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: 125I-LDL or an 125I-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 125I-LDL and for 125I-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 125I-LDL and 125I-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-depent dissociation of 125I-LDL and 125I-IgG-HL1 is used 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) 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 50 amino acids projecting 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-C5C8 (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 (pLDLR-2) (13). COS-M6 cells were obtained from Arnold Berk (Dept. of Microbiology, University of California, Los Angeles). [35S]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 Removawell Strips (Dynatech Laboratories, Catalogue No. 011-010-6301) that had been washed once with distilled water. An anti-LDL receptor monoclonal antibody was absorbed 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 (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
Plasmid Construction—The LDL receptor coding region in pDC295 (Fig. 6) was obtained from a previously prepared plasmid (pLDLR-4A6) 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 HindIII that bridged the deletion site. To introduce a stop codon at residue 295, pLDLR-4A6 was digested with HindIII and Tsp50I and ligated to pT7 Blue (Stratagene). The plasmid was then digested with HindIII and BgIII to remove the 1-kbp fragment that contained the HindIII site. The HindIII/XbaI fragment was then ligated to the HindIII/XbaI fragment containing 295-1 bp from pBR322. This plasmid was designated pLDLR-15.

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, standards were myosin (205,000), ovalbumin (45,000), IgG-C7, and phosphorylase b (97,000), bovine serum albumin (68,000), 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 800-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, 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 Tris containing 1 mM phenylmethylsulfonyl fluoride, 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 onto a column with a diameter of 1 cm. Proteins were eluted with successive 2-ml aliquots of 50 mM Tris maleate, pH 6.0, containing 2 mM calcium chloride, 0.5% Triton X-100, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM sodium chloride. The solid phase radioprecipitation assay was employed to determine which fractions contained LDL receptors. The concentration of purified receptor samples was estimated by measuring 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. Monoclonal 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 bound 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

![Figure 1](image1.png)

**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 LDL receptor as a function of the amount of LDL receptor added to immobilized antibodies. Each point represents the average of triplicate incubations.

![Figure 2](image2.png)

**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 microtiter wells 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. 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.
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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 (see "Experimental Procedures"). In the solid phase assay, the human receptor bound ligands rose linearly in proportion to the amount of LDL.

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 125I-LDL to 125I-IgG-HL1 was 1:1. This calculation was based on M, 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 125I-LDL and 125I-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 125I-LDL and 125I-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 125I-IgG-15C8 (open symbols).

![Fig. 3. Saturation curves for binding of 125I-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 purified 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, 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.)](image-url)

![Fig. 4. Acid-dependent release of 125I-LDL and 125I-IgG-15C8 from purified bovine LDL receptors. Purified bovine LDL receptors (20 ng/well) were bound to IgG-4A4-coated wells overnight at 4 °C. 125I-LDL at 16 nm protein (panel A), 212 cpm/fmol of protein; panel B, 143 cpm/fmol of protein) or 125I-IgG-15C8 at 34 nm (panel A, 92 cpm/fmol; panel B, 45 cpm/fmol) were 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 (O, O) or 50 mM Tris succinate (O, O) 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 125I-ligand bound in the presence of EDTA from that bound in its absence. The values for nonspecific binding in the absence of EDTA did not exceed 10% (A) or 17% (B) of total binding. The amount of released 125I-ligand is expressed as a percentage of total ligand recovered from buffer plus wells. The "100% of total" values for 125I-LDL and 125I-IgG-15C8 averaged 43 and 136 fmol/well, respectively (A), and 41 and 149 fmol/well, respectively (B).](image-url)
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Fig. 5. Time course of acid-dependent release of LDL from LDL receptor at pH 7.0 (A) and pH 5.5 (B). Purified bovine LDL receptor (~10 ng/well), was bound to IgG-4A4-coated wells overnight at 4°C. [125I]LDL at 9 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. Unbound LDL 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 295 amino acids of the receptor followed by a COOH-terminal serine and a TAG nonsense codon, the polyadenylation signal from the human growth hormone 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 [35S]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 125I-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, nonenlarged 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 125I-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 125I-LDL and 125I-IgG-HL1 in a 1:1 molar ratio (Fig. 3 and Table
tor, the linker between repeats 4 and 5, therefore strongly suggest that 1 mol of LDL binds to 1 mol of the first ligand binding repeat (8). Moreover, Gavigan et al. (22) reported that maximum binding of LDL to cultured bovine LDL receptors was performed with 125I-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 (55 cpm/fmol) was added to the wells in the absence (O, □) or presence (C, △) of 1.7 μM 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.

In addition to binding LDL, which has a single copy of the 512-kDa apoB-100, the LDL receptor also binds apoE-HDL, 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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