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A von Eckardstein, … , A Benninghoven, G Assmann

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Research Article

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Apolipoprotein A-I Variants

Naturally Occurring Substitutions of Proline Residues Affect Plasma Concentration of Apolipoprotein A-I

Arnold von Eckardstein,* Harald Funke,‡ Anne Henke,‡ Klaus Altland,§ Alfred Benninghoven,‖ and Gerd Assmann*‡
With the technical assistance of **Sabine Welp,* Alois Roetrige,* and Renate Kock***
**Institut für Arteriosklerosеforschung an der Universität Münster, D-4400 Münster, Federal Republic of Germany; ‡Institut für Klinische Chemie und Laboratoriumsmedizin, Zentrallaboratorium, Westfälische Wilhelms-Universität Münster, D-4400 Münster, Federal Republic of Germany; §Institut für Humangenetik, Justus-Liebig-Universität Giessen, D-6300 Giessen, Federal Republic of Germany; and ‖Physikalisches Institut, Westfälische Wilhelms-Universität Münster, D-4400 Münster, Federal Republic of Germany*

Abstract

Six unrelated families with genetically determined structural variants of apo A-I were found in the course of an electrophoretic screening program for apo A-I variants in dried blood samples of newborns. The following structural variations were identified by the combined use of HPLC, time-of-flight secondary ion mass spectrometry (TOF-SIMS), and automated gas phase sequencing: Pro_3 → Arg (1×), Pro_4 → Arg (1×), and Pro_{165} → Arg (4×). All variant carriers were heterozygous for their mutant of apo A-I.

Subjects heterozygous for apo A-I(Pro_{165} → Arg) (n = 12) were found to exhibit lower mean values for apo A-I (109±16 mg/dl) and HDL cholesterol (37±9 mg/dl) than unaffected family members (n = 9): 176±41 and 64±18 mg/dl, respectively ($P < 0.001$). In 9 of 12 apo A-I(Pro_{165} → Arg) variant carriers the concentrations of apo A-I were below the fifth percentile of sex-matched controls. By two-dimensional immunoelectrophoresis as well as by densitometry the relative concentration of the variant apo A-I in heterozygous carriers of apo A-I(Pro_{165} → Arg) was determined to account for only 30% of the total plasma apo A-I mass instead of the expected 50%. Thus, the observed apo A-I deficiency may be largely a consequence of the decreased concentration of the variant apo A-I.

In the case of the apo A-I(Pro_3 → Arg) mutant, densitometry of HDL apolipoproteins demonstrated a distinctly increased concentration of the variant proapo A-I relative to normal proapo A-I. This phenomenon was not observed in the apo A-I(Pro_4 → Arg) mutant or in other mutants. This suggests that the interspecies conserved proline residue in position 3 of mature apo A-I is functionally important for the regular enzymatic conversion of proapo A-I to mature apo A-I.

Introduction

Apo A-I, in its mature form a polypeptide of 243 amino acids of known sequence (1, 2), is the major protein component of plasma HDL. It is secreted as proapo A-I by hepatic and intestinal cells (3, 4) with an aminoterminal hexapeptide that is cleaved extracellularly by a propeptidase (5, 6). Neither this proapo A-I-propeptidase nor its recognition site in proapo A-I have been identified to date. Analysis of synthetic peptides (7–10) and structural variants of apo A-I (11, 12) revealed that amino acid residues that are located between positions 99 and 186 and are supposed to form repetitive amphipathic alpha-helices (13) mediate biological functions of apo A-I: solubilization of lipids in an aqueous environment (14), and activation of the cholesterol esterifying enzyme lecithin:cholesterol acyltransferase (15). In vitro HDL as well as apo A-I containing proteoliposomes have been demonstrated to bind specifically to various cells and promote cholesterol efflux (16–19). Because of its in vitro functions apo A-I is supposed to be a key protein in reverse cholesterol transport (20), by which excess cholesterol is transported from peripheral cells to the liver for final deposition into the biliary tract. Results from epidemiological studies and clinical data have supported this hypothesis; decreased levels of HDL cholesterol (21) as well as low levels (22) or absence of apo A-I (23, 24) have been found associated with premature atherosclerosis. Previously, genetically determined structural variants of apo A-I have been identified by electrophoretic screening. They were informative to characterize structure–function relationships and to better understand the role of apo A-I in the pathomechanism leading to dyslipidemia or atherosclerosis (11, 12, 25–29).

Recently our laboratory reported a simple and rapid method for the structural analysis of mutant apolipoproteins. This method combines the isolation of variant isoproteins from plasma or serum by isoelectric focusing in immobilized pH gradients, separation of proteolytic peptides by reversed phase HPLC and molecular weight determination by time-of-flight secondary ion mass spectrometry (TOF-SIMS; 28).[1] This technique allows the precise identification of amino acid substitutions predicted from mass analysis without sequencing the whole variant protein. Using this procedure we analyzed the amino acid substitutions in the structural variants of apo A-I, which were found in 16 families by an electrophoretic mutation screening of 32,000 newborns. Here we report three different mutants assessed in six unrelated families in which proline residues were substituted by arginine. Because proline has great impact on the folding of proteins (30), its substitution could alter the secondary and tertiary structure of the protein. Conformational changes should influence structure–function relationships and thus could have impact on the metabolism of apo A-I. We further studied the in vivo effects of the variant protein by comparing the relative concentrations of the normal and the variant apo A-I isoproteins in individuals heterozygous for the variants.

Address correspondence to Prof. Dr. med. G. Assmann, Institut für Klinische Chemie und Laboratoriumsmedizin, Zentrallaboratorium, Westfälische Wilhelms-Universität Münster, Albert-Schweitzer-Strasse 33, D-4400 Münster, FRG.

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1. *Abbreviations used in this paper:* LPDS, lipoprotein-depleted serum; PROCAM, Prospective Cardiovascular Münster; TOF-SIMS, time-of-flight secondary ion mass spectrometry.

Methods

Subjects. 6 unrelated families with genetic variants of apo A-I reported here were found together with 10 other variants in a mutation screening program of 32,000 dried blood samples of newborns (von Eckardstein, A., T. Sgraja, and K. Altland, unpublished observations). For the structural analysis of the mutant proteins the parental carriers of the variants, who were determined in the course of the screening, were asked for a donation of 100 ml of blood. Family studies were performed in three families in which an apo A-I(Pro_{165} → Arg) mutation was vertically transmitted. For this purpose, depending on the age of the investigated subjects, 10–50 ml EDTA blood were taken. A fourth family (Jo), which was affected by the same variation, did not agree to participate in a family study. Control populations for comparing plasma concentrations of lipids and apo were taken from the Prospective Cardiovascular Münster (PROCAM) study (31).

Quantitative analyses. Serum concentrations of triglycerides and cholesterol were determined with an autoanalyzer (Hitachi/Boehringer, Mannheim, FRG). HDL cholesterol was determined after precipitation with phosphotungstic acid (Boehringer Mannheim GmbH, Mannheim, FRG). Plasma concentrations of apo A-I were determined with a commercial turbidimetric assay (32; Boehringer Mannheim GmbH).

Analytical electrophoretic procedures. Separation of apo A-I isoproteins from plasma, lipoprotein fractions, or lipoprotein-depleted serum (LPDS) was done by isoelectric focusing in two different ways. A highly resolving demonstration of apo A-I isoproteins was performed by double one-dimensional electrophoresis as described by Altland and co-workers (33). PAGE was followed by isoelectric focusing in gels with immobilized pH gradients that were rehydrated with carrier ampholytes. Alternatively, for two-dimensional immunoelectrophoresis (34) or immunoblotting (35) samples were preseparated by a one-step procedure as described by Menzel and co-workers (34). Plasma samples were preincubated as described by Altland et al. (33) or Menzel et al. (34). VLDL, LDL, HDL, and LPDS (lipoprotein-free infranatant after ultracentrifugation at $d = 1.21$ kg/liter) were prepared by sequential ultracentrifugation (36). The fractions were dialyzed against 50 mM EDTA. After delipidation of 1 ml lipoprotein emulsion with ethanol/ether (3:1, vol/vol) (37) the precipitated apo were solubilized in 50 μl of a buffer containing 1% decylsulfate (wt/wt; Eastman Kodak Co., Rochester, NY), 20% glycerol (wt/wt), 0.01 M Tris HCl (pH 8.2), and 2% carrier ampholytes (wt/wt). 50 μl LPDS were preincubated either with 50 μl of a solution containing 0.4% SDS (wt/wt) and 20% glycerol (wt/wt) for the double one-dimensional electrophoresis or, for the one-step procedure, with 50 μl of a buffer containing 10% decylsulfate (wt/wt), 20% glycerol (wt/wt), Tris HCl (pH 8.2), and 2% carrier ampholytes (wt/wt). Sample volumes applied to one of the two alternative electrophoretic procedures were 5 μl for plasma, 50 μl for apo VLDL or apo LDL, 2 μl for apo HDL, and 100 μl for LPDS.

Estimation of relative concentrations of apo A-I isoproteins. The quantitative distribution of the isoproteins of apo A-I in plasma, VLDL, LDL, HDL, and LPDS was analyzed by two-dimensional immunoelectrophoresis (34) or by densitometry of Coomassie blue–stained gels subsequent to double one-dimensional electrophoresis. Scanning densitometry was performed with Ultrascan (LKB, Bromma, Sweden). Peak areas reflecting the intensity of the bands and thereby the concentration of demonstrated isoproteins were integrated automatically with SP 4270 (Spectra-Physics Inc., Mountain View, CA).

Preparation of isoproteins of apo A-I. Apo HDL isolated from 20 ml plasma by sequential ultracentrifugation (36) and delipidation (37) were solubilized in a buffer containing 5 mM sodium dihydrogen phosphate, 1% decylsulfate (wt/wt), 100 mM DTT, and 6 M urea (pH 8.0). Isoproteins of apo A-I were separated by isoelectric focusing in gels with immobilized pH gradients (Pharmacia-LKB, Bromma, Sweden). 5-mm-thick gels with pH gradients ranging from 5 to 6 were casted following the producer's recommendations (38); however, using only one-third of the recommended concentration of immobilines (33). The washed gels were rehydrated with 6 M urea and 15% glycerol (wt/vol). Separation of solubilized apo HDL was performed overnight at 2,000 V, 15 mA, and 5 W and for 4 more h at 3,000 V. The apo A-I isoforms were seen in the unstained gels as opalescent lines. Gel strips containing the variant isoproteins were cut out of the gel without prior staining. After electroelution for 24 h in a 0.05 M Tris HCl buffer (pH 9.0) the proteins were dialyzed against 0.01 M ammonium hydrogen carbonate (pH 7.8) for 24 h. Subsequent lyophilization and solubilization in 0.01 M ammonium hydrogen carbonate (pH 7.8) was followed by purification by reversed phase HPLC. A C18 column with a length of 250 mm and a diameter of 4 mm (E. Merck, Darmstadt, FRG) was run with a gradient from 0% acetonitrile in 0.1% trifluoroacetic acid (eluent A) to 100% acetonitrile without trifluoroacetic acid (eluent B). The flow rate was 1 ml/min. The acetonitrile content of the gradient was increased to 30% within the first 10 min, 45% within the next 15 min, and 100% within the last 10 min.

Proteolytic digestion of isoproteins. Lyophilized apo A-I isoproteins were solubilized in 0.01 M ammonium hydrogen carbonate (pH 7.8) and digested with trypsin (activity, 212 U/mg protein; Cooper Biomedical, Wiesbaden, FRG) at a ratio of 40:1 (wt/wt). The solution was incubated for 24 h at 37°C.

HPLC separation of proteolytic peptides. 500 μg of the proteolytic digest were separated by reversed phase HPLC using the LiChrospher CH18 HPLC column, the eluents, and the flow described above. The acetonitrile content of the gradient was increased to 40% within 55 min and to 100% in the following 2 min. A peak detector monitoring at 215 nm and a fraction collector (Pharmacia-LKB) were used to collect the peptides separately.

Characterization of proteolytic peptides by mass spectrometry and sequence analysis. HPLC fractions whose retention times or relative peak areas were different from those of normal apo A-I were analyzed by TOF-SIMS (28). Mass differences between peptides of normal apo A-I that were lost from the chromatogram, and peptides of variant apo A-I that were newly generated, were used to calculate possible amino acid substitutions. Predicted amino acid substitutions were confirmed by sequence analysis of the affected tryptic peptides. Sequence analysis was performed by automated gas phase protein sequencing (Applied Biosystems, Foster City, CA).

Results

Description of the investigated subjects. Six families with three different electrophoretic variants of apo A-I were found by a mutation screening program of newborns (n = 32,000; von Eckardstein, A., T. Sgraja, and K. Altland, unpublished observations). The apo A-I mutants were identified by isoelectric focusing by the presence of additional protein bands with distinct charge differences in comparison with normal apo A-I. It was proven by two-dimensional immunoelectrophoresis and by immunoblotting (data not shown) that the additional bands represented apo A-I. All investigated families were unrelated. The affected subjects were heterozygous for the variant apo A-I. Pedigrees of three families with an apo A-I(Pro_{165} → Arg) variant are presented in Fig. 1. Vertical transmission of the variant apo A-I was observed in all families.

Lipid values and apolipoprotein concentrations of all investigated subjects are given in Table I. Carriers of the apo A-I(Pro_{165} → Arg) variant exhibited significantly lower levels of HDL cholesterol (37±9 vs. 64±18 mg/dl, $P < 0.001$, two-tailed t test) and apo A-I (109±16 vs. 176±41 mg/dl, $P < 0.001$, two-tailed t test) than the unaffected members (see Table II). In families Ke (Fig. 1 *B*) and Ler (Fig. 1 *C*) the variant carriers exhibited apo A-I concentrations below the fifth percentile of PROCAM controls. In family Leh (Fig. 1 *A*) the variant carriers in generations I and III exhibited apo A-I

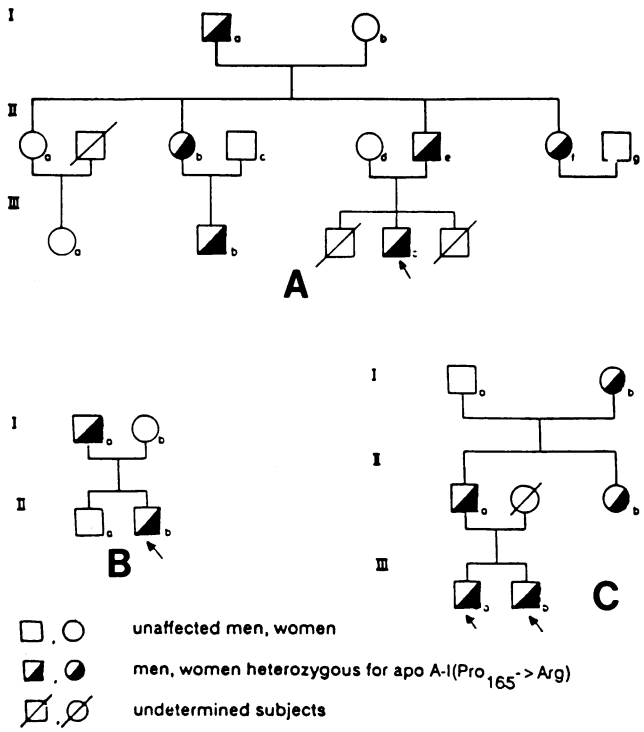


Figure 1. Pedigrees of three kindreds affected by apo A-I(Pro_{165} → Arg). *A*, Family Leh; *B*, family Ke; *C*, family Ler. The enumeration refers to Table I. Propositi marked with arrows were found in the original screening. In family Ler (*C*) only dried blood samples were provided to verify the presence of the variant apo A-I.

concentrations around the fifth percentile, whereas variant carriers of generation II exhibited higher apo A-I values, which, however, were lower than those of the unaffected individuals of the same generation.

In subject M. Gu, who was affected with an apo A-I (Pro_3 → Arg) variant, the plasma concentration of apo A-I was found above the 95th percentile.

Relative concentrations of normal and variant apo A-I isoproteins. The relative concentrations of normal and variant apo A-I isoproteins were investigated in whole plasma, apo VLDL, apo LDL, apo HDL, and LPDS by two-dimensional immunoelectrophoresis (Fig. 2). Apo A-I was undetectable in apo LDL of all investigated subjects. In plasma, apo VLDL, apo HDL, and LPDS of subjects Wi and Ler the peaks of normal and variant apo A-I were equal in altitude. However, in variant carriers of families Ler, Leh, Ke, and Jo total plasma, apo VLDL, and apo HDL shared distinctly smaller peak areas for the variant isoform than for the normal isoform. However, in LPDS of these probands both isoforms were found in equal concentrations. To exclude the possibility that reduced peak areas were due to changes in antigenicity of the variant apo A-I, apo A-I isoforms from all fractions were separated by double one-dimensional electrophoresis and analyzed by scanning densitometry of the stained bands in triplicate gels. Results of this analysis were confirmatory for those obtained with two-dimensional immunoelectrophoresis. In subjects M. Wi and M. Gu the ratios of variant to normal apo A-I were near one. In variant carriers of families Ler, Leh, Ke, and Jo the ratios of normal to variant apo A-I in total plasma, apo VLDL, or apo HDL were found near two, and they were found close to one in LPDS.

By isoelectric focusing and subsequent immunoblotting of total plasma from subject M. Gu the concentration of the variant proapo A-I appeared markedly higher than that of the normal proapo A-I. Relative concentrations of proapo A-I were found to be normal in all other mutants. This observation was validated by quantitative analysis. Fig. 3 shows the electrophoretic pattern and the corresponding densitogram of apo A-I isoforms separated from apo HDL of subject M. Gu. Concentrations of normal and variant mature apo A-I were similar. The ratio of variant to normal proapo A-I was approximately three. In heterozygous carriers of other variants this ratio was one. In apo VLDL and in LPDS proapo A-I concentrations were too low for reliable analysis.

Analysis of structural variations in the apo A-I variants. Fig. 4 shows the HPLC chromatogram of tryptic peptides and their location in the amino acid sequence of normal human apo A-I (2). Peptides were identified by the combination of molecular weight determination with TOF-SIMS and automated gas phase sequence analysis. HPLC-chromatograms obtained by separating the proteolytic peptides of variant proteins were compared with those from normal apo A-I. Fig. 5 shows the chromatogram of the variant apo A-I from a variant carrier of family Leh. The fraction containing peptide T26 was markedly reduced and two fractions containing peptides $T26_{x1}$ and $T26_{x2}$ were newly generated. The molecular masses of these peptides were 596 ($T26_{x1}$) and 781 D ($T26_{x2}$), respectively (Fig. 6). Assuming a single base change as the underlying defect, the replacement of proline at position 165 by arginine is the only amino acid substitution within T26, which can explain both an electrophoretic charge difference of +1 and the formation of two tryptic peptides with molecular masses of 596 and 781 D. $T26_{x1}$ consisted of Thr-His-Leu-Ala-Arg as shown by sequence analysis. In normal apo A-I the unique sequence Thr-His-Leu-Ala (residues 160 → 164) is followed by a proline residue in position 165. The predicted substitution Pro_{165} → Arg was thus verified. Comparable HPLC chromatograms, which were characterized by a loss of T26 and the generation of two peaks containing peptides with molecular masses of 596 and 781 D were seen in tryptic digests from the variant apo A-I of variant carriers of families Ler, Ke, and Jo. In all these cases the predicted substitution of proline at position 165 by arginine was verified by sequence analysis of $T26_{x1}$.

Fig. 7 shows the HPLC chromatograms of tryptic peptides obtained from variant apo A-I of subjects M. Wi and M. Gu. Both chromatograms differed from that obtained from normal apo A-I by the loss of the peak containing T1. The mutation underlying the variant apo A-I from subject M. Wi (Fig. 7 *a*) resulted in the generation of a new fraction containing a peptide with a molecular mass of 788 D. This molecular mass can be explained by a substitution of proline in position 4 by arginine. Substitution of proline by arginine in position 4 generates a new tryptic cleavage site producing two peptides, $T1_{x1}$ and $T1_{x2}$, with molecular masses of 515 and 788 D, respectively. Peptide $T1_{x1}$ with a mass of 515 D containing the substituted amino acid was not detected. To further confirm this finding the 10 aminoterminal amino acids of the variant apo A-I were sequenced. This analysis revealed Asp-Glu-Pro-Arg-Gln-Ser-Pro-Trp-Asp-Arg, thus verifying the predicted substitution Pro_4 → Arg. In the HPLC chromatogram obtained from subject M. Gu (Fig. 7 *b*) a fraction containing a peptide with a molecular mass of 1,284 D was newly generated. This

Table I. Lipid Values and Apo Concentrations from Subjects of Kindreds Affected with Apo A-I Variants

Subjects	Sex/age	Mutant apo A-I	Triglycerides	Cholesterol	HDL cholesterol*	LDL cholesterol	Apo A-I*
					mg/dl		
Family Leh							
Ia	**M/63**	**Pro_{165} → Arg**	**266**	**210**	**35 (<20)**	**122**	**100 (<5)**
Ib	F/63	—	109	276	80 (<95)	174	245 (>95)
IIa	F/38	—	174	247	79 (<95)	133	219 (>95)
IIb	**F/36**	**Pro_{165} → Arg**	**179**	**204**	**37 (<10)**	**131**	**128 (<25)**
IIc	M/41	—	47	141	70 (>95)	61	166 (<90)
IId	F/30	—	101	165	55 (<50)	90	142 (<45)
IIe	**M/33**	**Pro_{165} → Arg**	**102**	**129**	**47 (<65)**	**62**	**119 (<15)**
IIf	**F/32**	**Pro_{165} → Arg**	**73**	**169**	**53 (<45)**	**101**	**134 (<30)**
IIg	M/37	—	125	204	58 (<90)	121	179 (<95)
IIIa	F/11	—	56	168	67 (<80)	90	152 (<65)
IIIb	**M/5**	**Pro_{165} → Arg**	**108**	**156**	**42 (<45)**	**102**	**107 (<5)**
IIIb	**M/11**	**Pro_{165} → Arg**	**68**	**136**	**35 (<20)**	**67**	**104 (<5)**
Family Ke							
Ia (1988)	**M/36**	**Pro_{165} → Arg**	**101**	**169**	**36 (<25)**	**113**	**112 (<10)**
(1989)			**253**	**190**	**30 (<10)**	**110**	**107 (<5)**
Ib	F/34	—	128	190	89 (>95)	75	211 (>95)
IIa	M/9	—	270	218	46 (<60)	118	150 (<70)
IIb	**M/5**	**Pro_{165} → Arg**	**155**	**170**	**34 (<15)**	**105**	**104 (<5)**
Family Ler							
Ia	M/65	—	229	256	31 (<10)	179	118 (<20)
Ib	**F/65**	**Pro_{165} → Arg**	**167**	**198**	**27 (<5)**	**165**	**95 (<5)**
IIa	**M/40**	**Pro_{165} → Arg**	**329**	**222**	**27 (<5)**	**129**	**91 (<5)**
IIb	**F/35**	**Pro_{165} → Arg**	**134**	**187**	**39 (<10)**	**121**	**109 (<5)**
P.Jo	**F/35**	**Pro_{165} → Arg**	**186**	**176**	**35 (<5)**	**104**	**102 (<5)**
M.Wi	**F/32**	**Pro_4 → Arg**	**62**	**208**	**58 (<60)**	**138**	**124 (<20)**
M.Gu	**F/27**	**Pro_3 → Arg**	**97**	**205**	**79 (<95)**	**106**	**210 (>95)**

Values of variant carriers are printed in boldface. The enumeration refers to Fig. 1. * Values in parentheses refer to percentiles of sex-matched controls from the PROCAM study (31).

molecular mass theoretically could be explained by substitutions Pro_3 → Arg, Pro_4 → Arg, or Pro_7 → Arg, anticipating that the expected new tryptic cleavage site in the variant apo A-I was not effective. Sequence analysis of $T1_x$ revealed a sequence Asp-Glu-Arg-Pro-Gln-Ser-Pro-Trp-Asp-Arg, thus verifying a substitution of proline by arginine at residue 3.

Discussion

Six mutants of apo A-I described in this report were detected because of their distinct electrophoretic charge difference compared with normal apo A-I. Structural analysis revealed that they differed from normal apo A-I by substitutions of proline residues by arginine. Three different phenotypes were characterized. Apo A-I(Pro_4 → Arg) in proband M. Wi is the second case described (26). Apo A-I(Pro_3 → Arg) in proband M. Gu and apo A-I(Pro_{165} → Arg) in families Ler, Leh, Jo, and Ke represent novel findings.

Apo A-I(Pro_{165} → Arg). This amino acid substitution was found in four unrelated families by screening of 32,000 samples from newborns, corresponding to a mutant frequency of 1:8,000 in the German population. Family studies helped to identify 12 heterozygous carriers of the apo A-I(Pro_{165} → Arg) variant and revealed that this variant apo A-I is associated with mean plasma levels of apo A-I below the 10th percentile of PROCAM controls and decreased concentrations of HDL

Table II. Mean Lipid Values and Apo Concentrations of Members from Four Kindreds with the Apo A-I (Pro_{165} → Arg) Variant

Phenotype	Triglycerides*	Cholesterol‡	HDL Cholesterol§	LDL Cholesterol‖	Apo A-I¶
			mg/dl		
Normals (n = 9)	138±75	207±49	64±18	116±41	176±41
Apo A-I (Pro_{165} → Arg) Heterozygotes (n = 12)	156±78	177±29	37±9	110±25	109±16

P was calculated by two-tailed t test. * P = NS; ‡ $P < 0.05$; § $P < 0.001$; ‖ P = NS; ¶ $P < 0.001$.

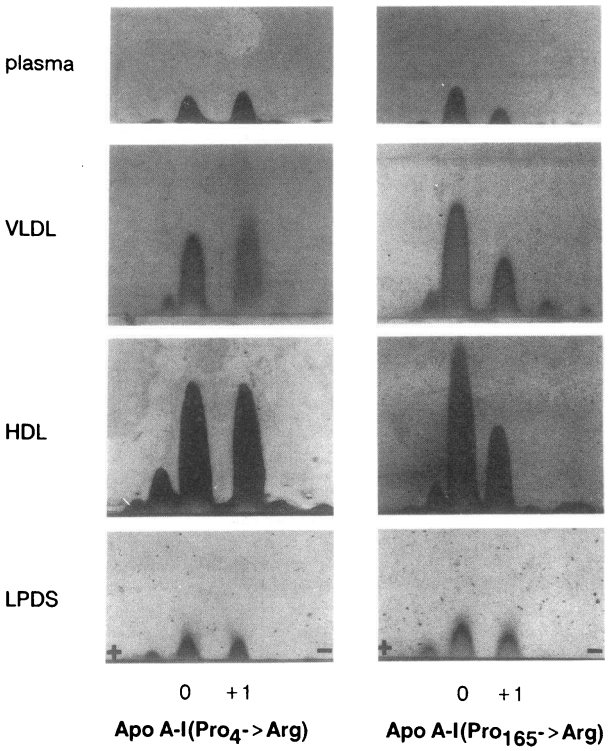


Figure 2. Separation of samples from subject M. Wi with apo A-I(Pro_4 → Arg) (*left*) and subject IIa of family Ler with apo A-I(Pro_{165} → Arg) (*right*) from total plasma, VLDL, HDL, and LPDS by two-dimensional immunoelectrophoresis. The position of normal apo A-I is marked by 0, the position of the variant by +1. Note that the concentration of apo A-I(Pro_{165} → Arg) is relatively diminished in plasma, VLDL, and HDL. The make-believe also decreased concentration of the apo A-I(Pro_{165} → Arg) in LPDS due to the fact that the anodic band of the variant apo A-I (apo $A\text{-}I_2$) is projected onto the cathodic band of normal apo A-I (apo $A\text{-}I_1$). By densitometry of apo A-I separated by isoelectric focusing in a highly resolving immobilized pH gradient the concentrations of variant and normal apo A-I were found equal in LPDS.

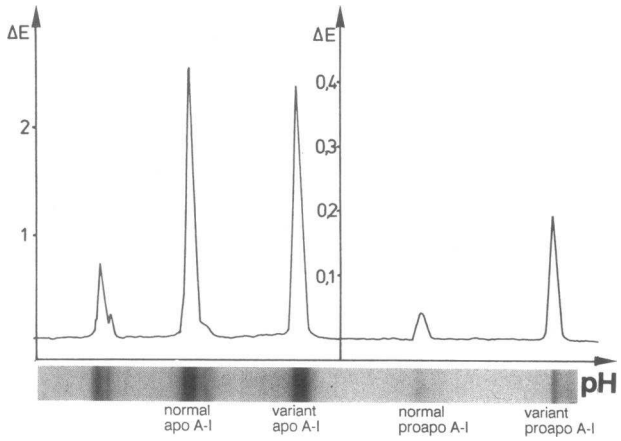
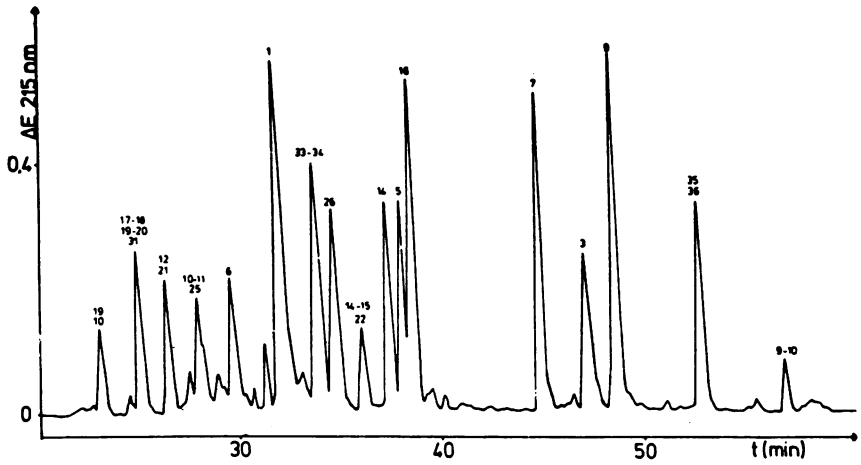


Figure 3. Separation of HDL apo by double one-dimensional electrophoresis in the sequence PAGE → isoelectric focusing in an immobilized pH gradient pH 4-7 and densitometry of apo A-I isoproteins from subject Gu heterozygous for apo A-I(Pro_3 → Arg). The concentrations of normal and variant apo A-I were almost equal, while the concentration of proapo A-I(Pro_3 → Arg) was relatively increased.

cholesterol compared with unaffected family members as well as normal controls from the PROCAM study. 9 of 12 cases represented apo A-I concentrations below the fifth percentiles of sex- and age-matched controls. Normal apo A-I values were only found in the variant carriers belonging to generation II of family Leh. This might be related to an HDL cholesterol-increasing gene effect transmitted by the mother of these subjects. In summary, besides apo A-I(Milano), in which an arginine in position 173 is replaced by a cysteine (25, 39), apo A-I(Pro_{165} → Arg) is the second case of an apo A-I variant, which has been shown to quantitatively affect the plasma concentrations of apo A-I and HDL cholesterol.

In general, the concentration of the variant protein was found diminished in total plasma, isolated VLDL, and isolated HDL, but not in LPDS of affected subjects. Detailed electrophoretic and immunochemical analysis showed a reduction of the mutant isoform in the plasma of these probands to ~ 50% of the normal isoform's concentration. It thus appears that the observed apo A-I deficiency in carriers of apo A-I(Pro_{165} → Arg) can largely be attributed to the diminished concentration of this variant. At present it remains unclear whether the observed reduction of plasma apo A-I associated with this variant is related to alterations in its anabolism or its catabolism. The Pro_{165} → Arg substitution affects a putative beta turn between two neighboring helices of the alpha-helical domain (13). In a similar variant, apo A-I(Pro_{143} → Arg), such a localization has been proposed to be responsible for the diminished lipid binding and lecithin:cholesterol acyltransferase cofactor activity of this variant (12). Application of the Chou-Fasman algorithm (40–42) reveals that the Pro_{165} → Arg substitution lowers the beta turn probability of the tetrapeptide Ala-Pro-Tyr-Ser (residues 164 → 167). Therefore it is reasonable to speculate that this substitution may result in an altered conformation of the protein's alpha-helical domain. Although Chou and Fasman defined the cutoff for the prediction of a beta turn at 0.5×10^{-4}, the dramatic lowering of the beta turn probability by a factor of almost 3, from 2.18×10^{-4} to 0.77×10^{-4}, to a value near the cutoff suggests that an effect of this mutation on the stability of the protein's conformation is possible.

Apo A-I(Pro_3 → Arg) and apo A-I(Pro_4 → Arg). The relative concentration of the variant proapo A-I was shown to be distinctly increased compared with normal proapo A-I in an individual heterozygous for apo A-I(Pro_3 → Arg), whereas in a person heterozygous for apo A-I(Pro_4 → Arg) this phenomenon was not observed. In a further subject heterozygous for apo A-I(Pro_3 → His) (26) the variant proform was also found overrepresented (unpublished observations). A relative enrichment of the proapo A-I in plasma is characteristic for Tangier disease, a heritable disorder of lipoprotein metabolism with extremely low plasma concentrations of HDL, apo A-I, and apo A-II (43, 44). In this condition the overrepresentation of proapo A-I is probably due to hypercatabolism of HDL and apo A-I (45, 46). In proband M. Gu heterozygous for apo A-I(Pro_3 → Arg) the products of both the normal and the mutant allele were nearly equal in plasma concentration. Total plasma concentrations of apo A-I and HDL cholesterol in M. Gu were not reduced. Therefore accelerated catabolism probably does not account for the enrichment of proapo A-I in this subject. Rather, proapo A-I(Pro_3 → Arg) and proapo A-I (Pro_3 → His) are apparently less efficiently converted into the mature plasma protein compared with normal proapo A-I and proapo A-I(Pro_4 → Arg). Probably, therefore, proline at posi-



a

T 1 (1226) Asp–Glu–Pro–Pro–Gln–Ser–Pro–Trp–Asp–Arg | Val–Lys | T 3 (1235) Asp–Leu–Ala–Thr–Val–Tyr–Val–Asp–
T 4 (433) Val–Leu–Lys | Asp–Ser–Gly–Arg | T 5 (1400) Asp–Tyr–Val–Ser–Gln–Phe–Glu–Gly–Ser–Ala–Leu–Gly–Lys
T 6 (614) Gln–Leu–Asn–Leu–Lys | T 7 (1612) Leu–Leu–Asp–Asn–Trp–Asp–Ser–Val–Thr–Ser–Thr–Phe–Ser–Lys | Leu–
T 9 (1932) Arg | Glu–Gln–Leu–Gly–Pro–Val–Thr–Gln–Glu–Phe–Trp–Asp–Asn–Leu–Glu–Lys | T 10 (703) Glu–Thr–Glu–
T 11 (621) Gly–Leu–Arg | Gln–Glu–Met–Ser–Lys | T 12 (731) Asp–Leu–Glu–Glu–Val–Lys | Ala–Lys | T 14 (1252) Val–Gln–Pro–Tyr–
T 16 (1283) Leu–Asp–Asp–Phe–Gln–Lys | Lys | Trp–Gln–Glu–Glu–Met–Glu–Leu–Tyr–Arg | Gln–Lys | T 18 (612) Val–Glu–
T 19 (872) Pro–Leu–Arg | Ala–Glu–Leu–Gln–Glu–Gly–Ala–Arg | Gln–Lys | T 21 (895) Leu–His–Glu–Leu–Gln–Glu–Lys
T 22 (1031) Leu–Ser–Pro–Leu–Gly–Glu–Glu–Met–Arg | Asp–Arg | Ala–Arg | T 25 (780) Ala–His–Val–Asp–Ala–Leu–Arg
T 26 (1301) Thr–His–Leu–Ala–Pro–Tyr–Ser–Asp–Glu–Leu–Arg | Gln–Arg | T 28 (429) Leu–Ala–Ala–Arg | T 29 (572) Leu–Glu–Ala–
T 30 (602) Leu–Lys | Glu–Asn–Gly–Gly–Ala–Arg | T 31 (830) Leu–Ala–Glu–Tyr–His–Ala–Lys | T 32 (1215) Ala–Thr–Glu–His–Leu–
T 34 (812) Ser–Thr–Leu–Ser–Glu–Lys | Ala–Lys | Pro–Ala–Leu–Glu–Asp–Leu–Arg | T 35 (1230) Gln–Gly–Leu–Leu–Pro–
T 36 (1386) Val–Leu–Glu–Ser–Phe–Lys | Val–Ser–Phe–Leu–Ser–Ala–Leu–Glu–Glu–Tyr–Thr–Lys | Lys | T 38 Leu–
(474) Asn–Thr–Gln

b

Figure 4. a, HPLC chromatograms of normal apo A-I obtained by reversed phase HPLC of its tryptic peptides digested with trypsin in a ratio of 40:1 (wt/wt) for 24 h at 37°C. Proteolytic peptides were eluted with a gradient from 0 to 45% acetonitrile within 55 min. Numbers at the top of the peaks indicate sequence positions of tryptic peptides. *b,* Alignment of tryptic peptides to the sequence of apo A-I (2). Numbers and molecular masses (in parentheses) of the tryptic peptides are indicated.

tion 3 is essential in the regular propeptide cleavage process. According to the rules described by Chou and Fasman for the prediction of secondary structures (40–42) the prosegment of normal apo A-I consists of an alpha helix. Subsequent proline residues at positions 3 or 4 of mature apo A-I can form a beta turn that may fulfill the structural requirement for the formation of a recognition site for the proapo A-I-propeptidase. As proline residues mostly occupy the second position in a tetrapeptide forming a beta turn, in the case of proapo A-I the two neighboring proline residues in positions 3 and 4 give rise to two possible formations of a beta turn (Table III). Application of the Chou-Fasman algorithm (40–42) reveals that the beta turn probability is higher for the tetrapeptide Pro-Pro-Gln-Ser (residues 3 → 6 of apo A-I) than for the tetrapeptide Glu-Pro-Pro-Gln (residues 2 → 5). However, replacement of proline in position 3 by arginine or histidine, but not of proline in position 4 by arginine apparently interferes with the formation of a beta turn at the aminoterminal end of apo A-I (Table III). For this reason, and because in several species including pig (47), dog (48), rat (49), rabbit (50), and chicken (51, 52) only one of the two prolines (position 3) is present in an otherwise highly conserved region, it appears more likely that a beta turn is formed by the proline immediately after the glutamic acid residue.

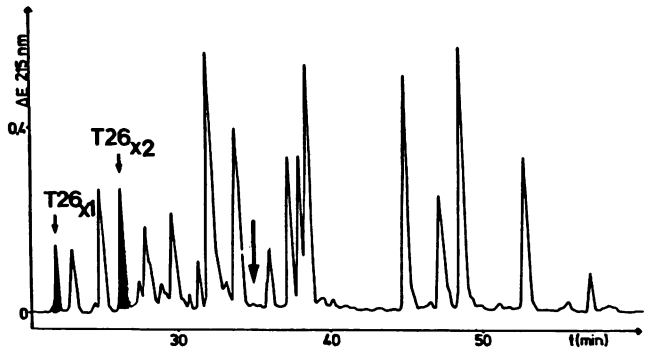


Figure 5. HPLC chromatogram of tryptic peptides obtained from the mutant apo A-I from a variant carrier of family Leh (Ile). Peptide T26 was lost. The hatched fractions $T26_{x1}$ and $T26_{x2}$ were newly generated.

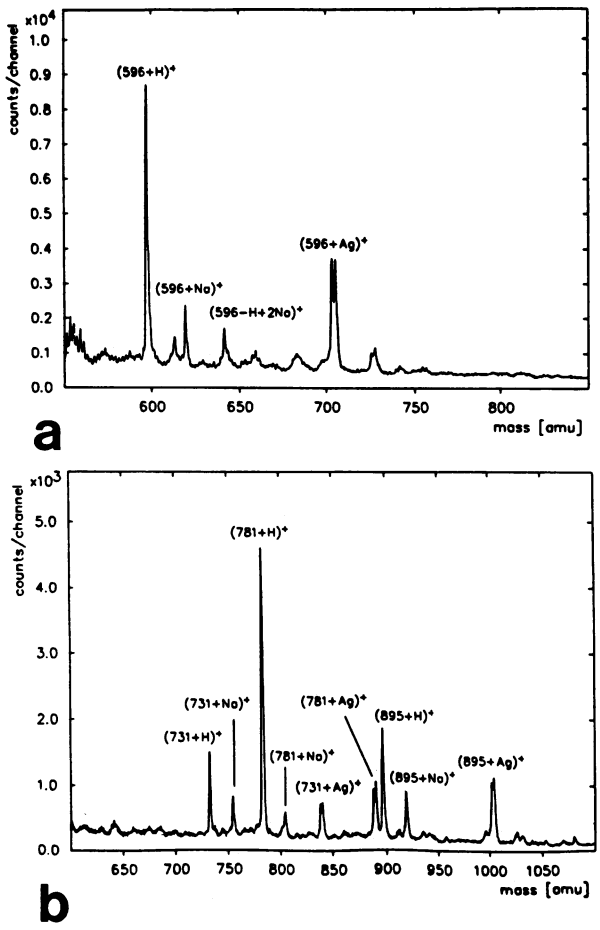


Figure 6. Molecular weight analysis of peptides $T26_{x1}$ (*a*) and $T26_{x2}$ (*b*) by TOF-SIMS. Molecular ion region in the positive secondary ion spectrum obtained from 1 nmol of tryptic peptides deposited on a 100-mm² silver target. Primary ion bombardement: 2.5×10^{-10} A, 12 keV in 60 s. The masses of peptides $T26_x$ are indicated. The additional masses of 731 and 895 D in *b* represent peptides T12 and T21, respectively, which were coeluted with $T26_{x2}$.

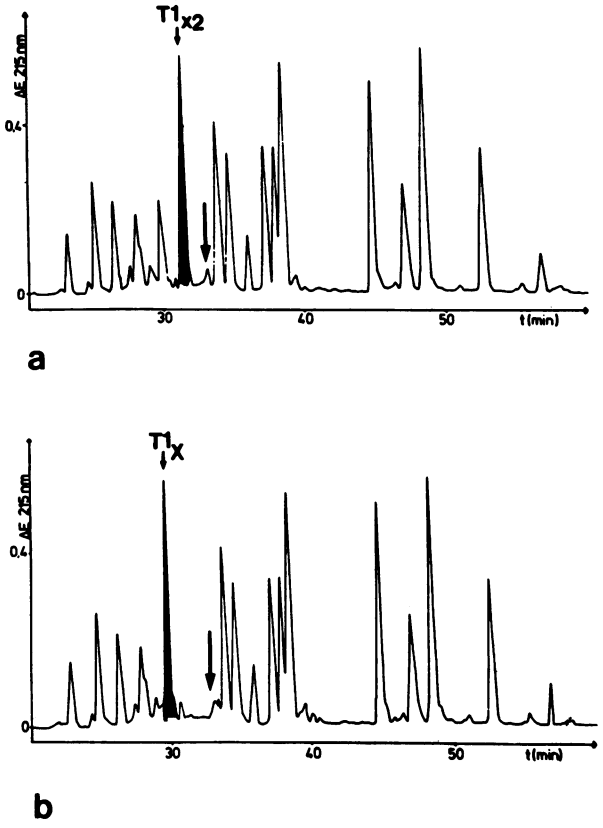


Figure 7. HPLC chromatograms of tryptic peptides of apo A-I variants obtained from subjects M. Wi (*a*) and M. Gu (*b*). Peptide T1 was lost in both chromatograms. Hatched peaks $T1_{x2}$ (*a*) containing a peptide with a molecular mass of 788 D, and $T1_x$ (*b*) containing a peptide with a molecular mass of 1,284 D were newly generated.

Overrepresentation of proline substitutions in apo A-I variants. The present study and two previous reports (12, 26) led to the identification of five apo A-I mutants involving proline residues. In each case the proline in the wild type sequence was replaced by another amino acid; four times proline was substituted by arginine and once it was changed to histidine. In apo A-I no switch of any amino acid to proline has been reported to date, although based on codon frequencies in the apo A-I gene one should expect as many mutations in the reverse direction. Barker and co-workers identified CpG dinucleotides as hotspots for cytosine to thymidine transitions (53). All possible mutations causing changes between proline and histidine or arginine are transversions. Interestingly, four of the five proline mutations observed in apo A-I on the DNA level occur within a stretch of at least five cytosine residues. Future analysis will show whether such areas can favor the formation of transversions. Apo A-I mutants reported so far were detected by isoelectric focusing in carrier ampholytes containing pH gradients under denaturing conditions. This method has a high sensitivity for the detection of mutants that result from the loss or gain of complete net charges. Although this limited sensitivity does not bias towards the significant overrepresentation of proline substitutions in apo A-I, a more detailed analysis of point mutations occurring in certain codons requires screening techniques, which are also sensitive for electrically neutral amino acid substitutions.

Neither the crystallographic structure of apo A-I nor the functional characteristics of the propeptidase are known today. However, as shown in this study, the combined use of secondary structure predictions, interspecies comparisons, and experimental data derived from naturally occurring structural variants may yield information on structure–function requirements in the processing of apo A-I. At present it is not known whether the apo abnormalities reported here put their carriers

Table III. Influence of Amino Acid Substitutions on Beta Turn Probabilities of Tetrapeptides Possibly Forming Aminoterminal Beta Turn Structures in Proapo A-I

Sequence of the tetrapeptide	Beta-turn probabilities ($\times 10^{-4}$)*			
	Normal	$Pro_3 \rightarrow Arg$	$Pro_3 \rightarrow His$	$Pro_4 \rightarrow Arg$
Glu_2-**Pro_3**-Pro_4-Gln_5	0.56	0.20	0.09	1.64
Pro_3-**Pro_4**-Gln_5-Ser_6	1.20	0.87	1.69	0.42

Beta turn–causing proline residues are printed in boldface. * Beta turn probabilities were calculated as described by Fasman (42). Cut-off was given by Fasman with 0.5×10^{-4}.

at increased risk for coronary heart disease. Long-term evaluation of the probands' health and family studies will help to gain further insight into the clinical relevance of these apolipoproteinopathies.

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