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- I. VAN DRIEL ET AL.: "Self-association of the low density lipoprotein receptor mediated by the cytoplasmic domain". THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol 262, No. 33, 16127-16134, 1987.
- I. VAN DRIEL ET AL.: "First Cysteine-rich repeat in ligand-binding domain of low density lipoprotein receptor binds CA^{2+} and monoclonal antibodies, but not lipoproteins". THE JOURNAL OF BIOLOGICAL CHEMISTRY, Vol 262, No. 36, 17443-17449, 1987.

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Stoichiometric Binding of Low Density Lipoprotein (LDL) and Monoclonal Antibodies to LDL Receptors in a Solid Phase Assay*

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The current paper describes a solid phase ligand binding assay for the low density lipoprotein (LDL) receptor that takes advantage of the domain structure of the protein. An antibody directed against one domain, e.g. the cytoplasmic tail, is adsorbed to a microtiter well. A detergent solution containing the LDL receptor is added, and the receptor is allowed to bind to the antibody. The wells are then washed, and one of the following radiolabeled ligands is added: ^{125}I -LDL or an ^{125}I -labeled monoclonal antibody directed against a different domain than the antibody adsorbed to the well. Under these conditions, the human LDL receptor shows high affinity for ^{125}I -LDL and for ^{125}I -IgG-HL1, a monoclonal antipeptide antibody directed against a 10-amino-acid "linker" between repeats 4 and 5 in the ligand binding domain. The binding affinity is the same at 4 °C and 37 °C. The binding of ^{125}I -LDL and ^{125}I -IgG-HL1 occurs with 1:1 molar stoichiometry, suggesting that the human LDL receptor binds 1 mol of LDL per mol of receptor. The acid-dependent dissociation of ^{125}I -LDL and ^{125}I -labeled monoclonal antibody from LDL receptors that is observed in intact cells was also shown to occur in the solid phase binding assay. We used the solid phase assay to demonstrate the secretion of LDL receptors from monkey cells that have been transfected with a cDNA encoding a truncated form of the human receptor that lacks the membrane-spanning domain. This assay may be useful in measuring the relative amounts of the intact LDL receptor in tissue extracts and the secreted receptor in transfected cells.

The low density lipoprotein (LDL)¹ receptor is a single-chain transmembrane glycoprotein that mediates the cell surface binding and endocytosis of plasma lipoproteins containing apoproteins B-100 or E (1). The receptor is oriented with its NH₂-terminal 767 amino acids facing the extracellular environment and its COOH-terminal 80 amino acids project-

ing into the cytoplasm. The NH₂-terminal portion contains seven cysteine-rich repeats of 40 amino acids each that constitute the ligand binding domain. This is followed by 400 amino acids that are homologous to the precursor for epidermal growth factor (EGF) (1). The EGF precursor region is required for the binding of LDL on the cell surface, and it also plays a role in the acid-dependent release of LDL from the receptor, an event that is necessary for receptor recycling (2). Next is the O-linked sugar region, followed by the transmembrane region and the COOH-terminal cytoplasmic tail (1).

The binding activity of the LDL receptor has been measured in intact cultured cells (3), in plasma membrane vesicles (4), and in solubilized receptors co-precipitated with phospholipids (5). The receptor can also be assayed by ligand blotting following sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis and transfer to nitrocellulose (6).

In the current paper, we report a new solid phase assay that can be used to study LDL binding activity of receptors in crude membrane extracts as well as in purified form. The assay takes advantage of the domain structure of the receptor. Antibodies against one domain are adsorbed to plastic wells, the receptor in solution is allowed to bind to the antibody, and a radiolabeled ligand or second antibody directed at a different domain is added. We have used this assay to demonstrate the secretion of a truncated form of the LDL receptor from cultured cells that were transfected with a cDNA containing a premature terminator codon preceding the membrane-spanning region.

EXPERIMENTAL PROCEDURES

Materials—The epitopes recognized by the four anti-LDL receptor monoclonal antibodies used in these studies are shown in Fig. 1 (see Ref. 7). Monoclonal anti-LDL receptor antibodies IgG-C7 (8, 9), IgG-15C8 (8, 10), IgG-HL1 (11), and IgG-4A4 (8) and a monoclonal antibody to an irrelevant antigen IgG-2001 (12) were prepared as described in the indicated reference. They were purified from mouse ascites fluid and iodinated as previously described (9, 12). Human LDL (d 1.019–1.063 g/ml) was prepared by ultracentrifugation and radiolabeled by the iodine monochloride method (3). Lipoprotein-deficient serum (d >1.215 g/ml) was prepared as described (3). TR 715-19 is a Chinese hamster ovary cell line transfected with a plasmid encoding the entire human LDL receptor (pLDL-R-2) (13). COS-M6 cells were obtained from Arnold Berk (Dept. of Microbiology, University of California, Los Angeles). [^{35}S]Methionine (800 Ci/mmol) and ENHANCE were purchased from DuPont-New England Nuclear.

Solid Phase Radioimmunoassay for LDL Receptors—Assays were performed in Immulon 1 Removawall Strips (Dynatech Laboratories, Catalogue No. 011-010-6301) that had been washed once with distilled water. An anti-LDL receptor monoclonal antibody was adsorbed to each well by incubation at a concentration of 0.1 mg/ml in buffer A (50 mM Tris-HCl at pH 8.0 containing 2 mM CaCl₂). After 30–60 min at 37 °C, the solution was removed. Nonspecific binding sites were saturated by filling the wells completely with buffer A containing 1% (w/v) bovine serum albumin (BSA) and incubating them at 37 °C for

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¹ The abbreviations used are: LDL, low density lipoprotein; HDL, high density lipoprotein; apo, apoprotein; BSA, bovine serum albumin; EGF, epidermal growth factor; bp, base pairs; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

1 h. The wells were then washed four times with 0.25 ml of ice-cold buffer A containing 0.5% BSA (buffer B), and solutions containing LDL receptors (3–30 ng of purified receptor or 100–125 µg of partially purified proteins) were added in a volume of 0.1 ml. Purified bovine adrenal LDL receptor (Ref. 14 and see below) was diluted 1000- to 2000-fold in buffer B before addition to the wells. Cell extracts containing partially purified human LDL receptors and culture medium containing a secreted form of the human LDL receptor (see below) were diluted 10- to 100-fold in buffer B containing 0.5% (w/v) Triton X-100 and 1 mM phenylmethylsulfonyl fluoride and were centrifuged at 10,000 × g for 10 min at 4 °C. The supernatants were added to the wells, which were then incubated overnight at 4 °C. Wells that received partially purified receptors were washed once with buffer B containing 0.5% Triton X-100 and then five times with buffer B. Wells that received purified bovine LDL receptors or culture medium were washed four times with buffer B. ¹²⁵I-labeled LDL was added to the wells in 0.1 ml of buffer B and incubated for 1 h at the indicated temperature. Unbound ¹²⁵I-ligand was removed by one fast wash followed by four 3-min washes with ice-cold buffer B. Wells were then individually separated from the strip, and radioactivity was determined. The procedure for binding of ¹²⁵I-labeled monoclonal antibodies was identical with that described above for ¹²⁵I-lipoproteins except that the binding and subsequent washes were performed in buffer B containing 0.05% (v/v) Tween 20.

Plasmid Construction.—The LDL receptor coding region in pDC295 (Fig. 6) was obtained from a previously prepared plasmid (pLDLR-4Δ6) from which the DNA corresponding to exons 7 and 8 (A and B repeats in EGF receptor region) had been deleted. This deletion created a restriction site for *Hind*III that bridged the deletion site. To introduce a stop codon at residue 295, pLDLR-4Δ6 was digested with *Hind*III, and the cohesive end was filled in with the Klenow fragment of *Escherichia coli* DNA polymerase. The plasmid was then rejoined by blunt and ligation. This changed the codon at residue 295 from TTC to TAG (stop). It also changed the codon at residue 294 from threonine (ACC) to serine (AGC). This plasmid was designated pTAGVII. The ~1570-base-pair *Sal*I/*Eco*RV fragment of pTAGVII and the ~4170-bp *Sal*I/*Sma*I fragment of pLDLR-15 were ligated to produce pC295-1. pLDLR-15 is a plasmid (provided by David W. Russell, University of Texas Southwestern Medical Center) in which transcription of an LDL receptor cDNA is driven by an enhancer-promoter derived from a human cytomegalovirus (Towne strain) immediate early region (15). pDC295 was produced by ligating the ~2220-bp *Sal*I/*Eco*RV fragment from pC295-1 and the ~5500-bp *Spe*I/*Sma*I fragment of pDL-6. pDL-6 is similar to pLDLR-15 but also contains an SV40 early region/dihydrofolate reductase transcription unit that was derived from pFR400 (16).

Miscellaneous Methods.—Immunoprecipitation of metabolically labeled LDL receptors from extracts of cultured cells and SDS-polyacrylamide gel electrophoresis of the immunoprecipitates were performed as previously described (8, 17). *M_r* standards were myosin (200,000), β-galactosidase (116,000), phosphorylase B (97,000), bovine serum albumin (66,000), IgG-C7 heavy chain (55,000), and ovalbumin (45,000). Bovine LDL receptors were purified to homogeneity from adrenal glands as previously described (14). Human LDL receptors were partially purified from cultured cells as follows. Transfected TR 715-19 cells were trypsinized from stock cells and seeded on day 0 into ten 850-cm² roller bottles (~1 × 10⁷ cells/bottle) in medium C (Ham's F-12 medium containing 2 mM glutamine, 100 units/ml penicillin, 100 µg/ml streptomycin sulfate, 5% (v/v) fetal calf serum, and 10 mM HEPES, pH 7.4). On day 2, the cells were refed with medium C and harvested on day 4 by scraping in buffer D (50 mM HEPES, pH 7.0 containing 2 mM calcium chloride, 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM leupeptin). The cellular debris was removed by centrifugation at 3000 × g for 10 min at 4 °C. Approximately 5 ml of DEAE-cellulose (Whatman DE52) equilibrated in buffer D was added to the lysate, and the mixture was agitated overnight on a rotary shaker at 4 °C. The DEAE-cellulose was isolated by centrifugation at 1000 × g for 2 min at 4 °C, washed three times with 50 ml of buffer D, and poured into a column with a diameter of 1 cm. Proteins were eluted with successive 2-ml aliquots of 50 mM Tris maleate, pH 8.0, containing 2 mM calcium chloride, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.5 M sodium chloride. The solid phase radioimmunoassay was employed to determine which fractions contained LDL receptors. The concentration of purified receptor samples was estimated by subjecting aliquots to electrophoresis on SDS-polyacrylamide gels and comparing the intensity of Coomassie Blue staining to that of known amounts of

BSA. Other protein determinations were made by the method of Lowry (18) using BSA as a standard.

RESULTS

Fig. 1 shows the five domains of the LDL receptor and denotes the binding sites for the four monoclonal antibodies that were used in these studies. Monoclonals IgG-15C8 and IgG-C7 bind primarily to the first repeat in the ligand binding domain (8). IgG-HL1 is a monoclonal anti-peptide antibody directed against a 10-amino-acid "linker" sequence that separates repeats 4 and 5 (11). A 10-amino-acid linker is found in the same position in the rabbit, hamster, and human LDL receptors (1, 7, 19). Whereas the sequence of the cysteine-rich repeats is strongly conserved among these species, the linker sequence varies, and it is thus a convenient site for the preparation of species-specific monoclonal antibodies. IgG-4A4 is an anti-peptide monoclonal antibody directed against the COOH-terminal 14 amino acids of the LDL receptor (8).

In the experiment shown in Fig. 2, IgG-4A4 was adsorbed to plastic wells, and varying amounts of highly purified bovine LDL receptors were added. After washing, the wells were incubated with either ¹²⁵I-IgG-15C8 (panel A) or with ¹²⁵I-LDL (panel B). The incubations were carried out in the absence and presence of EDTA since specific binding of both of these ligands requires calcium (1). Specific binding of both

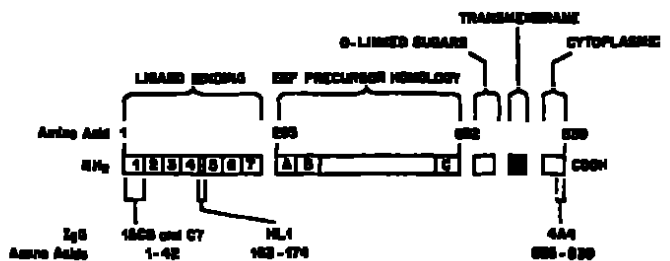


FIG. 1. Epitopes of the human LDL receptor recognized by monoclonal antibodies IgG-15C8, IgG-C7, IgG-HL1, and IgG-4A4. The figure shows the seven cysteine-rich repeat sequences of the ligand binding domain (designated 1-7) and the three cysteine-rich growth factor repeats in the EGF homology domain (designated A, B, and C).

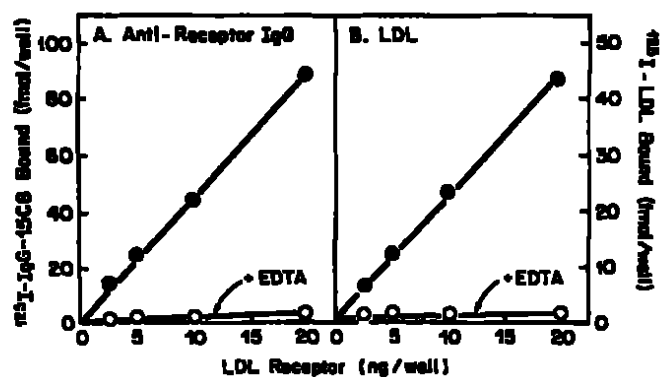


FIG. 2. Binding of ¹²⁵I-labeled ligands to immobilized LDL receptors as a function of the amount of LDL receptor added to each well. Varying amounts of purified bovine LDL receptor were added to IgG-4A4-coated wells and incubated overnight at 4 °C as described under "Experimental Procedures." After washing, either ¹²⁵I-IgG-15C8 (34 nM; 65 cpm/fmol) (panel A) or ¹²⁵I-LDL (20 nM protein; 225 cpm/fmol protein) (panel B) was added to the wells in the absence (●) or presence (○) of 10 mM EDTA. After incubation for 1 h at 4 °C, the total amount of bound radioactivity was determined. All assays were carried out as described under "Experimental Procedures" except that Tween 20 was not included in the antibody binding or washing solutions. Each value represents the average of duplicate incubations.

ligands rose linearly in proportion to the amount of LDL receptor added to each well.

The solid phase assay was used to study the human LDL receptor that was produced in Chinese hamster ovary fibroblasts that had been transfected with a cDNA encoding the human receptor (Fig. 3). The transfected cells were solubilized with detergent, and the LDL receptor was partially purified by adsorption to DEAE-cellulose (see "Experimental Procedures"). In the solid phase assay, the human receptor bound ^{125}I -LDL with equally high affinity at 4 °C and 37 °C (Fig. 3A). Half-maximal binding was obtained at approximately 1.8 nM at both temperatures. This differs from the findings in intact cells in which the receptor has much lower affinity at 37 °C (20). The affinity in the solid phase assay corresponds to the high affinity state of the receptor, which is observed in cultured cells only at 4 °C. IgG-HL1 also showed identical affinities at 4 °C and 37 °C in the solid phase assay (Fig. 3B). At saturation, the molar ratio for binding of ^{125}I -LDL to ^{125}I -IgG-HL1 was 1:1. This calculation was based on M_r values of 512,000 for the apoB-100 of LDL (one molecule of apoB-100 per mol of LDL) (21) and 147,000 for monoclonal IgG. The specific activity values used for calculating the binding of ^{125}I -LDL and ^{125}I -IgG-HL1 were based on protein measurements made by the Lowry method (18). We also measured the protein concentration by quantitative amino acid analysis. The Lowry method overestimated the concentration of both ligands by a similar factor of 1.5 (LDL) and 1.3 (IgG-HL1) and hence did not significantly change the calculation of relative stoichiometry.

Table I shows the relative stoichiometry of ^{125}I -LDL and ^{125}I -IgG-HL1 binding at saturation to four different preparations of human LDL receptor expressed in transfected hamster cells. The mean ratio was 1.1 mol/mol (range: 0.8–1.8), indicating that 1 mol of LDL receptor binds 1 mol of apoB-100 in LDL.

A characteristic property of the LDL receptor is its release of LDL preferentially at pH values less than 6 (2). Fig. 4 shows that this property is retained by the purified bovine LDL receptor in the solid phase immunoassay both at 4 °C (panel A) and at 37 °C (panel B). The acid treatment also caused the preferential release of ^{125}I -IgG-15C8 (open symbols

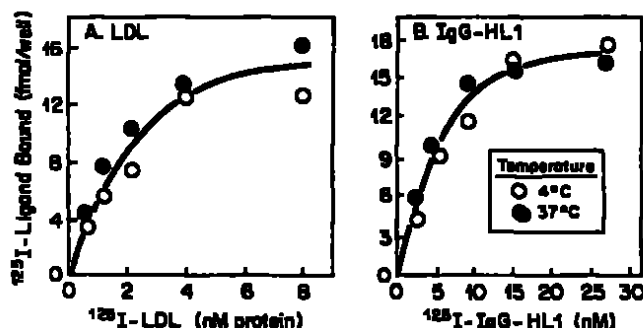


FIG. 3. Saturation curves for binding of ^{125}I -LDL and monoclonal antibodies to LDL receptors at 4 °C and 37 °C. A partially purified extract from TR 715-19 cells expressing the human LDL receptor (100 μg of protein/well) was bound to IgG-4A4-coated wells overnight at 4 °C. The wells then received the indicated concentration of ^{125}I -LDL (314 cpm/fmol protein) (A) or ^{125}I -IgG-HL1 (81 cpm/fmol) (B) and were incubated at 4 °C (○) or 37 °C (●) for 1 h before the amount of bound radioactivity was determined. All washing was performed at 4 °C. Each value represents the average of triplicate incubations. Nonspecific binding was subtracted from each value and was determined by performing parallel incubations in the presence of 10 mM EDTA (A) or a 50-fold excess of unlabeled IgG-HL1 (B). The nonspecific values ranged from 4–11% (A) and 3–11% (B) of total bound ligand.

TABLE I

Stoichiometric comparison of binding of ^{125}I -LDL and ^{125}I -labeled monoclonal anti-receptor IgG to human LDL receptors at 4 °C

Partially purified preparation of human LDL receptors (100–125 μg of protein) was bound to IgG-4A4-coated wells overnight at 4 °C. The wells then received either ^{125}I -LDL (19 nM protein in Experiment A; 13 nM in Experiments B–D) or 34 nM ^{125}I -IgG-HL1 (Experiments A–D). After incubation for 1 h at 4 °C, the total amount of bound radioactivity was determined. Each value represents the average of triplicate incubations. Nonspecific binding was subtracted from each value and was determined as described in the legend to Fig. 2. Human LDL receptors were partially purified from TR715-19 cells on DEAE-cellulose as described under "Experimental Procedures." Calculations of ligand concentration were based on M_r values of 512,000 for apoB-100 in LDL and 147,000 for a monoclonal antibody with IgG2b heavy chains and κ light chains. (The heavy chain of IgG-HL1 is subclass IgG2b.)

Experiment	^{125}I -Ligand bound		Ratio of bound ^{125}I -ligands (a/b)
	LDL (a)	IgG-HL1 (b)	
	fmol/well		
A	23	13	1.8
B	14	15	0.93
C	16	20	0.80
D	24	28	0.92
	Mean		1.1

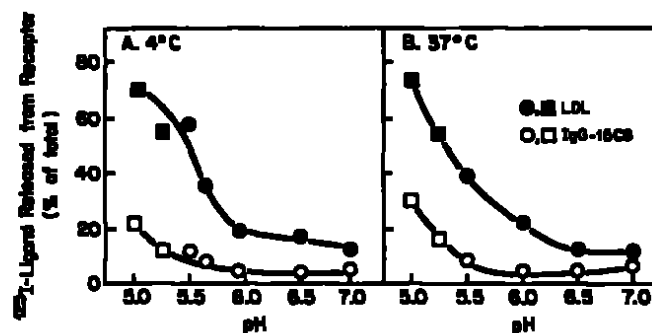


FIG. 4. Acid-dependent release of ^{125}I -LDL and ^{125}I -IgG-15C8 from purified bovine LDL receptors. Purified bovine LDL receptors (~20 μg /well) were bound to IgG-4A4-coated wells overnight at 4 °C. ^{125}I -LDL at 10 nM protein (panel A, 312 cpm/fmol of protein; panel B, 143 cpm/fmol of protein) or ^{125}I -IgG-15C8 at 34 nM (panel A, 89 cpm/fmol; panel B, 45 cpm/fmol) was then added to the wells at 4 °C for 1 h in the absence or presence of 10 mM EDTA, after which the wells were washed at 4 °C to remove unbound ligand as described under "Experimental Procedures." The wells in panel B then received 0.25 ml of buffer B warmed to 37 °C and were incubated for 3 min at 37 °C. All wells then received 0.25 ml of either 50 mM Tris maleate (●, ○) or 50 mM Tris succinate (■, □) at the indicated pH containing 2 mM calcium chloride and 0.5% BSA; buffers in panel A were ice-cold and in panel B were prewarmed to 37 °C. After 30 min at 4 °C (A) or 5 min at 37 °C (B), the buffer in the wells was removed, and the radioactivity in wells and buffer was determined. All values are the average of triplicate incubations. The data shown represent specific values that were obtained by subtracting the values for ^{125}I -ligand bound in the presence of EDTA from that bound in its absence. The values for nonspecific binding in the presence of EDTA did not exceed 10% (A) or 17% (B) of total binding. The amount of released ^{125}I -ligand is expressed as a percentage of total ligand recovered from buffer plus wells. The "100% of total" values for ^{125}I -LDL and ^{125}I -IgG-15C8 averaged 43 and 138 fmol/well, respectively (A), and 41 and 149 fmol/well, respectively (B).

in Fig. 4), but the extent of release was not as great as was observed with ^{125}I -LDL. Fig. 5 compares the rates of release of ^{125}I -LDL from the purified bovine LDL receptor at pH 7.0 and pH 5.5 at 4 °C and at 37 °C. At both temperatures, the initial rate of release was much greater at the acidic pH. At both temperatures, the release was biphasic, with 50–60% of

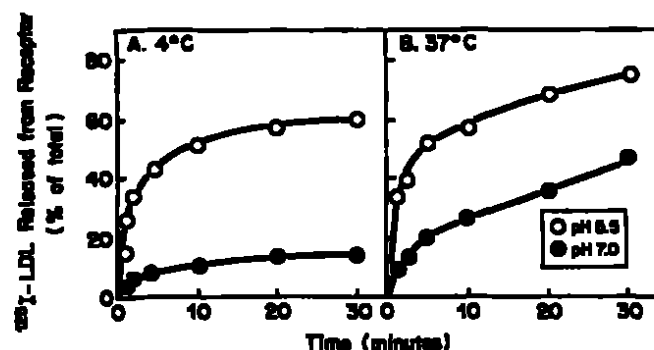


FIG. 5. Time course of acid-dependent release of LDL from LDL receptor at pH 7.0 (●) and pH 5.5 (○). Purified bovine LDL receptor (~10 ng/well) was bound to IgG-4A4-coated wells overnight at 4°C. ^{125} I-LDL at 8 nM protein (145 cpm/fmol of protein) was added to the wells for 1 h at 4°C in the absence or presence of 10 mM EDTA, and unbound ligand was removed as described in the legend to Fig. 4. The wells were then incubated for the indicated time at either 4°C (A) or 37°C (B) with Tris maleate at pH 7.0 (●) or pH 5.5 (○) containing 2 mM calcium chloride and 0.5% BSA. Specific binding and ligand release were measured as described in the legend to Fig. 4. All values are the average of triplicate incubations. The values for nonspecific binding in the presence of EDTA did not exceed 6% of total binding. The "100% of total" value was 27 fmol/well.

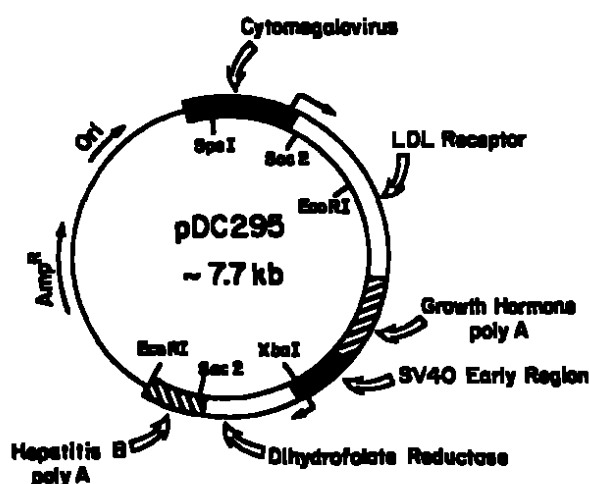


FIG. 6. Structure of pDC295, a plasmid encoding a secreted form of the human LDL receptor. The plasmid contains a human cytomegalovirus immediate early region enhancer/promoter, a truncated LDL receptor cDNA sequence that encodes the first 293 amino acids of the receptor followed by a COOH-terminal earline and a TAG nonsense codon, the polyadenylation signal from the human growth hormone gene, the SV40 virus early region enhancer/origin, a murine dihydrofolate reductase coding sequence, and a hepatitis B virus surface antigen gene polyadenylation site. The directions of transcription are indicated by the filled arrows. The plasmid was constructed as described under "Experimental Procedures."

the release occurring within 10 min, followed by a much slower second phase that was similar at both pH values.

Fig. 6 shows the structure of a recombinant plasmid that was designed to achieve high level expression of a soluble, secreted form of the human LDL receptor. The vector uses the promoter from cytomegalovirus fused to an LDL receptor cDNA with a premature termination codon at residue 295. This receptor lacks the EGF precursor, O-linked sugar, membrane-spanning, and cytoplasmic regions, and thus it should be secreted from the cell. The vector also contains the early region of the SV40 virus, and it therefore is replicated to high levels when transfected into monkey COS-M6 cells that produce SV40 large T antigen.

Fig. 7, left panel, shows an experiment in which COS-M6 cells were transfected with this plasmid and labeled with [^{35} S] methionine. Cells and medium were then subjected to immunoprecipitation with IgG-C7. After the 24-h labeling period, some of the receptor was still contained within the cell (lane 3). Much of the receptor had been secreted into the medium (lane 4). The secreted receptor was more diffuse in mobility and was larger than the intracellular form, possibly owing to the processing of N-linked carbohydrates. The truncated receptor contains two Asn-X-Ser sequences that are potential targets for N-linked glycosylation (7).

To estimate the amount of secreted receptor, we modified the solid phase assay to circumvent the fact that the secreted receptor lacked the COOH terminus and therefore cannot react with monoclonal IgG-4A4. Therefore, we coated the wells with IgG-15C8, which mainly recognizes the first ligand binding repeat (8). Various amounts of culture medium were then added to the wells in order to allow secreted receptors to bind to the IgG-15C8. After washing the wells, we added ^{125} I-labeled IgG-HL1, which bound to the wells in proportion to the amount of medium that had been added (Fig. 7, right, panel A). Binding was competed with an excess of unlabeled IgG-HL1. No binding was observed when we used medium from control cells that were transfected only with salmon sperm DNA (Fig. 7, right, panel B). Assuming that 1 mol of IgG-HL1 binds to 1 mol of receptor, the data indicate that the concentration of receptor in the culture medium is 0.08 mg/liter.

DISCUSSION

In the current paper, we describe a solid phase ligand binding assay that can be used to characterize purified LDL receptors, impure receptors in crude extracts of solubilized membranes, and secreted, truncated receptors that lack the membrane-spanning region. In contrast to the nitrocellulose-based ligand binding assay, which requires SDS-polyacrylamide gel electrophoresis (6), the current assay measures binding to the native, nondenatured form of the receptor. Moreover, the binding activity in the current assay is quantified by scintillation counting, which is more precise than the autoradiographic procedure used in the ligand blotting assay (6).

The LDL receptor in this assay shows high affinity for ^{125} I-LDL at 37°C as well as at 4°C. In intact cells, the receptor shows much lower affinity at 37°C, apparently owing to a more rapid dissociation rate (20). It is possible that the high rate of dissociation at 37°C depends on the mobility of the receptor in the plane of the membrane or upon its interaction with another protein. It does not seem to be an intrinsic property of the receptor, since it is not manifest in the solid phase assay.

In vivo, LDL is released from the receptor in an acidic endosomal compartment, and this allows the receptor to recycle to the cell surface (1). The acid-dependent release of bound ligands that is observed for receptors in intact cells was reproduced in the solid phase system. The data in Figs. 4 and 5 demonstrating the release of LDL from immobilized receptors *in vitro* strongly suggest that the release phenomenon is due to a pH-dependent effect on the receptor molecule itself. Previous mutational analyses showed that the EGF precursor domain of the receptor mediates this conformational change (2). It is therefore likely that this domain, like the ligand binding domain, retains its structural integrity during purification of the receptor.

In the current assays, the human LDL receptor bound ^{125} I-LDL and ^{125} I-IgG-HL1 in a 1:1 molar ratio (Fig. 3 and Table

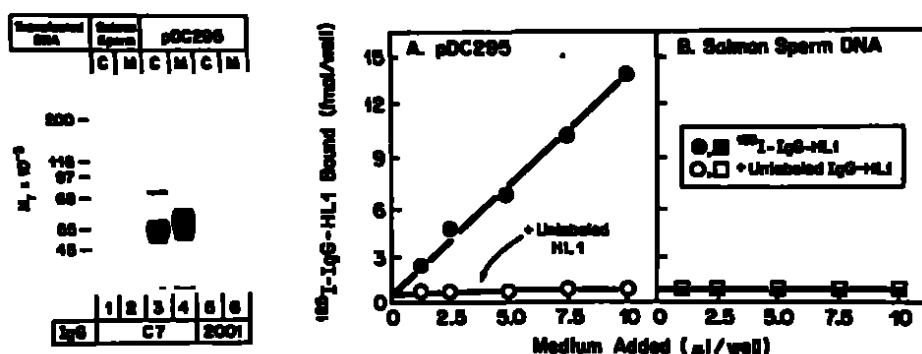


FIG. 7. Detection of secreted form of the LDL receptor in the culture medium of COS-M6 cells. On day 0, 3×10^6 COS-M6 cells were seeded into each of the five 60-mm culture dishes in 3 ml of medium E (Dulbecco's modified Eagle's medium containing 10% fetal calf serum, 100 units/ml penicillin, 100 μ g/ml streptomycin sulfate, and 10 mM HEPES, pH 7.5). On day 1, dishes were transfected with salmon sperm DNA (two dishes) or with pDC295 (three dishes) as previously described (24). All dishes received 3 ml of medium E. On day 3, one of the dishes transfected with salmon sperm DNA and two dishes transfected with pDC295 received 1.5 ml of methionine-free medium E containing 0.25 mCi of [³⁵S]methionine (300 Ci/mmol) and 2 μ M unlabeled methionine. The medium from all dishes and the cells of the [³⁵S]methionine-labeled dishes were harvested on day 3. Left panel, the cells (lanes 1, 3, and 5) and medium (2, 4, and 6) of the [³⁵S]methionine-labeled dishes that were transfected with either salmon sperm DNA (lanes 1 and 5) or pDC295 (lanes 3–6) were immunoprecipitated (one dish per lane) with an anti-LDL receptor antibody, IgG-C7 (lanes 1–4), or a control antibody IgG-2001 (lanes 5 and 6), and the immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis and fluorography. The ENHANCE-treated and dried gel was exposed to Kodak X-Omat XRP film for 2 h at -70°C . The mobility of *M*, standards are indicated. C, cells; M, medium. Right panel, the medium from dishes that did not receive [³⁵S]methionine was diluted 10-fold with buffer B containing 0.5% Triton X-100 after which the indicated volume of medium was added to IgG-15C8-coated wells and incubated overnight at 4°C . ¹²⁵I-IgG-HL1 at 34 nM (85 cpm/fmol) was added to the wells in the absence (●, ■) or presence (○, □) of 1.7 μ M unlabeled IgG-HL1. After incubation for 1 h at 4°C , the amount of total bound radioactivity was determined. All values are the average of triplicate incubations.

I). IgG-HL1 has only one binding site per human LDL receptor, the linker between repeats 4 and 5 (Fig. 1). These data therefore strongly suggest that 1 mol of LDL binds to 1 mol of human LDL receptor. Beisiegel *et al.* (8) observed a similar 1:1 stoichiometry of binding to intact human fibroblasts of LDL and IgG-C7, a monoclonal antibody that reacts primarily with the first ligand binding repeat (8). Moreover, Gavigan *et al.* (22) reported that maximum binding of LDL to cultured human fibroblasts at 4°C was the same as that of an anti-receptor monoclonal antibody (10A2) and of Fab fragments prepared from the antibody.

In addition to binding LDL, which has a single copy of the 512-kDa apoB-100, the LDL receptor also binds apoE-HDL₂, which contains multiple copies of the 33-kDa apoE (1). The affinity for apoE-containing particles is much higher than it is for the apoB-100-containing particles, apparently owing to the multiplicity of binding sites on the apoE-containing particles (23). At saturation, however, the LDL receptors bind only one-fourth as many apoE-containing particles as LDL (14, 23). If each molecule of receptor binds a single LDL particle, as suggested by the current study and earlier studies reviewed above, these data suggest that each apoE-containing particle (which contains multiple copies of apoE) must bind to four receptors. Most of the LDL receptors on the cell surface are believed to exist as dimers, as revealed by chemical cross-linking techniques (11). Each of these dimers may bind two LDL particles, and a pair of dimers might bind to a single apoE-containing particle.

We have used the solid phase ligand binding assay to estimate the amounts of receptor secreted by cultured cells that were transfected with an expressible cDNA encoding a truncated form of the receptor which lacks the membrane-spanning domain. The transfected COS cells produced approximately 0.08 mg of truncated LDL receptor per liter of culture medium. It is likely that this result represents an underestimate, since not all of the receptor may adhere to the

antibody-coated wells in a form that is competent to bind a second antibody. Nevertheless, this assay should reveal the relative amount of receptor when different subclones of permanently transfected cells are examined. The vector that contains the LDL receptor cDNA also contains an expressible gene for dihydrofolate reductase, which confers resistance to high levels of methotrexate. Through growth in increasing amounts of methotrexate, we hope to be able to increase the expression of the truncated LDL receptor so as to purify large amounts of it. This in turn will allow us to perform a standard curve so that we can use the solid phase ligand binding assay for absolute quantification.

In studying the human LDL receptor, we have used monoclonal ¹²⁵I-IgG-HL1, which reacts with a single site on the receptor. This antibody does not react with LDL receptors from cows or other species, and therefore the assays with bovine LDL receptors were performed with ¹²⁵I-IgG-15C8. The latter antibody binds with high affinity to the first repeat in the ligand binding domain of the human LDL receptor (8). It also reacts to some extent with other repeats, and therefore more than 1 mol of IgG-15C8 may bind to each mol of receptor. For this reason, IgG-15C8 is not useful for calculating the absolute stoichiometry of binding. Nevertheless, when this antibody is used at a standard concentration, it binds to receptors in proportion to the number of receptors present (Fig. 2), and thus it can be used to measure the relative number of LDL receptors in a given cell extract.

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Hepatitis C virus and other Flaviviridae viruses enter cells via low density lipoprotein receptor

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Endocytosis of the Flaviviridae viruses, hepatitis C virus, GB virus C/hepatitis G virus, and bovine viral diarrhoea virus (BVDV) was shown to be mediated by low density lipoprotein (LDL) receptors on cultured cells by several lines of evidence: by the demonstration that endocytosis of these virus correlated with LDL receptor activity, by complete inhibition of detectable endocytosis by anti-LDL receptor antibody, by inhibition with anti-apolipoprotein E and -apolipoprotein B antibodies, by chemical methods abrogating lipoprotein/LDL receptor interactions, and by inhibition with the endocytosis inhibitor phenylarsine oxide. Confirmatory evidence was provided by the lack of detectable LDL receptor on cells known to be resistant to BVDV infection. Endocytosis via the LDL receptor was shown to be mediated by complexing of the virus to very low density lipoprotein or LDL but not high density lipoprotein. Studies using LDL receptor-deficient cells or a cytolytic BVDV system indicated that the LDL receptor may be the main but not exclusive means of cell entry of these viruses. Studies on other types of viruses indicated that this mechanism may not be exclusive to Flaviviridae but may be used by viruses that associate with lipoprotein in the blood. These findings provide evidence that the family of LDL receptors may serve as viral receptors.

Hepatitis C virus (HCV), the principal viral cause of chronic hepatitis, is not readily replicated in cell culture systems, and, as yet, no information on cell receptors for the virus is available. However, several observations from studies on the role of HCV in mixed cryoglobulinemia (1–3) have provided some insights to HCV entry into cells.

Mixed cryoglobulinemia is a systemic vasculitis associated with cold-precipitable immunoglobulins in the blood. During the past 5 years, a strong association of HCV infection with mixed cryoglobulins has been established (4), and the specific concentration of HCV in type II mixed cryoglobulins that consist of polyclonal IgG and monoclonal IgM has been demonstrated (1). It also was shown that very low density lipoprotein (VLDL) is selectively associated with HCV in type II cryoglobulins (2). In studies on the cutaneous vasculitic lesions in type II cryoglobulinemia using *in situ* hybridization (ISH), the HCV RNA virion form (positive strand) but not the putative replicative form (negative strand) of the virus was detected in keratinocytes in lesions but not normal skin of the same patients (3). Furthermore, it was demonstrated that LDL receptors were up-regulated on keratinocytes in cutaneous vasculitis lesions compared with normal skin (3). These observations and the finding that anti- β lipoprotein precipitates HCV from infected serum (5) suggested that the low density lipoprotein (LDL) receptor also may be a receptor for HCV complexed to VLDL or LDL. This hypothesis led to this study to determine whether the LDL receptor is also a receptor for HCV and other members of the family of viruses Flaviviridae.

Materials and Methods

Reagents, Antisera Virus Stocks, and Cell Lines. Cyclohexanedione, phenylarsine oxide (PAO), heparin sulfate, and EGTA were purchased from Sigma; 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine iodine (Dil) was purchased from Molecular Probes.

Purified IgG 2a mouse monoclonal anti-LDL receptor antibody (C7 clone) was obtained from Oncogene Scientific Products (Cambridge, MA). Anti-bovine viral diarrhoea virus (BVDV) envelope antibody bovine serum, α 49, was provided by Marc S. Collett (Viro Pharma, Malvern, PA). Mouse monoclonal IgG 2a anti-CD-16, anti-CD-19, and anti-transferrin (CD71) were purchased from Immunotech (Hialeah, FL). Anti- μ was purchased from Jackson ImmunoResearch. Anti-apolipoprotein (apo) E and apo A-I were purchased from Cortex Pharmaceuticals (San Leandro, CA); apo B was purchased from Sigma. Purified mouse monoclonal IgG apo E (1D7), apo A-I (3G10), and apo B (4G3) were purchased from the University of Ottawa Heart Institute. F(ab')₂, an antibody fragment with two antigen-combining sites, preparations of mouse IgG were prepared by treating the mouse monoclonal antibodies from 30 minutes to 10 hours with 3% pepsin (Sigma) (pH 3.5) at 37°C. The F(ab')₂ fragments were isolated by column chromatography using a HR 10/30 Superose 12 column (Amersham Pharmacia). Bovine viral diarrhoea virus-free donor calf serum was purchased from Boyl Veterinary Laboratory (Neosho, MO). Potassium bromide density gradient ultracentrifugation was used for preparation of VLDL, LDL, and high density lipoprotein (HDL) from normal sera, and these lipoproteins complexed to HCV from infected sera. The VLDL band [density (d) = 0.95–1.006 g/ml], the LDL band (d = 1.019–1.063), the HDL band (d = 1.063–1.21 g/ml), and HCV free of lipoproteins (d > 1.21) were isolated by aspiration and then dialyzed against Hank's balanced salt solution (Sigma) containing 0.01% EDTA. Isolated HCV-VLDL was dissociated to HCV and VLDL by treatment with deoxycholate and fractionated by sucrose density gradient ultracentrifugation as described (6). The high density HCV fraction, free of lipoproteins, was further fractionated by column chromatography on a lecithin pretreated Superose 6 (Amersham Pharmacia). The peak of HCV present in the void volume was contaminated with small amounts of immunoglobulins that were removed by using immobilized rProtein A (Repligen). Immunoblotting (dot blots) to detect small amounts of protein was performed as described (7). Sensitivity of the assay was 100 pg for IgG and IgM and 200 pg for apolipoproteins B and E. Lipoproteins were quantitated by Lowry

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Abbreviations: apo, anti-apolipoprotein; BT, bovine turbinate; BVDV, bovine viral diarrhoea virus; CRR, cells resistant to infection with BVDV; d, density; Dil, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine iodine; gE, genomic equivalents; HDL, high density lipoprotein; HCV, hepatitis C virus; HGV, hepatitis G virus; HSV, herpes simplex virus; ISH, *in situ* hybridization; LDL, low density lipoprotein; NADL, National Animal Disease Laboratory; PAO, phenylarsine oxide; RT, reverse transcriptase; VLDL, very low density lipoprotein; VSV, vesicular stomatitis virus.

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assay using commercial kits (Sigma). Highly purified VLDL, LDL, and HDL were purchased from Cortex. Labeling of LDL with DiI was performed as described (8).

Infected human sera were used as stocks for HCV (3×10^8 genomic equivalents per milliliter [gE/ml]), GB virus C/hepatitis G virus (HGV) (2×10^8 gE/ml), and herpes simplex virus (HSV). BVDV strains NY-1 and National Animal Disease Laboratory (NADL) and vesicular stomatitis virus (VSV), Indiana strain, and respiratory syncytial virus were obtained from American Type Culture Collection (Rockville, MD). Bovine turbinate (BT) and kidney (MDBK) cell lines, Hep G2, a hepatoma cell line that is biochemically similar to hepatocytes (9), Daudi, a B cell lymphoblastoid cell line, the Molt-4 T cell line, HEp 2, a squamous carcinoma cell line, and normal fibroblasts (MRC-5) were obtained from the American Type Culture Collection. The B lymphocyte lines G4 and E11 were generated from fusion of F3B6 human-mouse heterohybridoma with peripheral B cells from patients with type II cryoglobulinemia and rheumatoid arthritis, respectively. Development of the 35G6 peripheral B cell line, cloned from a normal patient, was previously described (10). Four LDL receptor negative cell lines, GM00488C, GM02000G, GM00701C, and GM03040B, were obtained from the National Institute of General Medical Sciences, Human Genetics Mutant Cell Repository, Coriell Institute for Medical Research (Camden, NJ). Cells resistant to infection with BVDV (CRIB) were provided by R. O. Donis (University of Nebraska, Lincoln, NE).

LDL Receptor Assays. Cells were cultured in RPMI medium 1640 supplemented with 10% BVDV-free bovine calf serum or expression of the LDL receptor was up-regulated by culturing for 24 hours in RPMI medium 1640 supplemented with 10% lipoprotein-deficient BVDV-free medium. The cells then were washed twice with PBS (pH 7.2). Cytospin preparations were made, fixed with acetone, and blocked with 5% normal mouse serum, and the LDL receptor was visualized by incubating the slides with 5 μ g/ml purified IgG 2a monoclonal anti-LDL receptor antibody followed by a 1:50 dilution of FITC-labeled goat anti-mouse [F(ab')₂] second antibody (Jackson ImmunoResearch). The demonstration of LDL receptors on adherent cells, MDBK, CRIB, fibroblasts, Hep G2, and HEp 2 was performed in the same manner except monolayers of cells were cultured and fixed on slides.

Demonstration of endocytosis of DiI-LDL by cells was performed by incubation of 2×10^5 cells for 2 hours at 37°C in 5% CO₂ with 20 μ g/ml DiI-LDL as described (11). The cells were washed twice with cold PBS and were fixed with 1% buffered paraformaldehyde, and cytospin preparations were made for fluorescent microscopic studies or cells in suspension were analyzed by flow cytometry. Flow cytometric analysis was performed by using the Epic XL-MCL cytometer (Coulter) using a 575 BP filter. Nonspecific binding of DiI-LDL was determined by using DiI-LDL treated with cyclohexanedione and was subtracted from the DiI-LDL binding to give specific DiI-LDL binding to cultured cells.

HCV RNA and Endocytosis Assays. HCV RNA, reverse transcriptase (RT)-PCR, and ISH assays were performed as described (12). Specificity of the ISH method for HCV was determined by comparing monolayers of human fibroblasts inoculated with either 3×10^7 gE/ml HCV or dilutions of adenovirus or respiratory syncytial virus that produced pathologic changes in cells at 24 hours. After incubation for 24 hours at 37°C, the cultures were assayed for HCV RNA by ISH. The endocytosis assay for HCV was performed as described (12). Daudi cells (5×10^5 cells) were inoculated with 3×10^7 gE HCV or GB virus C/HGV, were incubated for 3 hours at 37°C, were washed three times, and were assayed for intracytoplasmic HCV RNA or GB virus C/HGV RNA by ISH. RT-PCR and ISH assays for GB virus C/HGV RNA were performed as described (13). The same

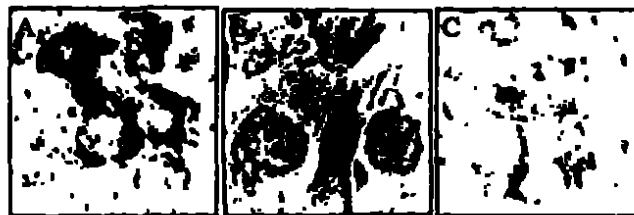


Fig. 1. Demonstration of the specificity of the ISH method for HCV. (A) HEp 2 cells 24 hours after inoculation with HCV. The brown staining indicates the presence of positive-strand HCV. (B and C) HEp 2 cells incubated with respiratory syncytial virus or adenovirus, respectively, show no staining for HCV using the same ISH method as in A. Note the cytopathic effect of these viruses on the HEp 2 monolayers. Original magnification, $\times 500$.

methodology also was used for studies with HCV-lipoprotein recombinants. One hundred micrograms each of normal VLDL, LDL, HDL or cyclohexanedione-treated VLDL, or LDL were incubated with 10^6 gE HCV free of lipoproteins and immunoglobulins for 30 minutes at 37°C and then were added to the Daudi cells.

For cytolytic viruses, BVDV, NADL, VSV, and HSV, various dilutions of the respective viruses were incubated with monolayers of cells at 4°C for 1 hour, were washed three times with cold PBS, and were incubated with fresh medium. Virus dilutions that produced complete cytolysis at 72 hours for BVDV and VSV and 48 hours for HSV were selected. Immunofluorescent detection of intracytoplasmic BVDV was performed on acetone-fixed slides by using 1:50 dilutions of anti-BVDV serum and a FITC-labeled anti-bovine second antibody. The presence of BVDV in cells was confirmed by RT-PCR using BVDV specific primers (14).

Inhibition Studies. Blocking of LDL receptor with various dilutions of antibodies (anti-LDL receptor, 5–20 μ g/ml; control antisera, 5–20 μ g/ml) or inhibitors was performed by pretreatment of cells with various concentrations of antisera or inhibitor for 15 minutes at 37°C and inoculating with virus without washing the cells. Additions of antisera during incubation period were made at 45-minute intervals. Treatment of LDL and VLDL with cyclohexanedione was performed as described (15). In experiments with cytopathic virus, cells were pretreated with 50 μ g/ml of anti-LDL receptor antibody for 30 minutes at 4°C before inoculation with virus at 4°C.

Inhibition of endocytosis by PAO was assessed by pretreating cells with a range of final PAO concentration of 0.1–100 μ M as described (16), and then endocytosis of LDL or HCV was evaluated by the DiI-LDL assay or HCV-ISH, respectively, as described earlier.

Results

Endocytosis of HCV via the LDL Receptor. We previously demonstrated that endocytosis of HCV *in vitro* correlates with the titer of HCV in the inoculum. The percentage of cells positive for HCV RNA by ISH correlated directly with the number of gE of HCV per cell as determined by RT-PCR (12). There was also a crude correlation between intensity of ISH staining for HCV RNA and gE HCV per cell by RT-PCR. The specificity of this ISH assay for HCV is shown in Fig. 1. Cell cultures infected with viruses other than HCV were negative using this assay.

For a further investigation of endocytosis of HCV by cells *in vitro*, a variety of human cell cultures (see *Materials and Methods*) were demonstrated to have LDL receptors with the use of anti-LDL receptor antibody or DiI-LDL uptake. These cell lines then were inoculated with a high titer HCV-positive human serum. Intracellular HCV RNA then was detected by using ISH. To determine whether endocytosis of HCV correlated with the level of LDL

Table 1. Uptake of LDL by cultured cells

Cell line	Culture medium	Treatment	Mean fluorescence
G4	Routine*	None	0.8 ± 0.4
G4	Routine	Dil-LDL	4 ± 2.0
G4	Lipoprotein deficient†	Dil-LDL	16 ± 7
Hep G2	Routine	None	3 ± 1
Hep G2	Routine	Dil-LDL	16 ± 5

*RPMI with 10% fetal bovine serum.
†RPMI with lipoprotein-deficient serum.

receptor expression on cells, the well known modulatory effect of lipoproteins on the LDL receptor was used to increase the number of LDL receptors (up-regulate) on cells by culturing in lipoprotein-deficient media. Relative differences in endocytosis of LDL by various cultured cell lines could be demonstrated by the specific uptake of Dil-LDL. The specific Dil-LDL uptake of Hep G2 cells as shown in Table 1 was four times greater than of the peripheral B cell line, G4, without up-regulation.

Up-regulating these B cells produced a LDL uptake equivalent to that of the Hep G2 cells without up-regulation. These results were confirmed by immunofluorescent studies using anti-LDL receptor antibody staining and Dil-LDL uptake (Fig. 2A and B).

Up-regulation of the LDL receptors of G4 cells resulted in 70–80% of cells staining positive for the LDL receptor, a result that was similar to the 70–80% cells positive for the LDL receptor on Hep G2 cells (Fig. 3E) and Daudi cells (Fig. 3G), also known to have higher densities of LDL receptor (11).

The percentage of cells positive for HCV by ISH was shown to correlate with the percentage of cells positive for LDL receptor by immunofluorescence using anti-LDL receptor antibody or Dil-labeled LDL. Endocytosis of HCV by peripheral B cells that showed 5–30% weakly positive cells in routine culture (Fig. 3A) when up-regulated showed a percentage of positive cells and an intensity of staining (Fig. 3B) comparable to Hep G2 cells (Fig. 3E) and Daudi cells (Fig. 3G) that were each 70–80% positive.

Direct evidence that the LDL receptor-mediated endocytosis of HCV was obtained by inhibiting endocytosis with anti-LDL receptor antibody. The endocytosis of HCV could be inhibited in a dose-dependent manner by preincubating the cells with anti-LDL receptor antibody. At sufficient concentrations of anti-LDL receptor antibody, complete inhibition of endocytosis of the virus could be demonstrated for both G4 and Hep G2 cells (Fig. 3 C–F). Similar results were obtained by using infected serum or VLDL-HCV complexes isolated from type II cryoglobulins as the inoculum in these experiments. No inhibition was observed with control mouse IgG 2a or antisera to specific cell surface antigens at a concentration up to 20 times the lowest inhibiting concentration of anti-LDL receptor: antisera to μ heavy chain, CD-19, and CD-16 surface antigens on peripheral B cells and Daudi cells and antiserum to transferrin receptor on



Fig. 2. (A) Demonstration of the LDL receptor on up-regulated G4 cell using anti-LDL receptor antibody (green fluorescent staining). The signal was very weak in ~30% of G4 cells without up-regulation of the LDL receptor (not shown). (B) Demonstration of the uptake of 5 μ g of Dil-LDL by G4 cells that have up-regulated LDL receptors as in A. (C) Inhibition of 5 μ g of Dil-LDL endocytosis by 30X excess of unlabeled LDL. (D) Same field as C by phase contrast microscopy. Original magnification, $\times 500$.

Hep G2 cells did not inhibit the endocytosis of HCV by these cells. Moreover, treatment of Daudi cells with the endocytosis inhibitor PAO at 2 μ M concentration completely inhibited the endocytosis of HCV (Fig. 3 G and H).

The role of the LDL receptor in the endocytosis of HCV was confirmed by demonstrating competitive inhibition with LDL and VLDL but not HDL, which is known not to bind to the LDL receptor. With use of hepatoma cells (Hep G2) or B cells (G4 and E11), 25–100 μ g/ml of LDL or VLDL completely inhibited endocytosis of HCV whereas concentrations of HDL up to 200 μ g/ml (a 5- to 20- and 10- to 40-fold molar excess over LDL and VLDL, respectively) did not inhibit. Treatment of LDL or VLDL with cyclohexanedione, which is known to alter a critical arginine residue in the LDL receptor binding site of apolipoproteins B and B (15), the main apolipoproteins found in VLDL and LDL, respectively, eliminated the inhibition by LDL or VLDL. Moreover, 25 units/ml of heparin sulfate or 2 μ M EGTA inhibited endocytosis of HCV. Both are known inhibitors of LDL receptor endocytosis of lipoprotein (17).

To determine whether lipoproteins were involved in the endocytosis of HCV, we performed inhibition studies using various previously characterized antisera to apolipoproteins [α apo E, ID7 (18); α apo B, 4G3 (19); and α apo A-I, 3G10 (20)]. F(ab')₂ fragments were prepared and were used for all of the studies; inhibitory activities of the preparations were tested against Dil-labeled VLDL, LDL, and HDL isolated from a normal serum. Optimum F(ab')₂ antibody concentrations and conditions for inhibition of endocytosis were determined. Optimum conditions required addition of F(ab')₂ after pretreatment during the incubation period, and both α apo E and α apo B were required for maximal inhibition of VLDL endocytosis, whereas α apo B was sufficient for maximal inhibition of LDL endocytosis. Under these conditions, the maximum inhibition of HCV endocytosis achieved was 65%, with the remaining positive cells showing only trace staining. Pretreatment with α apo A-I gave 10% inhibition, with the remaining positive cells showing no decrease of staining compared with the control. The addition of α apo A-I during incubation did not increase inhibition. The finding that both α apo E and α apo B were required and that additional F(ab')₂ during the incubation increased inhibition was most likely attributable to the complexity of VLDL metabolism and dissociation of F(ab')₂ binding at 37°C. Hence, it could not be determined whether VLDL alone or both VLDL and LDL mediated endocytosis of HCV. Moreover, because complete inhibition could not be achieved, direct endocytosis of HCV by the LDL receptor could not be excluded.

Endocytosis experiments of isolated HCV-lipoprotein complexes and recombination experiments with HCV and lipoproteins provided more definitive data on the role of lipoproteins in endocytosis of HCV via the LDL receptor. Isolation of HCV by dissociation of HCV-VLDL complexes was unsuccessful; however, density gradient fractionation of a serum containing a high concentration of HCV produced not only HCV lipoprotein fractions but also a high density HCV fraction free of lipoprotein. Immunoglobulins contaminating the latter fraction were removed, providing a "free" HCV fraction for recombinant studies. Comparison of endocytosis of the various fractions is shown in Table 2. The HCV-VLDL and HCV-LDL, but not the HCV-HDL or high density HCV, fractions were endocytosed. Addition of VLDL or LDL but not HDL, isolated from normal serum, to the free HCV resulted in restoration of endocytosis. Cyclohexanedione treatment of the VLDL or LDL abrogated the rescue (data not shown).

Further studies were performed by using the LDL receptor-deficient fibroblast cells (21). Inoculation of these cells with HCV showed only weak endocytosis that could not be increased with preincubation of cells in lipoprotein deficient medium nor inhibited by anti-LDL receptor antibody (data not shown).

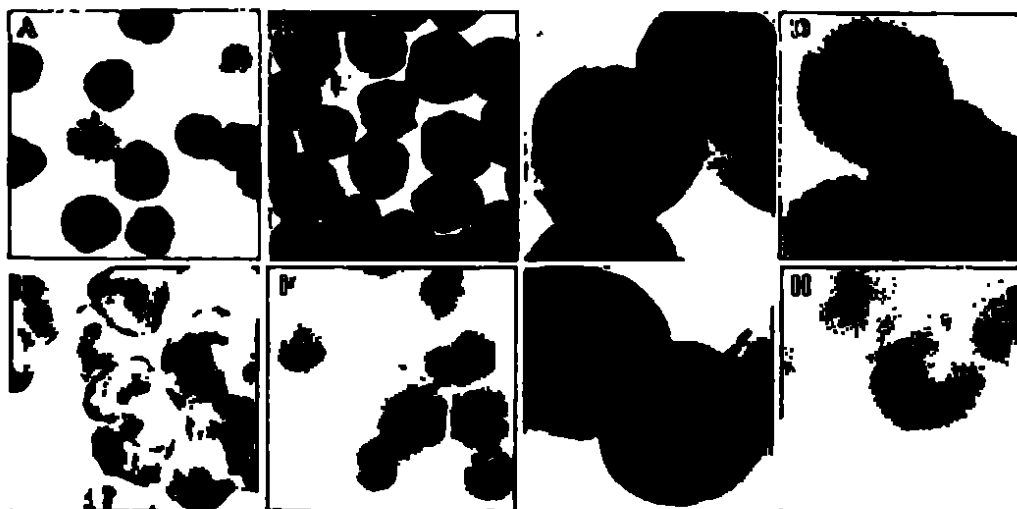


Fig. 3. (A) HCV ISH of HCV-inoculated G4 cells that were not up-regulated. Dark brown cytoplasmic staining indicates the presence of the virion form of HCV; only ~30% of the cells are weakly positive. (B) By contrast, HCV was present in most cells in cultures in which the LDL receptor was up-regulated before HCV exposure. Original magnification for A and B, $\times 500$. (C) High-power view of HCV-infected G4 cells with up-regulated LDL receptor as in A. (D) G4 cells prepared as in B and C were pretreated with anti-LDL receptor antibody. Blocking of the receptor by the antibodies decreased the uptake of the virus below the detection limit of ISH. Control antisera (see text) did not have such blocking effect (not shown). Original magnification for C and D, $\times 1,250$. (E) Uptake of HCV by Hep G2 hepatoma cell line is shown by ISH. (F) Blocking the LDL receptor with its specific antibody completely prevented the endocytosis of the virus, as demonstrated by ISH in E. Original magnification for E and F, $\times 500$. (G) Incubation of Daudi cells with HCV-positive serum resulted in positive staining by ISH. (H) Pretreatment of Daudi cells with 2 μ M PAO completely inhibited the endocytosis of HCV, as illustrated in G. Original magnification for G and H, $\times 1,250$.

Furthermore, this low level endocytosis could not be competitively inhibited with excess VLDL.

Replication of Endocytosed HCV. Replication of HCV has been reported in Hep G2 and Daudi cell cultures. Extended cultures of Hep G2, Daudi, and G4 cells were tested serially by ISH for evidence of replication. In the Hep G2 cells, only positive-strand HCV was detected in the cells up to 1 week, but, at 3 weeks, 85% of the cells contained positive-strand HCV and 65% contained negative-strand HCV (data not shown). At 4 weeks, the cells were negative for HCV. In Daudi cells, only positive strand was detected through day 10, but on days 15 and 20, both positive- and negative-strand genome sequences were present in 80% cells. The cells died in the fourth week of culture. Only the positive strand of HCV was detected in G4 cells up to 1 week; the cells died after 1 week.

Endocytosis of Other Flaviviridae Viruses. We investigated commercial bovine sera that were known to be contaminated with the pestivirus BVDV (22, 23). Human cell lines routinely cultured in

media containing bovine serum were found to be positive for intracytoplasmic BVDV by immunofluorescence using anti-BVDV antibody. The presence of BVDV was confirmed by RT-PCR using BVDV-specific primers. Negative-strand BVDV was not detected in cells nonpermissive to infection. BVDV-positive human nonpermissive cells became negative over a 4-week culture period in noncontaminated media. Endocytosis of BVDV by nonpermissive cultured cells could be inhibited completely with anti-LDL receptor antibody but not with the control anti-transferrin receptor antibody (data not shown for this set of experiments).

With use of the cytopathic NADL strain of BVDV and permissive cells, BT or bovine kidney (MDBK) cells, anti-LDL receptor antibody but not control antiserum inhibited the cytopathic effect and positive fluorescence at 3 days (Fig. 4 A–D). Five days after infection, there was complete cytolysis of both the inhibited and control cells. Similar studies using VSV and HSV showed no inhibition of infection by anti-LDL receptor antibody for HSV (Fig. 4 E, F, and I) but some inhibition for VSV at 48 hours, which was no longer apparent at 72 hours (Fig. 4 G–J).

Additional evidence for endocytosis of BVDV by LDL receptor was obtained by using a cell line (CRIB) resistant to BVDV that was derived from a permissive bovine kidney cell line (MDBK). As illustrated in Fig. 5, the CRIB cells that do not permit entry of BVDV (24) also do not endocytose LDL. The CRIB cells were negative for LDL receptors by immunofluorescence using anti-LDL receptor antibody (data not shown). However, the absence of DiI-LDL staining (see Fig. 5) is a more sensitive indication of the absence of LDL receptor and LDL receptor endocytosis because accumulation of DiI-LDL occurs from the rapid turnover of LDL by LDL receptor in the course of cholesterol metabolism.

A third member of the Flaviviridae family, GB virus C/HGV, was reported to associate with lipoproteins in the blood (25). Evidence also was obtained for LDL receptor-mediated endocytosis of this virus, as illustrated in Fig. 6.

Discussion

Several lines of evidence were presented supporting the hypothesis that HCV and other members of Flaviviridae are endocytosed via

Table 2. Comparison of endocytosis of HCV in lipoprotein fractions and the high density HCV fraction

Fraction	HCV, gE/ml	Lipoprotein, concentration, mg/ml	Endocytosis of 5 μ g DiI-labeled fraction, mean fluorescence, log scale	Endocytosis of 1×10^6 gE HCV from each fraction, percent cell positive, intensity of staining
VLDL	2.5×10^4	0.48	8.49	90%, ++
LDL	2.9×10^4	1.47	9.98	75%, +
HDL	8.3×10^4	2.56	1.88	0
d > 1.21	3.9×10^4	—	—	0

++, Moderately positive; +, weakly positive.

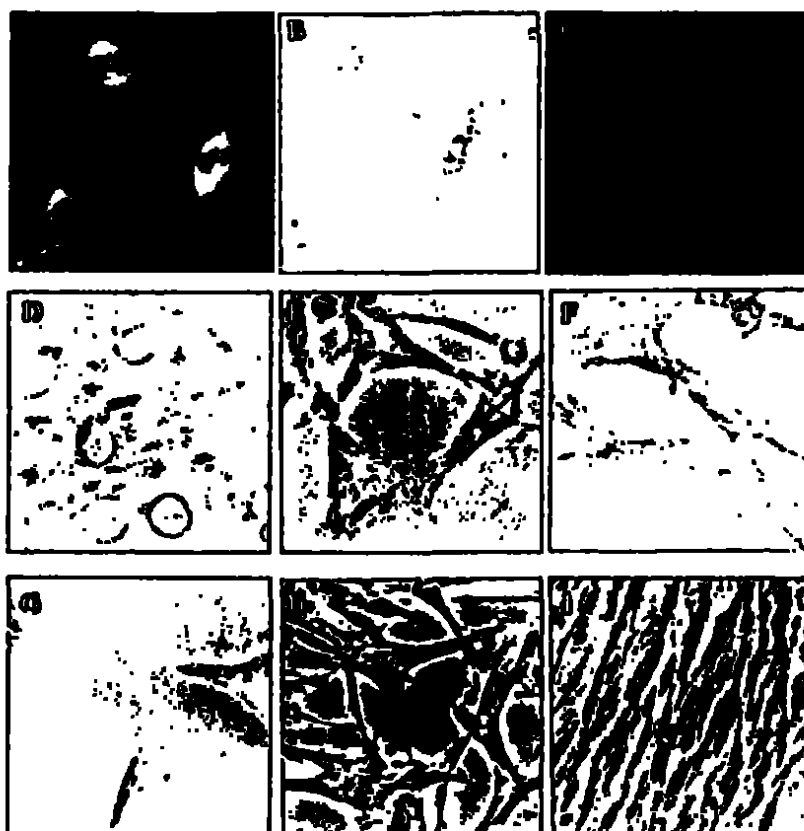


Fig. 4. (A) Demonstration of infection of BT cell monolayers by cytopathic BVDV (NADL strain) after 72 hours of incubation by immunofluorescence using anti-BVDV (green fluorescent staining). (B) Same field as in A by phase contrast microscopy. (C) Preincubation of BT cell monolayers with anti-LDL receptor antibody has completely prevented the infection (shown in A) of BT cells, as seen after 72 hours of culturing. Same detection method as in A. (D) Phase contrast photomicrograph of the same field as in C. Original magnification for A–D, $\times 500$. (E) Inoculation of MRC-5 fibroblasts with HSV resulted in widespread cytolysis and destruction of the monolayer after 48 hours. (F) Pretreatment of the monolayers with anti-LDL receptor antibody, using the same protocol as in C, did not prevent cytolysis and cell death. (G) Incubation of MRC-5 cells with dilutions of VSV also resulted in cytopathy and deterioration of the monolayer. (H) Pretreatment with anti-LDL receptor showed some inhibition of the destruction of the cells. Pretreatment and incubation conditions were as in C and F. (I) Control MRC-5 cell monolayer treated with anti-LDL receptor antibody as in C, F, and H but not inoculated subsequently with virus. Note the uniformity and healthy appearance of these fibroblasts. Original magnification for E–I, $\times 250$.

the LDL receptor. Endocytosis of HCV was shown to correlate with cell LDL receptor activity by experimental modulation of LDL



Fig. 5. (A) Intense uptake of DiI-LDL by a monolayer of the MDCK cells. (B) Phase contrast microscopy of the same field as A. (C) The lack of endocytosis of DiI-LDL was demonstrated in the CRB cell line that is resistant to BVDV infection. (D) Same field as in C with phase contrast. (E) Demonstration of the infection of MDCK cells with the NY-1 noncytopathic strain of BVDV after 72 hours of incubation by immunofluorescence using anti-BVDV (green fluorescent staining). (F) Phase contrast picture of E. (G) No BVDV was demonstrated by immunofluorescence in the BVDV-resistant CRB cells that have been incubated with the virus. Conditions were as in E–F. (H) Phase contrast for G. Original magnification for A–H, $\times 500$.

receptor activity and with the striking demonstration that the CRIB cell line, resistant to infection with BVDV for unknown reasons (24), lacked detectable LDL receptor activity. Direct evidence for the hypothesis was provided by inhibiting endocytosis of HCV, BVDV, and GB virus C/HCV with anti-LDL receptor antibody. Moreover, known biochemical inhibitors of LDL endocytosis and the endocytosis inhibitor, PAO, inhibited LDL receptor-mediated

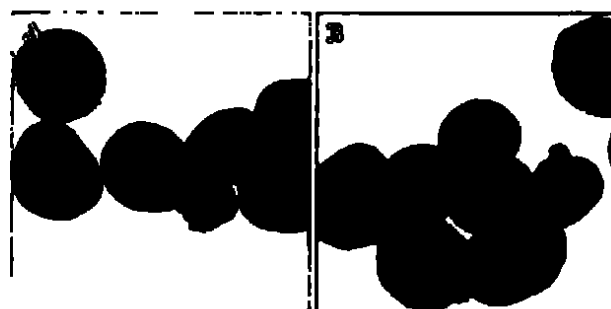


Fig. 6. (A) Daudi cells inoculated with HGV show the presence of HGV virion in the cytoplasm using ISH specific for this virus. (B) Preincubation of the cells with anti-LDL receptor antibody decreased the uptake of this virus below the detection limit of ISH. Original magnification for A and B, $\times 1,250$.

endocytosis of these viruses. The reports that up-regulation of LDL receptors facilitated persistent HCV infection *in vivo* (26) and the induction of HCV binding with transfection of the LDL receptor gene in COS-7 cells that were unable to bind HCV (27) are consistent with these findings.

Evidence also was presented that endocytosis of HCV via the LDL receptor was mediated by the complexing of HCV to VLDL or LDL. Although, HCV also appeared to complex with HDL in the serum to the same extent as VLDL and LDL, there was no evidence of HDL-mediated endocytosis of HCV. It could not be determined from the studies performed whether the low level of endocytosis of HDL compared with those of VLDL and LDL, as shown by cytometric studies with DII-labeled lipoproteins, was responsible for the absence of detectable levels of HDL-mediated endocytosis of HCV.

The complete inhibition by anti-LDL receptor antibody of HCV endocytosis by a variety of cells *in vitro* suggests that the LDL receptor may be the main mechanism for entry of HCV into cells. However, the detection of small amounts of intracellular HCV in LDL-deficient fibroblasts inoculated with HCV that could not be inhibited by anti-LDL receptor antibody suggested that receptors not related to the LDL receptor mediate viral entry in these cells. Furthermore, the finding that there was only a delaying effect of anti-LDL receptor antibody on the cytopathic effect of the NADL strain of BVDV indicates that receptors for BVDV other than LDL receptors or low affinity LDL receptors were present. This finding is consistent with the observations of Flores and Donis (24) that CRIB cells could be infected with high concentrations of BVDV. Hence, the LDL receptor may be the main but not exclusive means of entry of these viruses into these cells.

The evidence of HCV replication in Hep G2 and Daudi cells, although transient, suggests that endocytosis of HCV mediated by the LDL receptor can result in infection. Whether this route of entry can result in a productive infection *in vivo* and whether all members of the Flaviviridae family of viruses use this mechanism of cell entry remain to be determined. The evidence presented in this study that VSV also may be endocytosed by the LDL receptor appears to be contrary to an earlier study (28) that found interferon- α induces a soluble LDL receptor fragment that inhibits VSV infection but concluded that the LDL receptor was not a receptor for VSV. The latter conclusion was reached because VSV was found to infect LDL receptor-deficient fibroblasts. From our finding that entry of HCV into LDL receptor-deficient fibroblast was independent of the LDL receptors, it is apparent that Fischer *et al.* (28) did not present definitive

evidence that VSV was not endocytosed by means of the LDL receptor because receptors for VSV other than LDL receptors may have been present on those cells.

LDL receptor-mediated endocytosis may not be exclusive to Flaviviridae. Rather, this mechanism may be used by all viruses that can associate with VLDL or LDL in the blood. The reported association of VSV with lipoproteins in the blood, particularly VLDL (29), is consistent with this notion.

Thus far, there only has been indirect evidence of *in vivo* endocytosis of HCV mediated by the LDL receptor, that is, the apparent endocytosis of HCV by keratinocytes in the inflammatory skin lesions of patients with type II cryoglobulinemia (3). In this study, evidence that BVDV contaminated media from bovine serum is endocytosed by a wide variety of human cells nonpermissive to BVDV infection suggests that this mechanism may explain the presence of positive-strand HCV that has been widely reported in human nonhepatic cells, particularly peripheral blood mononuclear cells. This mechanism must be considered before assigning HCV tropism for tissues other than the liver where clear evidence for HCV replication has been demonstrated.

The potential involvement of the LDL receptor in HCV infection has implications for the current therapy of HCV infection and also provides the rationale for a new approach to therapy—specifically, administration of anti-LDL receptor antibody or analogues of the LDL receptor binding site of apolipoproteins B and E. The effect of IFN α , the current therapy for HCV infection, may be mediated in part by the down-regulation of LDL receptors. IFN α is known to induce IL-1 receptor antagonist (30), which blocks the IL-1 receptor-mediated stimulation by IL-1. Because IL-1 is known to increase LDL receptor activity (31), IFN α would indirectly cause a down-regulation of LDL receptor activity by stimulating IL-1 receptor antagonist production, thereby decreasing IL-1 receptor-mediated stimulation by IL-1.

It has been reported that a minor group of human rhinovirus (HRV2) (32) and Rous sarcoma virus A (33) enters cells via the LDL receptor-related protein. In the latter report, evidence was presented that a viral envelope moiety binds to LDL receptor-related protein. Our study provides evidence that the family of LDL receptors may serve as viral receptors.

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Self-association of the Low Density Lipoprotein Receptor Mediated by the Cytoplasmic Domain*

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When the low density lipoprotein (LDL) receptor was solubilized from bovine adrenal cortex membranes and subjected to electrophoresis in the absence of reducing agents, a disulfide-bonded dimeric species was demonstrated. Formation of these covalent bonds was blocked when the tissue was homogenized in the presence of sulfhydryl alkylating agents, indicating that the native receptor was self-associated noncovalently and that the disulfide bond formation occurred only after homogenization. The disulfide-linked dimers were disrupted and the receptor was restored to a monomeric form when inside-out adrenal vesicles were treated with trypsin, suggesting that the disulfide bond formation involved the 50-amino acid cytoplasmic domain of the receptor. When the receptor was solubilized from bovine adrenal cortex membranes and then purified by ion exchange and affinity chromatography, it could be covalently coupled into dimers and trimers in the presence of bivalent cross-linking agents. Receptor dimers could also be demonstrated by chemical cross-linking of intact cells that were transfected with an expressible cDNA encoding the normal human LDL receptor. Dimer formation was markedly reduced in transfected cells expressing mutated cDNAs that had premature termination codons at positions 792, 807, and 812, which produced shortened receptors that retained 2, 17, and 22 of the original 50 amino acids of the cytoplasmic domain, respectively. The first two mutant receptors, which did not form oligomers, did not enter coated pits and were not rapidly internalized by cells. However, the mutant receptor that terminates at position 812 was internalized normally even though oligomer formation was greatly reduced. Moreover, a mutant receptor with a cysteine substituted for a tyrosine at position 807, which internalized very slowly, showed a normal susceptibility to chemical cross-linking. Deletion of external domains of the LDL receptor, including the epidermal growth factor homology region and the O-linked sugar domain, did not alter susceptibility to chemical cross-linking. We conclude that the cytoplasmic domain of the LDL receptor is responsible both for self-association into oligomers and for clustering in coated pits, but the available data do not establish a causal connection between these two events.

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Many of the cell surface receptors that participate in receptor-mediated endocytosis exist as multimers. Examples include the transferrin receptor, which is a disulfide-linked dimer of identical subunits (1), and the insulin receptor, which consists of complexes of two nonidentical subunits, designated α and β , that are disulfide-linked (2). The hepatic receptors for analogoglycoproteins in several mammalian species (3) and for *N*-acetylglucosamine-terminated glycoproteins in chickens (4) behave as noncovalently associated oligomers. Recently this oligomerization has been directly demonstrated for the chicken hepatic receptor by the use of chemical cross-linking agents (4). Another receptor, the receptor for epidermal growth factor (EGF),¹ is believed to form noncovalent dimers after the binding of EGF (5).

The receptors that mediate endocytosis cluster in coated pits (6). No relation between multimer formation and clustering has yet been established. Indeed, the chicken hepatic lectin had the same distribution of multimers when chemical cross-linking was performed in plasma membranes, coated vesicles, or membranes of the microsomal fraction (4).

One system that may provide information to link oligomer formation and endocytosis is the low density lipoprotein (LDL) receptor system. The LDL receptor mediates the binding and uptake of cholesterol-carrying lipoproteins from blood, thereby supplying cholesterol to cells. The LDL receptor is a single-chain glycoprotein of 839 amino acids that spans the membrane once (7) and is oriented with its NH_2 terminus facing the extracellular environment and its COOH -terminal 50 amino acids facing the cytoplasm (8). The extracellular portion is composed of three discrete domains: 1) an NH_2 -terminal ligand binding domain of 292 amino acids that is composed of seven imperfect repeats of a 40-amino acid cysteine-rich sequence (9); 2) a sequence of ~400 amino acids that is homologous to the precursor for epidermal growth factor and that plays a role in receptor recycling (10); and 3) a sequence of 58 amino acids immediately external to the membrane that contains clustered O-linked carbohydrates (11).

Monomers of the LDL receptor, which have been isolated by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis, are able to bind LDL on ligand blots after transfer to nitrocellulose (12). Moreover, target inactivation studies of the receptor in intact fibroblasts reveal a molecular weight of 106,000 for the active binding species, which is consistent with a monomeric form of the receptor (13). However, these

¹ The abbreviations used are: EGF, epidermal growth factor; β -VLDL, β -migrating very low density lipoproteins; DSS, dithionitrosyl suberate; DFDNB, 1,5-difluoro-2,4-dinitrobenzene; DST, dithionitrosyl tartarate; DTSSP, 3,3'-dithiobis(sulfosuccinimidylpropionate); LDL, low density lipoprotein; SDS, sodium dodecyl sulfate; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, [ethylenbis(oxyethylenetriole)]tetraacetic acid.

studies can only estimate the size of the ligand binding subunit and do not rule out the presence of noncovalently associated multimers in the membrane environment. Pitas et al. (14) showed that a lipoprotein particle that contains multiple apoprotein E molecules can engage more than one LDL receptor, which supports the possibility that the LDL receptor exists in an oligomeric state in the membrane.

In the steady state in cultured human fibroblasts, about 70–80% of the cell surface LDL receptors are located in coated pits, a clustering reaction that does not require the binding of ligand (6). Rather, this localization is mediated by the juxta-membranous 22 amino acids in the 50-amino acid cytoplasmic domain of the receptor (15). Naturally occurring human mutations that eliminate this segment or alter its amino acid sequence destroy the ability of the receptor to cluster in coated pits (16, 17). Detailed studies of artificially produced mutations in an expressible LDL receptor cDNA have revealed a strict requirement for an aromatic residue at position 807 in the cytoplasmic domain (15).

In the current studies we have sought to determine whether the LDL receptor is capable of forming dimers or higher order oligomers in solution, in membranes, or in intact cells and to determine whether this self-association is mediated by the cytoplasmic domain.

EXPERIMENTAL PROCEDURES

Materials. Oligonucleotides were synthesized by the phosphoramidite method (18) with an automated DNA synthesizer (model 380A, Applied Biosystems). The synthetic peptide, Cys-Arg-Gly-Leu-Tyr-Val-Phe-Gln-Gly-Asp-Ser-Ser, which corresponds to residues 163–174 of the human LDL receptor (7), was synthesized by Peninsula Laboratories. The chemical cross-linking agents 3,3'-di-thiobis(sulfosuccinimidylpropionate) (DTSSP), disuccinimidyl tartrate (DST), disuccinimidyl suberate (DSS), and 1,5-difluoro-2,4-dinitrobenzene (DFDNB) were purchased from Pierce Chemical Co. β -VLDL ($d < 1.006$ g/ml) was isolated from the plasma of cholesterol-fed rabbits and radiolabeled with ^{125} I as previously described (12).

Antibodies. Mouse monoclonal antibodies IgG-C7 (19) and IgG-15C8 (20), which both react with the NH₂-terminal domains of the human and bovine LDL receptors, were prepared as described in the indicated references. Mouse monoclonal IgG-8001, a control antibody directed against an irrelevant antigen, was described previously (21). A polyclonal antibody directed against the COOH-terminal 14 amino acids of the human and bovine LDL receptor was prepared as previously described (20).

To produce monoclonal antibody IgG-HL1, a synthetic peptide corresponding to residues 163–174 of the human LDL receptor (7) was coupled to keyhole limpet hemocyanin (8) and injected into the foot pads of BALB/c mice according to the protocol of Brodsky (22). Popliteal lymph node cells were then fused (23) to NS1/1 Ag 4.1 myeloma cells. Hybridomas were initially screened for the production of antibodies that reacted with the above peptide coupled to bovine serum albumin. We used a solid-phase enzyme-linked immunosorbent assay according to the manufacturer's instructions (HyClone Laboratories, cat. no. E-5010-K). Strongly positive hybridoma supernatants were then assayed for anti-LDL receptor antibodies by immunoblot analysis of cell lysates prepared from TR 715-19 cells (see below). The heavy chain of IgG-HL1 is IgG γ h, and the light chain is κ .

All antibodies were purified from mouse ascites fluid or rabbit serum by affinity chromatography on *Staphylococcus* Protein A-Sepharose (19).

Plasmids and Transfected Cell Lines. The plasmids encoding mutant forms of the LDL receptor and the names of the transfected cell lines expressing these plasmids are shown in Fig. 8 and Table I, respectively. All plasmids and transfected cell lines have been described previously (10, 11, 15, 16). The plasmids were transfected into a Chinese hamster ovary cell mutant that lacks a functional LDL receptor (*hLA-7* cells) (24).

Cell Culture. All transfected hamster *hLA-7* cells were grown in monolayer culture in an atmosphere of 5–7% CO₂ in air. Cells were set up for experiments according to a standard format in which cells from stock flasks were first dissociated with trypsin/EDTA. On day 0, 5×10^5 cells were seeded into a 100-mm Petri dish in 10 ml of

medium A (Ham's F-12 containing 20 mM Hapes (pH 7.4), 1% (v/v) fetal calf serum, 4% (v/v) calf lipoprotein-deficient serum (25), 100 units/ml penicillin, 100 μ g/ml streptomycin, and 2 mM glutamine). On day 2, cells received 10 ml of medium A containing 10 μ g/ml cholesterol and 0.1 μ g/ml 25-hydroxycholesterol to suppress the expression of the endogenous receptor in *hLA-7* cells. On day 3, cells were used for experiments.

Isolation and Iodination of Adrenal Cortex Membranes. Bovine adrenal cortex tissue (5 g) was obtained (26) and homogenized in a glass-Teflon homogenizer in 5 volumes (w/v) of buffer B (50 mM Hapes (pH 7.4) and 100 mM NaCl) containing 1 mM phenylmethanesulfonyl fluoride and other additions as indicated in the figure legends. The homogenate was centrifuged at $470 \times g$ for 10 min at 4°C in a Sorvall SA-600 rotor. The supernatant was filtered through one layer of gauze cloth and centrifuged at $100,000 \times g$ for 1 h at 4°C in a Sorvall TPT 50.3 rotor. The pellet was resuspended by aspiration through a 21-gauge needle and sonicated (26) in 5 ml of the above buffer.

To iodinate these vesicles, the protein concentration was diluted to 5 mg/ml in a final volume of 100 μ l of buffer B. Then three IODO-BEADS (Pierce Chemical Co.) and 2 mCi of Na ^{125} I (Amersham Corp.) were added. After a 30-min incubation at room temperature, the suspension was separated from the IODO-BEADS and placed into a microcentrifuge tube (Kontes), after which the vesicles were pelleted by centrifugation at $10,000 \times g$ for 15 min at 4°C. The pellet was homogenized in the tube using 10 strokes of a plastic pestle (Kontes) in 500 μ l of buffer B, recentrifuged, and finally homogenized as above in 500 μ l of 10 mM Hapes (pH 7.4) containing 200 mM NaCl, 2.5 mM MgCl₂, 2 mM CaCl₂, 1% (v/v) Triton X-100, 1 mM phenylmethanesulfonyl fluoride, and 0.1 mM leupeptin.

Cross-linking Reactions. Purified bovine LDL receptor (26) was cross-linked at a concentration of 10 μ g/ml in 0.1 ml of buffer B containing 0.5% Triton X-100 for 2 h at 0°C. The SDS sample buffer was then added, and the solution was subjected to electrophoresis. Stock solutions of the cross-linking agent DFDNB were made in dimethyl sulfoxide and added to the receptor solution so that the final solvent concentration was 1%.

Intact cells were cross-linked and analyzed by immunoblotting as follows. All operations were performed at 0°C. The medium was removed from the Petri dishes, and cells were washed twice with buffer B. Cross-linking was initiated by the addition of 4 ml of buffer B containing the cross-linker indicated in the figure legends. The reactions were terminated at the times specified in the figure legends by adding 40 μ l of 1 M glycine followed by a further 10-min incubation. Dishes were continuously agitated during incubations. The cells were then washed twice with buffer B and scraped from the dishes with a rubber policeman in 1 ml of buffer C (buffer B containing 1 mM phenylmethanesulfonyl fluoride, 0.5 mM leupeptin, 10 mM EDTA, 10 mM EGTA, 10 mM *N*-ethylmaleimide, and 2.5% dimethyl sulfoxide). After centrifugation at $10,000 \times g$ for 30 s, the cells were lysed in 100 μ l of buffer C containing 1% Triton X-100 for 10 min. Cellular debris was pelleted by centrifugation at $10,000 \times g$ for 5 min, and lysates were either subjected to SDS-gel electrophoresis directly or stored at -70°C. One half of each lysate was used for each immunoblot analysis.

Immunoblot and Ligand Blot Analysis. Proteins were transferred from SDS-polyacrylamide gels (see below) to either nitrocellulose (Schleicher and Schell) or Immobilon (Millipore) membranes as described by Dunn (27). LDL receptors were detected using antibodies that were labeled with ^{125} I or conjugated with alkaline phosphatase. For detection with ^{125} I-labeled antibodies, the procedure was as follows. After transfer of proteins from the gels the membranes were incubated in buffer D (50 mM Tris-HCl (pH 8.0), 80 mM NaCl, 3 mM CaCl₂, 0.05% (v/v) polyoxyethylene sorbitan monolaurate) containing 3% (w/v) bovine serum albumin for 1 h at room temperature. Membranes were then incubated with either monoclonal or polyclonal anti-LDL receptor antibody at a concentration of 5 or 10 μ g/ml, respectively, in buffer D for 1 h at room temperature. After two 15-min washes in buffer D, ^{125} I-labeled rabbit anti-mouse or goat anti-rabbit IgG antibodies (IgG fraction, Cooper Biomedical, Inc.) were incubated with the membranes (3×10^5 cpm/ml in buffer D; specific activity, $1-2 \times 10^4$ cpm/ng) for 1 h at room temperature. After three 15-min washes in buffer D, the membranes were dried and set up for autoradiography. For detection with alkaline phosphatase-conjugated antibodies, the membranes were incubated with monoclonal anti-LDL receptor antibody as described above, followed by incubation with alkaline phosphatase-conjugated goat anti-mouse IgG and color

development reagents according to the manufacturer's instructions (Bio-Rad).

Ligand blotting with ^{125} I- β -VLDL (5 μ g of protein/ml, 440 cpm/ng protein) was performed as described (12).

Miscellaneous Methods—SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (28). Samples did not receive sulfhydryl reducing agent unless otherwise stated. Apparent molecular masses were calculated by references to the migration of the following marker proteins: myosin (200 kDa), β -galactosidase (116 kDa), phosphorylase b (97 kDa), and bovine serum albumin (66 kDa). Marker proteins were always reduced with 3% (v/v) 2-mercaptoethanol prior to electrophoresis.

^{125} I-labeled proteins were visualized by fluorography at room temperature using Kodak X-Omat RP-1 film and Du Pont Lightning Plus intensifying screens unless otherwise stated.

RESULTS

In previous studies we isolated the LDL receptor from membranes of bovine adrenal cortex by solubilization in non-denaturing detergents followed by ion exchange and affinity chromatography (26). The true M_r of the mature LDL receptor including the protein component (93,000) and the carbohydrate component ($\sim$ 22,000) is $\sim$ 115,000 (7, 29). The purified LDL receptor migrates anomalously on SDS gels with an apparent molecular weight of 160,000 in the presence of sulfhydryl reducing agents. The anomalously slow migration of this glycoprotein is caused by the clustered O-linked sugars (11, 29). In the absence of reducing agents, the LDL receptor migrates faster (apparent molecular weight, $\sim$ 130,000) (12) apparently because of the globular structure created by the multiple intramolecular disulfide bonds in the cysteine-rich repeats (7).

To determine whether the membrane form of the LDL receptor is a monomer, we subjected bovine adrenal membranes to radioiodination, then solubilized and immunoprecipitated the LDL receptors with a monoclonal antibody (Fig. 1). The immunoprecipitates were subjected to electrophoresis either without reduction (Fig. 1A) or after treatment with 5% 2-mercaptoethanol (Fig. 1B). In the nonreduced form most of the receptors migrated with a molecular weight of approximately 220,000 (Fig. 1A), which was reduced to 160,000 in the presence of 2-mercaptoethanol (Fig. 1B). No other major bands became apparent upon reduction even when samples were electrophoresed in a higher percentage polyacrylamide

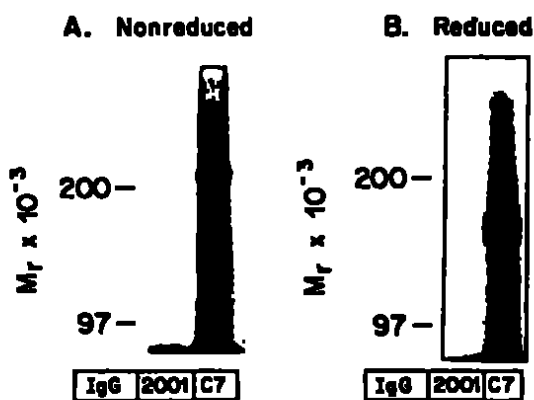


FIG. 1. Immunoprecipitation of LDL receptors from ^{125} I-labeled bovine adrenal cortex. Membranes were isolated and iodinated as described under "Experimental Procedures." Aliquots of the ^{125} I-labeled membrane proteins (500 μ g of protein) were incubated with insoluble immune complexes containing either IgG-2001 or IgG-C7 as previously described (33). The immunoprecipitates were subjected to electrophoresis in a 4% SDS-polyacrylamide gel in the absence (A) or presence (B) of 5% (v/v) 2-mercaptoethanol. The migration of marker proteins is indicated. The dried gels were exposed to XAR-5 film for 16 h.

gel (data not shown). These data raised the possibility that the bulk of the receptors in the radioiodinated membranes was present as disulfide-linked dimers.

To determine whether the intermolecular disulfide bond formed before or after homogenization, we homogenized the adrenal cortex in the absence or presence of an alkylating agent (either *N*-ethylmaleimide or iodoacetic acid) (Fig. 2). The receptors were then solubilized, subjected to electrophoresis in the absence or presence of 2-mercaptoethanol, and visualized by immunoblotting. When the homogenates were prepared in the absence of alkylating agents, most of the receptor was present as dimers (Fig. 2A), which were reduced to monomers in the presence of 2-mercaptoethanol (Fig. 2D). When homogenization was performed in the presence of *N*-ethylmaleimide (Fig. 2B) or iodoacetic acid (Fig. 2C), the amount of dimer was much reduced.

We interpret the experiment of Fig. 2 to indicate that the receptors in the adrenal membranes exist largely as noncovalently associated dimers and that covalent cross-linking occurs by intermolecular disulfide bond formation after the tissue is homogenized. The formation of disulfide bonds is prevented when cysteines are alkylated at the time of homogenization.

On nitrocellulose blots the disulfide-bonded dimeric receptor was able to bind not only the monoclonal antibody IgG-15C8 (Fig. 3B) but also the physiologic ligand β -VLDL (Fig. 3D). When the homogenization was carried out in the presence of *N*-ethylmaleimide, dimer formation was prevented, and the receptors remained as monomers. These monomers bound both IgG-15C8 (Fig. 3A) and β -VLDL (Fig. 3C). Thus, the dimerization reaction did not affect the binding of β -VLDL. Similar results were obtained with LDL (data not shown).

To determine whether covalent dimer formation requires



FIG. 2. Immunoblot analysis of LDL receptors from bovine adrenal membranes prepared in the presence of alkylating agent. Membranes were prepared as described under "Experimental Procedures" except the following agents were present during the homogenization and centrifugation steps. A and D, none; B and E, 10 mM *N*-ethylmaleimide, 10 mM EDTA, and 10 mM EGTA; C and F, 10 mM iodoacetic acid, 10 mM EDTA, and 10 mM EGTA. Aliquots of the membranes (100 μ g of protein) were added to SDS sample buffer either in the absence (A-C) or presence (D-F) of 5% 2-mercaptoethanol and subjected to electrophoresis in a 4% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes, incubated with mouse monoclonal anti-receptor IgG-15C8 (A-C) or a polyclonal rabbit anti-LDL receptor COOH-terminal antibody (D-F), and then probed with the appropriate ^{125} I-labeled second antibody as described under "Experimental Procedures." The mobilities of standard proteins are indicated. The dried nitrocellulose was exposed to film for 6 h (A-C) or 16 h (D-F). The anti COOH-terminal antibody was used in D-E because IgG-15C8 reacts poorly with the reduced form of the receptor.

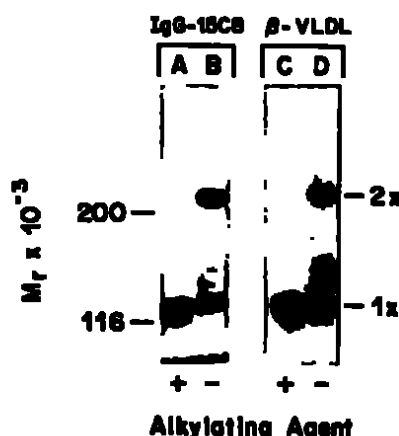


FIG. 3. Ligand blot analysis of LDL receptors from bovine adrenal membranes. Membranes were prepared as described under "Experimental Procedures" except that the following agents were present during the homogenization and centrifugation steps: A and C, 10 mM *N*-ethylmaleimide, 10 mM EDTA, and 10 mM EGTA; B and D, none. Aliquots of the membranes (100 μ g of protein) were added to SDS sample buffer that did not contain reducing agent and subjected to electrophoresis in a 4% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated with either ^{125} I-labeled mouse monoclonal anti-receptor IgG-15C8 (A and B) or ^{125} I-labeled rabbit β -VLDL (C and D) as described under "Experimental Procedures." The symbols 1x and 2x indicate the proposed monomeric and dimeric forms of the LDL receptor. M_r standards are shown. The nitrocellulose was exposed to film for 36 h.

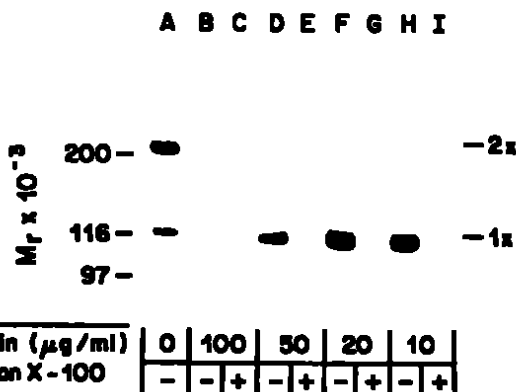


FIG. 4. Immunoblotting of LDL receptors in bovine adrenal membranes after digestion with trypsin. Membranes (270 μ g of protein) were resuspended in 100 μ l of buffer B containing 2 mM CaCl_2 , the indicated concentration of trypsin, and 0.5% Triton X-100 as indicated. After a 1-h incubation at 37°C, all samples received 10 μ l of 3 mg/ml soybean trypsin inhibitor and 2 μ l of 0.5 M phenylmethanesulfonyl fluoride. One-half of each sample was subjected to electrophoresis in a 4% SDS-polyacrylamide gel in the absence of reducing agents. After electrophoresis, proteins were transferred to nitrocellulose membranes, incubated with mouse monoclonal anti-receptor IgG-15C8, and then probed with ^{125} I-labeled rabbit anti-mouse IgG. The symbols 1x and 2x indicate the proposed monomeric and dimeric forms of the receptor. M_r standards are indicated. The nitrocellulose was exposed to film for 1 h.

the cytoplasmic portion of the receptor, we prepared adrenal membrane vesicles in the absence of alkylating agents and digested the vesicles with trypsin. The receptors were then subjected to solubilization, electrophoresis, and visualization by immunoblotting (Fig. 4). When the membranes were incubated in the absence of trypsin, most of the receptor was in the dimer form (Fig. 4A). When the membranes were incubated with 10 μ g/ml trypsin, there was a marked decline in the amount of dimer and a corresponding increase in the

amount of monomer (Fig. 4H). The molecular weight of the monomeric form was reduced by about 5000 (Fig. 4H), and it no longer reacted with an anti-peptide antibody that is specific for the COOH terminus (data not shown). When the digestion was performed in the presence of Triton X-100, nearly all of the receptors were totally destroyed (Fig. 4I). Higher concentrations of trypsin gave similar results, except that at very high concentrations of trypsin the receptor was destroyed even in the absence of Triton X-100, apparently due to entry of trypsin into the membrane vesicles (Fig. 4B). We interpret these results to indicate that most of the adrenal membrane vesicles are inside-out, with the 5000-dalton cytoplasmic domain exposed to the protease. Removal of this cytoplasmic domain with trypsin reduces the molecular weight by 5000 and causes the dimer to dissociate into monomers.

We have performed experiments similar to those described above with purified coated vesicles from bovine adrenal cortex and rat liver. In both cases most of the receptor was found to be a disulfide-bonded dimer. In the bovine coated vesicles the cytoplasmic domain was required for dimer formation as indicated by proteolysis experiments similar to those in Fig. 4 (data not shown).

To determine whether the solubilized purified LDL receptor was capable of forming multimers, we purified the receptor to homogeneity by ion exchange and immunoaffinity chromatography in the presence of detergents (26). We then incubated the receptor with varying concentrations of a chemical cross-linking agent, DFDNB, in the presence of 0.5% Triton X-100 (Fig. 5). The receptor that was purified in this fashion ran as a monomer on SDS polyacrylamide gels even in the absence of reducing agent (Fig. 5A). Addition of DFDNB at 0.13 mg/ml caused a high proportion of the receptor to be cross-linked into species that migrated at approximately 200,000 daltons and approximately 300,000 daltons, suggesting the existence of dimeric and trimeric forms of the receptor. Further increases in the concentration of DFDNB did not cause further cross-linking, but the mobility of all forms of the receptor progressively increased, probably because of the formation of a more tightly packed globular structure through the formation of intramolecular cross-links. A similar increase

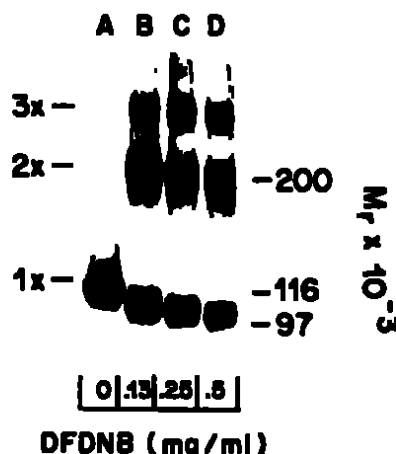


FIG. 5. Cross-linking of purified bovine LDL receptors at different concentrations of DFDNB. Purified bovine LDL receptors in a solution containing 0.5% Triton X-100 were cross-linked as described under "Experimental Procedures" with the indicated concentrations of DFDNB. Cross-linked products (0.5 μ g) were separated in a 4% SDS-polyacrylamide gel in the absence of reducing agent. After electrophoresis, immunoblotting analysis was carried out as described in the legend to Fig. 4. The symbols 1x, 2x, and 3x indicate the proposed monomeric, dimeric, and trimeric forms of the receptor. The nitrocellulose was exposed to film for 30 min.

in mobility was reported by Loeh and Drickamer (4) in studies of the chicken hepatic lectin. The cross-linking of purified LDL receptor was not affected by the addition of 5 mM EDTA, 5 mM EGTA, 5 mM *N*-ethylmaleimide, or 0.5 M NaCl to the reaction mixture (data not shown).

To determine whether the LDL receptor could be cross-linked into a multimeric form in intact cells, we examined mutant hamster cells that had been transfected with a plasmid encoding the normal human LDL receptor (pLDLR-2) under control of the SV40 early region promoter. These cells, designated TR-715 cells, produce higher levels of human LDL receptors than do normal cultured fibroblasts (11). The results in Fig. 6A show that in the absence of cross-linking agent the LDL receptor migrated as a monomeric species, even in the absence of sulfhydryl reducing agents. In the presence of any of three different cross-linking reagents, DTSSP (Fig. 6B), DST (Fig. 6C), and DSS (Fig. 6D), a fraction of the receptors was cross-linked so that they migrated as dimers. The most potent cross-linker was DTSSP (Fig. 6B).

Fig. 7 shows an experiment in which TR-715 cells were incubated with increasing concentrations of DTSSP followed by solubilization, electrophoresis, and visualization of the LDL receptor by immunoblotting. DTSSP was able to cross-link a maximum of about 20% of the receptors into a dimeric form. The efficiency of cross-linking in several experiments varied from ~15 to 25% as judged by densitometric scans of autoradiograms. The failure to achieve a higher level of cross-linking is likely attributable to the low efficiency of chemical cross-linking agents in intact cells (4, 30).

We next sought to determine whether the susceptibility to cross-linking in intact cells required the presence of the cytoplasmic domain in the receptor. For this purpose we employed cells that were transfected with expressible cDNAs from which segments encoding various regions of the LDL receptor were either deleted or altered by mutagenesis (Fig. 8 and Table I). The results of these experiments are presented in Figs. 9 and 10. The LDL receptor expressed by the normal cDNA was cross-linked into a dimeric form (Fig. 9B). A similar cross-linking was obtained with the receptor that terminated at residue 829, which eliminates the COOH-terminal 14 amino acids (Fig. 9D). In contrast, the receptor that terminated at residue 812 showed no cross-linking (Fig. 9F).

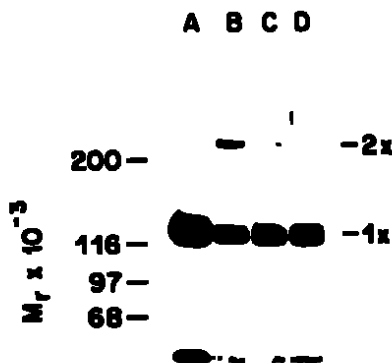


FIG. 6. Use of three agents to cross-link LDL receptors in intact cultured fibroblasts. Monolayers of hamster fibroblasts transfected with pLDLR-2 were treated for 1 h at 4°C with one of the following chemicals: A, none; B, 2 mg/ml of DTSSP; C, 2 mg/ml of DST; or D, 1 mg/ml of DSS. The samples were solubilized and subjected to electrophoresis in a 4% SDS-polyacrylamide gel in the absence of reducing agents. After electrophoresis, proteins were transferred to nitrocellulose membranes, incubated with mouse monoclonal anti-receptor IgG-HL1, and probed with ¹²⁵I-labeled rabbit anti-mouse IgG. The symbols 1x and 2x indicate the proposed monomeric and dimeric forms of the receptor. M, standard are shown. The nitrocellulose was exposed to film for 16 h.

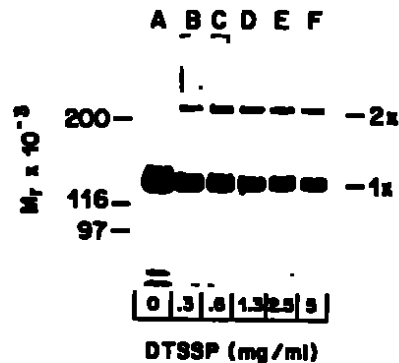


FIG. 7. Cross-linking of LDL receptors in intact cultured fibroblasts at different concentrations of DTSSP. Monolayers of hamster cells transfected with pLDLR-2 were treated with the indicated concentration of DTSSP for 30 min at 4°C, solubilized, and subjected to electrophoresis in a 4% SDS-polyacrylamide gel in the absence of reducing agents. After electrophoresis, immunoblotting analysis for the detection of LDL receptors was carried out as described in the legend to Fig. 6. The symbols 1x and 2x indicate the proposed monomeric and dimeric forms of the receptor. M, standards are indicated.

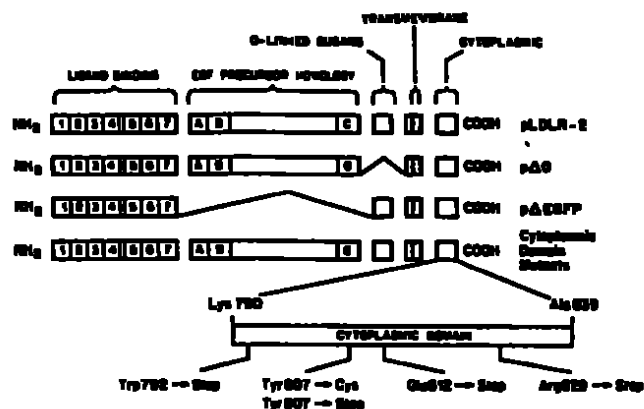


FIG. 8. Structures of mutant LDL receptors. Plasmid pLDLR-2 encodes the entire sequence of the human LDL receptor. The seven cysteine-rich repeat sequences of the ligand binding domain (1-7), the three growth factor repeats in the EGF homology region (A, B, and C), the O-linked sugar domain, the transmembrane domain, and the cytoplasmic domain are indicated. The remaining plasmids encode LDL receptors from which various portions have been deleted or in which premature terminator codons or amino acid substitutions have been made.

TABLE I
Description of LDL receptor mutants

Plasmid	Mutation	Transfected cell line	Reference
pLDLR-2	None	TR 715-19	11
pΔO*	Deletion of residues 700-747	TR 722-18A	11
pΔEGFP	Deletion of residues 293-692	TR 841A-2A	10
pStop792	Trp ⁷⁹² → Stop	TR 807-3	15
pStop807	Tyr ⁸⁰⁷ → Stop	TR 989	15
pCys807	Tyr ⁸⁰⁷ → Cys	TR 735-7A	15, 16
pStop812	Glu ⁸¹² → Stop	TR 937-1	15
pStop829	Arg ⁸²⁹ → Stop	TR 964-10	15

* Formerly named pLDLR-2 Δ(Thr⁷⁹²-Ser⁷⁹⁷) (11).

In other experiments we have observed a trace amount of a dimeric form of this mutant receptor after cross-linking. However, the ratio of dimeric to monomeric receptors was never more than one-tenth of the ratio found for the normal receptor in the same experiments (data not shown). Despite

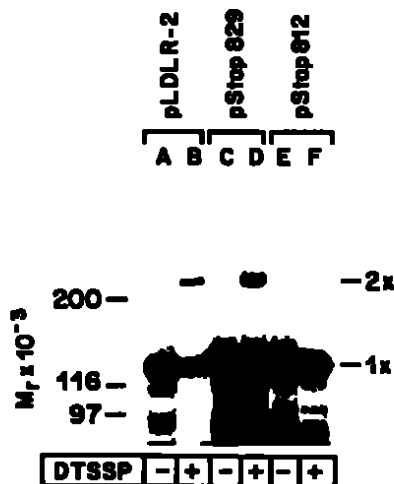


FIG. 9. Cross-linking of mutant LDL receptors in intact cultured fibroblasts. Monolayers of hamster cells transfected with the indicated plasmids were incubated for 30 min at 4 °C in the absence or presence of 2 mg/ml DTSSP as indicated. The samples were solubilized and subjected to electrophoresis in a 4% SDS-polyacrylamide gel in the absence of reducing agents. After electrophoresis, immunoblotting analysis for detection of LDL receptors was carried out as described in the legend to Fig. 6. The symbols 1x and 2x indicate the proposed monomeric and dimeric forms of the receptor. M, standards are indicated. The nitrocellulose was exposed to film for 6 h.

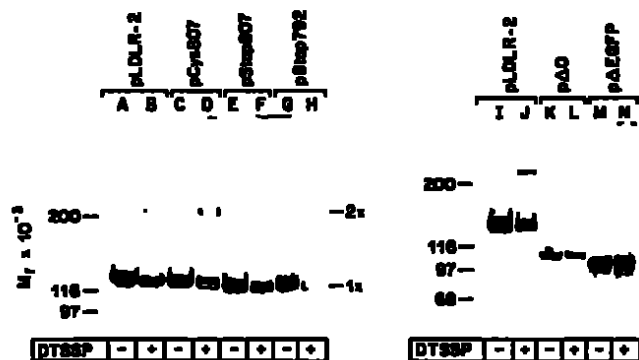


FIG. 10. Cross-linking of mutant LDL receptors in intact cultured fibroblasts. Monolayers of hamster cells transfected with the indicated plasmids were incubated for 30 min at 4 °C in the absence or presence of 2 mg/ml DTSSP as indicated. The samples were solubilized and subjected to electrophoresis in a 4% SDS-polyacrylamide gel in the absence of reducing agents. After electrophoresis, proteins were transferred to Immobilon membranes, incubated with mouse monoclonal anti-receptor IgG-HL1, and probed with alkaline phosphatase-coupled goat anti-mouse IgG. The symbols 1x and 2x indicate the proposed monomeric and dimeric forms of the receptor. M, standards are shown.

this resistance to cross-linking, the receptor that terminates at position 812 is internalized by cells at a normal rate (15).

As shown in the left panel of Fig. 10, LDL receptors that terminate at position 807 (Fig. 10F) or position 792 (Fig. 10H) showed no susceptibility to cross-linking. These receptors retain only 17 and 2 of the original 50 amino acids in the cytoplasmic domain, respectively. Both of these receptors have a markedly reduced ability to cluster in coated pits and internalize receptor-bound ¹²⁵I-LDL (16). On the other hand, the receptor with a cysteine substituted for tyrosine at residue 807 showed a normal susceptibility to cross-linking (Fig. 10D). This amino acid substitution markedly reduces the rate of clustering in coated pits (15, 16).

Extensive alterations in the extracellular domain of the

LDL receptor did not abolish cross-linking. Thus, the receptor lacking the clustered O-linked sugar region was cross-linked in a normal fashion by DTSSP (Fig. 10L). Similarly, the receptor that lacked the entire EGF precursor region was also susceptible to cross-linking (Fig. 10N), although the proportion of cross-linked receptors appeared to be somewhat less than was observed in the cells expressing the normal receptor (Fig. 10J). The receptors that lack the O-linked sugar region and the EGF precursor region enter cells at normal rates (10, 11). The latter receptor returns to the cell surface sluggishly, apparently because of a defective ability to dissociate from the lipoprotein ligand at acid pH (10).

DISCUSSION

The current results indicate that bovine and human LDL receptors have the capacity to undergo noncovalent self-association to form dimers and higher order structures. When the receptors were immunoprecipitated from bovine adrenal membranes, many of the dimers were covalently joined by disulfide bonds that formed after homogenization. When the free cysteine residues were blocked by reaction with N-ethylmaleimide or with iodoacetic acid at the time of homogenization, covalent dimer formation was markedly reduced.

When LDL receptors were solubilized from the adrenal membranes and then purified by DEAE-cellulose chromatography and affinity chromatography with a monoclonal antibody, the disulfide-linked species was not apparent (Fig. 5), perhaps because of reduction of disulfide bonds during the purification procedure. However, the purified receptor could be covalently linked into dimeric and trimeric forms by the addition of bivalent cross-linking agents (Fig. 5), indicating that it was still present as oligomers. We believe that this cross-linking represents the attachment of the receptor to itself and not to some other protein for three reasons: 1) the size of the cross-linked product corresponds to the size expected for a dimeric species; 2) the size of the cross-linked receptor in crude preparations of membranes (Figs. 1-4) or cells (Figs. 6, 7, and 10) was similar to the size of the dimer that was formed when purified receptor was chemically cross-linked (Fig. 5); and 3) reduction of the ¹²⁵I-labeled immunoprecipitated 220-kDa dimers resulted in the appearance on SDS gels of only one protein, which corresponded in size to the 160-kDa receptor monomer (Fig. 1). No other protein was liberated from this complex.

Covalent cross-linking by disulfide bond formation in the adrenal membranes appeared to be dependent on the presence of the cytoplasmic domain of the receptor. Thus, digestion of inside-out membrane vesicles with trypsin removed the cytoplasmic domain and caused the dimeric species to disappear. Simultaneously, there was a marked increase in the amount of monomer (Fig. 4H). The cytoplasmic domain of the LDL receptor contains one free cysteine located 29 amino acids from the transmembrane domain. It is possible that these cysteine residues on two adjacent receptors are brought into juxtaposition by self-association and that they form a disulfide bond when the cell is homogenized and the cytoplasmic domain of the receptor is no longer exposed to the strong sulfhydryl reducing environment of the cytoplasm.

To gain additional insight into the role of the cytoplasmic domain in the self-association process, we turned to the use of chemical cross-linkers in intact cells that were transfected with expressible cDNAs encoding normal and mutant forms of the LDL receptor. In hamster fibroblasts that were transfected with the normal human cDNA but not treated with chemical cross-linking agents, we only occasionally observed trace quantities of a disulfide-linked receptor dimer (data not

shown). Nevertheless, approximately 15–25% of the LDL receptors were susceptible to cross-linking with a variety of cross-linking agents (Fig. 6, B–D). One of these agents (DTSSP) was effective even though it is unable to penetrate cells (31). Thus, the LDL receptors of intact cells, like those of isolated membranes, were at least partly associated into noncovalently joined multimers.

The susceptibility to cross-linking in the cultured cells depended on the presence of the cytoplasmic domain. Thus, cross-linking was markedly reduced or undetectable with receptors that were encoded by cDNAs that produced premature termination at positions 792, 807, or 812. These receptors retained 2, 17, and 22 of the original 50 amino acids in the cytoplasmic domain, respectively. Termination at position 829 did not abolish cross-linking. Moreover, deletions of the extracellular domain, including deletion of the O-linked sugar region or the EGF precursor homology region, did not abolish cross-linking.

The most likely explanation for these findings is that the juxtamembranous portion of the cytoplasmic domain contains the site that undergoes self-recognition to bind the receptor into dimers or higher order multimers. It is likely that the transmembrane region plays a permissive role in the oligomerization, but we doubt that this region is the primary site for self-recognition because this sequence shows relatively little conservation among different animal species (6, 32). Through proteolytic cleavage experiments, Loeb and Drickamer (4) found that self-association of the chicken hepatic lectin was mediated by a sequence in or near the transmembrane region, but they were not able to determine which side of the membrane contained this information.

The number of LDL receptors present in each oligomeric complex cannot be resolved from the current studies. The solubilized purified receptor was present at least partially in a trimeric form (Fig. 5). However, in the intact cells we could only obtain evidence for dimers. Given the low efficiency of cross-linking, however, we would not expect to observe trimers in intact cells, even if they existed. A similar problem was encountered by Loeb and Drickamer (4), who were able to produce only dimers and trimers of the chicken hepatic lectin when cross-linking was performed in liver membranes, even though hexamers could be produced when the purified receptor was cross-linked.

We do not know the proportion of cell surface receptors in intact cells that are in the oligomeric form at any time. The maximal percentage of cross-linked receptors was only ~25%. However, it is likely that the percentage of oligomeric species is higher in view of the known inefficiency of chemical cross-linking agents when added to intact cells. Furthermore, in the bovine adrenal membrane preparations, nearly all of the receptors could be shown to participate in intermolecular disulfide bonds, indicating that the bulk of the receptors was self-associated.

It appears that the functional role of oligomerization is not to promote ligand binding. Thus, the receptor binds LDL or β -VLDL after SDS-polyacrylamide gel electrophoresis, during which it migrates in a monomeric form, followed by transfer to nitrocellulose and incubation with ligand (12). It is formally possible, though highly unlikely, that the receptor, after immobilization in nitrocellulose, would be able to form oligomers when the SDS is removed. In addition, LDL binds with normal affinity on the cell surface to the receptors produced by the cDNAs with stop codons at positions 792 and 807 (15), neither of which can be shown to form oligomers.

If oligomerization is not necessary for LDL binding, might it play some role in receptor clustering and internalization?

We cannot reach a firm conclusion on this point at present. Although the cytoplasmic domain is necessary for oligomer formation as well as clustering in coated pits, the data on mutated receptors reveal a poor correlation between these two events. Oligomerization may be necessary for internalization, but it cannot be sufficient. Thus, the mutant receptor containing a cysteine at position 807 is susceptible to cross-linking, indicating that a normal portion of these receptors exists as oligomers, yet these receptors are poorly internalized. On the other hand, the mutant receptor that terminates at position 812, which is internalized at a normal rate (15), shows a very low susceptibility to cross-linking. It is possible that these truncated receptors are able to undergo sufficient oligomerization to allow internalization but that the association constant of the oligomers is so low as to prevent efficient cross-linking with the agents used. Alternatively, it is possible that this receptor enters coated pits despite its existence as a monomer and that self-association is not necessary for internalization. Further studies will be necessary to clarify this point.

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First Cysteine-rich Repeat in Ligand-binding Domain of Low Density Lipoprotein Receptor Binds Ca^{2+} and Monoclonal Antibodies, but Not Lipoproteins*

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The ligand binding domain of the low density lipoprotein receptor consists of seven cysteine-rich repeats of ~40 amino acids each. These repeats, which are located at the NH_2 terminus of the protein, are homologous to sequences in complement components C8 and C9. To determine the role of the first repeat (amino acids 2-49), we prepared two plasmids containing expressible low density lipoprotein receptor cDNAs. The first plasmid, pAR1, lacks only the nucleotides encoding the first repeat. It produced a receptor that bound and internalized lipoproteins and recycled to the cell surface with the same efficiency as the normal receptor. This deleted receptor failed to bind two monoclonal antibodies, IgG-C7 and IgG-15C8, which were shown previously to react with the ligand-binding domain. The second plasmid, pR1, encodes a markedly truncated protein whose extracellular domain consists of the first repeat joined to the transmembrane and cytoplasmic domains. This protein bound the two monoclonal antibodies with the same affinity as the normal receptor, but failed to bind lipoproteins. Binding of IgG-15C8 to the normal receptor and the pR1-encoded protein was Ca^{2+} -dependent, indicating that the first repeat binds Ca^{2+} . We conclude that repeats 2-6 in the ligand-binding domain are sufficient for binding lipoproteins and that the first repeat is highly immunogenic, but is not required for lipoprotein binding.

The low density lipoprotein (LDL)¹ receptor is a recycling cell surface protein that mediates the uptake of cholesterol-carrying lipoproteins in coated pits (1). The receptor is a single-chain transmembrane glycoprotein with five modular domains. These domains are encoded by 18 exons, many of whose borders coincide with the structural and functional limits of the domains (1). The exons corresponding to each of these domains have been deleted, either by naturally occurring human mutations (2-4) or through *in vitro* mutagenesis of an expressible cDNA (5, 6).² These deletions abolish the function

normally performed by that domain, but leave the other functions of the receptor intact. This deletional analysis has allowed the function of each domain to be assigned.

The first domain of the LDL receptor consists of 292 amino acids at the NH_2 terminus, which is located outside the cell membrane. This domain is composed of seven copies of an imperfect repeat of a ~40-amino acid cysteine-rich sequence (1). This domain contains binding sites for two proteins, apoprotein (apo) B-100 and apoE, which are the active components of LDL and β -migrating very low density lipoprotein (β -VLDL), two lipoprotein ligands for the receptor (3).³ The cysteine-rich repeats are closely related to a 40-amino acid, cysteine-rich sequence found in several proteins of the blood complement system, namely C8 α , C8 β , and C9 (1, 7, 8). This structural motif occurs only once in each of the complement components and its function is unknown. The second domain in the LDL receptor consists of 400 amino acids that are homologous to a sequence in the precursor for epidermal growth factor, a peptide hormone. This sequence includes three copies of a 40-amino acid cysteine-rich repeat that differs from the repeat unit in the binding domain. This "growth factor" repeat has been found in more than a dozen extracellular proteins, including proteases of the blood coagulation system, components of complement, cell surface receptors, viral proteins, and differentiation-associated proteins in invertebrates (8, 9). When the growth factor homology domain is deleted from the LDL receptor, the receptor loses the ability to discharge its ligand at acid pH, and concomitantly the receptor shows a diminished ability to leave the endosome and recycle to the surface after internalization (6). The third domain, located on the external surface near the plasma membrane, is a region of 58 amino acids that is rich in serine and threonine residues, which serve as the sites of attachment for clustered O-linked sugars. Deletion of this domain does not affect the function of the receptor in cultured fibroblasts (5).

The fourth domain of the LDL receptor is a 23-amino acid hydrophobic sequence that spans the membrane and anchors the protein in the lipid bilayer (1, 2, 10). The fifth domain is a 50-amino acid sequence, located at the COOH terminus, that penetrates into the cytoplasm (1). This domain plays a role in allowing the receptor to self-associate into dimers and higher order multimers (11). It also plays a crucial role in clustering of the receptor in coated pits, an event that is required for efficient internalization (4, 12, 13).

Previous studies of the LDL receptor have been facilitated by the availability of monoclonal antibodies that were raised by immunizing mice with the receptor purified from bovine adrenal cortex. Two monoclonal antibodies, designated IgG-C7 (14) and IgG-15C8 (15), have been of particular impor-

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‡ The abbreviations used are: LDL, low density lipoprotein; β -VLDL, β -migrating very low density lipoprotein; apo, apoprotein; SDS, sodium dodecyl sulfate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

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tance. These antibodies are thought to bind to the ligand-binding domain of the LDL receptor since they partially block the binding of LDL. Moreover, these antibodies react with mutant receptors that lack either the O-linked sugar region (5) or the epidermal growth factor precursor homology region (6). The precise sequence within the ligand-binding domain that is recognized is not known.

In the current studies we have sought to functionally dissect the ligand-binding domain by deleting the exon that encodes the first 40-amino acid cysteine-rich repeat. When this repeat was deleted, the receptor lost the ability to bind the antireceptor monoclonal antibodies IgG-C7 and IgG-15C8, but it retained the ability to bind apoB-100 and apoE-containing lipoproteins. We have also prepared a severely deleted receptor in which the first cysteine-rich repeat is essentially the only remaining external sequence. This receptor binds the two monoclonal antibodies and undergoes slow internalization, but it does not bind lipoproteins. The antibody binding to this truncated receptor showed an absolute dependence on Ca^{2+} , indicating that the first repeat of the LDL receptor contains a Ca^{2+} -binding site.

EXPERIMENTAL PROCEDURES

Materials.—Human LDL (d 1.019–1.033 g/ml) (16) and rabbit β -VLDL (d < 1.006 g/ml) (17) were prepared by ultracentrifugation and radiolabeled by the iodine monochloride method. Lipoprotein-deficient serum (d > 1.215 g/ml) was prepared as described (18). Oligonucleotides were synthesized with an automated DNA synthesizer (model 380A, Applied Biosystems, Inc.). *hLA-7* cells, a Chinese hamster ovary cell mutant lacking a functional LDL receptor (18), were obtained from Monty Krieger (Department of Biology, Massachusetts Institute of Technology, Cambridge, MA). A synthetic peptide corresponding to the COOH-terminal 14 amino acids of the human LDL receptor was prepared as previously described (15).

Antibodies.—Mouse monoclonal antibodies IgG-C7 (14), IgG-15C8 (15), and IgG-HL1 (11) directed against the LDL receptor were prepared as described in the indicated reference. A control mouse monoclonal, IgG-3001, is directed against an irrelevant antigen (19). IgG-4A4 is a mouse monoclonal antibody directed against a synthetic peptide corresponding to the COOH-terminal 14 amino acids of the human LDL receptor. A hybridoma producing this antibody was prepared by fusion of popliteal lymph nodes from immunized mice to NS1/1 Ag 4.1 myeloma cells as described (11, 20). The heavy chain of IgG-4A4 is IgG₁, and the light chain is κ . The rabbit polyclonal antibody directed against the COOH-terminal 14 amino acids of the human LDL receptor has been previously described (15). All antibodies were purified from mouse ascites fluid or rabbit serum by affinity chromatography on *Staphylococcus* protein A-Sepharose (14). Monoclonal antibodies were radiolabeled with ^{125}I as previously described (14).

Plasmids and Transfected Cell Lines.—pLDLR-2, a plasmid containing a cDNA encoding the human LDL receptor under the control of the SV40 early promoter (21), and TR-715, a stable line of hamster *hLA-7* cells that were transfected with pLDLR-2, have been described previously (8). pR1 encodes a truncated LDL receptor cDNA in which the first repeat of the ligand-binding domain (amino acids 1–42) is attached via four foreign amino acids (Gly-Thr-Arg-Ser) to the COOH-terminal 89 amino acids consisting of the 17 amino acids immediately above the membrane, the 23 amino acids of the membrane-spanning domain and the 50 amino acids of the cytoplasmic domain (amino acids 751–838). This plasmid was constructed by standard procedures (22) from two plasmids (pAX3-6 and pBTC) derived from pLDLR-2. pAX3-6 was constructed by deleting the DNA-encoding amino acids 43–382 of the LDL receptor and introducing a *KpnI* recognition sequence immediately 3' to the deletion site. This was achieved using a single 48-mer oligonucleotide that bridged the deletion site. pBTC was constructed by deleting the DNA-encoding amino acids 5–746 of the LDL receptor and introducing a *BglII* recognition sequence that bridged the deletion site. To facilitate the introduction of the *BglII* recognition sequence, the codon for Ala-750 of the LDL receptor (GCT) was changed to TCT (Ser) by using a single 48-mer oligonucleotide. To construct pR1, the 234-base pair *XbaI-KpnI* fragment of pAX3-6, the 5623-base pair *XbaI-BglII* fragment of pBTC, and a 5'-phosphorylated oligonucleotide 5'-

GATCTGGTAC-3' were ligated together. The oligonucleotide was included to partially link the noncomplementary *BglII* and *KpnI* ends. The product of the ligation had two bases unpaired, which were filled using the Klenow fragment of *Escherichia coli* DNA polymerase I.

pAR1 encodes a truncated LDL receptor from which the first repeat of the ligand-binding domain (amino acids 2–42) was deleted. This plasmid was constructed by deleting DNA corresponding to exon 2 of the LDL receptor from pLDLR-2 (21, 23), using oligonucleotide-directed mutagenesis (24).

The constructs pR1 and pAR1 were used to transform *E. coli* TG-1, after which they were characterized by DNA sequencing and transfected into *hLA-7* cells as described (8). Stable cell lines were selected by indirect immunofluorescence with monoclonal antireceptor IgG-C7. The cell lines transfected with pR1 and pAR1 are designated TR-1011-25 and TR-884-1 cells, respectively. All cell lines were maintained in monolayer culture at 37 °C in an atmosphere of 5–7% CO_2 .

LDL Receptor Assays.—For assays of LDL receptor activity, 4×10^4 cells from trypsinized stock cultures were seeded into 60-mm Petri dishes in medium A (Ham's F-12 medium containing 2 mM glutamine, 100 units/ml penicillin, and 100 $\mu\text{g}/\text{ml}$ streptomycin sulfate) supplemented with 10% (v/v) fetal calf serum (day 0). On day 2, cells were refed with medium of identical composition. On the day prior to experiments (day 3), cells received medium A supplemented with 10% calf lipoprotein-deficient serum, 0.1 $\mu\text{g}/\text{ml}$ 25-hydroxycholesterol, and 10 $\mu\text{g}/\text{ml}$ cholesterol. The steroids were added to suppress any endogenous defective hamster LDL receptors (8). Experiments were performed on day 4. Cell surface binding of ^{125}I -LDL, ^{125}I - β -VLDL, and ^{125}I -labeled antireceptor monoclonal antibodies by monolayers at 4 °C was determined as described previously (14, 15). Metabolism of ^{125}I -ligands by monolayers at 37 °C was determined as previously described (16). Surface-bound and internalized ^{125}I -LDL were separately measured by the dextran-sulfate release technique (18). Surface-bound and internalized ^{125}I -IgG-15C8 were separately measured by a minor modification of the trypsin release technique (25).

Immunochemical Procedures.—Immunoblot analysis of proteins transferred to nitrocellulose membranes (11) and immunoprecipitation of LDL receptors from [^{35}S]cysteine-labeled cells (5) were performed as described.

Miscellaneous Methods.—SDS-polyacrylamide gel electrophoresis was performed by the method of Laemmli (26). Samples did not receive reducing agent unless otherwise stated. Apparent molecular masses were calculated by reference to the migration of the following marker proteins: myosin (900 kDa), β -galactosidase (116 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), egg albumin (45 kDa), carbonic anhydrase (29 kDa), soybean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). Marker proteins were always reduced with 3% (v/v) 2-mercaptoethanol prior to electrophoresis. Proteins were visualized by fluorography using Kodak X-Omat RP-1 or XAR-5 film and Du Pont Lightning Plus intensifying screens as stated in the figure legends.

RESULTS

Deletion of First Repeat Eliminates Binding of Monoclonal Antibodies but Not Lipoproteins.—Fig. 1 shows the binding of various ligands to hamster cells that were transfected with an expressible cDNA (pLDLR-2) encoding the normal human LDL receptor or a mutant cDNA (pAR1) encoding a receptor that lacks the first cysteine-rich repeat in the ligand-binding domain (amino acids 2–42). As recipients for the transfections we used mutant cells that do not express LDL receptors on their surface (18). At 4 °C the cells expressing the deleted receptor showed a normal ability to bind ^{125}I -LDL (Fig. 1A) and ^{125}I - β -VLDL (Fig. 1B), but no ability to bind ^{125}I -IgG-C7 with high affinity (Fig. 1C). IgG-C7 is one of the monoclonal antibodies that recognizes the ligand-binding domain. In other experiments, the receptor encoded by pAR1 again bound ^{125}I - β -VLDL normally at 4 °C (Fig. 2A), but did not bind ^{125}I -IgG-15C8 (Figs. 2B and 3A), the other monoclonal antibody that recognizes the ligand-binding domain.

The receptor encoded by pAR1 showed a normal affinity for ^{125}I -IgG-HL1 (Fig. 2B). This monoclonal antipeptide an-

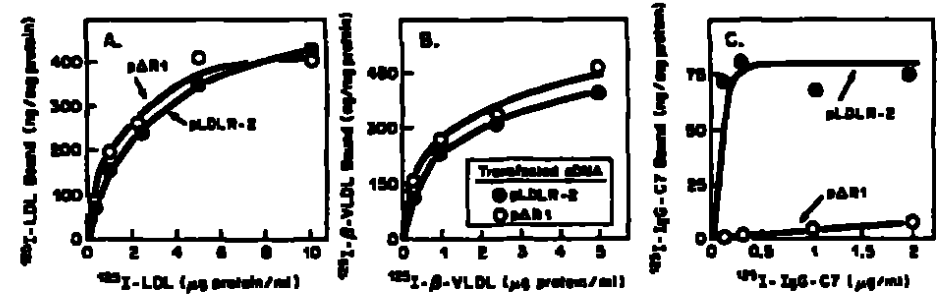


FIG. 1. Surface binding at 4 °C of ¹²⁵I-LDL (A), ¹²⁵I-β-VLDL (B), and ¹²⁵I-IgG-C7 (C) to monolayers of cells transfected with pAR1. On day 4 of cell growth, cells expressing the indicated cDNA received 1.5 ml of ice-cold medium B (Eagle's minimum essential medium without bicarbonate (GIBCO) containing 5% human lipoprotein-deficient serum and 20 mM HEPES at pH 7.4) and the indicated concentration of ¹²⁵I-LDL (401 cpm/ng protein), ¹²⁵I-β-VLDL (267 cpm/ng protein), or ¹²⁵I-IgG-C7 (801 cpm/ng). After 2 h at 4 °C, the total radioactivity bound to the cells was determined. Each value represents the average of duplicate incubations.

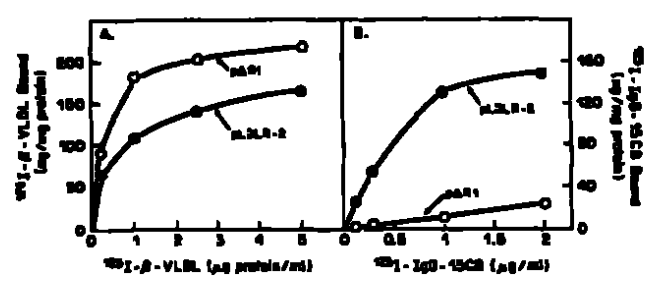


FIG. 2. Surface binding at 4 °C of ¹²⁵I-β-VLDL (A) and ¹²⁵I-IgG-15C8 (B) to monolayers of cells transfected with pAR1. On day 4 of cell growth, cells expressing the indicated cDNA received 1.5 ml of ice-cold medium B and the indicated concentration of ¹²⁵I-β-VLDL (261 cpm/ng protein) or ¹²⁵I-IgG-15C8 (820 cpm/ng). After 2 h at 4 °C, the total radioactivity bound to the cells was determined. Each value represents the average of duplicate incubations.

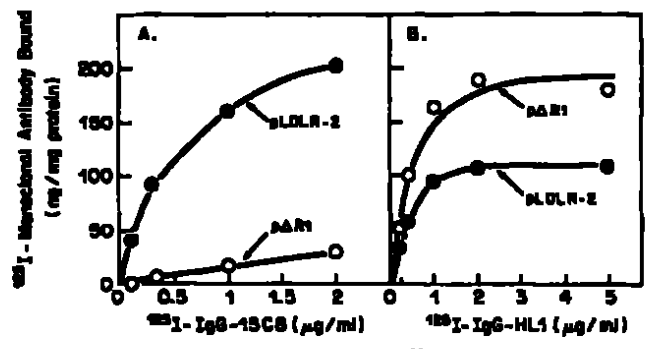


FIG. 3. Surface binding at 4 °C of ¹²⁵I-IgG-15C8 (A) and ¹²⁵I-IgG-HL1 (B) to monolayers of cells transfected with pAR1. On day 4 of cell growth, cells expressing the indicated cDNA received 1.5 ml of ice-cold medium B and the indicated concentration of ¹²⁵I-IgG-15C8 (810 cpm/ng) or ¹²⁵I-IgG-HL1 (434 cpm/ng). After 3 h at 4 °C, the total radioactivity bound to the cells was determined. Each value represents the average of duplicate determinations.

tibody was raised against a synthetic peptide corresponding to amino acids 163-174 in the ligand-binding domain (11). These amino acids constitute a linker between repeats 4 and 5 (23).

At 37 °C the receptor produced by pAR1 was able to mediate the binding (Fig. 4A), internalization (Fig. 4B), and lysosomal degradation (Fig. 4C) of ¹²⁵I-LDL with normal affinity. The deleted receptor was also able to mediate the binding, uptake, and degradation of ¹²⁵I-β-VLDL (Table I), but there was no binding, uptake, or degradation of ¹²⁵I-IgG-15C8 (Table I).

Fig. 5 shows a series of immunoblots of extracts from cells transfected with either the normal cDNA or pAR1. In the

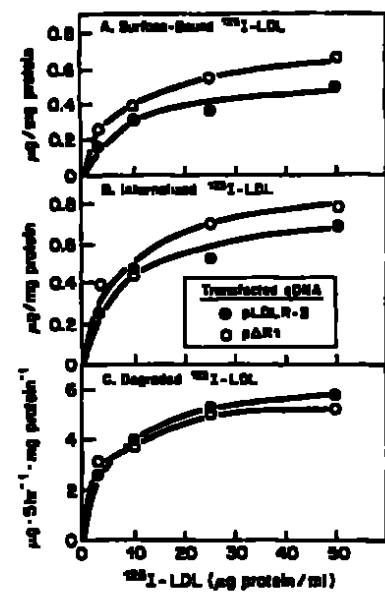


FIG. 4. Metabolism of ¹²⁵I-LDL at 37 °C by monolayers of cell transfected with pAR1 as a function of the concentration of ¹²⁵I-LDL. On day 4 of cell growth, cells expressing the indicated cDNA received 2 ml of warm minimum Eagle's medium (GIBCO) supplemented with 5% human lipoprotein-deficient serum and the indicated concentration of ¹²⁵I-LDL (78 cpm/ng protein) in the absence or presence of 1 mg of protein/ml of unlabeled LDL. After incubation for 5 h at 37 °C, the total amounts of surface-bound ¹²⁵I-LDL, internalized ¹²⁵I-LDL, and degraded ¹²⁵I-LDL were measured. The values shown represent high affinity values, which were determined by subtracting the values for ¹²⁵I-LDL in the presence of unlabeled LDL (nonspecific value) from that in its absence (total value). Each value represents the average of duplicate incubations.

presence of Ca²⁺, the normal receptor bound IgG-15C8 (lane A), but the deleted receptor did not (lane B). Binding of IgG-15C8 to the normal receptor was abolished by EDTA (lane G). The deleted receptor and the normal receptor bound IgG-HL1, the antibody directed against the linker peptide (lanes C and D), and binding was not abolished by EDTA (lanes I and J). Both receptors also bound IgG-4A4, a monoclonal antipeptide antibody directed against the COOH-terminal 14 amino acids of the cytoplasmic domain (lanes E and F).

The above data indicate that the first repeat of the ligand-binding domain is the site at which IgG-C7 and IgG-15C8 bind to the receptor, but that this repeat is not necessary for the binding of LDL or β-VLDL, nor is it necessary for internalization and recycling of LDL receptors.

TABLE 1
Metabolism at 37 °C of ¹²⁵I-β-VLDL and ¹²⁵I-IgG-15C8 in cells transfected with pDLR1

On day 4 of cell growth, cells expressing the indicated cDNA received 2 ml of warm Eagle's minimum medium (GIBCO) supplemented with 5% human lipoprotein-deficient serum and one of the indicated ¹²⁵I-ligands: 10 μg of protein/ml of ¹²⁵I-β-VLDL (267 cpm/ng protein) in the absence or presence of 1 mg of protein/ml of unlabeled β-VLDL or 10 μg/ml ¹²⁵I-IgG-15C8 (339 cpm/ng) in the absence or presence of 1 mg/ml unlabeled IgG-15C8. After incubation for 5 h at 37 °C, the total amounts of surface-bound, internalized, and degraded ¹²⁵I-ligand were determined. High affinity values were calculated by subtracting the values obtained in the presence of unlabeled ligand from the values obtained in the absence of unlabeled ligand. Each value represents the mean of duplicate incubations.

Transfected cDNA	¹²⁵ I-β-VLDL			¹²⁵ I-IgG-15C8	
	Surface-bound	Internalized	Degraded	Surface-bound + Internalized	Degraded
	ng/mg	ng/mg	ng · 5 h ⁻¹ · mg ⁻¹	ng/mg	ng · 5 h ⁻¹ · mg ⁻¹
pDLR-2	127	826	3438	104	553
pDLR1	192	824	3048	0	0

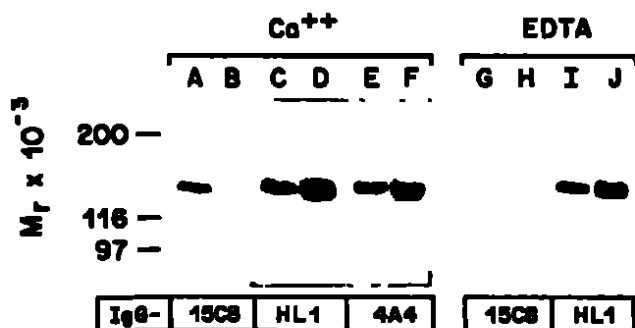


FIG. 5. Immunoblotting of solubilized LDL receptors from transfected cells. Detergent-solubilized extracts of cells transfected with pDLR-2 (A, C, E, G, I) or pDLR1 (B, D, F, H, J) were subjected to electrophoresis on a 6% SDS-polyacrylamide gel. After electrophoresis, proteins were transferred to nitrocellulose membranes and incubated with the indicated mouse monoclonal anti-receptor antibodies in the presence of either 2 mM CaCl₂ or 10 mM EDTA as indicated. The membranes were then probed with 3 × 10⁵ cpm/ml ¹²⁵I-labeled rabbit anti-mouse IgG (10⁴ cpm/ng). The nitrocellulose was exposed to RP-1 film for 2 h at room temperature with an intensifying screen. The migration of molecular weight markers is shown.

First Repeat Binds Antibodies but Not Lipoproteins—We next prepared a plasmid containing a cDNA from which the entire external domain of the LDL receptor was deleted except for the first repeat (amino acids 2–42) plus 17 amino acids immediately external to the membrane. This plasmid, designated pR1, was predicted to produce a small protein with an apparent molecular weight of ~16,000. When cells were pulse-labeled with [³⁵S]cysteine and then subjected to immunoprecipitation with a rabbit polyclonal anti-COOH-terminal antibody, the normal receptor encoded by pDLR-2 appeared as a 120,000-D precursor that was rapidly processed to the 160,000-Da mature form (left lane of Fig. 6). The receptor was stable for 4 h, but after a 16-h chase, most of the receptors were destroyed. The receptor encoded by pR1 was also precipitated by the rabbit anti-COOH-terminal antibody (right panel of Fig. 6). This receptor was synthesized as an M_r 16,000 precursor that did not undergo any increase in apparent molecular weight, indicating that this protein does not serve as an acceptor for oligosaccharide moieties. The receptor had approximately the same stability as the normal receptor. It was intact at 4 h, but was degraded 16 h after synthesis.

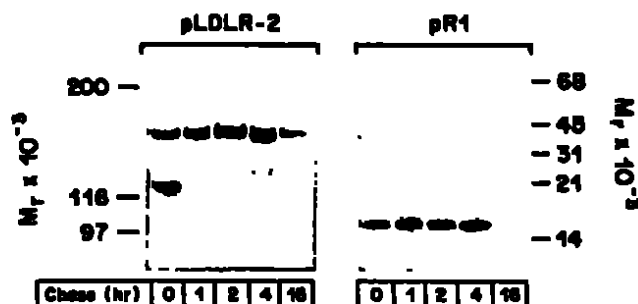


FIG. 6. Biosynthesis of ¹²⁵S-labeled LDL receptors in cells transfected with pDLR-2 and pR1. Cells transfected with the indicated plasmid were pulse-labeled for 1 h with 0.5 mCi of [³⁵S]cysteine in cysteine-free Dulbecco's modified Eagle's medium containing 0.3 mM L-proline and 10% lipoprotein-deficient serum, followed by a chase for the indicated time in medium A containing 10% lipoprotein-deficient serum. Receptors were immunoprecipitated from detergent lysates with a rabbit anti-COOH-terminal antibody, reduced with 5% (v/v) 2-mercaptoethanol, and subjected to electrophoresis on either a 6% (pDLR-2) or a 10% (pR1) SDS-polyacrylamide gel. The migration of molecular weight markers is shown. Gels were treated with Amplify (Amersham Corp.), dried, and exposed to XAR-5 film for 16 h at -70 °C.

At 4 °C cells expressing the receptor encoded by pR1 bound ¹²⁵I-IgG-C7 but not ¹²⁵I-LDL or ¹²⁵I-β-VLDL (Fig. 7). The amount of binding of ¹²⁵I-IgG-C7 to the cells expressing the mutant receptor was about half the amount that was bound to the cells expressing the normal receptor cDNA. This ratio was variable from experiment to experiment; in other experiments the amount of ¹²⁵I-IgG-C7 binding was the same in the two transfected cell lines. The apparent affinity for ¹²⁵I-IgG-C7 was not significantly affected by the deletion (Fig. 7A).

Binding of IgG-C7 and IgG-15C8 has previously been shown to be Ca²⁺-dependent on immunoblots after SDS-polyacrylamide gel electrophoresis (see Fig. 5) as well as in intact cells.² Cells expressing the receptor encoded by pR1 showed the same Ca²⁺ requirement for binding ¹²⁵I-IgG-C7 as did the normal receptor (Fig. 8).

At 37 °C the receptor encoded by pR1 bound ¹²⁵I-IgG-15C8 normally, but internalization and degradation of the bound antibody were sluggish. Fig. 9 shows an experiment in which cells expressing pDLR-2 and pR1 were incubated with ¹²⁵I-IgG-15C8 at 37 °C. After various times, the medium was removed for measurement of trichloroacetic acid-soluble degradation products, the cells were washed, and the amount of ¹²⁵I-IgG-15C8 releasable by trypsin was determined and used as a measure of surface-bound antibody. The material that was resistant to trypsin release is considered to be intracellular. In the steady state the pDLR-2 cells contained nearly 3 times more antibody inside the cell than was bound at the surface. The dynamic nature of this steady state was revealed by the progressive increase in trichloroacetic acid-soluble radioactivity which reflects ongoing binding, internalization, and degradation. After 5 h the internalization index, defined as the sum of internalized plus degraded antibody divided by the amount of surface-bound antibody (13), was 17. This indicates that each receptor had cycled into and out of the cell approximately 17 times during the 5-h period. In the cells expressing pR1, the amount of surface binding was higher than in the normal cells (Fig. 9B). The amount of internalized ¹²⁵I-IgG-15C8 rose rapidly but reached a plateau level that was no higher than the level of surface binding. The amount

² W. J. Schneider, J. L. Goldstein, and M. S. Brown, unpublished observations.

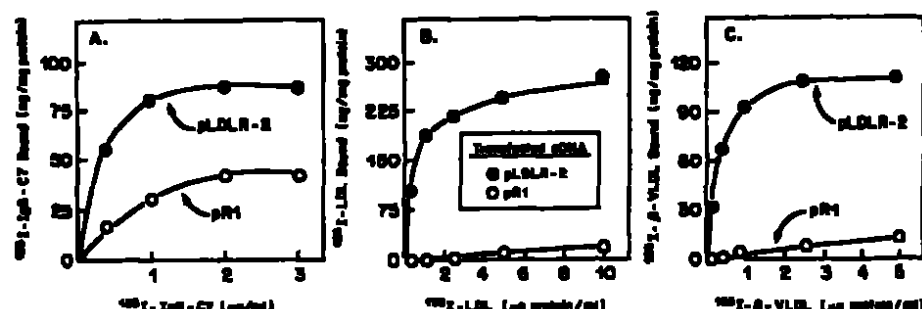


FIG. 7. Surface binding at 4 °C of ^{125}I -IgG-C7 (A), ^{125}I -LDL (B), and ^{125}I - β -VLDL (C) to monolayers of cells transfected with pR1. On day 4 of cell growth, cells expressing the indicated cDNA received 1.5 ml of ice-cold medium B and the indicated concentration of ^{125}I -IgG-C7 (738 cpm/ng), ^{125}I -LDL (328 cpm/ng protein), or ^{125}I - β -VLDL (305 cpm/ng protein). After 3 h at 4 °C, the total radioactivity bound to the cells was determined. Each value represents the average of duplicate incubations.

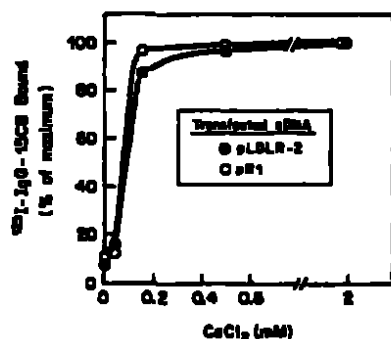


FIG. 8. Surface binding at 4 °C of ^{125}I -IgG-15C8 to monolayers of cells transfected with pR1 as a function of the concentration of Ca^{2+} . On day 4 of cell growth, cells expressing the indicated cDNA were washed with 3 ml of ice-cold phosphate-buffered saline (Ca^{2+} - and Mg^{2+} -free) and then incubated with 1.5 ml of ice-cold phosphate-buffered saline supplemented with 5% human lipoprotein-deficient serum, 180 μM sodium EDTA (pH 7.4), 2 $\mu\text{g}/\text{ml}$ ^{125}I -IgG-15C8 (618 cpm/ng), and the indicated concentration of CaCl_2 . After incubation for 3 h at 4 °C, the total radioactivity bound to the cells was determined. Each value is the average of duplicate incubations. The "100% of maximum" values were 161 and 86 ng of ^{125}I -IgG-15C8 bound/mg of protein for cells transfected with pLDLR-2 and pR1, respectively.

of degradation was also much lower than expected, based on the amount of surface binding. The internalization index was 2.9, which was only one-sixth the normal.

To confirm the defective internalization and degradation in the pR1 cells, we incubated cells expressing the normal and deleted cDNAs with ^{125}I -IgG-15C8 at two different concentrations for 5 h (Fig. 10). As a control, we performed the same experiment with TR-718 cells, a stable line of receptor-negative hamster cells that had been transfected only with salmon sperm DNA. The values for binding, internalization, and degradation of ^{125}I -IgG-15C8 in the TR-718 cells were subtracted from the values observed in the cells transfected with the LDL receptor cDNAs. These control values are given in the legend to Fig. 10. In this experiment cells transfected with pR1 bound the same amount of ^{125}I -IgG-15C8 at the cell surface as did the cells expressing the normal cDNA (Fig. 10A). However, the amount of internalized ^{125}I -IgG-15C8 in the pR1 cells was only half the amount in the pLDLR-2 cells (Fig. 10B). The amount of degradation was even more severely reduced (Fig. 10C). The calculated internalization indexes at both concentrations of ^{125}I -IgG-15C8 were similar to those in the experiment of Fig. 9 (i.e. 2.1 for the pLDLR-2 cells and 3.0 for the cells expressing pR1).

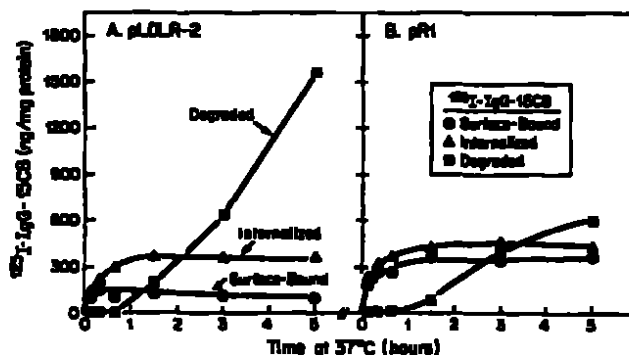


FIG. 9. Time course of metabolism of ^{125}I -IgG-15C8 at 37 °C in cells transfected with pR1. On day 4 of cell growth, cells transfected with the indicated cDNA received 3 ml of warm Eagle's minimum medium supplemented with 5% human lipoprotein-deficient serum and 30 $\mu\text{g}/\text{ml}$ either ^{125}I -IgG-15C8 (305 cpm/ng) or ^{125}I -IgG-3001 (306 cpm/ng). After incubation for the indicated time at 37 °C, the total amounts of surface-bound (●), internalized (▲), and degraded (■) ^{125}I -ligand were determined. The values shown represent specific values for ^{125}I -IgG-15C8, which were determined by subtracting the values for ^{125}I -IgG-3001 (monoclonal antibody against irrelevant antigen) from those for ^{125}I -IgG-15C8 (monoclonal antibody against LDL receptor). Each value represents the average of duplicate incubations.

DISCUSSION

The current experiments localize the binding sites for monoclonal antibodies IgG-C7 and IgG-15C8 to the first of the seven cysteine-rich repeats in the ligand-binding domain of the LDL receptor. When this repeat was deleted through oligonucleotide-directed mutagenesis, the receptor could no longer bind either of the two monoclonal antibodies. Somewhat surprisingly, this deleted receptor continued to bind LDL and β -VLDL, the two major ligands for the LDL receptor, with normal affinity. A severely truncated receptor in which the first cysteine-rich repeat was essentially the only external domain bound the two monoclonal antibodies, but it did not bind lipoproteins. These data establish that the first cysteine-rich repeat is both necessary and sufficient for binding of the two monoclonal antibodies.

The seven repeats in the LDL receptor show conservation in the spacing of the cysteine residues and in certain amino acid positions. The sequence Asp-Cys-X-Asp-Gly-Ser-Asp-Glu near the COOH terminus of each repeat is especially well conserved. None of the seven repeats has more than a single substitution in this sequence (1, 27). This negatively charged sequence has been postulated to be involved in the binding of apoB-100 and apoE (1), which are known to contain positively

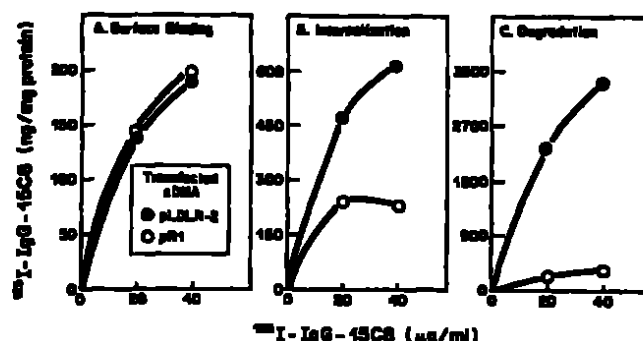


FIG. 10. Concentration curves for metabolism of ^{125}I -IgG-15C8 and 37°C in cells transfected with pR1. On day 4 of cell growth, cells transfected with the indicated cDNA received 2 ml of warm Eagle's minimum medium supplemented with 5% human lipoprotein-deficient serum and the indicated concentration of ^{125}I -IgG-15C8 (181 cpm/ng). After incubation for 5 h at 37°C , the total amounts of surface-bound (A), internalized (B), and degraded (C) ^{125}I -IgG-15C8 were determined as described under "Experimental Procedures." In parallel incubations carried out in the same experiment, a clone of hamster hLA-7 cells that had been stably transfected with salmon sperm DNA (designated TR-718 cells) were incubated with the same concentrations of ^{125}I -IgG-15C8. The values shown represent specific receptor-dependent values for metabolism of ^{125}I -IgG-15C8, which were determined by subtracting the values for the TR-718 cells from the values observed in the cells transfected with pLDLR-2 or pR1. These blank values in the TR-718 cells were as follows: A, 68 and 120 ng/mg at 20 and 40 $\mu\text{g/ml}$, respectively; B, 15 and 23 ng/mg at 20 and 40 $\mu\text{g/ml}$, respectively; and C, 65 and 94 ng/mg at 20 and 40 $\mu\text{g/ml}$, respectively. Each value represents the average of triplicate incubations.

charged residues that are crucial for binding (28, 29). Although the protein expressed by pR1 contains a single copy of this sequence, it did not bind lipoproteins, suggesting that multiple copies of this negatively charged region are necessary for ligand binding.

The two monoclonal antibodies, IgG-C7 and IgG-15C8, must both bind to a sequence in the first repeat that differs from the other six repeats. These two antibodies were produced independently; they were obtained from separate mouse immunization procedures. One of the antibodies (IgG-C7) was produced against the native, intact form of the LDL receptor, whereas the other antibody (IgG-15C8) was produced against a trypsin-treated receptor. IgG-C7 is of the IgG-2b subclass and IgG-15C8 is of the IgG-2a subclass. Both of these antibodies displace a maximum of 50–70% of LDL and β -VLDL from the binding site, and both of them compete completely with each other (14),⁴ suggesting that these antibodies bind to closely spaced or identical epitopes in the first repeat. Presumably, the first repeat is more antigenic than the other repeats, since we have not obtained monoclonal antibodies specific for the other cysteine-rich repeats.

Monoclonal antibodies IgG-C7 and IgG-15C8 bind to LDL receptors from humans and cows, but do not bind to LDL receptors from rodent species such as hamsters, mice, and rabbits (14). The amino acid sequence of the ligand-binding domain is available for four species (human, rabbit, cow, and hamster), and it shows considerable conservation in the COOH-terminal half of the first cysteine-rich repeat (27).⁴ However, there is a cluster of substitutions in the NH₂-terminal half of this repeat, suggesting that this may be the region that binds the monoclonal antibodies.

The binding sites for IgG-C7 and IgG-15C8 in the intact receptor are conformation-dependent, since binding is abol-

ished when the disulfide bonds are reduced (14, 30). Both antibodies require Ca^{2+} for binding. The binding of IgG-15C8 to the pR1-encoded protein was also Ca^{2+} -dependent. Since this receptor contains essentially no external domain other than the single cysteine-rich repeat, it is clear that this repeat must bind Ca^{2+} . Since lipoproteins bind to one or more of the six other repeats and since this binding also requires Ca^{2+} , then it is likely that all of the repeats bind Ca^{2+} . The final stages of assembly of the complement pore-forming complex is also dependent upon divalent cations (31). Therefore, it is possible that the 40-amino acid sequence in complement components C8 α , C8 β , and C9 that are homologous to the LDL receptor repeats also function as Ca^{2+} -binding sites.

When pR1-transfected cells were incubated with ^{125}I -IgG-15C8 at 37°C , the amount of surface binding and the apparent affinity were similar to those seen in cells transfected with the normal cDNA (Fig. 10). The number of receptors expressed in clones of cells permanently transfected with the normal cDNA or pR1 (as well as cells transfected with other mutant LDL receptor cDNAs) varies over at least a 3–4-fold range from experiment to experiment (13). For this reason, it is difficult to compare the absolute receptor number in cells transfected with different cDNAs. However, it is possible to measure the efficiency of internalization of a given receptor by calculating the internalization index, which is normalized for receptor number (13). This index was consistently reduced by about 85% in cells transfected with pR1 as opposed to pLDLR-2 (Figs. 9 and 10).

Two possible explanations might account for the observed sluggishness of internalization in the pR1-transfected cells. First, the receptor encoded by pR1 might not be incorporated normally into coated pits even though it has an intact cytoplasmic domain. Previous studies have shown that the juxta-membranous 22 amino acids of the cytoplasmic domain are required for normal incorporation into coated pits (13). Against this conclusion is the observation that the intracellular content of ^{125}I -IgG-15C8 rose promptly in the pR1-transfected cells, but reached an abnormally low plateau (Fig. 9). Degradation was more severely reduced than was internalization (Fig. 10). In previous studies with internalization-defective receptors with abnormal cytoplasmic tails, the intracellular content of ligand did not rise, and the rate of degradation was reduced proportionately to the lack of internalization (12, 13). These findings raise the alternate possibility that the pR1-encoded receptor may enter coated pits and endocytic vesicles at a relatively normal rate, but it might return to the surface more rapidly than normal without discharging its ligand in the cell.

The distinction between these two possibilities is difficult because the internalization studies in the pR1-transfected cells must be performed with monoclonal antibodies rather than with the natural lipoprotein ligands, which do not bind to this receptor. The affinity of IgG-C7 and IgG-15C8 for the normal LDL receptor is more than 75-fold lower at 37°C than it is at 4°C (see Ref. 14 and compare saturation curves at 4°C (Fig. 3A) and 37°C (Fig. 10A)). This is caused by a much more rapid dissociation rate at 37°C than at 4°C (14). Because of this low affinity, high concentrations of ^{125}I -IgG-C7 must be used at 37°C (Figs. 9 and 10) and this creates a significant blank value for nonspecific binding to the surface. We have attempted to correct for this blank by subtracting the binding at equivalent concentrations of ^{125}I -IgG-2001, an irrelevant antibody (Fig. 9), or by subtracting the binding of ^{125}I -IgG-15C8 to cells that do not express LDL receptors (Fig. 10). Although these corrections allow valid estimates of the amounts of cell surface binding to receptors in the steady

⁴ T. C. Sidhof, R. W. Bishop, D. W. Russell, M. S. Brown, and J. L. Goldstein, unpublished observations.

state, they make the performance of pulse-chase experiments difficult. We have therefore been unable to do warmup experiments to determine whether receptor-bound ^{125}I -IgG-16C8 transiently enters cells transfected with pR1. In the future we plan to use electron microscope histochemistry to clarify this issue.

The current data establish that the first repeat of the LDL receptor is not necessary for binding or uptake of lipoproteins in cultured fibroblasts, and this raises the question of the function of this repeat and the reason for its conservation during evolution. Clearly, the function of this repeat is not to bind monoclonal antibodies! It is likely that this repeat may play some role in LDL receptor function in some tissue other than fibroblasts. Alternatively, it may be an accident of evolution that is maintained because it has subtle beneficial effects on the protein structure.

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