Apolipoprotein E2-Dunedin (228 Arg→Cys): an apolipoprotein E2 variant with normal receptor-binding activity

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Abstract Homozygosity for the apolipoprotein (apo) E variant apoE2(158 Arg→Cys) invariably gives rise to dysbetalipoproteinemia, and when associated with obesity or a gene for hyperlipidemia, results in type III hyperlipoproteinemia. The association of the E2/2 phenotype with type IV/V hyperlipoproteinemia rather than type III hyperlipoproteinemia in identical twin brothers led us to investigate the primary structure of their apoE. Lipoprotein electrophoresis on agarose gels confirmed the presence of increased very low density lipoproteins (VLDL) and chylomicrons but little, if any, β-VLDL, indicating that these subjects did not have dysbetalipoproteinemia. When the apoE from these twins was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a system that can distinguish apoE2(158 Arg→Cys) from all other known apoE variants, it gave rise to two components. One had the unique mobility of apoE2(158 Arg→Cys), and one migrated in the position of the other variants of apoE (and normal apoE3), indicating that the brothers were heterozygous for apoE2(158 Arg→Cys) and a second apoE2 isoform. Cysteamine modification and isoelectric focusing showed that, like apoE2(158 Arg→Cys), the second apoE2 isoform also contained two cysteine residues. The structural mutation in the second apoE2 isoform was determined by peptide sequencing. Like normal apoE3, this variant had arginine at position 158, but differed from apoE3 by the substitution of cysteine for arginine at position 228. Total apoE isolated from the brothers had the same receptor-binding activity in a competitive binding assay as a 1:1 mixture of normal apoE3 and apoE2(158 Arg→Cys). Since the brothers possess approximately equal amounts of apoE2(158 Arg→Cys) and apoE2(228 Arg→Cys), this suggests that apoE2(228 Arg→Cys) has normal, or nearly normal, receptor-binding activity. Consequently, with respect to the common polymorphic sites in apoE and functional activity, these subjects are indistinguishable from E3/2 individuals, a finding consistent with the apparent absence of β-VLDL and other characteristics of type III hyperlipoproteinemia.—**Wardell, M. R., S. C. Rall, Jr., S. O. Brennan, E. R. Nye, P. M. George, E. D. Janus, and K. H. Weisgraber.** Apolipoprotein E2-Dunedin (228 Arg→Cys): an apolipoprotein E2 variant with normal receptor-binding activity. *J. Lipid Res.* 1990. **31:** 535-543.

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Apolipoprotein (apo) E is found on most of the major lipoprotein classes, including chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins, and high density lipoproteins (1). It serves as a specific high-affinity ligand for the low density lipoprotein (LDL) receptor (also referred to as the apoB,E(LDL) receptor), thereby mediating the binding of lipoprotein particles to cells (1, 2). The apoE present in human plasma is a single polypeptide of 299 amino acids (3). There are three major isoforms of apoE (apoE2, apoE3, and apoE4) (4), which are coded for by three common alleles at a single genetic locus (5, 6). The most common, apoE3, is considered the normal isoform and contains a single cysteine residue at position 112 (3). The other two isoforms differ from apoE3 by single amino acid substitutions (7): apoE4 has arginine rather than cysteine at position 112 (1), and apoE2 has cysteine rather than arginine at position 158 (3). Because each individual inherits two apoE alleles, there are six common apoE phenotypes in the population (8): three homozygous (E2/2, E3/3, and E4/4) and three heterozygous (E3/2, E4/3, and E4/2).

Homozygosity for apoE2(158 Arg→Cys) is associated with the lipid disorder type III hyperlipoproteinemia (9),

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoproteins; LDL, low density lipoproteins; β-VLDL, cholesteryl ester-rich remnant lipoproteins of both hepatic and intestinal origin that have identical ultracentrifugal properties as VLDL but have β rather than pre-β electrophoretic mobility; SDS, sodium dodecyl sulfate; DMPC, dimyristoylphosphatidylcholine; CM, carboxymethyl.

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which is characterized by the accumulation in the plasma of cholesteryl ester-rich remnants (β-VLDL) of both VLDL and chylomicrons (10, 11). These remnants accumulate because the substitution at residue 158 in apoE2 yields a protein that binds defectively to lipoprotein receptors, having only about 1% of normal activity (12). In addition to the common apoE2 isoform, six other rare apoE mutants with defective receptor-binding activity, [apoE2(145 Arg→Cys) (13), apoE2(146 Lys→Gln) (14), apoE1(146 Lys→Glu) (15), apoE2(136 Arg→Ser) (16), apoE3(112 Cys→Arg, 142 Arg→Cys) (17), and apoE3-Leiden (18–20)] are also associated with type III hyperlipoproteinemia, as is apoE deficiency (21, 22).

In 1986, Nye, Sutherland, and Janus (23) reported identical twin brothers with the E2/2 phenotype who had type V, rather than type III, hyperlipoproteinemia. Type V hyperlipoproteinemia is characterized by gross hypertriglyceridemia that is due to the presence of both fasting chylomicronemia and an elevation of the VLDL level (24). Upon weight loss, the brothers' chylomicronemia disappeared and their disorder converted to type IV hyperlipoproteinemia (23). Although many subjects homozygous for apoE2(158 Arg→Cys) do not develop hyperlipidemia (type III hyperlipoproteinemia), they invariably display β-VLDL (dysbetalipoproteinemia) (25). The combination of the apparent absence of β-VLDL and the occurrence of type IV/V hyperlipoproteinemia in the identical twin brothers led us to investigate the primary structure of their apoE. In this report we describe a novel apoE2 variant whose apparently normal receptor-binding activity is the likely explanation for the absence of dysbetalipoproteinemia in these subjects.

METHODS

Subject description

The plasma and lipoprotein lipids for obese identical twin brothers (BA and NA), age 47 years, were described previously (23). They had massive hypertriglyceridemia (2700–4000 mg/dl), with markedly elevated levels of both VLDL and chylomicrons in the fasting state. Plasma cholesterol was also elevated in both men (436 and 488 mg/dl), but their LDL cholesterol was low (34 and 83 mg/dl). The men were classified as having familial type V hyperlipoproteinemia (23). With weight loss, plasma cholesterol and triglyceride levels were reduced drastically in both subjects (to 185 and 294 mg/dl for cholesterol and 184 and 403 mg/dl for triglycerides, respectively), and there was no evidence of chylomicronemia. The VLDL ($d<1.006$ g/ml) cholesterol:plasma triglyceride ratio, measured on five separate occasions in one brother and on six occasions in the other, before and after weight loss in both cases, was always in the range of 0.04–0.26 (calculated from the data in ref. 23); a ratio greater than 0.3 is commonly indicative of type III hyperlipoproteinemia (26, 27). Both men had the homozygous E2/2 phenotype. The men are orphans and have no first-degree relatives (sibs or children).

Isolation and characterization of lipoproteins and apolipoprotein E

The characterization of ultracentrifugally isolated lipoprotein fractions and of the lipoprotein profile in whole plasma was performed on 1% agarose gels (Corning, Palo Alto, CA) in 0.05 M sodium barbital buffer, pH 8.6, for 35 min at 23°C and 90 V. Fatty acid-free bovine serum albumin (28) (100 mg/ml) was added to each sample in a ratio of 1:16 (v/v) to adsorb free fatty acids, which otherwise distort the migration positions of the lipoproteins to which they are bound. Agarose gels were stained with fat red 7B (Corning Medical and Scientific, Medfield, MA). Lipoproteins of $d<1.02$ g/ml were prepared from EDTA-plasma from both brothers, and apoE was isolated from this fraction as described previously (12, 29). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis was performed using the system of Neville (30) as adapted for apoE by Utermann et al. (31). The Tris-borate-SDS buffer, pH 8.64, was used in the upper reservoir chamber, and SDS was not included in either the upper or the lower gel solutions (31). Analytical isoelectric focusing was performed on 5% polyacrylamide gels containing 8 M urea and 2% Ampholine, pH 4–6 (Pharmacia LKB Biotechnology Inc., Piscataway, NJ), according to the method of Pagnan et al. (32), as modified (33). For cysteamine modification, apoE was dissolved at a concentration of 1.0 mg/ml in 10 mM Tris-HCl, pH 8.0, containing 8 M urea and 20 mM dithiothreitol, a solution that had previously been degassed and then deoxygenated for 2 h with nitrogen. The apoE was allowed to reduce for 4 h in this buffer under an atmosphere of nitrogen. After reduction, cysteamine was added at a 10-fold molar excess over total thiol groups in dithiothreitol, and the reaction was allowed to proceed for 16 h at 23°C and then for 4 h at 37°C. The cysteamine-treated apoE was exchanged into 0.1 M NH_4HCO_3 over Sephadex G-25 and lyophilized. It was then dissolved in 10 mM Tris-HCl, pH 8.0, containing 1% decyl sulfate and 20% sucrose and then subjected to isoelectric focusing.

Two-dimensional electrophoresis

To generate thrombolytic fragments, apoE (1 mg/ml in 0.1 M NH_4HCO_3) was incubated at 23°C with 1% thrombin (a gift from Dr. J. W. Fenton II of the New York Department of Health, Albany) (w/w) for 16 h. Cysteamine treatment of the thrombolytic digest was performed as for apoE. The thrombolytic fragments were separated in the first dimension by isoelectric focusing in a 0.75-mm-thick vertical slab gel of 5% acrylamide contain-

ing 8 M urea and 2% Ampholine, pH 4–6; 0.4% (v/v) ethylenediamine was used as the upper (cathode) electrolyte and 0.2% (v/v) H_2SO_4 as the lower (anode) electrolyte. Focusing was performed for 1 h at 400 V and then for 2.5 h at 800 V. Individual lanes were excised from the slabs and loaded onto the top of the second-dimension gel, which consisted of a 1.5-mm-thick 10–20% gradient of polyacrylamide containing SDS with a 4.4% stacking gel. Electrophoresis in the second dimension was performed using the buffer system of Laemmli (34) in which 2-amino-2-methyl-1,3-propanediol was substituted for Tris. Immunoblotting analysis (35) of the two-dimensional gels was performed using an affinity-purified ^{125}I-labeled immunoglobulin G fraction of a rabbit polyclonal antiserum to human apoE (10^6 cpm/ml).

Structural analysis

Apolipoprotein E and its 12-kDa thrombolytic fragment (isolated as previously described) (29) were reduced and carboxymethylated according to the method of Nelson et al. (36), as modified (16). Digestion with L-(1-tosylamido-2-phenyl) ethyl chloromethyl ketone-trypsin (Cooper Biomedical, Freehold, NJ) and separation of the tryptic peptides by two-dimensional paper mapping were performed as previously described (16), with 0.5 mg of digest applied to each map. Amino acid analyses were performed using two systems. Phenylthiocarbamyl amino acid analysis (37) was performed on all peptides from the two-dimensional paper maps. For the larger thrombolytic fragments and the intact protein, amino acid analyses were performed on a Beckman 121MB analyzer (Beckman Instruments, Fullerton, CA) as described previously for apoE (7). Cysteine was determined as cysteic acid according to the method of Moore (38).

Automated peptide sequencing was performed on either a Beckman model 890M spinning cup sequencer as previously described (7) or on an Applied Biosystems 477A pulsed liquid sequencer (Applied Biosystems, Foster City, CA) equipped with an on-line 120A analyzer. Tryptic peptides that had been located on the paper map with fluorescamine (Aldrich Chemical Co., Milwaukee, WI) were eluted with acetic acid–ethanol–water (1:1:8, v/v/v) and sequenced.

Receptor binding assays were carried out with apoE · dimyristoylphosphatidylcholine (DMPC) complexes and cultured human fibroblasts using ^{125}I-labeled LDL as the competitor (39).

RESULTS

Lipoprotein electrophoresis

Electrophoresis of the plasma and the d<1.02 g/ml lipoproteins from each of the brothers on 1% agarose demonstrated an apparent lack of β-VLDL. **Fig. 1** (lane 1) shows the agarose electrophoretic pattern of the lipoproteins in the fasting plasma from a subject with type III hyperlipoproteinemia. A prominent β-migrating band is present, as well as chylomicrons that streak up from the origin. The fasting plasma from one of the brothers (NA) has a prominent pre-β band, a less intense β-migrating band, and chylomicrons (Fig. 1, lane 2). As indicated in Fig. 1, lane 3, the d<1.02 g/ml lipoprotein fraction from NA contains the particles with pre-β electrophoretic mobility and the chylomicrons. The d 1.02–1.063 g/ml lipoprotein fraction (Fig. 1, lane 4) suggests that the LDL accounts for the β-migrating band seen in the fasting plasma (lane 2).

Electrophoretic analysis of apoE

The apoE variant apoE2(158 Arg→Cys) has a different electrophoretic mobility than all other apoE variants but one thus far studied on the sodium dodecyl sulfate (SDS)-polyacrylamide gel system described by Utermann et al. (31). All other variants except apoE3-Leiden migrate with a mobility identical to normal apoE3; apoE3-Leiden has a mobility between that of apoE3 and apoE2(158 Arg→Cys) (19). The apoE from both brothers displayed two bands of approximately equal intensity (**Fig. 2**); one comigrating with apoE2(158 Arg→Cys) and the other with apoE3. This indicated that these brothers are compound heterozygotes, having one usual E2 variant and a second E2 variant.

Isoelectric focusing analysis of purified apoE confirmed the E2/2 phenotype (**Fig. 3**, lane 3). After cysteamine treatment, the isoelectric focusing position of the total apoE from both brothers shifted two charge units more cathodally, i.e., from the E2 to the E4 position (Fig. 3, lane

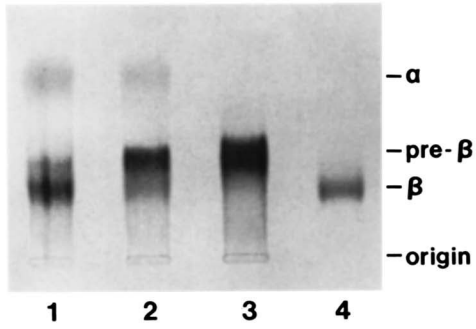

Fig. 1. Lipoprotein electrophoresis of fasting plasma and isolated lipoprotein fractions on 1% agarose. Lane 1, fasting plasma from a subject with type III hyperlipoproteinemia; lane 2, fasting plasma from one of the brothers (NA); lane 3, ultracentrifugally isolated d<1.02 g/ml lipoproteins from NA; lane 4, ultracentrifugally isolated d 1.02–1.063 g/ml lipoproteins (LDL) from NA. Defatted bovine serum albumin was added to all samples to remove free fatty acids. The origin and electrophoretic migration positions of the major lipoprotein classes are indicated. Chylomicrons remain on or streak up from the origin, LDL migrate with β mobility, VLDL migrate with pre-β mobility, and HDL migrate with α mobility.

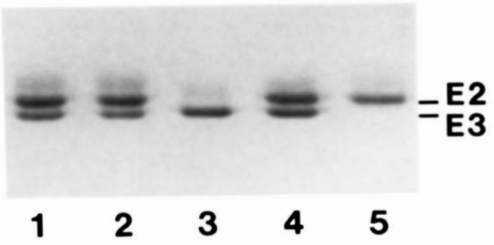

Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of total apoE2 isolated from the identical twin brothers. Lanes 1 and 2, 2 μg of the total apoE isolated from each of the two brothers (lane 1 is BA and lane 2 is NA); lane 3, 1 μg of normal apoE3; lane 4, a 1:1 mixture of samples used in lanes 3 and 5 (2 μg); lane 5, 1 μg of apoE2(158 Arg→Cys). The migration position labeled E2 is unique to the variant apoE2(158 Arg→Cys) (31). The position labeled E3 is where normal apoE3 and all other known variants migrate except apoE3-Leiden, which has a migration position between the E2 and E3 positions (19). The diffuse bands with higher apparent molecular weights than the major bands in each lane represent glycosylated isoforms.

4). This indicated that like the usual apoE2(158 Arg→Cys) isoform, the second apoE2 isoform also possessed two cysteine residues. This result was confirmed by amino acid analysis, which indicated two residues of cysteine (determined as cysteic acid) per molecule (data not shown).

Because the second apoE2 isoform contains two cysteine residues (Fig. 3) but apparently does not have cysteine at residue 158 (Fig. 2), an attempt was made to locate the putative new cysteine site. To determine whether the mutation occurred in the amino-terminal or carboxy-terminal domain of the second isoform, purified total apoE2 from the brothers was digested with thrombin and the fragments were mapped by two-dimensional electrophoresis. A parallel digestion of normal apoE3 was performed and also subjected to two-dimensional electrophoresis. Thrombin digestion of apoE3 generated a single amino-terminal 22-kDa fragment (residues 1–191) and a single carboxy-terminal 10-kDa fragment (residues 216–299) (**Fig. 4**, panel A). Under these conditions, the intermediate 12-kDa carboxy-terminal product (residues 192–299) was not observed. In contrast, digestion of the apoE2 from the two brothers resulted in the generation of two amino-terminal species (22-kDa fragments) and four carboxy-terminal species (two 12-kDa and two 10-kDa fragments) (Fig. 4, panel B). One of the 22-kDa species from the brothers corresponds to the apoE3 22-kDa fragment, and the other focuses in the position of the apoE2 22-kDa fragment. Since one of the apoE2 isoforms contained an amino-terminal fragment that focused in the position of the apoE3 22-kDa fragment, it was anticipated that the carboxy-terminal fragment would possess a substitution responsible for the mobility of the intact variant in the apoE2 position. This is indeed the case; a new carboxy-terminal species, labeled 10k-Dn in Fig. 4 (panel B), has a more acidic focusing position than the normal 10-kDa thrombolytic fragment. When the thrombin digest of apoE from the two brothers was treated with cysteamine before two-dimensional electrophoresis, the new 10k-Dn fragment shifted to the same position as the normal 10-kDa thrombolytic fragment, indicating that it contains cysteine (Fig. 4, panel C). The same is true for the 12k-Dn fragment (Fig. 4, panels B and C). For the apoE of the brothers, the rate of thrombin cleavage at arginine-215 is much slower than normal, yielding a considerable amount of the 12-kDa thrombolytic fragment (labeled 12k and 12k-Dn in Fig. 4).

Structural analysis

The two apoE isoforms could not be separated using the gel system described in Fig. 2 because of the poor resolution achieved when preparative scale isolation was attempted. Therefore, the structural analysis was performed on a mixture of the two apoE components. The two-dimensional tryptic peptide map of carboxymethyl (CM) apoE from these subjects (**Fig. 5**) differed from the map of normal apoE3 in two respects. First, both peptides T22 and CM-T22* were present. As reported previously (16), T22 represents the tryptic peptide from normal apoE3 that encompasses amino acid residues 159–167 (Leu-Ala-Val-Tyr-Gln-Ala-Gly-Ala-Arg). In apoE2(158 Arg→Cys), the presence of cysteine rather than arginine at position 158 leads to the loss of a tryptic cleavage site after position

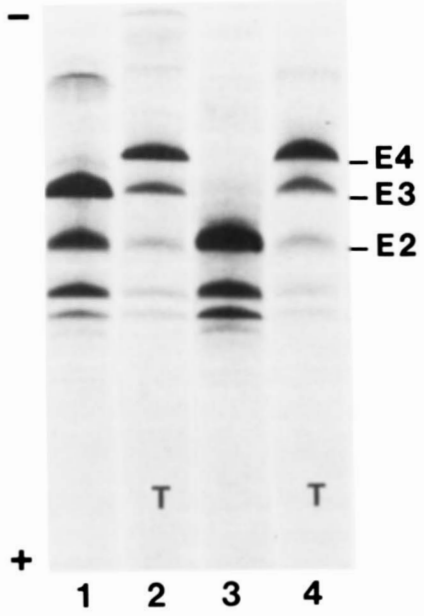

Fig. 3. Analytical isoelectric focusing of total apoE2 from one of the brothers. Lane 1, 10 μg of untreated normal apoE3; lane 2, 10 μg of cysteamine-treated (T) apoE3; lane 3, 10 μg of untreated total apoE isolated from one of the brothers (BA); lane 4, 10 μg of cysteamine-treated (T) total apoE isolated from BA. The cathode (−) is at the top, and the anode (+) is at the bottom. The focusing positions of apoE2, apoE3, and apoE4 are indicated.

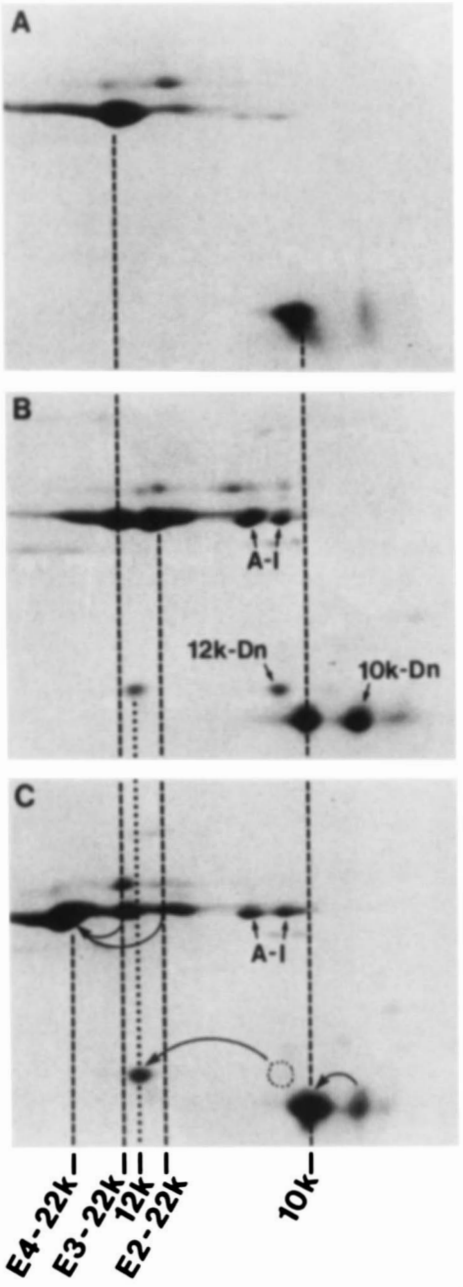

Fig. 4. Two-dimensional gel electrophoresis of the thrombolytic fragments of total apoE2 from one of the brothers. Panel A, thrombolytic fragments from 15 μg of normal apoE3. Digestion was with 1% (w/w) thrombin for 18 h at 23°C. In addition to the 22-kDa and 10-kDa thrombolytic fragments, a small amount of deamidated 10-kDa fragment with a more acidic pI can be seen; panel B, thrombolytic fragments from 15 μg of total apoE isolated from one of the brothers (BA). Although digestion conditions were identical to those for apoE3, some 12-kDa thrombolytic fragment is still present (confirmed by immunoblotting), indicating incomplete hydrolysis at arginine-215. The 12k-Dn and 10k-Dn are the 12-kDa and 10-kDa thrombolytic fragments generated from the apoE2-Dunedin isoform. Some apoA-I was added to the apoE to serve as a marker in this experiment; panel C, cysteamine-treated thrombolytic fragments from 15 μg of total apoE isolated from BA. The arrows indicate the movement of those fragments that contain cysteine after modification with cysteamine. The cathode is at the left, and the anode is at the right. The focusing positions of the 22-kDa thrombolytic fragments of apoE4, apoE3, and apoE2 (E4-22k, E3-22k, and E2-22k) and the normal 12-kDa and 10-kDa thrombolytic fragments (12k and 10k) are indicated.

158, and trypsin now cleaves after lysine-157. This means that in apoE2(158 Arg→Cys) the peptide corresponding to T22 from normal apoE3 has an extra cysteine residue at its amino terminus, and it has been designated T22* (16). When the cysteine is carboxymethylated, this peptide (now designated CM-T22*) gains negative charge, which alters its electrophoretic migration position from the basic to the neutral region of the peptide map. The presence of both T22* and CM-T22* on the map in Fig. 5 indicates that cysteine-158 was incompletely carboxymethylated. Because only T22* (or CM-T22*) is expected on the tryptic map of a homozygous E2/2 subject, the additional presence of T22 on the maps from the brothers indicated immediately that there was heterogeneity at residue 158.

The second difference on the apoE maps was the presence of an acidic peptide that is not found in normal apoE3. The sequence analysis of this peptide, CM-T32-Dn, is given in **Table 1**. This new peptide represents amino acid residues 225–233, in which cysteine substitutes for the normally occurring arginine at position 228. A separate sequence analysis of CM-T32-Dn isolated from a tryptic peptide map of the carboxymethylated 12-kDa thrombolytic fragment confirmed this sequence, definitively showing CM-cysteine at the fourth cycle (using a CM-cysteine standard to determine the retention time of this amino acid). The presence of cysteine in this peptide was also shown by amino acid analysis (determined as cysteic acid). Thus, the two brothers are compound heterozygotes: they have one usual ε2 allele that codes for cysteine at position 158 and one new ε2 allele that has arginine at position 158 and cysteine replacing the normally occurring arginine at position 228.

Receptor-binding activity of apoE2(228 Arg→Cys)

Receptor binding assays were performed on the total mixture of the two apoE components, apoE2(158 Arg→Cys) and apoE2(228 Arg→Cys), which were present in approximately equal amounts. As shown in **Fig. 6**, the apoE·DMPC complexes were defective in binding to the LDL receptors on cultured human fibroblasts when compared with normal apoE3, but were not as defective as pure apoE2(158 Arg→Cys), which in this experiment possessed less than 1% of the binding activity of the normal apoE3 control (Fig. 6). The binding activity of the mixture of the two apoE2 isoforms from one of the brothers (BA) was nearly identical to that observed in a 1:1 mixture of normal apoE3 and apoE2(158 Arg→Cys) (Fig. 6). Based on the 50% competition point from a logit-log plot of the binding data from two separate experiments for the apoE from each brother, their apoE displayed 21% and 25% of normal binding, respectively, while the 1:1 apoE3/apoE2 mixture had 23% of normal binding; this indicates that the binding activity of the variant apoE2(228 Arg→Cys) is normal or nearly normal.

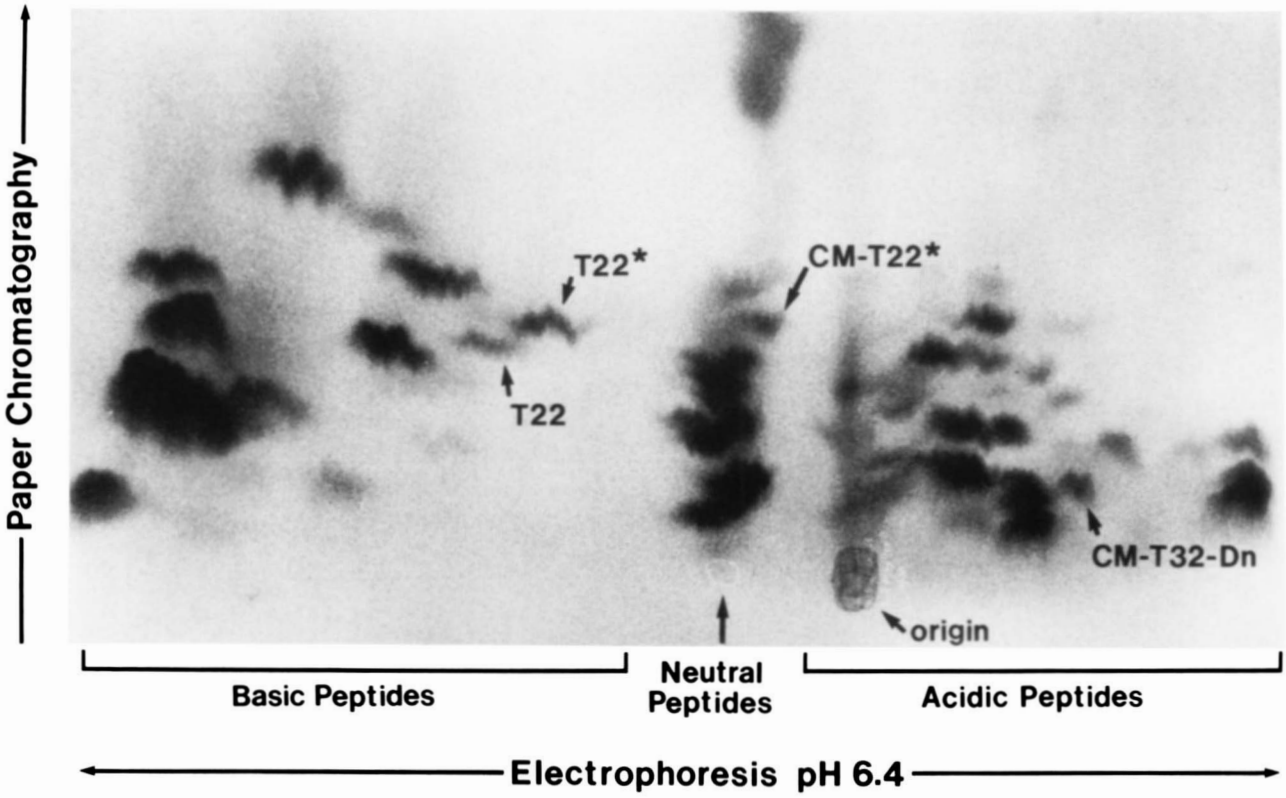

Fig. 5. Fluorescamine-stained two-dimensional tryptic peptide map of carboxymethylated (CM) apoE2 from one of the brothers (BA). The tryptic digest (0.5 mg) was applied at the origin and electrophoresed at 3 kV for 55 min in a pH 6.4 pyridine–acetate buffer (pyridine–acetic acid–water, 100:4:900, v/v/v) in the first dimension and chromatographed in the ascending direction in the second dimension for 20 h in the upper phase of pyridine–isoamyl alcohol–water 6:6:7 (v/v/v) (17). Basic, neutral, and acidic peptides are indicated. The peptides are designated as in Rall et al. (3) and Wardell et al. (16). Peptide T22* is present as a result of incomplete carboxymethylation of cysteine-158 and has the same sequence as CM-T22*. These peptides are derived from apoE2(158 Arg→Cys). Peptides T22 and CM-T32-Dn are derived from apoE2-Dunedin.

DISCUSSION

These results have demonstrated that identical twin brothers with type IV/V hyperlipoproteinemia are compound heterozygotes for apoE. They each possess one usual variant ε2 allele coding for apoE2(158 Arg→Cys) and one novel mutant ε2 allele coding for a protein that differs from normal apoE3 by having cysteine rather than arginine at position 228. Therefore, it is likely that apoE2(228 Arg→Cys) arose as a result of a point mutation, CGC to TGC, in the ε3 allele.

Apolipoprotein E2(228 Arg→Cys) is the fourth natural apoE variant that has resulted from a cysteine-for-arginine substitution, the others being apoE2(158 Arg→Cys) (3), apoE2(145 Arg→Cys) (13), and apoE3(112 Cys→Arg, 142 Arg→Cys) (17). In all these cases the first two nucleotides in the arginine codons are CG, a dinucleotide that is ten times more likely to mutate (a mutational "hot spot") than any other dinucleotide in mammalian DNA (40, 41). This is due to the fact that cytosine residues 5' to guanine are methylated in mammalian DNA and this methylation promotes cytosine deamination and cytosine-to-thymine transitions. Because 23 of the 34 arginine codons in the apoE3 gene are CG^{C}_{T} (42), the frequency of arginine-to-cysteine mutations in apoE is not unexpected.

TABLE 1. Sequence analysis of CM-T32-Dn demonstrating the substitution at residue 228

Cycle	Amino Acid Identified	Amount
		pmol
1	Thr	50
2	Arg	119
3	Asp	177
4	(CM-Cys)[a]	26
5	Leu	228
6	Asp	183
7	Glu	261
8	Val	183
9	Lys	21

[a]Presumed to be CM-Cys based on the retention time of the peak, but no CM-Cys standard was run with this analysis. However, the presence of cysteine was confirmed at this site (see text).

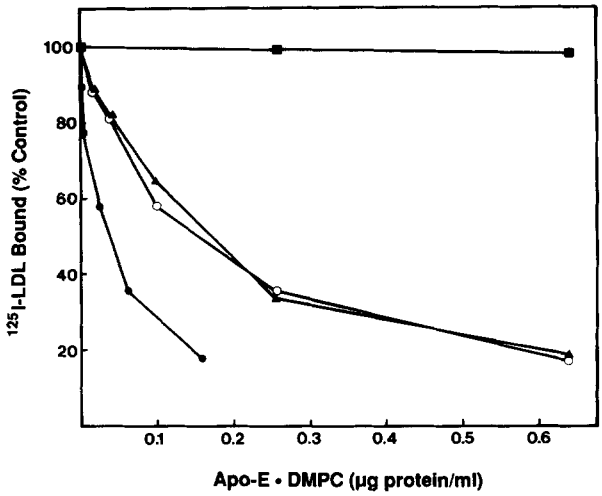

Fig. 6. Receptor-binding activity of apoE2 from one of the brothers heterozygous for apoE2(158 Arg→Cys) and apoE2(228 Arg→Cys). The apoE·DMPC complexes were mixed at various concentrations with 2 μg of ^{125}I-labeled LDL and incubated with cultured human fibroblasts on 35-mm culture dishes for 4 h at 4°C; (●) normal apoE3; (■) apoE2(158 Arg→Cys); (▲) a 1:1 mixture of normal apoE3 and apoE2(158 Arg→Cys); (○) total apoE isolated from one of the brothers (BA) heterozygous for apoE2(158 Arg→Cys) and apoE2(228 Arg→Cys).

The apoE2(228 Arg→Cys) variant described in this report does not appear to be defective in its interaction with the LDL receptor as determined by a competition assay of apoE·DMPC complexes with ^{125}I-labeled LDL in cultured normal human fibroblasts (Fig. 6). Because we were not able to separate apoE2(228 Arg→Cys) from apoE2(158 Arg→Cys) the apoE used in the binding study comprised a mixture of both. There are approximately equal amounts of the two isoforms in the d<1.02 g/ml lipoprotein fraction from the two subjects, as determined by examining several different apoE preparations on SDS-polyacrylamide gels (Fig. 2) and from tryptic peptides specific for each isoform (Fig. 5). The receptor-binding activity of the apoE from the two brothers (21 and 25% of that of apoE3) was effectively the same as that of a 1:1 mixture of apoE3 and apoE2(158 Arg→Cys) (23% of that of apoE3) (Fig. 6). In contrast, apoE2(158 Arg→Cys) had less than 1% of normal binding activity (Fig. 6). Previous studies have indicated that mixtures containing equal amounts of active and inactive apoE have about 20-30% of normal binding activity (43, 44), a finding confirmed here with the apoE3/apoE2 mixture. Furthermore, it has been demonstrated that a 1:1 mixture of apoE2(158 Arg→Cys) and apoE2(145 Arg→Cys) has a binding activity of about 10% of that of normal apoE3 (12, 13), which is clearly distinguishable from the 21-25% of the activity of the apoE of the brothers. Since the apoE2(145 Arg→Cys) variant itself has about half of normal binding activity (13), any defect in apoE2(228 Arg→Cys) binding must be slight, and certainly less than that of apoE2(145 Arg→Cys). Thus, essentially all of the binding activity displayed by the apoE from the two brothers is contributed by the apoE2(228 Arg→Cys) isoform. Therefore, it appears that this is the first apoE2 isoform to have normal receptor-binding activity. While the conformation of the region of the molecule responsible for receptor binding is probably not influenced by the mutation in this variant, the cysteine-for-arginine substitution at position 228 does reduce the rate of thrombin cleavage at argine-215. Therefore, the substitution may alter the local environment or conformation of a region of the molecule that is thought to have a major lipid-binding function (2).

All subjects with hyperlipidemia and known genotypic homozygosity for apoE2(158 Arg→Cys) have type III hyperlipoproteinemia, whereas the association of the E2/2 phenotype with type IV/V hyperlipoproteinemia is very rare. In fact, a number of studies have suggested that the ε4 allele may be involved in the development of type V hyperlipoproteinemia (45-47), although there is some controversy over this concept (48, 49). With respect to the two common polymorphic sites in apoE, positions 112 and 158, the brothers reported here are really analogous to an E3/2 phenotype, in which one allele codes for arginine and one for cysteine at position 158. The frequency of the E3/2 phenotype varies between 10 and 20% in different populations (50), and while people with this phenotype may have slightly lower plasma cholesterol levels than an age- and sex-matched group of phenotype E3/3 (50), they rarely have dysbetalipoproteinemia (i.e., the presence of β-VLDL) (9, 51). More importantly, the E3/2 phenotype is almost never associated with type III hyperlipoproteinemia (9, 51). Thus, the presence of one apoE2 isoform with normal or nearly normal receptor-binding activity in the twin brothers is the apparent explanation for the absence of dysbetalipoproteinemia.

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