Identification and properties of the proline$_{664}$-leucine mutant LDL receptor in South Africans of Indian origin

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Abstract The incidence of familial hypercholesterolemia (FH) is high among South African Indians. The proline$_{664}$-leucine low density lipoprotein (LDL)-receptor mutation was detected in four apparently unrelated Indian FH families in South Africa. This mutation was originally described in an FH subject (MM) of Indian (Gujerat province) origin (Soutar et al. 1989. Proc. Natl. Acad. Sci. **86:** 4166–4170). All four South African families trace their origin to the vicinity of Surat in the Gujerat province of India. Haplotype analyses revealed that both LDL receptor genes in one of the homozygous patients are the same as those in the subject MM. The phenotype of the mutant protein was analyzed in skin fibroblasts of homozygous patients. [^{35}S]methionine pulse-chase experiments revealed that the receptor precursors were slowly processed to mature receptors. Mature mutant receptors were degraded at faster than normal rates. This mutation, which is in the epidermal growth factor (EGF)-precursor-like domain of the LDL receptor, was previously reported to yield binding-defective receptors. Here we report that the affinity of the mutant LDL receptor for both LDL and β-very low density lipoprotein (β-VLDL) was normal and that the steady-state level of mutant receptors was about 20% of normal. Thus, the disease FH in these subjects is presumably due to the low steady-state level of receptor molecules that are functionally normal but exhibit accelerated turnover.—**D. C. Rubinsztein, G. A. Coetzee, A. D. Marais, E. Leitersdorf, H. C. Seftel, and D. R. van der Westhuyzen.** Identification and properties of the proline$_{664}$-leucine mutant LDL receptor in South Africans of Indian origin. *J. Lipid Res.* 1992. **33:** 1647–1655.

Supplementary key words familial hypercholesterolemia • coronary heart disease • cholesterol • atherosclerosis • low density lipoprotein receptor

Familial hypercholesterolemia (FH) is an autosomal dominant disease caused by mutations in the LDL-receptor gene (1). More than 40 different LDL-receptor gene mutations have been identified. These have been grouped into various classes according to the effect the particular mutation has on LDL-receptor expression or function. These mutations have been informative in defining structure–function relationships in the LDL-receptor (2).

It has been observed that there is a relatively high incidence of FH among Indians in South Africa (H. C. Seftel, M. S. Asvat, unpublished data). The approximately one million Indians in South Africa are almost entirely descended from persons who came from India to South Africa between 1860 and 1911, mainly as indentured laborers but also as artisans and traders (3). This group has remained isolated as a whole and also within its different communities, primarily as a result of religious and cultural practices. An LDL-receptor mutation (C to T transition in codon 664) has been identified by Soutar, Knight, and Patel (4) in an FH subject of Indian origin (MM) who resided in Zambia. The mutation has been designated FH-Zambia (2). This point mutation alters a single amino acid in the EGF-precursor-like domain of the LDL receptor, distant from the repeats known to be essential for ligand binding. Interestingly, however, it was reported that the mutation markedly diminished the affinity of the receptor for LDL (5).

We report here the identification of the same mutation in four South African Indian families. The families are all Muslim and trace their origin to the vicinity of Surat in

Abbreviations: LDL, low density lipoprotein; LDL-R gene, LDL-receptor gene; β-VLDL, β-very low density lipoprotein; EGF, epidermal growth factor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; FH, familial hypercholesterolemia; RFLP, restriction fragment length polymorphism; LPDS, lipoprotein-deficient serum.

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the Gujerat province of India. When fibroblasts from subjects homozygous for this mutation were studied, the mutant LDL receptors were found to have normal affinities for the ligands LDL and β-VLDL. The disease FH in these subjects is presumably due to low steady-state levels of receptor molecules that are functionally normal but which exhibit accelerated turnover.

METHODS

Materials

Human LDL (6), rabbit β-VLDL (7), Ig-G C7 (8), and human lipoprotein-deficient serum (6) were prepared as described. LDL (6) and Ig-G C7 (8) were radioiodinated with ^{125}I. The specific activity of iodinated LDL and Ig-G C7 ranged from 214 to 748 cpm/ng and 514 to 1442 cpm/ng, respectively.

Patients

The FH subjects were patients of the Lipid Clinics of the Johannesburg General and Groote Schuur Hospitals. Clinical details are provided in **Table 1.**

Detection of the FH-Zambia mutation

Genomic DNA was prepared from fibroblasts or whole blood (9). The mutation was identified in genomic DNA according to Soutar et al. (4) using either oligonucleotides 541 and 542 (kindly provided by Dr. A. Soutar, MRC Lipoprotein team, Hammersmith Hospital, London, UK) or oligonucleotides G29 and 542. Oligonucleotides 541 (5'-AATGTCGACGTCATCTTCCTTGCTGCCTGT-3') and 542 (5'-TATGTCCAGAAACAAGGCGTGTGCCAC-3') hybridize to the 3' end of intron 12 and the 5' end of intron 14, respectively, while G29 (5'-AAAGAATTCAGTGCCAACCGCCTCAC-3') hybridizes to a sequence in exon 13. Each 50-μl reaction mix contained 500 ng genomic DNA, 2.5 U Sequencing Grade Taq Polymerase (Promega, Madison, WI), Taq Polymerase buffer (Promega), 100 μg/μl acetylated BSA (Promega), 100 μM of each dNTP, and 20 pmol of each primer. Amplification was with 35 two-stage cycles of 93°C for 35 sec and 60°C for 3 min followed by one cycle of 93°C for 35 sec and 60°C for 6 min. After analyzing 15 μl of the product by electrophoresis in 1% agarose (Sigma, St. Louis, MO), the remaining 35 μl was digested overnight at 37°C with 10 U Pst 1 (Boehringer Mannheim, Mannheim, Germany) and then analyzed by electrophoresis on a 6% acrylamide gel.

Haplotype analysis

The LDL-receptor RFLP's were determined using PCR amplification with oligonucleotides flanking the

TABLE 1. Biochemical and clinical data for patients and affected relatives

Individual	Sex	Age	TC	LDL-C	HDL-C	TG	FH664 Status	Sequelae
		yr		*mg/dl*				
Family V								
AV (proband)	M	10	836	778	35	115	homozygote	Arcus, Xanthomata (at age 3), Xanthelasmata
Mrs. SV (mother)	F	42	294	224	54	53	heterozygote	Nil
Mr. MV (father)	M	47	275	221	43	62	heterozygote	Arcus, Xanthomata
Family A								
AA (proband)	F	16	685	631	35	97	homozygote	Xanthomata (at age 5), Bruits
Mrs. HA (mother)	F	43	310	236	43	159	heterozygote	
Family MA								
FMA	F	47	321	248	66	44	heterozygote	Arcus, Xanthelasmata, IHD
Family MT								
AMT	M	36	356	283	46	150	heterozygote	Arcus, Xanthelasmata, IHD

TC, total cholesterol; LDL-C, LDL-cholesterol; HDL-C, HDL-cholesterol; TG, triglyceride, all measured in mg/dl. IHD, ischaemic heart disease.

variable restriction sites for Taq 1, Stu 1, Hinc II, Ava II and Nco I (10–12).

Cell culture

Skin fibroblasts were seeded at 60,000 cells per 60-mm diameter Petri dish (ligand binding experiments) or at 20,000 cells per 35-mm diameter Petri dish (immunoprecipitation experiments) in Dulbecco's MEM (DMEM) (Flow Laboratories, Ayrshire, Scotland, UK) containing 10% (v/v) fetal calf serum (day 0). On day 3 this medium was replenished. On day 5, the medium was changed to Dulbecco's MEM containing lipoprotein-deficient serum (LPDS) (5 mg protein/ml). This medium was replenished on day 6. Experiments were performed on day 7.

Surface binding of ^{125}I-labeled Ig-C7 or ^{125}I-labeled LDL at 4°C

Surface binding of ^{125}I-labeled IgG-C7 (13) and ^{125}I-labeled LDL (14) was performed at 4°C using the indicated concentrations of the respective ligands. Incubations were done in DMEM/LPDS buffered at pH 7.4 with 20 mM HEPES. After the incubation the cells were washed 4 times with Dulbecco's phosphate-buffered saline (PBS) (Ca^{2+}- and Mg^{2+}-free), containing 0.2% bovine serum albumin followed by 3 washes with PBS at 4°C. In the ^{125}I-labeled LDL binding experiments, 2 ml of heparin (4 mg/ml) was added to the cells for 40 min at 4°C and the ^{125}I-labeled LDL released was taken as the bound fraction. In the ^{125}I-labeled IgG-C7 experiments, the cell-associated radioactivity was quantitated after the cells were dissolved in 1 N NaOH.

β-VLDL affinities at 4°C

The affinities of the LDL receptors for apoE were estimated by measuring the ability of unlabeled β-VLDL to compete for ^{125}I-labeled LDL binding at 4°C. Rabbit β-VLDL was added at the indicated concentrations to 2 μg/ml ^{125}I-labeled LDL in DMEM/LPDS buffered at pH 7.4 with 20 mM HEPES. These premixes were added to dishes and ^{125}I-labeled LDL binding was determined as above. The β-VLDL concentration required for half-maximal inhibition of ^{125}I-labeled LDL binding was taken to reflect the affinity of the receptors for β-VLDL. This value was determined by nonlinear regression analysis (Enzfitter; Elsevier Biosoft, Cambridge, U.K.).

^{125}I-labeled LDL metabolism at 37°C

The ability of cells to bind, internalize, and degrade ^{125}I-labeled LDL at 37°C was measured at the indicated ^{125}I-labeled LDL concentrations as described previously (6). After the cells were incubated with the desired concentration of ^{125}I-labeled LDL in DMEM/LPDS at 37°C for 4 h, they were put on a cold metal tray in the 4°C cold room. The medium was immediately harvested and its content of non-iodide trichloroacetic acid-soluble radioactivity was measured (LDL degradation). The cells were washed four times with PBS-albumin, three times with PBS, and then treated with heparin as described in the 4°C LDL binding assay. The heparin-releasable radioactivity was taken as the bound fraction. The cells were then solubilized in 1 M NaOH and the radioactivity was quantitated (intracellular ^{125}I-labeled LDL). Cellular protein in the solubilized extract was also determined (15).

Calculation of B_{max} and K_d values

The B_{max} and K_d values were calculated from binding curves using a commercial software package (Enzfitter; Elsevier Biosoft, Cambridge UK) for nonlinear regression analysis assuming one saturable site plus nonsaturable processes. In some cases specific values were determined by subtracting from the total activity the nonspecific values obtained either in the presence of excess unlabeled LDL (200 μg/ml) or in cells in which LDL-receptor activity had been fully down-regulated by treatment with 1 μg/ml 25-OH cholesterol for 48 h. The resultant curves of specific high-affinity activity were analyzed using Enzfitter, assuming only one site of specific binding.

[^{35}S]methionine pulse-chase experiments

These experiments were done essentially as described (16). On day 7, up-regulated cells were pre-incubated for 30 min in methionine-free Eagle's MEM at 37°C, after which cells were pulse-labeled at 37°C with [^{35}S]methionine (50–100 μCi/ml; L-[^{35}S]methionine, DuPont, Wilmington, DE). The medium was then changed to DMEM/LPDS containing 200 μM unlabeled methionine and the cells were chased for varying times. The cells were then washed and solubilized and the [^{35}S]methionine-labeled LDL receptors were immunoprecipitated with a preformed immune complex containing IgG-C7. Immunoprecipitates were electrophoresed on 7% SDS polyacrylamide gels that were enhanced with salicylate, dried, and exposed to X-ray film. The ^{35}S radioactivity in the mature and precursor LDL-receptors was quantified by densitometric scanning of the fluorograms.

RESULTS

The CCG (proline) to CTG (leucine) mutation at codon 664 creates a Pst 1 restriction site in the LDL-R gene (4). This mutation was detected by amplifying genomic DNA by PCR using primers flanking exons 13 and 14, and digesting the 496 bp product with Pst 1 (**Fig. 1**). The generation of a 381 bp and a 115 bp fragment indicated the presence of the mutant sequence CTG in exon 14 at codon 664. The possibility that two such fragments result from a mutation that creates a Pst I site at the 5'-end of the amplified fragment (i.e., in exon 13) was ruled out; when genomic DNA was amplified using an

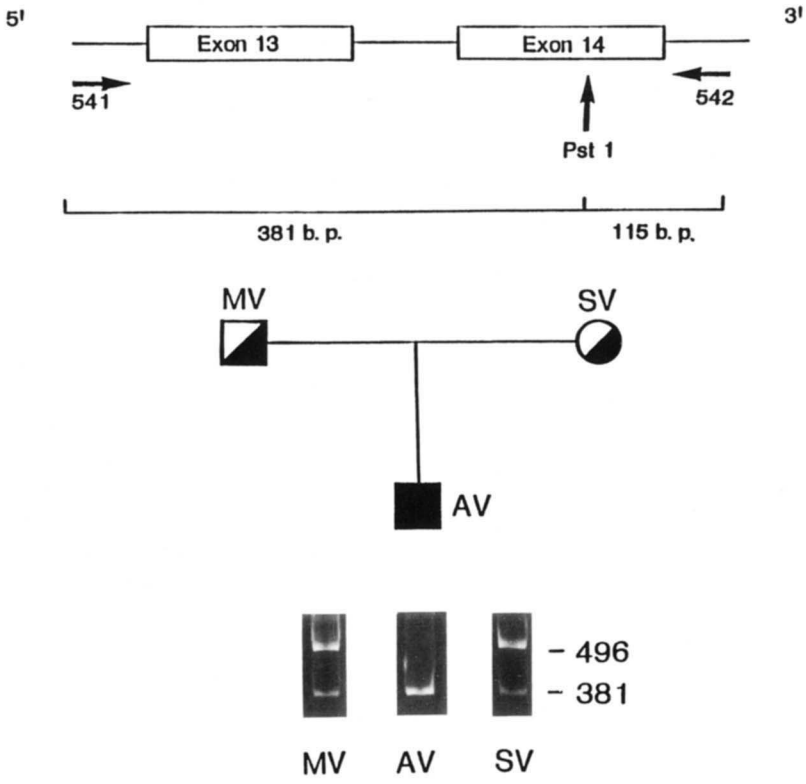

Fig. 1. Top: Diagram of the LDL-receptor gene showing the positions of oligonucleotides 541 and 542 and the Pst 1 site created by the Leu_{664} mutation. Bottom: Polyacrylamide gel stained with ethidium bromide showing the Pst 1 digests of the 541-542 PCR products in the V family.

upstream primer within rather than flanking exon 13 and the same downstream primer flanking exon 14, the Pst 1 digest of this product again released a 115 bp fragment. We have identified the $proline_{664}$-leucine mutation in FH patients from four unrelated families of Indian origin (Table 1). All the families are Muslims and all originate from the vicinity of Surat in the Gujerat province on the west coast of India. The unrelated homozygous probands AV and AA are offspring of consanguinous marriages between first cousins. This mutation was confirmed in the homozygotes AV and AA by direct sequencing of the above PCR products (results not shown).

Subsequent to its initial identification in a Zambian Indian, MM, the mutation was also detected in English subjects on a different LDL receptor haplotype and in a Norwegian family with the same haplotype as MM (17). The five-enzyme haplotype of both LDL receptor genes in the FH patient, AV, was the same as that of MM (Ava II++, HincII--, Stu 1++. Taq 1--, Nco 1++) and distinct from the haplotype of the English subjects, suggesting that AV and MM inherited the same mutant LDL-receptor alleles. The haplotype of the British subjects differed from that of AV and MM at three restriction sites.

LDL-receptor synthesis and processing was studied using [^{35}S]methionine pulse-chase experiments in cultured fibroblasts. The precursor receptors in AV cells were abnormally slowly processed to mature forms with a half-life of conversion of about 2 h (**Fig. 2** and **Fig. 3**). Most of these precursor receptors were converted to the mature form (Fig. 3) although the possibility that some precursor degradation may have occurred could not be ruled out. By contrast, in the same experiments, normal receptor precursors (120 kDa) were rapidly and completely converted to the mature form (160 kDa) within 30 min of a chase period (data not shown). The retarded processing of the mutant receptors was presumably a consequence of a structural abnormality. We determined whether such an abnormality would affect the stability of the mature mutant receptors. The half-life of AV's mature LDL receptors was about 3.5 h, in comparison to a half-life of about 9 h for normal receptors (**Fig. 4**).

In similar experiments, the degradation rate of the mutant receptors measured over 4 h was not abnormally enhanced when β-VLDL (10 μg/ml) was added to the chase medium (data not shown).

AV and AA fibroblasts were able to sustain internalization and degradation of ^{125}I-labeled LDL at 37°C for 4 h

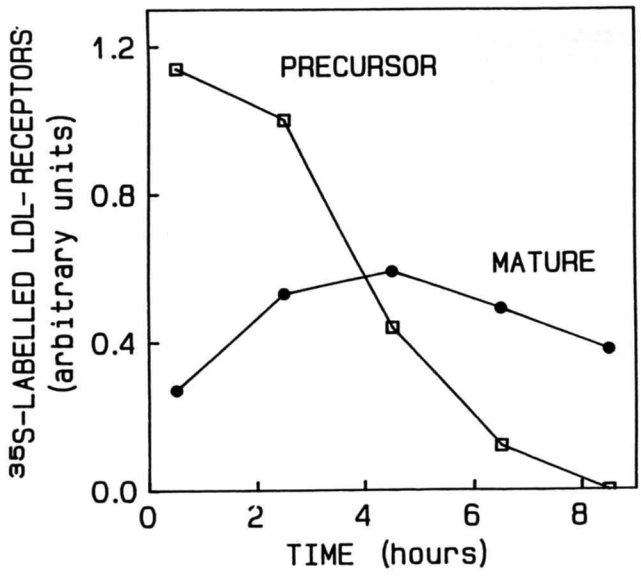

Fig. 2. Decay of radioactivity from pulse-labeled LDL-receptor protein. Up-regulated fibroblasts from AV were pulse-labeled with [^{35}S]methionine for 1.5 h and chased in DMEM/LPDS for the indicated times. The radioactivity in the 120 kDa precursor and 160 kDa mature receptors was quantified by densitometric scanning of autoradiographs of gels of immunoprecipitated LDL-receptors. All the values except those at 30 min are means of two experiments done in duplicate dishes. The values at 30 min are from one experiment only. In each experiment the intensity of the bands was expressed relative to the precursor band at 2.5 h. In the same and other control experiments, normal receptor precursors were rapidly and completely converted to mature forms within 30 min of the chase period.

at rates commensurate with their steady-state binding activity of about 25% of normal levels (**Table 2**). The internalization index for the mutant cells was normal, therefore indicating that the mutant cells internalized and degraded LDL normally and that their receptors recycled back to the surface appropriately. The rapid degradation of mutant receptors (see above) was therefore not further accelerated by the presence of β-VLDL or LDL. The ^{125}I-labeled LDL concentrations that resulted in half-maximal ^{125}I-labeled LDL degradation were similar in normal and mutant cells (**Fig. 5**). This suggested that the mutation does not affect the affinity of the receptor for LDL at 37°C. The maximum binding of AV cells at ligand saturation was 23% of normal.

The binding of ^{125}I-labeled LDL and ^{125}I-labeled IgG-C7 to AV cells was also studied at 4°C. The maximum number of surface receptors of the mutant fibroblasts that bind these ligands was about 20% of that in normal cells (**Fig. 6**). These binding curves indicated that the affinity of AV receptors for both ligands was essentially normal (Fig. 6).

The K_d values for ^{125}I-labeled LDL binding to normal and mutant receptors were 1.8 ± 0.4 μg/ml and 1.8 ± 0.9 μg/ml, respectively. AA and AV fibroblasts bound ^{125}I-labeled LDL with a similar affinity (results not shown).

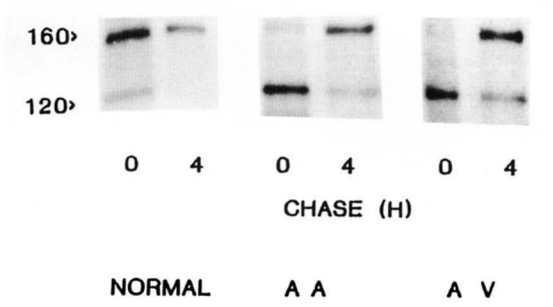

Fig. 3. Autoradiographs of immunoprecipitated ^{35}S-labeled LDL receptors from normal, AV, and AA fibroblasts. Up-regulated fibroblasts were pulse-labeled with [^{35}S]methionine for 1 h and chased in DMEM/LPDS for the indicated times. LDL receptors were immunoprecipitated and visualized on autoradiographs as indicated in Methods. The apparent molecular masses of the precursor (120 kDa) and mature (160 kDa) receptors are indicated.

These values were calculated by nonlinear-regression analysis of the total ligand binding values assuming one specific high-affinity binding site and a nonsaturable component. Similar K_d values for normal and mutant cells were obtained when specific receptor binding was determined by two alternative approaches: nonspecific binding was measured either in the presence of excess unlabeled ligand or in down-regulated cells and subtracted from to-

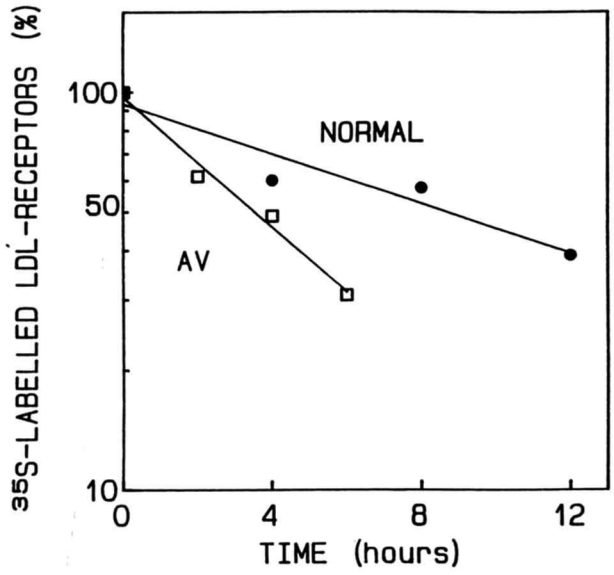

Fig. 4. Half-lives of mature LDL receptors from normal and AV fibroblasts. Normal and AV up-regulated fibroblasts were pulse-labeled with [^{35}S]methionine for 40 min and 90 min, respectively. The medium was then changed to DMEM/LPDS and the normal cells were chased for 0.5 h and the AV cells were chased for 6.5 h before starting to measure the half-life of mature LDL-receptors at time 0. LDL receptors were immunoprecipitated, run on 7% acrylamide, and visualized on autoradiographs as indicated in Methods. The points are the mean values from two experiments. Normal and AV fibroblasts were compared in each experiment in which each point was the mean of the values obtained in duplicate dishes. The half-lives of the mature LDL receptors from normal and AV fibroblasts were determined in the same two experiments.

TABLE 2. High affinity binding, intracellular content, and rate of degradation of ^{125}I-labeled LDL in cultured fibroblasts

Cell Strain	^{125}I-Labeled LDL			Internalization Index
	Bound	Intracellular	Degraded	
	ng/mg cell protein			
Normal	198 ± 55	1200 ± 750	2990 ± 1150	17–26
	% normal			
AV	15 ± 3	24 ± 9	25 ± 7	29–42
AA	25 ± 9	27 ± 9	29 ± 8	20–27

Studies on LDL metabolism at 37°C were done at 10 μg/ml ^{125}I-labeled LDL as described in Methods. Values are the means of three experiments, each done in duplicate dishes. The internalization index was calculated by dividing the sum of the ^{125}I-labeled LDL degraded in 4 h and the intracellular ^{125}I-labeled LDL by the ^{125}I-labeled LDL bound.

tal binding values. In these cases the calculated specific binding values were plotted and analyzed as single component curves (**Fig. 7**). The binding of an alternate lipoprotein ligand, β-VLDL, was also examined. The β-VLDL concentration required for half-maximal inhibition of ^{125}I-labeled LDL binding at 4°C was similar in normal and AV cells, indicating that the affinity of the mutant receptor for this ligand was also normal (**Fig. 8**).

Our results differ in one fundamental respect from those previously reported: Soutar et al. (4) and Knight et al. (5) reported that MM's receptors exhibited a decreased affinity for the ligands β-VLDL (4) and LDL (4, 5). The decreased affinity for LDL was apparent both at 37°C and particularly at 4°C, where the mutant receptor's K_d was 5 times the value obtained for normal receptors. Our affinity measurements indicate that the mutant receptors

DISCUSSION

The abnormal function of many mutant LDL receptors can be predicted from the known structure-function correlations in the normal receptor (2). The EGF-precursor-like repeat C contains 6 cysteines that are thought to be disulfide bonded to each other. The proline at amino acid 664 is in a highly conserved region between cysteine 3 and cysteine 4 (18) and, being an amino acid that causes fixed kinks in polypeptide chains, probably plays an important role in determining the conformation of this repeat. The substitution of leucine at this site presumably results in aberrant folding of the mutant protein, which in turn could account for the slow processing and decreased stability of this receptor. However, the report that the pro to leu substitution at amino acid 664, more than 350 amino acids away from the ligand binding domain, affected the affinity of the receptor for LDL and β-VLDL was unexpected (4, 5). It suggested a new role for repeat C either in the maintenance of the correct conformation of the binding domain or in controlling appropriate interactions between LDL-receptor molecules. Previously, the regions shown to be critical for LDL binding were repeats 2 to 7 of the ligand-binding domain and repeat A of the EGF-precursor-like domain (19). The identification of patients who were homozygous for this mutation allowed us to re-examine its phenotype.

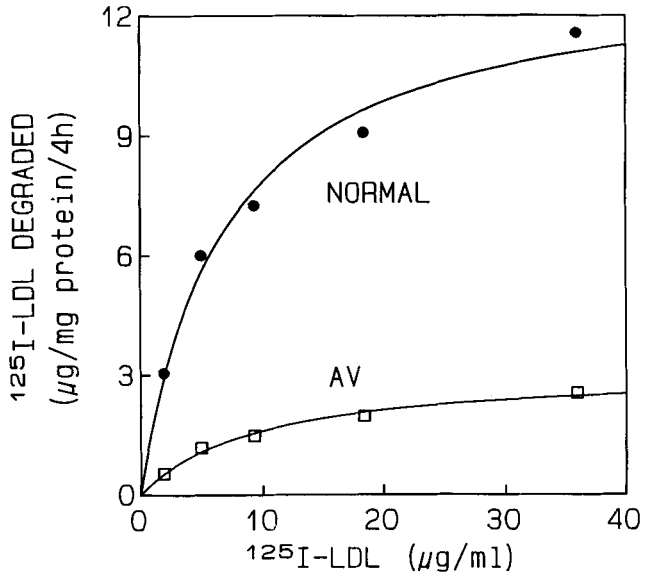

Fig. 5. ^{125}I-labeled LDL degradation in normal and AV fibroblasts. Normal and AV up-regulated fibroblasts were incubated with the indicated concentrations of ^{125}I-labeled LDL for 4 h at 37°C. The medium was collected and its content of non-iodide trichloracetic acid-soluble radioactivity was determined and considered as the ^{125}I-labeled LDL degraded in 4 h (each point is the mean value of duplicate incubations). AV's fibroblasts degraded ^{125}I-labeled LDL with a B_{max} of 23% normal. The ^{125}I-labeled LDL concentration that resulted in half-maximal ^{125}I-labeled LDL degradation was 5.1 μg/ml and 3.6 μg/ml in the mutant and the normal fibroblasts, respectively.

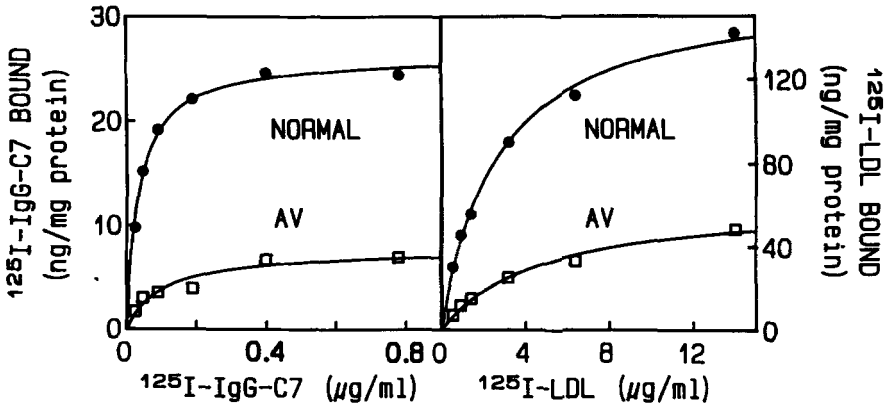

Fig. 6. 4°C binding of ^{125}I-labeled LDL and ^{125}I-labeled IgG-C7 to normal and AV fibroblasts. Up-regulated cells were incubated at 4°C for 2 h and the total ^{125}I-labeled ligand bound was determined as described in Methods. Each value represents the average of duplicate incubation. AV fibroblasts bound IgG-C7 with a B_{max} of 20% of the normal fibroblasts and with a K_d of 0.05 μg/ml (normal K_d = 0.04 μg/ml). AV fibroblasts bound ^{125}I-labeled LDL with a B_{max} of 25% of the normal fibroblasts and with a K_d of 1.97 μg/ml (normal K_d = 1.81 μg/ml). When these results were pooled with those from two other similar experiments, AV fibroblasts bound ^{125}I-labeled LDL with a B_{max} of 20 ± 9% of normal fibroblasts and with a K_d of 1.8 ± 0.9 μg/ml as compared to 1.8 ± 0.4 μg/ml in normal fibroblasts (mean ± standard deviation values).

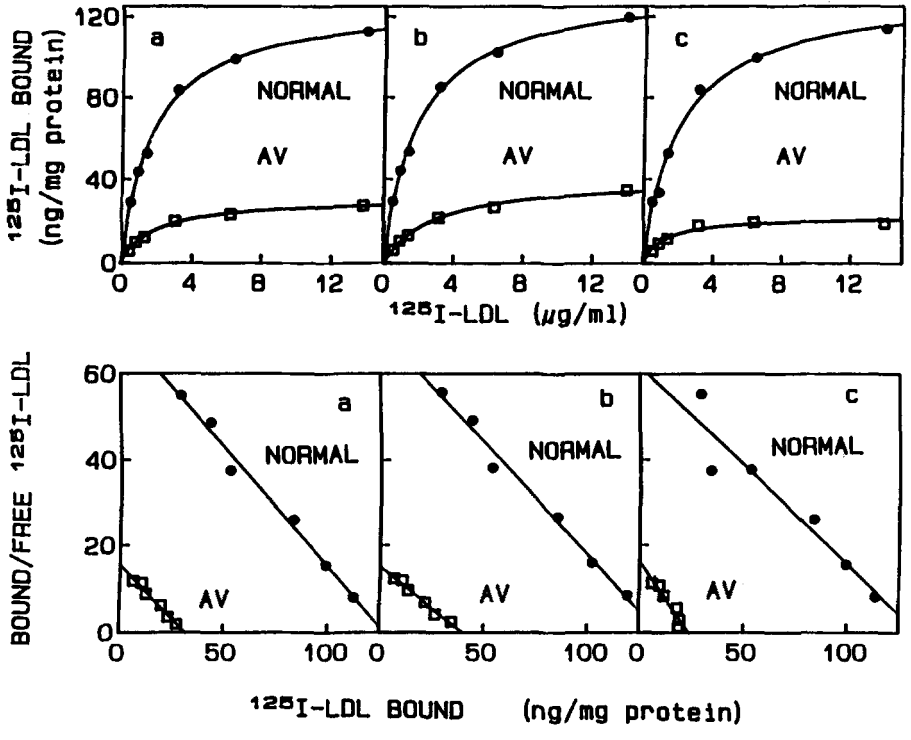

Fig. 7. Comparison of specific binding of ^{125}I-labeled LDL at 4°C derived by three different methods. Fibroblasts, in a single experiment, were up-regulated (a, b) or down-regulated (c) as described in Methods and incubated at 4°C for 2 h with different concentrations of ^{125}I-labeled LDL. a) Total binding values were analyzed using nonlinear regression analysis assuming one specific binding site and a nonspecific component (Enzfitter, Elsevier Biosoft). Specific values are plotted. The calculated K_d values for the normal and mutant cells were 1.81 μg/ml and 1.97 μg/ml, respectively. b) Nonspecific binding was determined in the presence of excess unlabeled LDL (200 μg/ml). Nonspecific binding was subtracted from total binding and the resultant specific binding values are plotted. The calculated K_d values of specific binding for normal and mutant cells were 1.99 μg/ml and 2.5 μg/ml, respectively. c) Nonspecific binding was measured in cells that had been down-regulated with 25-OH cholesterol for 48 h. Nonspecific binding values were subtracted from total binding and the resultant specific binding values are plotted. The calculated K_d values of specific binding for normal and mutant cells were 1.84 μg/ml and 1.35 μg/ml, respectively. The bottom panel in this figure shows the Scatchard plots of the corresponding binding data represented in the top panel.

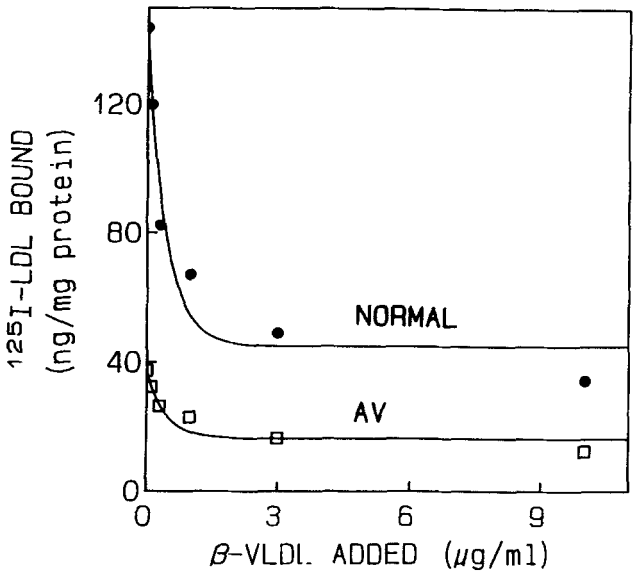

Fig. 8. β-VLDL competition for ^{125}I-labeled LDL bound to normal and AV fibroblasts. AV and normal fibroblasts were incubated for 2 h at 4°C with 2 μg/ml ^{125}I-labeled LDL plus the indicated concentration of unlabeled β-VLDL. Heparin-releasable ^{125}I-labeled LDL binding was determined as in Methods. Each value is the mean of duplicate incubations. Similar results were obtained in a separate experiment. The mean concentration of β-VLDL required for half-maximal inhibition of ^{125}I-labeled LDL binding to AV's fibroblasts calculated by nonlinear regression (from two experiments) was 0.44 μg/ml as compared to 0.32 μg/ml in the normal fibroblasts.

have a normal affinity for IgG-C7, LDL, and β-VLDL at 4°C. The ^{125}I-labeled LDL concentration that resulted in half-maximal ^{125}I-labeled LDL degradation at 37°C was similar in normal and in mutant fibroblasts. This suggested that the affinity of these receptors for LDL at 37°C was also normal. The reason for the differences between our results and those previously reported is not clear.

The low number of surface receptors in AV's fibroblasts is at least partially due to the increased rate of receptor turnover. The enhanced degradation rate, also reported by Knight and coworkers (5), was observed in the absence of lipoprotein ligand. The abnormally fast degradation rate of the mutant receptors was not further enhanced by the presence of LDL or β-VLDL, as there was no gross depletion of mutant receptors after 4 h of incubation of cells with either ligand at 37°C. Therefore, an impairment of acid-dependent ligand-receptor dissociation and a consequent trapping of receptors in endosomes do not explain the accelerated receptor degradation. The newly defined Class 5 type of receptor defect comprises mutant receptors that are rapidly degraded due to impaired acid-dependent ligand-receptor dissociation (2). Perhaps the entrance criteria for this class should be extended to include mutations like the pro_{664}-leu mutation and the previously described FH-Afrikaner-2 (2, 13) and FH Cape Town-2 (20) mutations that each result in receptors that are inherently unstable and that have increased turnover rates even in the absence of ligands.

The diagnosis of this mutation in AV and AA relied on the creation of a Pst 1 restriction site and direct sequencing of exon 14. It is unlikely, but possible, that another LDL-receptor mutation might co-exist on the same allele(s) as the FH-Zambia mutation in our patients. We have established that our subject AV has the same haplotype as MM. It is unlikely that a second mutation could correct for a defect in binding caused by an initial mutation at amino acid 664.

The pro_{664}-leu mutation was originally identified in MM, a Zambian who originated from Gujerat on the West Coast of India. Subsequently this mutation was identified in another family of the same racial and geographical origin (17). We have identified this mutation in four families. They are of Gujerati Muslim origin from the vicinity of Surat. The frequent identification of this mutation in Indian emigrants of Gujerati/Surat origin suggests that this mutation might be very common in this area of India.

Muslim Gujeratis, until very recently, only married descendants from their own village or locality in India. Therefore this group was genetically isolated both in India and abroad. It is thus unlikely that this mutation would have been uniformly distributed among all Indians in South Africa of which the majority are Hindus. However, since Muslim Gujeratis comprise less than 1/5 of South Africa's 800,000–1,000,000 Indians (21), the finding of four unrelated kindreds with this mutation suggests that this mutation is indeed very common in this group.

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