Identification of homozygosity for a human apolipoprotein A-I variant

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Abstract An apolipoprotein (apo) A-I variant, previously described in two Norwegian families (Schamaun et al. 1983. *Hum. Genet.* **64:** 380–383), represents a mutation in apoA-I in which a single amino acid substitution of lysine for glutamic acid has taken place at residue 136. An offspring resulting from intermarriage between the two families is genotypically homozygous for this variant. He is the first individual discovered to be homozygous for any of the apoA-I variants. Analysis of lipid data collected from these families indicates one or more lipid abnormalities. The low density lipoproteins (LDL) of subjects having this apoA-I variant demonstrate a compositional abnormality. The plasma cholesterol concentration in the homozygous subject is low because of the extremely reduced levels of LDL and apoB, a property shared by some of his first-degree relatives. However, because of the presence of apoE2 in this family, it is not possible to definitively link these lipid abnormalities to the presence of the A-I variant. —**Rall, S. C., Jr., K. H. Weisgraber, R. W. Mahley, C. Ehnholm, O. Schamaun, B. Olaisen, J. P. Blomhoff, and P. Teisberg.** Identification of homozygosity for a human apolipoprotein A-I variant. *J. Lipid Res.* 1986. **27:** 436–441.

Supplementary key words dyslipoproteinemia • lecithin:cholesterol acyltransferase • genetic marker • hypocholesterolemia • lipid transfer activity

Apolipoprotein (apo) A-I is the major protein constituent of human high density lipoproteins (HDL) and is also associated with newly secreted chylomicrons. Apolipoprotein A-I also serves, at least in vitro, as a potent activator of the plasma cholesterol-esterifying enzyme lecithin:cholesterol acyltransferase (LCAT) (EC 2.3.1.43) (1). In humans, apoA-I is secreted as a 249-amino acid proprotein, having six extra amino acids at its amino terminus, as compared with the circulating plasma protein of 243 amino acids (2). The proprotein has a charge of +2 compared with the mature apoA-I (3) and can be detected in some HDL preparations by isoelectric focusing (4).

Genetically determined electrophoretic variants of human apoA-I have been detected at a low frequency (usually ≤0.1%) in the human population (5, 6). In several of these variants, the amino acid substitutions that account for the differences in the isoelectric focusing pattern have been identified (4, 7–10). Only individuals heterozygous for these variants have been found. Recently, however, an electrophoretic variant of apoA-I was discovered in two Norwegian families, and one homozygous individual was detected, an offspring resulting from intermarriage between the two families carrying this variant (11). Linkage data from these same two families have also recently established a close linkage between the apoA-I and apoA-IV loci (12).

In this report, we describe the amino acid substitution in apoA-I that is responsible for this variant. Although it has a +2 charge relative to normal apoA-I on isoelectric focusing gels and comigrates with normal proapoA-I, this variant is not proapoA-I. We also demonstrate that the phenotypically homozygous individual is indeed genotypically homozygous for this apoA-I variant.

METHODS

Subject description

The detection of the apoA-I variant in two Norwegian families and a pedigree analysis have been described (11).

Lipid, lipoprotein, and apolipoprotein determinations

Plasma samples were drawn into containers having EDTA for lipid and lipoprotein determinations and were

Abbreviations: apo, apolipoprotein; VLDL, very low density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; LCAT, lecithin:cholesterol acyltransferase; PTH-, phenylthiohydantoin.

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stored on ice and separated for lipoprotein analyses within 48 hr. Lipoprotein separation was performed by preparative ultracentrifugation at 4°C in a Beckman L3-50 centrifuge with a 50 Ti rotor (13). Cholesterol and triglyceride concentrations in serum and lipoprotein fractions were determined by enzymatic methods (Boehringer, West Germany). Plasma apolipoprotein levels were determined by radial immunodiffusion (14).

HDL and apoA-I preparation

The HDL were prepared from plasma or serum by sequential ultracentrifugation. The plasma density was raised to 1.063 g/ml by addition of solid KBr and centrifuged at 59,000 rpm for 18 hr at 4°C in a Beckman L8-70 ultracentrifuge (60 Ti rotor). The d 1.063 g/ml top fraction was discarded, and the density of the infranatant was raised to 1.21 g/ml and recentrifuged at 59,000 rpm for 40 hr in the same rotor. The d 1.21 g/ml top fraction was recentrifuged at d 1.21 g/ml at 59,000 rpm for 24 hr. The HDL were delipidated with chloroform–methanol, and the apolipoproteins were solubilized in 0.01 M Tris (pH 8), 1% decyl sulfate, 6 M urea, and 20% sucrose for preparative isoelectric focusing on Immobiline gels (15). Apolipoprotein A-I isoforms were isolated by this technique as previously described (4).

ApoA-I structural analysis

The major apoA-I isoform (purified by Immobiline gels) from the homozygous subject (no. 542 in ref. 11) was subjected to CNBr digestion. The apoA-I (6.3 mg in 1.8 ml of 70% HCOOH) was reacted with 208 mg of CNBr (33-fold weight excess) for 24 hr at room temperature. Peptides were isolated by Sephadex G-50 chromatography as previously described (4, 8) and were identified by amino acid analysis. Amino acid analyses and peptide sequencing were performed as previously described (16).

LCAT activity

The plasma LCAT activity of several subjects was measured according to the method of Stokke and Norum (17), as well as by the method of Glomset and Wright (18). Heat-inactivated plasma from one healthy subject served as a common substrate source for the latter LCAT analyses.

The LCAT cofactor activity of the isolated apoA-I from the homozygous subject was measured in an in vitro assay as previously described (8).

RESULTS

Family clinical data

As judged from the history and physical examination, both families with this apoA-I variant were considered to be generally healthy. There was no indication of premature cardiovascular disease or high blood pressure. However, adult onset diabetes was present in descendants of EB25-580-581 (see Fig. 3 of ref. 11). Two subjects in the second generation, reportedly with diabetes, had died from stroke. Also, subject no. 574 (the paternal grandmother of subject no. 542, who is homozygous for the A-I variant) has been reported to have adult onset diabetes.

Table 1 gives plasma and lipoprotein cholesterol values for various members of the two families. There is a wide range of cholesterol levels among these individuals, but there is no trend that is associated with the presence of the apoA-I variant. The homozygous subject (no. 542) has a low plasma cholesterol concentration that is attributable to an extremely low LDL cholesterol level. He also has an extremely low level of plasma apoB, a phenomenon shared by his first-degree relatives (nos. 536, 540, and 541). Interpretation of these data is further complicated by the presence of the gene for the receptor-defective apoE2 in this family (Table 1). Low LDL levels have been correlated with the presence of apoE2 (19). The homozygous subject (no. 542) and several of his first- and second-degree relatives (nos. 541, 573, 574, and 576) are heterozygous for E2. One first-degree relative (no. 540) and one second-degree relative (no. 575) are homozygous for E2.

As shown in **Table 2**, there does appear to be an abnormality in LDL composition that may correlate with the presence of the A-I variant. The LDL triglyceride to cholesterol (or cholesteryl ester) ratios within LDL are increased in the subject homozygous for the apoA-I variant (type 2), as compared with the ratios in subjects homozygous for normal apoA-I (type 1). The ratios in heterozygous individuals (type 2-1) are intermediate. On the other hand, there appears to be no abnormality in the HDL. High density lipoprotein cholesterol and apoA-I levels of family members fall within the normal range (Table 1), and HDL chemical compositions and apoA-II levels are also normal (not shown).

Characterization of the apoA-I variant

A partial characterization of the apoA-I variant has been published (11). This variant has a more alkaline isoelectric point than does normal apoA-I. Although not stated explicitly in ref. 11, it is apparent that the variant has a charge of +2 relative to normal apoA-I (Fig. 2b of ref. 11). Furthermore, as shown in **Fig. 1**, the apoA-I variant isolated from the homozygote comigrates with normal proapoA-I, which has a known charge of +2 relative to normal apoA-I. However, this variant is not proapoA-I. The possible molecular weight difference suggested previously (11) has not been confirmed in a one-dimensional sodium dodecyl sulfate–polyacrylamide gel analysis (not shown). Furthermore, 24 cycles of sequence analysis on 7 nmol of the isoform shown in lane a in Fig. 1 demonstrated an amino-terminal sequence identical to that of

TABLE 1. Lipid data (non-fasting values) related to the different apoA-I phenotypes

EB25 Pedigree Number[a]	ApoA-I Type[a]	Age/Sex	Cholesterol				% Recovery	ApoA-I	ApoB	ApoE Phenotype
			Total	VLDL	LDL	HDL				
			mg/dl					*mg/dl*		
311	1	29/F	193	11	111	43	85	121	80	4/3
314	1	15/F	246	3	138	78	89	117	86	3/3
525	1	60/F	298	23	221	54	100			3/3
526	1	59/M	206	34	142	30	100			4/3
528	1	55/M	261	21	200	40	100			4/4
530	1	51/F	267	24	201	42	100			4/4
531	1	47/F	312	28	227	57	100			4/3
537	1	31/M	144	30	74	40	100			4/3
573	1	69/M	212	25	77	34	64	130	92	4/2
576	1	38/F	210	18	93	31	68	123	100	4/2
589	1	41/M	174	20	71	29	69	106	97	3/3
310	2-1	49/F	213	5	74	91	80	143	101	4/3
313	2-1	20/M	163	15	84	45	88	107	75	3/3
524	2-1	62/M	267	8	225	34	100			4/3
527	2-1	57/F	205	24	153	28	100			4/3
536	2-1	34/F	159	7	110	42	100	127	61	4/3
540	2-1	39/M						112	53	2/2
541	2-1	16/M						103	34	3/2
574	2-1	67/F	330	134	114	22	82	93	124	3/2
575	2-1	39/F	251	47	104	27	71	130	92	2/2
583	2-1	81/F	246	18	104	48	69	141	112	3/2
542[b]	2	14/M	103	8	37	58	100	98	53	3/2

[a] As in ref. 11: 1 indicates individuals with only normal apoA-I isoproteins; 2-1 indicates individuals heterozygous for normal and variant apoA-I; 2 indicates the individual homozygous for the variant apoA-I.
[b] Values confirmed in a fasting blood sample obtained on another occasion (total cholesterol = 101; VLDL cholesterol = 8; LDL cholesterol = 40; HDL cholesterol = 38; % recovery = 85).

normal apoA-I, i.e., Asp-Glu-Pro-Pro-Gln-Ser-Pro-Trp-, etc. (20). There was no evidence of the proapoA-I sequence, i.e., Arg-His-Phe-Trp-Gln-Gln- (21).

Identification of the mutation in the variant apoA-I

Amino acid analysis of the variant apoA-I prepared from the HDL of the homozygous subject was compared with that of normal apoA-I isolated from the HDL of a normal subject. As shown in **Table 3**, this variant apoA-I had a composition very similar to that of normal apoA-I, except that the variant appeared to have one more residue of lysine and one less residue of glutamic acid.

The variant apoA-I was subjected to CNBr digestion, and the resultant peptides were separated on Sephadex G-50. The elution profile was the same as published previously for other apoA-I variants (4, 8). Each peptide was identified by amino acid analysis (Table 3). When the compositions of the CNBr peptides of the variant apoA-I were compared with the compositions expected for CNBr peptides of normal apoA-I, the only significant difference seen was in peptide CB3 (Table 3). This peptide appeared to have one more lysine residue and one less glutamic acid residue than normal.

The amino acid difference suggested by the amino acid analyses was confirmed by complete sequence analysis of peptide CB3. At cycle 24, which corresponds to residue 136 in apoA-I (20), lysine was found rather than the usual glutamic acid (**Fig. 2**). Furthermore, the complete absence of any glutamic acid at cycle 24 (Fig. 2) indicated that all the apoA-I from the homozygous subject had the

TABLE 2. Lipid ratios in LDL and HDL[a]

ApoA-I Type[b]	LDL			HDL
	Tg/TC	Tg/CE	CE/TC	CE/TC
1 (n = 6)	0.140 ± 0.059	0.191 ± 0.081	0.738 ± 0.037	0.845 ± 0.073
2-1 (n = 4)	0.258 ± 0.074[c]	0.398 ± 0.182[c]	0.683 ± 0.122	0.842 ± 0.051
2 (n = 2)[d]	0.392	0.538	0.727	0.803

[a] Tg, triglycerides; TC, total cholesterol; CE, cholesteryl ester.
[b] See footnote *a* of Table 1 for a definition of 1, 2-1, and 2.
[c] $P < 0.05$, compared with type 1 subjects.
[d] Average of two determinations on separate samples from the one homozygous (type 2) subject.

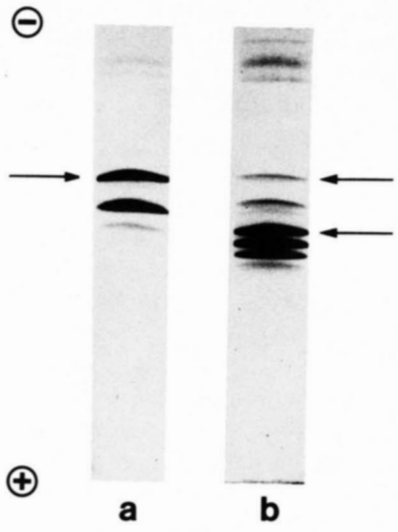

Fig. 1. Analytical isoelectric focusing gels (pH 4–6) of apoA-I. The cathode (−) and anode (+) are indicated. Lane a, variant apoA-I isoform (arrow) isolated by preparative Immobiline gels from the homozygous subject; lane b, apoA-I from a normal subject. In lane b, the upper arrow indicates proapoA-I, which has a relative charge of +2 compared with the major normal apoA-I isoform (lower arrow). The more acidic isoforms appear with time after manipulation and storage, probably by deamidation (24).

Glu→Lys substitution at this site. This indicated that the phenotypically homozygous subject was also genotypically homozygous for this particular variant apoA-I.

Association of LCAT activity with the variant apoA-I

The plasma LCAT activity of several family members is depicted in **Table 4**. As measured by two methods, there is no apparent effect associated with the presence of the apoA-I variant. The plasma of the homozygote (type 2) displayed a high percent of esterification (Table 4), but also a low starting value for total cholesterol (Table 1). Furthermore, the HDL and LDL of family members had a normal cholesteryl ester to total cholesterol ratio (Table 2), regardless of the apoA-I type, suggesting no abnormality of plasma cholesterol esterification.

The ability of the variant apoA-I to serve as a cofactor for LCAT was tested in an in vitro liposome assay (8). This variant demonstrated no significant difference compared with normal apoA-I in this regard. In this assay, at 5 and 7 μg of added apoA-I, 13.2 and 14.6 μg of cholesteryl ester per ml per hr, respectively, were measured with the variant apoA-I, whereas 13.4 and 13.6 μg, respectively, were measured with normal apoA-I.

DISCUSSION

The apoA-I variant detected in two Norwegian families (11) has been shown in the present report to represent a previously undescribed mutation in apoA-I in which a single amino acid substitution of lysine for glutamic acid occurs at residue 136. Therefore, this variant can be described as apoA-I(Glu_{136}→Lys). It is significant that this

TABLE 3. Amino acid compositions of the variant apoA-I and its CNBr peptides[a]

Amino Acid	Normal A-I (n = 3)	Variant A-I (n = 3)	Variant A-I CNBr Peptides[b]			
			CB1	CB2	CB3	CB4
Asp	20.9 (21)	21.0	11.5 (12)	3.1 (3)	0.4 (0)	6.2 (6)
Thr	9.6 (10)	9.6	4.9 (5)	0 (0)	0.1 (0)	4.8 (5)
Ser	13.6 (15)	13.4	6.6 (7)	1.0 (1)	1.0 (1)	5.4 (6)
Glu	46.1 (46)	**45.1**	14.5 (14)	7.0 (7)	**11.1** (12)	13.6 (13)
Pro	10.6 (10)	10.5	4.2 (4)	1.1 (1)	2.2 (2)	3.3 (3)
Gly	10.6 (10)	10.6	4.9 (5)	0.2 (0)	2.0 (2)	3.2 (3)
Ala	19.4 (19)	19.3	2.2 (2)	1.1 (1)	2.0 (2)	14.0 (14)
Cys	n.d. (0)	n.d.	n.d. (0)	n.d. (0)	n.d. (0)	n.d. (0)
Val	13.2 (13)	13.1	7.3 (7)	1.9 (2)	1.3 (1)	3.2 (3)
Met	2.9 (3)	3.1	+ (1)[c]	+ (1)[c]	+ (1)[c]	0 (0)
Ile	0 (0)	0	0 (0)	0 (0)	0 (0)	0 (0)
Leu	37.7 (37)	37.7	11.0 (11)	2.1 (2)	6.9 (7)	16.9 (17)
Tyr	7.1 (7)	7.1	2.0 (2)	1.0 (1)	0.9 (1)	2.9 (3)
Phe	6.2 (6)	6.1	2.9 (3)	1.0 (1)	0 (0)	2.0 (2)
Lys	20.6 (21)	**21.7**	5.9 (6)	4.6 (5)	**3.8** (3)	6.9 (7)
His	4.9 (5)	4.8	0.1 (0)	0 (0)	0.9 (1)	3.7 (4)
Trp	n.d. (4)	n.d.	n.d. (3)	n.d. (1)	n.d. (0)	n.d. (0)
Arg	15.7 (16)	15.8	3.8 (4)	0 (0)	2.8 (3)	8.6 (9)

[a] Compositions are given in residues per molecule, with no corrections for hydrolytic destruction; numbers in parentheses are the theoretical numbers derived from the sequence of normal apoA-I (20); n.d. = not determined; **boldface** indicates the difference from normal apoA-I.

[b] CB1 (residues 1–86) was recovered in 32% yield; CB2 (residues 87–112), 29%; CB3 (residues 113–148), 51%; CB4 (residues 149–243), 61%. Also recovered in good yield was the partial digestion peptide CB1,2 (residues 1–112), 37% yield.

[c] Both homoserine and homoserine lactone were detected.

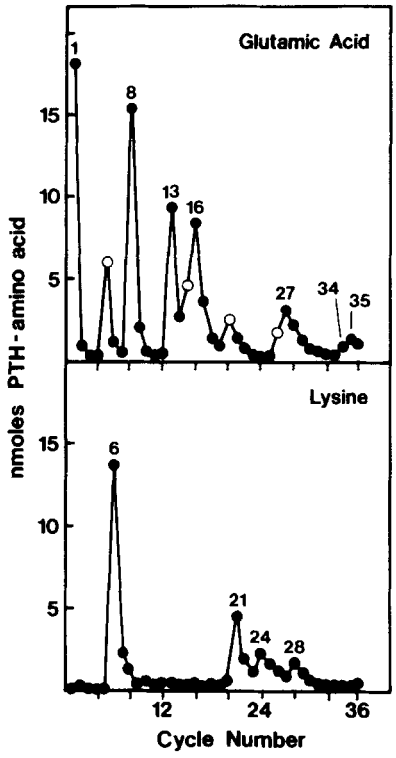

Fig. 2. Sequence of peptide CB3 from the variant apoA-I. The peptide (34 nmol) was degraded in a Beckman 890C Sequencer using a standard 0.1 M Quadrol program in the presence of 2 mg of Polybrene. The cycle-by-cycle recoveries, as ascertained by high-performance liquid chromatography, of the PTH-amino acids glutamic acid and lysine are shown. Open circles (○) mark the positions of identification of PTH-Gln. Under the conversion conditions used, about 50% of PTH-Gln is deamidated to PTH-Glu, which accounts for the appearance of PTH-Glu at cycles 5, 15, 20, and 26. In peptide CB3 from normal apoA-I, glutamic acid occurs at cycles 1, 8, 13, 16, 24, 27, 34, and 35; lysine occurs at cycles 6, 21, and 28.

single replacement of a basic for an acidic amino acid accounts for the +2 charge difference of the variant compared with normal apoA-I.

This variant is the first reported case of an apoA-I variant expressed in the homozygous state. Previous identification (11) of the phenotypic homozygosity (as ascertained by isoelectric focusing) would not a priori indicate homozygosity for just one apoA-I variant. However, the present sequence analysis of the apoA-I variant from the homozygous subject has shown that the subject is indeed genotypically homozygous for the variant designated apoA-I-(Glu_{136}→Lys). Thus far, no subject has been identified who expresses two different apoA-I variants.

In most cases, apoA-I variants (4, 8, 9, 22) cannot be positively linked to any lipoprotein abnormality. The only variant in which a structural mutation has been identified that appears to be directly associated with a lipoprotein abnormality is A-I_{Milano} (7, 23). Even in two cases where a dysfunctional apoA-I variant has been identified, there is no apparent in vivo consequence; two variants of apoA-I have been shown to be deficient in their ability to activate LCAT in vitro (8, 9), but these dysfunctional variants appear to have no detrimental effect on the lipoprotein metabolism of the individuals possessing these variants.

In the present case, it is also not clear whether the apoA-I(Glu_{136}→Lys) variant is associated with any of the described abnormalities in the subjects. The low apoB levels appear to be confined to the homozygous subject and his first-degree relatives (nos. 536, 540, and 541 in Table 1). Although the presence of apoE2 in the family may partially account for this characteristic, it may not be the full explanation, since not all of these subjects with low apoB levels (e.g., no. 536) have apoE2. Therefore, the low level of apoB does not seem to correlate with the presence of either the A-I variant or apoE2, but may represent an unrelated additional abnormality of lipoprotein metabolism in these subjects.

The abnormal LDL composition (increased triglyceride to cholesterol ratio) does seem to correlate with the presence of the A-I variant, but this also must be viewed with caution. Some of the subjects have apoE2, which increases the likelihood that intermediate density-like lipoprotein particles may be contributing to the LDL density fraction. Furthermore, the plasma samples were taken under nonfasting conditions, introducing a possible further complication to the interpretation. Although it is possible that the LDL compositional abnormality is related to a defect

TABLE 4. LCAT activity related to the different apoA-I phenotypes

EB25 Pedigree Number[a]	ApoA-I Type[a]	Age/Sex	% Cholesterol Esterified per hr[b]	μmol/L per hr[b]	μmol/L per hr[c]
573	1	69/M	3.1	46	26
576	1	38/F	3.1	47	29
589	1	41/M	4.2	47	29
574	2-1	67/F	3.2	83	28
575	2-1	39/F	3.2	57	35
583	2-1	81/F	2.0	38	18
542	2	14/M	5.6	37	22

[a] See footnote *a* of Table 1 for definition of 1, 2-1, and 2.
[b] As determined by the Stokke-Norum method (17).
[c] As determined by the Glomset-Wright method (18).

in lipid transfer between HDL and LDL, it is not possible at this time to state whether there is a causal relationship between the A-I variant and this lipid abnormality. A further detailed analysis of the lipoproteins of these subjects must be carried out before such a correlation can be shown to be valid.

The authors thank Drs. Yasushi Ogawa and Christopher J. Fielding for performing the in vitro LCAT analyses, Barbara Allen, Al Averbach, and Sally Gullatt Seehafer for editorial assistance, James X. Warger and Norma Jean Gargasz for graphics, and Kerry Humphrey for manuscript preparation. This work was supported in part by Grant HL 27455 from the U.S. Public Health Service and by the Sigrid Juselius Foundation (Finland).

Manuscript received 12 December 1985.

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