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ORIGINAL ARTICLE

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The important role for βVLDLs binding at the fourth cysteine of first ligand-binding domain in the low-density lipoprotein receptor

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Abstract The low-density lipoprotein (LDL) receptor (LDLR) is a crucial role for binding and uptaking apolipoprotein (apo) B-containing lipoproteins, such as very-low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and LDL. The defect function of the LDLR causes familial hypercholesterolemia (FH), the phenotype of which is elevated plasma cholesterol and premature coronary heart disease (CHD). In the present study, we characterize the role of the cysteine residue of the ligand-binding domain of the LDLR. The mutant LDLR protein of cysteine for serine at codon 25 (25S-LDLR) was expressed in Chinese hamster ovary (CHO) cell line, *ldl*-A7. By Western blot analysis, the 25S-LDLR was detected with monoclonal antibody IgG-12D10, which reacts with the linker site of the LDLR but not with IgG-C7, which reacts with the NH_2 terminus of the receptor. The 25S-LDLR bound LDL similarly to the wild-type LDLR, but the rate of uptake of LDL by the mutant receptor was only about half of that by the wild-type receptor. In contrast, the 25S-LDLR bound and internalized β VLDL more avidly than LDL. These results suggest that the fourth cysteine residue of the first ligand-binding domain of the LDLR might be important for the internalization of atherogenic lipoproteins by vascular cells despite reduced LDL uptake, leading to atherosclerosis and premature cardiovascular disease.

Keywords Familial hypercholesterolemia · Low-density lipoprotein receptor · Mutation · Ischemic heart disease · Atherosclerosis

Abbreviations *FH* Familial hypercholesterolemia · *LDL* Low-density lipoprotein · *VLDL* Very-low-density lipoprotein · *LDLR* Low-density lipoprotein receptor · *VR* Very-low-density lipoprotein receptor · *ER2* Apolipoprotein E receptor 2 · *IHD* Ischemic heart disease · *DiI* 3,3′-Dioctadecylindocarbocyanine iodide

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Introduction

Familial hypercholesterolemia (FH) is an autosomal-dominant inherited disease caused by mutations in the low-density lipoprotein (LDL) receptor (LDLR) gene. Heterozygous FH has a population frequency of one in 500 (Goldstein et al. 1995). The clinical features of FH are an elevated plasma cholesterol due to impaired clearance of plasma LDL, the presence of xanthomas, and increased risk of coronary heart disease (CHD) as a consequence of premature atherosclerosis (Brown and Goldstein 1986). It is thought that the high incidence of

CHD in FH might be caused by the mechanism out of LDLR because of its functional defect.

The LDLR protein contains five domains, which include a ligand-binding domain, an epidermal growth factor precursor homology domain, an *o*-linked sugar domain, a membrane-spanning domain, and a cytoplasmic tail domain (Goldstein et al. 1995; Russell et al. 1989a). The ligand-binding region of LDLR consists of seven contiguous ligand-binding repeats each approximately 40 amino acids long with a repeat of six cysteine residues (Sudhof et al. 1985). This combination of repeats folds a cluster of conserved negatively charged sequences (Ser-Asp-Glu) with disulfide bond connections (Esser et al. 1988; Russell et al. 1989b; Bieri et al. 1995a) and allows LDLR to bind plasma lipoproteins containing apolipoprotein (apo) B-100 and apoE (Brown and Goldstein 1986; Mahley 1988). The first two ligand-binding repeats (LB1 and LB2) of the human LDLR are autonomously folding domains that contain three disulfide bonds with a Cys(I)-Cys(III), Cys(II)-Cys(V) and Cys(IV)-Cys(VI) connectivity (Bieri et al. 1995a,b). Mutations deleting one of the third to the seventh repeats in the ligand-binding domain of the LDLR result in a marked reduction of LDL binding (Russell et al. 1989b). The LDLR requires calcium ions for the physiologic binding of lipoprotein particles, which is eliminated in the presence of EDTA (Kita et al. 1981). The recognition of the first repeat, LB1, by a conformationally specific monoclonal antibody IgG-C7, is also dependent on the presence of calcium (van Driel et al. 1987). The ligand-binding repeat is thought to function as a protein-binding domain, which interacts with Lys and Arg residues, resembling the positively charged receptor-binding regions of apo B-100 and apoE. Differences in the number and rearrangement of these repeated sequences are thought to be responsible for the diversity of ligands that bind to the LDLR (Hobbs et al. 1990). We have previously reported a mutation at the fourth cysteine of the first ligand-binding domain in the LDLR gene in a homozygous FH patient (Takahashi et al. 2001). In this study, we have generated a mutant protein in CHO cells and examined its functional activity toward lipoproteins.

Materials and methods

Lipoprotein preparation

Human LDL ($d = 1.006$–1.063 g/ml) and rabbit β-very-low-density lipoprotein (VLDL) ($d < 1.006$ g/ml) was prepared by sequential preparative ultracentrifugation as previously described (Kujiraoka et al. 2000; Kosaka et al. 2001). Each lipoprotein (1 mg) was labeled with 1 mg/ml 3,3′-dioctadecylindocarbocyanine iodide (DiI; Molecular Probes, MO) by incubation for 3 h at room temperature (Corsetti et al. 1991), and after ultracentrifugation at the same density, fluorescent-labeled lipoproteins were isolated and exhaustively dialyzed against 150 mmol/l NaCl and 0.24 mmol/l EDTA (pH 7.4). Proteins were measured according to the method of Lowry (Lowry et al. 1951).

Engineering and cloning of human LDLR

The human LDLR cDNA (pLDLR3; ATCC 57004) in the pEF321 vector (Kim et al. 1990) was used as a template for PCR. The mutant cDNA of the LDLR was cloned from peripheral blood lymphocytes of a proband by RT-PCR using a paired primer 5′ complementary forward primer (5′-GACTCTAGACAATTGATGGGGCCCT-GGGGCTGGAAATTGC-3′) and 3′ reverse primer (5′-GACTGCGACCAATTGTCACGCCACGTCATC-CTCCAGACTG-3′) for the C25S mutation of the LDLR; 5′ complementary forward primer (5′-CTGG-GGGTCTTCCTTCTATGGTAGAACTGGCGGCTT-AAGAAC-3′). and 3′ reverse primer (5′-GTTCTTA-AGCCGCCAGTTCTCACCATAGAAGGAAGACC-CCCAG-3′) for the K790X mutation of the LDLR as control. The resultant approximately 500 bp human LDLR fragment was ligated into pBluescript II-SK vector (Stratagen) by digestion with *Xba*I and *Sal*I, and each vector was transformed into chemically competent DH5 cells (Toyobo, Osaka, Japan). The entire LDLR cDNA sequence was sequenced in both directions for three individual clones using an ABI autosequencer (Applied Biosystems, CA, USA). The three clones were found to be identical, and one was selected for further use. The hLDLR plasmid for transfection was amplified in LB culture medium (containing 100 μg/ml ampicillin) and purified using QIAGEN plasmid kits. Isolated plasmid stocks were stored at −20°C.

Generation of stable cell line

Chinese hamster ovary (CHO) cells (*ldl*A7) were co-transfected with hLDLR plasmids of wild and mutant types and pSV2-neo by the calcium phosphate transfection method using a ratio of 19:1 (pEF321-hLDLR:pSV2-neo). Transfected cells were selected using 700 μg/ml G418 (Sigma), and several clones were screened and selected for LDLR expression by a flow-cytometric procedure with antibody IgG-12D10 (Hattori et al. 2002). Each clone was established by two rounds of dilution cloning and identified as the highest protein-expressing clone. Each cell line for wild type and mutant LDLR was maintained under continuous selection using 700 μg/ml of G418 in DMEM/ham's F12 (Nissui Parmaceutical, Tokyo, Japan), 10% heat-inactivated fetal bovine serum, and 0.01% penicillin-streptomycin. The amount of LDLR protein on the surfaces of transfected CHO cells was measured using a specific monoclonal antibody (mAb) against LDLR by flow cytometry, as below.

Measurement of cell-surface LDLR protein and its functional activity by fluorescence-activated cell-sorter (FACS) flow cytometry

The LDLR protein on the cell surface and LDLR functional activity was measured by a flow cytometry, as previously described (Hattori et al. 2002). For the expression of the LDLR in CHO cells, the amount of LDLR protein on the cell surfaces was measured using a specific mAb against the LDLR—IgG-C7 (Amersham Pharmacia Biotech, Buckinghamshire, UK) or IgG-12D10, the latter of which is raised against the synthetic peptide WPQRCRGLYVFQGDSSPC, representing 158–175 amino acid residues of the human LDLR (Kosaka et al. 2001). The binding and uptake of lipoproteins in cells was measured using DiI-labeled LDL (DiI-LDL) or DiI-βVLDL. All results were expressed as mean intensity of fluorescence (MIF) after subtracting the background values (MIF typically less than 50) obtained with murine IgGs or in the presence of 2 mM EGTA or excess 50-fold unlabeled LDL or βVLDL.

Protein isolation and Western blot analysis

Cell protein was prepared according to a standard method (Kosaka et al. 2001) and quantified using BCA protein assay kit (Pierce, CA, USA). Total cell protein (1 μg) was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) with 5–20% slab gels containing 0.1% SDS. Immunoblotting was performed, as previously described (Kujiraoka et al. 2000).

Results

Expression of mutant LDLR protein in CHO cells

To analyze the function of the mutant LDLR, wild-type mutant 25S and 790X human LDLR cDNA were separately transfected into *ldl*-A7 cells, a line of mutant CHO cells that do not express LDLRs (Kingsley and Krieger 1984). The transfection was carried out with pSV2-Neo, and G418-resistant clones were selected. Several clones of each transfected CHO cell were established, and the representative results below shown in the transfected cells expressed the receptor equally determined by the Western blotting and flowcytomeric procedure.

The expression levels of cell-surface LDLR were examined in cell lysates (10 μg protein) from each transfectant by SDS-PAGE using monoclonal antibodies specific for the LDLR IgG-12D10, which reacts with the linker site between repeats 4 and 5 of the ligand-binding domain (Kosaka et al. 2001), and IgG-C7, which reacts with amino-acid residues 1–17 of the NH_2 terminus of the LDLR (Beisiegel et al. 1981). The wild-type and 790X LDLR protein were detected equally with IgG-C7 and IgG-12D10 while 25S-LDLR was detected only with IgG-12D10 (Fig. 1).

The cell-surface LDLR protein in the transfected cells was also examined by the flowcytometric procedure. The expression level of membrane-associated LDLR in the mutant 25S-LDLR and 790X-LDLR CHO cell was 86% and 92% of that of the wild type by IgG-12D10, respectively, and 3.9% and 104% by IgG-C7 (Fig. 2).

Functional activity of mutant LDLR

The binding and uptake activity of lipoproteins in mutant LDLR cells was analyzed using DiI-LDL and DiI-βVLDL in a flow cytometer. The binding and uptake activity of DiI-LDL were 91% and 48% for 25S-LDLR and 118% and 39% for 790X-LDLR, respectively (Fig. 3). The binding and uptake activity of DiI-βVLDL were 71% and 92% for 25S-LDLR and 54% and 44% for 790X-LDLR, respectively (Fig. 4). The internalization indexes (the MIF value internalized divided by the MIF value bound on the surface) for LDL and βVLDL were 3.3 and 2.6 for the wild type, 1.7 and 3.2 for 25S-LDLR, and 1.1 and 2.0 for 790X-LDLR, respectively (Fig. 5).

Discussion

LDLR plays an essential role in lipoprotein metabolism, and defective function of the receptor causes an autosomal dominant disease, FH. Homozygous FH is rare, but heterozygous FH has a frequency of about one in 500 (Goldstein et al. 1995). FH patients have frequently

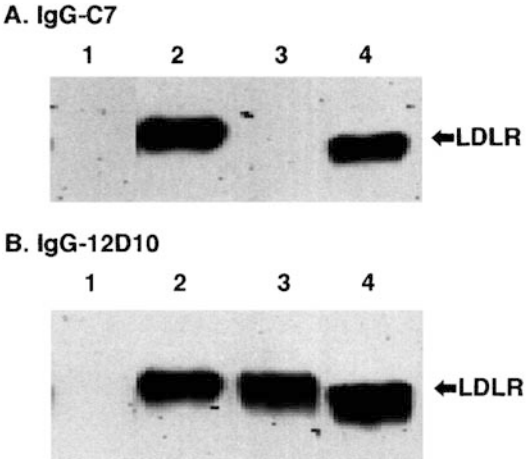


Fig. 1 The expression of the low-density lipoprotein receptor (LDLR) protein in the transfectant by Western blotting. Membrane protein (10 μg) from each transfected Chinese hamster ovary (CHO) cell was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The immunoblotting was carried out using monoclonal antibody **a** IgG-C7 or **b** IgG-12D10. *Lane 1*, CHO/Neo; *lane 2*, wild-type LDLR; *lane 3*, 25S-LDLR; *lane 4*, 790X-LDLR

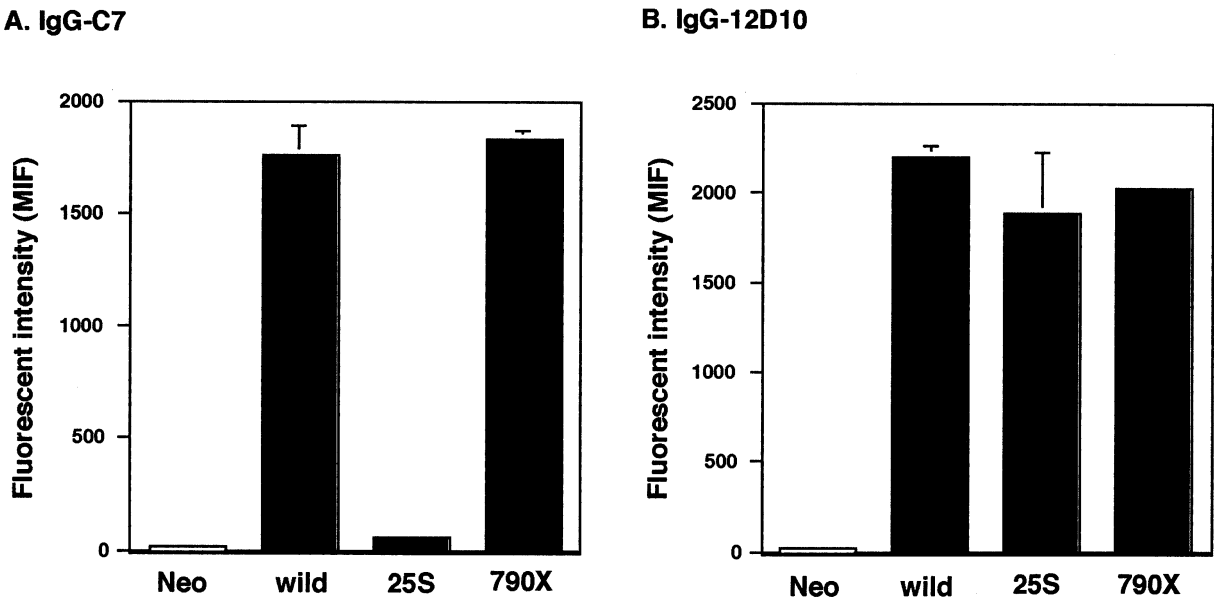
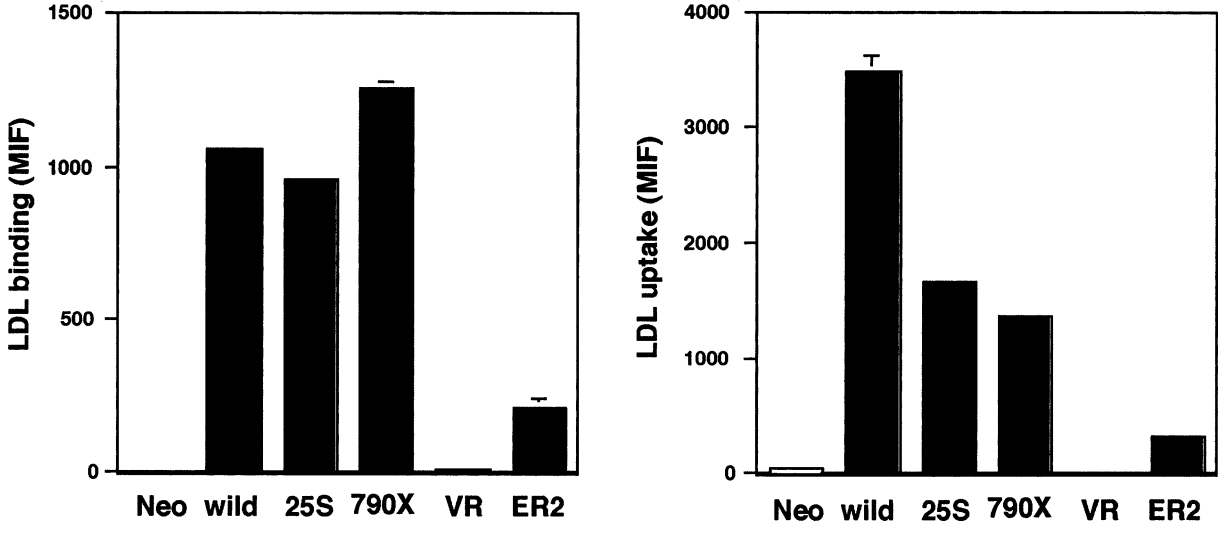


Fig. 2 Cell-surface low-density lipoprotein receptor (LDLR) protein in transfected Chinese hamster ovary (CHO) cells, quantified in a fluorescence-activated cell-sorter (FACS) flow cytometer using monoclonal antibody **a** IgG-C7 or **b** IgG-12D10. Results are the mean fluorescent intensities in transfected cells after subtraction of nonspecific bound IgG (obtained by incubating cells with mouse control IgG). Results represent the mean ± SD of triplicate determinations. The experiment was performed twice with essentially identical results

progressed to CHD, and it is believed that a mutant receptor does not contribute the pathogenesis of CHD. Most of the LDLR gene mutations were identified by a substitution of nucleotide, and the functional defects of LDLR protein were not well characterized. In this study, we have characterized the function of the first ligand-binding domain of the LDLR protein.

By Western blot analysis and flowcytometric procedure, the mutant 25S-LDLR protein expressed in CHO cells, which is a substitution of serine for cysteine at the fourth cysteine (codon 25) of the first cysteine-rich repeat in the ligand-binding domain, was detected with IgG-12D10, which reacts with the linker site between repeats 4 and 5 of the ligand-binding domain of the LDLR (Kosaka et al. 2001) but not with IgG-C7, which reacts with amino-acid residues 1–17 of the NH_2 terminus of the LDLR (Beisiegel et al. 1981). These results indicated that the mutant protein was expressed normally on the cell surface. The substitution at the fourth cysteine of the first ligand-binding domain would create a conformational change of the ligand-binding domain, resulting in no recognition by IgG-C7. Yamamoto et al. (1984) and Goldstein et al. (1985) have previously shown that most of the cysteines in the LDLR form disulfide

Fig. 3 Binding and uptake of DiI-labeled low-density lipoprotein (DiI-LDL) by low-density lipoprotein receptors (LDLR). Results are the mean intensity of fluorescence (MIF) in transfected Chinese hamster ovary (CHO) cells after subtraction of nonspecific binding or uptake of DiI-LDL in the presence of excess unlabeled LDL. Results represent the mean ± SD of triplicate determinations. The experiment was performed twice with essentially identical results



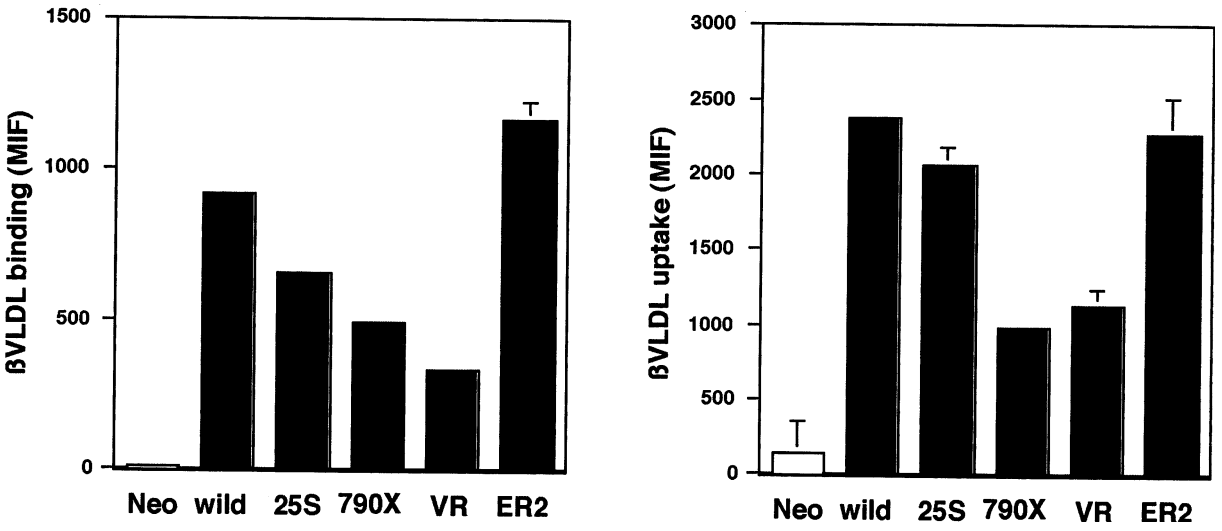
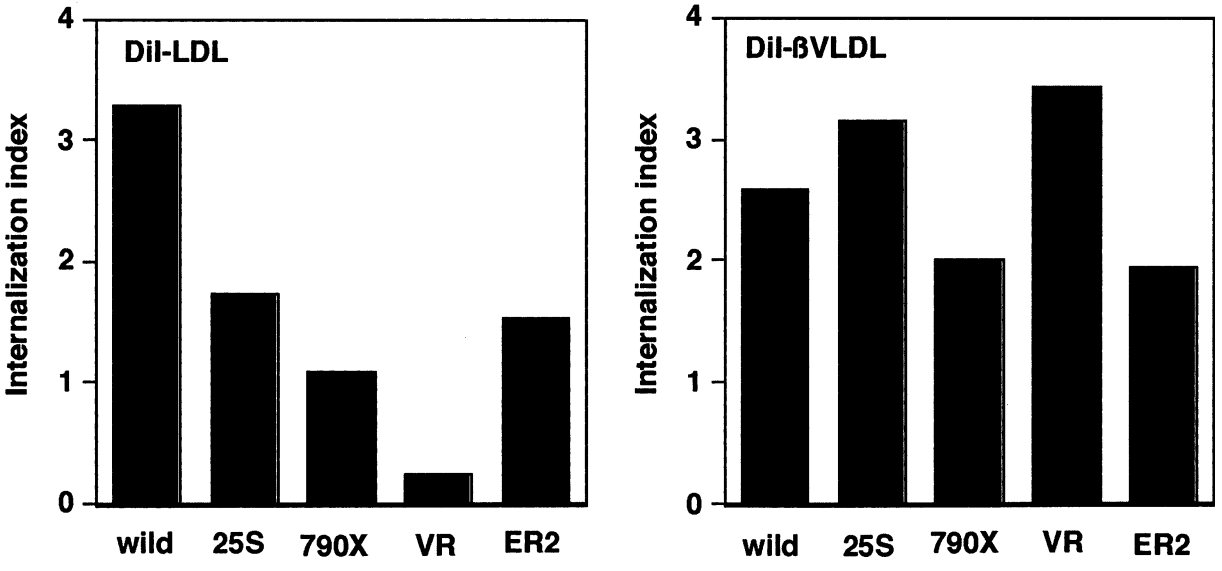


Fig. 4 Binding and uptake of DiI-labeled β very-[low-density-lipoprotein (DiI-βVLDL) by the low-density lipoprotein receptor (LDLR). Results are the mean intensity of fluorescence (MIF) in transfected Chinese hamster ovary (CHO) cells after subtraction of nonspecific binding or uptake of DiI-βVLDL in the presence of excess unlabeled βVLDL. Results represent the mean $\pm$ SD of triplicate determinations. The experiment was performed twice with essentially identical results

bridges. The disulfide bond connections between Cys(I) and Cys(III), Cys(IV) and Cys(VI), and Cys(II) and Cys(V) in the first cysteine-rich repeat fold a cluster of negatively charged residues, including the conserved Ser-Asp-Glu sequence, in the first ligand-binding repeat of the LDLR (Bieri et al. 1995a). Moreover, IgG-C7 binds only to the calcium complex of the repeat (Bieri et al. 1998). Therefore, the fourth cysteine residue may have an important function for the ability to bind calcium in the first ligand-binding repeat.

The LDL binding activity of the 25S-LDLR was similar to that of the wild-type LDLR, but the uptake of LDL was only about half of that mediated by the wild type. In contrast, the binding of βVLDL was slightly decreased (70% of the wild type), and βVLDL uptake was almost the same as that by the wild type. Mutational analysis of the ligand-binding domain of the LDLR revealed that mutations of the first ligand-binding repeat, Cys6Ala and Cys18Ala, which are the first and third cysteines, had no defect in the binding of antibody IgG-15C8 against the first repeat of the ligand-binding domain (residues 2–42) or of LDL and βVLDL (Esser et al. 1988). The deletion of the first cysteine-rich repeat also had no defect in the binding of LDL or βVLDL. Together with their report and the results in the present study, the alteration of disulfide bond connection has no affect on the ligand binding of the receptor. It has been

Fig. 5 Internalization index of low-density lipoprotein (LDL) and β very-low-density lipoprotein (βVLDL) by the low-density lipoprotein receptor (LDLR). Internalization index was calculated by dividing mean intensity fluorescence (MIF) internalized fluorescence-labeled lipoproteins by that of surface-bound lipoproteins. The result shown are representative of two independent experiments



reported that LDL receptor with the deletion of the exon encoding the sixth repeat, which has no recognition by IgG-C7, abolishes the binding of LDL but not βVLDL (Hobbs et al. 1986; Russell et al. 1989b). They concluded that the fifth cysteine-rich repeat of the ligand-binding domain would be a crucial role for binding of LDL but not βVLDL. Our results showed that the 25S-LDLR bound LDL as well as βVLDL with high affinity and took up βVLDL more rapidly than LDL. These results indicate that the disulfide bond of the fourth cysteine of the first ligand-binding domain might also be a crucial role for binding and uptake of atherogenic lipoproteins, such as remnant lipoproteins and chylomicron remnants, despite reduced LDL uptake, and suggest the enhancement of progression for CHD.

Autosomal-recessive hypercholesterolemia, which is caused by a mutation of a putative LDL receptor adaptor protein, has been recently identified. Studies of this disorder have shown that signaling through the adaptor protein in the cytosol is required for the endocytosis of receptor-bound LDL (Garcia et al. 2001). Decreased uptake of LDL or βVLDL via the mutant receptor may be affected by defect signaling for the endocytosis. Although the mutant 25S-LDLR had the similar binding activity for LDL as the wild-type LDLR and mutant K790X LDLR, which is lacking the cytoplasmic domain involving the phosphotyrosine binding (PTB) domain (Garcia et al. 2001) and has defective endocytosis of LDL, the 25S-mutant had defective uptake of LDL like the 790X-mutant. These results suggest that the NH_2 terminus of the ligand-binding domain may have a role in signaling for the endocytosis. The precise mechanism needs further investigation.

In addition, the LDLR binds apoB-100 and apoE, whereas the VLDLR or apoER2 binds only apoE (Takahashi et al. 1992, 1996; Kim et al. 1996). The phenotype of apoE has no effect on binding to the VLDLR (Bieri et al. 1998), and the ligand-binding site of apoE has not been identified. These considerations suggest that LDLR binds LDL and βVLDL at different sites. Our results showed that uptake of LDL but not βVLDL was affected in the mutant receptor, suggesting that the cytoplasmic signaling for the endocytosis may be mediated by at least two or more systems. The precise mechanism also needs further investigation.

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