (4). Each apoprotein associates with a specific class or classes of lipoprotein particles possessing distinct compositional and size differences. It is this remarkable specificity that determines the function and metabolic fate of each class of lipoprotein particles. The present study focuses on apoE and apoA-I, two apoproteins that associate with different classes of lipoprotein particles. It is well known that apoE associates with VLDL and the larger classes of HDL, such as HDLc and HDL1, whereas apoA-I associates predominantly with the smaller HDL subclasses, HDL2c and HDL3 (5). We have constructed chimeric apoproteins from these two human apoproteins in order to determine which domains are responsible for their lipoprotein particle segregation patterns. We examined the association of the apoproteins with nascent hepatic lipoproteins secreted from the MCA-RH7777 rat hepatoma cell line transfected with expression vectors containing the chimeric cDNAs. These cells are capable of vigorous triglyceride and apolipoprotein production, and their apolipoproteins are secreted as lipoprotein particles resembling VLDL and HDL of rat plasma (6). Electron microscopy reveals that these particles are primarily spherical in shape (7). Because this is a rat cell line, we were able to differentiate between endogenous and transfected apoproteins using species-specific antibodies. Therefore, we felt that these cells would provide a good model in which to study the lipoprotein class targeting of human apoE, human apoA-I, and apoA-II.

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The abbreviations used are: apo, apolipoprotein; VLDL, very low density lipoprotein; HDL, high density lipoprotein; n, third exon encoded amino acids; c, fourth exon encoded amino acids; MCA-RH7777, McArdle rat hepatoma cells; CMV, cytomegalovirus; LPDS, lipoprotein-deficient serum; DMEM, Dulbecco’s modified Eagle’s medium.
apoA-I, and chimeric proteins containing sequences of each. Gilbert (8) has suggested that the division of genes into exons separated by introns may correspond to coding regions for polypeptides with "related or differing functions" (8). Several well known proteins exhibit this kind of gene organization such as the LDL receptor (9), immunoglobulin genes (10), and the chick pyruvate kinase gene (11). This evidence makes our chimeric approach a logical one. By exchanging homologous exonic regions between structurally related but functionally different apoproteins, we hoped to begin to identify those regions of apoE and apoA-I responsible for their specific lipoprotein class distribution. Chimeric apoproteins were expressed by creating cDNAs in which the corresponding third and fourth exon regions of human apoE and apoA-I were exchanged. This approach allows the isolation and study of a specific apoprotein domain in the context of an overall apoprotein structure.

EXPERIMENTAL PROCEDURES

Materials—The rat hepatoma cell line MA-RH7777 (ATCC CRL 1601) was obtained from American Type Culture Collection, MD. The isopes [35S]dATP (370 MBq/ml), [32P]dCTP (740 MBq/ml), [38S]orthophosphate (carrier-free, 370 MBq/ml), 5'-32P-end-labeled dATP (>600Ci/mmol) and an oligonucleotide corresponding to apoB-48 nucleotide sequence 1708–1720 upstream from the site of mutagenesis. The final 890-base pair cDNA chimera was subcloned into the HindIII–XbaI site of pBR322 DNA (obtained from J. Breslow, Mount Sinai School of Medicine, New York, with the gift of Dr. Eric Rassart at the Universite de Montreal as a clone). The 5'-32P-end labeled DNA was subcloned into the unique restriction site of the polylinker region of the eukaryotic expression vector pCMV4. The pCMV4–huapoE parent was subcloned into the polylinker region of pBSK II (nucleotide 469), producing pBSK II–huapoE. The pCMV4–huapoE fragment was subcloned upstream of a neomycin analogue G418 (400 μg/ml) and the media of the individual colonies were screened for the presence of the chimeric proteins by overlaying the plate with ampicillin and testing for proper translation initiation (17) and containing a Sall restriction site at the 5'-end. The SalI–EcoRI restriction site at the 3'-end of the pBSK II–huapoE parent was subcloned into the polylinker region of pCMV4. The pCMV4–huapoE fragment, containing a full-length human apoE cDNA, was prepared as described.2

Stable Cell Lines—MA-RH7777 rat hepatoma cells were stably transfected with the recombinant expression vectors and psi2-neo using the calcium phosphate co-precipitation method described by Graham and van der Eb (19). Stable transfecents were selected using the neomycin analogue G418 (400 μg/ml), and the media of the individual colonies were screened for the presence of the chimeric proteins by metabolic labeling and immunoprecipitation. Stable cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 4500 μl/g glucose, 10% horse serum, 10% bovine fetal serum, 1% penicillin/streptomycin, 1% penicillin/streptomycin, 1% glutamine and 200 μg/ml of G418. All cell lines were maintained in the 13-cm2, 24–base pair fragment containing the Klenow fragment of DNA polymerase. This blunt-ended piece was then subcloned into the SalI site of the pUC19 polylinker region, and the resultant colonies were screened for plasmids in which the 5'-end of the cDNA was oriented near the BamHI site of the pUC19 polylinker region (pUC19-AIc, see Fig. 1A). The apoE signal peptide region and third exon were isolated from the cDNA plasmid pHEBB6, which contains the human apoA-I coding amino acids 40–243 of human apoA-I plus 24 base pairs of the 3'-untranslated region. The 5'-32P-end labeled DNA was subcloned into the polylinker region of pCMV4. The pCMV4–huapoE fragment, containing a full-length human apoE cDNA, was prepared as described.2

Preparation of Lipoprotein-deficient Serum (LPS)—Lipoproteins were removed from fetal bovine serum by centrifugation in NaBr, d 1.25 g/ml, twice. The bottom two-thirds of the serum was removed and dialyzed extensively against phosphate-buffered saline. Protein concentration was determined by Lowry assay (20) using bovine serum albumin as a standard. LPS was used in media at a final concentration of 2.5 mg/ml (10%). Cell Culture Protocol—Cells were seeded at 1.5 × 105 cells/25 ml. After 24 h, the medium was removed, and the cells were washed three times with phosphate-buffered saline. The supernatant was washed with 5 ml of 1 M NaOH, and the radiolabeled media was then added to the cells. This media was removed, incubated with 3 ml of DMEM/high glucose, 10% LPS (2.5 mg/ml), 1% penicillin/streptomycin, and 1% glucose. After an overnight incubation, the LPS-containing cell medium was removed, spun at 3000 rpm in a table top centrifuge for 5 min to remove cell debris, and supplemented with 21 μg/ml aspirin, 0.1 μM phenylmethylsulfonyl fluoride, 0.1% EDTA, 0.1 M Tris–HCl (pH 7.4). The media was then added to the cells, analyzed by gradient centrifugation or nondenaturing gradient gels.

Gradient Centrifugal Separation of Lipoproteins from the Medium—2 ml of the medium, harvested as described in the preceding paragraph, was layered at the interface of a 3–20 or 10–20% NaBr gradient and centrifuged in a Beckman SW41 Ti rotor at 38,000 rpm for 66 h at 15°C (21). The densities of individual fractions were determined.

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by refractometry of a treatment medium blank included in the same run. 26 fractions were collected using an ISCO gradient collector with UV monitor (Instrument Specialties Co., Lincoln, NE), dialyzed against Tris-buffered saline (10 mM Tris, 150 mM NaCl, 0.01% EDTA, 20 mM NaN3, pH 7.4), and used for the apoprotein analysis via Western blotting using species-specific antibodies and 125I-protein A. 125I-Labeled chimeric protein bands were cut out from the blots, counted, and graphed. The area under the curve defined by two density ranges ($d_{1.063–1.125}$ g/ml and $d_{1.125–1.210}$ g/ml) was quantitated by a computer digitizer (Sigma Scan, Scientific Measurement System; Jandel Scientific) and expressed as a percentage of total area described by the gradient profile for each stable cell line.

Nondenaturing Gradient Gels—The LPDS-treated cell medium was loaded (60–100 μl/lane) directly onto 4–30% nondenaturing gradient gels. The standards (high molecular weight electrophoresis calibration kit, Pharmacia Biotech Inc.) had the following radii: thyroglobulin, 8.5 nm; ferritin, 6.1 nm; catalase, 4.6 nm; lactate dehydrogenase, 4.1 nm; albumin, 3.55 nm. Gels were electrophoretically separated in 90 mM Tris, 80 mM boric acid, 2.5 mM sodium azide, EDTA, pH 8.4, for 24 h at 360 mA/gel. The next day, the proteins were electrotransferred to Immobilon P membrane overnight and then probed with species-specific antibodies followed by ECL detection.

32P Labeling of Phospholipids of Lipoproteins Secreted by McA-RH7777 Cells—Cells were seeded at a density of 0.75 × 10⁶ cells per T25 flask. After 24 h, the cells were rinsed 3 times with sterile phosphate-buffered saline and then incubated overnight with 5 ml/flask of a pulse medium containing phosphate-free DMEM/high glucose, 10% horse serum, 10% fetal calf serum, 1% each penicillin, streptomycin, glutamine, 3.75 mg/liter Na₄HPO₄·7H₂O, and 20 μCi/ml [32P]orthophosphate (Amersham Corp., carrier-free, 370 MBq/ml). After 24 h, the pulse medium was removed, and cells were washed 3 times with sterile phosphate-buffered saline and chased for an additional 24 h with 3 ml of chase medium (DMEM/high glucose, 10% LPDS, 1% each penicillin, streptomycin, glutamine). The chase medium was subjected to equilibrium density gradient centrifugation as described above. The peak fractions for chimeric apoproteins and wild-type apoproteins, as determined by quantitative Western blotting with 125I-protein A, were dialedyzed and run on 4–30% nondenaturing gradient gels. The gel was exposed to film at 4°C for 7 days.

Determination of Apoprotein Association with VLDL via Metabolic Labeling and Column Fractionation—McA-RH7777 stable cell lines were plated at a cell density of 1.5 × 10⁶ cells/T25 flask. After 24 h, the cells were pulsed with 100 μCi/ml Tran35S-label in methionine-free DMEM supplemented with 10% LPDS, 1% each penicillin, streptomycin, and glutamine, and 40 μM cold methionine for 24 h. 5 ml of labeled medium from cell lines expressing each chimeric apoprotein was fractionated on 1.5 × 120 cm Biogel A-5m column equilibrated with 10 mM Tris, 0.3 M NaCl, pH 7.4, 1 mM EDTA, 0.02% azide. The VLDL fractions were counted and graphed, and the peak fractions were immunoprecipitated with the appropriate species-specific antibodies to ascertain the presence of human apoproteins and endogenous rat apoE and apoA-I. Equal trichloroacetic acid precipitable counts of the unfractionated medium were similarly immunoprecipitated, and all samples were run on 9–15% gradient SDS-PAGE and subject to fluorography (22). Unlabeled human plasma had been previously applied to these columns to demonstrate that VLDL was separable from LDL and HDL using this method.

Antibody Production—Antibodies were prepared in rabbits as described previously (12). To prepare antibodies that distinguish between rat and human apoE and apoA-I, polyclonal antibodies to human apoE and apoA-I were passed through a column containing rat HDL. The antibodies to the human apoproteins that cross-react with rat apoproteins remained on the column, and human-specific antibodies were eluted. Antibodies to the rat apoproteins were purified in a similar fashion. The specificity of the antibodies was confirmed via Western blotting against human and rat VLDL and HDL.
RESULTS

In an effort to understand the difference in lipoprotein association patterns of apoE and apoA-I, we have constructed and expressed chimeric apoproteins using cDNAs in which the corresponding third and fourth exon regions of human apoE and human apoA-I are exchanged. These two exons code for the amino acids found in the mature protein. We refer to the amino acids encoded by the third exon as the N terminus (designated as n) and the amino acids encoded by the fourth exon as the C terminus (designated as c). Chimera apoEnA-Ic is encoded by a construct that specifies the apoE signal peptide and residues 1–61 followed by apoA-I residues 44–243. The complementary chimera, apoA-InEc, contains the apoA-I signal peptide, propeptide, and residues 1–43 followed by apoE residues 62–299. This chimera was constructed with the E3 allele, i.e. containing a cysteine at position 112. It is not known whether McA-RH7777 secrete apoA-I as a proprotein or a mature apoprotein; however, experiments with HepG2 cells show that apoA-I is secreted into the culture medium as a proprotein (22).

We included the propeptide in the apoInEc chimera for the sake of experimental consistency, assuming that whether or not the propeptide is removed in our cell system, the wild-type human apoA-I and apoA-InEc transfected cells would be processed in the same manner. McA-RH7777 cells are derived from a rat hepatoma cell line and secrete VLDL- and HDL-like particles (6). We prepared individual stable transfectants of this cell line expressing human wild-type apoE and apoA-I and the chimeric apoproteins.

Expression and Secretion of Chimeric Apoproteins and Wild-type Human Apoproteins—

To determine the relative levels of secretion of the chimeric apoproteins, wild-type human apoproteins, and the endogenous rat apoE and apoA-I, the transfected cells were labeled with Tran35S-label, the media were immunoprecipitated with the species-specific antibodies, and the regions of the gels corresponding to each apoprotein band were cut out and counted. The counts were corrected for the number of methionine residues in each protein, and the levels were expressed relative to rat apoE, which was set at 1.00 (Table I, part A). Rat apoE levels were unaffected by the expression of the human apoproteins. With the possible exception of the cells transfected with human apoA-I cDNA, the rat apoE/rat apoA-I ratios appear to be unaffected by the presence of the human apoproteins and chimeric apoA-I expressed by the transfected cells. The level of apoEnA-Ic secreted from the stably transfected cells is approximately 40% higher than the amount of rat apoE secreted from the same cell. ApoA-InEc is secreted at slightly lower levels, at approximately 60% the level of rat apoE. Table I also includes the relative secretion levels of the human apoE and human apoA-I transfected clones. Both of these wild-type human apoproteins were expressed at higher levels than the chimeric apoproteins. In part B of Table I, human apoA-I and apoEnA-Ic levels were also calculated relative to rat apoA-I for comparison. Human apoA-I is overexpressed relative to the other transfected cells and appears to be associated with a decrease in endogenous rat apoA-I levels, but the effect of this overexpression on endogenous rat apoproteins was not fully explored.

**TABLE I**

| Relative expression levels of human, chimeric, and rat apoproteins |
|-------------------------------------------------|
| Stable transfected McA-RH7777 cells expressing human apoE, human apoA-I, apoEnA-Ic, and apoA-InEc were labeled with Tran35S-label, immunoprecipitated with species-specific antibodies, and the immunoprecipitates were analyzed by fluorography as described in Fig. 2. The bands corresponding to each immunoprecipitated protein were excised and counted. The disintegrations/min were corrected for the number of methionine residues in each protein. The level of expression of each of the human apoproteins was expressed relative to rat apoE (A) or rat apoA-I (B). Rat apoE levels were not affected by the expression of the human apoproteins. For apoEnA-Ic transfected cells, rat apoE, n = 5; chimera, n = 4; rat apoA-I, n = 3. For apoA-InEc transfected cells, rat apoE, n = 6; chimera, n = 6; rat apoA-I, n = 6. For human apoE transfected cells, rat apoE, n = 7; human apoE, n = 9; rat apoA-I, n = 7. For human apoA-I transfected cells: rat apoE, n = 3; human apoE, n = 3; rat apoA-I, n = 2. |
| **A. Relative to rat apoE** | **B. Relative to rat apoA-I** |
| Rat apoE | 1.00 | 1.00 |
| Human transfected | 1.38 | 1.00 |
| Rat apoA-I | 0.82 | 0.89 |
| Human transfected | 1.68 | 1.68 |

*Note:* Values are normalized to 1.00 for rat apoE in part A and rat apoA-I in part B. The relative expression levels of human, chimeric, and rat apoproteins are expressed as a fraction of the level of rat apoE or rat apoA-I, respectively. Rat apoE and rat apoA-I levels were normalized to 1.00. Human apoE and human apoA-I levels were normalized to 1.00. Rat apoE and rat apoA-I levels were normalized to 1.00. Human apoE and human apoA-I levels were normalized to 1.00.
FIG. 3. Distribution of apoproteins on nascent lipoproteins separated by equilibrium NaBr density gradients. McA-RH7777 cells transfected with pSV2-neo or the human apoprotein constructs were seeded at $1.5 \times 10^6$ cells/T25 flask. After 24 h, the medium was replaced with 3 ml of DMEM/high glucose plus 10% LPDS for an additional 24 h. Two ml of medium was fractionated on a NaBr density gradient as described under "Experimental Procedures." 120 $\mu$l of each fraction was subject to Western blotting using anti-rat or human specific antibodies to apoE and apoA-I and 125I-protein A. After exposure to X-ray film, the apoprotein bands were excised and counted, and cpms from each fraction were graphed. The density of each fraction was determined by refractometry. Panel A, single representative distribution profile of rat apoE; panel B, single
The peak density of the nascent particles is consistent with the presence of one major subclass of HDL in rat plasma in contrast with the multiple HDL subclasses present in human plasma. Rat apoE was not consistently detected in the VLDL fractions obtained from the gradient centrifugation of McA-RH7777 cell medium. Thus, these equilibrium gradients were used primarily to examine apoprotein association with HDL particles.

The association of the human apoE and apoA-I with nascent HDL particles separated by gradient centrifugation is shown in Fig. 3, C and D, respectively. The majority of both of these apoproteins is found associated with the nascent HDL particles. Like rat apoE, human apoE is found on lipoprotein particles that distribute as a single peak within the HDL density range of the gradient. The peak density is 1.106 g/ml, similar to the peak density of the endogenous rat apoproteins. However, the human apoE distribution is broader than that of either rat apoE, human apoA-I, and the broadening of this peak is toward the lighter and presumably larger lipoprotein fractions. In contrast to rat apoA-I and rat and human apoE, human apoA-I has a broader distribution and associates with lipoproteins of two distinct peak densities. The first peak has a density of 1.111 g/ml and the second peak has a density of 1.164 g/ml. The broadness of the peaks is probably due to the high expression of human apoA-I. This polydisperse distribution pattern is characteristic of human apoA-I in the human HDL subclasses.

The association of the chimeric apoproteins with nascent hepatic lipoproteins secreted from the McA-RH7777 cells is shown in Fig. 3, E and F. apoA-InEc is found on particles that distribute as a single peak with a peak density of 1.116 g/ml. Very little of this chimeric apoprotein was found in the lipoprotein “bottom fractions.” This distribution pattern is very similar to that of wild-type human apoE (Fig. 3C). Similar to the results with human apoA-I, apoEnA-Ic (Fig. 3F) was found associated with two discrete particles within the HDL density range of these gradients. The peak density of the lighter particles is 1.098 g/ml, similar to the peak density of particles containing apoA-InEc. The peak density of the denser particles is 1.137 g/ml. These densities correspond approximately to the densities of human HDL2 and HDL3, respectively. As with the apoA-InEc chimera, very little of this chimeric apoprotein was found in the lipid-poor bottom fractions.

To quantitate the information from these graphs, the area under the curve, defined by the conventional HDL2 and HDL3 density ranges, was measured using the Sigma SCAN computer program. Areas between d 1.063–1.125 g/ml and d 1.125–1.210 g/ml were expressed as a percentage of total area described by the specific HDL apoprotein distribution obtained from the medium of each stable cell line. As shown in Table II, the percentage of the total apoEnA-Ic and human apoA-I found in the HDL2 and HDL3 density ranges is similar, with these apoproteins being distributed approximately equally in these fractions. By contrast, the major proportion of apoA-InEc and human apoE is found on particles in the HDL2 density range.

Non-denaturing Gradient Gel Electrophoresis of Nascent Lipoproteins—To determine the size of the particles secreted from the stable transfected cells, the cells were labeled with [32P]orthophosphate for 24 h, and the phospholipid-labeled lipoproteins secreted from cells during a 24 h chase were separated on NaBr equilibrium gradients. The peak fractions for each of the apoproteins were analyzed on nondenaturing gradient gels (Fig. 4). The rat HDL particles secreted from neo-transfected cells have a radius of 4.50 nm. Two discrete sized

TABLE II
Quantitation of the area under the HDL peaks obtained by density gradient centrifugation

| Stable McA-RH7777 cell line | HDL2 range (1.063–1.125 g/ml) | HDL3 range (1.125–1.210 g/ml) |
|-----------------------------|-------------------------------|-------------------------------|
| ApoEnA-Ic                   | 53.2 ± 2.9                    | 45.1 ± 5.3                    |
| Human apoA-I                | 44.5 ± 4.6                    | 53.1 ± 3.4                    |
| ApoA-InEc                   | 76.7 ± 9.2                    | 22.1 ± 9.0                    |
| Human apoE                  | 77.4 ± 5.3                    | 18.0 ± 9.2                    |

Determined by Western blotting. The distributions of endogenous rat apoE and apoA-I on the lipoprotein secreted by the pSV2-neo transfected McA-RH7777 cells are shown in Fig. 3, A and B, respectively. Both apoproteins were detected on particles in the HDL density range and in the lipopoor fractions. The peak density of the rat apoE-containing nascent particles is 1.099 g/ml and that of rat apoA-I is 1.089 g/ml. The profiles shown in Fig. 3, A and B, are single examples of the distribution pattern seen repeatedly in these experiments. This relative monodispersity of apoprotein distribution is consistent with the presence of one major subclass of HDL in rat plasma in contrast with the multiple HDL subclasses present in human plasma. Rat apoE was not consistently detected in the VLDL fractions obtained from the gradient centrifugation of McA-RH7777 cell medium. Thus, these equilibrium gradients were used primarily to examine apoprotein association with HDL particles.

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particles were observed for human apoA-I and apoEnA-Ic. The lighter fractions on the gradients (lanes 1 and 3) contain particles with radii of 4.65 nm, similar to the particle in the neo-transfected cells, while the particle sizes in the more dense fractions (lanes 2 and 4) are 3.75 and 3.90 nm, respectively, for apoA-I and apoEnA-Ic. When compared with the radii of the labeled particles in the peak fraction obtained from control neo-transfected cells (lane 7, at d 1.0987 g/ml, radius = 4.50 nm), human apoE appears to transform this particle species to a larger size (lane 5, at d 1.0987 g/ml, radii range = 5.45–8.85 nm). ApoEnA-Ic also induces a similar transformation (lane 6, at d 1.0987 g/ml, radii range = 4.85–8.20 nm), although not as effectively.

To confirm that the 32P-labeled lipoproteins contain the human wild-type and chimeric apoproteins, unfractionated media from the stable transfected cells were analyzed on nondenaturing gradient gels, which were subsequently immunoblotted with species-specific antibodies (Fig. 5). Human apoA-I and apoEnA-Ic are both found on two distinct sized particles. apoA-I is found on particles within the broad range of 5.90–4.50 nm in radii and in particles with radii of 3.90 nm. ApoEnA-Ic is found on particles with radii of 5.80–4.75 nm and 4.00 nm. Human apoE was detected on particles with radii ranging from 8.60 to 5.70 nm and a minor band at 4.3 nm. ApoEnA-Ic has a similar pattern with this apoprotein detected on particles with radii ranging from 8.50 to 5.15 nm and a minor band at 4.1 nm.

Association with Nascent VLDL. Particles—Sodium bromide gradients were not useful for analyzing apoprotein association with VLDL, since we could not consistently demonstrate endogenous rat apoE in the top part of the gradient. The prolonged centrifugation was probably responsible for loss of apoE from the nascent VLDL. To characterize VLDL-sized particle association, we isolated the VLDL particles by gel filtration chromatography using Biogel A-5m columns. Upon separation of human plasma on these columns, we detected apoB and apoE in fractions 60–70, apoB in fractions 70–90, and primarily albumin and other proteins in fractions >100 (data not shown). We infer from this pattern that plasma VLDL is readily separated from plasma LDL on these columns.

Hepatoma cell medium containing chimeric proteins labeled with Tran35S-label was fractionated on these columns, and the VLDL peak was identified by counting aliquots of each fraction. Equal levels of radioactivity in media or fractions were immunoprecipitated with anti-rat apoE antibodies, antihuman apoA-I antibodies, and anti-human apoA-I, simultaneously. A, lane 1, unfractionated medium from apoA-InE-expressing cells; lanes 2–4, peak three VLDL fractions from apoA-InE-expressing cells; lane 5, unfractionated medium from apoEnA-Ic-expressing cells; lanes 6–8, peak VLDL fractions from apoEnA-Ic-expressing cells. B, samples from human apoE expressing MGH-RH7777 cells: total medium and combined peak fractions 55/56, 57/58, 59/60, immunoprecipitated separately for human apoE and rat apoE.

Fig. 6. Association of wild-type and chimeric apoprotein with VLDL particles. Cells were seeded and labeled with Tran35S-label as described under “Experimental Procedures.” 5 ml of medium was fractionated on Biogel A-5m columns, and the VLDL peak was identified by counting an aliquot of each fraction. Equal levels of radioactivity in media or fractions were immunoprecipitated with anti-rat apoE antibodies, anti-rat apoA-I antibodies, and anti-human apoA-I, simultaneously. A, lane 1, unfractionated medium from apoEnA-Ic-expressing cells; lanes 2–4, peak three VLDL fractions from apoEnA-Ic-expressing cells; lane 5, unfractionated medium from apoEnA-Ic-expressing cells; lanes 6–8, peak VLDL fractions from apoEnA-Ic-expressing cells. B, samples from human apoE expressing MGH-RH7777 cells: total medium and combined peak fractions 55/56, 57/58, 59/60, immunoprecipitated separately for human apoE and rat apoE.

FIG. 5. Western blotting of unfractionated media run on 4–30% nondenaturating gradient gels. McA-RH7777 cell lines expressing human apoproteins, and chimeras were seeded at a density of 1.5 × 106 cells/750 ml flask. After 24 h, the medium was replaced with DMEM/high glucose with 10% LPDS for an additional 24 h. 60 μl of medium from each cell line was then loaded onto 4–30% nondenaturating gradient gels, transferred to Immobilon P membrane, and probed with human specific antibodies, followed by ECL detection. Comparison of the chimeric apoprotein lipoprotein distributions with that of the wild-type human apoproteins is shown. Lane 1, human apoA-I medium; lane 2, apoEnA-Ic medium; lane 3, human apoE medium; lane 4, apoEnA-Ic medium. Note, lanes 1 and 2 were probed with anti-human specific apoA-I antibodies, and lanes 3 and 4 were probed with anti-human specific apoE antibodies. The following standards were used to generate a standard curve to calculate the following radii: thyroglobulin, 8.5 nm; ferritin, 6.1 nm; catalase, 4.6 nm; lactate dehydrogenase, 4.1 nm; albumin, 3.55 nm.

DISCUSSION

In this study, we have set out to determine whether the domains of human apoproteins E and A-I encoded by the third
or fourth exons account for their selective targeting to VLDL and subsets of HDL. Our results indicate that the selective targeting of apoproteins E and A-I to nascent lipoproteins secreted from hepatoma cells is attributable largely to the domains of these apoproteins encoded by the fourth exons, which specify a series of amphipathic α helices. Preliminary studies utilizing the same chimeric proteins as used in this study produced in Escherichia coli indicate that similar domains function to target the apoproteins to mature human plasma lipoproteins. However, we cannot discount a small contribution from domains encoded by the third exons, although any effects these domains must be quite subtle. Our results also suggest quite strongly that the domains encoded by the fourth exon of the apoA-I gene account for the observed differences in the HDL subclass present in humans and rodents.

The majority of the human apoE associated with HDL-like particles with a density similar to those particles secreted by the neo transfected cells, as observed on equilibrium density gradients, although there does appear to be a broader distribution of particles on the HDL population containing human apoE than those containing rat apoE. This broadening affects both a subpopulation of higher density particles that also contain human apoE as well as a set of lighter subfractions (fractions of density 1.07 and 1.08 g/ml, Fig. 3C). Thus, human apoE is capable of associating with particles of similar density as the rat apoproteins. However, when the sizes of the particles were examined on nondenaturing gels, the majority of the human apoE associated with HDL-like particles were larger (radii = 8.85–5.45 nm) than the HDL particles secreted from the neo-transfected cells (radius = 4.50 nm). The density of the particles with which apoA-InEc associates is again similar to that of human apoE with a monodisperse distribution, although not quite as diffuse as the latter. Thus it appears that the fourth exon encoded amino acids of human apoE allow the formation of these larger HDL particles without significantly altering the density of the particles.

In contrast to the distribution of rat apoA-I, human apoA-I distributes onto particles with two distinct densities and sizes. The particles in the first peak of the NaBr density gradients have the same density and size as those in the single peak of the control cells, while the particles in the second peak are more dense and smaller in size. ApoEnA-Ic, which contains the fourth exon of apoA-I, also associates with two different particles of distinct size and density. These results suggest that the fourth exon-encoded amino acids of apoA-I are sufficient to determine HDL association and the formation of HDL subclasses. In addition, they strongly suggest that amino acid differences in the fourth exon-encoded amino acids of rat and human apoA-I may account for the different HDL subclass formation in humans and rodents.

The different lipoprotein distribution patterns seen for rat apoA-I compared with human apoA-I, and as shown here apoEnA-Ic, have been observed previously in mice expressing human apoA-I as a transgene (23). In control mice, the HDL profile was monodisperse, and the mouse apoA-I associated with particles 4.6 nm in radius. By contrast, the transgenic mice exhibited a biphasic HDL profile and the human apoA-I appeared in particles with radii of 5.1 and 4.15 nm. An explanation for these differences may reside in the primary sequence of the fourth exon-encoded amino acids. Since rat and mouse both appear to have monodisperse HDL distributions, while human HDL distributions are polydisperse (biphasic), the important contribution to the difference between rat and human must be shared between rat and mouse. There are 56 positions in the fourth exon in which both rat and mouse sequences differ from the human (Fig. 7). At 12 of these positions, there is either a proline or a charge change, and at five a change in hydrophylicity. The change clusters are centered in helices 6 and 7, both of which are class A helices (24, 25). The single proline change is at position +187 (using human sequence numbering) and may be the best candidate.

Studies by Leroy et al. (26) have suggested that differences in the helical repeat regions of apoA-I, apoE, and apoA-IV may account for the spontaneous formation of discrete sized reconstituted HDL (rHDL) particles in the presence of varying amounts of palmitoyl oleoyl phosphatidylcholine. Generally, it was observed that the apoprotein with the greatest number of repeats, apoA-IV, was capable of forming the largest HDL, while apoE formed larger rHDL than did apoA-I. This phenomenon may explain the formation of larger particles by the McA-RH7777 cells in the presence of human apoE. Human apoE is found on the cholesterol ester-enriched classes of HDL, HDL2c, and HDL, (18.27, 28), and its ability to conform to these larger particles, relative to apoA-I, may play an important role in reverse cholesterol transport.

In our cell culture model, human apoE and apoA-InEc were also found associated with the VLDL-like particles secreted by the McA-RH7777 cells. By contrast, neither human apoA-I nor apoENa-Ic associated with VLDL particles. Thus the third exon of each of these parent apoprotein genes does not encode a strong VLDL targeting domain. In addition, the rat apoE levels in the peak fractions from apoA-InEc transfected cells appeared to be lower than in the apoENa-Ic transfected cell line.
This difference suggests that apoA-InEc and rat apoE may be competing for association with the VLDL-sized particles. However, the wild-type human apoE does not appear to displace rat apoE despite being expressed at higher levels than apoA-InEc. There may be a subtle effect of the apoA-I third exon region in the apoA-InEc chimera, which needs to be further explored before a full explanation can be reached.

In conclusion, we have established a model for efficient expression of chimeric apoproteins and have shown that the chimeric nature of these apoproteins is not detrimental to their expression or secretion. Our results support the conclusion that the fourth exon-encoded amino acids of apoE contain sequences that target apoE to VLDL and larger HDL classes. The fourth exons of both apoE and apoA-I also encode amino acid sequences that determine not only the association of each apoprotein with HDL but also the ability to form different subclasses of HDL particles. Thus it appears that it is the nature of the apoprotein secreted that is primarily responsible for the size and density of particles produced by hepatoma cells. It remains to be established whether sequences encoded by exon 4 alone are sufficient to determine lipoprotein association. The role of exon 3-encoded sequences, even though highly conserved across species in the case of apoE, is uncertain, since this region is not the primary or major determinant of lipoprotein association and also does not impede lipoprotein association directed by exon 4 sequences.

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