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RECEPTOR-MEDIATED ENDOCYTOSIS: Concepts Emerging from the LDL Receptor System

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INTRODUCTION

The concept of *receptor-mediated endocytosis* was formulated in 1974 to explain the observation that regulation of cellular cholesterol metabolism

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depended on the sequential cell surface binding, internalization, and intracellular degradation of plasma low density lipoprotein (LDL) (Goldstein & Brown 1974, Goldstein et al 1976). This uptake mechanism was postulated on the basis of biochemical studies; it was soon verified morphologically when the receptors for LDL were observed to be clustered in coated pits that pinched off from the surface to form coated vesicles that carried the LDL into the cell (Anderson et al 1976, 1977a).

Coated pits and coated vesicles had been recognized by electron microscopy in the mid-1960s (Roth & Porter 1964, Fawcett 1965, Friend & Farquhar 1967). Their role in receptor-mediated endocytosis was appreciated a decade later as a result of the convergence of twin events: 1) the demonstration that coated vesicles were the sites at which LDL receptors were concentrated, and 2) the demonstration by Pearse (1975) that a single protein, clathrin, formed the cytoplasmic coat, an observation that provided a biochemical definition of coated vesicles. The biological implications of receptor-mediated endocytosis were vividly underscored by the finding that genetic defects in the LDL receptor preclude cellular uptake of LDL, producing hypercholesterolemia and heart attacks (Brown & Goldstein 1984).

During the last decade, receptor-mediated endocytosis was recognized as a mechanism by which animal cells internalize many macromolecules in addition to LDL (Goldstein et al 1979a, Pastan & Willingham 1981, Bretscher & Pearse 1984). The process is initiated when receptors on the cell surface bind macromolecules and slide laterally into clathrin-coated pits. Within minutes the coated pits invaginate into the cell and pinch off to form coated endocytic vesicles. After shedding their clathrin coats the vesicles fuse with one another to form endosomes whose contents are acidified by ATP-driven proton pumps (Tycko & Maxfield 1982, Helenius et al 1983, Pastan & Willingham 1983). Within the endosome the ligand and receptor part company. Often, but not always, the ligand is carried to lysosomes for degradation, while the receptor cycles back to the cell surface to bind new ligand (Brown et al 1983).

More than 25 specific receptors have been observed to participate in receptor-mediated endocytosis. These include receptors for transport proteins that deliver nutrients to cells, such as the cholesterol-carrying lipoprotein LDL, the iron transport protein transferrin, and the vitamin B_{12} transport protein transcobalamin II. Receptor-mediated endocytosis also applies to many nontransport plasma proteins, including asialoglycoproteins, α-2-macroglobulin, and immune complexes. Moreover, the process mediates the cellular uptake of lysosomal enzymes, which occurs when these enzymes bind to receptors that recognize mannose-6-phosphate residues uniquely attached to this class of proteins. Certain viruses and

toxins use receptor-mediated endocytosis to enter cells, apparently by binding opportunistically to receptors that normally function in the uptake of other substances.

Protein growth factors, such as epidermal growth factor (EGF) and platelet-derived growth factor (PDGF), as well as classic polypeptide hormones, such as insulin and luteinizing hormone, also enter cells by receptor-mediated endocytosis. The same receptors that mediate endocytosis of these proteins mediate their physiologic actions. However, frequently cellular entry of the ligand does not seem to be required for the action of the growth factor or the hormone. Rather, the entry mechanism functions in the rapid control of receptor number and in the removal of the growth factor or hormone from the circulation (Carpenter & Cohen 1979, Terris et al 1979).

Progress in this field has been rapid. Within a single year—1984—complementary DNAs (cDNAs) for five different coated pit receptors were isolated, and their nucleotide and corresponding amino acid sequences were determined (Mostov et al 1984, Ullrich et al 1984, Russell et al 1984, Yamamoto et al 1984, McClelland et al 1984, Schneider et al 1984, Holland et al 1984). The cDNA cloning and structure of a sixth coated pit receptor, the insulin receptor, was reported early in 1985 (Ullrich et al 1985, Ebina et al 1985). In this review we summarize the information that is emerging from study of the amino acid sequences of the receptor proteins, with emphasis on the LDL receptor.

PATHWAYS OF RECEPTOR-MEDIATED ENDOCYTOSIS

Entry Into Coated Pits

The various pathways of receptor-mediated endocytosis share one common feature: in each case the receptors move to coated pits and coated vesicles. However, there are differences in the mechanisms that trigger movement to coated pits as well as differences in the routes the ligands and receptors follow after entering the cell. We can divide the process of receptor-mediated endocytosis into subcategories according to these differences, as described below.

The first distinction is whether the receptors spontaneously move to coated pits and enter cells continuously (even in the absence of ligand), or whether the receptors wait on the surface until a ligand is bound, whereupon they are captured by coated pits. The receptors in the first category include those for LDL (Anderson et al 1982, Basu et al 1981), transferrin (Hopkins & Trowbridge 1983, Hopkins 1985), α-2-macroglobulin (Hopkins 1982, Via et al 1982), asialoglycoproteins (Wall et

al 1980, Berg et al 1983), and insulin (Krupp & Lane 1982). Conversely, the receptor for EGF is diffusely distributed on the cell surface, and is not trapped in coated pits unless it is occupied with ligand (Schlessinger 1980, Dunn & Hubbard 1984).

The propulsive force for movement of receptors to coated pits may be simple diffusion, or it may involve a more directed type of propulsion (Bretscher 1984). The rate of diffusion of receptors on cell surfaces is sufficiently fast in itself to explain movement into coated pits (Goldstein et al 1981, Barak & Webb 1982). However, considerable evidence suggests that membrane lipids are continuously flowing toward coated pits (Bretscher 1984). This lipid flow may carry membrane proteins along passively (Bretscher & Pearse 1984, Hopkins 1985), but why are only certain cell surface proteins trapped in coated pits? One possibility is that receptors are marked for such entry by the attachment of prosthetic groups. Many receptors (such as those for transferrin, asialoglycoproteins, EGF, PDGF, and insulin) have phosphate groups attached to serine, threonine, or tyrosine residues in their cytoplasmic domains (see Table 1 in Brown et al 1983).

Recent attention has focused on phosphorylation or dephosphorylation as a potential mechanism for signaling entry, perhaps through induction of receptor binding to clathrin, the protein that covers the cytoplasmic surface of coated structures. Phosphorylation of the receptors for EGF (Hunter 1984), transferrin (Klausner et al 1984), and insulin (Jacobs et al 1983) can be enhanced by treatment of cells with phorbol esters, which activate protein kinase C. Phorbol esters cause transferrin receptors in K562 cells to become trapped within the cell, which suggests that phosphorylation either increases the rate of their cellular entry or slows their return to the cell surface, or both (Klausner et al 1984).

A few receptors undergo acylation of cysteine residues with fatty acids, but this modification does not apply to all receptors that participate in endocytosis. Moreover, in the one case that has been studied in detail, that of the transferrin receptor, the turnover of the fatty acid moiety is much slower than the internalization rate (Omary & Trowbridge 1981), which implies that acylation-deacylation does not occur during each recycling event.

Intracellular Routes

A second variation in the systems of receptor-mediated endocytosis is the fate of the ligand and receptor. It appears that all endocytotic receptors enter cells in the same coated pits, and are delivered to the same acidified endosomes (Pastan & Willingham 1981, Via et al 1982, Carpentier et al 1982). Thereafter, the pathways diverge. The receptor-ligand complex may

follow one of four routes, which are discussed below and illustrated schematically in Figure 1.

ROUTE 1: RECEPTOR RECYCLES, LIGAND DEGRADED This pathway is the classic one described for the endocytosis of LDL, asialoglycoproteins, and α-2-macroglobulin, as well as for insulin and luteinizing hormone. In following this route, ligands dissociate from their receptors within the endosome, apparently as a result of the drop in pH (Brown et al 1983, Helenius et al 1983). The ligand is carried further to lysosomes, where it is degraded. The receptor leaves the endosome, apparently via incorporation into the membrane of a vesicle that buds from the endosome surface. These recycling vesicles may originate as tubular extensions of the endosome, which gather receptors and then pinch off from the main body of the endosome (Geuze et al 1983, 1984). After their return to the surface, LDL receptors are said to remain clustered so that they can be incorporated rapidly into newly formed coated pits (Robenek & Hesz 1983). Conversely,

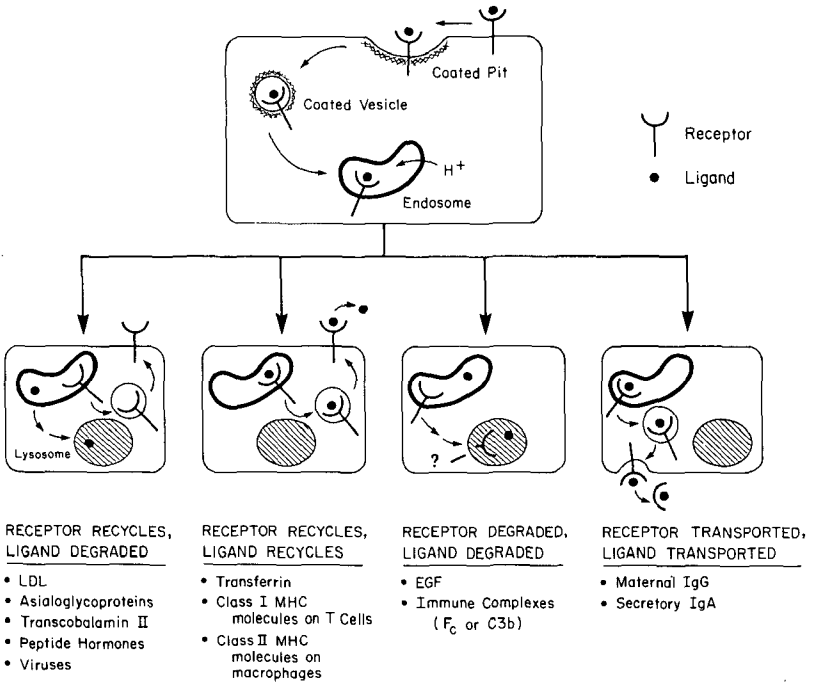

Figure 1 Four pathways of receptor-mediated endocytosis. The initial steps (clustering of receptors in coated pits, internalization of coated vesicles, and fusion of vesicles to form endosomes) are common to the four pathways. After entry into acidic endosomes, a receptor-ligand complex can follow any of the four pathways shown in the figure.

recycling transferrin receptors seem to go through a transient phase of monomolecular dispersion on the cell surface before clustering and internalizing again (Hopkins 1985).

Route 1 seems ideally adapted for use by receptors that transport ligands into cells at a high rate: It allows reuse of receptors once every 10–20 min. Thus, one receptor can mediate the uptake of hundreds of ligands during its usual lifespan of 10–30 hr. Recycling requires that the receptors have a stable structure that will permit them to pass repeatedly through the acidic environment of the endosome without denaturation. In the acidic endosome the receptors must undergo sufficient conformational change to release their ligands (DiPaola & Maxfield 1984), but they must not become irreversibly denatured. The LDL receptor, for example, can make up to 150 trips through the endosome without losing its function (Goldstein et al 1979a, Brown et al 1982). Maintenance of stability may require unusual protein structures, some of which are detailed below.

ROUTE 2: RECEPTOR RECYCLES, LIGAND RECYCLES This pathway was originally described for the transferrin receptor (Octave et al 1983). When the transferrin/receptor complex reaches the endosome the two proteins do not dissociate. In vitro binding studies show that the transferrin receptor, in contrast to the receptors for LDL, asialoglycoproteins, and EGF, fails to dissociate from its ligand at pH 5 (Klausner et al 1983, Dautry-Varsat et al 1983). However, iron does dissociate from transferrin at acidic pH. Thus, in the endosome the iron is stripped from transferrin while the apo-transferrin remains attached to the receptor. The apo-transferrin/receptor complex then returns to the cell surface. The recycling transferrin receptor seems to leave the endosome by a network of membrane tubules and vesicles that eventually leads it back to the cell surface (Geuze et al 1984). Once on the surface and again at neutral pH, the apo-transferrin dissociates from the receptor. (Iron-containing transferrin binds to the receptor at neutral pH, but apo-transferrin dissociates from the receptor at this pH). The transferrin receptor is now free to bind another molecule of iron-containing transferrin and to repeat the cycle. Like the LDL receptor, the transferrin receptor is degraded very slowly with a half life of >30 hr (Omary & Trowbridge 1981), even though it enters the cell every 15–20 min (Bleil & Bretscher 1982, Ciechanover et al 1983).

Recent studies suggest a new role for internalization and recycling of ligands by this route. Such recycling may provide the mechanism by which cells of the immune system process antigen and "present" it to effector cells (for reviews see Unanue 1984, Pernis 1985, Pernis & Tse 1985). Macrophages and certain B lymphocytes internalize foreign antigens by receptor-mediated endocytosis. The receptors responsible for this uptake

are poorly characterized. Their function is to deliver the antigen to an acidic intracellular compartment where the antigen undergoes partial proteolysis. The proteolytic fragments are transported back to the cell surface where they are presented to the well-characterized antigen receptors on neighboring T lymphocytes. Presentation requires that the surface of the antigen-presenting cell express Class II major histocompatibility (MHC) proteins of the same genotype as the T lymphocyte. The Class II MHC proteins are dimers of two nonidentical transmembrane glycoproteins, which are continuously internalized and recycled without degradation. One reason for such recycling may be that after proteolysis the antigen and the Class II molecules must pass through the same acidic compartment so they can form a complex that presents the fragmented antigen to the T-cell antigen receptor (Brodsky 1984).

Once a responding T cell is stimulated by exposure to antigen, it begins to internalize and recycle its own MHC molecules, which are of the Class I type (Pernis 1985). These Class I MHC molecules are a complex of a transmembrane glycoprotein and another protein (β_2-microglobulin) that is adherent to the outer surface of the cell membrane. The Class I MHC molecules are not internalized by the T cells unless the cells are activated (i.e. stimulated by antigen). After activation the internalized Class I molecules are delivered to acidic endosomes and cycled back to the cell surface every 15 min (Pernis 1985). Each Class I molecule makes many trips in and out of the activated T cell during its half-life of 14 hr (Tse & Pernis 1984, Tse et al 1985). Monoclonal antibodies directed against the Class I molecules do not affect this recycling. In fact, the bound antibody enters the cell and cycles back to the cell surface with the Class I molecule still attached, in a manner analogous to the co-recycling of apo-transferrin and the transferrin receptor (Tse et al 1985).

The specificity of regulation of internalization of Class I MHC molecules is striking. Internalization and recycling occur only on activated T lymphocytes and not on resting T lymphocytes, B lymphocytes (resting or activated), or on any other known cell type. It is not possible to induce rapid internalization of these molecules in B cells, even when the Class I molecules have been cross-linked by exposure to a monoclonal anti-Class I antibody followed by a second layer of polyclonal antibodies (Pernis & Tse 1985). Moreover, nonlymphoid cells, such as fibroblasts and mouse L cells, do not rapidly internalize their Class I MHC molecules even though they rapidly internalize other membrane molecules, such as the receptors for LDL and transferrin (Pernis & Tse 1985). Internalization of Class I molecules by mouse L cells or fibroblasts can be observed when the molecules have been cross-linked by a double layer of antibodies, but the rate is considerably slower than receptor-mediated endocytosis—it takes hours rather than

minutes. Moreover, this internalization leads to lysosomal degradation of the Class I molecules rather than to recycling (Pernis & Tse 1985, Huet et al 1980).

Do the internalized antigens and MHC molecules follow the classic coated pit to coated vesicle to endosome to lysosome pathway described for LDL? There are no electron microscopic data that address this question; however, there is circumstantial evidence that coated pits and vesicles are involved. For instance, recycling of MHC molecules is strictly regulated, has rapid kinetics, and is inhibited by drugs that raise the pH of endosomes (Tse & Pernis 1984, Tse et al 1985, Unanue 1984), all of which are features of endocytosis via coated pits and vesicles (Brown et al 1983).

ROUTE 3: RECEPTOR DEGRADED, LIGAND DEGRADED This pathway has been described in greatest detail for EGF. After the EGF/receptor complex reaches the endosome both components are degraded, probably as a result of subsequent cotransport to the lysosome (Carpenter & Cohen 1979). The mechanism for this cotransport is unclear. Since EGF dissociates from its receptor at acidic pH (DiPaola & Maxfield 1984), it would presumably dissociate in the endosome. Somehow this dissociation does not allow the EGF receptor to return to the surface, but rather it is carried further into lysosomes. If the EGF receptor is delivered to lysosomes by vesicular fusion then the cytoplasmic domain of the receptor would remain outside of the lysosome, facing the cytoplasm. This domain of the EGF receptor consists of 542 amino acids and contains tyrosine kinase activity (Hunter 1984, Ullrich et al 1984). It is tempting to speculate that this tyrosine kinase domain of the receptor might be liberated from its hydrophobic anchor through proteolytic cleavage and then migrate elsewhere in the cell, where it could phosphorylate proteins that trigger cell division. The selective release of such a cytoplasmic fragment has not yet been demonstrated.

In certain cells, one population of EGF receptors may escape degradation and recycle. In cultured fibroblasts (Carpenter & Cohen 1979) and in the perfused rat liver (Dunn & Hubbard 1984) the addition of EGF causes a decrease of up to 80% in EGF receptors, apparently due to ligand-induced internalization and degradation. However, the remaining 20% of EGF receptors continue to bind, internalize, and degrade EGF with kinetics that suggest recycling.

ROUTE 4: RECEPTOR TRANSPORTED, LIGAND TRANSPORTED This pathway has been described most clearly for the receptor that carries polymeric immunoglobulin A (IgA) and immunoglobulin M (IgM) across epithelial surfaces, such as across liver cells for excretion into the bile, and across mammary epithelia for excretion into milk (Solari & Kraehenbuhl 1984, Mostov et al 1984). In the liver the newly synthesized receptor appears on

the sinusoidal surface of the hepatocyte, where it binds dimeric IgA. The receptor/immunoglobulin complex is incorporated into vesicles and carried into the cell (Renston et al 1980). Coated vesicles have not been implicated formally, but such involvement seems likely. At some point after internalization, the receptor is clipped proteolytically so that part of the receptor with the immunoglobulin still bound to it is released from the membrane. This released receptor fragment is the so-called secretory component. The IgA-containing vesicle eventually migrates to the bile canalicular face of the hepatocyte, where it discharges the IgA/secretory component adduct into the bile.

In neonatal animals, transepithelial transport of maternal IgG from the lumen of the intestine to the interstitial space is mediated by a receptor that binds to the Fc domain of the IgG. This transport probably does not involve cleavage of the receptor, since a secretory component has not been identified (Rodewald & Abrahamson 1982).

The delineation of four routes for disposal of receptors and ligands (Figure 1) implies that cells have multiple mechanisms for sorting receptors after they enter the cell. These mechanisms must be regulated so as to allow different cells to process the same receptor by different routes or a single cell to process the same receptor by different routes at different times. In some cases sorting involves passage of the receptors through vesicles that are located near the Golgi complex. However, the receptors do not seem to transit through classic Golgi stacks, which are the sites of sorting in the exocytotic pathway. Rather, they pass through nearby vesicles that may or may not contain Golgi-associated enzymes (Dunn & Hubbard 1984, Hanover et al 1984).

To assure accuracy of the multiple sorting and targeting events, each receptor must contain multiple functional domains. It must contain a binding domain that is specific for a given set of ligands, and regions that allow it to interact with other macromolecules so it can be transported to various sites within the cell. Often a receptor will proceed successively from one compartment to another, at each stage being sorted from other membrane molecules that are stationary or are moving to different sites. Therefore, each receptor must contain multiple sorting signals that act sequentially. These signals will be revealed only when the complete structures of the receptors are known.

THE LDL RECEPTOR: STRUCTURE-FUNCTION RELATIONSHIPS

We recently carried out detailed studies of the structure and biosynthesis of the LDL receptor. This receptor performs a simple function: it carries

cholesterol-bearing lipoproteins into cells. To accomplish this task, the receptor must move from its site of synthesis in the membranes of the endoplasmic reticulum (ER) through the Golgi complex to the cell surface, where it is targeted to coated pits. It must then recycle from the endosome to the cell surface. Naturally occurring mutations in the gene for the LDL receptor disrupt several of these transport steps and produce a clinical condition of receptor deficiency known as familial hypercholesterolemia (FH).

Protein Purification and cDNA Cloning

The LDL receptor was purified from the bovine adrenal cortex, an organ that contains a relative abundance of LDL receptors ($\sim 10^5$ molecules per cell), which it uses to supply cholesterol for conversion to steroid hormones (Schneider et al 1982). Biochemical tools were developed that permitted cloning of cDNAs for the receptor. Thus, polyclonal antibodies raised against the purified bovine protein were used to enrich for the rare LDL-receptor mRNA by polysome immune purification. A cDNA library was constructed from the purified mRNA of bovine adrenal cortex, and was screened with two families of oligonucleotides derived from the amino acid sequence of a cyanogen bromide fragment of the bovine protein. These methods led to the isolation of a partial cDNA for the bovine receptor (Russell et al 1983).

The bovine cDNA was used as a probe to isolate a fragment of the human LDL-receptor gene. In turn, an exon probe from this genomic fragment was employed to isolate a cDNA clone representing the complete 5.3-kilobase (kb) human LDL-receptor mRNA. Transfection studies indicated that this cDNA could direct the expression of functional human LDL receptors in simian COS cells (Yamamoto et al 1984).

The nucleotide sequence of this cDNA was used to derive the complete amino acid sequence of the human LDL receptor (Yamamoto et al 1984). This sequence, together with biochemical experiments (Russell et al 1984), revealed that the mature receptor is divided into five distinguishable domains. A model of the domain structure is shown in Figure 2.

A Single Polypeptide Chain With Five Domains

FIRST DOMAIN: LIGAND BINDING The extreme NH_2-terminus of the LDL receptor consists of a hydrophobic sequence of 21 amino acids that is cleaved from the receptor and is not present in the mature protein. This segment presumably functions as a classic signal sequence to direct the receptor-synthesizing ribosomes to the ER membrane. Because it does not appear in the mature receptor, the signal sequence is omitted from the numbering system that is described below.

The mature receptor (without the signal sequence) consists of 839 amino acids. The first domain of the mature LDL receptor consists of the NH_2-terminal 292 amino acids, which are extremely rich in cysteines (42 out of 292 amino acids). Studies with anti-peptide antibodies revealed that this domain is located on the external surface of the plasma membrane (Schneider et al 1983b). The cysteines are spaced at intervals of 4–7 amino acids (Figure 3). An initial computer analysis suggested that the first domain was made up of eight repeat sequences (Yamamoto et al 1984). More recent analysis of the sequence, considered together with exon/intron mapping data (see below and Figure 2), suggests that the number of repeats is only seven, as shown in Figure 3.

Each of the seven repeats consists of ~40 amino acids and contains 6 cysteine residues, which are essentially in register for all of the repeats. The receptor cannot be labeled with [^{3}H]iodoacetamide without prior reduction, suggesting that all of these cysteines are involved in disulfide bonds. This region of the receptor must therefore exist in a tightly cross-linked, convoluted state.

A striking feature of the COOH-terminus of each repeat sequence is a cluster of negatively-charged amino acids (Figure 3). These sequences are complementary to positively-charged sequences in the best-characterized ligand for the LDL receptor, apolipoprotein E (apo E), a 33-kilodalton protein component of the plasma lipid transport system. Apo E contains a cluster of positively charged residues that are believed to face one side of a single α-helix (Innerarity et al 1984). Studies with mutant and proteolyzed forms of apo E, and with monoclonal antibodies against different regions of apo E showed that the positively charged region contains the site by which

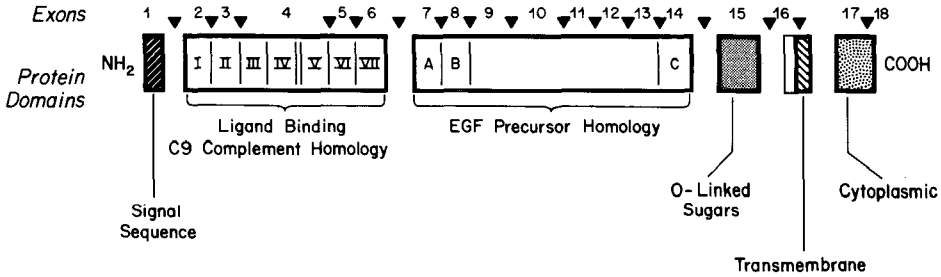

Figure 2 Exon organization and protein domains in the human LDL receptor. The domains of the protein are delimited by thick black lines and are labeled in the lower portion of the figure. The 7 cysteine-rich, 40–amino acid repeats in the LDL binding domain (see also Figure 3) are assigned roman numerals I–VII. Repeats IV and V are separated by 8 amino acids. The 3 cysteine-rich repeats in the EGF precursor homology domain are lettered A–C. The positions at which introns interrupt the coding region are indicated by arrow heads. Exon numbers are shown between the arrow heads. (Reprinted from Südhof et al 1985a with permission.)

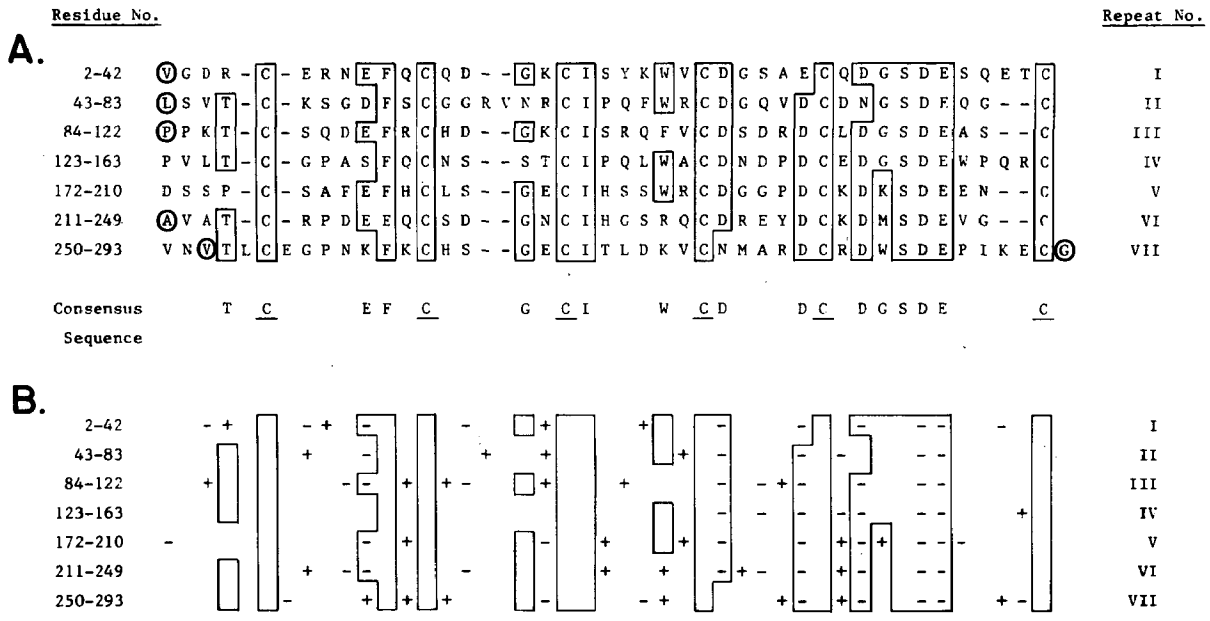

Figure 3 The first domain of the human LDL receptor is composed of seven cysteine-rich repeats. The amino acids constituting each repeat unit are numbered in the left column according to the sequence data of Yamamoto et al (1984). *Panel A*: Optimal alignment was made by the computer programs ALIGN and RELATE with slight modifications based on the location of intervening sequences (Yamamoto et al 1984, Südhof et al 1985a). Amino acids that are present at a given position in more than 50% of the repeats are boxed and shown as a consensus on the bottom line. Cysteine residues (*C*) are underlined. The positions at which intervening sequences interrupt the coding sequence of the gene are denoted by the encircled amino acids. 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. *Panel B*: The net charge of each of the amino acids in Panel *A* is shown. All of the charged amino acids that are conserved bear a negative charge; none are positively charged. (Reprinted from Südhof et al 1985a with permission.)

this protein binds to the LDL receptor (Innerarity et al 1984). It is therefore tempting to speculate that the negatively charged clusters of amino acids within the cysteine-rich repeat sequence of the LDL receptor constitute multiple binding sites, each of which binds a single apo E molecule by attaching to its positively-charged α-helix (Table 1).

This speculation is supported by several observations. First, Innerarity & Mahley (1978) and Pitas et al (1980) showed through kinetic analysis that multiple apo E molecules (4–8) bind to a single LDL receptor. Second, receptor binding of apo E and of apo B (the other ligand for the LDL receptor) is blocked by modification of lysine or arginine residues of the two ligands, a reaction that is achieved with acetylation or cyclohexanedione treatment, respectively (Basu et al 1976, Mahley & Innerarity 1983). Third, although both apo E and apo B have a net negative charge, they nevertheless bind tightly to polyanions, such as heparin, which suggests that both have exposed clusters of basic residues. Fourth, the binding of ^{125}I-LDL to the LDL receptor is inhibited by negatively and positively charged molecules such as heparin, suramin, protamine, and platelet factor 4 (Goldstein et al 1976, Brown et al 1978, Schneider et al 1982). Fifth, the binding activity of the LDL receptor is destroyed by reduction of the disulfide bonds. Sixth, proteolytic treatment of the purified bovine LDL receptor with thrombin yields a 60-kilodalton fragment (isolated on SDS polyacrylamide gels) that is recognized by an anti-peptide antibody directed against the NH_2-terminus of the receptor, and that also specifically binds LDL (Schneider et al, manuscript in preparation). Thus, the LDL binding site is located within the NH_2-terminal 60 kilodaltons of protein, which includes the cysteine-rich acidic region.

The disulfide bonds confer great stability upon the binding site of the

Table 1 Complementarity between amino acid sequences in the LDL receptor and in one of its ligands

LDL Receptor	(-Cys-*Asp*-X-X-X-*Asp*-Cys-X-*Asp*-Gly-Ser-*Asp*-*Glu*-)$_7$	
Apo E	-*His*-Leu-*Arg*-*Lys*-Leu-*Arg*-*Lys*-*Arg*-Leu-Leu-*Arg*-	
	140	150

The LDL receptor sequence corresponds to the consensus from the most conserved part of the seven repeat units shown in Figure 3. The apo E sequence (amino acids 140–150) has been identified as being responsible for binding to the LDL receptor (McLean et al 1984, Innerarity et al 1984). Negatively charged amino acids in the LDL receptor sequence and positively charged amino acids in the apo E sequence are italicized. No amino acid sequence data are available for apo B, the other ligand for the LDL receptor.

receptor. The receptor can be boiled in SDS or guanidine and still retain binding activity as long as the disulfide bonds are not reduced (Daniel et al 1983). The disulfide bonds in this region may preserve the stability of the receptor when it delivers LDL to endosomes. In this acidic environment, the negatively-charged residues of the receptor become protonated and lose their charges, allowing the LDL to be released. (LDL is known to dissociate from the receptor in vitro when the pH falls below 6.5) (Basu et al 1978). Despite the titration of its carboxyl groups, the receptor is not irreversibly denatured by this acid exposure, apparently because of the structural stability afforded by the multiple disulfide bonds.

Each of the seven 40–amino acid repeats in the LDL receptor is strongly homologous to a single 40-residue sequence that occurs within the cysteine-rich region of human complement component C9, a plasma protein of 537 amino acids (Stanley et al 1985, DiScipio et al 1984). Of the 19 conserved amino acids in the LDL receptor repeats, 14 are found in the C9 sequence, including the highly conserved negatively charged cluster:

LDL Receptor Consensus (Figure 3):

x x T [C] x x x E [F] x [C] x x [G] x [C I] x x x W x [C] D x x x [D C] x [D] G [S D E] x x [C]

E D D [C] - G N D [F] Q [C] S T [G] R [C I] K M R L R [C] N G D N [D C] G [D] F [S D E] D D [C]

Complement factor C9 (residues 77–113) (above).

This finding raises the possibility that C9 might have measurable binding affinity for lipoproteins containing apo B or E, the two ligands for the LDL receptor.

SECOND DOMAIN: HOMOLOGY WITH THE EGF PRECURSOR Epidermal growth factor (EGF) is a peptide of 53 amino acids that is synthesized as a large precursor of 1217 amino acids (Scott et al 1983, Gray et al 1983). Analysis of the amino acid sequence, as revealed from the sequence of the cloned cDNA, suggests that the EGF precursor is synthesized as a membrane-bound molecule (Doolittle et al 1984). During synthesis, the first 1038 amino acids of the precursor penetrate into the lumen of the ER, whereupon a stretch of 22 hydrophobic amino acids is encountered. According to the current view of protein synthesis in the ER, such a hydrophobic stretch should become anchored in the membrane and stop further transfer (Sabatini et al 1982). Upon completion of translation a short tail of 158 amino acids would face the cytoplasm, constituting the cytoplasmic domain of the precursor. The 53–amino acid EGF sequence lies just outside the membrane-spanning region in the external domain of 1038 amino acids. This external sequence also contains multiple repeats of the EGF sequence that have diverged during evolution, as well as spacer sequences that are

not related to EGF. EGF is presumably liberated from this putative membrane-bound precursor by proteolysis, and the peptide is then released, and eventually gains access to receptors on epithelial cells, which it stimulates to divide.

The second domain of the LDL receptor, consisting of ~400 amino acids (Figure 2), is homologous to a portion of the extracellular domain of the EGF precursor (Russell et al 1984, Yamamoto et al 1984, Südhof et al 1985b). Within this region approximately 35% of the amino acids are identical, with a few short gaps (Russell et al 1984, Yamamoto et al 1984). This overall domain is flanked by several short repetitive sequences of ~40 amino acids each that are designated A, B, and C in Figure 2. Each of these repeats contains six cysteine residues spaced at similar intervals. The A, B, and C sequences are homologous to four repeat sequences in the EGF precursor (Scott et al 1983, Doolittle et al 1984, Südhof et al 1985a). Repeats A, B, and C in the LDL receptor are also homologous to certain proteins of the blood clotting system, including Factor IX, Factor X, and protein C (Doolittle et al 1984, Südhof et al 1985a).

The existence of these homologies implies that all of these proteins contain regions derived from a common ancestral protein. Does this observation bear any further significance? Does the homology imply that the functions of these regions are conserved among the proteins? It is difficult to imagine functions that would be conserved among a blood clotting factor, the EGF precursor, and the LDL receptor. All of these proteins are made in the ER and reach the cell surface, but it is unlikely that the homology relates to this shared characteristic since other proteins that follow similar routes do not have homologous regions.

Another possibility is that the EGF precursor and the LDL receptor evolved from the duplication of a single ancestral gene that played roles in growth control as well as nutrient delivery (Russell et al 1984). Comparison of the structures of the genes for the human LDL receptor and the human EGF precursor reveals that the region of homology is encoded by eight contiguous exons in each respective gene (Südhof et at 1985b). Of the nine introns that separate these exons, five are located in identical positions in the two protein sequences. This finding strongly suggests that the homologous region arose by a duplication of an ancestral gene. Each copy of the duplicated gene would have further evolved by recruitment of exons from other genes, which provided specialized functions, i.e. the provision of a nutrient (cholesterol) or the signaling of cell growth via the secretion of a peptide (EGF). It is even possible that at some phase of its life cycle the EGF precursor exists in the intact form on the cell surface, where it functions as a receptor, thus increasing the analogy with the LDL receptor. In this regard, Rall et al (1985) have recently shown that the EGF precursor is synthesized

in the distal tubules of the mouse kidney where it accumulates in the intact form and is not detectably processed to EGF.

THIRD DOMAIN : O-LINKED SUGARS Immediately external to the membrane-spanning domain of the human LDL receptor is a sequence of 58 amino acids that contains 18 serine or threonine residues (Yamamoto et al 1984). This domain is encoded within a single exon (see below). Proteolysis studies reveal that this region contains carbohydrate chains attached by O-linkage (Cummings et al 1983, Russell et al 1984). Each O-linked sugar chain consists of a core N-acetylgalactosamine, plus a single galactose and one or two sialic acids. In this respect, the LDL receptor resembles glycophorin, a red-cell membrane protein that contains short O-linked sugar chains attached to clusters of serines and threonines (Marchesi et al 1976). Another cell surface receptor, that for interleukin-2 (IL-2) on T lymphocytes, contains O-linked sugars and a cluster of serine and threonine residues immediately external to the membrane-spanning region (Leonard et al 1984, Nikaido et al 1984).

What is the function of the clustered O-linked sugars? Their similar location in two plasma membrane receptors suggests that these sugars may function as struts to keep the receptors extended from the membrane surface so they can bind their ligands. Why only certain receptors require such struts is not known.

FOURTH DOMAIN : MEMBRANE-SPANNING REGION This domain consists of a stretch of 22 hydrophobic amino acids. Proteolysis experiments (see below) confirmed that this domain spans the membrane (Russell et al 1984). Comparison of the amino acid sequences of the bovine and human LDL receptors reveals that the membrane-spanning region is relatively poorly conserved (Figure 4). Of the 22 amino acids in this region, 7 differ between human and cow, but all of the substitutions are also hydrophobic. The human receptor contains a single cysteine in the membrane-spanning region. In the bovine receptor this cysteine is replaced by an alanine (Figure 4). Since the bovine and the human receptors function similarly, it seems likely that this intramembraneous cysteine exists in a reduced state in the human LDL receptor.

FIFTH DOMAIN : CYTOPLASMIC TAIL The human and bovine LDL receptors both contain a COOH-terminal segment of 50 amino acids that projects into the cytoplasm. This sequence is strongly conserved ; only four of the 50 amino acids differ between the two species, and each of these substitutions is conservative with respect to the charge of the amino acid (Figure 4). Localization of this domain to the cytoplasmic side of the membrane was determined through use of an anti-peptide antibody directed against the

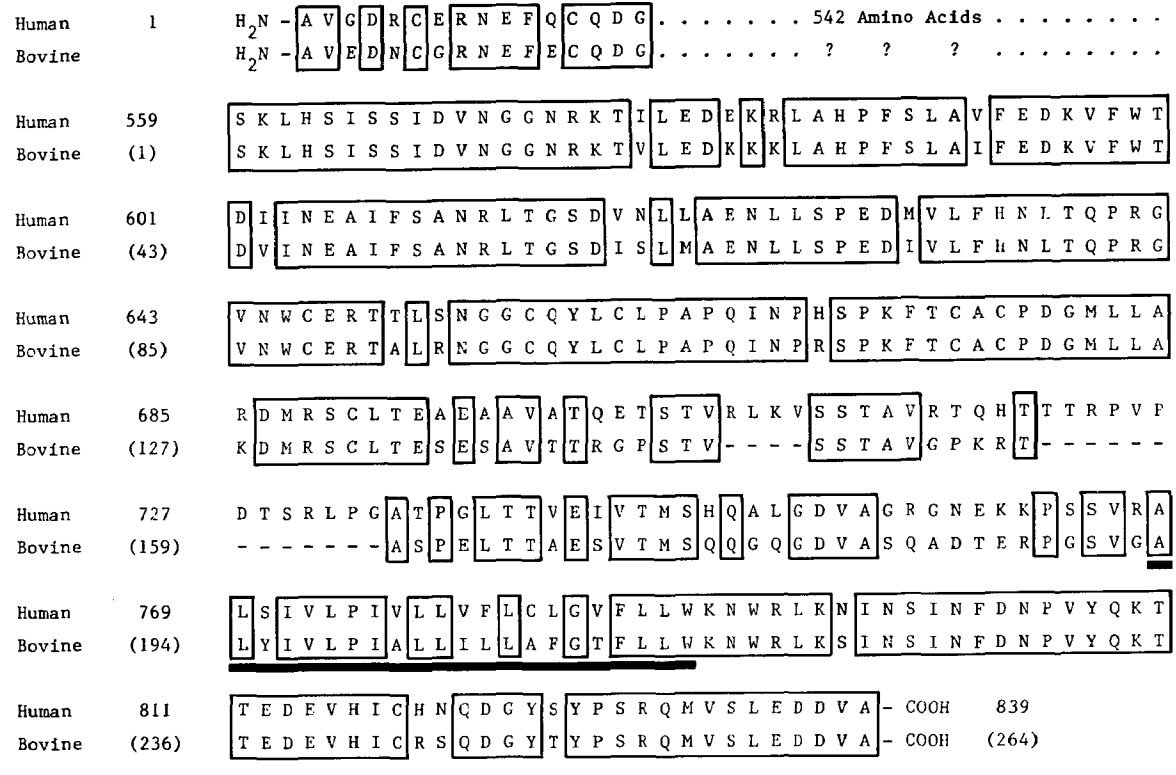

Figure 4 Comparison of the amino acid sequences of the bovine and human LDL receptors. The single letter amino acid code is used (see legend Figure 3). Regions of identity are boxed. The amino acid sequence of the human receptor was deduced from the nucleotide sequence of a full-length cDNA (Yamamoto et al 1984). The NH_2-terminal sequence (16 amino acids) of the bovine receptor was obtained by chemical sequencing of purified receptor protein (Schneider et al 1983b). The COOH-terminal sequence of the bovine receptor (264 amino acids) was deduced from the nucleotide sequence of a partial cDNA (Russell et al 1984). The membrane-spanning region of the two receptors is indicated by the heavy underline.

COOH-terminal sequence (Russell et al 1984). When inside-out membrane vesicles containing receptor were digested with pronase, the antibody-reactive material was removed, and the molecular weight of the receptor was reduced by approximately 5000.

Immediately internal to the membrane the cytoplasmic tail contains a cluster of positively charged amino acids (3 of the first 6 residues are lysines or arginines). This is a frequent feature of plasma membrane proteins (Sabatini et al 1982). Near the COOH-terminal end of the receptor lies a cluster of negatively charged residues (glutamic-aspartic-aspartic) (Figure 4). The cytoplasmic segment also contains several serine and threonine residues and three tyrosines, which may be sites for phosphorylation. This domain also contains a single cysteine, which may be a site for disulfide bond formation or for fatty acylation. None of these modifications have been detected as yet.

The cytoplasmic domain of the LDL receptor plays an important role in clustering in coated pits, either through interaction with clathrin itself or with some protein associated with clathrin on the cytoplasmic side of the membrane (see below). For this reason, it is important to compare the sequences of the cytoplasmic domain of the LDL receptor with those of other receptors known to enter coated pits.

Comparison With Five Other Coated Pit Receptors

In 1984 and early 1985 complete cDNA sequences for 6 coated pit receptors were reported. When the predicted amino acid sequences are compared, no obvious conserved feature is apparent (Figure 5). In particular, the cytoplasmic domains show tremendous differences, varying in size from 38 amino acids (asialoglycoprotein receptor) to 542 amino acids with tyrosine kinase activity (EGF receptor). In addition, the orientations of the receptors are different. Four receptors, those for LDL, EGF, insulin, and polymeric IgA/IgM, are oriented with their NH_2-termini outside the cell and their COOH-termini in the cytoplasm. Two receptors, for transferrin and asialoglycoprotein, exhibit an inverted orientation with their NH_2-termini in the cytoplasm and their COOH-termini outside the cell. The cytoplasmic domains of the 4 receptors that lack tyrosine kinase domains contain clusters of negatively charged amino acids (glutamic and aspartic), generally in regions predicted to have an α-helical conformation. These acidic residues may play some role in interaction with coated pits.

The transferrin receptor exists as a homodimer, linked by a disulfide bond between two cysteine residues that are immediately external to the plasma membrane (McClelland et al 1984, Schneider et al 1984). The insulin receptor is composed of two α-subunits (ligand binding domain) and two β-subunits (tyrosine kinase domain) linked by disulfide bonds. The α and β

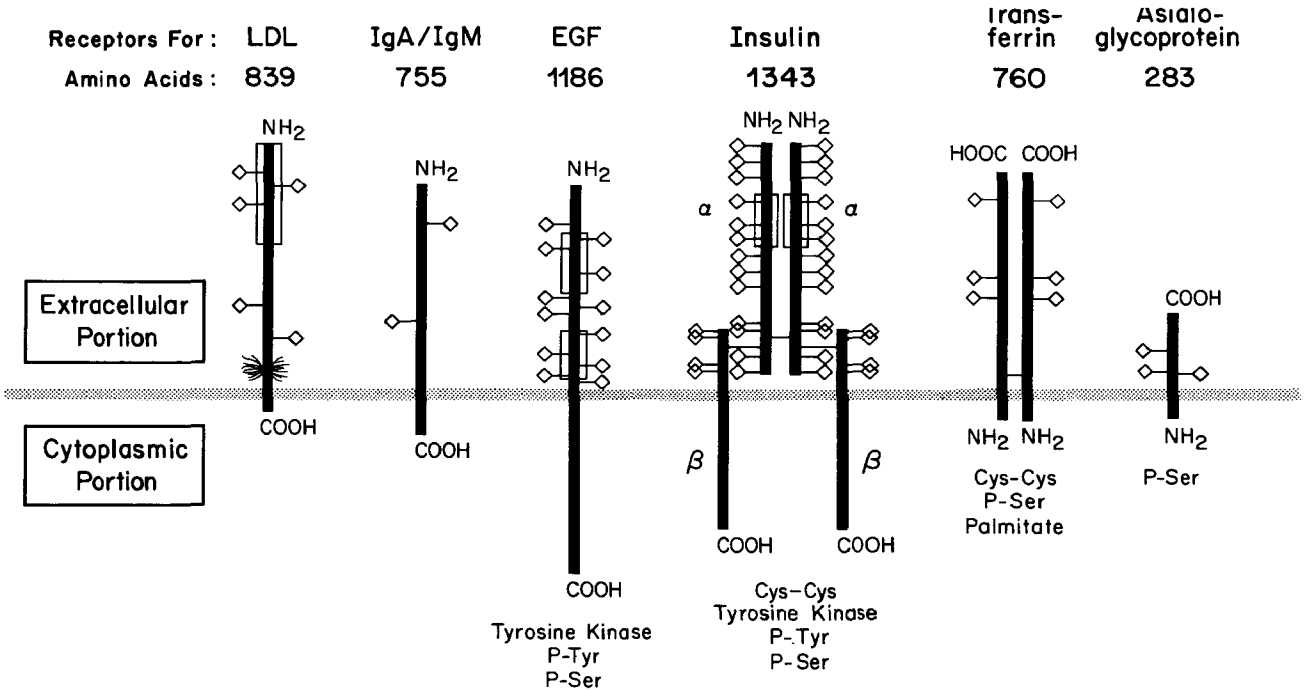

Figure 5 Comparison of the structures of the LDL receptor and 5 other receptors that enter coated pits. Each protein spans the plasma membrane once and has the indicated orientation. The number of amino acids in the cytoplasmic portion of each receptor is as follows: human LDL receptor, 50 residues; rabbit IgA/IgM receptor, 105; human EGF receptor, 542; human insulin receptor, 402; human transferrin receptor, 61; rat asialoglycoprotein receptor, 38. The potential sites of attachment of N-linked oligosaccharide chains are indicated by diamond symbols. The sites of attachment of O-linked oligosaccharide chains in the LDL receptor are shown by a cluster of horizontal lines. Regions rich in cysteine residues in the receptor for LDL, EGF, and insulin are boxed. The insulin receptor is shown as a disulfide-linked heterodimer (Cys-Cys). The transferrin receptor is shown as a disulfide-linked homodimer (Cys-Cys). The cytoplasmic domain of the receptors for EGF and insulin express tyrosine kinase activity. P-Tyr (phosphotyrosine), P-Ser (phosphoserine), and palmitate denote posttranslational covalent modifications that occur on the cytoplasmic domain of the indicated receptor. (For original sequence data see Yamamoto et al 1984; Mostov et al 1984; Ullrich et al 1984, 1985; Ebina et al 1985; Schneider et al 1984; McClelland et al 1984; Drickamer et al 1984; Chiacchia & Drickamer 1984.)

chains are derived from a single precursor molecule that undergoes proteolytic cleavage to assume the configuration shown in Figure 5 (Ullrich et al 1985, Ebina et al 1985). There is no published evidence that the other 4 coated pit receptors form disulfide-linked dimers. Four coated pit receptors (those for LDL, IgA/IgM, transferrin, and asialoglycoproteins) have a single cysteine residue in their cytoplasmic regions; the EGF and insulin receptors have several such residues. This observation suggests that a novel cytoplasmic interchain disulfide bond may play a role in clustering in coated pits.

As mentioned above, the amino acid sequences of the cytoplasmic domains of the bovine and human LDL receptors are highly conserved. In contrast, no significant conservation is observed between the cytoplasmic domains of the asialoglycoprotein receptor in the rat and the analogous receptor in the chicken, even though the extracellular domains of these two receptors are conserved (Drickamer et al 1984).

THE LDL RECEPTOR AT A GENETIC LEVEL

mRNA Structure

In the human tissues studied so far (cultured diploid fibroblasts, SV40-transformed fibroblasts, A-431 epidermal carcinoma cells, fetal and adult adrenal glands, and fetal and adult liver) the LDL-receptor mRNA appears on Northern blots as a single species of approximately 5.3 kb (Yamamoto et al 1984, unpublished observations). (Minor heterogeneity in size cannot be excluded by this technique.) About half of the mRNA consists of an unusually long 3′ untranslated region of 2.5 kb. It terminates with a poly $(A)^+$ tract that is about 15 nucleotides downstream from a likely polyadenylation signal (AAUUAAA).

An unusual feature of the 3′ untranslated region is the presence of $2\frac{1}{2}$ RNA copies of a middle repetitive sequence present in mammalian genomes. This sequence, designated *Alu*, occurs on average once in every 5000 base pairs (bp) in the human genome, for a total of $\sim$300,000 copies (Schmid & Jelinek 1982). Each *Alu* repeat is about 300 bp long, and consists of a tandem repeat of two monomeric units—a left monomer of 130 bp and a right monomer 160 bp long, owing to a 30 bp insertion. The human LDL-receptor mRNA has two complete *Alu* sequences and an extra right monomeric unit, all clustered together within a region of about 750 nucleotides (Yamamoto et al 1984).

The bovine mRNA does not contain these *Alu* sequences or any other repetitive sequences (Hobbs et al, manuscript in preparation). The sequences on either side of the *Alu* repeats are conserved in the human and the cow, suggesting that the *Alu* sequences were inserted after the human

and bovine evolutionary lines diverged. Restriction maps of genomic DNA suggest that the *Alu* repeats are present at the same location in the 3′ untranslated regions of the LDL-receptor genes of the gorilla and chimpanzee, but not the baboon (unpublished observations). If this finding is confirmed by direct cloning studies, it would suggest that the *Alu* sequences have inserted into this location late in the evolution of the primates. Whether this insertion has any functional consequence for the processing, translation, or stability of the receptor mRNA is unknown.

Gene Structure

Southern blotting of genomic DNA demonstrated that the haploid human genome contains a single copy of the LDL-receptor gene (Lehrman et al 1985). This gene resides on chromosome 19, as determined by somatic cell genetic techniques (Francke et al 1984). The gene spans more than 45 kb. Sequences representing almost the entire gene have been isolated from bacteriophage lambda and cosmid libraries (Südhof et al 1985a,b). The position of each intron within the gene has been mapped, and the sequence of each exon-intron junction has been determined.

These studies reveal that the receptor gene is made up of 18 exons. The sites of the introns in relation to the protein sequence are indicated in Figure 2 (Südhof et al 1985a,b). Most of the introns separate regions of the protein that correspond to domains that were identified through the protein chemistry studies described above. The first intron is located just at the end of the DNA encoding the cleaved signal sequence of the protein. Within the binding domain of the receptor (which contains the seven cysteine-rich repeats), introns occur precisely between repeats I and II; II and III; V and VI; and VI and VII (Figures 2, 3). Repeats III, IV, and V are included in one exon. The binding domain is terminated by an intron at amino acid 292, the last residue in the seventh repeat.

The next domain, the region of homology with the EGF precursor, is encoded in 8 contiguous exons. Within this 400–amino acid region of homology are located 3 copies of a repeated sequence (repeats A, B, and C in Figure 2), each of which is encoded by a single exon (Südhof et al 1985a,b). (The striking similarity in the exon-intron organization of this region of the LDL receptor gene and the EGF precursor gene is discussed in the preceding section).

The O-linked sugar domain is also demarcated neatly by two introns (Figure 2). However, not all domains of the protein are encoded by single exons. Thus, the membrane-spanning region is interrupted by an intron. Another intron interrupts the coding region for the cytoplasmic tail 11 amino acids from the COOH-terminus.

The placement of the introns is consistent with the notion that the human

LDL receptor gene was constructed by the stepwise assembly of exons that encode useful protein sequences. Thirteen of the 18 exons comprising the LDL receptor gene encode protein sequences that are homologous to sequences in other proteins: 5 of these exons encode a sequence similar to one in the C9 component of complement; 3 exons encode a sequence similar to a repeat sequence in the EGF precursor and in 3 proteins of the blood clotting system; and 5 other exons encode nonrepeated sequences that are shared only with the EGF precursor. The LDL receptor thus appears to be a mosaic protein built up of exons shared with different proteins (Südhof et al 1985a,b).

The 5′ untranslated region of the receptor gene is less than 100 base pairs, and it is not interrupted by an intron (Südhof et al 1985a). Two TATA-like boxes occur 20–30 base pairs to the 5′ side of the two major sites of transcription initiation located between nucleotides −79 to −93. Analysis of the upstream promoter region will be of interest because transcription of the gene into mRNA appears to be regulated by a feedback mechanism. When cholesterol accumulates in cells, the level of cytoplasmic mRNA for the receptor declines dramatically (Russell et al 1983, Yamamoto et al 1984), and this leads to a decrease in the rate of synthesis of the receptor protein (Goldstein & Brown 1977). It is likely, but not yet proven, that the decrease in cytoplasmic mRNA is due to a cholesterol-mediated suppression of transcription of the receptor gene.

BIOSYNTHESIS OF THE HUMAN LDL RECEPTOR

The presence of a cleaved NH_2-terminal hydrophobic signal sequence suggests that the LDL receptor is synthesized on membrane-bound ribosomes. At the earliest time point that can be studied (15 min after the addition of [^{35}S]methionine to cultured human fibroblasts) the receptor appears in immunoprecipitates as a protein with an apparent molecular weight of 120,000, as estimated on SDS polyacrylamide gels (Tolleshaug et al 1982). This precursor contains asparagine-linked (N-linked), high-mannose oligosaccharide chains, which are sensitive to endoglycosidase-H (endo-H) (Tolleshaug et al 1983). According to the best estimates available, there are two N-linked sugar chains on the purified bovine LDL receptor (Cummings et al 1983). Although the protein sequence for the human receptor shows five potential N-linked glycosylation sites (Figure 5), it is possible that the three N-linked sites in the cysteine-rich, disulfide-linked region of the receptor are not glycosylated (Yamamoto et al 1984).

The earliest detectable receptor precursor also contains N-acetylgalactosamine (GalNAc) residues attached to serines and threonines

by O linkage. This finding emerged from experiments in which the [^{3}H]glucosamine-labeled 120-kilodalton precursor of human A-431 epidermal carcinoma cells was isolated by SDS gel electrophoresis and digested with pronase. Multiple GalNAc residues were found on a single pronase-resistant fragment (Cummings et al 1983).

The presence of O-linked GalNAc residues at a time when the N-linked sugar chains are still in the high-mannose (endo-H sensitive) configuration implies that the GalNAc transferase that initiates synthesis of O-linked sugar chains is proximal to the *cis*-Golgi stacks. This follows from the observation that once a protein reaches the *cis*-Golgi the mannose residues are trimmed from the N-linked sugars and the chains become endo-H resistant (Hubbard & Ivatt 1981). Whether the O-linked GalNAc residues are added in the ER, or whether they are added in some transitional zone between the ER and the *cis*-Golgi is not known.

Between 30 and 60 min after synthesis, the LDL receptor precursor undergoes a sudden shift in apparent molecular weight from 120,000 to 160,000 (Tolleshaug et al 1982, 1983; Schneider et al 1983a). The timing of this shift coincides with the maturation of the N-linked and O-linked chains. The shift is not the result of the alteration in N-linked sugars, because a change of nearly equal magnitude occurs in cells that are treated with tunicamycin, which blocks the addition of N-linked chains. Conversely, the increase in apparent molecular weight is minimized in a mutant strain of Chinese hamster ovary (CHO) cells that is unable to add galactose to the core GalNAc residues of the O-linked chains (Cummings et al 1983). These findings suggest that the 40,000 change in apparent molecular weight is attributable to the elongation of the O-linked chains. This elongation consists of the addition of a single galactose and one or two sialic acids to the GalNAc core sugar of each O-linked chain.

The apparent molecular weight of the mature receptor is reduced by only about 10,000 when the sialic acids are completely removed with neuraminidase (Schneider et al 1982, Cummings et al 1983). Thus, most of the change from 120,000 to 160,000 daltons is contributed not by the sialic acids, but by the simple addition of galactose residues to the GalNAc core sugars. This change selectively retards the mobility of the receptor on SDS gels, so that the mature receptor migrates more slowly than would be appropriate for its true molecular weight. The calculated molecular weight of the protein component of the receptor is 93,102. When the mature carbohydrates are included, the molecular weight will be $\sim$115,000, not 160,000 as observed on SDS gels. Aberrant migration of other membrane glycoproteins that contain clustered O-linked sugars has been previously documented (Marchesi et al 1976).

The increase in apparent molecular weight of the LDL receptor is

partially blocked when cells are incubated with monensin, an ionophore that blocks vesicular transport in the Golgi complex (unpublished observations). Under these conditions there is no longer a discrete jump from apparent molecular weight of 120,000 to 160,000. Rather, the receptor appears as a smear between these two extremes.

The LDL receptor in human fibroblasts can be labeled with ^{35}S-sulfate, which attaches to N-linked sugars; incorporation is blocked by tunicamycin (Cummings et al 1983). The receptor synthesized in tunicamycin-treated fibroblasts appears to undergo normal internalization and recycling, but subtle changes in receptor half-life have not been ruled out (unpublished observations). Since these tunicamycin-treated receptors do not contain sulfate, it is unlikely that sulfate performs a crucial function in the LDL receptor. Incorporation of ^{35}S-sulfate into the receptor in human A-431 carcinoma cells could not be demonstrated (Cummings et al 1983).

NATURALLY OCCURRING MUTATIONS IN THE LDL RECEPTOR

The power of the LDL receptor as a system for the study of receptor-mediated endocytosis derives from the existence of many naturally occurring mutations in the LDL receptor gene that disrupt receptor function in revealing ways. The mutations occur in individuals with familial hypercholesterolemia (FH) (Goldstein & Brown 1983). Those who inherit one mutant LDL-receptor gene produce half the usual number of normal receptors. In tissue culture their cells degrade LDL at about half the normal rate. In the body the receptor deficiency causes LDL to build up in plasma to levels about twofold greater than normal. Eventually, the high plasma LDL levels lead to atherosclerosis and heart attacks as early as 40 years of age (Brown & Goldstein 1984).

Individuals with two mutant LDL-receptor genes are termed FH homozygotes. Their cells produce few or no functional LDL receptors. As a result, plasma LDL accumulates to levels eight to ten times greater than normal, and they develop atherosclerosis and heart attacks in childhood.

At least ten different mutant alleles at the LDL-receptor locus have been described (Goldstein & Brown 1983, Tolleshaug 1982, 1983, Schneider et al 1983a, Lehrman et al 1985). Many of the phenotypic FH homozygotes actually represent compound heterozygotes who inherit different mutant alleles of the receptor gene from each parent. Study of cultured skin fibroblasts from 104 FH homozygotes revealed that the mutations could be divided into four broad classes based upon their effects on receptor structure and function. These mutations are summarized in Table 2 and discussed below.

Class 1 Mutations: No Detectable Precursor

These alleles, designated *R-0* for "receptor-zero," are the most frequent of the mutant alleles. It is difficult to determine their frequency directly (since they cannot be identified unequivocally in the heterozygous state), but they probably account for about one-third to one-half of all mutant alleles at the LDL-receptor locus. Class 1 alleles fail to express receptor proteins as measured by functional assays (binding of ^{125}I-LDL) or immunological assays (immunoblotting or precipitation by a variety of monoclonal and polyclonal antibodies directed against the LDL receptor). It is likely that this class includes nonsense mutations, which introduce termination codons early in the protein coding region. It may also include: point mutations in the promoter that block transcription of mRNA; point mutations in intron-exon junctions that alter the splicing of mRNA; and large deletions.

Class 2 Mutations: Precursor Not Processed

These alleles encode receptor precursors that are synthesized in normal or reduced amounts, but that do not undergo any apparent increase in molecular weight after synthesis. These receptors remain in the endo-H sensitive form, and they do not receive sialic acid, as indicated by their lack of susceptibility to neuraminidase. Thus, we believe these receptors are not transported to the Golgi complex. Receptors specified by these alleles never reach the cell surface, and hence they are protected when the surface of intact cells is treated with pronase (Tolleshaug et al 1983).

Most of the alleles in this class encode receptors with apparent molecular weights on SDS gels of 120,000, which is similar to the apparent molecular weight of the normal precursor. These alleles are designated *R-120* (Tolleshaug et al 1982, 1983). Detailed structural analysis of the oligosaccharide chains of one mutant receptor encoded by the *R-120* allele showed it to contain N-linked high mannose chains and O-linked core GalNAc residues indistinguishable from the normal 120-kilodalton receptor precursor (Cummings et al 1983). We have also observed precursor proteins with abnormal apparent molecular weights of 100,000 and 135,000 that fall into this class (designated *R-100* and *R-135* alleles, respectively). These molecular weight abnormalities may result from alterations in the length of the protein chain, rather than from alterations in carbohydrate, since the molecular weight remains abnormal after endo-H treatment (Tolleshaug et al 1983).

Variants of the Class 2 mutation were observed in a consanguineous black American family, in several Afrikaners, and in a strain of rabbits that has a syndrome similar to FH, i.e. Watanabe heritable hyperlipidemic

Table 2 Mutations at the LDL receptor locus that produce familial hypercholesterolemia (FH)

Class of mutation	Allele designation[1]	Apparent receptor mass on SDS gels (kDa)		Receptor location				LDL binding to intact cells	Frequency in FH patients
					Plasma membrane				
		Precursor	Mature	Intracellular	Coated pits	Noncoated regions	Extracellular		
Class 1: No detectable precursor	*R-0*	None	None					None	Common
Class 2: Precursor not processed	*R-100*	100	100	+				None	Rare; found in Lebanese
	R-120	120	120	+				None	Common
	R-135	135	135	+				None	Rare
Class 2 variant: Precursor processed slowly, mature receptor binds LDL poorly	*R-120^{slow}160 b$^-$*	120	160	+	(+)[2]			Reduced	Rare; found in Afrikaners and in WHHL rabbits

Class 3:	*R-140 b*$^-$	100	140	+			Reduced	Rare
Precursor processed normally, mature receptor binds LDL poorly	*R-160 b*$^-$	120	160	+			Reduced	Common
	R-210 b$^-$	170	210	+			Reduced	Rare
Class 4:	*R-150 i*$^-$, *sec*	110	150		(+)	+	Normal binding; defective internalization	Rare
Precursor processed normally, mature receptor binds LDL normally, but does not enter coated pits	*R-160 i*$^-$	120	160		+		Normal binding; defective internalization	Rare
	R-155 i$^-$	115	155		+			

[1] Allele designations are based on the apparent molecular weight (in kilodaltons) on SDS polyacrylamide gel electrophoresis of the mature form of the receptor, i.e. the predominant form observed after a 2 hr pulse followed by a 2 hr chase. b^- denotes defective LDL binding; i^- denotes defective internalization; *sec* denotes secretion from the cell.

[2] Symbols in parentheses refer to minor populations.

(WHHL) rabbits (Schneider et al 1983a, unpublished observations). In these variants the receptor is produced as a 120-kilodalton precursor that is processed to the mature form at a slow but finite rate. Eventually about 10% of the receptors appear on the cell surface as 160-kilodalton mature proteins. Even after they reach the surface, these receptors have a reduced ability to bind LDL. Normal receptors bind equimolar amounts of IgG-C7 (a monoclonal antibody against the external domain of the LDL receptor) and LDL protein (Beisiegel et al 1981). In the Class 2 variants the ratio of LDL binding to monoclonal antibody binding is reduced, suggesting that these receptors have an abnormality in the LDL binding site as well as a slower rate of transport to the surface (Schneider et al 1983a).

The molecular basis of the defect in the Class 2 mutations is not known. These receptors are all recognized by monoclonal and polyclonal antibodies against the receptor, so their structures are not drastically different from the normal receptor. It seems likely that the failure of transport arises from some subtle alteration in structure. Elucidation of this change should lead to new insights into the signals that govern transport of proteins from the ER to the *cis*-Golgi.

Scheckman and co-workers have described a mutation in yeast invertase, a secreted enzyme, that is analogous to the Class 2 mutations (Schauer et al 1985). The defect in invertase results from the alteration of a single amino acid at the site at which the hydrophobic NH_2-terminal signal sequence is cleaved from the protein. In the absence of cleavage, invertase is transported to the Golgi at 2% of the normal rate. A similar transport defect has been created in yeast by in vitro mutagenesis of the gene for acid phosphatase (Haguenauer-Tsapis & Hinnen 1984). It seems likely that some of the Class 2 mutations in FH may result from the failure to cleave the signal sequence from the protein.

Class 3 Mutations: Precursor Processed, Abnormal Binding of LDL by Receptor

Receptors specified by Class 3 mutant alleles reach the surface at a normal rate and are recognized on the surface by monoclonal anti-receptor antibody (IgG-C7). However, these receptors bind less than 15% of the normal amount of ^{125}I-LDL (Goldstein & Brown 1983, Beisiegel et al 1981, Tolleshaug et al 1983).

Most commonly, the receptors produced by the Class 3 alleles have a normal molecular weight on SDS gel electrophoresis. This allele is designated *R-160 b*$^-$. Receptors with molecular weights of 140,000 (*R-140 b*$^-$) (Tolleshaug et al 1983) and 210,000 (*R-210 b*$^-$) (Tolleshaug et al 1982) have also been described. Both of these proteins originate as precursors

with apparent molecular weights that are 40,000 less than their mature species, i.e. 100,000 and 170,000, respectively. The correct increase in apparent molecular weight suggests that the carbohydrate processing reactions occur normally. Structural analysis of the carbohydrates of the receptor specified by the *R-210* b^- allele showed no abnormality (Cummings et al 1983). We believe, therefore, that the abnormal molecular weight is due to alterations in the amino acid sequence, and not to changes in carbohydrate content.

An explanation for the abnormally sized receptors is suggested by the structure of the binding domain for LDL. As discussed above, this binding domain is made up of seven repeats of a 40 amino acid sequence. Because of this internal homology, the DNA encoding such a repeat structure would be susceptible to deletion or duplication following "slipped mispairing" and recombination during meiosis. Such duplications or deletions would change the size of the receptor and might reduce LDL binding without affecting the binding of monoclonal antibody IgG-C7 (which does not recognize the LDL binding site). This hypothesis is presently being tested with the available cDNA and genomic probes.

Class 4 Mutations: Precursor Processed, Receptor Binds LDL But Does Not Cluster In Coated Pits

These are the so-called internalization-defective mutations. The original example was patient JD (Brown & Goldstein 1976). Biochemical studies showed that patient JD is a compound heterozygote (Goldstein et al 1977). From his mother he inherited a gene that produces a nonfunctional receptor (*R-0* allele). From his father he inherited a gene that produces a receptor of normal size that reaches the surface and binds LDL normally, but is not able to carry the bound LDL into the cell (*R-160* i^- allele). Electron microscopic studies revealed that the internalization-defective receptors in JD and his father are present on the surface in small clusters, but they are not sequestered in coated pits, even though coated pits are present in these cells (Anderson et al 1977b) and the coated pits function normally in the receptor-mediated endocytosis of other ligands such as EGF (Goldstein et al 1978).

Subsequently, four other FH patients with internalization defects have been identified. One is a young man from Minnesota (designated FH 274 or BH) who is also a compound heterozygote with an internalization-defective allele inherited from his mother and a nonfunctional allele from his father (Goldstein et al 1982, Lehrman et al 1985). Another is a patient from Japan, born of consanguineous parents, who appears to be homozygous for an internalization-defective allele (Miyake et al 1981). The third and fourth

patients are Arab siblings from a consanguineous marriage, who appear to be homozygous for an internalization-defective allele (Lehrman et al 1985a).

We have recently elucidated the molecular defect in the internalization-defective allele in patient FH 274 (Lehrman et al 1985b). Protein chemistry studies demonstrated that his mutant receptor has two abnormal properties. First, it is about 10,000 daltons smaller in apparent molecular weight than the normal receptor. Second, about 80% of the receptors are secreted into the culture medium, and only about 20% remain associated with the cell. (In normal fibroblasts no detectable amounts of receptor are secreted.) The allele giving rise to this abnormal receptor was inherited from the mother. Since the mother's cells had the internalization-defective phenotype, the shortened receptor must be responsible for the internalization-defective state.

Through restriction endonuclease mapping of genomic DNA and subsequent cloning of the relevant genomic fragments into bacteriophage lambda, Lehrman et al (1985b) demonstrated that the mutant internalization-defective allele in FH 274 cells (designated *R-150 i*$^{-}$, *sec*) had undergone a large deletion. The deletion resulted from a recombination between one of the *Alu* sequences in the 3′-untranslated region of the mRNA and an *Alu* sequence that is located 5 kb upstream in the intervening sequence that separates the exon encoding the O-linked sugar region from the exon encoding the membrane-spanning domain. This deletion eliminated the DNA sequences that encode the membrane-spanning region and the cytoplasmic domain of the receptor.

According to the above results the protein produced by the deleted gene is expected to have a normal sequence from the NH_2-terminus through the O-linked sugar region. Thereafter, the protein should terminate because the deletion joint should produce a random sequence of nucleotides in the mRNA. By chance, a protein termination codon is expected to be reached within 20 codons. These predictions derived from the genomic cloning were confirmed by studies of the FH 274 protein. The truncated receptor precursor (110 kilodaltons) showed the normal 40,000 increase in molecular weight after synthesis, indicating that the O-linked sugars were present (Lehrman et al 1985b). Moreover, the FH 274 protein reacted with anti-peptide antibodies directed against the NH_2-terminal domain of the receptor, but failed to react with anti-peptide antibodies directed against the COOH-terminal cytoplasmic tail, which confirms that the cytoplasmic domain was eliminated (Lehrman et al 1985b).

The secretion of the truncated receptor from the FH 274 cells is an expected result based on earlier studies with truncated mutants for viral

envelope proteins, such as that of the influenza hemagglutinin (Gething & Sambrook 1982) and the G protein of vesicular stomatitis virus (Florkiewicz et al 1983). When such truncated proteins are synthesized on membrane-bound ribosomes, the lack of a hydrophobic membrane-spanning region allows the entire protein to translocate across the ER membrane and to appear in the lumen of the ER, from which it is eventually incorporated into secretory vesicles.

Why are some of the mutant receptors produced by the FH 274 cells found attached to the cell surface? These molecules appear to be firmly attached since they cannot be washed off easily by high salt, EDTA, highly charged polymers, or reducing agents (Lehrman et al 1985b, unpublished observations). The membrane-adherent receptors are found in noncoated regions of the cell surface where they bind LDL (Anderson et al, unpublished observations) and give rise to the internalization-defective phenotype by which the FH 274 cells were originally identified (Goldstein et al 1982). It is possible that these truncated receptors bind with high affinity to some other protein that keeps them anchored to the membrane. Alternatively, perhaps some of the mutant receptors have acquired a hydrophobic sequence by chance, as a result of alternative splicing of the mRNA within the intervening sequence that contains the deletion joint. Such splicing might lead to a random sequence of nucleotides that happens to encode a stretch of hydrophobic amino acids prior to the termination codon. Thus, some of the receptors might have sticky hydrophobic COOH-terminal tails that make them adhere to the membrane.

We have recently identified defects in two other internalization-defective LDL receptor mutations (Lehrman et al 1985a and manuscript in preparation). Both of these mutations involve single base substitutions in the exon encoding the majority of the cytoplasmic domain of the receptor (exon 17 in Figure 2), and both were identified through cloning and sequencing of genomic DNA fragments that contain this exon. One of these point mutations, found in the Arab family mentioned above, results from a guanosine to adenosine transition, which changes a tryptophan codon (UGG) to a termination codon (UGA). The receptor produced by this gene terminates at a position corresponding to the tryptophan that is just at the beginning of the cytoplasmic domain of the receptor (Figure 4). The resulting protein has a truncated cytoplasmic domain of only 2 (rather than 50) amino acids. This protein moves to the cell surface, but the lack of a cytoplasmic domain renders it incapable of clustering in coated pits.

A different point mutation occurs in JD, the first internalization-defective FH patient to be described (Brown & Goldstein 1976, Goldstein et al 1977). In this case, an adenosine to guanosine transition converts a codon for

tyrosine (UAU) into a codon for cysteine (UGU). This mutation occurs in the cytoplasmic domain 33 residues from the COOH-terminus (Lehrman et al, manuscript in preparation). The mutant receptor has 2 cysteine residues in its cytoplasmic domain: the normal one at position 818 and a new one at position 807. We do not yet know whether the failure to cluster in coated pits and the abnormal internalization are attributable to the acquisition of an extra cysteine or to the loss of a crucial tyrosine.

The finding of defects in the cytoplasmic domain in three internalization-defective mutations (one deletion, one nonsense mutation, and one missense mutation) supports our earlier proposal that this domain is crucial in directing the LDL receptor to coated pits (Anderson et al 1977, Goldstein et al 1979).

EXPERIMENTALLY INDUCED MUTATIONS IN THE LDL RECEPTOR

To increase the repertoire of available mutations, Krieger et al (1981, 1983) developed two methods for creating LDL-receptor mutations in tissue culture cells. In the first procedure the cholesteryl esters of LDL are extracted and replaced with hydrophobic molecules that convert the LDL into either toxic or fluorescent particles (Krieger et al 1981). Mutagen-treated Chinese hamster ovary (CHO) cells are incubated with reconstituted LDL-containing toxic 25-hydroxycholesteryl oleate. Wild-type cells take up this lipoprotein via the LDL receptor, liberate the 25-hydroxycholesterol in lysosomes, and die. The few surviving clones are incubated with LDL reconstituted with fluorescent cholesteryl ester, and the colonies that fail to accumulate fluorescence are picked. This two-step isolation procedure yielded LDL-receptor-deficient cells at a frequency of 1×10^{-5} (Krieger et al 1981).

The second selection procedure takes advantage of two fungal metabolites: compactin, a potent inhibitor of cholesterol biosynthesis, and amphotericin B, a polyene antibiotic that forms toxic complexes with sterols in membranes (Krieger et al 1983). Mutagen-treated CHO cells are preincubated in a medium containing compactin, LDL, and small amounts of mevalonate, a combination that makes CHO cells dependent on the LDL receptor for obtaining cholesterol (Goldstein et al 1979b). After the preincubation, mutant cells that cannot utilize the cholesterol of LDL become cholesterol-deficient. Subsequent incubation with amphotericin B kills the wild-type cells through formation of complexes with membrane cholesterol. The receptor-deficient clones, which are depleted in cholesterol, do not bind amphotericin B and therefore they survive. With this procedure

mutant cells are isolated at a frequency of approximately 2.6×10^{-5} (Krieger et al 1983).

All of the mutants obtained to date, by either of the two procedures, express an LDL-receptor-deficient or negative phenotype, i.e. there is a proportional reduction in the binding, internalization, and degradation of LDL. Complementation studies suggest that the mutants fall into four groups, designated *ldlA*, *ldlB*, *ldlC*, and *ldlD* (Kingsley & Krieger 1984). The *ldlA* locus appears to be the structural gene for the LDL receptor on the basis of two observations: 1) fusion of these cells with normal human fibroblasts, but not FH homozygote fibroblasts, leads to complementation; and 2) mutants in the *ldlA* group show abnormal LDL receptors by immunoprecipitation and SDS gel electrophoresis (Kozarsky & Krieger, manuscript in preparation). The *ldlA* locus appears to be diploid in CHO cells, as defined by the isolation of a heterozygous revertant of a homozygous mutant (Kingsley & Krieger 1984).

The biochemical basis of the LDL-receptor deficiency in the *ldlB*, *ldlC*, and *ldlD* mutants is unclear at the present time; it may involve defects in the posttranslational processing of the LDL receptor. Most of these mutants exhibit altered sensitivity to plant lectins, which suggests they harbor a pleiotropic abnormality in glycoprotein processing (Krieger et al, unpublished observations). The *ldlD* mutants are unique in that the receptor-negative phenotype can be corrected by co-cultivation with other mammalian cells (Krieger 1983). LDL-receptor activity can also be induced in the *ldlD* cells by addition of a factor found in human or bovine serum (Krieger 1983).

Sege et al (1984) recently used the technique of calcium phosphate–mediated gene transfer to introduce human genomic DNA into the *ldlA* cells. After transfection the *ldlA* cells were incubated in the presence of compactin, LDL, and mevalonate, a combination that selects for functional LDL receptors. One of the clones that survived this selection was shown to express the human LDL receptor on its cell surface, as determined by immunoprecipitation with monoclonal antibody IgG-C7, which reacts with the receptor of human but not of hamster origin (Beisiegel et al 1981). The transfected human receptor gene was functional in the binding, uptake, and degradation of LDL, and its expression was suppressed by cholesterol (Sege et al 1984).

The ability to introduce a functional human LDL-receptor gene into mutant CHO cells illustrates the power of the LDL-receptor system for use in studies of experimentally induced mutations. Not only are efficient selection systems available for the production of LDL-receptor-deficient mutants, but efficient systems are also now available for selecting revertants

of these mutants, and for introducing transfected genes into these cells. Finally, the ability to express functional LDL receptors from cloned cDNAs following transfection of cultured cells (Yamamoto et al 1984), together with the techniques of in vitro mutagenesis, should provide much exciting information regarding the LDL receptor in particular, and the process of receptor-mediated endocytosis in general.

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