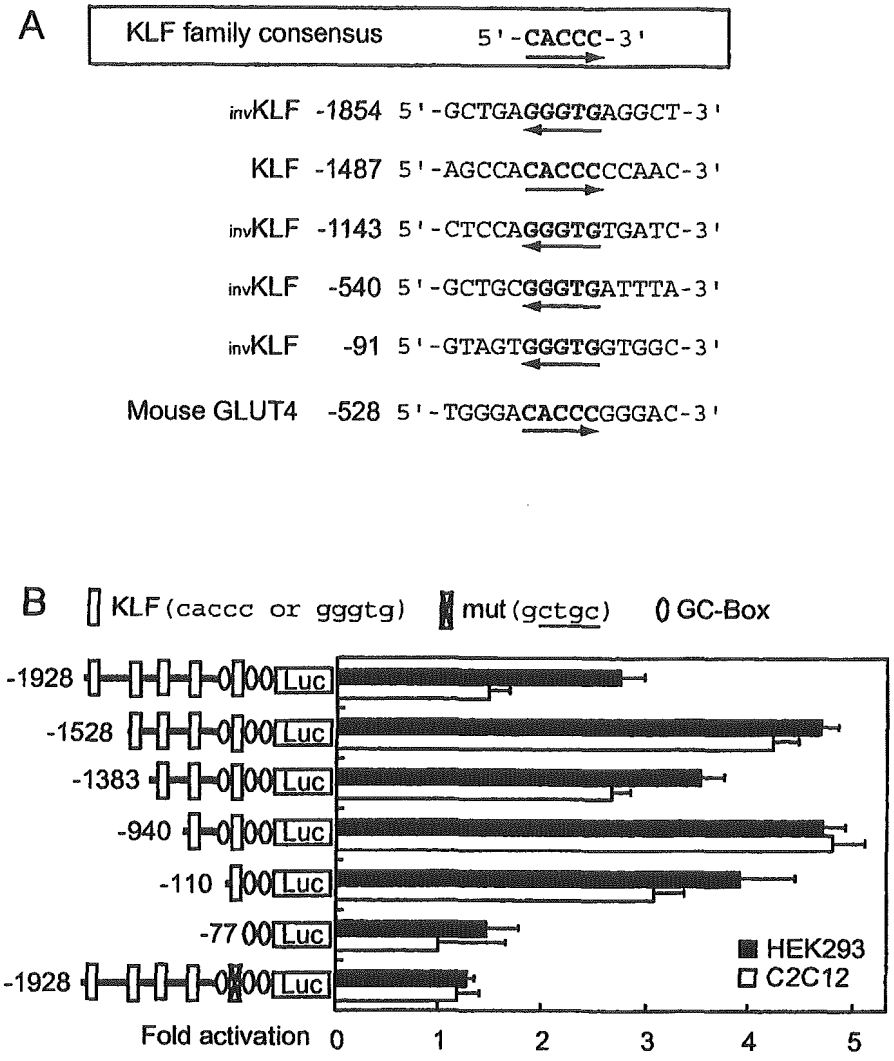


FIG. 4. **KLF15 motifs in the *AcecS2* promoter.** *A*, alignment of five KLF motifs in the *AceCS2* promoter. The sequences of potential KLF motifs at −1854, −1487, −1143, −540, and −91 and their surroundings in the AceCS2 promoter are compared with KLF15 binding sequence in the mouse *GLUT4* promoter. The KLF motifs in the inverted orientation are labeled with inv. *B*, localization of KLF15 responsive element by progressive deletion and mutation analyses. On day 0, HEK293 cells were seeded at a density of $2 \times 10^4$ cells/well in 24-well plates. On day 1, the cells were transfected with 0.8 μg of the indicated AceCS2-luciferase reporter and 0.1 μg of pRL-TK together with 0.01 μg of pCMV-KLF15 or pcDNA3.1. On day 2, the cells were harvested, and firefly and *Renilla* luciferase activities were determined as described under "Experimental Procedures." The -fold activation (luciferase activity co-transfected with pCMV-KLF15 *versus* pcDNA3.1) is shown. The *error bars* represent mean ± S.E. of triplicate incubations.

II, and PDK4 mRNAs were determined by quantitative real time PCR. As shown in Fig. 1*A*, insulin treatment did not alter the levels of AceCS2 mRNA, whereas those of hexokinase II and PDK4 were, respectively, induced and reduced by insulin treatment. Similarly, the levels of AceCS2 mRNA in human skeletal myotubes were not altered (Fig. 1*B*), suggesting that fasting-induced *AceCS2* gene expression is not directly dependent on insulin action.

*5′-Flanking Region of the Mouse AceCS2 Gene*—To determine the mechanism regulating fasting-induced expression of the *AceCS2* gene, we initially isolated and characterized the 5′-flanking region of the mouse *AceCS2* gene. Approximately 2.4-kb upstream of the translation initiation ATG was isolated by PCR (see "Experimental Procedures") and was sequenced (Fig. 2*A*). The transcription initiation sites were determined by 5′-RACE experiments on poly(A)$^+$ RNA from mouse skeletal muscle using an AceCS2 transcript-specific primer (see "Experimental Procedures"). Sequence analysis of the 5′-RACE products revealed that the *AceCS2* gene has multiple transcription start sites: two major points at −23 and −28 and four minor points at −12, −17, −19, and −53 (Fig. 2*A*); the A of the translation initiation ATG of the gene is designated +1.

*Trans-activation of AceCS2 Promoter by KLF15*—Potential elements for various transcription factors were identified by computer-assisted search using AliBaba2.1 (www.gene-regulation.com/pub/programs/alibaba2/index.html) and TFSEARCH (www.cbrc.jp/research/db/TFSEARCHJ.html). As summarized in Fig. 2*B*, the 5′-upstream of the most 5′ transcription initiation site (−53) consists of potential sites for MyoD (−1509, −1379, and −698), IRS (−1509 and −1243), c-Ets-1 (−1435 and −1409), HNF1 (−802), MEF2 (−261), and KLF (−1854, −1487, −1143, −540, and −91). There are three GC-boxes located at −65, −76, and −132.

To determine the effects of these transcription factors, the genomic DNA fragment containing the 5′-flanking region (−2347) was ligated with the firefly luciferase gene in pGL3 basic to create pAceCS2(2347). This promoter-reporter was transiently transfected into non-muscle HEK293 cells along with the indicated transcription factor(s) listed in Fig. 3. All the expression plasmids used in this study were well characterized by the original investigators from whom we obtained. All the plasmids were confirmed by restriction enzyme digestions and partial sequencing. For the expression of AFX and FKHR, since they contain a FLAG epitope tag, we performed immunoblot analyses using an anti-FLAG antibody and confirmed that protein were expressed properly (data not shown). For the other transcription (co-) factors, we have performed real time PCR analysis, because of the unavailability of their antibodies. The transfection efficiencies were also examined by *Renilla* luciferase activities as described under "Experimental Procedure." As shown in Fig. 3, among 13 transcription factors tested, only KLF15 trans-activated the *AceCS2* promoter re-

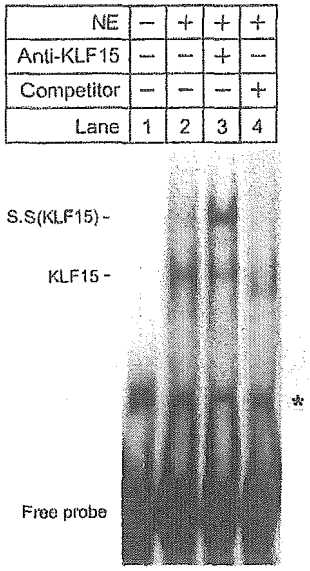


FIG. 5. **EMSA of KLF15-binding to the *AceCS2* gene promoter.** Nuclear extracts were prepared from C2C12 cells stably expressing KLF15 and incubated with labeled double-stranded oligonucleotide containing a putative KLF binding site (at −91), followed by EMSA as described under "Experimental Procedures." The KLF15-specific shifted band and the supershifted band (*S.S*) specific to the KLF15 antibody are indicated on the *left*. Nonspecific band is denoted by an *asterisk*.

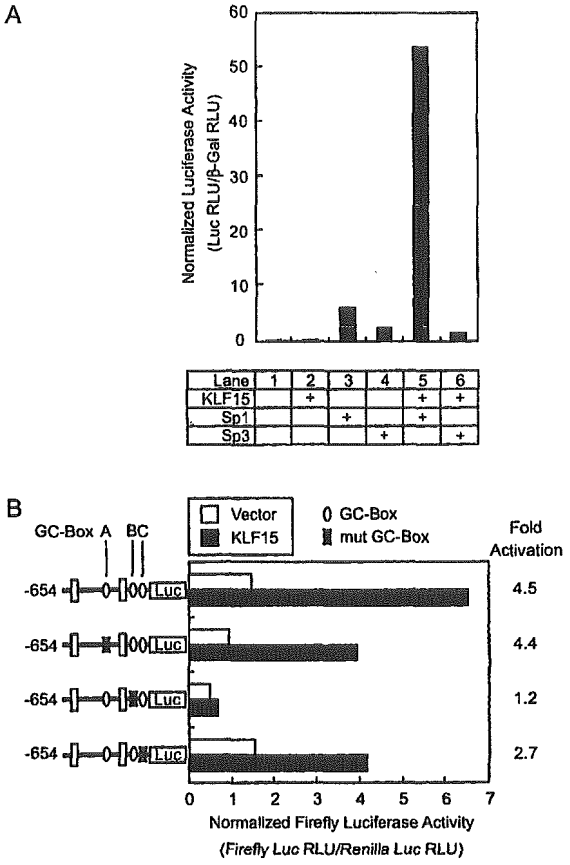


FIG. 6. **KLF15 and Sp1 cooperate to induce *AceCS2* promoter activity.** *A*, KLF15 functions synergistically with Sp1. On day 0, SL2 cells were plated at a density of $2 \times 10^5$ cells/well in 24-well plates. On day 1, the cells were transfected with 0.5 μg of pAceCS2 (654) and 0.1 μg of pPac-β-gal (internal reference encoding the β-galactosidase gene) together with 0.01 μg of the indicated plasmid. On day 2, the cells were harvested, and luciferase activity was measured and normalized to β-galactosidase activity. Each value represents the average of duplicate incubations. *B*, mutation analyses of three GC-boxes in *AceCS2* promoter. pAceCS2(654) and its mutants, in which each GC-box was mutated, were constructed as described under "Experimental Procedures." On day 0, $2 \times 10^4$ cells/well of HEK293 cells were set up in 24-well plates. On day 1, the cells were transfected with 0.8 μg of the indicated luciferase reporter construct and 0.1 μg of pRL-TK together with 0.01 μg of pCMV-KLF15 or pcDNA3.1. On day 2, the cells were harvested, and firefly luciferase activity was measured and normalized to *Renilla* luciferase activity. The -fold activation (luciferase activity co-transfected with pCMV-KLF15 *versus* pcDNA3.1) is shown. Each value represents the average of duplicate incubations.

porter activities: ~5.5-fold induction was detected by co-transfection with KLF15. Other KLFs, KLF6 or KLF9 had almost no effects.

KLF15 is known to form a heterodimer complex with MEF2A and the resulting complex synergistically induces the transcription of *GLUT4* gene (23). On the other hand, MEF2 family of transcription factors MEF2A, 2B, 2C, and 2D had no synergistic effects on the AceCS2 reporter activities when coupled with KLF15. Each of the MEF2 family of transcription factors, MEF2A-2D, alone had almost no effects.

We also examined the effects of peroxisome proliferator-activated receptor γ co-activator 1 (PGC-1α) in the absence or presence of KLF15 or MEF2 family of transcription factors, MEF2A-2D. Neither PGC-1α alone, PGC-1α in the presence of KLF15, nor PGC-1α with each of the MEF2 family of transcription factors had stimulatory effects on the promoter reporter activities. The forkhead box transcription factors FKHR (also known as FOXO1) (24, 25) and AFX (FOXO4) (11) are involved in insulin-responsive gene activation. Thus we examined the effects of FKHR and AFX on the *AceCS2* promoter activities. As a positive control experiment, we transfected AFX and FKHR together with a reporter-luciferase under the control of three copies of insulin responsive sequence (IRS) in tandem. This configuration, which is based on the *IGFBP-1* promoter/enhancer elements, gave rise to abundant luciferase activity (Fig. 3, *inset*) (11). Consistent with the lack of insulin-dependence, neither FKHR nor AFX had stimulatory effects on the *AceCS2* promoter in the absence or presence of KLF15.

Other factors MyoD, cEts-1, HNF1α, and HNF1β in the absence or presence of KLF15 were inactive. Although these transcription factors were not active in this reporter assay and we cannot exclude the possibility of their significance in the regulation of *AceCS2* gene *in vivo*, these data indicate that KLF15 plays a key role in the trans-activation of the *AceCS2* gene.

*Crucial KLF Binding Site in the AceCS2 Promoter*—There are five potential KLF15 binding sites within 2.4-kb region of the *AceCS2* promoter. Fig. 4*A* compares the sequences of five potential KLF15 sites and their surroundings in the *AceCS2* promoter with that present in the *GLUT4* gene. Although four of the five KLF15 sites are inversely orientated, all the five sequences contain an identical sequence of CACCC that is specifically bound by KLF15 (23) and other KLF family of transcription factors (26–28).

To determine the most crucial KLF binding site, a series of 5′-deletions was introduced into the promoter region. Deletion of the sequence from −1928 to −110 did not change the trans-activation by KLF15 (Fig. 4*B*). A further deletion to −77 resulted in almost complete loss of induction by KLF15. Similar results were obtained using a myogenic line, C2C12 cells. Furthermore, the reporter construct containing a mutated KLF binding site lacked inducible activity. Similarly, in the context of pAceCS2 (−1928), the mutation in the most proximal KLF binding site lacked the inducible activity, indicating that the most proximal KLF site is a curtail site for the trans-activation of the *AceCS2* gene by KLF15.

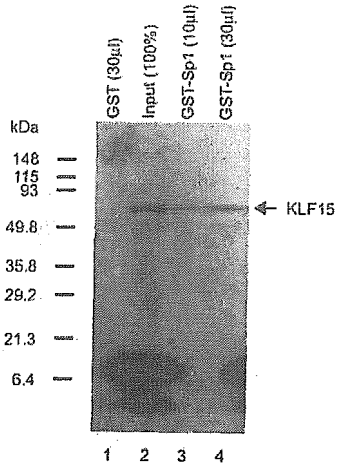


FIG. 7. **KLF15 interacts with Sp1 *in vitro*.** Glutathione beads bound with *E. coli* cell-expressed GST-Sp1 (10 and 30 μl) or GST (30 μl) were incubated with *in vitro* transcribed/translated $^{35}$S-labeled KLF15 at 4 °C for 1 h. After washing extensively, the proteins bound on the beads were analyzed by SDS-PAGE and visualized by autoradiography.

TABLE II
*Quantitative real time PCR of various KLF transcripts in skeletal muscle of fasted and fed mice*

Total RNAs from skeletal muscles of fasted (48 h) or fed mice were subjected to quantitative real-time PCR as described under "Experimental Procedures." Cyclophilin mRNA was used as the invariant control. Values represent the relative amount of mRNA in skeletal muscle of fasted mice relative to that of fed mice, which is arbitrarily defined as 1. The values are mean of three mice.

| Krüppel-like factor | Fold induction (Fasted/Fed) |
|---|---|
| KLF1 | 2.11 |
| KLF2 | 1.69 |
| KLF3 | 2.28 |
| KLF4 | 1.88 |
| KLF5 | 2.62 |
| KLF6 | 6.88 |
| KLF7 | 1.24 |
| KLF8 | 1.69 |
| KLF9 | 5.50 |
| KLF10 | 2.29 |
| KLF11 | 3.72 |
| KLF12 | 1.29 |
| KLF13 | 1.86 |
| KLF14 | 1.67 |
| KLF15 | 27.9 |
| KLF16 | 1.19 |

*EMSA of KLF15-Binding Site in the AceCS2 Promoter*—To determine whether KLF15 is capable of binding to the most proximal KLF site of the *AceCS2* promoter, we performed EMSA using labeled probe (double-stranded 36-mer corresponding −70 to −105) with the nuclear extracts from C2C12 cells stably expressing KLF15. As shown in Fig. 5, the nuclear extracts containing KLF15 produced a single band of DNA-protein complex. This complex was specific as it can be competed by unlabeled probe. Furthermore, this complex can be supershifted with an antibody against KLF15, indicating that KLF15 is able to bind to the most proximal KLF site to the transcription start point of the *AceCS2* gene.

*Synergistic Transcriptional Activation by KLF15 and Sp1*—In contrast to the synergic transcriptional activation of the *GLUT4* gene by KLF15 and MEF2 (23), none of MEF family transcription factors in combination with KLF15 synergistically trans-activated the *AceCS2* promoter (see Fig. 3). The proximity of the KLF15 binding site and GC boxes raised the possibility that KLF15 and Sp1 may function in a coordinated manner to induce the *AceCS2* promoter. To assess this possibility, we performed co-transfection studies using Sp-null *Drosophila* SL2 cells. As shown in Fig. 6*A*, we observed that the combination of KLF15 and Sp1 resulted in a synergistic activation of the *AceCS2* promoter (*lanes 1*, *3*, and *5*): ~10-fold by comparison to Sp1 alone. Although Sp3 recognizes the same sequence as Sp1, and Sp3 alone trans-activated the *AceCS2* promoter (Fig. 6*A*, *lane 4*), the combination of KLF15 and Sp3 did not synergistically induce the *AceCS2* promoter (Fig. 6*A*, *lane 6*).

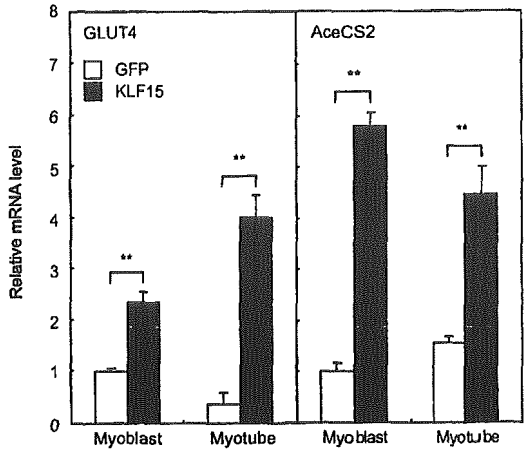


FIG. 8. **Induction of GLUT4 and AceCS2 transcripts in C2C12 cells stably expressing KLF15.** On day 0, C2C12 stably expressing KLF15 (C2C12/KLF15) and GFP (C2C12/GFP) were individually plated in 6-well plates at a density of $1 \times 10^5$ cells/well and cultured in medium A. On day 2, the cells were either harvested (myoblasts) for RNA extraction, or induced to differentiate into myotubes by incubating in medium B for an additional 7 days. Medium was changed every other day. On day 9, myotubes were harvested for RNA extraction. Total RNA from myoblasts and myotubes were subjected to quantitative real time PCR with specific primers for GLUT4 (*A*) or AceCS2 (*B*). Cyclophilin was used as the invariant control. Values represent the amount of mRNA relative to that in C2C12/GFP myoblasts, which is arbitrarily defined as 1. The *error bars* represent mean ± S.E. of triplicate incubations.

There are three GC-boxes, termed as GC-boxes A, B, and C, within 100 bases upstream of the most 5′ transcriptional initiation site (−53) of the *AceCS2* gene. To further define the role of these GC-boxes in the synergistic trans-activation with KLF15, we introduced mutations in each of the three GC-boxes in pAceCS (654) (see "Experimental Procedures" and Fig. 6*B*). For this co-transfection experiment with these mutant promoter-reporters, we used HEK293 cells. As shown in Fig. 6*B*, mutations in GC-box B in the *AceCS2* promoter almost completely abolished the trans-activation by KLF15, while other mutants containing mutated GC-boxes A or C retained the trans-activation activity by KLF15. These data indicate that GC-box B, located 8 bases downstream of the most crucial KLF15 site, is the most important site in the synergistic trans-activation of the *AceCS2* gene by KLF15 and Sp1.

To determine whether KLF15 and Sp1 interact directly to activate the *AceCS2* promoter, we carried out GST pull-down assays. As shown in Fig. 7, *in vitro* transcribed/translated $^{35}$S-labeled KLF15 was bound with GST-Sp1 fusion protein, but not with GST, indicating that KLF15 interacts with Sp1 *in vitro*.

*Induction of KLF15 Transcripts in Skeletal Muscle of Fasted Animals*—To test whether fasting induces KLF15 expression, like the *AceCS2* gene, we carried out quantitative real time RT-PCR analysis of KLF transcripts. As shown in Table II, 48 h fasting robustly induced the KLF15 transcripts in the skeletal muscle of mice: the levels of KLF15 transcripts in fasted animals were ~28-fold higher than those in fed animals. Together

with the trans-activation of the *AceCS2* promoter, it is suggested that this fasting-induced AceCS2 expression is largely contributed by KLF15.

*Overexpression of KLF15 in C2C12 Myogenic Cells Induces the Expression of AceCS2 Transcripts*—We next examined the effects of KLF15 overexpression on the levels of AceCS2 transcripts in C2C12 myogenic cells. C2C12/KLF15 and C2C12/GFP cells were cultured in non-differentiation medium (medium A) or differentiation medium (medium B) and the levels of AceCS2 transcripts were determined by quantitative real time RT-PCR. As shown in Fig. 8, KLF15 overexpression induced the levels of AceCS2 transcripts by severalfold both in myoblasts and in myotubes, indicating that *AceCS2* gene expression *in vivo* is indeed induced by KLF15.

## DISCUSSION

In the current study, we provide evidence that fasting-induced transcriptional activation of the *AceCS2* gene in the skeletal muscle is contributed largely by a unique transcription factor KLF15. KLF15 is a recently characterized member of the Sp1-like/KLF family and regulates *GLUT4* gene expression in both adipose and muscle cells (23). Consistent with the induction of AceCS2 transcripts, the levels of KLF15 transcripts in the skeletal muscle are also robustly induced by fasting. Together with the trans-activation of the *AceCS2* promoter by KLF15, this co-induction of KLF15 by fasting may indicate that fasting-induced AceCS2 transcription is largely contributed by KLF15.

KLF15 is a member of the Sp1-like/KLF family, a family of highly related zinc-finger DNA binding proteins that are important regulators of cellular development, differentiation, and activation (29). It is highly expressed in the liver, kidney, adipose tissue, heart, and skeletal muscle (23, 30). Gray *et al.* demonstrated that KLF15 specifically induces the expression of the insulin-sensitive glucose transporter GLUT4 and glucose uptake in 3T3-L1 cells. It was also shown that KLF15 directly interacts with MEF2, a known activator of the *GLUT4* promoter, and activates the *GLUT4* promoter in a synergistic manner. On the other hand, our current data show that KLF15 in combination with a general transcription factor Sp1, synergistically activates the *AceCS2* promoter. In this context, it is interesting to note that the two AceCSs require Sp1 for their trans-activation; activation of the *AceCS1* gene is synergistically regulated by SREBPs and Sp1 (3).

Fasting-induced gene expression is of current interest. Especially, the induction of hepatic gluconeogenic genes during prolonged fasting or starvation is important for survival. Recently, Puigserver *et al.* (25) showed that insulin-regulated hepatic induction of gluconeogenic genes is mediated through interaction of FKHR and PGC-1$\alpha$. In the skeletal muscle, genes involved in fatty acid oxidation and PDK4 are up-regulated during fasting in order to suppress glucose utilization (4, 5). We recently showed evidence that the PPAR$\delta$ together with PGC-1$\alpha$ mediates the induction of genes involved in fatty acid transport, $\beta$-oxidation, and mitochondrial respiration in the skeletal muscle (31). The gene expression profile induced by PPAR$\delta$ is very similar to those induced by fasting in the skeletal muscle (4, 5). Therefore, PPAR$\delta$ together with PGC-1$\alpha$ may play a part in the muscle expression of genes induced by fasting.

Our current data provide evidence for a unique role of KLF15 in the activation of fasting-induced genes in the skeletal muscle. In the skeletal muscle, KLF15 is the most abundant KLF and its induction by fasting is most potent among 16 KLFs. To our knowledge, AceCS2 and GLUT4 are the only known targets for KLF15. It is therefore important to identify target genes driven by KLF15. These studies are currently in progress.

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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 $\beta$-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

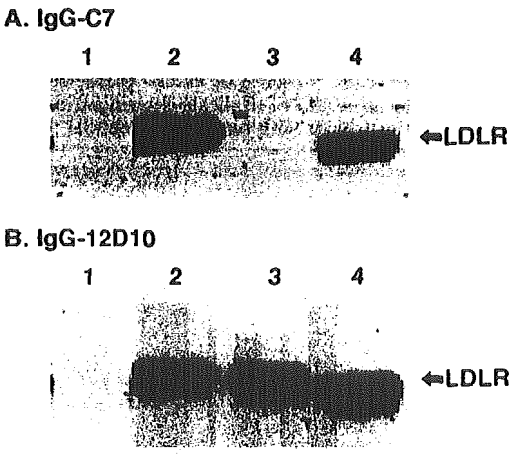



**Fig. 1** The expression of the low-density lipoprotein receptor (LDLR) protein in the transfectant by Western blotting. Membrane protein (10 μg) from each transfected Chinese hamster ovary (CHO) cell was subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE). The immunoblotting 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

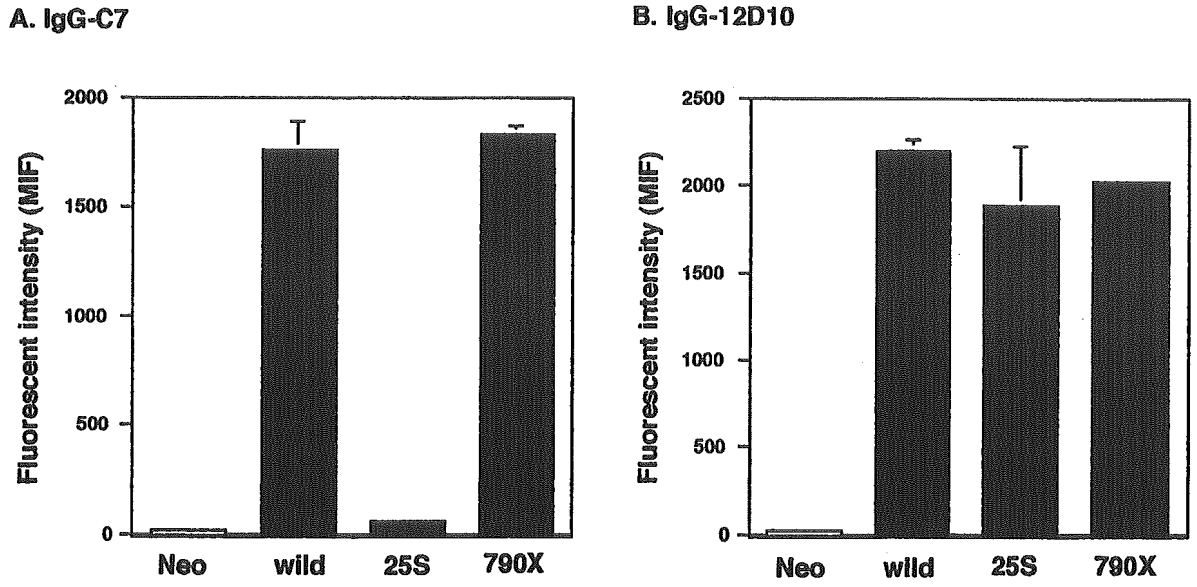
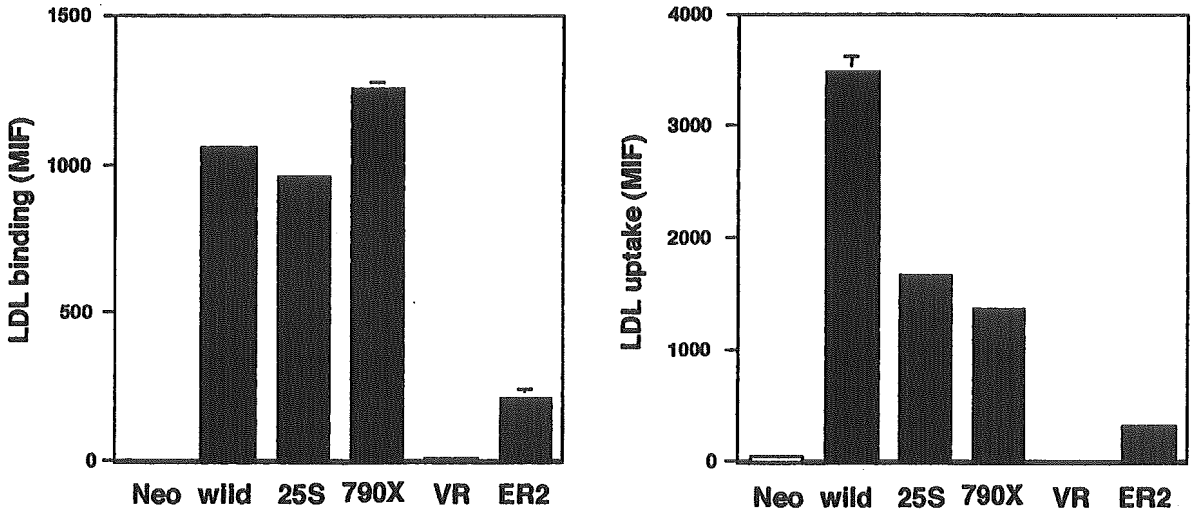


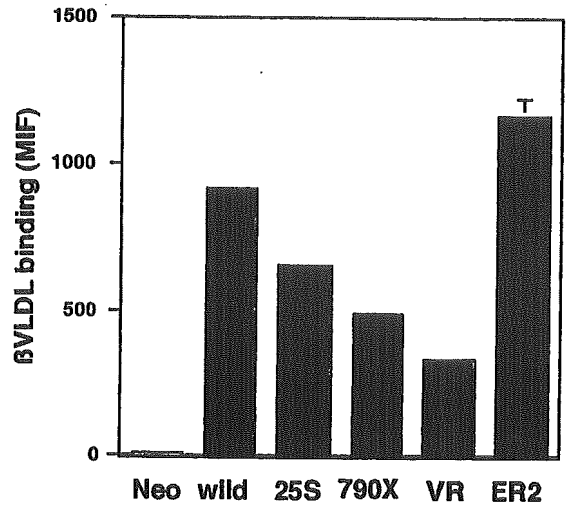

Fig. 2 Cell-surface low-density lipoprotein receptor (LDLR) protein in transfected Chinese hamster ovary (CHO) cells, quantified in a fluorescence-activated cell-sorter (FACS) flow cytometer using monoclonal antibody **a** IgG-C7 or **b** IgG-12D10. Results are the mean fluorescent intensities in transfected 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





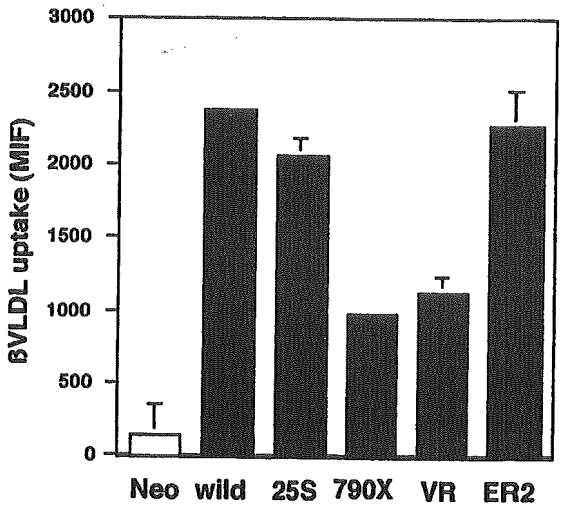
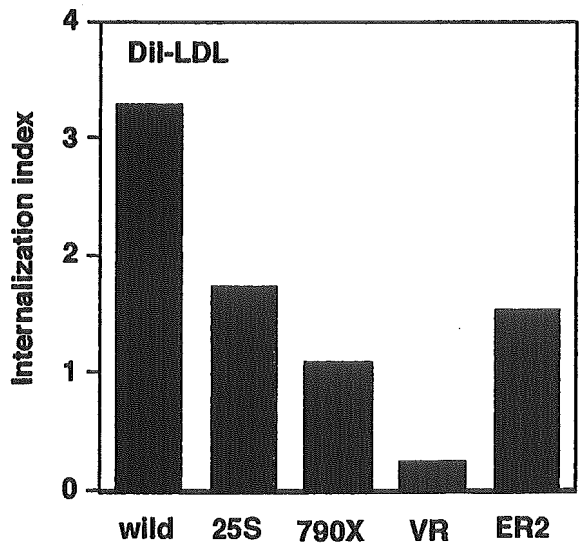



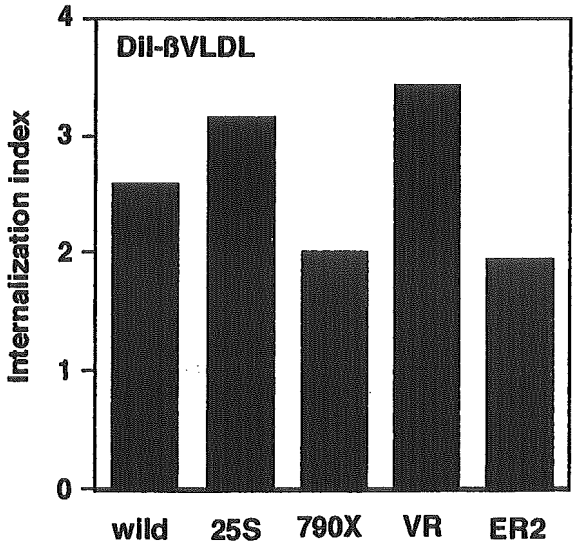

**Fig. 4** Binding and uptake of DiI-labeled $\beta$ very-[low-density-lipoprotein (DiI-$\beta$VLDL) by the low-density lipoprotein receptor (LDLR). Results are the mean intensity of fluorescence (MIF) in transfected Chinese hamster ovary (CHO) cells after subtraction of nonspecific binding or uptake of DiI-$\beta$VLDL in the presence of excess unlabeled $\beta$VLDL. Results represent the mean ± 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 $\beta$VLDL was slightly decreased (70% of the wild type), and $\beta$VLDL 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 $\beta$VLDL (Esser et al. 1988). The deletion of the first cysteine-rich repeat also had no defect in the binding of LDL or $\beta$VLDL. 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 $\beta$ very-low-density lipoprotein ($\beta$VLDL) 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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