Analysis of two duplications of the LDL receptor gene affecting intracellular transport, catabolism, and surface binding of the LDL receptor

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Abstract **Two novel mutations of the low density lipoprotein (LDL)-receptor gene were found in two Italian familial hypercholesterolemia (FH)-heterozygotes. The first mutation was an 18 nucleotide duplication in exon 8 which is preceded by an A→T transversion. The translation product of the mutant allele was predicted to be a receptor with an in-frame insertion of 6 amino acids in repeat B of the epidermal growth factor precursor homology domain. Analysis of LDL-receptor activity in the proband's fibroblasts showed a 50% reduction of ^{125}I-labeled LDL binding and pulse-chase studies suggested that little, if any, of the mutant protein was processed to the mature form. The second mutation was a 7 kb duplication (from intron 2 to intron 6) of exons 3 through 6, predicted to encode an elongated receptor with the duplication of repeats 2–7 of the ligand binding domain. The elongated receptor was processed slightly more slowly than the normal receptor, but was converted to a mature form of the expected size. This mature, mutant receptor was degraded more rapidly than the normal receptor. On ligand blotting the elongated receptor bound twice as much LDL or beta-very low density lipoprotein (βVLDL) as the normal receptor. In contrast, maximum binding of LDL to proband's cells was decreased to approximately 70% of the normal cells with a significant increase in apparent affinity. Cell association at 37°C, internalization, and degradation showed a similar reduced maximum. Thus these mutations demonstrate that duplications of amino acid sequences in the low density lipoprotein LDL-receptor may disrupt the LDL-receptor pathway at different levels.—**Patel, D. D., N. Lelli, R. Garuti, S. Li Volti, S. Bertolini, B. L. Knight, and S. Calandra. **Analysis of two duplications of the LDL-receptor gene affecting intracellular transport, catabolism, and surface binding of the LDL receptor. *J. Lipid Res.* 1998.** 39: **1466–1475.**

Supplementary key words familial hypercholesterolemia • amino acid sequence

Familial hypercholesterolemia (FH) is a common autosomal co-dominant disorder characterized by elevated plasma low density lipoprotein (LDL) cholesterol, tendon xanthoma, and an increased risk of premature coronary artery disease (1). The primary defect in FH is caused by mutations in the gene encoding the low density lipoprotein receptor (LDL-receptor) (1). In most populations the frequency of heterozygous FH is estimated to be 1: 500 (1). FH is heterogeneous at the molecular level, with more than 200 mutations having been reported so far (1, 2). The majority of these mutations have been investigated at the DNA level, but only in a relatively small number of cases, has DNA analysis been corroborated by cellular studies to discover the functional defects of the receptor protein. In the present study we report the characterization of two novel partial duplications (a minute and a large duplication, respectively) of the LDL-receptor gene found in two Italian FH heterozygotes. These duplications do not disrupt the reading frame of LDL-receptor mRNA but cause the formation of two abnormal receptors whose functional and structural properties were examined in cultured fibroblasts. One resulted in a duplication of most of the binding region and had unexpected consequences for the cellular behavior of the protein and for the binding of LDL to receptors on the cell surface.

METHODS

Subjects

Proband C.D. was a 41-year-old male who was known to have primary hypercholesterolemia since adolescence. At the time of

Abbreviations: VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; LPDS, lipoprotein-deficient serum; apoA-I, apolipoprotein A-I; apoB, apolipoprotein B; SSCP, single-strand conformation polymorphism; FH, familial hypercholesterolemia; EGF, epidermal growth factor; PCR, polymerase chain reaction; RT, reverse transcription; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

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DNA analysis he had no clinical manifestations of overt coronary heart disease nor tendon xanthomas. Other members of his family, including his daughters, had severe hypercholesterolemia (Table 1). Proband C.D.'s family has been living for several generations in the city of Caltanissetta, in the eastern part of Sicily.

Proband B.N. was a 56-year-old male who was found to have hypercholesterolemia at the age of 20. At the age of 52 he suffered a myocardial infarction. His family, which includes several other hypercholesterolemic subjects, has been living for several generations near the city of Chieti, in central Italy.

Informed consent was obtained from the patients or, in the case of children, from their parents. The study protocol was approved by the institutional human investigation committee of the participating institutions.

Southern blot and SCSP analysis

Genomic DNA, extracted from peripheral blood leukocytes, was digested with several restriction enzymes, separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with LDL-receptor cDNA probes as previously described (3). Single-strand conformation polymorphism (SSCP) analysis of the promoter region and the exons of the LDL-receptor was performed as previously reported (4) using the primers reported by Leitersdorf et al. (5) unless otherwise specified.

Polymerase chain reaction (PCR) amplification of genomic DNA in proband C.D.

In order to define the boundaries of the duplication we amplified the wild type introns 2 and 6 from patient C.D. and from a control subject, and the "hybrid" intron, generated by the joining of the 5′ end of intron 6 with the partially duplicated intron 2, from proband C.D. (see restriction map in Fig. 1). To amplify the wild type intron 2, 1 μg of genomic DNA was incubated in 40 μl of 0.1 mm of each dNTP, 40 pmole of each primer and 2.5 units of Taq DNA polymerase in 1 × PCR buffer (50 mm KCl, 10 mm Tris-HCl, 1.5 mm $MgCl_2$). The following primers were used : 5′ CCT TTC TCC TTT TCC TCT CTC TCA G 3′ (intron 1 forward primer, SP57) (5) and 5′ AAT AGC AAA GGC AGG GCC ACA CTT A 3′ (intron 3 reverse primer, SP 60) (5). The conditions were: 95°C for 20 sec and 70°C for 5 min for 35 cycles. To amplify the wild type intron 6 we used the same conditions with the primers: 5′ TCC TTC CTC TCT CTG GCT TCT ACA G 3′ (intron 5 forward primer, SP64) (5) and 5′ AGG GCT CAG TCC ACC GGG GAA TCA C 3′ (intron 7 reverse primer, SP67) (5). To amplify the "hybrid" intron in proband C.D., we used SP64 (intron 5 forward primer) and SP60 (intron 3 reverse primer)(5) with the amplification conditions of 95°C for 1 min and 70°C for 2 min for 30 cycles. All amplified introns were subjected to KpnI and EcoRI digestion and the fragments were separated by agarose gel electrophoresis, transferred to nylon membranes, and hybridized with the 5′ ^{32}P-labeled oligonucleotide probe SP58 (5), a primer corresponding to the 5′ end of intron 2. This primer was labeled at its 5′ end with ^{32}P- γ ATP (Amersham, UK) using T4 polynucleotide kinase (Boehringer, Mannheim, Germany), according to manufacturers instructions.

Northern blot analysis

Total cellular RNA was isolated by extraction in guanidinethiocyanate (6) from cultured skin fibroblasts that had been maintained in a lipoprotein-deficient serum (LPDS) for 15 h (3). Total RNA (15 μg) was denatured in 50 μl of 50% formamide and separated by electrophoresis in 1.2% agarose gels and then transferred to Hybond N membranes, which were then hybridized with the full-size cDNA probe (3, 4). The cDNA clone pHFβA-1 of human β-actin was used to normalize the RNA filters. Pre-hybridization and hybridization were performed as previously described (3).

Reverse transcription (RT) and PCR amplification

RNA (1 μg) isolated from cultured fibroblasts of proband C.D., proband B.N., and control subjects were reverse-transcribed in 20 μl reaction mixture containing 5 mm $MgCl_2$, 1 mm of each dNTP, 1 unit of RNAse inhibitor, 100 pmol random hexamers, and 12 units of AMV reverse transcriptase in 1 × PCR buffer (see above) (4). After heating the sample at 95°C for 5 min, 80 μl of 1 × PCR buffer containing 20 pmol of each primer and 2.5 units of Taq polymerase were added (4).

RT-PCR in proband C.D. To amplify the exon 1–exon 4 region in proband C.D., the following primers were used: 5′ GCT GGA AAT TGC GCT GGA CCG TCG C 3′ (forward primer complementary to exon 1)(E1s) and 5′ ACG AAC TGC CGA GAG ATG CAC 3′ (E4as) (reverse primer complementary to exon 4)(E4as). The conditions were: 95°C for 1 min, 65°C for 2 min for 30 cycles. To amplify the junction between exon 6 and exon 3bis (i.e., duplicated exon 3) (Fig. 1) the following primers were used: 5′AAT GCA TCA CCC TGG AGA AAG TCT G 3′ (E6s) (exon 6 forward primer) and primer E4as (see above). The conditions were: 95°C for 1.30 min and 65°C for 1.30 min for 30 cycles. This RT-PCR fragment was sequenced directly using primer E6s. To amplify the exon 6–exon 7 junction in a control subject, the following primers were used: E6s (see above) as forward primer and 5′CGT CAG ATC ATT CTC TGG GA 3′ (E9s) as reverse primer. The conditions were: 95°C for 1 min, 50°C for 1.30 min, 72°C for 2 min, for 30 cycles. This RT-PCR fragment was sequenced directly using primer E9as (4).

RT-PCR in proband B.N. The exon 6–exon 9 region in proband B.N. was amplified as specified above, using primer E6s and E9as (see above). The RT-PCR product was digested with EcoRV and the fragments were separated by 10% polyacrylamide gel electrophoresis. Gels were stained with ethidium bromide.

Cloning of the mutant allele in proband B.N.

PCR products containing exon 8 amplified from genomic DNA, were cloned into the pCR™ II vector using the TA cloning kit (Invitrogen, San Diego, CA). Twenty colonies of transformed bacterial cells were selected. Plasmid DNA was subjected to a double digestion with EcoRI (to release the insert from the vector) and with EcoRV (which cuts the insert into two fragments). The length of the EcoRV fragments allowed us to discriminate the wild type from the mutant insert and to define their 5′→3′ orientation (see results). Two positive clones were selected, purified by using a Quiagen plasmid purification kit (Diagen GmbH, Dusserdorf, Germany) and sequenced directly using primer 5′ CCA CCC GCC GCC TTC CCG TGC TCA C 3′ (primer SP69) (5).

Nucleotide sequence of exon 8 in proband B.N.

Exon 8 of proband B.N. and a control subject were amplified by PCR using 5′ CCA AGC CTC TTT CTC TCT CTT CCA G 3′ (primer SP68) (5), as forward primer and primer SP69 (as reverse primer) (5). The conditions were: 95°C for 1.30 min, 70°C for 1.30 min for 30 cycles. The PCR fragments were sequenced directly using primer SP69 (5).

Cellular studies

Human low density lipoprotein (LDL, relative density 1.019–1.063 g/ml) and rabbit very low density lipoprotein (βVLDL, relative density < 1.006 g/ml) were prepared and radioiodinated as described before (7). For the assay of lipoprotein bind-

TABLE 1. Clinical features of FH patients

Subjects	Sex	Age	CH	TG	ApoB	ApoAI
			mg/dl			*mg/dl*
Proband C.D.	M	41	409	194	295	98
Spouse S.G.	F	37	136	100	96	151
Daughter C.V.	F	13	478	229	317	143
Daughter C.C.	F	11	354	100	232	123
Proband B.N.	M	56	466	151	278	82
Spouse M.M.	F	48	235	128	nd	nd
Son B.R.	M	18	213	100	117	125
Son B.S	M	17	330	95	195	107

ing at 4°C or cell-association and degradation of lipoprotein at 37°C, cells in 35-mm Multi-wells were pre-incubated for 40 h in medium containing 5% (v/v) lipoprotein-deficient serum (LPDS). Conditions used for incubation and washing the cells were essentially those described by Goldstein et al. (8, 9). Values were corrected for non-saturable binding observed in the presence of an excess of unlabeled lipoprotein. For ligand- and immunoblotting, cells in 90-mm dishes were washed and solubilized as described by van Driel et al. (10). When required, cells were pretreated at 4°C with 5 μg/ml Pronase or with the bifunctional cross-linking reagent DTSSP [3,3′-dithiobis (sulfosuccinimidyl proprionate)] as described previously (11, 12). Proteins were separated by SDS PAGE, transferred to nitrocellulose membranes, and the LDL-receptor proteins were detected with monoclonal antibodies to the LDL receptor (13) or ^{125}I-labeled LDL or βVLDL as described before (13, 14). One of the antibodies (mAb4B3) is thought to bind to an epitope at, or near, the second binding repeat of the receptor (15). Incorporation of [^{35}S]methionine into immunoprecipitable LDL-receptor protein by cells in 90-mm dishes was determined using complexes of mAb4B3 with anti-mouse IgG as described previously (16).

RESULTS

Genomic DNA analysis

The clinical and biochemical features of probands C.D. and B.N., shown in **Table 1**, were consistent with the diagnosis of familial hypercholesterolemia.

To exclude the presence of major structural rearrangements of the LDL-receptor gene, genomic DNA was digested with several restriction enzymes. After Southern blotting and hybridization with various LDL-receptor cDNA probes (3), abnormal digestion fragments were seen only in proband C.D.

Proband C.D. The hybridization of genomic DNA of proband C.D. with a cDNA probe complementary to exons 1–11 revealed the presence of the normal bands (with the same relative intensity as those observed in control DNA) and an additional band of variable size depending on the restriction enzyme used (BgIII, EcoRI, BamHI, and KpnI). These preliminary restriction data suggested that proband C.D. was heterozygous for a partial duplication involving a region spanning from the exon 2–intron 2 junction to intron 6. Southern blot results, combined with PCR amplification and restriction mapping of normal introns 2 and 6 and the "hybrid" intron, derived from the joining of intron 6 to intron 2bis (see Methods for details), allowed us to draw the restriction map shown in **Fig. 1**. This map shows that proband C.D. was heterozygous for a 7 kb insertion resulting from the duplication of the region containing exon 3–exon 6. This duplication, which was also present in proband's hypercholesterolemic daughters, was named FH-Caltanissetta after the city in Sicily where proband C.D. was living.

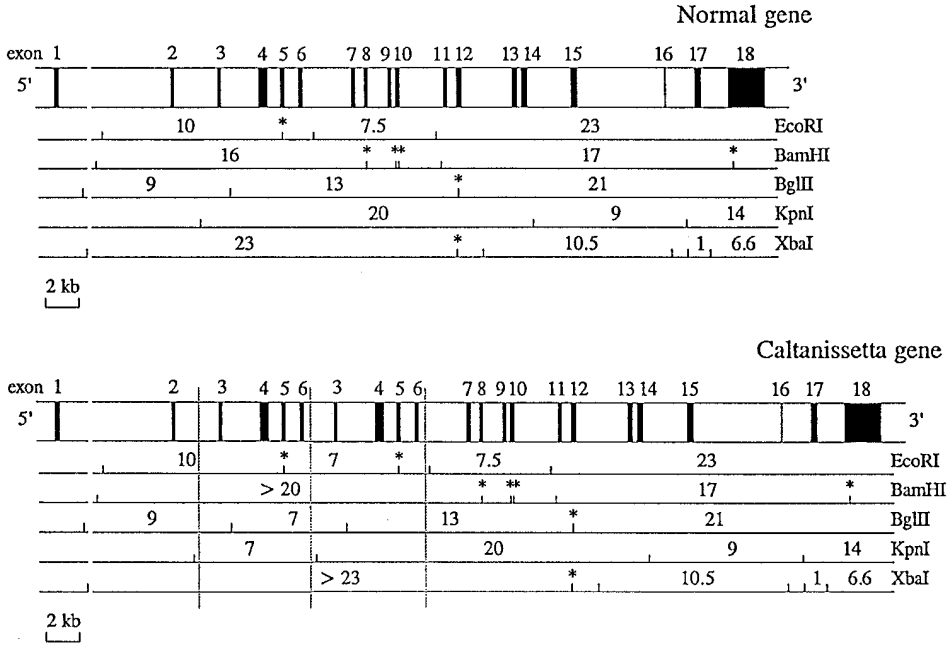

Fig. 1. Partial restriction map of the LDL receptor gene in proband C.D. (FH-Caltanissetta gene). The proband was found to be heterozygous for a 7 kb insertion due to a duplication of a region spanning from the 5′ end of intron 2 to the 5′ end of intron 6. Stars indicate the restriction sites in the exons.

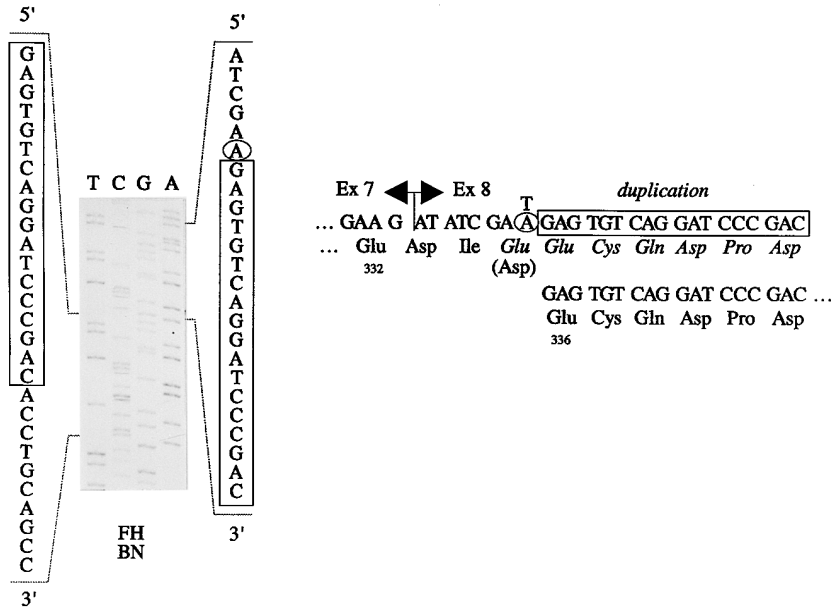

Fig. 2. Nucleotide sequence of mutant exon 8 in proband B.N. (FH-Chieti 2). Exon 8 was amplified by PCR from genomic DNA and cloned in a pCR TM vector. The inserts corresponding to the normal and mutant allele were isolated and sequenced. The sequence of the mutant allele, shown here, demonstrated the presence of two identical 18 bp sequences (boxed), one of which results from a duplication. The putative duplicated sequence is preceded by an A→T transversion (circle). The scheme shows the position of the duplicated sequence in exon 8. The mutation causes an in-frame duplication of six amino acids (shown in italics) that is preceded by an Asp→Glu substitution at codon 335 (shown in italics).

Proband B.N. SSCP analysis of all exons and the promoter region showed a grossly abnormal electrophoretic pattern of exon 8. The nucleotide sequence of this exon suggested that the mutant allele contained a duplication of 18 nucleotides (from the 9th to the 26th nucleotide of exon 8). To define this duplication in more detail, we separated the two alleles by cloning PCR amplified exon 8 in a pCR TM vector. EcoRV digestion generated fragments of 176 bp and 27 bp in the wild type insert, as opposed to 194 bp and 27 bp in the mutant insert. The sequence of the insert corresponding to the mutant allele (**Fig. 2**) confirmed the presence of a duplication of 18 nucleotides preceded by an A→T transversion. This duplication could be easily detected by electrophoretic separation of PCR amplified exon 8 in 2% agarose gel. Using this screening procedure we demonstrated that one of proband's sons (B.S.) was a carrier of the duplication. Other hypercholesterolemic family members (not indicated in Table 1) were also carriers of the same mutation.

Northern blot and reverse transcription PCR

Proband C.D. (FH-Caltanissetta). In order to ascertain whether the mRNA generated by the mutant allele was present in proband's cells, Northern blot analysis and RT-PCR were performed using RNA extracted from cultured skin fibroblasts. Northern blot analysis revealed the presence of a broad band, in the 5.3–5.9 kD region. RT-PCR of the exon 1–exon 4 region produced a single 367 bp fragment in control subjects, but two fragments (367 and 1117 bp, respectively) in proband C.D. (**Fig. 3**). The size of the larger fragment (1117 bp) was consistent with the presence of a duplication of exons 3 through 6 (750 bp) in the mRNA. To define whether in this abnormal mRNA exon 6 was followed by exon 3 bis (as suggested by the restriction map shown in Fig. 1) we performed a PCR amplification of the appropriate region of the cDNA, using a forward primer complementary to exon 6 and a reverse primer complementary to exon 4. The size of the RT-PCR fragment (268 bp) was consistent with the amplification of exon 6 and exons 3bis and 4bis. The nucleotide sequence of this fragment showed that exon 6 was followed by exon 3bis with no disruption of the reading frame. Only a single amino acid change (a valine for leucine substitution) was observed at the exon 6–exon 3bis junction (**Fig. 4**). Thus the receptor encoded by the mutant allele was predicted to contain an in-frame duplication of 250 amino acids (from residue 43 to residue 292) corresponding to repeats 2–7 of the ligand binding domain.

Proband B.N. (FH-Chieti-2). RT-PCR analysis of the exon 6-exon 9 region revealed the presence of a single fragment of 496 bp in mRNA from fibroblasts of a control and two fragments of 496 and 514 in mRNA from the proband (**Fig. 5**). In order to discriminate the two RT-PCR fragments seen in the proband, the RT-PCR products were digested with EcoRV. This digestion generated two fragments of 198 and 298 in the control and three fragments of 298, 316 and 198 bp in the proband (Fig. 5). As the intensities of the 316 bp and 298 bp bands were similar, we inferred that the mutant and wild type allele were equally expressed in proband's fibroblasts.

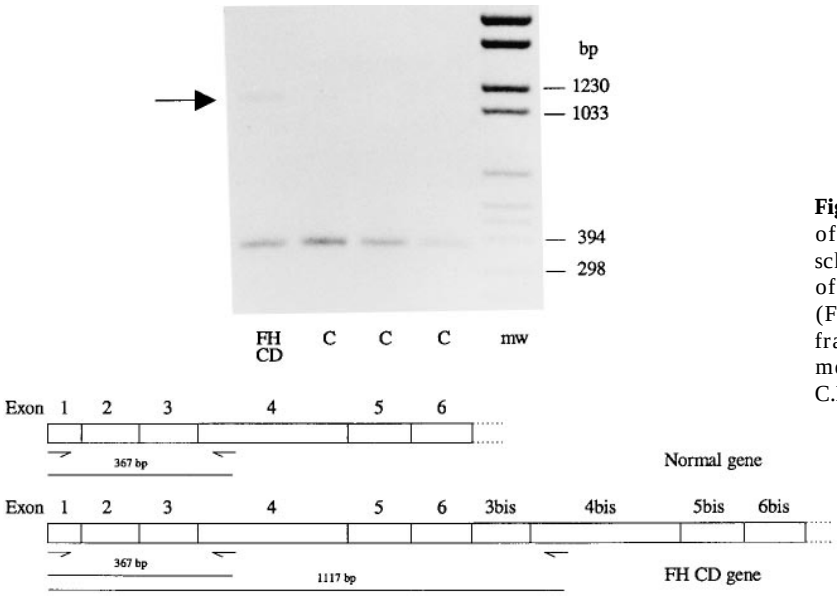

Fig. 3. Reverse transcription and PCR amplification of the LDL-receptor mRNA in proband C.D. The scheme shows the position of the primers and the size of the expected RT-PCR fragments in proband C.D. (FH-CD) and in control subjects (C). A single 367 bp fragment was observed in controls (C) and two fragments (367 bp and 1117 bp) were observed in proband C.D.

Blotting and immunoprecipitation of mutant receptors

Extracts of cultured fibroblasts from probands C.D. and B.N. were subjected to immunoblotting with two monoclonal antibodies against the LDL-receptor and to ligand blotting with LDL and βVLDL (**Fig. 6**). With mAb4B3, which recognizes an epitope in the second repeat of the binding region, cells from B.N. showed only the normal LDL receptor band (apparent M_r 128,000), whereas cells from C.D. gave an extra band of slower mobility with an apparent M_r of 160,000 ± 1.400 (mean ± SE of 9 observations) under non-reducing conditions. The same high molecular weight band was recognized on ligand blots with LDL and βVLDL. In both cases, as with mAb4B3, the higher molecular weight band bound more ligand than the normal band. The ratio of densities of the mutant to the normal band was 1.49 ± 0.13 (mean ± SE of 8 separate blots) for mAb4B3, 1.61 ± 0.18 for LDL (3 blots), and 1.51 ± 0.05 for βVLDL (3 blots). In contrast, mAb10A2 bound more to the normal band, with a ratio of 0.76 ± 0.04 (7 blots) (Fig. 6, lane h). Increasing the concentration of ligand or antibody did not affect the ratio observed.

To check that the mutant receptors reached the cell surface, cells from C.D. were treated with pronase at 4°C. Under these conditions both the normal and mutant receptors in cells from C.D. were digested. During 3 experiments, pronase reduced the density of the mutant band to 43 ± 10% (±SE) of the untreated value. The normal band in cells from C.D. was reduced to 33% ± 5% and the normal receptor band in normal cells to 36% ± 4%. Pronase digested the receptor on normal cells to a relatively stable intermediate of apparent M_r 115,000 that was de-

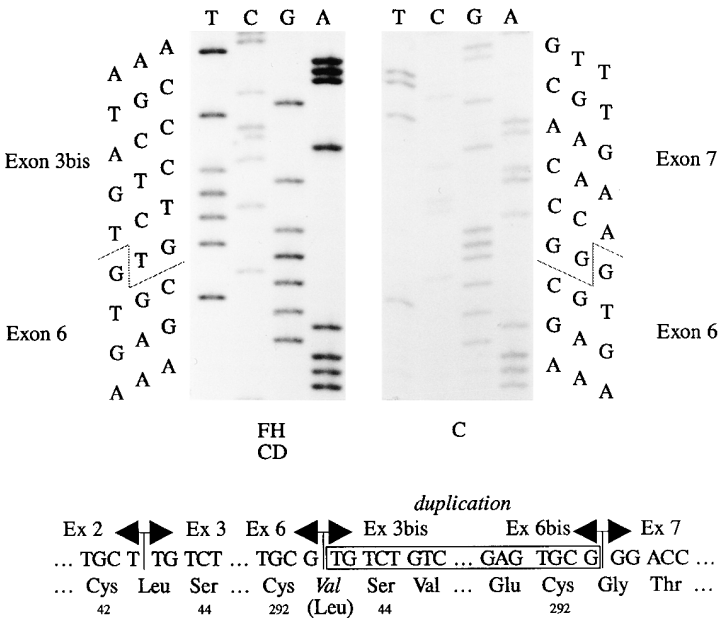

Fig. 4. Sequence of exon 6–exon 3 junction in proband C.D. Using a forward primer complementary to exon 6 and a reverse primer complementary to exon 4, a 268 bp region was obtained from mRNA by RT-PCR (see the scheme in Fig. 3). The figure shows the 5′ end of this RT-PCR fragment corresponding to the exon 6–exon 3bis junction (FH-CD). The control sequence (C) shows the normal exon 6–exon 7 junction. The scheme shows that exon 3–exon 6 duplication does not disrupt the reading frame. The joining of exon 6 with exon 3 bis causes a Leu→Val substitution (shown in italics) at codon 293.

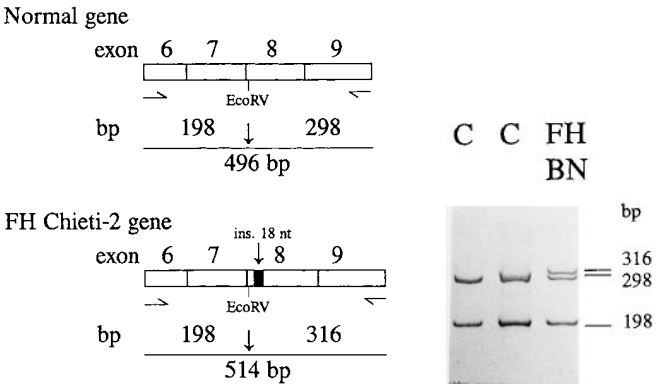

Fig. 5. Reverse transcription and PCR amplification of exon 6–exon 9 region of LDL receptor mRNA in proband B.N (FH Chieti-2 gene). The amplified fragment was digested with EcoRV whose restriction site is located at the 5′ end of exon 8. The digestion products were separated by 10% polyacrylamide gel electrophoresis. FH-BN, proband B.N.; C , control subjects.

tected by mAb10A2 but not by mAb4B3 (ref. 15 and Fig. 6 lane, g). With cells from C.D. there was no evidence for a larger degradation product and the densities of the bands indicated that both the normal and mutant receptors had been digested to the same-sized intermediate (Fig. 6, lane i). To discover whether the normal and mutant receptors could interact with each other on the cell surface, cells from C.D. were treated with cross-linking agent DTSSP at 4°C, which cross-linked receptors on normal cells to a dimer of apparent M_r 215,000 (ref. 12 and Fig. 6, lane j). With cells from C.D. this normal dimer was visible, together with two other bands of similar intensity, of apparent M_r 230,000 and 241,000, which are presumably the normal-mutant heterodimer and the mutant-mutant homodimer (Fig. 6, lane k).

To examine the synthesis and breakdown of the mutant receptors, cells were incubated with [^{35}S]methionine and the radioactivity incorporated into LDL-receptor protein was determined after immunoprecipitation with mAb4B3 and SDS-PAGE under reducing conditions. After a 2-h incubation, cells from C.D. showed a band at 120,000 M_r that corresponded to the normal precursor protein and a broad band of about 160,000 M_r in the position of the normal mature protein (**Fig. 7**, lane a). In addition there was a band of apparent M_r 193,000 that increased in intensity during a 1-h chase period with non-radioactive methionine (Fig. 7, lanes a–c), suggesting that it represented the mature form of the mutant protein with the extra binding region. In the same experiment, cells from B.N. showed an accumulation of radioactivity at the position of the normal precursor band, which decayed only slowly during the chase period (Fig 7, lanes d–f). A detailed time course showed a much slower loss of radioactivity from the precursor band in cells from B.N. than in normal cells, but a similar rise of radioactivity in the mature band (Fig. 7A).

In subsequent experiments (e.g., Fig. 7, lane j), it became clear that the broad central band observed with extracts of cells from C.D. was made up of two bands, one corresponding to the normal mature receptor and one with an apparent M_r of 171,000, which was identified as the precursor of the large mutant protein. After incubation with [^{35}S]methionine for only 30 min, there was little radioactivity incorporated into the mature proteins, which allowed the precursor to be seen more clearly (Fig. 7, lane g). During a chase period with unlabeled methionine, radioactivity was lost from the precursor and appeared in

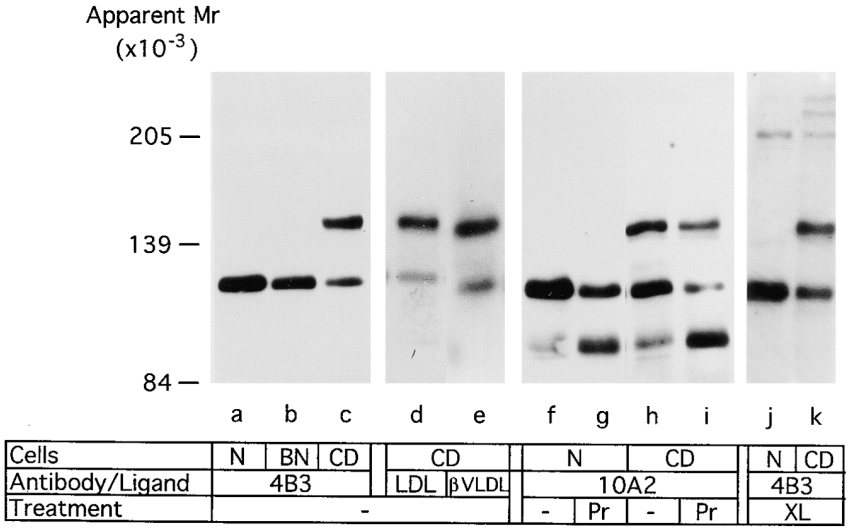

Fig. 6. Immuno- and ligand blotting of LDL receptors from cells from C.D. and B.N. Cultured fibroblasts form the heterozygous FH subjects C.D. and B.N. were pre-incubated for 40 h in a medium containing LPDS and then at 4°C with 5 μg/ml pronase (Pr) or 1 mg/ml DTSSP (XL) as required. Triton X-100-soluble proteins were separated by SDS-PAGE under non-reducing conditions, transferred to nitrocellulose membranes, and the receptors were detected with monoclonal antibodies 4B3 and 10A2 or with LDL and βVLDL as indicated. Values of apparent M_r are those of protein standards run on the same gels.

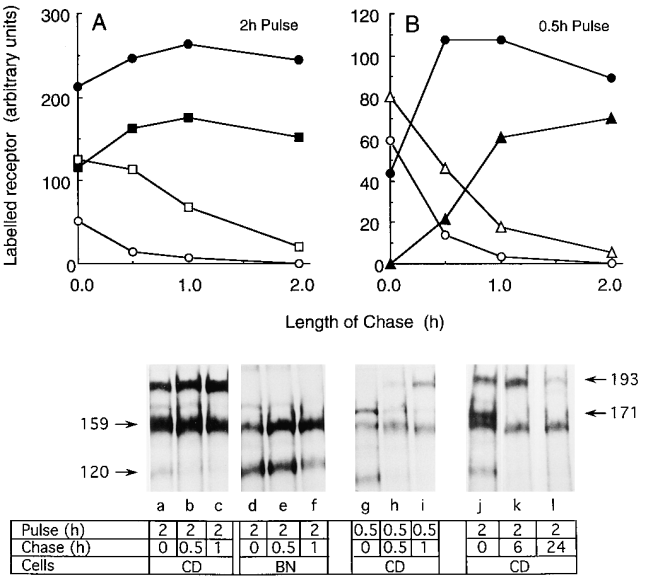

Fig. 7. Time course of incorporation of [^{35}S]methionine into the precursor (open symbols) and mature (closed symbols) forms of the LDL receptor. A: incorporation into the receptors of normal cells (○, ●) or cells from B.N. (□, ■). B: incorporation into the normal (○, ●) or mutant (△, ▲) receptors of cells from C.D. Fibroblasts were incubated for 14 h in medium containing LPDS and then for a 2 h (A) or 30 min (B) pulse incubation with [^{35}S]methionine, followed by a chase incubation with non-radioactive methionine as shown. Cell extracts were immunoprecipitated with mAb4B3 and the precipitated proteins were solubilized and separated by SDS-PAGE under reducing conditions. Relative amounts of radioactivity in the various receptor bands were estimated from densitometric scans of autoradiograms of the dried gels, some of which are shown beneath the graphs. Values were adjusted for the protein content of the cell extracts. Figures beside the autoradiograms refer to the values of apparent M_r ($\times 10^{-3}$), obtained from known standards, for the normal precursor (120) and mature (159) receptors and for the mutant precursor (171) and mature (193) receptors of cells from C.D.

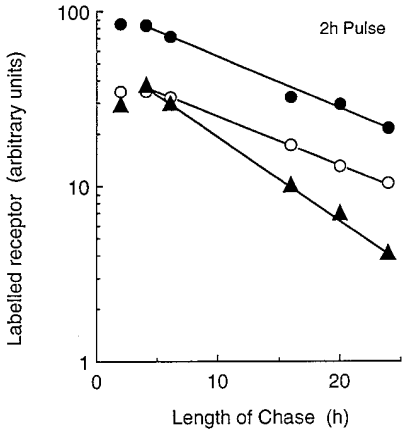

Fig. 8. Decay of radioactivity from pre-labeled LDL receptors of normal cells (●) and from normal (○) and mutant (▲) receptors of cells from C.D. Fibroblasts were pre-incubated for 14 h with medium containing LPDS and for 2 h with [^{35}S]methionine, before being incubated for the indicated periods with 200 μm unlabeled methionine. LDL-receptor proteins were immunoprecipitated with mAb4B3 and separated by SDS-PAGE under reducing conditions. The radioactivity in each band was estimated from densitometric scans of autoradiograms of the dried gels. Values were corrected for the protein content of the original cell extracts.

the mature protein slightly more slowly than for the normal receptor in the same cells, although essentially all was eventually transferred (Fig. 7B). After a 4-h chase there were roughly equal amounts of radioactivity in the mature mutant and mature normal receptor bands (Fig. 7, lane k). However, 20 h later, the mutant band was clearly lighter (Fig. 7, lane l) and a detailed curve showed that the mutant receptor was degraded more rapidly than the normal receptor in the same cells (**Fig. 8**). The half life of the decay of radioactivity from the normal receptor in cells from C.D. was approximately 11 h, which is similar to that in normal cells, whereas the half-life for the mutant receptor was approximately 6 h.

Binding and catabolism of lipoproteins

Binding of LDL or βVLDL to fibroblasts from the probands C.D. and B.N. was assayed at 4°C. **Figure 9** shows the Scatchard (17) plots of values obtained with different concentrations of ligand. Maximum binding of LDL to cells from C.D. and B.N. was much lower than binding to normal cells. However, whereas the apparent affinity of the receptors on cells from B.N. was similar to normal, that of the receptors on cells from C.D. was greater. During 4 experiments there was a statistically significant reduction in maximum binding of LDL to cells from C.D. to about 70% of that of the normal cells, with a significant increase in apparent affinity (**Table 2**). These differences were also observed at 37°C. Heparin-releasable binding of LDL (**Fig. 10A**) as well as LDL internalization and degradation (Fig. 10 and Table 2) all showed a reduced maximum with a decrease in the concentration required to give one-half of the maximum. There was no such increase in affinity when βVLDL was used as ligand (Fig. 9B). Indeed at both 4C and 37°C there was an apparent decrease in the affinity of the receptors on cells from C.D. for βVLDL, with little effect on maximum binding or degradation (Table 2).

DISCUSSION

In this study we analyzed two mutations of the LDL-receptor gene and studied their effects on the LDL-receptor pathway in two Italian FH-heterozygotes. Proband B.N. was found to carry a minute duplication of 18 nt, (preceded by a single base substitution) at the 5′ end of exon 8. This duplication is located in a nucleotide sequence that contains several di-trinucleotide direct repeats which are thought to be hot spots for intragenic recombination (18). Several examples of minute rearrangements of the LDL-receptor gene involving regions containing short direct or inverted repeats have been reported (19–21). As the analysis of LDL-receptor mRNA in fibroblasts from B.N. revealed that the mutant mRNA (i.e., that generated

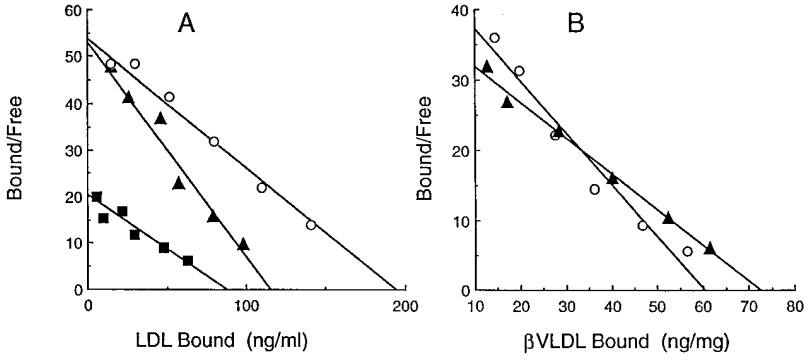

Fig. 9. Binding of LDL (A) and βVLDL (B) to fibroblasts from a normal subject (○) and heterozygous FH subjects B.N. (■) and C.D. (▲). Cells in 35-mm Multi-wells were pre-incubated for 40 h in medium containing LPDS and for 2 h at 4°C with 0.31, 0.63, 1.25, 2.5, 5.0, or 10.0 μg protein/ml of ^{125}I-labeled LDL or βVLDL. The amount of radioactivity bound was determined after the cells had been extensively washed and was corrected for non-saturable binding determined in the presence of an excess of the appropriate unlabeled lipoprotein. Values are presented according to the analysis described by Scatchard (ref. 17) and are the averages of duplicate incubations. The intercept on the x-axis gives the maximum binding, while the slope of the line is proportional to the reciprocal of the dissociation constant.

by the mutant allele) was expressed in similar amounts as its normal counterpart, and that the duplication did not disrupt the reading frame, we predicted that fibroblasts from B.N. should synthesize a receptor protein containing 6 additional amino acids in repeat B of the EGF precursor homology domain. This protein would not be separated from the normal protein in the electrophoretic system we have used. However cells from B.N. showed an abnormal accumulation of radioactivity in the precursor band during a pulse incubation with [^{35}S]methionine. Assuming that the normal protein in cells from B.N. behaved as it did in normal cells, the pattern of radioactivity lost from the precursor band and appearing in the mature band (Fig. 7A) suggests that little, if any, of the mutant protein was processed to the mature form. This is consistent with the binding results, which show binding of LDL with normal affinity and approximately one-half of the maximum of normal cells. Taken together, the results suggest that the mutant protein with the small duplication produced by cells from B.N. is rapidly degraded before it matures and does not reach the cell surface.

The analysis of the LDL receptor gene in proband C.D. revealed the presence of a 7 kb insertion due to the duplication of a region spanning from intron 2 to intron 6. The mutant allele was transcribed into an mRNA that was detectable in proband's fibroblasts and whose sequence demonstrated the presence of a duplication of exons 3 through 6, with no disruption of the reading frame. The receptor protein encoded by this mutant allele was predicted to be an elongated protein containing a duplication of repeats 2–7 of the binding domain of the LDL-receptor. This is the second large duplication involving the binding domain reported so far in the literature. Lehrman et al. (22) have previously reported a 14 kb duplication that encompassed exons 2 through 8. It is possible that the duplication found in proband C.D. resulted from an unequal crossing over

TABLE 2. Binding and uptake of LDL and βVLDL by fibroblasts from a normal subject and from heterozygous FH subject C.D.

		Maximum (Conc. for half-maximum)		
	Ligand	Normal Cells	C.D. Cells	C.D./Normal
		ng/mg (μg/ml)		*%*
4°C Binding	LDL	147 ± 43 (3.19 ± 0.05)	99 ± 27 (2.37 ± 0.18[a])	68.8 ± 2.6[a]
37°C Cell-association	LDL	1858 ± 370 (8.39 ± 0.40)	1355 ± 167 (5.91 ± 0.88[a])	78.3 ± 10.3
37°C Degradation	LDL	3574 ± 299 (10.45 ± 1.74)	2622 ± 259 (7.33 ± 0.90)	73.0 ± 2.8[a]
4°C Binding	βVLDL	99.0 ± 26.5 (1.60 ± 0.22)	108.3 ± 20.7 (1.83 ± 0.23)	114.1 ± 9.5
37°C Degradation	βVLDL	1268 ± 54 (1.99 ± 0.30)	1100.3 ± 66.4 (3.07 ± 0.53[b])	85.6 ± 1.3[a]

Values were obtained from experiments such as those shown in Fig. 9 and Fig. 10. Maximum binding and apparent K_d values at 4°C were obtained by Scatchard analysis (17). Maximum values for cell-association and degradation at 37°C were estimated assuming that the concentration curves followed a rectangular hyperbola. The concentrations that gave half-maximum values shown in parentheses were read from the curves. Values are means ± SE for 4 experiments with LDL and 3 experiments with βVLDL. The ratio of the maximum value given by cells from C.D. were expressed as a percentage of that given by the normal cells in the same experiment, and the mean ± SE of these is given in the right-hand column.

[a] Significant ($P < 0.05$) difference between C.D. and normal (unpaired *t*-test).

[b] Significant ($P < 0.05$) difference between C.D. and normal (paired *t*-test).

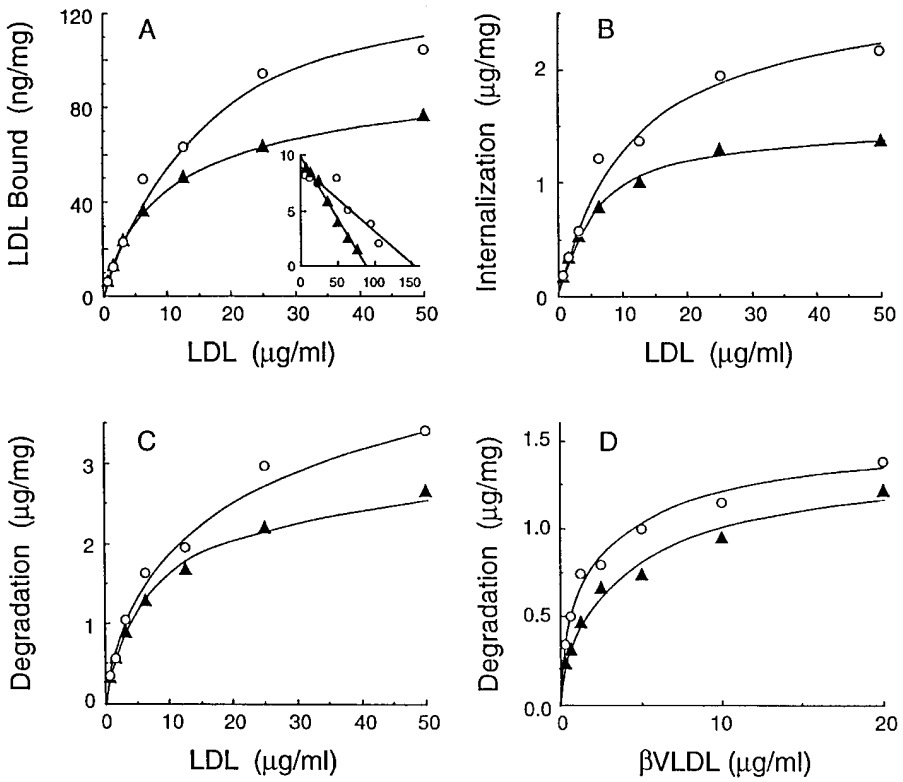

Fig. 10. Cell-association and degradation of LDL and βVLDL by normal (○) and cells from C.D. (▲) at 37°C. Fibroblasts were pre-incubated for 40 h with medium containing LPDS and then for 4 h at 37°C with the indicated concentration of ^{125}I-labeled LDL or βVLDL. With LDL as ligand the cells were extensively washed and assayed for heparin-releasable (bound, A) or non-heparin-releasable (internalized, B) radioactivity. With both ligands the medium was assayed for trichloroacetic acid-soluble, non-iodide degradation products. Values are the averages of duplicate incubations and are corrected for non-saturable association or degradation observed in the presence of an excess of the appropriate unlabeled lipoprotein. A Scatchard (17) analysis of the binding (bound/free against bound, see Fig. 9) is shown as an insert in A.

between intron 6 and intron 2 in two LDL-receptor genes, possibly due to the presence of direct or inverted repeats (23). One product of this unequal crossing over would correspond to the mutant allele found in proband C.D (Fig. 1) whereas the other product is expected to carry a deletion of exons 2 through 6. It is of interest that this deleted allele was reported in a Danish FH-heterozygote (FH-DK-2) carrying a 9 kb deletion spanning from intron 2 to the 5′ end of intron 6 (24).

The mutant LDL-receptor protein in cells from C.D. was processed slightly more slowly than normal, but all was apparently converted to a mature form of a size consistent with a protein containing 6 extra cysteine-rich repeats. There was no significant difference in the susceptibility of the mutant and normal proteins to pronase at 4°C, suggesting that the mutant receptor was distributed normally between the cell surface and endocytic vesicles.

The mature, mutant receptor in cells from C.D. was degraded more rapidly than the normal LDL-receptor. This is a further example of accelerated degradation of LDL-receptors that we originally observed with a receptor containing a large duplication of part of the EGF-precursor domain (25). Both reported examples of accelerated receptor degradation have been with proteins that could be easily distinguished on electrophoresis by their size. It is possible that this type of defect is relatively common but avoids detection because the majority of mutant LDL-receptor proteins are similar in size to the normal and cannot be readily separated. The reason for the accelerated degradation is not known. However, it is clear that neither the slightly delayed processing nor the accelerated degradation of the mutant protein had any effect on the properties of the normal receptors in the same cells. Thus in cells from C.D., as in those heterozygous for the EGF-precursor duplication (25), there is no evidence to suggest that the intracellular behavior of either the mutant or normal receptors is influenced by strong heterodimer formation.

Immunoblotting with mAb10A2, which recognizes an epitope outside the duplicated binding region, indicated that cells from C.D. contained only 75% as much of the mutant receptor protein as of the normal receptor protein. This is consistent with the more rapid breakdown of the mutant receptors. On ligand blots, the mutant receptors bound twice as much LDL or βVLDL as the normal receptors, indicating that, despite their close proximity, each of the binding regions was capable of binding lipoproteins simultaneously. If the mutant receptors behaved in intact cells as they did on blots, cells from C.D. should

have bound more LDL than normal cells. However, this was apparently not the case. The proband's family clearly exhibited hypercholesterolemia that segregated with the inheritance of the mutant gene. Furthermore, cultured fibroblasts from C.D. bound only 70% as much LDL as normal cells, although with much higher affinity. Thus there must have been interaction between receptors on the surface of the cell that blocked some of the binding sites and increased the affinity for LDL. The normal and mutant receptors were certainly close enough to interact, because heterodimers as well as homodimers were observed on cross-linking with DTSSP (Fig. 6, lanes j and k). The absolute values obtained from experiments such as these are not consistent enough to show which binding sites were blocked. Indeed, it may not be as simple as the blocking of a single site, as the binding and catabolism of βVLDL showed a decrease rather than an increase in affinity to cells from C.D., with a smaller effect on the amount bound. Thus these results provide further evidence that the way in which receptors can interact on the cell surface can be important in determining the ability of cells to bind and degrade LDL, and in some instances can be the main factor responsible for the observed phenotype.

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