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Receptor-mediated endocytosis: Insights from the lipoprotein receptor system*

(lysosomes/coated pits and vesicles/cholesterol/familial hypercholesterolemia/hormone action)

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ABSTRACT **The low density lipoprotein (LDL) receptor system coordinates the metabolism of cholesterol, an essential component of the plasma membrane of all mammalian cells. Study of this system has led to an enhanced understanding of the cellular basis of cholesterol homeostasis. It has also brought into focus an important mechanism of metabolic regulation—the process of receptor-mediated endocytosis. In this article, we first describe the receptor-mediated endocytosis of LDL, a sequence of events in which receptor binding and internalization are coupled in specialized regions of the plasma membrane called coated pits. Second, we trace the cellular functions of the cholesterol derived from internalized LDL. Third, genetic evidence is presented to indicate that both the binding and internalization of LDL are mediated by a single receptor molecule that contains two active sites, one mediating binding and the other internalization. Finally, the characteristics of the LDL receptor system are used to suggest models for receptor systems in general.**

One of the central problems in modern biology is the understanding of the mechanism by which extracellular macromolecules interact with surface receptors to regulate intracellular metabolic events. Recent advances in the field of cell biology have made it possible to study receptor-mediated events directly in living mammalian cells. We have now come to realize that cell surface receptors operate through a network of subcellular organelles. Receptors on the plasma membrane communicate with proteins in the nucleus, endoplasmic reticulum, lysosomes, mitochondria, and other structures. How is this cell-wide communication articulated? How does a cell regulate a metabolic pathway whose active components reside in different subcellular compartments?

Answers to some of these fundamental questions have recently become available through studies of the lipoprotein receptor system (1, 2). This cell-wide regulatory system coordinates the metabolism of cholesterol, an essential component of the plasma membrane of all mammalian cells. But above and beyond its importance in cholesterol homeostasis, the lipoprotein receptor system has become recognized as a prototype for a mechanism of metabolic regulation—the process of receptor-mediated endocytosis.

Before the lipoprotein receptor system is described at the cellular level, a few preliminary comments about cholesterol metabolism seem appropriate. All animal cells require cholesterol for their plasma membranes, yet the body cannot tolerate excessive cholesterol in blood because the insoluble sterol deposits in artery walls, producing atherosclerosis. Thus, animals have evolved a mechanism for transporting cholesterol through the blood and delivering it to cells, while at the same time avoiding its excessive accumulation. For this purpose, cholesterol is packaged into lipoprotein particles in the liver and intestine, and the soluble lipoproteins are secreted into plasma and carried to target tissues. In man, the most abundant of these particles is low density lipoprotein (LDL).

Two-thirds of the cholesterol in human plasma is contained within LDL. The bulk of the cholesterol resides in an apolar core in which each sterol molecule is esterified with a long-chain fatty acid. This core of cholesteryl esters is surrounded by a polar coat that contains phospholipid, small amounts of unesterified cholesterol, and a protein called apoprotein B (3). In synthesizing their membranes, cells require unesterified cholesterol. To use the cholesterol of LDL, cells must be able to take apart the lipoprotein and hydrolyze the cholesteryl esters. This is where the need for multiple subcellular organelles comes in. The key to the process is a cell surface receptor that binds LDL and facilitates its entry into cells. The sequence of reactions by which cells use the receptor to obtain cholesterol is called the LDL receptor pathway.

Receptor-mediated endocytosis of LDL: Binding coupled to internalization via coated pits

The biochemical steps in the LDL receptor pathway are initiated by the binding of LDL to a specific receptor on the cell surface. The LDL receptor was discovered in 1973 through studies of human skin fibroblasts in tissue culture (4–6). The background for these studies lay in the observations of Bailey (7) and Rothblat (8), who in the 1960s showed that cultured animal cells did not synthesize their own cholesterol, but rather took it up from lipoproteins that were present in the serum of the culture medium.

In 1973 our laboratory observed that even though human serum contains a mixture of cholesterol-carrying lipoproteins, the fibroblasts could derive cholesterol only from LDL (4, 9). This was the first clue that cells might have a specific mechanism for interacting with a specific lipoprotein. We then found that the specificity of the LDL effect was due to the existence of a cell surface receptor for LDL (6, 10).

To study the receptor, we labeled LDL with ^{125}I so that the iodine was attached only to the protein component. When monolayers of intact fibroblasts are incubated at 4°C with ^{125}I-LDL, binding occurs but internalization is prevented (6, 10, 11). The ^{125}I-LDL binds to a single population of surface receptor sites. At 37°C half-maximal binding is achieved at an LDL concentration of about 1 nM (6, 12). Human fibroblasts produce a maximum of 20,000–50,000 receptors per cell. Binding requires a divalent cation, either calcium or manganese (13). The receptor is exquisitely sensitive to proteases such as Pronase, trypsin, and chymotrypsin, but is resistant to glycosidases and other hydrolytic enzymes (10, 13). The receptor is also inactivated by protein-modification reactions, such as acetylation and glutaraldehyde treatment. We therefore believe that the receptor is a protein. The receptor recognizes the apoprotein B component of LDL; it does not recognize apoproteins A-I and A-II, the proteins found in high density

Abbreviations: FH, familial hypercholesterolemia; LDL, low density lipoprotein; HDL, high density lipoprotein.

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lipoprotein (HDL), the other major cholesterol-carrying lipoprotein in human plasma (6, 14, 15). In certain animals that are fed large amounts of cholesterol, another lipoprotein, called HDL_c, accumulates in plasma (16). Although HDL_c does not contain apoprotein B, it contains a protein called apoprotein E that shares a number of physical and chemical properties with apoprotein B. The LDL receptor of fibroblasts recognizes apoprotein E and therefore it binds HDL_c as well as LDL (16, 17).

An important property of the LDL receptor is that LDL can be dissociated from it by exposure to heparin and other sulfated glycosaminoglycans (11). The ability to release LDL from the receptor with heparin has provided a powerful tool with which to dissect the receptor pathway. Having bound LDL at the cell surface, how does the cell extract the cholesteryl esters from the core of the lipoprotein? The answer came when the cells were warmed to 37°C and the entire LDL particle was found to be internalized and degraded (10).

Fig. 1 shows a biochemical experiment designed to follow the internalization of receptor-bound ^{125}I-LDL. Prior to time zero, the fibroblasts were incubated with ^{125}I-LDL at 4°C so that binding occurred. The cells were then washed to remove unbound LDL. One set of cells was then exposed to heparin at zero time. Because these cells had not been warmed, nearly all of the bound ^{125}I-LDL was released from the receptor by heparin. Only a small amount of ^{125}I-LDL was resistant to heparin release. The rest of the cells were then warmed to 37°C. After various times, individual dishes of cells were chilled to 4°C to stop the internalization and then they were exposed to heparin. During the warm-up period, the amount of LDL that was on the surface and subsequently releasable by heparin declined rapidly, and this was balanced by an increase in the LDL that had entered the cell and was no longer releasable by heparin. This internalized ^{125}I-LDL did not remain in the cell

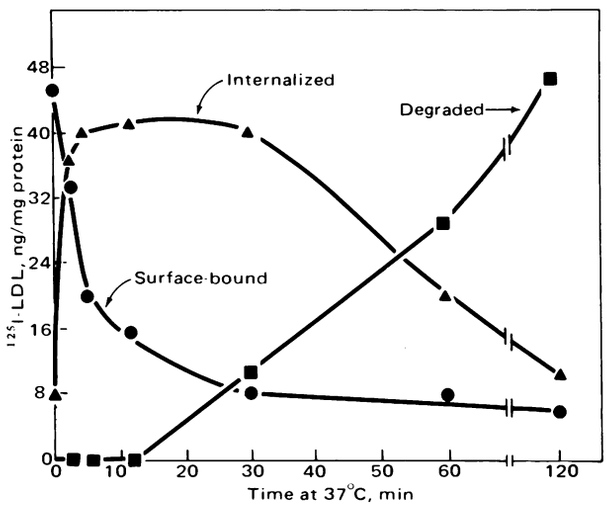

FIG. 1. Internalization and degradation at 37°C of ^{125}I-LDL previously bound to the LDL receptor at 4°C in normal human fibroblasts. Cells were incubated in growth medium containing 10% lipoprotein-deficient serum for 48 hr prior to the experiment. On the day of the experiment, each dish received 2 ml of ice-cold medium containing 10 μg of protein per ml of ^{125}I-LDL. The ^{125}I-LDL was allowed to bind to the cells at 4°C, after which each monolayer was washed extensively. Each dish then received 2 ml of medium containing 10 μg of protein per ml of unlabeled LDL, and all dishes were incubated at 37°C. After the indicated time at 37°C, groups of dishes were rapidly chilled to 4°C, the medium was removed, and its content of ^{125}I-labeled trichloroacetic acid-soluble material (■) was measured (10). The amounts of surface-bound (heparin-releasable) ^{125}I-LDL (●) and internalized (heparin-resistant) ^{125}I-LDL that remained associated with the cells (▲) were also determined (11).

for a long period. After about 30 min the LDL within the cell declined. At the same time, trichloroacetic acid-soluble ^{125}I-labeled material appeared in the culture medium. These acid-soluble products consisted almost entirely of mono[^{125}I]-iodotyrosine, indicating that the protein component of the internalized LDL had been digested completely to amino acids (10). This hydrolysis has been shown to occur within lysosomes (18, 19). At the same time that the protein of LDL is hydrolyzed, the cholesteryl esters are also hydrolyzed (19). The cholesterol released within the lysosome enters the cytoplasmic compartment, where it becomes the central regulator of cholesterol metabolism (discussed below).

One aspect of the warm-up experiment in Fig. 1 was striking. That was the remarkable efficiency of the internalization. Nearly all of the receptor-bound ^{125}I-LDL was internalized within 15 min (20). Fibroblasts must have an efficient mechanism for coupling the binding and internalization events. The key to this coupling turned out to be an intriguing structure called the coated pit. Coated pits on the surface of mosquito oocytes were described in 1964 by Roth and Porter (21), and subsequently these structures have been observed in virtually all animal cells (22). They represent specialized regions of the cell surface where the plasma membrane is indented and coated on its cytoplasmic surface by a fuzzy material. The role of the coated pit in receptor-mediated endocytosis was recognized in studies of LDL uptake that were carried out in collaboration with Richard Anderson (23, 24).

The visualization of LDL receptors was made possible through the use of LDL that had been covalently coupled to the iron-containing protein ferritin so that the lipoprotein could be seen in the electron microscope (23). Coated pits cover only 2% of the cell surface of fibroblasts, yet they contain 50–80% of the LDL receptors (23, 25). When fibroblasts are first allowed to bind LDL-ferritin at 4°C and are then warmed to 37°C, within 1 min the LDL-ferritin can be seen in coated pits that have invaginated into the cell and have begun to form coated endocytic vesicles (Fig. 2). Within 3 min after warming, these coated vesicles detach from the membrane and can be seen carrying the LDL-ferritin into the cell. Within 6–8 min after warming, the LDL-ferritin is observed within lysosomes where the LDL is being degraded (24). The time course of LDL-ferritin uptake in the electron microscope is identical to the rapid uptake of ^{125}I-LDL observed in the biochemical studies. The clustering of LDL receptors in coated pits explains the extraordinary efficiency of the LDL internalization process. The properties of coated pits are listed in Table 1.

At the same time that we, together with Richard Anderson, were carrying out functional studies of coated pits, Barbara Pearse was isolating coated vesicles from pig brain. Pearse

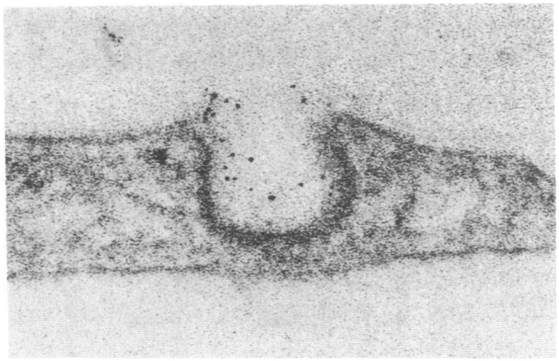

FIG. 2. Electron micrograph showing the localization of LDL-ferritin in a coated pit on the cell surface of a normal human fibroblast. The black dots denote the LDL-ferritin particles. (×115,500.)

Table 1. Properties of coated pits

1. Coated pits are transient structures that continually form and pinch off to form coated vesicles.
2. Life span is less than 5 min.
3. Rate of formation and internalization are not influenced by presence of either LDL or LDL receptors.
4. Each coated pit contains receptors for many different macromolecules.
5. Coat is composed predominantly of a protein of 180,000 molecular weight, called clathrin.

showed that the fuzzy coat is composed predominantly of a protein of 180,000 molecular weight that she named clathrin (22, 26, 27). It seemed likely that the same protein was present in the coated pits of human fibroblasts. Therefore, we isolated clathrin from bovine brain coated vesicles, prepared an antibody to it, and used this antibody to stain coated pits and vesicles in human fibroblasts (28). By indirect immunoperoxidase electron microscopy, the anti-clathrin antibody was observed to bind specifically to coated pits on the surface of human fibroblasts and to coated vesicles within the cell (28). In more recent electron microscopic studies (29), Anderson has demonstrated that LDL-ferritin and clathrin are both localized to the same coated pits and vesicles. These findings raise a crucial question: How do the LDL receptors find their way to coated pits? The answer has been suggested by genetic studies that are discussed later in the article.

Fig. 3 shows a schematic representation of the sequential steps in the LDL receptor pathway as they emerged from the biochemical and ultrastructural studies. The LDL receptors are located in coated pits. Binding of LDL is followed by internalization within coated vesicles, which rapidly fuse with lysosomes. The protein of LDL is hydrolyzed to amino acids, and the cholesteryl esters are hydrolyzed by a lysosomal acid lipase. The resulting unesterified cholesterol crosses the lysosomal membrane and enters the cytoplasmic compartment, where it fulfills its cellular functions.

Functions of cholesterol derived from internalized LDL: Regulatory and structural roles

The cholesterol derived from internalized LDL is the central agent mediating a complex system of feedback control that stabilizes the cellular cholesterol concentration. This stabilization is achieved by the three regulatory reactions illustrated in Fig. 3. First, the incoming cholesterol suppresses the microsomal enzyme 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA reductase), the rate-controlling enzyme in cholesterol biosynthesis, thereby turning off cholesterol synthesis in the cell (4, 9). Second, the incoming cholesterol activates a microsomal cholesterol esterifying enzyme called

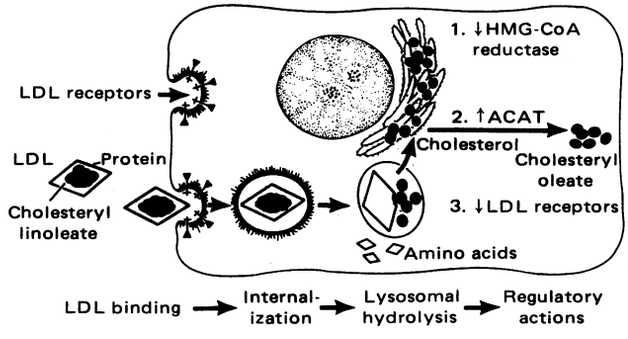

FIG. 3. Sequential steps in the LDL pathway in cultured mammalian cells. HMG-CoA reductase denotes 3-hydroxy-3-methylglutaryl-coenzyme A reductase; ACAT denotes acyl-CoA:cholesterol acyltransferase; vertical arrows suggest regulatory effects.

acyl-CoA:cholesterol acyltransferase (ACAT) so that excess cholesterol can be reesterified and stored as cholesteryl esters (30, 31). And, most striking of all, the incoming cholesterol turns off the synthesis of the LDL receptor, preventing further entry of LDL and protecting cells against an overaccumulation of cholesterol (32).

The overall effect of this system is to coordinate the intracellular and extracellular sources of cholesterol so as to maintain a constant level of unesterified cholesterol within the cell in the face of fluctuations in the external supply of lipoproteins. Human fibroblasts grow in the absence of lipoproteins because they can synthesize cholesterol from acetyl-CoA. On the other hand, when LDL is available, the cells preferentially use the receptor to take up LDL and keep their own cholesterol synthesis suppressed.

In addition to its regulatory roles, LDL-derived cholesterol fulfills two other roles in cultured cells: it is an essential component of cell membranes (33, 34) and it is a substrate for the synthesis of steroid hormones (35, 36). Table 2 lists the types of cultured cells in which the LDL receptor pathway has been demonstrated. These cells come from humans, dogs, swine, mice, hamsters, and cows. In each cell type, LDL-derived cholesterol is used for membrane synthesis and for the regulation of cholesterol homeostasis. Adrenal cells from the mouse and cow have especially large numbers of LDL receptors (35, 36). In these adrenal cells, LDL-cholesterol is transferred from the lysosome to still another compartment—the mitochondria, where the side chain is cleaved and the cholesterol is converted to steroid hormones.

As expected from its widespread occurrence in cultured cells, the LDL receptor plays a role in a wide variety of cells in the body. Ho *et al.* (37, 38) found that the entire LDL pathway functions in human lymphocytes immediately after their isolation from blood. Our colleagues Basu *et al.* (13) and Kovanen *et al.* (39) recently developed a binding assay that measures LDL receptor activity in membranes prepared from fresh tissue homogenates. They have used this assay to measure receptor activity in 16 tissues of the cow. As predicted from the cell culture studies, the number of high-affinity LDL binding sites was highest in the membranes of the adrenal cortex and the ovarian corpus luteum, the two tissues that synthesize steroid hormones and have the highest requirements for cholesterol. In contrast, the adrenal medulla and the ovarian interstitium, which do not produce large amounts of steroids, showed much less LDL binding activity. High-affinity binding was also detected in many other tissues, including adipose tissue, myocardium, skeletal muscle, etc. No significant high-affinity LDL binding was detected in mature erythrocytes (39).

The regulatory and structural roles of the LDL receptor in cultured cells and its widespread distribution in animal tissues have suggested a model for the function of LDL in the body.

Table 2. Cultured cells that obtain cholesterol through the LDL receptor pathway

Human	Mouse
Fibroblasts	Adrenal cells (Y-1 clone)
Fibroblasts transformed by simian virus 40	Teratocarcinoma cells
Smooth muscle cells	L cells
Endothelial cells	L 1210 leukemia cells
Lymphoblasts	Hamster
Burkitt lymphoma cells (Raji)	Chinese hamster ovary (CHO) cells
Acute myelogenous leukemia cells	Fibroblasts
HeLa cells	
Choriocarcinoma cells	
Dog and Swine	Cow
Fibroblasts	Adrenocortical cells
Smooth muscle cells	Endothelial cells

The salient feature of this model is that LDL carries cholesterol from its sites of synthesis and absorption in the liver and intestine to its sites of utilization in extrahepatic cells. As it delivers cholesterol to cells, the LDL particle is degraded. Thus, the LDL receptor serves a dual function: it allows cells to obtain cholesterol and at the same time it removes LDL from plasma (40).

Genetic dissection of the LDL receptor: A single protein mediating both binding and internalization

The biochemical and morphological experiments show that binding and internalization of LDL are coupled events that are mediated through the coated pit. Understanding of the molecular basis of this coupling has been facilitated by the existence of naturally occurring human mutations in the gene for the LDL receptor. These mutations are found among patients who have a common disease called familial hypercholesterolemia (FH) (41).

FH is an autosomal dominant disease that exhibits a gene dosage effect. Affected individuals show one of two clinical phenotypes: heterozygotes or homozygotes (41). Heterozygotes occur in the population at a frequency of 1 in 500 persons, placing FH among the most common inherited diseases in man. Heterozygotes have a 2- to 3-fold elevation in the plasma level of LDL and they often develop myocardial infarctions as early as 35 to 45 years of age. Rarely, two heterozygotes marry and produce a homozygote who inherits two doses of the mutant gene. Homozygotes are affected in childhood with severe hypercholesterolemia with plasma LDL levels 6-fold above normal. They typically suffer myocardial infarctions before the age of 15. Although homozygotes are rare, their fibroblasts made genetic analysis of the LDL receptor possible.

We have now studied fibroblasts from a large number of patients with the clinical diagnosis of "homozygous" FH. In each case the cells showed evidence of a primary abnormality in the gene for the LDL receptor (42, 43). Three classes of mutant alleles have been identified. One of these, R^{b^0}, specifies a receptor that has no binding activity. The second class of alleles, R^{b^-}, specifies a receptor that has reduced but detectable binding activity. The third mutant allele, R^{b^+,i^0}, specifies a fascinating receptor that can bind LDL normally but that cannot carry the LDL into the cell.

Table 3. Genetic analysis of fibroblasts cultured from 50 individuals with the clinical phenotype of homozygous FH

Subjects	Genotype at LDL receptor locus	Cellular phenotype
29	R^{b^0}/R^{b^0}	Receptor-negative
20	R^{b^-}/R^{b^0} or R^{b^-}/R^{b^-}	Receptor-defective
1	$R^{b^0}/R^{b^+,i^0}$	Internalization-defective

Table 3 shows the distribution of these alleles in fibroblasts from 50 patients with homozygous FH. In each case the cells were found to display evidence for two mutant alleles at the receptor locus. Twenty-nine of the subjects show no binding activity and are presumed to be homozygous for the R^{b^0} allele (genotype R^{b^0}/R^{b^0}). Twenty of the subjects show detectable but markedly reduced binding activity. Each of these subjects is postulated to have one R^{b^-} allele and either one R^{b^0} allele or a second R^{b^-} (genotype either R^{b^-}/R^{b^0} or R^{b^-}/R^{b^-}). The cells from one patient have an internalization defect. His cells retain the ability to bind LDL but are unable to internalize the receptor-bound lipoprotein (see below).

The fibroblasts from a large number of FH heterozygotes have also been studied. In each case, the cells show evidence of one normal allele and one of the three mutant alleles at the receptor locus (42, 43). Thus, FH, like most inborn errors of metabolism, appears to be genetically heterogeneous in that several different mutations at a single locus produce a similar clinical syndrome.

Fig. 4 illustrates the biochemical consequences of the LDL receptor defect in fibroblasts from one of the patients who is homozygous for the R^{b^0} allele and whose cells show the complete binding defect. In each panel, the actions of LDL are

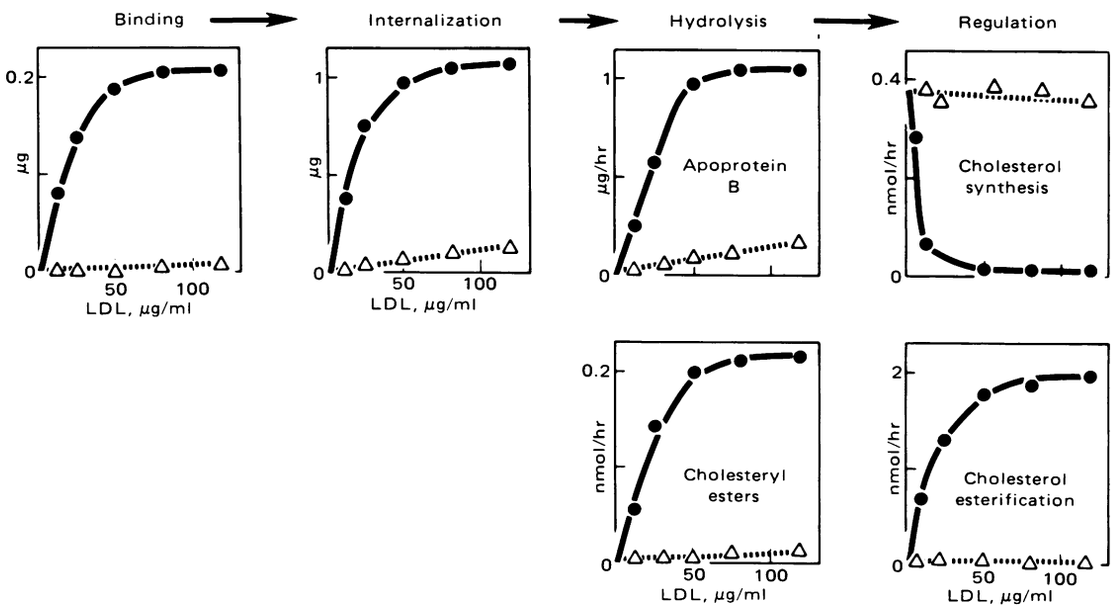

FIG. 4. LDL receptor actions in normal fibroblasts (●) and receptor-negative FH homozygote fibroblasts (△) incubated with ^{125}I-LDL or unlabeled LDL at 37°C for 5 hr. Assays were performed in growing cells in monolayers as described (1, 44). All data are normalized to 1 mg of total cell protein. The units for each assay are as follows: *Binding*, μg of ^{125}I-LDL bound to cell surface; *Internalization*, μg of ^{125}I-LDL contained within the cell; *Hydrolysis of apoprotein B*, μg of ^{125}I-LDL degraded to mono[^{125}I]iodotyrosine per hr; *Hydrolysis of cholesteryl esters*, nmol of [^{3}H]cholesterol formed per hr from the hydrolysis of [^{3}H]cholesteryl linoleate-labeled LDL; *Cholesterol synthesis*, nmol of [^{14}C]acetate incorporated into [^{14}C]cholesterol per hr by intact cells; *Cholesterol esterification*, nmol of [^{14}C]oleate incorporated into cholesteryl [^{14}C]oleate per hr by intact cells.

plotted as a function of the concentration of the lipoprotein in the culture medium. In normal cells as the concentration of ^{125}I-LDL in the medium is increased, the amount of LDL bound to the receptor increases until all of the sites become occupied. The rate of uptake of LDL is proportional to the surface binding. In the normal cells the rates of hydrolysis of both the apoprotein and cholesteryl esters are proportional to the surface binding and cellular uptake of the lipoprotein. In normal cells, LDL uptake suppresses cholesterol synthesis and stimulates cholesterol esterification.

The results in cells from the receptor-negative FH homozygote are in striking contrast. No surface binding of ^{125}I-LDL is detected in the mutant cells. As a result, high-affinity uptake of LDL does not occur (6, 11), and the apoprotein and cholesteryl ester components are not hydrolyzed at normal rates (10, 44). Because it cannot enter the cell, LDL fails to suppress cholesterol synthesis (5, 9), and it does not stimulate cholesterol esterification (30).

The intracellular messenger in the LDL pathway is cholesterol derived from the lysosomal hydrolysis of LDL (1, 19). To confirm the specificity of the LDL binding defect in these FH homozygote cells, we incubated the cells with unesterified cholesterol dissolved in ethanol. This cholesterol enters cells by diffusing through the membrane without a requirement for the LDL receptor. When cholesterol was added in this form to the FH homozygote cells, it bypassed the receptor defect and suppressed the synthesis of cholesterol and stimulated the esterification of cholesterol normally (9, 31, 33).

The major conclusion from the above experiments is that FH homozygote cells have a primary defect in the LDL receptor that prevents them from degrading LDL and using its cholesterol. As a result, the FH homozygote cells must synthesize their own cholesterol in order to grow.

The studies of the receptor-negative FH homozygote cells confirmed that binding of LDL to specific receptors is essential for the cellular action of LDL. But what about internalization? Is any separate action of the receptor required, or is internalization simply the inevitable fate of any molecule that becomes bound to the cell surface? The initial studies gave no evidence for a role for the receptor other than binding. In all normal subjects, internalization inevitably followed binding, and in all patients with binding defects internalization was reduced in proportion to the decrease in binding (42, 45). But then we encountered a type of mutation that challenged this simple notion. This mutation was observed in the fibroblasts of the unique patient with the internalization defect (initials J.D.). J.D. is a 14-year-old boy who has a 6-fold elevation in his plasma LDL level and all of the other clinical features of homozygous FH (20).

Fig. 5 compares LDL metabolism in the fibroblasts of J.D. with those of a normal subject as a function of the LDL concentration in the medium. In the J.D. cells the surface binding of LDL is only slightly lower than in the normal cells. However, the J.D. cells are unable to internalize the bound LDL. As a result of this uptake defect, the J.D. cells do not hydrolyze the protein and cholesteryl ester components of receptor-bound LDL. Cholesterol synthesis remains high and cholesterol esterification is not activated. The failure of the J.D. cells to internalize LDL is highly specific. These cells show normal endocytosis of inert molecules such as sucrose and gamma globulin (20), and they also have a normal ability to take up and degrade ^{125}I-labeled epidermal growth factor, a molecule that is bound to surface receptors, internalized, and degraded in a manner similar to LDL (2).

The mechanism for the internalization defect was disclosed by the electron microscope. When J.D.'s fibroblasts were examined by electron microscopy, they were observed to contain the same number of coated pits and vesicles as normal cells and the appearance of the coated pits was normal. However, in contrast to normal fibroblasts in which 50–80% of the LDL receptors are located in the coated pits, virtually no LDL-ferritin was bound to the coated pits of the J.D. cells (46).

Instead of binding to coated pits in the J.D. cells, the LDL-ferritin binds to LDL receptors that are scattered along noncoated segments of membrane (46). The binding properties of these receptors are the same as those of normal cells (20); they are simply in the wrong place. The striking electron microscopic findings in the J.D. cells have been confirmed by Carpentier *et al.* (47), who used the technique of quantitative ^{125}I-LDL electron microscopic autoradiography. It is clear that the J.D. cells fail to internalize LDL because their LDL receptors cannot be incorporated into coated pits.

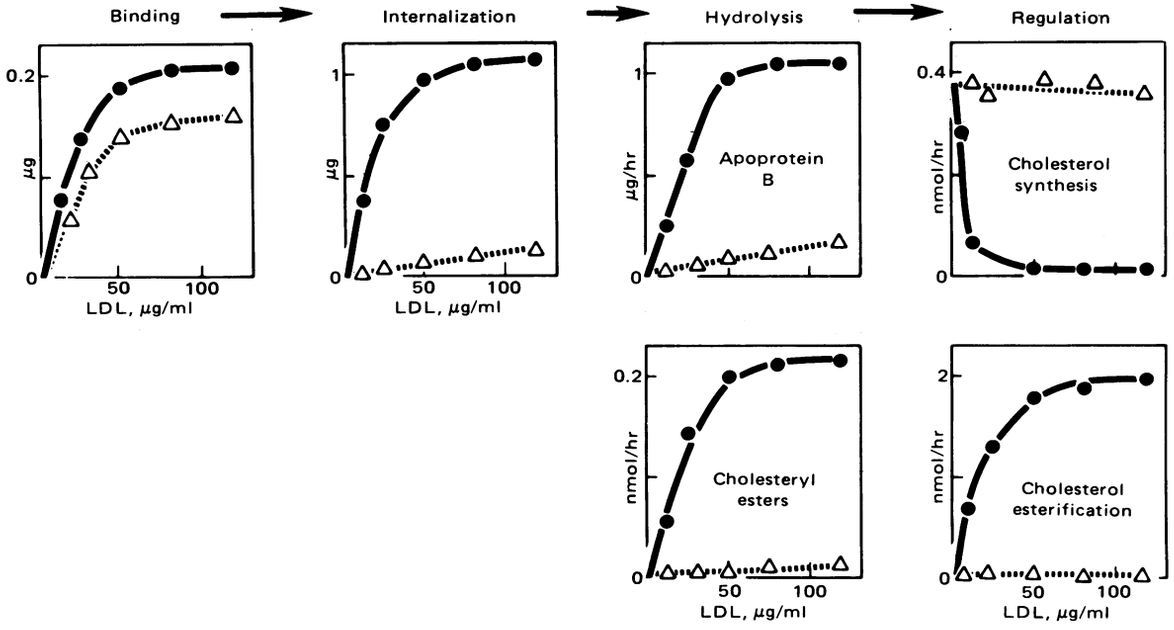

FIG. 5. LDL receptor actions in normal fibroblasts (●) and internalization-defective FH homozygote fibroblasts (△) incubated with ^{125}I-LDL or unlabeled LDL at 37°C for 5 hr. See legend to Fig. 4 for experimental details.

An explanation for the genetic defect in the J.D. cells has been advanced on the basis of studies of fibroblasts from J.D.'s parents (2, 43). Both parents exhibit hypercholesterolemia and other clinical features typical of heterozygous FH. However, the two parents exhibit different mutations at the receptor locus. When the mother's fibroblasts were incubated with ^{125}I-LDL, they bound half the normal amount of lipoprotein (43). Studies with LDL-ferritin showed that a normal proportion of her receptors were in coated pits (46). When her cells were warmed to 37°C, all of the receptor-bound ^{125}I-LDL entered the cell within 10 min (43). Thus, the mother's cells were biochemically identical to those of the usual heterozygote for the receptor-negative allele (genotype $+/R^{b0}$).

A different genetic situation was apparent in cells from J.D.'s father (42). Although he had the clinical phenotype of heterozygous FH, his cells bound about 1.5-fold more ^{125}I-LDL than normal cells (43). Half of the receptor-bound ^{125}I-LDL was internalized within 10 min and the other half remained on the surface for more than 30 min (43). Studies with LDL-ferritin confirmed that the father's cells had two populations of LDL receptors. One of these populations was located in coated pits and the other population was located along noncoated segments of membrane (46). Both populations of receptors bound LDL-ferritin, but when the cells were warmed only the LDL-ferritin that was bound to receptors in the coated pits entered the cell. The LDL-ferritin bound to receptors in the noncoated membrane remained on the cell surface.

The finding of two populations of receptors in the father's cells suggested that he also had one normal allele and one mutant allele at the receptor locus. The receptors produced by the normal allele were located in coated pits and carried their bound LDL into the cell. The receptors specified by the mutant allele were able to bind LDL but were unable to become incorporated into coated pits. As a result, the LDL bound to these receptors was not internalized by the cells. This allele was designated $R^{b+,i0}$ (binding positive, internalization negative), and the genotype of the father was designated $+/R^{b+,i0}$ (43).

The pedigree of the J.D. family is shown in Fig. 6. Study of the offspring of this marriage support the notion that the R^{b0} mutation in the mother and the $R^{b+,i0}$ mutation in the father are allelic (2, 43). The index case, J.D., has severe hypercholesterolemia and the homozygous phenotype as discussed above. The sister of J.D. is clinically normal. Her fibroblasts had a normal number of LDL receptors and internalized the bound LDL normally. Presumably she inherited a normal allele from each parent. The brother of J.D. has a moderately elevated plasma cholesterol level and clinically appears to be a heterozygote. His fibroblasts behaved identically to the father's. They bound a somewhat higher than normal amount of LDL, but only half the bound lipoprotein was internalized. Moreover, by electron microscopy, his cells, like the father's, had only half the normal amount of receptors in coated pits. Thus, the brother of J.D. appeared to have inherited the normal allele from the mother and the $R^{b+,i0}$ allele from the father (Fig. 6).

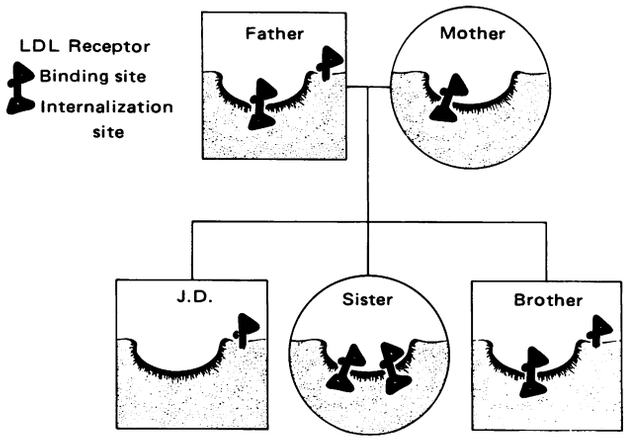

FIG. 6. Pedigree of the J.D. family. The diagram illustrates the genetic basis of the internalization defect in this family. A normal cell (genotype +/+) is exemplified by the sister of J.D. and is shown as having two functional LDL receptors. The presumed genotypes of the other family members are as follows: Father, $+/R^{b+,i0}$; Mother, $+/R^{b0}$; J.D., $R^{b0}/R^{b+,i0}$; and Brother, $+/R^{b+,i0}$.

As described above, J.D.'s cells bound slightly less LDL than normal. None of J.D.'s receptors were located in coated pits and none of the bound LDL was internalized. This pattern would be explained if J.D. were a genetic compound (genotype $R^{b0}/R^{b+,i0}$). From his mother, he inherited the silent allele, R^{b0}. From his father, he inherited the internalization-defective allele, $R^{b+,i0}$. All of the receptors that can bind LDL are of the $R^{b+,i0}$ type and hence none of the bound LDL is internalized (Fig. 6).

The above mechanism implies that the binding mutation and the internalization mutation are alternate alleles at a single locus specifying the LDL receptor. Other more complex mechanisms might be invoked to account for the findings in this pedigree. For example, the LDL receptor might have multiple subunits encoded by independent genes. The mother might be heterozygous for a mutation in the gene for the binding subunit and the father might be heterozygous for a mutation in another gene specifying the internalization subunit. In this case J.D. would be a double heterozygote; i.e., he would carry single mutant alleles at two genetic loci. If each receptor were composed of a single binding subunit and a single internalization subunit, such a double heterozygote should have a half-normal number of binding sites, but half of these should be associated with normal internalization subunits. As a result, half of the bound LDL should be internalized normally. Thus, the overall rate of internalization of ^{125}I-LDL in these cells should be one-fourth that of normal (half-normal binding × half-normal internalization). However, direct measurements show that ^{125}I-LDL internalization in J.D.'s cells is less than 5% of the normal rate (20, 43). This finding tends to exclude the possibility that the two mutations involve genes for different subunits.

A second alternative hypothesis is that the internalization defect is due not to a defect in the receptor itself, but to a defect in a protein that functions catalytically to incorporate LDL receptors into coated pits. By this hypothesis, the heterozygous father would have half the normal amount of this internalization enzyme. If this were the case, the father's cells should internalize all of the bound LDL, but the rate of internalization should be half normal. However, as summarized above, the father's cells behaved in a different fashion; they internalized half the bound LDL at a normal rate and failed to internalize the other half (43). Such a finding suggests a stoichiometric defect in the receptor and not a half-normal amount of a catalytic protein.

On the basis of the above considerations, we believe that the hypothesis of a single genetic locus with two alleles is most likely. Its proof will require isolation of the receptor protein from J.D.'s cells and demonstration that its primary amino acid sequence is altered as a result of both mutations. In view of the small number of receptors per cell, such definitive proof may be a long way off. Nevertheless, the single-locus hypothesis is useful in that it allows one to construct a testable model for the mechanism by which the LDL receptors are incorporated into coated pits.

Our working model is shown in Fig. 7. By analogy with other membrane proteins, the LDL receptor is likely to be synthesized

on membrane-bound polyribosomes and glycosylated in the Golgi apparatus. The receptor is initially inserted into the plasma membrane at random sites. The genetic data on the internalization defect suggest that the receptor has two active sites. One of these, the binding site for LDL, must be on the external surface of the membrane. The second site, the internalization site, allows the receptor to be recognized as a component of coated pits. We envision that this internalization site is on the cytoplasmic surface of the membrane. Receptors that contain a functional internalization site migrate laterally in the plane of the membrane and cluster together in coated pits. Our hypothesis is that this clustering occurs on the cytoplasmic surface of the membrane as a result of a specific interaction of the internalization site of the receptor with the coat protein clathrin or with some other protein that is itself bound to clathrin (46). The clustering of receptors does not appear to be induced by the binding of LDL. All of the data indicate that the entire sequence of receptor insertion into the plasma membrane, clustering into coated pits, and internalization proceeds continously in fibroblasts whether or not LDL is present (23, 46). Although the LDL receptors are likely to be internalized when coated pits pinch off to form coated vesicles, they appear to escape degradation in the lysosome (11). When synthesis of new receptors is blocked by cycloheximide, fibroblasts continue to bind and internalize LDL at maximal rates for hours. This finding suggests that the receptors are recycled; that is, after they deposit their LDL in lysosomes the receptors return to the cell surface, where they again cluster in coated pits.

The model in Fig. 7 may be of importance for several reasons. First, it explains the internalization defect in J.D. Because his abnormal receptors lack functional internalization sites, they are not recognized by the cytoplasmic proteins that form coated pits and hence they remain where they were initially inserted—scattered at random on the cell surface. Second, the model may have implications for the structure and function of other cell surface receptors that carry protein ligands into cells.

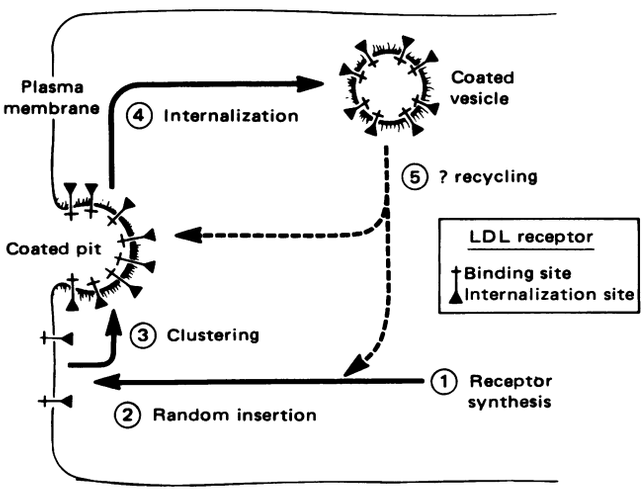

FIG. 7. Proposed mechanism by which LDL receptors become localized to coated pits on the plasma membrane of human fibroblasts. The sequential steps are as follows: 1, synthesis of LDL receptors on polyribosomes; 2, insertion of LDL receptors at random sites along noncoated segments of plasma membrane; 3, clustering of LDL receptors in coated pits; 4, internalization of LDL receptors as coated pits invaginate to form coated endocytic vesicles; and 5, recycling of internalized LDL receptors back to the plasma membrane. Reprinted from ref. 46 with permission.

Models for other cell surface receptor systems

In multicellular organisms, signals are transmitted from one cell to another in the form of protein hormones. Like LDL and other plasma transport proteins, protein hormones bind to receptors on the surface of target cells and elicit regulatory responses. A recent and surprising finding in receptor research is that some of the protein hormones previously thought to act only on the surface of target cells are in fact internalized and degraded just as the transport proteins are. The most striking examples include insulin (48), epidermal growth factor (49), and chorionic gonadotropin (50). In each case the uptake and degradation of the hormone in the target cells is mediated by the same receptor that mediates the hormone's action. In each case degradation occurs within lysosomes. In the case of epidermal growth factor, the uptake occurs through coated pits that are the same as those that carry LDL into cells (51, 52). These findings raise the possibility that some hormone receptors have internalization sites that allow them to become incorporated into coated pits so as to carry their ligands into cells. The question then arises: what is the relation between the internalization and degradation of a hormone and its regulatory action?

Fig. 8 shows two models that can be advanced to explain the relation between hormone action and hormone degradation. The first, or sequential model, is exemplified by LDL. Binding leads to internalization, which in turn leads to degradation. The products of the degradation mediate the regulatory action. In the second model, binding to the receptor is sufficient to produce the regulatory action. But in a parallel action the receptor also mediates the internalization and lysosomal degradation of the hormone. The second model differs from the first in that degradation is not required for the regulatory action of the hormone. Nevertheless the degradation serves a physiologic function by destroying the hormone so that each hormone molecule can act only one time. In some cases the receptor may be destroyed as well.

For most protein hormones, it is not yet possible to choose between these two models. To date, no regulatory action of a protein hormone has been shown to require hormone degradation. However, only a few studies have specifically addressed this point. It is possible that a hormone with multiple actions may act through both of the mechanisms shown in Fig. 8, some regulatory actions requiring hormone degradation and others being independent.

In the LDL receptor system, the first model in Fig. 8 was documented through the use of several experimental tools. First, there was a system, the cultured human fibroblast, in which the initial event—binding—and the final event—enzyme regulation—could be studied simultaneously in whole cells. Second, there were mutants with blocks at each step in the pathway and it was clear that a block at any one of these steps caused a failure of regulation. Although only the binding and internalization

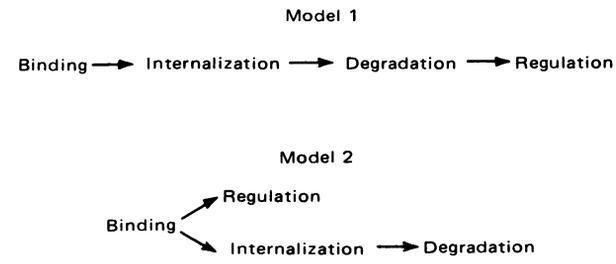

FIG. 8. Models advanced to explain the relationship between the receptor-mediated endocytosis of a protein hormone and its regulatory action. The salient features of each model are discussed in the text.

mutations have been discussed, we have also studied fibroblasts from patients who have a genetic block in LDL degradation. These mutant cells lack the lysosomal acid lipase and thus cannot respond to LDL even though they can bind and internalize it normally (19). Finally, once it was clear that unesterified cholesterol was the intracellular messenger, exogenous cholesterol could be added to the mutant cells to reproduce the actions of LDL and thus to bypass the metabolic blocks, confirming their specificity. All of these tools are not yet available for any single protein hormone system. Nevertheless, imaginative studies are being carried out in many laboratories to see whether or not either of these models applies to any of the protein hormones.

In a broader sense, the observation that some protein hormones are internalized by receptor-mediated endocytosis and degraded within lysosomes brings us back to our initial theme—namely, that metabolic regulation must now be considered a problem in cell biology as well as in classical biochemistry.

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