Deletion in Cysteine-Rich Region of LDL Receptor Impedes Transport to Cell Surface in WHHL Rabbit

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The Watanabe heritable hyperlipidemic (WHHL) rabbit, an animal with familial hypercholesterolemia, produces a mutant receptor for plasma low-density lipoprotein (LDL) that is not transported to the cell surface at a normal rate. Cloning and sequencing of complementary DNA's from normal and WHHL rabbits, shows that this defect arises from an in-frame deletion of 12 nucleotides that eliminates four amino acids from the cysteine-rich ligand binding domain of the LDL receptor. A similar mutation, detected by S1 nuclease mapping of LDL receptor messenger RNA, occurred in a patient with familial hypercholesterolemia whose receptor also fails to be transported to the cell surface. These findings suggest that animal cells may have fail-safe mechanisms that prevent the surface expression of improperly folded proteins with unpaired or improperly bonded cysteine residues.

RECEPTORS AND OTHER CELL SURFACE PROTEINS FOLLOW a complex path from their sites of synthesis in the rough endoplasmic reticulum to their sites of function in the plasma membrane (*1*). The orderly progression of such proteins through the endoplasmic reticulum and the Golgi complex was elucidated through study of model proteins, such as those of lipid-enveloped viruses. The signals that direct this movement and the fail-safe mechanisms that prevent denatured proteins from reaching the cell surface are still largely unknown. One way to solve the problem is by studying mutant proteins that do not reach the cell surface. Such mutations have been created artificially through in vitro mutagenesis of genes encoding viral envelope proteins (*2*). A second approach is through study of naturally occurring mutations in which transport is blocked. In human and animal cells, the most informative group of these mutations occurs in the gene for the low density lipoprotein (LDL) receptor and gives rise to a genetic disease called familial hypercholesterolemia (FH) (*3*, *4*).

FH is an autosomal dominant disorder characterized by an elevation of cholesterol in plasma and severe atherosclerosis (*3*, *4*). The disease is caused by defects in the cell surface receptor for LDL, which is a cholesterol transport protein (*5*). When the LDL receptor is defective, LDL cannot enter cells by receptor-mediated endocytosis and the lipoprotein accumulates in plasma, eventually producing atherosclerosis (*3*). Some mutations alter the receptor in such a way that it cannot move from the rough endoplasmic reticulum to the Golgi complex (*6*, *7*). Such transport-deficient mutations have been observed frequently in humans with FH (*5–7*) and in Watanabe heritable hyperlipidemic (WHHL) rabbits, an animal counterpart of FH (*7*).

WHHL rabbits are homozygous for a mutant allele that produces an LDL receptor that is of normal apparent molecular size but is transported to the cell surface at only one-tenth the normal rate (*7*). The newly synthesized receptor receives its normal complement of high mannose N-linked sugars and the core *N*-acetylgalactosamine of the O-linked sugars (*5*). However, these carbohydrate chains are not processed to their mature form, apparently because the mutant receptor does not reach the relevant parts of the Golgi complex. Instead, most of the receptors are destroyed without ever reaching the cell surface. To date the molecular abnormality responsible for this type of cellular transport defect is unidentified.

We now report the complementary DNA (cDNA) cloning of LDL receptor messenger RNA's (mRNA's) from the normal and WHHL rabbit. We show that the receptor defect in the WHHL rabbit is brought about by a deletion of four amino acids in a region of the receptor that protrudes from the cell surface and is extraordinarily rich in disulfide-bonded cysteine residues. We present evidence of a deletion in another cysteine-rich region of the LDL receptor gene in a patient with FH who also produces a receptor that is not transported to the cell surface at a normal rate. These findings suggest that transport of membrane molecules to the cell surface is contingent on proper folding of the polypeptide chain and on proper formation of disulfide bonds.

cDNA cloning of normal rabbit LDL receptor. In rabbit liver the mRNA for the LDL receptor is normally present at about 80 copies per cell (*8*). To enrich for this low abundance mRNA, New Zealand White rabbits were treated with pharmacological doses of ethinyl estradiol for 10 days, a regimen that induces, by an unknown mechanism, levels of LDL receptor mRNA in the liver that approach 500 copies per cell (*8*). The hepatic poly(A)$^+$ RNA (2 μg) was used to construct a cDNA library with the techniques and vectors of Okayama and Berg (*9*). Total plasmid DNA from the library was linearized and subjected to agarose gel electrophoresis. The region of the gel containing near full-length rabbit LDL receptor cDNA's was localized by cross-hybridization with a ^{32}P-labeled bovine LDL receptor cDNA probe (*10*). The plasmids were eluted from this region of the gel, recircularized, and used to transform *Escherichia coli* HB101 cells. Colonies were screened by

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hybridization with the ^{32}P-labeled bovine receptor cDNA. In this manner we obtained a plasmid, designated pLDLR-11 (Fig. 1), that contained a cDNA insert of about 2.9 kilobases (kb).

Restriction endonuclease mapping and DNA sequencing (Fig. 1) indicated that pLDLR-11 did not extend to the 5′ end of the mRNA. Accordingly, an oligonucleotide corresponding to a sequence near the 5′ end of the cDNA insert in pLDLR-11 was used to prime cDNA synthesis on liver RNA. The extended products were converted to double-stranded DNA's and propagated in a pBR322 vector (*11*). A ^{32}P-labeled oligonucleotide probe located 5′ to the primer sequence was used to screen this library. By these methods, we isolated a plasmid, designated pR52, that contained an additional 78 nucleotides derived from the 5′ end of the mRNA.

Nucleotide and deduced protein sequence of rabbit LDL receptor cDNA. The strategy used to determine the primary sequence of the cDNA inserts in pLDLR-11 and pR52 is shown in Fig. 1, and the amino acid sequence of the rabbit LDL receptor predicted from the nucleotide sequence of the two cDNA's is shown in Fig. 2. The first eight amino acids in the longest open reading frame are nonpolar and of a type that occurs in hydrophobic signal sequences, such as the one in the human LDL receptor (*11*). At the ninth residue (alanine), which is designated residue 1 in Figs. 2 and 3, the sequence becomes closely homologous to the sequence of the mature human LDL receptor (Fig. 3). This sequence is also homologous (10 of 16 identical residues) to the sequence determined for the NH_2-terminus of the mature bovine LDL receptor (*12*). We believe, therefore, that this alanine is the first residue of the mature rabbit LDL receptor and that the eight residues on the NH_2-terminal side of this point represent part of the signal sequence, which is cleaved proteolytically after synthesis. Multiple attempts to further extend the cDNA to reach the initiator methionine were not successful.

If the conclusion regarding the signal sequence is correct, the mature rabbit LDL receptor would consist of 829 amino acids with a molecular size of 90,775 daltons. This is ten amino acids shorter than the mature human receptor (*11*). Like the human receptor, the NH_2-terminal half of the rabbit protein is very rich in cysteines (14.5 percent), and the COOH-terminal half is relatively depleted in these residues (1.7 percent). Three potential N-linked carbohydrate sites (Asn-X-Ser or Asn-X-Thr) are present in the rabbit sequence. There is also a stretch of 25 hydrophobic residues located about 50 residues from the COOH-terminus. By analogy with the bovine (*10*) and human (*11*) LDL receptors, this sequence represents the region of the protein that spans the plasma membrane.

Comparison of human and rabbit LDL receptor sequences. The mature human LDL receptor consists of five discrete domains (*10*, *11*). These are (i) an NH_2-terminal ligand binding domain of 292 amino acids; (ii) a region of about 400 residues homologous to the EGF precursor; (iii) a domain of 58 amino acids containing clustered O-linked sugars; (iv) a hydrophobic transmembrane domain of 22 amino acids; and (v) a cytoplasmic domain of 50 amino acids at the COOH-terminus.

The NH_2-terminal ligand binding domain of the human receptor is composed of a single 40–amino acid unit that is repeated seven times (*5*, *13*). Each repeat contains (i) six cysteine residues that form intrachain disulfide bonds; (ii) a conserved acidic sequence (Asp-Gly-Ser-Asp-Glu) at the COOH-terminal end, which is thought to be involved in ligand binding; and (iii) a characteristic pattern on hydropathy plots (*14*), in which a central hydrophobic core is flanked by hydrophilic regions at both ends. Between the fourth and fifth repeats there is an insertion of eight amino acids (residues 164 to 171) that is not part of the repeat structure.

All of these features are conserved in the rabbit receptor (Fig. 3A). The ligand binding domain is composed of seven cysteine-rich repeats, which span 292 amino acids. An alignment of repeat sequences (Fig. 4) reveals that the spacing of the six cysteine residues is identical to that of the human receptor. The negatively charged residues at the COOH-terminus of each repeat, which are proposed to mediate the binding of LDL (*5*), are highly conserved, a finding that agrees with the observation that the rabbit receptor and the human receptor have similar affinities for human LDL (*15*). The seven repeats in the rabbit protein sequence are even more homogeneous than those of the human; each contains a hydrophobic core centered around the sequence Cys-Ile or Cys-Val (Fig. 4). One feature, however is strikingly different in the rabbit and human LDL receptors. Although the eight–amino acid insertion between repeats

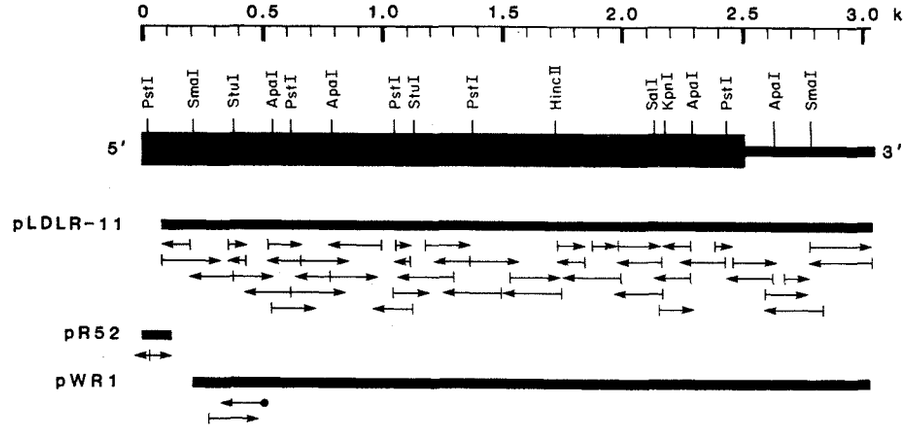

Fig. 1. Restriction endonuclease map and sequencing strategy for normal and WHHL rabbit LDL receptor cDNA's. Nucleotide positions in kilobases are indicated by the top scale. The coding region of the mRNA is indicated by the thick black line and the 3′ untranslated region is represented by the thin line in the restriction endonuclease map. Only selected restriction endonuclease sites are shown. The regions of the mRNA corresponding to each of the three partial cDNA clones are indicated below the map. Plasmids pLDLR-11 and pR52 are derived from mRNA of normal New Zealand White rabbits; plasmid pWR1 is derived from mRNA isolated from a WHHL rabbit. Arrows emanating from vertical hatch marks indicate the direction and extent of DNA sequence established by the dideoxy chain termination method. The arrow emanating from the dot indicates a region of DNA sequenced by the chemical method. Poly(A)$^+$ RNA was isolated from the livers of estradiol-treated (*8*) normal or WHHL rabbits by means of a guanidinium isothiocyanate–CsCl procedure (*28*) followed by oligo dT–cellulose chromatography (*29*). Complementary DNA libraries containing more than 10^6 independent transformants in *E. coli* HB101 were constructed (*9*) and size-fractionated (*30*). Plasmids containing LDL receptor cDNA inserts were identified by cross-hybridization under reduced stringency (*11*) with a ^{32}P-labeled bovine LDL receptor cDNA probe (*10*). Plasmid pR52, a cDNA clone representing the 5′ end of the normal rabbit LDL receptor mRNA, was isolated by primer extension cloning (*11*) in a pBR322 vector. DNA sequencing was done either chemically (*31*) or enzymatically (*32*) with specific oligonucleotide primers after subcloning into bacteriophage M13 vectors (*33*). Routine sequencing gels contained 10 percent polyacrylamide, 100 m*M* tris-borate (*p*H 8.3), 7*M* urea, and 25 percent (v/v) formamide. Bacteriophage M13 templates giving rise to intransigent compressions under these conditions were resequenced and subjected to electrophoresis in a warm room (37°C) on 9 percent polyacrylamide gels containing 100 m*M* tris-borate (*p*H 8.3), 7*M* urea, and 38 percent (v/v) formamide (*34*).

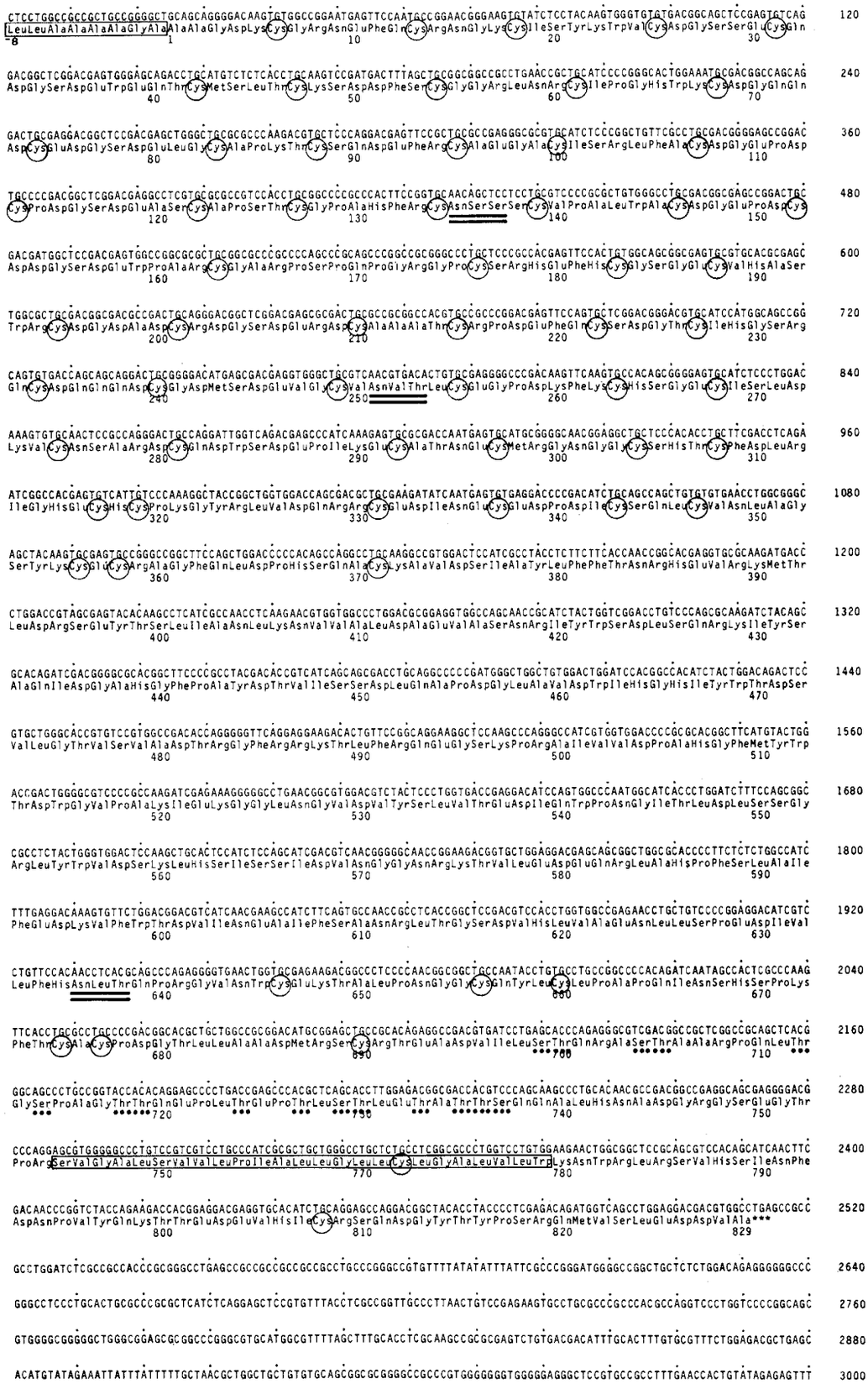

Fig. 2. Nucleotide sequence and predicted amino acid sequence of the normal rabbit LDL receptor. DNA sequences derived from the partial cDNA plasmids pLDLR-11 and pR52 were determined as described in Fig. 1. Nucleotides are numbered on the right side; position 1 is the most 5′ nucleotide cloned in pR52. Dots are placed above every tenth nucleotide in the sequence. Amino acids are numbered below the sequence; residue 1 is the alanine that is believed to constitute the NH_2-terminus of the mature protein; negative numbers refer to residues in the cleaved signal sequence (boxed, NH_2-terminus). Cysteine residues are circled. Three potential sites of N-linked glycosylation (Asn-X-Ser or Asn-X-Thr) are indicated by double solid underlines. Serine and threonine residues in a region that corresponds to a domain of the bovine and human LDL receptor that was previously shown to contain clustered O-linked sugars (*5, 18*) are indicated by dotted underlines. The 25-residue transmembrane segment located toward the COOH-terminus of the protein is boxed. A potential polyadenylation signal at the extreme 3′ end of the sequence is overlined. The sequence of the 3′-untranslated region of the rabbit LDL receptor mRNA has been described (*35*), and is shown here for the sake of completion.

4 and 5 is present in the rabbit protein (residues 164 to 171), only one of the eight amino acids is the same in the human and rabbit receptors. From this finding it seems likely that the insertion sequence forms a link between two subdomains of the receptor that form the LDL binding sites (repeats 1 to 4 and repeats 5 to 7). The length of this linker may be important but its composition is not critical.

The domain of the human LDL receptor that is homologous to the epidermal growth factor (EGF) precursor encompasses 400 amino acids in the center of the protein. About 35 percent of these residues are identical in the two human proteins (*5*, *16*). This region includes three copies of a disulfide-bonded, cysteine-rich repeat of approximately 40 amino acids. A similar repeat is present in three proteins of the blood clotting system—factor IX, factor X, and protein C (*16*). In comparing the rabbit and human LDL receptors, we find that the EGF precursor–like domain is the most highly conserved extended sequence (Fig. 3); 84 percent of the amino acids are identical. The three cysteine-rich repeat units are present, and once again there are no insertions or deletions in the rabbit sequence relative to that of the human. This conservation points to a fundamental role for this domain in the function of the receptor, but so far that function is unknown.

In the human LDL receptor the clustered O-linked sugars are found in a domain of 58 amino acids that are encoded by a single exon (*13*). This region contains 18 serine and threonine residues, most of which serve as attachment sites for carbohydrate chains (*10*,

Fig. 3. Alignment of the normal rabbit and human LDL receptor protein sequences. Rabbit and human LDL receptor sequences were aligned for maximum homology with the use of a Beckman Microgenie Align program. (A) Alignment of the amino acid sequences in the five domains of the mature rabbit and human LDL receptors. Asterisks denote identical residues. Amino acids in the respective proteins are numbered on the left and right. Dots are placed above every tenth position in a given comparison line. The single-letter amino acid code translates to the three-letter code as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; Y, Tyr. (B) Model representing five domains in the mature LDL receptor. The percentage homology between the human and rabbit proteins in a given domain is indicated above the schematic.

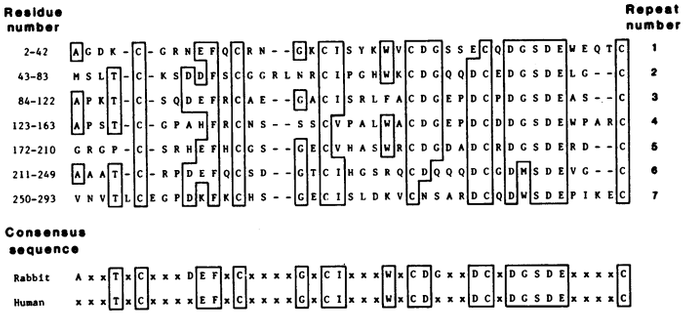

Fig. 4. Alignment of repeat amino acid sequences in the ligand binding domain of the normal rabbit LDL receptor. The sequences of the seven cysteine-rich repeats in the ligand binding domain of the rabbit protein were aligned for maximum homology based on computer-assisted analyses and the structure of the human LDL receptor gene (*13*). Conserved residues are boxed. A consensus sequence representing amino acids that occur in a given position in four or more repeats is shown below the alignment. The consensus sequence derived from a similar alignment of repeats in the human LDL receptor (*13*) is also shown. The single-letter abbreviations for amino acid residues are given in the legend to Fig. 3.

17). Deletion of this region from the cDNA leads to the production of a receptor that performs normally in cultured hamster fibroblasts (*18*); thus at present the function of this domain is not known. We have previously reported that there is little sequence homology in the clustered O-linked sugar domains of the bovine and human LDL receptors (*5*). This lack of conservation also applies to the rabbit and human receptors (Fig. 3). Only 40 percent of the amino acids are identical, and the rabbit domain is ten residues shorter than the human. The only conserved feature of the two domains is their serine- and threonine-rich character; there are 18 such residues in this region of the bovine and human receptors and 16 in the rabbit. The signals that specify the addition of O-linked sugars to a polypeptide backbone are not well defined (*19*). The fact that this region is glycosylated in receptors from the three species, even though the amino acids are only 40 percent identical, suggests that a long, conserved primary sequence within this region is not the signal that leads to O-linked glycosylation.

The transmembrane domain of the human LDL receptor anchors the protein in the membrane; its deletion in certain FH alleles causes the receptor to be secreted into the culture medium (*20*). In general, transmembrane domains that serve only as anchors vary in length and show little sequence homology other than an absence of amino acids with charged side chains (*1*). These characteristics pertain to the transmembrane segments of the rabbit and human LDL receptors (Fig. 3), which are hydrophobic, only 68 percent homologous, and differ in length (25 residues in the rabbit, 22 in the human, and 27 in the cow) (*5*, *10*).

The cytoplasmic domain of the human LDL receptor, consisting of 50 amino acids, contains signals responsible for targeting the protein to coated pits on the plasma membrane (*5*, *21*). This crucial role is reflected in the high degree of sequence conservation (86 percent) that exists between the rabbit and human cytoplasmic domains (Fig. 3). The sequence of this region is also highly conserved in the cow (*5*, *10*).

Cloning and analysis of LDL receptor cDNA from the WHHL rabbit and subsequent expression of the mutant mRNA. A cDNA library was constructed from mRNA isolated from livers of estradiol-treated WHHL rabbits as described above (*8*), except that a different plasmid vector was used. The clones were screened with a 5′ probe isolated from pLDLR-11, and a single plasmid, designated pWR1, was isolated. As shown in Fig. 1, pWR1 contained a cDNA insert of about 2.8 kb. Restriction endonuclease mapping revealed a small deletion centered around the most 5′ Stu I site when compared with the normal rabbit cDNA. DNA sequence analysis (Fig. 5) showed that 12 base pairs were absent from the WHHL cDNA clone in this region. The deletion results in the loss of four amino acids (residues 115 to 118 of Fig. 2) from the third cysteine-rich repeat of the ligand binding domain. Each of the seven repeats in the binding domain of the LDL receptor contains a highly conserved sequence (Asp-Gly-Ser-Asp-Glu) that is thought to be directly involved in binding LDL. The deletion in the WHHL receptor removes the first four amino acids of this sequence from the third repeat (Fig. 5).

To determine whether this alteration abolished the binding of lipoproteins, we performed ligand blotting experiments (Fig. 6). Proteins were solubilized from membranes isolated from the adrenal glands of normal and WHHL rabbits, separated by sodium dodecyl sulfate (SDS) electrophoresis on nonreducing polyacrylamide gels, and transferred to nitrocellulose filters. The filters were incubated with rabbit ^{125}I-labeled β-VLDL, a high affinity ligand for the LDL receptor (*22*). The mature LDL receptor from the normal rabbit binds β-VLDL (Fig. 6, lane 2). As previously demonstrated (*23*), much less of the precursor form of the receptor is visualized in these normal homogenates, owing to rapid intracellular processing and transport to the cell surface. The converse is true in extracts prepared from the WHHL rabbit. Only the precursor form of the receptor is seen; it binds the ^{125}I-labeled β-VLDL particle (Fig. 6, lane 4). In both extracts, binding was dependent on the presence of Ca^{2+} ions (lanes 1 and 3), a property shared with highly purified preparations of the bovine LDL receptor (*22*). These results demonstrate that the deletion in the WHHL receptor does not abolish binding of β-

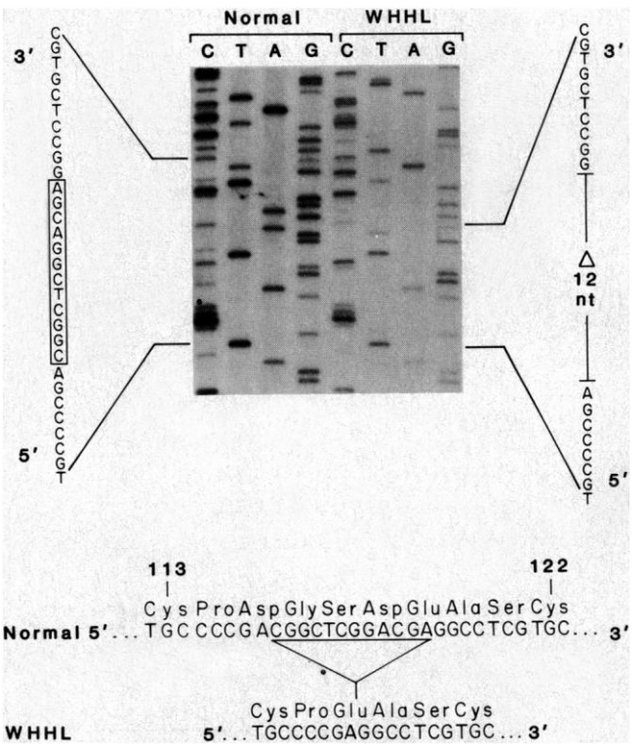

Fig. 5. Nucleotide sequences of LDL receptor cDNA's from normal and WHHL rabbits in the region between cysteines 113 and 122. DNA sequencing was done by the dideoxy chain termination method (*32*) with M13 templates (*33*) containing Pst I fragments isolated from the 5′ ends of plasmids pLDLR-11 and pWR1 (Fig. 1) and an LDL receptor–specific oligonucleotide primer. A 12-nt deletion is apparent in the sequence obtained from the WHHL-derived template. The consequences of this deletion for the LDL receptor protein are shown in the lower portion of the figure.

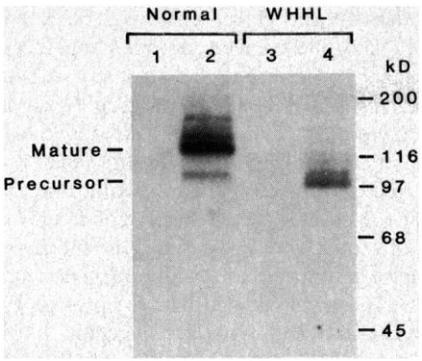

Fig. 6. Ligand blotting of LDL receptors from adrenal glands of normal (lanes 1 and 2) and WHHL (lanes 3 and 4) rabbits. One adrenal gland from the indicated rabbit was disrupted by Dounce homogenization (*23*) in buffer containing 20 m*M* tris-HCl (*p*H 8), 2 m*M* $CaCl_2$, 150 m*M* NaCl, 1 m*M* phenylmethylsulfonylfluoride, 1 m*M* 1,10 - phenanthroline, and 0.5 m*M* leupeptin in the absence (lanes 2 and 4) or presence (lanes 1 and 3) of 10 m*M* EGTA. Solubilized membrane fractions were prepared as described (*23*), and portions of each fraction (300 μg of protein) were subjected to 7 percent SDS gel electrophoresis under nonreducing conditions (*23*), except that the final sample buffer contained 5 percent (v/v) glycerol, 2.5 percent (w/v) sucrose, 4.9 percent (w/v) SDS, 2*M* urea, and 47 m*M* tris-HCl at *p*H 6.8. After electrophoretic transfer of proteins to nitrocellulose paper, the filter was incubated for 1 hour at 37°C in buffer A containing 2 m*M* $CaCl_2$ (*22*) with rabbit ^{125}I-labeled β-VLDL (protein, 8.5 μg/ml; ~6 × 10^5 count/min per microgram of protein), washed, dried, and exposed to x-ray film for 48 hours. Molecular size calibration was carried out as described (*22*).

VLDL. However, the ligand blotting technique does not lend itself to accurate measurements of the affinity for the ligand, and we therefore cannot determine whether the deletion in the WHHL rabbit lowers the affinity of the receptor for β-VLDL or LDL.

To estimate levels of LDL receptor mRNA in tissues of normal, WHHL homozygote, and WHHL heterozygote rabbits, we used S1 nuclease analysis (Fig. 7). Total RNA was extracted from the indicated tissue and hybridized with a uniformly labeled antisense probe derived from a bacteriophage M13 subclone of the normal cDNA (*8, 24*). Annealing of this probe to the normal LDL receptor mRNA should result in protection of a 553-nucleotide (nt) fragment from digestion by the single strand–specific S1 nuclease. If the probe annealed to mRNA derived from the WHHL receptor allele, the 12-nt deletion should give rise to an S1-sensitive loop, and thus the probe should be cleaved into fragments of 292 and 249 nt (Fig. 7).

In the mRNA samples from the normal rabbit, a single protected fragment (553 nt) was indeed visualized (Fig. 7, left). With mRNA from the WHHL rabbit no 553-nt protected fragment was detected. Instead, the 292- and 249-nt fragments were observed (Fig. 7, center). In the heterozygous WHHL rabbit the 553-nt fragment and the two smaller fragments were all seen, an observation consistent with the expression of one normal and one mutant allele. Densitometric scanning of the gels showed that in all animals the relative level of mRNA declined in the order: adrenal gland, liver, small bowel, testis, lung, and kidney. These results agree well with the relative amounts of functional receptors found in these tissues (*25*).

Transport-deficient mutants in human FH. To determine whether a mutation in the cysteine-rich region might be responsible for defective movement of the LDL receptor to the cell surface in a human subject with the clinical syndrome of homozygous FH, we examined fibroblasts isolated from patient FH 563 and his heterozygous parents.

Cultured fibroblasts from a normal individual and from FH 563 were labeled for 1 hour with [^{35}S]methionine and then an excess of unlabeled methionine was added for varying times (30 minutes to 2 hours) (Fig. 8). The LDL receptors were then immunoprecipitated with a monoclonal antibody and analyzed by SDS gel electrophoresis. In normal cells, 30 minutes after the addition of unlabeled methionine all of the receptors had moved through the Golgi complex where they were processed to the mature 160-kilodalton form (Fig. 8, left). In contrast, in the FH 563 cells about 95 percent of the receptors remained in the precursor form 2 hours after the addition of unlabeled methionine (Fig. 8, right). Study of the fibroblasts from the clinically heterozygous parents of FH 563 showed that 2 hours after unlabeled methionine was added about one-half of the ^{35}S-labeled receptors were processed to the mature form and one-half remained in the precursor form (Fig. 9). This result suggests that patient FH 563 inherited the same mutant allele

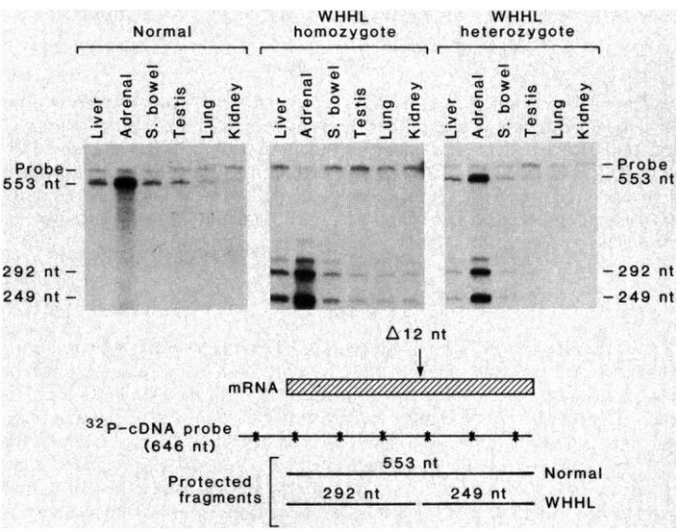

Fig. 7. Expression of LDL receptor mRNA's in different tissues of normal, WHHL homozygote, and WHHL heterozygote rabbits. LDL receptor mRNA's were detected with a quantitative solution hybridization–S1 nuclease assay (*8, 24*). The design of the experiments and the predicted results for the two mRNA's are indicated in the lower portion of the figure. A uniformly ^{32}P-labeled probe corresponding to nucleotides 78 to 629 in Fig. 2 plus 93 nucleotides of cloning and M13 polylinker sequences was hybridized to 25 μg of total RNA isolated from the indicated tissues of normal, WHHL heterozygous, or WHHL homozygous animals and then digested with S1 nuclease. Protected fragments were visualized by autoradiography after size-fractionation on denaturing polyacrylamide gels. Lengths of the protected fragments were estimated relative to standards generated from Hae III–digested bacteriophage φX174 DNA. The slightly larger band above the 292-nucleotide fragment derived from the WHHL allele is a consequence of incomplete S1 digestion of poly(G) tails (generated in the cloning procedure) present at the 5′ end of the probe. Total RNA was isolated from the indicated tissue by a guanidinium isothiocyanate–CsCl procedure (*28*). Uniformly ^{32}P-labeled single-stranded probes complementary to the mRNA were synthesized from bacteriophage M13 templates by the methods of Church and Gilbert (*36*). For hybridization, 25 μg of total RNA from various tissues was coprecipitated with the antisense probe (20,000 count/min) in 70 percent ethanol and resuspended in a buffer containing 80 percent (v/v) formamide, 400 m*M* NaCl, 5 m*M* EDTA, and 40 m*M* Pipes (*p*H 6.4). RNA-DNA hybrids were allowed to form for 16 hours at 50°C and then diluted with 470 μl of a buffer containing 250 m*M* NaCl, 30 m*M* potassium acetate (*p*H 4.5), 1 m*M* $ZnCl_2$, and 5 percent (v/v) glycerol. Nuclease S1 (250 units, Bethesda Research Laboratories) was added and the incubation was continued for 1 hour at 37°C. Nuclease-resistant hybrids were collected by ethanol precipitation in the presence of 1 μg of calf thymus DNA, resuspended in formamide, boiled, and resolved on denaturing polyacrylamide gels. After electrophoresis, gels were fixed with trichloroacetic acid, dried, and exposed to Kodak XAR-5 film. Results were quantified by densitometric scanning of the autoradiograms. Size standards were generated by electrophoresis of a labeled Hae III digest of φX174 DNA. nt, nucleotides.

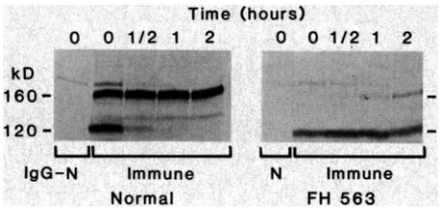

Fig. 8. Kinetics of processing of LDL receptors in normal fibroblasts (left) and in fibroblasts from FH homozygote 563 (right). Fibroblasts were cultured for 6 days and induced for synthesis of LDL receptors by incubation in lipoprotein-deficient serum for 16 hours (*6, 7*). Cells were pulse-labeled in methionine-free Dulbecco's modified Eagle medium (DMEM) with [^{35}S]methionine (102 μCi/ml) for 1 hour at 37°C (*6*). One set of dishes from each cell strain was processed for immunoprecipitation of labeled LDL receptors (zero time-point). For the remaining dishes, the medium was switched to complete DMEM for the indicated time. LDL receptors were immunoprecipitated from detergent-solubilized cell extracts with either an irrelevant monoclonal antibody IgG-2001 (N) or with a monoclonal antibody IgG-C7 directed against the LDL receptor (immune) (*6, 7*). The immunoprecipitates were processed for SDS gel electrophoresis under reducing conditions and autoradiography as described (*6, 7*). Apparent molecular sizes of the ^{35}S-labeled proteins were calculated from the position of migration of marker proteins (*6, 7*).

from each parent and is thus homozygous for an allele that produces a transport-deficient receptor whose behavior is similar to that of the WHHL rabbit.

We next used the S1 nuclease technique to examine RNA isolated from the fibroblasts from FH 563 and his parents. Antisense probes, uniformly labeled with ^{32}P, were derived from cDNA sequences that encode the cysteine-rich ligand binding domain of the human receptor. Figure 10 shows an experiment in which a probe encompassing exons 4 through 7 was used. In normal RNA, a protected fragment of 586 nt was detected (lane 2). However, in RNA isolated from FH 563 cells two smaller fragments of approximately 217 and 348 nt were detected together with a small amount of undigested heteroduplex (lane 5). RNA from the heterozygous parents of FH 563 showed roughly equal amounts of the normal protected fragment of 586 nt and the two abnormal protected fragments of 217 and 348 nt (lanes 3 and 4). These findings, together with those obtained with other probes, are consistent with the presence of a small deletion of about 7 to 12 amino acids in exon 4 of the LDL receptor gene. Inasmuch as exon 4 encodes the fifth repeat in the cysteine-rich binding domain (*13*), these data suggest that the transport-deficient receptor in FH 563 is produced by a mutation in this cysteine-rich region.

Implications for transport of cell surface proteins. The current findings indicate that a small in-frame deletion of 12 nt removes four amino acids from a cysteine-rich sequence of the LDL receptor and disrupts its movement from the endoplasmic reticulum to the Golgi complex in cells from WHHL rabbits. The mutation does not remove any of the cysteine residues, but its presence is likely to alter the folding pattern of the cysteine-rich region, thereby preventing formation of proper disulfide bonds. Evidence for a similar mutation affecting another cysteine-rich repeat was found through analysis of mRNA from a human FH subject who produces a receptor that fails to move to the Golgi complex at a normal rate. In this case, on the basis of predicted size of the deletion (21 to 36 nt, 7 to 12 amino acids), at least one cysteine residue is removed.

Precedent for mutant proteins that are blocked in transport from the endoplasmic reticulum to the Golgi complex has been obtained through study of induced mutations in expressible cDNA's introduced into cultured cells by transfection (*2, 26*). These studies have shown that various alterations in both the extracellular and cytoplasmic domains can block the movement of proteins to the cell surface.

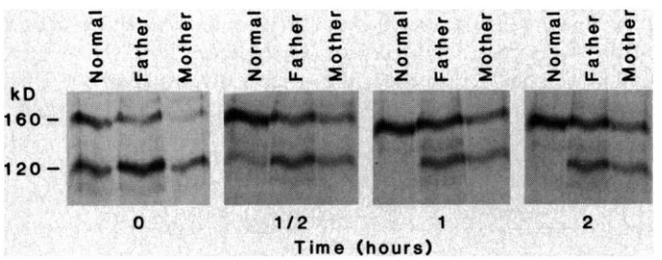

Fig. 9. Kinetics of processing of LDL receptors in normal fibroblasts and in fibroblasts from the parents of FH homozygote 563. Cells were cultured, and pulse-labeled in methionine-free medium with [^{35}S]methionine (125 μCi/ml) for 1 hour at 37°C; complete medium was added at the indicated time, and processed for immunoprecipitation of labeled LDL receptors with monoclonal antibody IgG-C7 and SDS gel electrophoresis as described in the legend to Fig. 8.

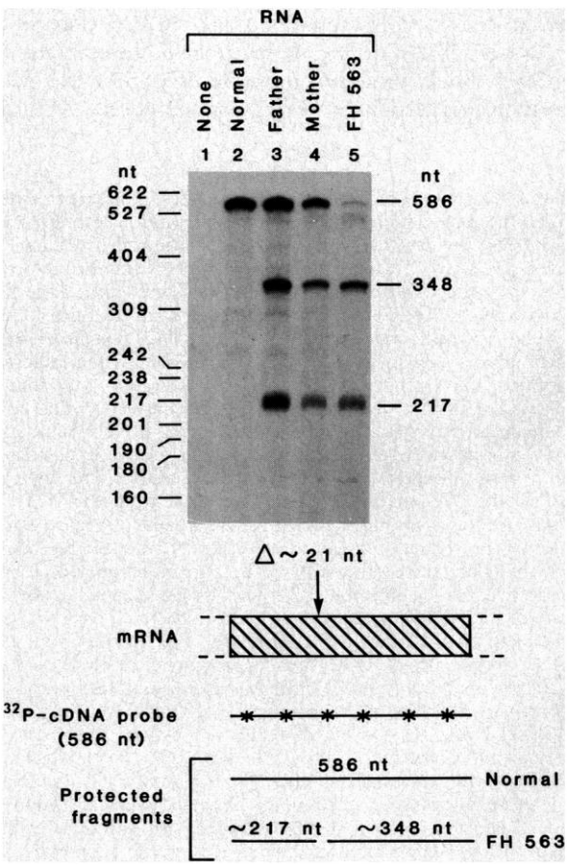

Fig. 10. Expression of normal and mutant LDL receptor mRNA's in human fibroblasts from FH 563 and his parents. LDL receptor mRNA's were detected by a solution hybridization–S1 nuclease assay. A uniformly ^{32}P-labeled probe encompassing nucleotides 422 to 1007 of the human receptor mRNA (*11*) was prepared as described in the legend to Fig. 7 and annealed to the indicated RNA at 39°C for 16 hours. After digestion with 700 units of S1 nuclease for 1 hour at 37°C and electrophoresis on denaturing polyacrylamide gels, nuclease-resistant hybrids were visualized by autoradiography. Each hybridization reaction contained RNA isolated from the following cell strains induced for LDL receptor expression (*11*): (lane 1) no RNA; (lane 2) 10 μg of total RNA from SV40-transformed normal human fibroblasts; (lane 3) 15 μg of total RNA from fibroblasts of the father of FH 563; (lane 4) 15 μg of total RNA from fibroblasts from the mother of FH 563; (lane 5) 10 μg of total RNA from fibroblasts of FH 563. The dried gel was exposed to Kodak XAR 5 film for 24 hours at −70°C with a Dupont Cronex Lightning Plus screen. Size standards were generated by electrophoresis of a labeled Msp I digest of pBR322 DNA.

Defective movement is also observed for many chimeric proteins composed of a secretory protein (for example, growth hormone) fused to a membrane-embedded protein (for example, influenza hemagglutinin) (*2, 26*). In many respects these chimeric proteins behave similarly to the transport-deficient receptors in the WHHL rabbit and in FH homozygotes. Recently, Rizzolo *et al.* (*26*) have shown that certain of these chimeric proteins are sequestered from the rough endoplasmic reticulum by incorporation into smooth-surfaced membrane cisternae that are in apparent continuity with the rough endoplasmic reticulum. Such cisternae may constitute transitional elements between the rough endoplasmic reticulum and the Golgi complex. It seems likely that the LDL receptors produced by transport-deficient alleles may be trapped in similar structures.

The mechanism by which abnormal proteins are blocked in their movement is obscure. It may be that these proteins are so denatured that they aggregate nonspecifically and hence fail to be transported. Such denaturation cannot be complete, however, because the proteins do undergo some processing. Thus, the LDL receptors of the WHHL rabbit and patient FH 563 undergo O-linked as well as N-linked glycosylation (*6, 7, 17*). In addition, the WHHL LDL receptor binds lipoprotein particles, implying that most structural features are intact. As mentioned above, certain chimeric proteins have been reported to move from the endoplasmic reticulum to transitional elements, or even to the Golgi complex, before becoming trapped and thus they, too, cannot be totally immobile.

In view of these data, it seems reasonable to propose that the mutant proteins are trapped by detection mechanisms that are designed specifically to prevent the movement of abnormally folded proteins to the cell surface. Such fail-safe mechanisms might be crucial in biology because the surface appearance or secretion of even a small number of denatured molecules might lead to a harmful immune response. It is possible that one of these fail-safe mechanisms detects free cysteine residues that would normally be disulfide-bonded. If such free cysteine residues were exposed on the protein surface, they might form disulfide bonds with a gatekeeper protein that would trap the abnormal protein in intracellular structures and prevent movement to the cell surface.

Many secretory and plasma membrane proteins contain clusters of disulfide-bonded cysteines of the type present in the LDL receptor. The widespread occurrence of this structural motif has been appreciated only recently as a result of cDNA cloning, which has revealed the sequence of many extracellular protein domains. The disulfide bonds are believed to form co-translationally as a result of the intrinsic folding of such proteins and as a result of the action of protein disulfide isomerase, an enzyme of the endoplasmic reticulum that catalyzes the formation of disulfide bonds (*27*). The hypothetical gatekeeper protein would block transport of these proteins to the cell surface whenever their disulfide bonding patterns were incorrect.

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