Preferential association of apolipoprotein E Leiden with very low density lipoproteins of human plasma

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Abstract Apolipoprotein (apo) E Leiden is a rare variant of human apoE characterized by defective receptor binding and associated with dominant transmission of type III hyperlipoproteinemia. In heterozygotes, apoE Leiden is present in higher concentrations in both total plasma and very low density lipoproteins (VLDL) than the other apoE allele product. In the present study we analyzed cell expression and plasma lipoprotein association of apoE Leiden to determine whether the unequal concentration of the two apoE allele products could be explained by differences in secretion rate from the hepatocyte or by preferential association with VLDL. We transfected the rat hepatoma cell line McA-RH7777 with apoE Leiden or normal human apoE3, and studied their secretion and media distribution. In pulse-chase experiments, the secretion of apoE Leiden was comparable to that of both human apoE3 and rat endogenous apoE, approaching 100% in 90 min. In similar transfection experiments, secreted apoE Leiden was significantly less glycosylated than normal apoE3 (21.7% vs. 36.6%, $P < 0.005$, n = 4), a finding also noted for apoE Leiden in human plasma. In in vitro incubation experiments, apoE Leiden showed a markedly higher preference for VLDL of normolipidemic human plasma when compared to both apoE3 (2.6-fold, $P < 0.001$) and apoE4 (1.6-fold, $P < 0.001$). These results suggest that the accumulation of apoE Leiden in VLDL derives from a high affinity of the mutant protein for the VLDL. This enrichment in defective apoE probably exacerbates impairment of VLDL removal from the circulation, thus contributing to the dominant transmission of type III hyperlipoproteinemia.—**Fazio, S., Y. Horie, K. H. Weisgraber, L. M. Havekes, and S. C. Rall, Jr.** Preferential association of apolipoprotein E Leiden with very low density lipoproteins of human plasma. *J. Lipid Res.* 1993. **34:** 447-453.

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Apolipoprotein (apo) E, a polymorphic protein in humans, is the ligand for very low density lipoprotein (VLDL) and chylomicron remnant clearance by liver receptors (1). The polymorphic forms are termed apoE4(Arg112, Arg158), apoE3(Cys112, Arg158), and apoE2(Cys112, Cys158) (2). Several receptor-binding-defective variants of apoE have been identified that are associated with impaired catabolism of remnant particles, leading to the development of type III hyperlipoproteinemia (type III hlp) (3). Homozygosity for the polymorphic form apoE2(Cys112, Cys158), the most common of these defective variants, is associated with recessive transmission of type III hlp (4). In contrast, certain other apoE variants are associated with a dominant mode of inheritance (5–10), i.e., heterozygosity for these apoE variants is sufficient for the expression of type III hlp.

ApoE Leiden, one of the most thoroughly studied of the rare apoE variants (6, 11, 12), is characterized by the presence of a tandem repetition of amino acids 121–127 and has arginines at both polymorphic sites 112 and 158 (13, 14). In in vitro assays, apoE Leiden is defective in its affinity for the low density lipoprotein (LDL) receptor (6, 11, 14), having about 25% of normal apoE3 binding affinity. ApoE Leiden is considerably more active in receptor binding than apoE2 (6, 14), which exhibits less than 2% of normal apoE3 binding. All subjects carrying the apoE Leiden mutation are heterozygotes, but all demonstrate type III hlp, although to varying degrees (12). Interestingly, subjects heterozygous for the apoE Leiden mutation show a predominance of the variant apoE over the wild-type protein in total plasma, but especially in the VLDL fraction (4:1 ratio or higher) (6, 11, 12), a phenomenon for which there are several possible explanations. First, apoE Leiden could be secreted at an abnormally high rate compared to its normal apoE counterpart. Second, apoE Leiden could be catabolized relatively slower than normal apoE. In this latter instance, one mechanism that might lead to that result is preferen-

Abbreviations: VLDL, very low density lipoprotein; IDL, intermediate density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; DME, Dulbecco's modified Eagle's medium; apo, apolipoprotein; type III hlp, type III hyperlipoproteinemia.

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tial removal from circulation of lipoprotein particles having relatively more normal apoE, allowing the accumulation in plasma of particles having more of the defective apoE. This phenomenon could be attenuated or exacerbated depending on the relative preference for lipoproteins (in the case of type III hlp, it would be VLDL) of the mutant and normal apoE.

In the present study, we transfected the rat hepatoma cell line McA-RH7777 with an expression vector containing the cDNA for apoE Leiden to determine whether the unequal plasma representation of the two apoE allele products is due to hypersecretion of the mutant apoE relative to the normal apoE. We also investigated the in vitro lipoprotein distribution of apoE Leiden to determine whether the high ratio of mutant to normal apoE in VLDL is due to preferential association of the mutant apoE for the VLDL of human plasma. Our data show that while apoE Leiden is secreted at a normal rate by the liver cell, its accumulation on VLDL is due, at least in part, to very high affinity for this lipoprotein after secretion.

MATERIALS AND METHODS

Materials

The rat hepatoma cell line McA-RH7777 was obtained from American Type Culture Collection, Rockville, MD. All media (Dulbecco's modified Eagle's medium (DME) and methionine-free DME) were from GIBCO, Grand Island, NY. Fetal bovine serum and horse serum were obtained from HyClone, Logan, UT. Tran-S-Label ([^{35}S]methionine) was purchased from ICN Biomedicals, Costa Mesa, CA. Amplify was purchased from Amersham/Searle, Arlington Heights, IL. Tissue culture dishes and flasks were from Falcon Plastics, Oxnard, CA. Nitrocellulose paper was purchased from Schleicher & Schuell, Keene, NH. The ^{125}I-labeled secondary antiserum against rabbit antibodies and the Bolton-Hunter reagent were obtained from Amersham/Searle. The Superose 6 column was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden and was used on a Gilson Fast Protein Liquid Chromatography system. Cholesterol and triglyceride standards were from Abbott Laboratories, North Chicago, IL and Boehringer-Mannheim, Indianapolis, IN, respectively; the automated system for lipid analysis (Kinetic Microplate Reader) was from Molecular Devices, Menlo Park, CA. All the reagents for lipoprotein agarose gels were from Ciba Corning, Palo Alto, CA.

Vector construction

Two apoE Leiden expression vectors were constructed. Both contained the tandem insertion; one contained arginine at the polymorphic site 112 (as it is found in human subjects bearing this mutation), and the other contained cysteine at the same position. Genomic DNA from a subject heterozygous for the apoE Leiden mutation was amplified in the region of the fourth exon containing the insertional mutation, from codon 64 to codon 168 (15). The amplified material deriving from the mutated apoE allele was gel-purified and digested with either StyI/NarI or SacII/NarI, and the fragments were ligated into a similarly prepared pCMV (expression vector containing the cytomegalovirus promoter) already containing the full-length cDNA for apoE3 (16). The StyI/NarI ligation yields arginine at codon 112 and therefore recreates the original mutant, while the SacII/NarI ligation leaves the original cysteine at the same position. Positive transformants were identified by restriction analysis of plasmid DNA, by polymerase chain reaction of the mutated region and digestion with HhaI (17), and confirmed by sequencing of the mutated region and ligation boundaries. Upon transfection, the constructs produced proteins of the expected size and reacted with human-specific anti-apoE antibodies.

Transfection studies

For transient transfections, McA-RH7777 cells were first grown to 50% confluence in DME plus 10% fetal bovine serum, 10% horse serum. Fresh medium was added, and 20 μg of plasmid DNA was added as a calcium phosphate precipitate, as described (18). After overnight incubation the medium was removed and cells were incubated for 2 min with 15% glycerol, and then washed extensively in medium as previously described (16). The transient transfectants were analyzed the next day in metabolic labeling and immunoprecipitation experiments. Stably transfected cell lines with either of the two apoE Leiden constructs or a normal apoE3 construct were prepared using the same technique, except that cells were co-transfected with a plasmid expressing the neomycin resistance gene (pSV2Neo) at a ratio of 1:20 with the apoE plasmid.

Pulse-chase analyses

The experiments were performed in 60-mm dishes at 70% confluence in serum-containing DME. For the analysis of the secretion rate, a 2-h pulse with 100 μCi of [^{35}S]methionine (Tran-S-Label) per ml of methionine-free medium was followed by a 90-min chase in fresh, non-radioactive medium. Media and cells were collected at six time points: 10, 20, 30, 45, 60, and 90 min from the start of the chase period. Immunoprecipitation with antiserum against human apoE was conducted as described (19). An aliquot of the immunoprecipitated protein was separated on 12% SDS-polyacrylamide gels, which were then fixed in isopropanol-water-acetic acid 25:65:10, treated with Amplify, dried, and exposed to radiographic

film. The secretion rate of endogenous apoE also was studied in nontransfected McA-RH7777 cells using a rat-specific anti-apoE antiserum. In all experiments, the intensity of the bands was analyzed by both densitometry and counting.

Iodination and incubation experiments

Iodinations were performed using the Bolton-Hunter method according to a previously described protocol (20). Incubation of the iodinated protein with human plasma in vitro was done as reported previously (21). After Superose 6 chromatography, recovery of total radioactivity ranged from 74 to 94%.

Column chromatography and lipid analyses

Two hundred-μl aliquots of plasma that were preincubated with ^{125}I-labeled apoE, or 200 μl concentrated cell media were injected onto a Superose 6 column, as previously described (19, 22). Fractions were analyzed for cholesterol and triglyceride content, and for radioactivity. In some experiments, fractions were pooled together in four groups representing the major lipoprotein classes (19, 22), and apoE was immunoprecipitated using either human-specific or rat-specific anti-apoE antibodies. The immunoprecipitated apoE was then separated and visualized as described for pulse-chase analyses.

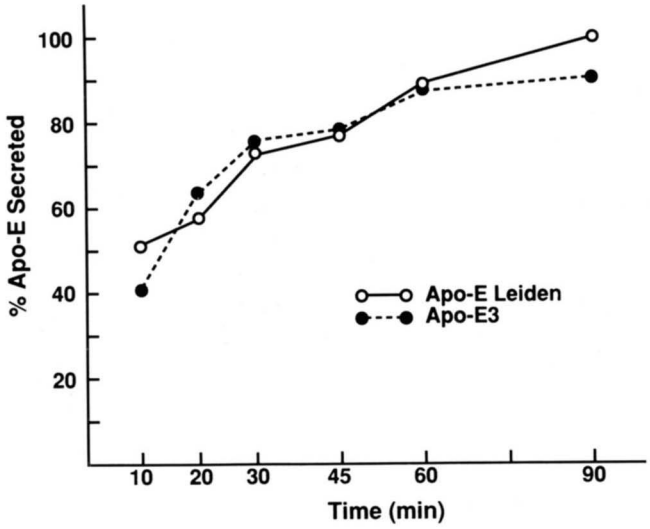

Fig. 1. Pulse-chase analysis of apoE synthesis and secretion in stably transfected McA-RH7777 cells. The experiment was performed in 60-mm dishes with cells at 70% confluence in serum-containing DME. A 2-h pulse in methionine-free DME containing 20% serum and 100 μCi/ml of [^{35}S]methionine was followed by a chase in nonradioactive medium. Media and cells were collected at the indicated time points, and apoE was immunoprecipitated and separated on a 12% SDS-polyacrylamide gel, which was then fixed, enhanced, dried, and exposed for 2 days to radiographic film (19). Values reported are the ratio between secreted and total apoE (total = secreted + intracellular) for one of three separate experiments conducted with each apoE, all of which yielded similar results.

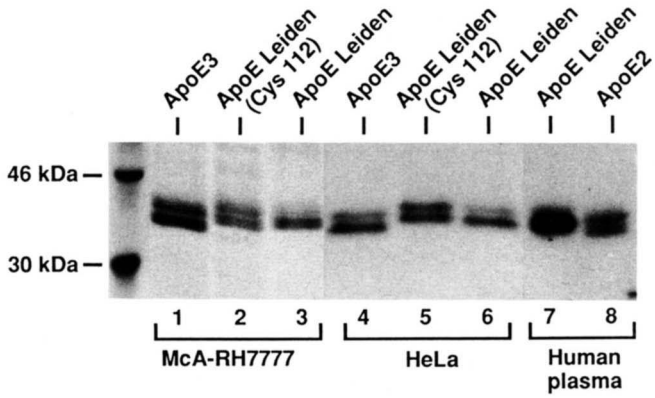

Fig. 2. Extent of glycosylation of apoE in media of transfected cells and in plasma of a heterozygous subject. Lanes 1–6: cells were transiently transfected with the expression vectors and metabolically labeled with [^{35}S]methionine (100 μCi/ml) for 12 h. ApoE was immunoprecipitated from the media, electrophoresed on a 12% SDS-polyacrylamide gel, and quantitated by densitometry (19). In each lane, the lower band is the nonglycosylated protein and the upper band represents the glycosylated form. ApoE Leiden has a slightly slower mobility because of the seven-amino acid insertion (6, 14). Lanes 7–8: plasma apoE from a heterozygous (apoE Leiden/apoE2) subject was passed through a thiopropyl column, which bound apoE2 but not apoE Leiden. Bound apoE2(Cys112, Cys158) was eluted with 50 mM dithiothreitol according to a published protocol (30), electrophoresed on a 12% SDS-polyacrylamide gel, transferred to nitrocellulose, and immunoblotted with a polyclonal anti-human apoE antisera (19).

Statistical analyses

All comparisons of means were done using the one-tail Student's t test.

RESULTS

We conducted our experiments using the rat hepatoma cell line McA-RH7777 transfected with apoE cDNA expression vectors driven by the constitutive cytomegalovirus promoter. **Fig. 1** shows that the secretion of apoE Leiden by transfected rat hepatoma cells was similar to the secretion of normal apoE3. In both instances the intracellular processing time of the transfected apoE was similar to that reported for endogenous apoE in HepG2 cells (23), with the protein appearing in the media 10 min after the beginning of the chase time. The same times of secretion were observed for rat endogenous apoE in nontransfected cells (not shown). The efficiency of secretion of apoE Leiden at different times was similar to that of normal apoE3, and approached 100% at 90 min (Fig. 1).

As shown in **Fig. 2,** the only difference noted in processing between the two transfected proteins was that secreted apoE Leiden was consistently underglycosylated compared with secreted normal apoE3 (21.7 $\pm$ 3.8% vs. 36.6 $\pm$ 4.5%, $P < 0.005$, n = 4). This phenomenon occurred not only in the rat hepatoma cell line (Fig. 2, lanes 1 and 3) but also in Hela cells (Fig. 2, lanes 4 and 6),

which do not make lipoproteins. A similar difference was true for the apoE Leiden purified from the plasma of a heterozygous subject when compared to the other plasma apoE allele product (Fig. 2, lanes 7 and 8). However, the degree of glycosylation of the mutant with cysteine (instead of arginine) at position 112 (Fig. 2, lanes 2 and 5) was normal (40.5 ± 4.0%, n = 4). Thus, while the polymorphism at site 112 does not affect the degree of glycosylation of apoE3 versus apoE4 (23), it does appear to modulate the glycosylation of apoE Leiden.

As mentioned before, patients heterozygous for the apoE Leiden mutation show a prevalence of the mutant over the normal protein in the plasma VLDL fraction. To determine whether this phenomenon derives from a preferential association of the mutant apoE with plasma VLDL, we studied the affinity of apoE Leiden for lipoproteins in vitro. We iodinated the two mutants, apoE Leiden and its counterpart with cysteine at site 112, and the two common polymorphic forms of normal apoE, apoE3 and apoE4, and incubated a trace amount of each labeled protein (200 ng) with fresh normolipidemic human plasma. It has been demonstrated previously that the polymorphism at position 112 affects the affinity of apoE for lipoproteins, with apoE4 (arginine 112) preferring VLDL to HDL relative to apoE3, which prefers HDL to VLDL (21). In our experiments (**Fig. 3, Table 1**), apoE Leiden showed a significant preference for VLDL not only when compared to apoE3 (2.6-fold), but also with respect to apoE4 (1.6-fold). The substitution of cysteine for arginine at position 112 did not change the affinity of the mutant protein for VLDL, indicating that the presence of the insertional mutation is responsible for directing apoE Leiden to VLDL.

Because McA-RH7777 cells are known to synthesize VLDL (24), we performed experiments to test whether apoE Leiden would displace endogenous apoE from VLDL. Secretion of transfected apoE was comparable in the two cell lines (5.71 μg/mg cell protein per h for apoE3 versus 5.35 for apoE Leiden) and did not modify secretion of endogenous rat apoE (about 0.6 μg/mg cell protein per h in both transfected cells and in the nontransfected line). Results shown in **Fig. 4** indicate that apoE Leiden

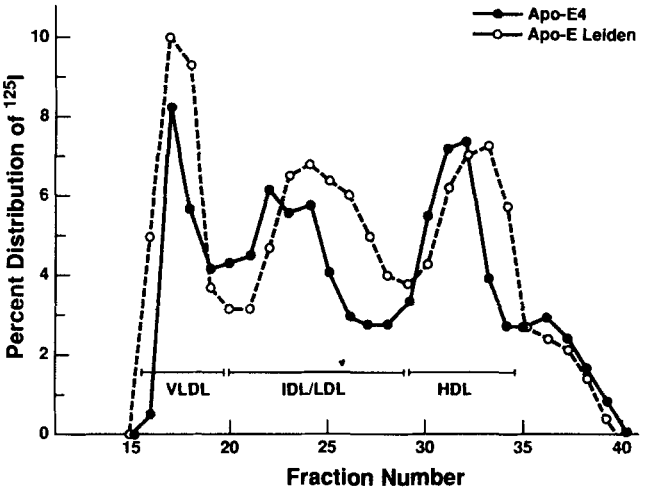

Fig. 3. Distribution of iodinated apoE Leiden in human plasma. A 200-ng aliquot of ^{125}I-labeled apoE was incubated with 200 μl of fresh human plasma for 2 h at 37°C and, after separation on a Superose 6 column, fractions were counted. The major lipoprotein classes are indicated. This experiment is one of the experiments included in Table 1.

produced by transfected cells had the same distribution pattern as normal human apoE3 (Fig. 4, panels C and D), and that rat endogenous apoE was not displaced from VLDL by either apoE3 or apoE Leiden (Fig. 4, panels A and B). Rat endogenous apoE in VLDL was 14.7%, 14.4%, and 13.8% of total rat apoE in apoE3-transfected, apoE Leiden-transfected, and nontransfected cells, respectively.

DISCUSSION

Our results show that the predominance of apoE Leiden in affected subjects' VLDL probably is not due to overexpression of the mutant apoE (Fig. 1). Instead, our results suggest that the preferential association of apoE Leiden with plasma VLDL is contributing to β-VLDL accumulation. About 28–30% of apoE Leiden associated with VLDL, compared to only 11% of apoE3 and 19% of apoE4 (Fig. 3, Table 1). The difference in lipoprotein distribution between apoE3 and apoE4 has been shown to be

TABLE 1. Percent lipoprotein distribution and overall recovery of ^{125}I-labeled apoE

Variant	VLDL[a]	IDL/LDL	HDL	Overall Recovery
ApoE3(Cys112)[b]	11.4 ± 1.2	44.9 ± 4.5	43.8 ± 4.7	83 ± 6
ApoE4(Arg112)[b]	18.9 ± 2.8	44.5 ± 2.2	36.6 ± 2.9	86 ± 6
ApoE Leiden (Cys112)[c]	28.2 ± 2.6	32.8 ± 3.6	38.0 ± 4.1	86 ± 4
ApoE Leiden (Arg112)[c]	29.8 ± 1.8	37.6 ± 2.7	32.6 ± 2.7	85 ± 6

[a] $P < 0.001$, apoE Leiden (Arg112) compared to apoE3 or to apoE4; $P < 0.001$, apoE Leiden (Cys112) compared to apoE3; $P < 0.005$, apoE Leiden (Cys112) compared to apoE4; $P < 0.001$, apoE3 compared to apoE4.
[b] Mean of five experiments ± SD.
[c] Mean of three experiments ± SD.

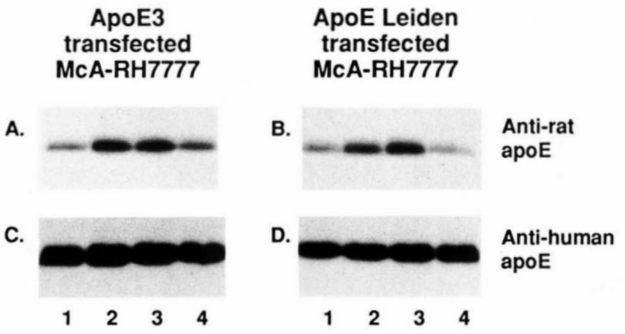

Fig. 4. Distribution of transfected apoE among McA-RH7777 media lipoproteins after Superose 6 chromatography. Each stably transfected cell line was incubated in a T75 flask with 10 ml of methionine-free medium containing 20% serum. Two hours after addition of [^{35}S]methionine (100 μCi/ml), the medium was collected, concentrated to 200 μl with Centricon filters, and separated on a Superose 6 column. Fractions corresponding to the major lipoprotein classes were pooled together, and apoE was immunoprecipitated using either anti-rat (panels A and B) or anti-human (panels C and D) antibodies. Panel A, endogenous apoE from apoE3-transfected McA-RH7777 cells. Panel B, endogenous apoE from apoE Leiden-transfected cells. Panel C, transfected apoE3. Panel D, transfected apoE Leiden. Lane 1: Superose fractions 16–19 (VLDL); lane 2: fractions 20–24; lane 3: fractions 25–28; lane 4: fractions 29–34 (HDL) (See Fig. 3).

due to the polymorphism at position 112 (21), where the presence of an arginine directs the apoprotein more to the VLDL. ApoE Leiden has an arginine at site 112, and therefore its preference for VLDL could be due solely to this substitution. However, somewhat unexpectedly, when we tested the apoE Leiden containing cysteine-112, we found that its association with VLDL was not significantly different compared to apoE Leiden(Arg112). Therefore, the insertional mutation appears to negate the influence exerted by the polymorphism at site 112 on the lipoprotein association of apoE Leiden. Moreover, the mutation directly determines an increased affinity of apoE Leiden for triglyceride-rich lipoproteins. The seven-amino acid insertion in apoE Leiden occurs in a loop that connects helix 3 to helix 4 in the 4-helical bundle of the amino-terminal domain of apoE (25). The insertion apparently disrupts the relationship of site 112 (which lies in helix 3) with the lipid-binding carboxyl-terminal domain, while at the same time causing an as yet unknown structural change that increases the preference of the protein for VLDL.

Since we found that the transfected apoE Leiden does not disturb the distribution of endogenous apoE among lipoproteins (Fig. 4), it appears that apoE Leiden does not associate with VLDL in the plasma of affected human subjects at the expense of the other allele product. Therefore, it is possible that the preponderance of apoE Leiden on the VLDL is achieved not by displacement of the other isoform but merely by its natural lipoprotein preference. These combined results indicate that part of the reason for the accumulation of apoE Leiden in affected subjects' VLDL is the preferential postsecretion association with this lipoprotein class in human plasma. This preferential association of a receptor-binding-defective apoE with the VLDL may help to explain the mode of inheritance of type III hlp in apoE Leiden subjects. Type III hlp can be inherited in either a recessive or a dominant fashion (3). The reason(s) for this difference is not known, but it cannot be attributed solely to differences in receptor binding of apoE. Since apoE2(Cys112, Cys158) is significantly more defective in binding to the LDL receptor compared with apoE Leiden (6, 14), one might predict that in apoE3/2 heterozygotes, the severe apoE2 binding defect would result in the preferential accumulation of apoE2 in their VLDL fraction. While this occurs to a modest extent in apoE3/2 subjects (apoE2 concentration is found to be about 40% higher than apoE3 in VLDL), these subjects do not develop type III hlp or demonstrate β-VLDL (26). Hence, apoE2(Cys112, Cys158) is associated with a recessive mode of inheritance of type III hlp. In contrast, both apoE Leiden and another apoE variant that shows a preferential in vitro association with VLDL (22) are found in much higher concentrations compared with normal apoE in affected subjects' VLDL, at a ratio of 4:1 or higher in the former instance (12) and 3:1 in the latter instance (22). We believe that in both these instances, the natural preference of the apoE variants for VLDL helps to exaggerate the differential catabolism of the normal and variant apoE, leading to accumulation of particles containing relatively more of the defective ligand and ultimately exacerbating β-VLDL formation and/or accumulation, a phenomenon that does not occur so readily in apoE3/2 heterozygotes. It is unlikely that all apoE variants associated with dominant transmission of type III hlp will demonstrate the lipoprotein preference phenomenon, since all others known to date do not have either insertions or Arg-112. Instead, it is probably that there are other properties of apoE or additional factors that help determine whether the mode of inheritance of type III hlp will be dominant or recessive (3, 12, 22).

The finding of a reduced glycosylation of apoE Leiden is of unknown physiologic relevance. This observation does not appear to be related to the ability of the cell to synthesize lipoproteins, since it is evident in both McA-RH7777 and HeLa cells. It is not known whether glycosylation has any role in the function of apoE, but glycosylation is not necessary for proper intracellular processing and secretion of the protein (16), it does not affect apoE binding to the LDL receptor (27, 28), and it does not influence apoE distribution among lipoproteins (data not shown). However, this phenomenon is interesting from a structural point of view; since the only glycosylation site in apoE is at threonine 194 (16), the underglycosylation of apoE Leiden probably is due to a change in accessibility of the glycosylation site to the cell's glycosylation

machinery. The interactions affecting the glycosylation of apoE Leiden appear to be complex, since both the insertion and the polymorphism at site 112 (where the presence of cysteine restores normal glycosylation) are involved. The existence of a communication between different portions of the apoE molecule is a phenomenon that has been described for other aspects of apoE function. One example is the polymorphism at position 112, which affects the preference for lipoproteins of the carboxyl-terminal lipoprotein-binding domain (21), and another is represented by the enhanced receptor-binding activity of the amino-terminal domain of both apoE2(Cys112, Cys158) (29) and apoE Leiden (14) after the proteolytic removal of their carboxyl-terminal domains.

In conclusion, the accumulation of apoE Leiden in VLDL appears to be a postsecretory phenomenon. The enrichment in defective apoE probably renders the VLDL less susceptible to receptor-mediated catabolism, exacerbating the accumulation of β-VLDL, and may help to explain the dominant transmission of type III hlp in subjects carrying the apoE Leiden mutation.

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