Apolipoprotein E2 (Arg^{136} → Cys) mutation in the receptor binding domain of apoE is not associated with dominant type III hyperlipoproteinemia[1]

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Abstract Using apoE phenotyping by immunoblotting and apoE genotyping we identified four heterozygous carriers of a rare apolipoprotein (apo) E2 variant, apoE2 (Arg^{136} → Cys). ApoE2 (Arg^{136} → Cys) was not distinct from apoE2 (Arg^{158} → Cys) by phenotyping, but produced a unique pattern of bands on *Cfo*I restriction typing of a 244 bp apoE gene fragment. Two of the four apoE2 (Arg^{136} → Cys)/3 heterozygotes had elevated triglycerides, two were normolipidemic. The composition of very low density lipoproteins (VLDL) was normal in each of the four apoE2 (Arg^{136} → Cys) carriers, regardless of the triglyceride concentrations. None of the apoE2 (Arg^{136} → Cys) carriers displayed a broad β-band and none revealed β-migrating particles in the VLDL. The two hypertriglyceridemic carriers of apoE2 (Arg^{136} → Cys) were, therefore, classified as having type IV rather than type III hyperlipoproteinemia. LDL receptor binding activities were studied using recombinant apoE loaded to dimyristoylphosphatidylcholine (DMPC) vesicles and to VLDL and from an apoE-deficient individual. LDL receptor binding of apoE2 (Arg^{136} → Cys) was 14% of apoE3 and was thus higher than that of apoE2 (Arg^{158} → Cys). Both apoE2 (Arg^{136} → Cys) and apoE2 (Arg^{158} → Cys) displayed substantial heparin binding (61 and 53% of apoE3, respectively). As the dominant apoE variants known so far are characterized by more pronounced reductions of heparin binding, we suggest that apoE2 (Arg^{136} → Cys) is not associated with dominant expression of type III hyperlipoproteinemia. These findings lend support to the concept that apoE variants predisposing to dominant type III hyperlipoproteinemia differ from recessive mutations by a more severe defect in heparin binding.—**März, W., M. M. Hoffmann, H. Scharnagl, E. Fisher, M. Chen, M. Nauck, G. Feussner, and H. Wieland**. Apolipoprotein E2 (Arg^{136} → Cys) mutation in the receptor binding domain of apoE is not associated with dominant type III hyperlipoproteinemia. *J. Lipid Res.* 1998. **39:** 658–669.

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Apolipoprotein (apo) E is a glycoprotein of 34 kDa (reviewed in refs. 1, 2). In plasma, it is associated with triglyceride-rich lipoproteins and high density lipoproteins.

ApoE is a ligand of members of the LDL receptor family of membrane proteins including the LDL receptor (1), the LDL receptor-related protein (3), the VLDL receptor (4), and others (5, 6). The best characterized function of apoE is to mediate the uptake of chylomicron and very low density lipoprotein (VLDL) remnants into the liver. ApoE is polymorphic. In humans, three common alleles exist that are designated ϵ2, ϵ3, ϵ4; their products differ from one another at positions 112 and 158 of the amino acid sequence (1, 2, 7). ApoE3, the most frequent isoform, has arginine at position 112 and cysteine at position 158. ApoE4 has arginine, and apoE2 has cysteine at both positions.

ApoE4 is associated with elevated LDL concentrations. It increases both the risk of artherosclerosis and Alzheimer's disease (2, 8, 9). ApoE2 is defective in its binding to lipoprotein receptors (10, 11). Homozygosity for apoE2 is necessary, but not sufficient by itself to precipitate type III hyperlipoproteinemia (HLP) (1, 2, 12), a disorder of lipoprotein metabolism characterized by the accumulation of cholesterol-rich remnant lipoproteins derived from the partial catabolism of chy-

Abbreviations: apo, apolipoprotein; DMPC, dimyristoylphosphatidylcholine; DOTAP, N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate; VLDL, LDL, HDL, very low, low, and high density lipoproteins; VLDL-C, LDL-C, HDL-C, cholesterol of VLDL, LDL, and HDL; HLP, hyperlipoproteinemia.

[1]This article is dedicated to Prof. Dr. Werner Gross on the occasion of his 60th birthday.

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lomicrons and very low density lipoproteins. More than 90% of the patients with type III HLP are homozygous for apoE2, but only about one in 20 individuals carrying the E2/2 phenotype finally develops type III HLP. This has led to the suggestion that further factors, genetic, metabolic, or environmental, are required for the phenotypic expression of type III HLP (12).

The receptor binding domain of apoE has been mapped to residues 136 through 150 (11). The replacement of cysteine for arginine at residue 158 which distinguishes the common recessive apoE2 isoform from apoE3 is outside this region and the mutation alters the conformation of the binding domain indirectly by compromising the salt bridge network formed by amino acid side chains of apoE (13, 14).

Rare apoE variants have been identified that have been implicated in a dominant mode of inheritance of type III HLP. This form of type III is considered to have a high degree of penetrance. To date, the dominant variants of apoE are apoE3-Leiden (15, 16), apoE2 ($Lys^{146} \rightarrow Gln$) (17, 18), apoE3 ($Cys^{112} \rightarrow Arg$, $Arg^{142} \rightarrow Cys$) (19–21), apoE4 ($Glu^{13} \rightarrow Lys$, $Arg^{145} \rightarrow Cys$) (22, 23), apoE2 ($Arg^{145} \rightarrow Cys$) (24), apoE1 ($Lys^{146} \rightarrow Glu$) (25, 26), and apoE1 ($Lys^{146} \rightarrow Asn$, $Arg^{147} \rightarrow Trp$) (27). With the exception of apoE3-Leiden (16) the dominant apoE variants involve substitutions of the basic residues at positions 142, 145, 146, and 147. These amino acids are all located within the first heparin binding domain of the apoE molecule which extends from residues 142 through 147 (28). Consistently, apoE mutants affecting positions 142, 145, or 146 have been shown to exhibit defective binding to cell surface heparan sulfate proteoglycans (HSPG) (29–31), whereas apoE2 ($Arg^{158} \rightarrow Cys$) possesses significant residual heparin binding (29, 31).

ApoE3 has arginine at position 136. Site-specific mutagenesis studies have implicated this residue in LDL receptor binding; conversion of Arg^{136} to Ser resulted in approximately 60% decrease in LDL receptor binding (11). As Arg^{136} lies outside the putative heparin binding domain of apoE, point mutations at this site would be supposed to result in recessive apoE variants. This is, however, controversial. Three point mutations affecting codon 136 of mature apoE have been described: apoE2-Christchurch ($Arg^{136} \rightarrow Ser$) (32), apoE3′ ($Arg^{136} \rightarrow His$) (33), and apoE2 ($Arg^{136} \rightarrow Cys$) (34). ApoE3′ ($Arg^{136} \rightarrow His$) is apparently not associated with dominant type III HLP (33). Both apoE2-Christchurch ($Arg^{136} \rightarrow Ser$) and apoE2 ($Arg^{136} \rightarrow Cys$) have been linked to incomplete, late-onset dominance of type III HLP (35, 36). Walden et al. (34), in contrast, identified four carriers of apoE2 ($Arg^{136} \rightarrow Cys$), one apoE2 ($Arg^{136} \rightarrow Cys$)/3 heterozygote and three apoE2 ($Arg^{136} \rightarrow Cys$)/2 ($Arg^{158} \rightarrow Cys$) heterozygotes; only one of them, a 39-year-old, obese apoE2 ($Arg^{136} \rightarrow Cys$)/2 ($Arg^{158} \rightarrow Cys$) heterozygote, presented with type III HLP. Hence, these investigators concluded that apoE2 ($Arg^{136} \rightarrow Cys$) contributes to type III HLP in a recessive rather than in a dominant fashion.

We here describe clinical findings in four other apoE2 ($Arg^{136} \rightarrow Cys$)/3 heterozygotes, two with a lipoprotein pattern resembling type IV hyperlipidemia and two having normal lipid concentrations. In an attempt to clarify whether or not apoE2 ($Arg^{136} \rightarrow Cys$) meets the biochemical criteria considered essential for a dominant apoE variant, we performed a comprehensive functional characterization of apoE2 ($Arg^{136} \rightarrow Cys$) utilizing recombinant apoE. Together, the results strongly suggest that apoE2 ($Arg^{136} \rightarrow Cys$) may contribute to recessive, but not dominant type III HLP in susceptible individuals.

MATERIALS AND METHODS

Human subjects

The first proband (U. T.) was a 47-yr-old German woman. She participated in a study that served to establish local reference ranges of lipoprotein and apolipoprotein levels. ApoE phenotyping and genotyping were performed routinely in this study. The proband was healthy and asymptomatic. Her clinical history was insignificant for hyperlipidemia, manifestations of atherosclerosis or other metabolic disorders. There was no family history of premature atherosclerosis. Her mother (H. K.) and her father (G. K.) were living at age 77 and 79, respectively, and were doing well clinically. Her sister and her daughter were also healthy. None of the family members revealed symptoms of coronary and/or peripheral vascular disease.

The second proband (J. S.) was a 33-yr-old male of Spanish descent. He was referred to the lipid outpatient clinic of the Frankfurt Medical School for evaluation of persistent hypercholesterolemia and hypertriglyceridemia. The patient did not smoke; he was reported to consume a typical Mid European diet and drank approximately 50 grams of alcohol per day. On physical examination the patient was normal, except that he was slightly overweight. His ECG was normal. He had no xanthomas, neither palmar nor tuberous. Routine blood biochemistry and hematology were normal, except for a mild elevation of the gamma-glutamyl transpeptidase at 44 U/l (normal range: <28 U/l). There was no evidence that his hyperlipidemia was due to diabetes mellitus, hypothyroidism, renal dysfunction, or severe liver disease. The family history of pre-

mature atherosclerosis was negative and it was not known whether relatives had elevated lipid levels. The family of this patient was not available for genetic and biochemical studies.

Lipids, lipoproteins, and apolipoproteins

Blood of fasting individuals was drawn into tubes containing EDTA·K_2 (final concentration: 1.5–2 g/l). Plasma was recovered by centrifugation. Cholesterol (total and non-esterified), triglycerides, and phospholipids were measured in duplicate using enzymatic reagents (Boehringer Mannheim, Mannheim, Germany and Wako, Neuss, Germany). VLDL ($d < 1.0063$ kg/l), IDL (1.0063 kg/l $< d <$ 1.019 kg/l), and LDL (1.019 kg/l $< d <$ 1.065 kg/l) were isolated by preparative ultracentrifugation (37, 38). HDL-C was determined by precipitating apoB-containing lipoproteins in the $d >$ 1.0063 kg/l infranate (39). Agarose gel lipoprotein electrophoresis was performed with a commercially available kit and a Rapid Electrophoresis System (Rep; both from Helena, Hartheim, Germany). Lipoproteins were separated at 400 V for 15 min. Their cholesterol and triglyceride moieties were enzymatically stained as described elsewhere (40, 41). βVLDL were assessed as the appearance of a band with β mobility in the less than 1.0063 kg/l density fraction on agarose electrophoresis. ApoA-I and apoB were measured by automated rate nephelometry (Array Protein System, Beckman Instruments, Fullerton, CA). ApoE phenotyping was carried out by immunoblotting in immobilized pH gradients (42).

Apolipoprotein E genotyping and sequencing

DNA was extracted from white blood cells using "blood PCR" DNA isolation cartridges (Diagen GmbH, Düsseldorf, Germany) according to the manufacturer's instructions. ApoE genotyping was performed by restriction isotyping (43). A 244 bp fragment of the apoE gene covering the codons for amino acids 112 and 158 was amplified by polymerase chain reaction using the primer pair F6 and F4 described by Emi et al. (44). The amplification reactions (100 μl) contained 100 ng genomic DNA, 100 ng oligonucleotide primers, 62.5 mmol/l KCl, 12.5 mmol/l Tris-HCl, pH 8.3, 2.125 mmol/l $MgCl_2$, 50 μmol/l, each, dATP, dCTP, dGTP, and dTTP, 10% (v/v) dimethyl sulfoxide, 0.11% (v/v) Tween-20, 0.11% (v/v) Nonidet P-40 and 2.5 units *Taq* polymerase (Perkin Elmer Cetus, Emeryville, CA). The thermal cycler protocol was: 1 min 95°C (denaturation), 1 min 60°C (annealing), 1 min 72°C (extension), 30 cycles. The amplification products were digested for 3 h with *Cfo*I (an isoschizomer of *Hha*I). Restriction fragments were analyzed by polyacrylamide gel electrophoresis and ethidium bromide staining. Direct sequencing of the double-stranded 244 bp PCR product was performed using the dideoxynucleotide chain termination method (45) and a fluorescently labeled forward primer. Reaction products were developed on an A.L.F. DNA sequencer (Pharmacia).

Recombinant apoE isoforms

Baculovirus recombinant apoE3 and apoE2 ($Arg^{158} \rightarrow$ Cys) was purchased from PanVera (Madison, WI). Recombinant apoE2 ($Arg^{136} \rightarrow$ Cys) was produced in the baculovirus system following the protocol of Gretch et al. (46). Previous experiments indicated that the LDL receptor binding of recombinant apoE3 from PanVera could not be distinguished from recombinant apoE3 made in our laboratory. To produce recombinant apoE2 ($Arg^{136} \rightarrow$ Cys), a 244 bp F6-F4 polymerase chain reaction product of the apoE gene was subcloned into the pCRII vector (Invitrogen, NV Leek, The Netherlands). pCRII harboring cDNA for apoE2 ($Arg^{136} \rightarrow$ Cys) was double digested with *Not*I and *Fsp*I (New England Biolabs, Schwalbach, Germany) to produce an 88 bp apoE cDNA fragment containing the mutant site. This fragment was exchanged against the wild type sequence of apoE3 in a pGEM-apoE3 cDNA clone. After digestion of the pGEM-apoE3 with *Bam*HI the mutant cDNA was exchanged against apoE3 cDNA of the baculovirus transfer vector pAc-E3 (kindly provided by Dr. Alan D. Attie, Department of Biochemistry, University of Madison) which is a derivative of pAcYM1 (46), to yield pAcYM1-apoE ($Arg^{136} \rightarrow$ Cys). The sequence of the mutant apoE cDNA construct was verified by DNA sequencing.

Cotransfection of BaculoGold™ baculovirus DNA (PharMingen) and pAcYM1-apoE ($Arg^{136} \rightarrow$ Cys) was performed using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP, Boehringer Mannheim, Mannheim) as transfection reagent. Sf21 cells (3×10^6) were seeded in a 60-mm tissue culture plate. After 15 min, the medium was exchanged against 1 ml fresh medium (Sf900II, Gibco BRL, Eggenstein, Germany). Linearized BaculoGold™ virus DNA (0.5 μg) and 2 μg recombinant plasmid DNA were diluted to a concentration of 0.1 g/l in 20 mmol/l HEPES buffer (pH 7.4). DOTAP (15 μl) was mixed with 35 μl HEPES buffer; the DNA was added to the DOTAP solution and the mixture was incubated for 15 min at room temperature. The resulting transfection solution was added dropwise to the cells. After 6 h of incubation at 27°C, 3 ml of fresh Sf900II medium was added to the cells. At day 4 after transfection, the supernatant was collected and single virus clones were isolated as described (47). To produce recombinant apoE2 ($Arg^{136} \rightarrow$ Cys) on a large scale, 2×10^6 Sf21 cells per milliliter were seeded in a spinner flask. The

cells were infected with a high-titer stock solution of recombinant baculovirus. The multiplicity of infection was 10. After incubation at 27°C for 72–96 h the apoE-containing supernatant was collected by centrifugation. Prior to storage (−80°C) or purification, a protease inhibitor cocktail was added to produce final concentrations of 32 mg/l benzamidine-HCl, 20 mg/l aprotinin, 10 mg/l leupeptin, and 1 mmol/l phenymethysulfonylfluoride. To purify the recombinant apoE, solid NH_4HCO_3 was added to yield a final concentration of 25 mmol/l. This solution was applied to a heparin-Sepharose 4B column (2 × 20 cm) previously equilibrated with 25 mmol/l NH_4HCO_3. The column was washed with 15 volumes of 25 mmol/l NH_4HCO_3 and 10 volumes of 300 mmol NH_4HCO_3. ApoE was eluted with 700 mmol/l NH_4HCO_3, dialyzed against 25 mm NH_4HCO_3, and re-chromatographed under identical conditions.

Preparation of apoE-dimyristoylphosphatidylcholine (DMPC) complexes

Recombinant apoE isoforms were reconstituted with DMPC essentially as described (11, 48–50). DMPC (15 mg) was suspended in 1 ml of 1 mmol/l Tris-HCl, 10 mmol/l EDTA·Na_2, pH 7.6, 150 mmol/l NaCl, incubated for 30 min at room temperature, and sonicated. Recombinant apoE isoforms (100 μg of each in 1.05 ml of 0.1 mol/l NH_4HCO_3, pH 8.1) were incubated overnight with 50 μl of the DMPC suspension. Resulting apoE–DMPC complexes were isolated by preparative ultracentrifugation at a density of 1.25 kg/l.

Binding, uptake, and degradation of ^{125}I-labeled lipoproteins in cultured cells

Lipoproteins were iodinated using the iodine monochloride method (51). Human skin fibroblasts were from skin biopsies of healthy, normolipidemic individuals. Cells were grown in 24-well polystyrene plates. Prior to the experiments, the cells were incubated for 40 h in medium containing 10% (v/v) human lipoprotein-deficient serum in order to up-regulate LDL receptors. Binding, uptake, and degradation of ^{125}I-labeled VLDL and ^{125}I-labeled LDL were measured as described (52) with slight modifications (53). To measure cell surface binding, lipoproteins were incubated with the cells for 1 h at 4°C in DMEM containing 10 mmol/l HEPES. To determine uptake (surface binding plus internalization) and degradation, cells were incubated for 4 h at 37°C with ^{125}I-labeled lipoproteins in DMEM containing 24 mmol/l bicarbonate (pH 7.4). The amount of ^{125}I-labeled material associated with the cells (binding plus internalization) was determined after lysis in 0.3 mm NaOH. Proteolytic degradation was determined as ^{125}I-labeled trichloracetic acid-soluble (non-iodine) material in the conditioned medium.

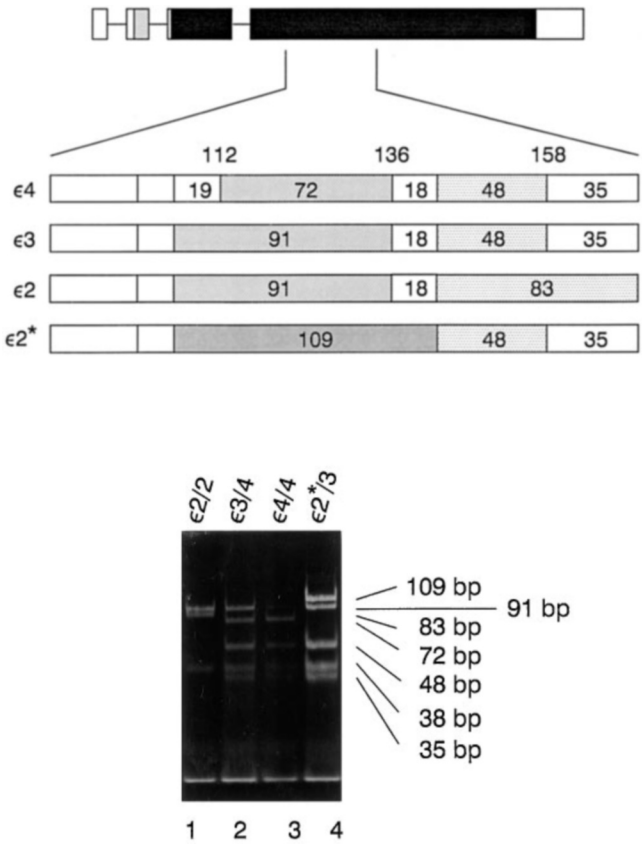

Fig. 1. Restriction isotyping of apoE. The apoE gene consists of four exons (wide bars) and three introns (thin lines). The third and fourth exon encode amino acids present in the mature protein (solid bars). Open bars: 5′ and 3′ untranslated regions of the mRNA. A 244-bp fragment of the fourth exon was amplified and digested with *Cfo*I. At the common polymorphic sites (amino acids 112 and 158), a substitution of cysteine (TGC) for arginine (CGC) abolishes the recognition site for *Cfo*I (GCGC). Thus, the common alleles ε2, ε3, and ε4 have different restriction maps (top panel). In the apoE gene of a 47-year-old woman with normal plasma lipoprotein concentrations, the third *Cfo*I cutting site, which is common to ε2, ε3, and ε4, was absent, giving rise to a unique 109 bp restriction fragment. The bottom panel shows polyacrylamide gel electrophoreses of *Cfo*I-digested 244 bp apoE gene fragments. Lane 1: ε2/2; lane 2: ε3/4; lane 3: ε4/4; lane 4: ε2 ($Arg^{136} \rightarrow Cys$)/3.

Binding of VLDL to heparin-Sepharose

Heparin binding was determined using the procedure of Mann et al. (29). ApoE–DMPC complexes were incubated with 20 mg heparin-Sepharose or 20 mg Sepharose, respectively, for 4 h on an overhead shaker in 200 μl Tris-buffer (20 mmol/l Tris HCl, pH 7.5, 50 mmol/l NaCl, 10 g/l BSA) containing 10 μg apoE. After centrifugation, the pellet was washed three times

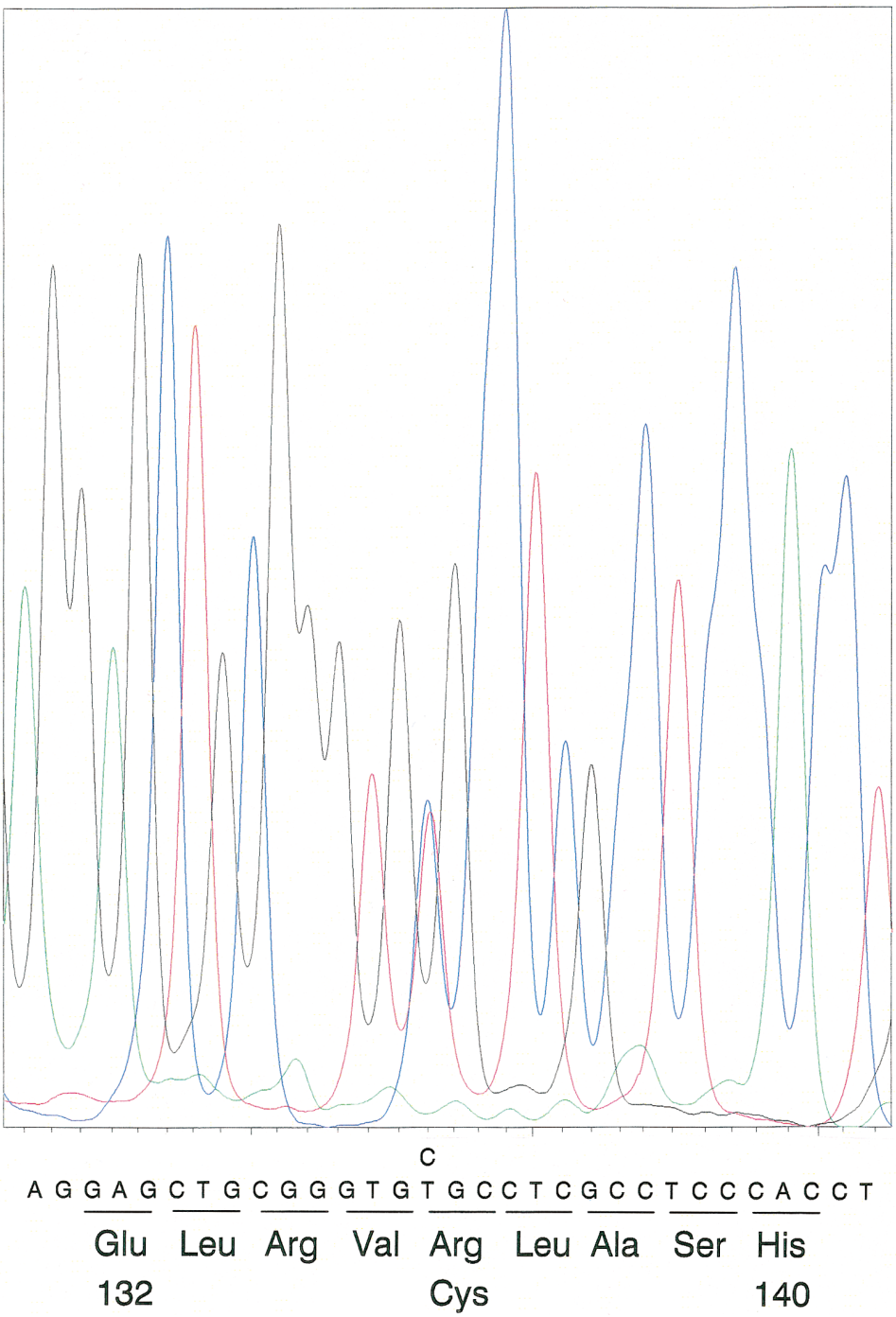

Fig. 2. Direct sequencing of the 244 bp apoE gene fragment from an individual with apoE2 ($Arg^{136} \rightarrow$ Cys). The nucleotide sequence (antisense strand) surrounding the codon for amino acid 136 is shown. The sequence indicates heterozygosity for a C to T transition changing codon 136 from CGC (arginine) to TGC (cysteine).

with Tris-buffer, and the heparin-Sepharose-associated radioactivity was counted. Results were corrected for binding to Sepharose alone and are expressed as ng apoE per mg heparin-Sepharose.

RESULTS

We determined the apoE phenotypes and genotypes in 114 plasma samples from healthy individuals. In a

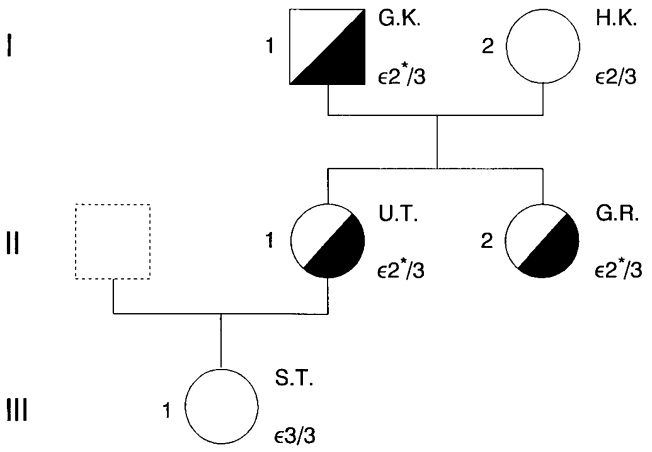

Fig. 3. Pedigree of the T. family. Pedigree members heterozygous for apoE2 ($Arg^{136} \rightarrow Cys$) are indicated by half-closed symbols. The proposita is heterozygous for the mutation. Clinical information on each individual is provided in Table 1. The dotted symbol represents a family member who has not been studied so far. Circles, females; squares, males.

healthy 47-year-old woman (U.T.) with normal plasma lipoprotein concentrations (cholesterol 1.75 g/l, triglycerides 1.17 g/l) the apoE phenotype was 2/3. When we amplified a 244 bp apoE gene fragment by polymerase chain reaction and analyzed the resulting product by cleavage with *Cfo*I, we found the fragments characteristic of the ε3 allele and a 109 bp fragment which was not seen with any of the common alleles ε2, ε3, or ε4 (**Fig. 1**). The same results were obtained in a male patient (J. S.) with cholesterol and triglycerides elevated at 2.60 g/l and 4.76 g/l, respectively, who was referred to the lipid outpatient clinic for differential diagnosis of hyperlipidemia. In both cases, direct sequencing of PCR amplified DNA revealed that the unusual 109 bp restriction fragment was due to a C to T transition that abolished a *Cfo*I cutting site (**Fig. 2**). This mutation converted the codon for arginine (CGC) at position 136 into cysteine (TGC), thus explaining that the newly identified apoE isoform possessed one net positive charge less than apoE3, the 'wild type' gene product.

To identify further carriers of the apoE2 ($Arg^{136} \rightarrow$ Cys) mutation we studied the first proband's (U.T.) family by apoE phenotyping and genotyping. The family of the second proband (J. S.) was not available for further investigation. The pedigree of the U.T. kindred is shown in **Fig. 3**; biochemical data are provided with **Table 1**. Together with the two index cases, a total of four heterozygous carriers of apoE2 ($Arg^{136} \rightarrow$ Cys) was identified.

In the normolipidemic apoE2 ($Arg^{136} \rightarrow$ Cys) carriers (U. T. and G. R.), total cholesterol and LDL choles-

TABLE 1. Clinical data and lipoproteins in the T family and in a non-related carrier of the apoE2 ($Arg^{136} \rightarrow$ Cys) variant

Subjects (Pedigree Position)	Age	Sex	Ht	Wt	BMI	ApoE[a]	TC	TG	LDL-C	HDL-C	VLDL-C	VLDL-TG	VLDL-C/TG	VLDL-C/VLDL-TG	ApoB	ApoA-I
	yr		*cm*	*kg*	*kg/m²*						*g/l*				*g/l*	
GK (I-1)	79	M	176	65	21.0	2*/3	1.96	3.23	1.06	0.41	0.49	2.70	0.15	0.18	0.85	1.38
HK (I-2)	77	F	164	75	27.9	3/4	2.76	6.50	1.42	0.37	0.97	5.13	0.15	0.19	1.48	1.44
UT (II-1)	47	F	159	57	22.5	2*/3	1.75	1.17	0.98	0.59	0.18	0.69	0.15	0.26	0.73	1.54
GR (II-2)	54	F	172	78	26.4	2*/3	2.10	1.31	1.12	0.82	0.16	0.86	0.12	0.19	0.78	1.87
ST (III-1)	26	F	164	62	23.0	3/3	1.87	1.05	0.97	0.79	0.11	0.40	0.10	0.28	0.76	1.88
JS[b]	30	M	178	89	28.1	2*/3	2.60	4.76	1.37	0.36	0.87	2.62	0.18	0.33	1.22	1.42
FE[b]	4	M	173	79	26.4	2/2	4.62	5.92	0.75	0.28	3.59	4.92	0.61	0.73	1.17	1.17

Ht, height; Wt, weight; BMI, body mass index.
[a] 2* denotes apoE2 ($Arg^{136} \rightarrow$ Cys).
[b] No family relationship with probands I-1 through III-1.

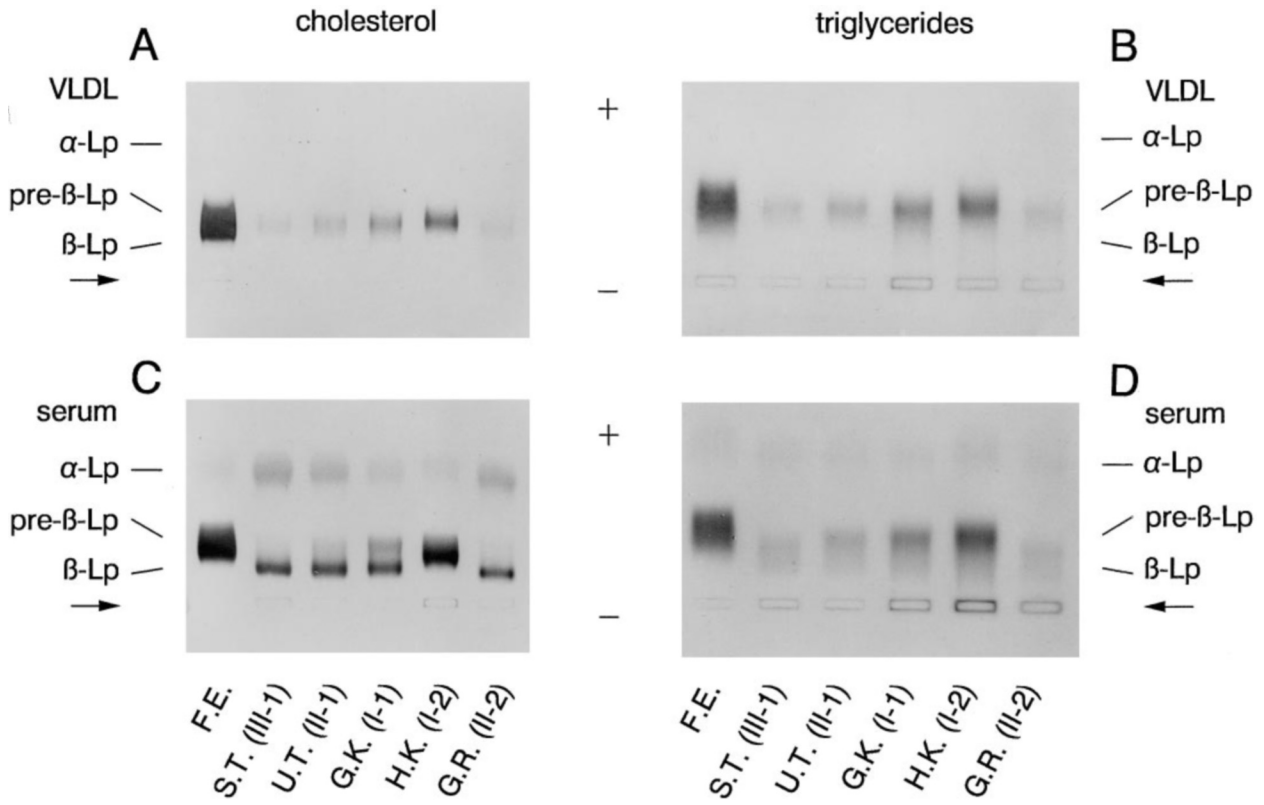

Fig. 4. Agarose gel electrophoresis of purified VLDL (panels A and B) and serum (panels C and D). The gels were stained enzymatically for cholesterol (panels A and C) and triglycerides (panels B and D), respectively. The samples are from the subjects in Table 1.

terol concentrations ranged from low to normal. Two carriers of apoE2 ($Arg^{136}\rightarrow$ Cys) (G. K. and J. S.) had elevated concentrations of triglycerides. In both individuals, however, the ratios of VLDL-C to total TG and of VLDL-C to VLDL-TG were normal (less than 0.30 and 0.42, respectively), whereas both ratios were markedly increased in an apoE2/2 homozygous individual (F. E.) with classical, recessive type III HLP who was included into the study for comparison (Table 1). Thus, the hypertriglyceridemic carriers of apoE2 ($Arg^{136} \rightarrow$ Cys) did not show the compositional changes of VLDL considered a hallmark of type III HLP. Another criterion required for the diagnosis of type III HLP is the occurrence of VLDL particles with β-electrophoretic mobility in agarose. The VLDL from all of our apoE2 ($Arg^{136} \rightarrow$ Cys) carriers revealed a single band with pre-β electrophoretic mobility, as expected for normal VLDL; βVLDL were not detected, regardless of whether staining was for cholesterol or triglycerides (**Fig. 4**). It should be noted that the electrophoretic mobility of VLDL from apoE2 ($Arg^{136} \rightarrow$ Cys) heterozygotes was absolutely identical to VLDL from family members lacking apoE2 ($Arg^{136} \rightarrow$ Cys). As the VLDL in the hyperlipidemic apoE2 ($Arg^{136} \rightarrow$ Cys) carriers did not show the compositional changes characteristic of type III HLP and did not reveal β-electrophoretic mobility, we classified these individuals as having Frederickson type IV hyperlipidemia.

We compared the LDL receptor binding activity of DMPC vesicles containing apoE2 ($Arg^{136} \rightarrow$ Cys), apoE2 ($Arg^{158} \rightarrow$ Cys) and apoE3 in cultured human fibroblasts. cDNA encoding apoE ($Arg^{136} \rightarrow$ Cys) was generated by site-specific mutagenesis, expressed in vitro and purified by heparin affinity chromatography, as described (46). Recombinant apoE2 ($Arg^{158} \rightarrow$ Cys) and apoE3 were used for comparison. Relative binding activities were derived from the apoE concentrations required to displace one-half of the iodinated LDL from receptor binding. As expected, binding activity of apoE2 ($Arg^{158} \rightarrow$ Cys) was less than 1% of apoE3. Residual binding activity of apoE2 ($Arg^{136} \rightarrow$ Cys) was 14% and thus exceeded apoE2 ($Arg^{158} \rightarrow$ Cys) (**Fig. 5**). ApoE-containing DMPC vesicles are artificial ligand particles and may not reflect the conditions in vivo. For this reason we wished to replicate the finding that apoE2 ($Arg^{136} \rightarrow$ Cys) possessed residual receptor bind-

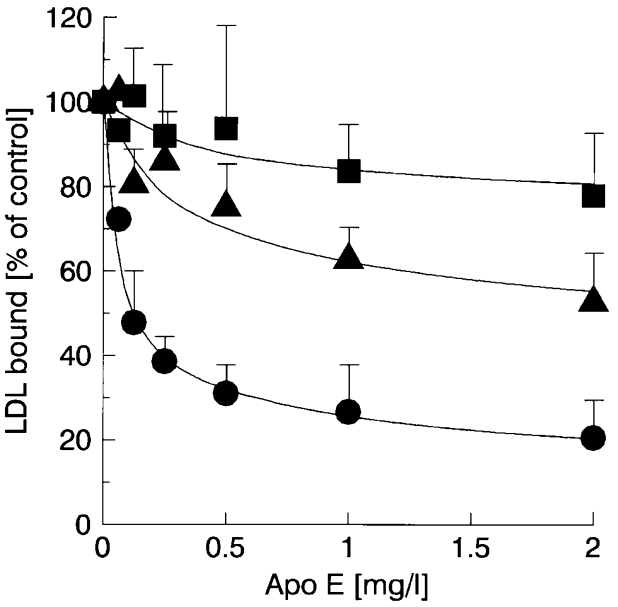

Fig. 5. Receptor binding activities of recombinant apoE2 ($Arg^{136} \rightarrow Cys$) (triangles), apoE2 ($Arg^{158} \rightarrow Cys$) (squares), and apoE3 (circles). The recombinant apoE isoforms were incorporated into DMPC complexes as described in Materials and Methods. Normal human skin fibroblasts were grown in 24-well polystyrene plates incubated for 40 h with medium containing 10% (v/v) human lipoprotein-deficient serum. The cells then received 5 mg/l ^{125}I-labeled LDL and apoE-DMPC complexes at apoE concentrations indicated on the abscissa for 1 h at 4°C. Each data point represents the average of two experiments, each performed in triplicate.

ing using remnant particles also occurring in vivo. Therefore, we loaded recombinant apoE isoforms to VLDL from an individual deficient in apoE due to homozygosity for a 10 bp deletion introducing a premature stop at codon 229 of the apoE mRNA (54). Addition of apoE3 to VLDL from this individual significantly enhanced receptor-mediated binding, uptake, and degradation. ApoE2 ($Arg^{158} \rightarrow Cys$) had no effect, and apoE2 ($Arg^{136} \rightarrow Cys$) slightly augmented the interaction of the VLDL with LDL receptors (**Fig. 6**).

Many dominant apoE variants display higher receptor binding activity than apoE2 ($Arg^{158} \rightarrow Cys$). A common feature of the dominant variants, however, is that they bind less effectively to heparin and cell surface heparan sulfate proteoglycans (HSPG). We, therefore, measured the interaction of DMPC vesicles containing recombinant apoE isoforms with heparin-Sepharose. On the average of three different concentrations of apoE-containing DMPC vesicles (**Fig. 7**), the heparin binding activities of apoE2 ($Arg^{136} \rightarrow Cys$) and apoE2 ($Arg^{158} \rightarrow Cys$) were 61% and 53.3% of normal, respectively. Thus, there was also no relevant difference in heparin binding between the two apoE2 isoforms.

DISCUSSION

We identified four individuals heterozygous for a C to T point mutation in the apoE gene replacing arginine by cysteine at position 136 of the amino acid sequence. Including this mutation, three naturally occurring point mutations affecting codon 136 of mature apoE are known so far: apoE2-Christchurch ($Arg^{136} \rightarrow Ser$) (32), apoE3′ ($Arg^{136} \rightarrow His$) (33), and apoE2 ($Arg^{136} \rightarrow Cys$) which has very recently also been found by others (34, 35). As both apoE2-Christchurch ($Arg^{136} \rightarrow Ser$) and apoE3′ ($Arg^{136} \rightarrow His$) would also have abolished the *Cfo*I restriction site at codon 136, we confirmed the presence of the C to T transition characteristic of apoE2 ($Arg^{136} \rightarrow Cys$) in both probands by direct sequencing.

There is substantial evidence that Arg^{136} is functionally important; it is the first residue of a basic cluster extending from amino acids 136 to 150 which is considered the receptor binding domain (11). The residue is conserved in eight of nine species (55). Substitutions of basic residues within the receptor binding region of apoE have been described to result in dominant expression of type III HLP (17–26). Feussner et al. (35) and Pocovi et al. (36) suggested that apoE2 ($Arg^{136} \rightarrow Cys$) and apoE2 ($Arg^{136} \rightarrow Ser$), respectively, were associated with late-onset dominant type III HLP. In the current study, two heterozygous apoE2 ($Arg^{136} \rightarrow Cys$) carriers had elevated triglyceride concentrations, indicating that hyperlipidemia-promoting factor(s) existed in these patients. Both patients, however, did not meet the common criteria of type III HLP and were consequentially diagnosed as having type IV HLP. This suggests that the possession of a single copy of the mutant apoE2 ($Arg^{136} \rightarrow Cys$) is not sufficient to cause type III HLP. An explanation resolving the apparent contradiction between the findings by Feussner et al. (35) and ours would be that another genetic or exogenous factor predisposing to HLP was present in the family studied by Feussner et al. (35) and that this factor was lacking in the apoE2 ($Arg^{136} \rightarrow Cys$)-carrying individuals studied here. Albeit impossible to rule out completely at present, this possibility is not very likely as, in the current study, two unrelated individuals heterozygous for apoE2 ($Arg^{136} \rightarrow Cys$) expressed HLP and thus must have had at least one such factor promoting HLP.

Clinical data provided by Walden et al. (34) are consistent with our results. These authors identified four carriers of apoE2 ($Arg^{136} \rightarrow Cys$), three apoE2 ($Arg^{136} \rightarrow Cys$)/2 compound heterozygotes and one apoE2 ($Arg^{136} \rightarrow Cys$)/3 heterozygote. Regardless of the lipid concentrations, βVLDL were detected in all of the apoE2 ($Arg^{136} \rightarrow Cys$)/2 compound heterozygotes, but not in the apoE2 ($Arg^{136} \rightarrow Cys$)/3 heterozygote, strongly

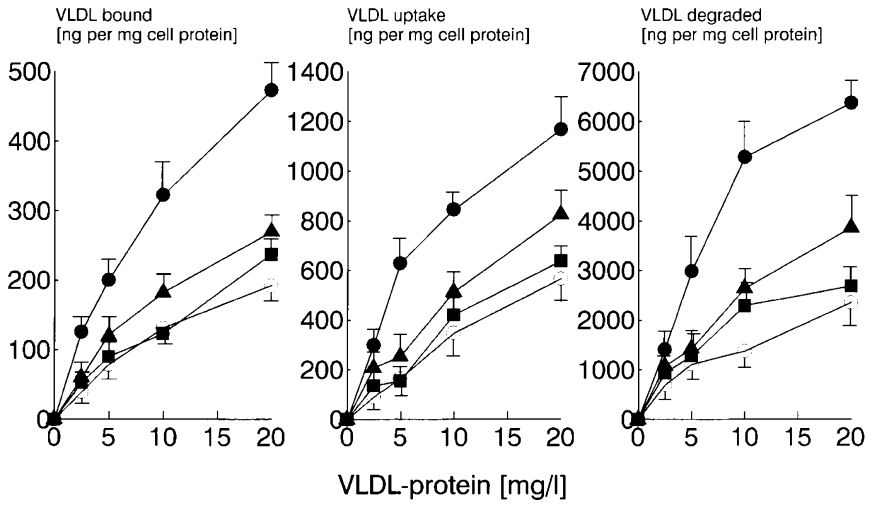

Fig. 6. Binding, uptake, and degradation of apoE-deficient VLDL enriched with apoE2 ($Arg^{136} \rightarrow Cys$) (triangles), apoE2 ($Arg^{158} \rightarrow Cys$) (squares), and apoE3 (circles). VLDL ($d < 1.00$ kg/l) were prepared by ultracentrifugation from a patient with apoE deficiency and type III hyperlipoproteinemia (54), labeled with ^{125}I, and loaded with recombinant apoE by incubation (37°C for 1 h) at a molar ratio of VLDL apoB to recombinant apoE of 1:4. The cells received ^{125}I-labeled VLDL at the indicated concentrations, either without recombinant apoE (open circles), or supplemented with apoE3 (circles), apoE2 ($Arg^{158} \rightarrow Cys$) (squares), or apoE2 ($Arg^{136} \rightarrow Cys$) (triangles). Binding (left), uptake (center), and degradation (right) were determined as described in Methods. Each data point represents the average of two experiments, each performed in triplicate.

suggesting that apoE2 ($Arg^{136} \rightarrow Cys$) causes dysbetalipoproteinemia in a recessive fashion.

Similarly, apoE3′ ($Arg^{136} \rightarrow His$) is most likely related to recessive expression of III HLP (33). Interestingly, heterozygous carriers of apoE3′ ($Arg^{136} \rightarrow His$) displayed a double pre-β band on agarose gel electrophoresis. One of the two bands migrated to the pre-β position as expected for VLDL; the other band had a mobility between LDL and VLDL. The latter fraction migrated slightly faster than βVLDL typically encountered in patients with type III HLP. When we studied the electrophoretic behavior of VLDL from both normolipidemic and hyperlipidemic apoE2 ($Arg^{136} \rightarrow Cys$) carriers, we did not detect a splitting of the pre-β band, suggesting that the double pre-β lipoprotein phenotype might be unique to apoE3′ ($Arg^{136} \rightarrow His$).

The biochemical characterization of apoE2 ($Arg^{136} \rightarrow Cys$) has so far been incomplete. Binding to LDL receptors (34) and to heparin (35) was inferred from studies using VLDL or VLDL apolipoproteins, respectively, from heterozygous carriers of the mutant. This is the first study in which the biochemical characterization apoE2 ($Arg^{136} \rightarrow Cys$) has been accomplished using the variant in a purified form. When loaded to DMPC vesicles, the LDL receptor binding of recombinant apoE2 ($Arg^{136} \rightarrow Cys$) was 14% of apoE3, which is higher than the activity of apoE2 ($Arg^{158} \rightarrow Cys$). Walden et al. (34) examined the ability of VLDL from an individual heterozygous for apoE2 ($Arg^{136} \rightarrow Cys$) and apoE2 ($Arg^{158} \rightarrow Cys$) to induce cholesterol esterification in the macrophage-like cell line J774 in comparison to VLDL from individuals with other apoE genotypes. The VLDL used in these experiments contained two apoE isoforms at an unknown ratio and their apolipoprotein composition was not characterized in more detail. The apoE2 ($Arg^{136} \rightarrow Cys$)/2 ($Arg^{158} \rightarrow Cys$) compound heterozygous VLDL displayed 33% of the normal activity, compared to 10% or less obtained with VLDL from a patient with classical, recessive type III HLP. Thus, despite the limitations of the experimental approach, the conclusions of Walden et al. (34) are in line with ours, indicating that the binding activity of apoE2 ($Arg^{136} \rightarrow Cys$) to LDL receptors was lower than normal, but greater compared to apoE2 ($Arg^{158} \rightarrow Cys$). The two other amino acid substitutions affecting residue 136 are even less defective in binding than apoE2 ($Arg^{136} \rightarrow Cys$), apoE3′ ($Arg^{136} \rightarrow His$) and apoE2 ($Arg^{136} \rightarrow Ser$) showing 80% and 40%, respectively, of the activity of apoE3 (11, 33).

The results are compatible with the known crystal structure of apoE (56, 57). In apoE2 ($Arg^{158} \rightarrow Cys$), the replacement of arginine disrupts a salt bridge between residue 158 and asparagine at position 154. As a consequence, a new salt bridge occurs between aspar-

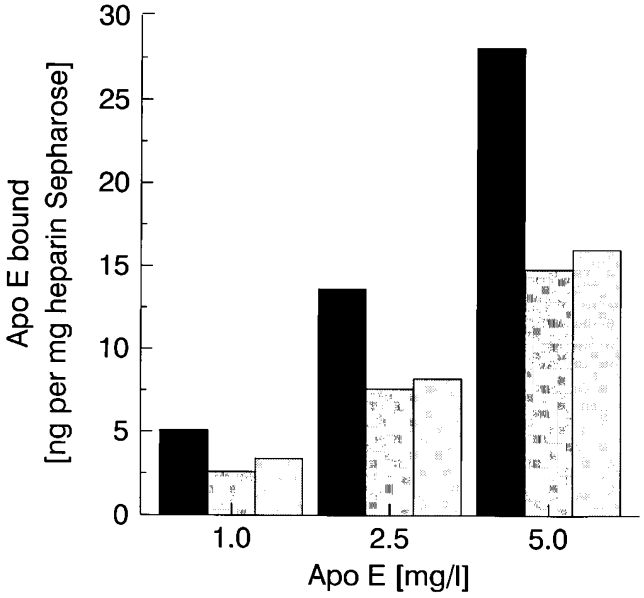

Fig. 7. Heparin binding of apoE3 (solid bars), apoE2 ($Arg^{158} \rightarrow Cys$) (dark grey bars), apoE2 ($Arg^{136} \rightarrow Cys$) (light grey bars). Recombinant ^{125}I-labeled apoE was incorporated into DMPC vesicles and incubated at the indicated apoE concentrations with heparin-Sepharose or Sepharose, respectively, and binding was measured as described in Methods. All incubations were performed in triplicate; results were corrected for binding to Sepharose alone and expressed as ng apoE per mg heparin-Sepharose.

agine 154 and arginine 150. Ultimately, this shifts arginine 150 out of the receptor binding domain. In contrast, ample conformational changes are unlikely to occur as a consequence of the Arg^{136} for Cys substitution. Arg^{136} does not participate in the formation of salt bridges; hence, its replacement by an uncharged residue would probably have only mild effects, if any, on the spatial orientation of other amino acids within the receptor binding domain of apoE.

Interaction of apoE with cell surface proteoglycans is thought to mediate the initial binding of apoE-containing particles to cells, preceding their delivery to endocytotic pathways (31, 58, 59). ApoE variants causing dominant transmission of type III HLP differ from apoE2 ($Arg^{158} \rightarrow Cys$) in that they not only exhibit reduced affinity for lipoprotein receptors, but are also largely defective in binding to cell surface proteoglycans (29–31). In this study, heparin binding of apoE2 ($Arg^{136} \rightarrow Cys$) was approximately two-thirds that of apoE3, which is close to the binding activity of apoE2 ($Arg^{158} \rightarrow Cys$) (29–31). The finding that apoE2 ($Arg^{136} \rightarrow Cys$) and apoE2 ($Arg^{158} \rightarrow Cys$) interact equally with heparin complies with the idea that apoE2 ($Arg^{136} \rightarrow Cys$) is not associated with dominant type III HLP. It is also consistent with data from Feussner et al. (35) who showed that these two isoforms co-eluted on heparin-Sepharose affinity chromatography. In addition, the two other apoE mutations involving residue 136, apoE2 ($Arg^{136} \rightarrow Ser$) and apoE3′ ($Arg^{136} \rightarrow His$), have also been reported to have only slightly reduced affinity to heparin (32, 33).

In conclusion, the present study provides clinical and experimental evidence suggesting that replacement of Cys for Arg at position 136 of apoE, albeit located in the putative receptor binding domain of the molecule, is not related to dominant transmission of type III HLP. As type III HLP was observed in an apoE2 ($Arg^{136} \rightarrow Cys$)/2 ($Arg^{158} \rightarrow Cys$) compound heterozygote by Walden et al. (34), apoE2 ($Arg^{136} \rightarrow Cys$) is obviously able to contribute to recessive type III HLP. Our findings do lend further support to the concept that dominant type III HLP is mainly caused by abnormalities of apoE, diminishing both the LDL receptor binding and heparin binding as it was demonstrated for substitutions of uncharged or acidic for basic residues at positions 142 (19–21), 145 (22–24), 146 (17, 18, 25, 26), or 147 (27) of the apoE molecule.

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