Unexpected Consequences of Deletion of the First Two
Repeats of the Ligand-binding Domain from the
Low Density Lipoprotein Receptor

EVIDENCE FROM A HUMAN MUTATION*

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Heterozygosity for a 5-kilobase (kb) deletion of the first two ligand-binding repeats (exons 2 and 3) of the
low density lipoprotein (LDL) receptor (R) gene (LDL-R Δ5kb) confers familial hypercholesterolemia (FH). The
FH phenotype is unexpected based on previous site-directed mutagenesis showing that deletion of exons 2
and 3 resulted in little or no defect in LDL-R activity. In the present study, we took unique advantage of the ability
to distinguish the LDL-R Δ5kb from the normal receptor on the basis of size, in order to resolve this apparent
discrepancy. Fibroblasts from heterozygotes for the LDL-R Δ5kb displayed 50% of normal capacity to
bind LDL and β-VLDL, apparently due to lower receptor number. Cellular mRNA for the Δ5kb allele was at least
as abundant as that for the normal allele. Immunoblotting and cell binding assays with anti-LDL-R antibody
IgG-4A4 demonstrated normal synthesis and transport of the Δ5kb receptor. Ligand blotting demonstrated that
the Δ5kb receptor displayed minimal or no ability to bind LDL or β-VLDL. Thus, in contrast to transfected
cell lines, in human fibroblasts, the first two cysteine-rich repeats of the LDL-R appear functionally necessary.
These characteristics of the LDL-R Δ5kb in human fibroblasts explain the in vivo phenotype of carriers.

The low density lipoprotein (LDL) receptor (R) binds and catabolizes apolipoprotein E-containing chylomicron
and VLDL remnants and LDL. In the liver, LDL-R functions to remove these lipoproteins from plasma for eventual excretion
of the cholesterol into the bile. In peripheral cells, it functions to provide the cell with cholesterol needed for membrane synthesis.
The LDL-R contains five major structural domains: a seven-repeat, cysteine-rich ligand binding domain encoded by
exons 2–6, an epidermal growth factor-precursor homology domain (exons 7–14), a glycosylation domain (exon 15), a mem-
brane-spanning domain (exon 16), and a cytoplasmic tail (exon 17) (1). The LDL-R is one of the few proteins for which knowledge
of the structure-function relationship has been generated both from site-directed mutagenesis and from numerous naturally
occurring human mutations.

Mutations in the LDL-R gene resulting in a dysfunctional receptor cause a codominantly inherited disorder of plasma
cholesterol catabolism known as familial hypercholesterolemia (FH). Human LDL-R mutations have been assigned to five
classes of defects based on their phenotypic effects on the receptor protein (1). We have previously described a deletion of
approximately 5 kb, which removes exons 2 and 3 of the LDL-R gene (LDL-R Δ5kb) (2). In site-directed mutagenesis experiments,
deletion of the first repeat (exon 2) has no effect on the binding or internalization of LDL or β-VLDL or recycling of
receptors in transfected mammalian cells (3). Simultaneous deletion of exons 2 and 3 has resulted in a receptor which binds
LDL 70% as well as the normal receptor and which binds β-VLDL equally as well (4). These results have led to the suggestion that
the first two repeats of the LDL-R ligand-binding domain are not necessary for LDL-R function.

Some studies have shown that the clinical phenotype resulting from LDL-R mutations correlates with biochemical pheno-
type or class (5, 6). As such, one would expect that heterozy-
gosity for the LDL-R Δ5kb would result in relatively mild or no expression of familial hypercholesterolemia (FH). However,
taking advantage of genetic founder effects among French Ca-
nadians, we have observed that plasma total and LDL choles-
terol levels among 8 probands for this deletion are indistin-
guishable from those in heterozygotes for a null LDL-R allele.

In the context of a clinical genetic study of a kindred with the
5-kb LDL-R gene deletion (7), we noted that heterozygote
(HTZ) fibroblasts displayed consistently 50–60% the maximal
receptor activity of normal cells. This was again unexpected
based on the apparent activity of the LDL-R lacking exons 2
and 3 in transfected cells.

Normally, the biochemical consequences of LDL-R mutations in cells from carriers are difficult to study in the absence of a
homozygote. Unlike the case for the vast majority of described mutations of the LDL-R, the LDL-R Δ5kb deleted protein is distinguishable
from the normal receptor on the basis of size. This situation offers a unique opportunity to examine and
compare the mRNA, protein, and ligand binding to the mutant receptor with those of the normal receptor in vivo within the
same cell. The present study provides evidence for important differences in the consequences of deletion of exons 2 and 3
from the LDL-R gene as assessed by site-directed mutagenesis and by analysis of heterozygous fibroblasts.
Cell Surface Binding of Lipoproteins and Antibodies—Human skin fibroblasts were obtained from normal subjects and from FH patients heterozygous for the LDL-R deletion. Receptor-negative fibroblasts were obtained from an FH patient homozygous for the >10-kb deletion (8). Lipoprotein-deficient serum (LPDS) and LDL were isolated from human plasma (9). β-VLDL were isolated from blood of cholesterol-fed rabbits (10). Lipoproteins were iodinated with carrier-free 125I-sodium (Amersham, Oakville, ON) with the IODOGEN (Pierce) method (11). Monoclonal anti-LDL-R antibody IgG-4A4 was isolated from hybridoma cells (American Type Culture Collection, Rockville, MD) as described (12), purified with MAb 1P-CA Protein A-Sepharose (Pharmacia) as recommended by the manufacturer, and iodinated with IODOBEADS® (13) (Pierce). Following a 48-h incubation of the cells in 10% LPDS, cell surface binding at 4°C of 125I-LDL, 125I-β-VLDL, and 125I-β-globulin were performed as described (9). Estimates of receptor number and affinity were calculated by the method of Scatchard.

LDL Immunoblotting—Cell protein extracts were prepared generally as described (14). Cells were washed with phosphate-buffered saline containing 1.5 mM phenylmethylsulfonyl fluoride. After centrifugation at 10,000 × g for 30 s, cells were lysed in 180 µl of 50 mM Tris-maleate, pH 6.5, 2 mM CaCl$_2$, 1% Triton X-100, and 1.5 mM phenylmethylsulfonyl fluoride for 20 min. Cellular debris was pelleted by centrifugation at 10,000 × g for 5 min, and the lysates were stored at −70°C. To obtain 50–100 µg of cell lysate, each sample was subjected to SDS-6% polyacrylamide gel electrophoresis and fluororescence detection and quantification on a model 373A Automated DNA Sequencer® (Applied Biosystems). The Hind1 polymorphism in exon 12 of the LDL-R gene (18) was detected in cDNA as follows. cDNA from fibroblasts or lymphocytes was amplified with 100 ng each of primers 5'-ctccggctgcaggccctct-ctgctgagagc-3' and 5'-ctgctggagcttcatctgctgtgc-3' in the presence of 10 µM fluorescein-12-labeled dUTP (Boehringer Mannheim, Laval, Quebec). Aliquots of the reactions were digested with Hind1. Fluorescence peak areas were compared to DNA fragments of molecular weight 140 and 204 base pairs, corresponding to the presence or absence of the Hind1 site, were quantified by integration with 672 GeneScanner® software (Applied Biosystems).

RESULTS

Phenotypic Expression of the LDL-R Δ5kb in Vivo and in Vitro—Plasma total and LDL cholesterol concentrations in 8 probands heterozygous for the LDL-R Δ5kb were not significantly different from those in heterozygotes for a deletion in the LDL-R gene which results in a null allele (8) (not shown).

Binding experiments with 125I-LDL at 4°C and Scatchard analysis (Fig. 1A) revealed that the apparent defect in LDL-R activity in LDL-R Δ5kb fibroblasts was due to an apparently lower number of binding sites (94 versus 159 ng of ligand/mg of cellular protein for LDL-R Δ5kb HTZ and normal fibroblasts, respectively) with no difference in receptor affinity (2.96 and 3.12 µg/μl) compared to normal subjects. A similar experiment with β-VLDL as ligand revealed a defect in binding by Δ5kb fibroblasts of approximately 50% that was also associated with lower receptor number (205 versus 457 ng/mg) and with higher affinity (0.42 versus 0.83 µg/μl) (Fig. 1B).

LDL-R mRNA in LDL-R Δ5kb Fibroblasts—Although LDL-R activity in LDL-R Δ5kb fibroblasts was only 50–60% that of normal cells, the ratio of LDL-R mRNA to that for β-actin in fibroblasts and in lymphocytes was similar in carriers and non-carriers (7). To compare the relative amount of LDL-R mRNA corresponding to the deleted allele with that of the normal allele, primers surrounding the deletion were designed (see “Materials and Methods”). Surprisingly, in fibroblast-derived cDNA from 5 related and 2 unrelated heterozygotes for the LDL-R Δ5kb, the deleted allele was consistently overexpressed compared to the normal allele by approximately 50% (Fig. 2). To see if the apparent overexpression of the deleted allele could result from more efficient amplification of a shorter DNA fragment, the Δ5kb and normal alleles were distinguished with a restriction polymorphism (18) in exon 12 of the LDL-R gene. Haplotype analysis in a kindred (7) containing LDL-R Δ5kb heterozygotes revealed that the LDL-R Δ5kb allele did not contain the polymorphic Hind1 site (data not shown). Digestion of the LDL-R exon 12 amplified from cDNA of LDL-R Δ5kb HTZ fibroblasts in which the normal LDL-R allele contained the Hind1 site revealed approximately 50% greater undigested than digested fragment (data not shown). Thus, low receptor activity in LDL-R Δ5kb HTZ fibroblasts cannot be attributed to defective transcription of the Δ5kb allele.

LDL-R Immunoblotting—Because the recognition epitope of IgG-C7 is the first repeat of the ligand-binding domain (3), this antibody was not expected to recognize the LDL-R Δ5kb. Consistent with this, only one band of apparent molecular mass of 140 kDa (average value from 9 independent experiments) was observed when blots of protein extracts from normal and LDL-R Δ5kb HTZ fibroblasts were probed with this antibody (Fig. 3, left, LDL-R IgG-C7). When similar blots were probed with monoclonal anti-LDL-R antibody IgG-4C4, raised against the carboxyl terminus of the LDL-R (3), only one band was seen for normal fibroblasts, while two bands were seen in LDL-R Δ5kb HTZ fibroblasts (Fig. 3, left, LDL-R IgG-4A4). The second band, not revealed with IgG-C7, appears at approximately 127 kDa, consistent with the deletion of amino acids encoded by 2 and 3 resulting in the loss of 83 amino acids (approximately

MATERIALS AND METHODS

Characterization of the FH Phenotype—Plasma total and LDL cholesterol were measured as described (7). The presence of the French-Canadian LDL-R >10- and 5-kb gene deletions was determined by Southern blotting as described (2).

Cell Surface Binding of Lipoproteins and Antibodies—Human skin fibroblasts were obtained from normal subjects and from FH patients heterozygous for the Δ5kb deletion. Receptor-negative fibroblasts were obtained from an FH patient homozygous for the >10-kb deletion (8). Lipoprotein-deficient serum (LPDS) and LDL were isolated from human plasma (9). β-VLDL were isolated from blood of cholesterol-fed rabbits (10). Lipoproteins were iodinated with carrier-free 125I-sodium (Amersham, Oakville, ON) with the IODOGEN (Pierce) method (11). Monoclonal anti-LDL-R antibody IgG-4A4 was isolated from hybridoma cells (American Type Culture Collection, Rockville, MD) as described (12), purified with MAb 1P-CA Protein A-Sepharose (Pharmacia) as recommended by the manufacturer, and iodinated with IODOBEADS® (13) (Pierce). Following a 48-h incubation of the cells in 10% LPDS, cell surface binding at 4°C of 125I-LDL, 125I-β-VLDL, and 125I-β-globulin were performed as described (9). Estimates of receptor number and affinity were calculated by the method of Scatchard.

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The relative amounts of LDL-R protein was assessed by densitometry in 4 independent experiments. The amount of total LDL-R protein was similar in normal and Δ5kb HTZ fibroblasts (Fig. 3, lower right). The amount of protein corresponding to the normal and LDL-R Δ5kb allele was approximately equal in fibroblasts from 4 LDL-R Δ5kb HTZ (Fig. 3, left, LDL-R IgG-4A4, and lower right panel). No LDL-R protein was detected with extracts from receptor-negative fibroblasts with either antibody (Fig. 3, left, Δ10kb HMZ). Thus, the LDL-R Δ5kb protein appears to be synthesized in normal amounts.

Treatment of cell protein extracts from LDL-R Δ5kb HTZ fibroblasts with neuraminidase reduced the apparent size of the normal receptor from 147 to 134 kDa (Fig. 4), consistent with previous reports of 10–15-kDa reduction (19, 20). A reduction of apparent molecular mass was also observed for the Δ5kb protein, from 134 to 117 kDa. The difference in size between the normal and deleted receptor was similar before and after neuraminidase treatment. Thus, no defect in glycosylation of the Δ5kb receptor was detected.

Cell Surface Binding of 125I-IgG-4A4 to LDL-R Δ5kb HTZ and Normal Fibroblasts—To address the possibility that low LDL-R activity in LDL-R Δ5kb HTZ fibroblasts with neuraminidase reduced the apparent size of the normal receptor from 147 to 134 kDa (Fig. 4), consistent with previous reports of 10–15-kDa reduction (19, 20). A reduction of apparent molecular mass was also observed for the Δ5kb protein, from 134 to 117 kDa. The difference in size between the normal and deleted receptor was similar before and after neuraminidase treatment. Thus, no defect in glycosylation of the Δ5kb receptor was detected.

Cell Surface Binding of 125I-IgG-4A4 to LDL-R Δ5kb HTZ and Normal Fibroblasts—To address the possibility that low LDL-R activity in LDL-R Δ5kb HTZ fibroblasts could be attributable to defective transport of the Δ5kb receptor to the cell surface, binding of anti-LDL-R antibodies to fibroblasts was studied. The number of binding sites for 125IgG-4A4, which recognizes both the normal and Δ5kb receptor, did not differ in normal and LDL-R Δ5kb HTZ fibroblasts (13 ng/mg cell protein, Fig. 5), despite lower affinity for the antibody in Δ5kb HTZ fibroblasts (0.43 versus 0.14 μg/ml). A similar result was obtained in a second experiment, in which specific binding of 1 and 5 μg of 125IgG-4A4 by LDL-R Δ5kb HTZ fibroblasts (measured in the presence of a 50-fold excess of unlabeled antibody) was 56 and 75% of normal (data not shown). Binding of 1 and 5 μg of 125IgG-4A4 to LDL receptor-negative fibroblasts was 19 and 21% of that in normal fibroblasts.

Ligand Blotting of LDL-R—Ligand blotting of fibroblast protein extracts with 125I-LDL resulted in a band of approximately 140 kDa in normal fibroblasts (Fig. 6, lanes 2 and 6) but none in LDL-R-deficient fibroblasts (Fig. 6, lanes 1, 5, and 10). Ligand blotting of cell protein from LDL-R Δ5kb HTZ fibroblasts revealed the same band as seen in normal subjects, but of lower intensity, and, in only 1 of 4 instances, a faint band of lower
molecular mass (Fig. 6, lane 9), corresponding to the Δ5kb form of the LDL-R. In 2 of 3 independent preparations, no binding of 125I-LDL to the Δ5kb receptor was detectable (Fig. 6, lanes 3, 4, 7, and 8), even after 6 days of exposure. Similar experiments with 125I-β-VLDL revealed interaction of this ligand with the deleted receptor which was detectable but weak (Fig. 6B). Immunoblotting performed in parallel on these same protein extracts revealed immunoreactive protein corresponding to both the normal and Δ5kb receptor in equal amounts. Thus, the Δ5kb receptor displays little or no ability to bind LDL or β-VLDL under these experimental conditions.

**DISCUSSION**

Site-directed mutagenesis experiments have shown minimal loss of LDL and β-VLDL binding, respectively, from an LDL receptor lacking exons 2 and 3 encoding the first two of seven ligand binding repeats (3, 4). Based on this information it is surprising that in French Canadians, with the exception of the e2 allele (7), heterozygosity for a 5-kb deletion of the LDL-R gene (LDL-R Δ5kb) encompassing exons 2 and 3 is associated with plasma LDL and total cholesterol levels which are equally as elevated as those associated with heterozygosity for an LDL-R null allele. The present study sought to resolve this apparent contradiction between in vivo phenotype and in vitro consequences of the LDL-R deletion.

Among possibilities to explain the association of the LDL-R Δ5kb with FH were decreased mRNA or protein synthesis, slow transport to the cell surface, or poor affinity of the receptor for LDL. The first two possibilities were ruled out by measurements of normal levels of mRNA and normal levels of LDL-R protein in LDL-R Δ5kb HTZ fibroblasts. The receptor appeared to be glycosylated normally, implying normal processing (21). LDL-R Δ5kb HTZ fibroblasts bound similar amounts of anti-LDL-R antibody IgG-4A4 as did normal cells, also suggesting normal transport of the Δ5kb receptor to the cell surface. However, ligand blotting of cell protein extracts from LDL-R Δ5kb HTZ fibroblasts revealed little or no LDL binding to the Δ5kb receptor. Thus, the apparent reduction of 50% in receptor num-

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**FIG. 3. Immunodetection of LDL-R in protein extracts from normal and LDL-R Δ5kb HTZ fibroblasts.** Forty μg of fibroblast protein extracts were applied to 6% SDS-polyacrylamide gels. Membranes were probed with IgG-C7 which recognizes the first repeat of the ligand-binding domain (upper left) or with IgG-4A4 directed against the carboxy-terminal 14 amino acids of the LDL-R (3) (lower left). Shown are the autoradiograms. The source of fibroblasts is indicated between panels: Δ10kb HMZ, receptor-negative fibroblasts from a homozygote for the French Canadian >10-kb LDL-R deletion (8); Δ5kb, from LDL-R Δ5kb HTZ fibroblasts; N, normal fibroblasts. Molecular mass in (kDa) is indicated at right; bands at 140 and 127 represent the normal and Δ5kb form of the LDL-R, respectively. Right panel shows the results calculated from densitometric scans of autoradiograms. Bars representing LDL-R Δ5kb HTZ fibroblasts are averaged band intensities relative to the normal form of 3–4 independent experiments with fibroblasts from 4 LDL-R Δ5kb HTZ and 2 normal subjects.

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**FIG. 4. Effect of neuraminidase on the molecular mass of LDL-R of LDL-R Δ5kb (Δ5kb) and normal (N) fibroblasts.** Forty μg of fibroblast protein extracts were treated with or without 0.016 unit of neuraminidase and subjected to immunoblotting with IgG-4A4 (see "Materials and Methods"). Molecular mass (kDa) is indicated at right; bands at 134 and 117 represent the normal and Δ5kb form of the LDL-R, respectively. Right panel shows the results calculated from densitometric scans of autoradiograms. Bars representing LDL-R Δ5kb HTZ fibroblasts are averaged band intensities relative to the normal form of 3–4 independent experiments with fibroblasts from 4 LDL-R Δ5kb HTZ and 2 normal subjects.

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**FIG. 5. Binding of anti LDL-R antibody IgG-4A4 to cell surface of normal and LDL-R Δ5kb HTZ fibroblasts.** Surface binding at of 125I-labeled IgG-4A4 to normal (NL, triangles) and LDL-R Δ5kb HTZ (Δ5kb, circles) fibroblasts. After a 48-h incubation with 10% LPDS, cells were incubated with 1 ml of medium containing the indicated concentrations of 125I-IgG-4A4 (485 cpm/ng). After 3 h at 4°C, the total radioactivity bound to cells was determined as described under "Materials and Methods." Shown is specific binding, obtained by subtraction of binding in the presence of a 20-fold excess of unlabeled antibody from the total. Nonspecific binding averaged 40–50% of total. Due to anomalously high nonspecific binding to NL fibroblasts at 1 μg/ml, this point was not included in the curve. Inset shows Scatchard analysis.
terminal sequence of Pro84-Pro85. The possible functional sig-
ificance of this difference is not clear but seems unlikely to account for differences in the ligand binding ability of the two deleted receptors. Other examples of discrepancies between apparent effects of gene deletions as assessed by in vitro studies and by phenotypic expression of a naturally occurring de-
letion are seen when domain 3 (O-linked sugar domain) is deleted in vitro by site-directed mutagenesis resulting in no defect in receptor activity (27), while a homozygote for such a mutation expresses FH (28, 29). A variant of lipoprotein lipase containing an Asn291→Ser substitution which is functionally
mildly abnormal in vitro (30) is associated with type IV hyper-
triglyceridemia in French Canadians (31). In the case of lip-
ipoprotein lipase, the unexpectedly profound clinical effect of heterozygosity for a mildly defective variant may be attribut-
able to a dominant negative mechanism, wherein the defective variant would interfere with lipoprotein lipase dimerization, which is necessary for function. Although the LDL-R is present on the cell surface as a monomer, one possible locus for a dominant negative effect of heterozygosity for a defective re-
ceptor could be in receptor clustering prior to internalization (14, 32).

However, such an explanation for the defective LDL-R activity observed in LDL-R Δ5kb HTZ fibroblasts is unlikely based on the observation that the Δ5kb receptor is poorly able to bind ligand. A more likely explanation for the discrepancies between results obtained from site-directed mutagenesis studies and HTZ fibroblasts is the amount of receptor expressed under each set of circumstances. Overexpression of proteins in transfected cells has been observed to result in unphysiological phenom-
ena, such as secretion of immature forms of apolipoprotein A-I (33) or constitutive activity of sterol regulatory element binding proteins 1 and 2 (34). Thus, prediction of in vivo phenotypic effects of gene mutations from their functional effects in trans-
fected cells may be complicated by the unphysiologically high levels of expression.

It has been estimated that 15–30% of “isolated” O-linked carbohydrate is located on the amino-terminal half of the re-
ceptor (27), more specifically, within the ~40-kDa ligand-bind-
ing domain (35). The absence of glycosylation in this domain in a monensin-resistant cell line has been shown to reduce LDL-R affinity for LDL by approximately 75% (35). Thus, loss of O-
linked carbohydrate may at least partially explain the absence of ligand binding of the LDL-R Δ5kb receptor observed in the present study. Although similar decreases in molecular mass after neuraminidase treatment between the LDL-R Δ5kb and normal protein in the present study may imply that significant O-linked glycosylation does not occur in the first two repeats of the LDL-R, it is questionable whether a difference would be detectable. O-Linked sialic acid and galactose residues are expected to contribute approximately 25 kDa to the molecular mass of the LDL-R (27). Therefore, if the carbohydrate was evenly distributed among repeats, the expected loss of molecu-
lar size after neuraminidase treatment due to glycosylation of the first two repeats is 3 kDa. Thus, it is possible that the effect of deletion of exons 2 and 3 on LDL-R activity is attributable to loss of carbohydrate and subsequent loss of receptor affinity for LDL. As such, possible differences in glycosylation patterns between human fibroblasts and transfected CHO cells may contribute to differences between the present and a previous study (4) on the functional consequence of the absence of these two repeats.

Another potentially interesting explanation for the surpris-
ingly severe effects of the LDL-R Δ5kb is a regulatory effect of a gene deletion that is not apparent in cells transfected with cDNA. Thus, one possibility is that deletion of a liver-specific enhancer in introns 1 or 2 causes a liver-specific regulatory
defect. An LDL-R gene deletion of exons 2 and 3 similar to the LDL-R Δ5kb has been reported to result from Alu recombination (36). Alu sequences have been known to act as enhancers (37) or repressors (37). In the present study, however, LDL-R mRNA corresponding to the Δ5kb allele was consistently higher in fibroblasts and lymphocytes than that of the normal allele, suggesting deletion of an element which may act as a repressor, at least in these cell types. Further studies will explore the regulatory consequences of the LDL-R Δ5kb.

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