# Binding characteristics of a panel of monoclonal antibodies against the ligand binding domain of the human LDLr

**Anh T. Nguyen,* Tomoko Hirama,† Vinita Chauhan,* Roger MacKenzie,† and Ross Milne[1,*,§]**

Lipoprotein and Atherosclerosis Research Group,* University of Ottawa Heart Institute, Ottawa, Ontario, Canada; Institute for Biological Sciences,† National Research Council, Ottawa, Ontario, Canada; and Department of Pathology and Laboratory Medicine,§ University of Ottawa, Ottawa, Ontario, Canada

**Abstract To obtain a panel of monoclonal antibodies (MAbs) to study the folding and conformation of the low density lipoprotein receptor (LDLr), we have generated hybridomas from LDLr-deficient mice that had been immunized with the extracellular domain of the human LDLr. The 12 MAbs were specific for the ligand binding domain of the LDLr, with individual MAbs recognizing epitopes in ligand binding repeats 1, 2, 3, 5, and 7. A subset of the MAbs failed to react with the LDLr when disulfide bonds were reduced, and one MAb, specific for an epitope that spans ligand binding repeats 1 and 2, recognized two conformational forms of the LDLr with different affinities. Antibodies specific for ligand binding repeats 3, 5, and 7 completely blocked the binding of LDL particles to the LDLr on cultured human fibroblasts, whereas MAbs with epitopes in ligand binding repeats 1 and 2 partially blocked the binding of LDL to the LDLr. These anti-LDLr MAbs will serve as useful probes for further analysis of LDLr conformation and LDLr-mediated lipoprotein binding.—**Nguyen, A. T., T. Hirama, V. Chauhan, R. MacKenzie, and R. Milne. **Binding characteristics of a panel of monoclonal antibodies against the ligand binding domain of the human LDLr. *J. Lipid Res.* 2006.** 47: **1399–1405.**

**Supplementary key words** low density lipoprotein receptor • knockout mice • structure-function analysis

The low density lipoprotein receptor (LDLr) is located in clathrin-coated pits on the cell surface and can bind and mediate the endocytosis of plasma lipoproteins that contain either apolipoprotein E (apoE) or apoB-100. It has an important role in regulating cholesterol homeostasis, and mutations in the LDLr gene can lead to familial hypercholesterolemia. The 839 amino acid human LDLr is organized into five structural domains (1, 2). The amino terminal 292 residues constitute the ligand binding domain (LBD) that is composed of seven imperfect 40 residue repeats (R1–R7) with a short linker between R4 and R5. The LBD is followed by the 400 residue epidermal growth factor (EGF) precursor homology domain (EGFPHD) that contains three EGF-like repeats, EGF-A, EGF-B, and EGF-C, and a β-propeller subdomain inserted between EGF-B and EGF-C (3, 4). The EGFPHD is necessary for the pH-dependent dissociation of receptor and lipoprotein in the endosome (1). The third domain (*O*-linked sugar domain) is rich in threonine and serine residues that become *O*-glycosylated during the intracellular maturation of the receptor. A short transmembrane domain is followed by a 50 residue cytoplasmic tail that is required to localize the LDLr in clathrin-coated pits on the cell surface and for the endocytosis of ligands.

Each of the LBD repeats contains a site for the coordination of a calcium ion and six cysteine residues that form three intrarepeat disulfide bonds (5–8). Folding of newly synthesized LDLr occurs posttranslationally and is nonvectorial with the formation of transient, nonnative, long-range disulfide bonds that are subsequently isomerized into the native intrarepeat disulfide bonds that characterize the LDLr LBD (9). Binding of lipoproteins to the LDLr appears to be mediated by an interaction between acidic residues in the LDLr LBD and basic residues of apoE and apoB-100. By systematic deletion of individual ligand binding repeats of the LDLr, it has been shown that the repeats contribute differently to apoB-100- and apoE-mediated lipoprotein binding to the LDLr (10, 11). Deletion of individual repeats R3–R7 results in a loss of LDL binding (apoB-100-mediated), whereas β-VLDL binding (apoE-mediated) is impaired only when R5 is deleted. An LDLr fragment consisting of R4 and R5 is sufficient to bind to apoE-phospholipid vesicles (12). The crystal structure of an LDLr segment composed of the LBD and EGFPHD has

*Manuscript received 17 March 2006.*

*Published, JLR Papers in Press, April 6, 2006.*
*DOI 10.1194/jlr.M600130-JLR200*

Abbreviations: apoE, apolipoprotein E; CETP, cholesteryl ester transfer protein; EGF, epidermal growth factor; EGFPHD, epidermal growth factor precursor homology domain; LBD, ligand binding domain; LDLr, low density lipoprotein receptor; MAb, monoclonal antibody.

[1] To whom correspondence should be addressed.
e-mail: rmilne@ottawaheart.ca

been solved at pH 5.3 (4). In this structure, R4 and R5 are docked onto the β-propeller with an interface that includes histidine 192 ($His^{192}$) in R5 and $His^{562}$ and $His^{586}$ in the β-propeller. It has been proposed that, on the cell surface, the extracellular domain of the LDLr would adopt an elongated structure, as has been visualized by cryo-electron microscopy (13) with R4 and R5 accessible for binding to ligand. When the LDLr-ligand complex is internalized and exposed to the acidic environment of the endosome (~pH 5.3), the LDLr would undergo a conformational change so that the extracellular domain would fold back on itself and the β-propeller could displace bound lipoprotein (14, 15). In support of this model, it has been demonstrated that mutation of $His^{192}$, $His^{562}$, and $His^{586}$ leads to an LDLr variant that binds LDL with high affinity at neutral pH but fails to release the ligand at pH 5.3 (16).

To obtain a panel of monoclonal antibody (MAb) probes to study the folding of the newly synthesized LDLr and the conformation of the mature receptor, we have generated hybridomas from LDLr-deficient ($Ldlr^{-/-}$) mice that had been immunized with the extracellular domain of the human LDLr. Here, we describe the binding characteristics of these antibodies.

## EXPERIMENTAL PROCEDURES

### Expression and purification of a soluble fragment of the LDLr

The mammalian expression plasmid, pCMV5-$LDLr^{1–692}$, encoding residues 1–692 of the LDLr ($LDLr^{1–692}$) was a gift from Dr. David Russell (University of Texas Southwestern Medical Center). Chinese hamster ovary K1 cells were cotransfected with pCMV5-$LDLr^{1–692}$ (15 μg of DNA) and pSV2neo (1.5 μg) by calcium phosphate precipitation (17). Stably transfected cell clones were selected using 700 μM G418 and were subsequently maintained with 500 μM G418. For the purification of $LDLr^{1–692}$ from cell supernatants, a high-expressing, $LDLr^{1–692}$-transfected clone was adapted for growth in CHO S-SFM II medium (Invitrogen, Burlington, Ontario, Canada) that was supplemented with 1% fetal bovine serum and cultured in a Spinner Basket 1 Cell Culture Bioreactor (New Brunswick Scientific, Edison, NJ) according to the manufacturer's recommendation. Cells were maintained for up to 1 month, and 400 ml of medium was harvested and replaced every 2 days. The anti-human LDLr MAb, C7 (18), was immobilized on cyanogen bromide-activated Sepharose 4B beads (Amersham, Baie D'Urfé, Québec, Canada) according to the manufacturer's instructions. The beads were washed with TBS containing 20 mM $CaCl_2$ (TBS-$CaCl_2$). Culture medium (400 ml) was passed over the beads, the column was washed with TBS-$CaCl_2$, and bound $LDLr^{1–692}$ was eluted with 0.1 M glycine, pH 3. The fractions were dialyzed against TBS-$CaCl_2$ and stored in liquid nitrogen. Characterization of $LDLr^{1–692}$ will be described in detail elsewhere.

### Monoclonal antibodies

$Ldlr^{-/-}$ mice (19) (a gift from Dr. Stewart Whitman, University of Ottawa Heart Institute) were immunized by subcutaneous injection of $LDLr^{1–692}$ (50 μg) emulsified in complete Freund's adjuvant. The mice received two additional boosts with 50 μg of $LDLr^{1–692}$ in incomplete Freund's adjuvant (Sigma-Aldrich, Oakville, Ontario, Canada) at 3 week intervals. Serum antibody titers were monitored 1 week after each boost by a solid-phase RIA with $LDLr^{1–692}$ as the immobilized antigen (20). Four days before the fusion and at least 3 weeks after the previous boost, a final boost (50 μg of $LDLr^{1–692}$ in 100 μl of PBS) was administered by tail vein injection. The protocol for the fusion of splenocytes from immunized mice with SP2-0 plasmacytoma cells has been described (20). Seven to 10 days after the fusion, hybridoma supernatants were tested for antibodies by solid-phase ELISA (20) with $LDLr^{1–692}$ as the immobilized antigen. Cells in positive wells were recloned twice on 96-well plates at a seeding density of one cell per well. Ascites was produced in BALB/c female mice by intraperitoneal injection of $5 \times 10^6$ hybridoma cells. The immunoglobulin isotype of MAbs was determined using a Mouse Monoclonal Antibody Isotyping Kit (Amersham). IgG was isolated from culture supernatant or from ascites by Sepharose Protein G affinity chromatography according to the manufacturer's recommendations (Amersham). Fab fragments of the IgG were produced by papain digestion and purified by Sepharose Protein A (Amersham) as described previously (21).

### Expression of LDLr variants in COS7 cells

The plasmids pLDLr17, pLDLr17ΔR2, pLDLr17ΔR3, pLDLr17ΔR4, pLDLr17Δlinker, pLDLr17ΔR5, pLDLr17ΔAB, pLDLr2ΔR1, pLDLr2ΔA, pLDLr2ΔB, pLDLr2ΔC, pLDLr2ΔAB, and pLDLr2ΔEGF (10, 11) were a gift from Dr. David Russell (University of Texas Southwestern Medical Center) (10, 11). The plasmids pLDLr17ΔR6 and pLDLr17ΔR7 were generated from pLDLr17 with the ExSite Mutagenesis Kit (Stratagene) using the primer pairs 5′-gtgacactctgcgagggacccaacaagttc-3′, 5′-gcagttttcct-cgtcagatttgtccttgca-3′ and 5′-gggaccaacgaatgcttggacaacaacggc-3′, 5′-attaacgcagccaacttcatcgctcatgtc-3′, respectively. COS7 cells were transfected with 2 μg of the plasmid to be tested using Lipofectamine™ (Invitrogen, Burlington, Ontario, Canada). Cells were cultured in the presence of 10% fetal bovine serum to suppress the expression of the endogenous LDLr, and after 48 h, the cells were harvested and lysed for analysis.

### Western blotting

Cell extracts were prepared and analyzed by Western blotting as described for the C7 anti-human LDLr monoclonal antibody (22). In certain experiments, cell extracts were subjected to SDS-PAGE under reducing conditions.

### Surface plasmon resonance

The kinetics of binding of the anti-LDLr MAb IgG and Fab fragments to $LDLr^{1–692}$ were determined by surface plasmon resonance using a BIACORE 3000 biosensor system (BIACORE, Inc., Piscataway, NJ). Immunopurified $LDLr^{1–692}$ (15 μg/ml in 10 mM sodium acetate, pH 4.5) was coupled to a research-grade CM5 sensorchip (BIACORE) using the amine-coupling kit supplied by the manufacturer to give surface densities of ~700 resonance units. Ethanolamine-blocked surfaces were used as references. Fab fragments were subjected to Supedex 75 (Amersham) gel filtration before analysis to remove any aggregates. Binding of the Fabs to the immobilized $LDLr^{1–692}$ was carried out using a running buffer of 10 mM HEPES, pH 7.4, containing 150 mM NaCl, 1.5 mM $CaCl_2$, and 0.005% Surfactant P20 at a flow rate of 40 μl/min. For MAbs 5G2 and 3D8, 20 mM acetate buffer, pH 5.3, containing 150 mM NaCl, 1.5 mM $CaCl_2$, and 0.005% Surfactant P20 was also used. Surfaces were regenerated with 50 mM HCl for 3 s. Data were evaluated using BIAevaluation 4.1 software (BIACORE).

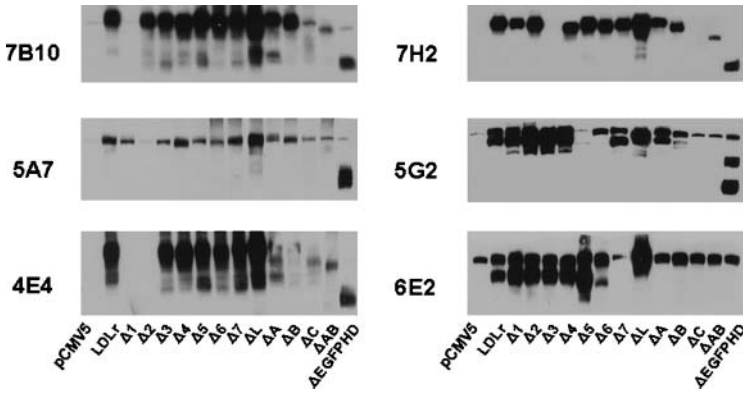


**Fig. 1.** Binding of anti-low density lipoprotein receptor (LDLr) monoclonal antibodies (MAbs) to the LDLr and to LDLr variants that lack individual ligand binding domain LBD repeats (Δ1–Δ7), the linker between R4 and R5 (ΔL), epidermal growth factor (EGF)-like repeats (ΔA, ΔB, ΔC, and ΔAB), or the entire epidermal growth factor precursor homology domain (ΔEGFPHD). COS7 cells were transfected with cDNA encoding the LDLr, LDLr variants, or the empty plasmid (pCMV5), and after 48 h, cell extracts were prepared and subjected to SDS-PAGE under nonreducing conditions. Migrated proteins were transferred to nitrocellulose membranes and tested for reactivity with the MAbs.

### Preparation and $^{125}$I labeling of LDL

Fresh plasma from normolipidemic subjects was supplemented with 1 mM EDTA, 0.02% $NaN_3$, 0.5 mM phenylmethylsufonyl fluoride, and 0.05 μg/ml leupeptin. LDL was isolated at 4°C by successive preparative ultracentrifugations between densities of 1.019 and 1.063 g/ml (23). LDL was dialyzed against PBS containing 1 mM EDTA and 0.02% $NaN_3$, sterilized by ultrafiltration, and stored at 4°C for up to 3 weeks. LDL was labeled with $^{125}$I as described by Bilheimer, Eisenberg, and Levy (24).

### Antibody-mediated inhibition of LDL to the LDL receptor

The protocol for measuring competition between the anti-LDLr MAbs and $^{125}$I-LDL for binding to the LDLr on the surface of cultured human fibroblasts was adapted from that previously described for determining the ability of anti-apoB MAbs to inhibit the binding of $^{125}$I-LDL to the LDLr (25). Anti-LDLr MAbs (50 μg IgG/ml) were used in place of the anti-apoB MAbs.

## RESULTS

From a single fusion, we obtained 12 stable hybridomas that secrete MAbs specific for the LDLr$^{1–692}$ fragment. To localize the epitopes recognized by the MAbs within the LDLr primary structure, we expressed, in COS7 cells, a series of LDLr variants that had deletions of individual LBD repeats, of the linker between R4 and R5, of individual or pairs of EGF-like repeats, or of the entire EGFPHD. Cell extracts containing the variant LDLrs were prepared and tested for reactivity with the MAbs by Western blotting. The reactivity of several of the MAbs is shown in **Fig. 1**, and a summary of the reactivities of all of the MAbs is presented in **Table 1**. All MAbs reacted with the wild-type receptor. MAbs 6B2, 7B10, and 8F11 did not react with an LDLr variant that lacked R1, and a variant lacking R2 was not recognized by MAbs 5A7 and 7A3. Deletion of either R1 or R2 prevented the binding of MAbs 3D8, 4C1, 4C6, and 4E4. The epitopes for MAbs 7H2 and 5G2 appear to be in R3 and R5, respectively, whereas the epitope recognized by 6E2 appears to be in R7. Deletion of the EGF-like repeats or the complete EGFPHD did not affect the binding of any of the antibodies. An epitope map based on these results is shown in **Fig. 2**. Reduction of disulfide bridges in the LDLr or removal of $Ca^{2+}$ from the medium has been reported to prevent the binding of both LDL and the well-characterized anti-LDLr MAb, C7, to the LDLr (22, 26). Therefore, we tested the new panel of MAbs for their reactivity with LDLr$^{1–692}$ that had been subjected to electrophoresis under reducing and nonreducing conditions (data not shown). Antibodies 6B2, 4E4, 7H2, 5G2, and 6E2 reacted only with the nonreduced LDLr$^{1–692}$ (indicated by asterisks in Fig. 2), whereas the other MAbs reacted under both reducing and nonreducing conditions. We confirmed that C7 did not recognize LDLr$^{1–692}$ after reduction. All MAbs recognized the LDLr in the presence of EDTA when analyzed by either Western blots or an ELISA assay (data not shown). Surprisingly, in our

TABLE 1. Mapping of the LDLr epitopes recognized by the panel of MAbs as determined by Western blotting

| | | Ligand Binding Repeats | | | | | | | | EGF Repeats | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Antibodies | LDLr | Δ1 | Δ2 | Δ3 | Δ4 | Δ Linker | Δ5 | Δ6 | Δ7 | ΔA | ΔB | ΔAB | ΔC | ΔEGF/β-Propeller |
| 6B2 | + | − | + | + | + | + | + | + | + | + | + | + | + | + |
| 7B10 | + | − | + | + | + | + | + | + | + | + | + | + | + | + |
| 8F11 | + | − | + | + | + | + | + | + | + | + | + | + | + | + |
| 5A7 | + | + | − | + | + | + | + | + | + | + | + | + | + | + |
| 7A3 | + | + | − | + | + | + | + | + | + | + | + | + | + | + |
| 3D8 | + | − | − | + | + | + | + | + | + | + | + | + | + | + |
| 4C1 | + | − | − | + | + | + | + | + | + | + | + | + | + | + |
| 4C6 | + | − | − | + | + | + | + | + | + | + | + | + | + | + |
| 4E4 | + | − | − | + | + | + | + | + | + | + | + | + | + | + |
| 7H2 | + | + | + | − | + | + | + | + | + | + | + | + | + | + |
| 5G2 | + | + | + | + | + | + | − | + | + | + | + | + | + | + |
| 6E2 | + | + | + | + | + | + | + | + | − | + | + | + | + | + |

EGF, epidermal growth factor; LDLr, low density lipoprotein receptor; MAb, monoclonal antibody.

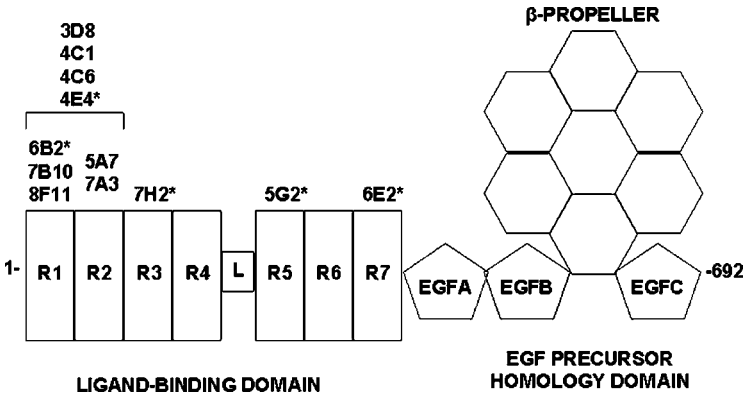


**Fig. 2.** An epitope map of LDLr$^{1-692}$. The epitopes recognized by the panel of anti-LDLr MAbs were assigned to regions within the primary structure of LDLr$^{1-692}$ based on the results shown in Table 1. Epitopes marked with asterisks are immunoreactive only under nonreducing conditions. See text for additional details.

hands, the binding of C7 to immobilized LDLr$^{1-692}$ also appeared to be $Ca^{2+}$-independent.

Six antibodies were selected to represent each of the specificity groups for further characterization (these antibodies can be obtained by contacting the corresponding author). The kinetics of binding of purified Fab fragments to immobilized LDLr$^{1-692}$ were determined with a BIACORE 3000 biosensor. The sensorgrams for MAbs 7H2, 5A7, 7B10, and 5G2 fitted well to a 1:1 binding model, allowing accurate kinetic constants for these antibodies to be calculated (**Table 2**). The data for MAb 3D8 best fitted a heterogeneous ligand model in which the antibody would recognize two conformations of the immobilized LDL receptor with different affinities (**Fig. 3**). Although the Fab monomers were isolated by gel filtration before analysis, the sensorgrams for MAb 6E2 nevertheless best fitted a bivalent analyte model. Fab fragments may have dimerized after purification or on the LDLr surface. In preliminary experiments with an IgG preparation, we had noted that MAb 3D8 bound to LDLr$^{1-692}$ with higher affinity at pH 5.3 than at pH 7.4 (data not shown). This was also the case for the 3D8 Fab fragment. As at pH 7.4, the binding data obtained at pH 5.3 best fitted a heterogeneous ligand model, with the increase in the affinity attributable to an increase in association rate and a decrease in dissociation rate (Table 2). In view of the recent structural model of the LDLr (12), we also tested the binding at pH 5.3 of the 5G2 Fab fragment that is specific for R5. In contrast to 3D8, 5G2 showed slower association and more rapid dissociation at pH 5.3 than at pH 7.4 (Table 2). Nevertheless, based on the surface capacity, the 5G2 epitope appeared to be equally accessible at pH 7.4 and pH 5.3 (data not shown).

The antibodies were also tested for their abilities to block the binding of LDL to the LDLr on the surface of cultured human fibroblasts (**Fig. 4**). MAbs specific for epitopes in R3 (7H2), R5 (5G2), and R7 (6E2) prevented the binding of LDL to the LDLr, whereas MAbs specific for epitopes in R1 (7B10) and R2 (5A7) blocked ~35% and 70% of binding, respectively. Surprisingly, MAb 3D8 reproducibly increased the binding of LDL to the LDLr. As had been reported previously (18), MAb C7, which is specific for an epitope in R1 (26), blocked ~50% of binding, whereas a control antibody, specific for human apoA-I (5F6) (27), did not influence LDL binding to the LDLr.

## DISCUSSION

We obtained a panel of MAbs from *Ldlr*$^{-/-}$ mice that had been immunized with a fragment of the human LDLr composed of the LBD and EGFPHD. We chose *Ldlr*$^{-/-}$ mice for immunization because the immune response should not be limited by immunological self-tolerance to the endogenous murine LDLr, as would be the case with wild-type mice. Therefore, we anticipated that a broad immune response would be elicited in *Ldlr*$^{-/-}$ mice after immunization with LDLr$^{1-692}$ that would include antibodies to highly conserved epitopes, such as functional sites on the molecule. For the LBD, this appeared to be the

TABLE 2. Rate and affinity constants for the interaction of the various MAbs with immobilized LDLr as determined by fitting sensorgram data to appropriate interaction models

| Fab | pH | $k_a1$ | $k_d1$ | $K_D1$ | $k_a2$ | $k_d2$ | $K_D2$ |
|---|---|---|---|---|---|---|---|
| | | *1/ms* | *1/s* | *M* | *1/ms* | *1/s* | *M* |
| 7B10 | 7.4 | $9 \times 10^4$ | $2 \times 10^{-3}$ | $2 \times 10^{-8}$ | | | |
| 5A7 | 7.4 | $3 \times 10$ | $3 \times 10^{-3}$ | $1 \times 10^{-8}$ | | | |
| 7H2 | 7.4 | $2 \times 10^6$ | $7 \times 10^{-3}$ | $4 \times 10^{-9}$ | | | |
| 6E2 | 7.4 | $6 \times 10^5$[a] | $4 \times 10^{-2}$ | | | $3 \times 10^{-4}$ | |
| 5G2 | 7.4 | $5 \times 10^5$ | $4 \times 10^{-3}$ | $1 \times 10^{-8}$ | | | |
| 5G2 | 5.3 | $2 \times 10^5$ | $1 \times 10^{-2}$ | $6 \times 10^{-8}$ | | | |
| 3D8 | 7.4 | $1 \times 10^4$ | $2 \times 10^{-3}$ | $<5 \times 10^{-7}$ | $7 \times 10^3$ | $<2 \times 10^{-4}$ | $3 \times 10^{-8}$ |
| 3D8 | 5.3 | $2 \times 10^4$ | $9 \times 10^{-4}$ | $5 \times 10^{-8}$ | $9 \times 10^4$ | $<1 \times 10^{-5}$ | $<1 \times 10^{-10}$ |

[a] $k_a1$ shows the association rate for total binding of 6E2 calculated from the observed rate constant plotted against Fab concentration. Individual $k_a1$ and $k_a2$ could not be determined.

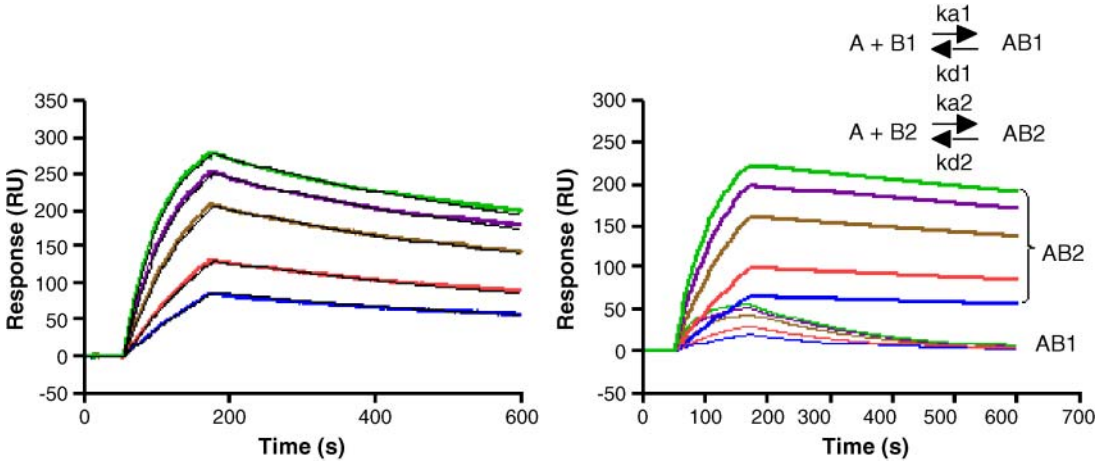


**Fig. 3.** Binding of MAb 3D8 to immobilized LDLr$^{1-692}$ as monitored by surface plasmon resonance. Sensorgrams for the binding of 3D8 to immobilized LDLr$^{1-692}$ when tested at concentrations of 300 nM (blue), 500 nM (red), 1,000 nM (brown), 1,500 nM (purple), and 2,000 nM (green) are shown at left. The data were fitted to a heterogeneous ligand model in which two conformations of LDLr$^{1-692}$ would exist, B1 and B2, that are recognized with different affinities by 3D8. The black lines represent the theoretical curves for each 3D8 concentration calculated according to the heterogeneous ligand model and given the rate and affinity constants presented in Table 2. In the right panel, the data were deconvoluted to illustrate the theoretical contributions to the sensorgrams of the binding of 3D8 to the B1 and B2 conformations of LDLr$^{1-692}$. RU, resonance unit.

case. Hybridomas were obtained that secrete MAbs specific for epitopes in five of the seven ligand binding repeats as well as an epitope(s) that appears to span the R1-R2 junction. In contrast, we failed to identify any antibodies that recognize epitopes in the EGFPHD. Algorithms to predict antigenic sites in proteins based on the primary structure (28, 29) indicate that the LDLr EGFPHD should be at least as immunogenic as the LBD. It is possible that immunological tolerance to EGFPHDs of other members of the LDLr gene family constrains the immune response to the human LDLr EGFPHD in *Ldlr*$^{-/-}$ mice and, as a consequence, the immune response is primarily directed against the LBD. We previously reported a panel of 20 murine MAbs to human cholesteryl ester transfer protein (CETP) that are specific for at least nine distinct epitopes, all of which are located in the C-terminal half of CETP (30, 31). As in the present case, it is not apparent why a large part of the molecule should not elicit a humoral immune response, as mice do not have a functional CETP gene (32).

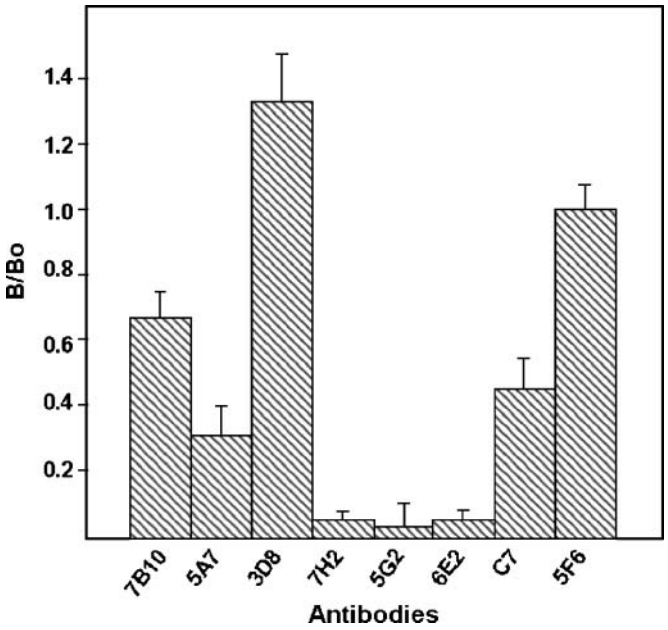


**Fig. 4.** Ability of anti-LDLr MAbs to block the binding of LDL to the LDLr on cultured human fibroblasts. Binding of $^{125}$I-LDL (3 μg/ml) at 4°C to cultured human fibroblasts was determined in the absence of antibody or in the presence of 50 μg/ml IgG of anti-LDLr MAbs 7B10, 5A7, 3D8, 7H2, 5G2, 6E2, and C7 or the anti-human apolipoprotein A-I 5F6. Results are presented as the ratio of binding in the presence of IgG (B) to binding in the absence of IgG (Bo). Each antibody was tested in triplicate in three separate experiments. Error bars represent the SD.

Our goal was to obtain anti-LDLr MAbs that could be used to study LDLr conformation and folding. Several of the MAbs do have specific conformational constraints for their binding to the LDLr. As certain MAbs only recognize the nonreduced LDLr, their epitopes likely require native disulfide bonds. Because these MAbs are specific for epitopes in R1, R3, R5, and R7, they will be useful probes to follow the folding of the LBD of newly synthesized LDLr. The binding of MAb 3D8 to immobilized LDLr$^{1-692}$ monitored by surface plasmon resonance best fits a heterogeneous ligand model indicating that 3D8 possibly recognizes two conformational forms of LDLr$^{1-692}$ with different affinities (Table 2). Moreover, 3D8 shows more rapid association and slower dissociation at pH 5.3 than at pH 7.4. As 3D8 does not recognize LDLr variants that lack either R1 or R2 (Table 1), MAb 3D8 appears to recognize an epitope that spans R1 and R2. The NMR structure of a concatemer of R1 and R2 shows that there are few intermodule interactions and that the four residue linker likely allows considerable flexibility between modules (8). The

antibody may preferentially recognize a conformational state of the LDLr that is in low abundance at neutral pH but is much more frequent at pH 5.3. Alternatively, at the lower pH, the increased protonation of residues in both $LDLr^{1–692}$ and in 3D8 may favor antibody-antigen association. The kinetic data cannot differentiate between these two possibilities. Based on the structural model of the extracellular domain of the LDLr at pH 5.3 (3), one might anticipate that the 5G2 epitope in R5 would be poorly accessible at pH 5.3. This was not the case. Thus, the 5G2 epitope may remain accessible when R5 is docked with the β-propeller at pH 5.3, or, when immobilized on the biosensor chip, $LDLr^{1–692}$ may maintain its extended conformation.

Antibodies specific for R3, R5, and R7 efficiently block the binding of LDL to the LDLr on cultured human fibroblasts, whereas partial blocking is seen with MAbs to R1 or R2. This roughly parallels the results reported for LDL binding to LDLr variants in which individual LBD repeats were deleted; LDLr variants lacking R3, R4, R5, R6, and R7 could not bind LDL, whereas deletion of R2 reduced LDL binding and deletion of R1 had little effect (10, 11). To our knowledge, this is the first report of MAbs that are capable of totally blocking LDL binding to the LDLr. The lack of inhibition of LDL binding to the LDLr by 3D8 may reflect its relatively low affinity at pH 7.4, although it is unclear why binding would be increased in the presence of the antibody.

In summary, we describe the production and characterization of a panel of MAbs to the LBD of the LDLr that should be useful probes to study the folding and conformation of the LDLr. Moreover, as certain of the antibodies can inhibit the binding of LDL to the LDLr, they should be valuable reagents for determining the contribution of the LDLr to lipoprotein binding. Finally, this study is another example (33, 34) of the utility of genetically modified mice for the production of monoclonal antibodies.

This work was supported by Operating Grant MGP-44361 from the Canadian Institutes of Health Research. The authors thank Dr. David Russell for his generous gift of plasmids and Dr. André Gauthier for intravenous injections of mice.

## REFERENCES


1. Brown, M. S., and J. L. Goldstein. 1986. A receptor-mediated pathway for cholesterol homeostasis. *Science.* **232:** 34–47.
2. Jeon, H., and S. C. Blacklow. 2005. Structure and physiologic function of the low-density lipoprotein receptor. *Annu. Rev. Biochem.* **74:** 535–562.
3. Jeon, H., W. Y. Meng, J. Takagi, M. J. Eck, T. A. Springer, and S. C. Blacklow. 2001. Implications for familial hypercholesterolemia from the structure of the LDL receptor YWTD-EGF domain pair. *Nat. Struct. Biol.* **8:** 499–504.
4. Rudenko, G., L. Henry, K. Henderson, K. Ichtchenko, M. S. Brown, J. L. Goldstein, and J. Deisenhofer. 2002. Structure of the LDL receptor extracellular domain at endosomal pH. *Science.* **298:** 2353–2358.
5. Blacklow, S. C., and P. S. Kim. 1996. Protein folding and calcium binding defects arising from familial hypercholesterolemia mutations of the LDL receptor. *Nat. Struct. Biol.* **3:** 758–762.
6. Bieri, S., J. T. Djordjevic, N. L. Daly, R. Smith, and P. A. Kroon. 1995. Disulfide bridges of a cysteine-rich repeat of the LDL receptor ligand-binding domain. *Biochemistry.* **34:** 13059–13065.
7. Fass, D., S. C. Blacklow, P. S. Kim, and J. M. Berger. 1997. Molecular basis of familial hypercholesterolaemia from structure of LDL receptor module. *Nature.* **388:** 691–693.
8. Kurniawan, N. D., A. R. Atkins, S. Bieri, C. J. Brown, I. M. Brereton, P. A. Kroon, and R. Smith. 2000. NMR structure of a concatemer of the first and second ligand-binding modules of the human low-density lipoprotein receptor. *Protein Sci.* **9:** 1282–1293.
9. Jansens, A., E. van Duijn, and I. Braakman. 2002. Coordinated nonvectorial folding in a newly synthesized multidomain protein. *Science.* **298:** 2401–2403.
10. Esser, V., L. E. Limbird, M. S. Brown, J. L. Goldstein, and D. W. Russell. 1988. Mutational analysis of the ligand binding domain of the low density lipoprotein receptor. *J. Biol. Chem.* **263:** 13282–13290.
11. Russell, D. W., M. S. Brown, and J. L. Goldstein. 1989. Different combinations of cysteine-rich repeats mediate binding of low density lipoprotein receptor to two different proteins. *J. Biol. Chem.* **264:** 21682–21688.
12. Fisher, C., D. Abdul-Aziz, and S. C. Blacklow. 2004. A two-module region of the low-density lipoprotein receptor sufficient for formation of complexes with apolipoprotein E ligands. *Biochemistry.* **43:** 1037–1044.
13. Jeon, H., and G. G. Shipley. 2000. Vesicle-reconstituted low density lipoprotein receptor. Visualization by cryoelectron microscopy. *J. Biol. Chem.* **275:** 30458–30464.
14. Innerarity, T. L. 2002. LDL receptor's β-propeller displaces LDL. *Science.* **298:** 2337–2339.
15. Rudenko, G., and J. Deisenhofer. 2003. The low density lipoprotein receptor: ligands, debates and lore. *Curr. Opin. Struct. Biol.* **13:** 683–689.
16. Beglova, N., H. Jeon, C. Fisher, and S. C. Blacklow. 2004. Cooperation between fixed and low pH-inducible interfaces controls lipoprotein release by the LDL receptor. *Mol. Cell.* **16:** 281–292.
17. Chen, C., and H. Okayama. 1987. High efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* **7:** 2745–2752.
18. Beisiegel, U., W. J. Schneider, J. L. Goldstein, R. G. W. Anderson, and M. S. Brown. 1981. Monoclonal antibodies to the low density lipoprotein receptor as probes for study of receptor-mediated endocytosis and the genetics of familial hypercholesterolemia. *J. Biol. Chem.* **256:** 11923–11931.
19. Ishibashi, S., M. S. Brown, J. L. Goldstein, R. D. Gerard, R. E. Hammer, and J. Herz. 1993. Hypercholesterolemia in low density lipoprotein receptor knockout mice and its reversal by adenovirus-mediated gene delivery. *J. Clin. Invest.* **92:** 883–893.
20. Milne, R. W., P. K. Weech, and Y. L. Marcel. 1992. Immunological methods for studying and quantifying lipoproteins and apolipoproteins. *In* Lipoprotein Analysis: A Practical Approach. C. Converse and E. Skinner, editors. Oxford University Press, Oxford. 61–84.
21. Milne, R. W., R. Theolis, R. B. Verdery, and Y. L. Marcel. 1983. Characterization of monoclonal antibodies against human low density lipoprotein. *Arteriosclerosis.* **3:** 23–30.
22. Beisiegel, U., W. J. Schneider, M. S. Brown, and J. L. Goldstein. 1982. Immunoblot analysis of low density lipoprotein receptors in fibroblasts from subjects with familial hypercholesterolemia. *J. Biol. Chem.* **257:** 13150–13156.
23. Havel, R. J., H. A. Eder, and J. H. Bragdon. 1955. The distribution and chemical composition of ultracentrifugally separated lipoproteins in human serum. *J. Clin. Invest.* **34:** 1345–1353.
24. Bilheimer, D. W., S. Eisenberg, and R. I. Levy. 1972. The metabolism of very low density lipoprotein proteins. *Biochim. Biophys. Acta.* **260:** 212–221.
25. Milne, R., R. Theolis, R. Maurice, R. J. Pease, P. K. Weech, E. Rassart, J. C. Fruchart, J. Scott, and Y. L. Marcel. 1989. The use of monoclonal antibodies to localize the low density lipoprotein receptor-binding domain of apolipoprotein B. *J. Biol. Chem.* **264:** 19754–19760.
26. van Driel, I. R., J. L. Goldstein, T. C. Südhof, and M. S. Brown. 1987. First cysteine-rich repeat in ligand-binding domain of low density lipoprotein receptor binds $Ca^{2+}$ and monoclonal antibodies, but not lipoproteins. *J. Biol. Chem.* **262:** 17443–17449.
27. Marcel, Y. L., P. R. Provost, H. Koa, E. Raffai, N. V. Dac, J. C. Fruchart, and E. Rassart. 1991. The epitopes of apolipoprotein A-I define distinct structural domains including a mobile middle region. *J. Biol. Chem.* **266:** 3644–3653.
28. Hopp, T. P., and K. R. Woods. 1983. A computer program for predicting antigenic determinants. *Mol. Immunol.* **20:** 483–489.

29. Kyte, J., and R. F. Doolittle. 1982. A simple method for displaying the hydropathic character of a protein. *J. Mol. Biol.* **157:** 105–132.
30. Roy, P., R. Mac, T. Kenzie, X. C. Hirama, P. Jiang, P. Kussie, A. Tall, E. Rassart, and R. Milne. 1996. Structure-function relationships of human cholesteryl ester transfer protein: analysis using monoclonal antibodies. *J. Lipid Res.* **37:** 22–34.
31. Guyard-Dangremont, V., V. Tenekjian, V. Chauhan, S. Walter, P. Roy, E. Rassart, and R. Milne. 1999. Immunochemical evidence that cholesteryl ester transfer protein and bactericidal/permeability-increasing protein share a similar tertiary structure. *Protein Sci.* **8:** 2392–2398.
32. Hogarth, C. A., A. Roy, and D. I. Ebert. 2003. Genomic evidence for the absence of a functional cholesteryl ester transfer protein gene in mice and rats. *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **135:** 219–229.
33. Zlot, C. H., L. M. Flynn, M. M. Véniant, E. Kim, M. Raabe, S. P. McCormick, P. Ambroziak, L. M. McEvoy, and S. G. Young. 1999. Generation of monoclonal antibodies specific for mouse apolipoprotein B-100 in apolipoprotein B-48-only mice. *J. Lipid Res.* **40:** 76–84.
34. Wang, X. Y., V. Chauhan, A. T. Nguyen, J. Schultz, J. Davignon, S. G. Young, J. Borén, T. L. Innerarity, R. T. Hui, and R. W. Milne. 2003. Immunochemical evidence that human apoB differs when expressed in rodent versus human cells. *J. Lipid Res.* **44:** 547–553.