Compensated endocytosis of LDL by hamster cells co-expressing the two distinct mutant LDL receptors defective in endocytosis and ligand binding

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Abstract The low density lipoprotein receptor (LDLR) regulates the plasma cholesterol level by mediating endocytosis of LDL. We established stable hamster cell lines expressing two LDLRs with distinct functional defects, i.e., endocytosis and ligand binding. In the cell line expressing only I189D h/r (human-rat chimeric) LDLR, defective in LDL binding, very little amount of LDL was internalized, although the receptor was endocytosed efficiently. In the cell line expressing Y807C LDLR solely, very few receptors were located in coated pits or endocytosed, while LDL binding to the receptor was not disrupted. In striking contrast, in the cells co-expressing both receptors, a much larger number of Y807C LDLR were internalized and co-located with I189D h/r LDLR in the perinuclear region. In these cells, LDL was bound exclusively to Y807C LDLR and its uptake was enhanced by 80% as compared to the cell expressing Y807C LDLR solely, whereas LDL binding affinity was not changed. These results suggest that a defect of the essential motif for endocytosis, cysteine 807, could be compensated by co-expression of I189D h/r LDLR, but the LDL binding was not affected.—Yoshida, H., M. Yokode, A. Yamamoto, R. Masaki, T. Murayama, H. Horiuchi, and T. Kita. **Compensated LDL receptors defective in endocytosis and ligand binding.** ***J. Lipid Res.*** **1999.** 40: **814–823.**

Supplementary key words LDL receptor • co-expression • endocytosis • familial hypercholesterolemia

The low density lipoprotein receptor (LDLR) is a single-chain transmembrane glycoprotein that regulates the plasma cholesterol level by mediating endocytosis of LDL (1–3). Intensive studies with transfected cells (4–6) and transgenic mice (7–9) have dissected endocytosis of LDL and revealed that it consists of sequential steps, i.e., binding of LDL to the LDLR, internalization of ligand–receptor complex, and intracellular degradation of LDL (10–12).

The pathophysiological significance of receptor-mediated endocytosis of LDL has been revealed by the studies on patients with familial hypercholesterolemia (FH). These patients show an increase in the plasma concentration of LDL and develop premature coronary atherosclerosis owing to the genetic defect in the LDLR activity. Most of these patients are either true heterozygotes expressing both mutant and wild-type LDLRs or compound heterozygotes with two different mutant alleles for the LDLR (12).

It is known that receptors for growth factors and cytokines such as platelet-derived growth factor and epidermal growth factor form oligomers on the cell surface, and that the signal transduction from these receptors involves a change in the oligomeric structure (13). van Driel et al. (14) reported previously that the LDLR was cross-linked chemically into oligomeric forms, most of which were dimeric. Also, Heuser and Anderson (15) demonstrated by scanning electronmicroscopy that many of the LDL particles appeared to be present in groups of two particles, estimating that 50–70% of the LDL particles were in dimeric form.

From these observations, it could be presumed that most of LDLRs would exist as an oligomeric form on the cell surface and that heterooligomers might be formed between the two different LDLRs in the FH heterozygote patients. However, despite those observations, most of the studies on LDLRs thus far have been based upon the experiments in which a single type of the LDLR gene was introduced into the cells or animals and hence it has not been examined whether co-expression of two distinct LDLR could have any effect on intracellular transport of either receptor or its ligand binding affinity.

Abbreviations: LDLR, low density lipoprotein receptor; FH, familial hypercholesterolemia; FCS, fetal calf serum; LPDS, lipoprotein-deficient serum; DTSSP, 3,3′-dithiobis sulfosuccinimidylpropionate; PMSF, phenylmethylsulfonyl fluoride; WT, wild-type.

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To elucidate the questions above, in the current study, we transfected *ldlA-7* cells, a Chinese hamster ovary cell line lacking functional LDLR, and prepared stable cell lines co-expressing two mutant LDLRs, I189D h/r (human–rat chimeric) LDLR and Y807C LDLR, which are defective in ligand binding and in clathrin-coated pit-mediated endocytosis, respectively. By both immunocytochemical study using selective antibody raised against each receptor and biochemical analysis of LDL incorporation into the cell, we report that co-expression of I189D h/r LDLRs could improve the efficiency of endocytosis of Y807C LDLR, but does not change the binding manner of LDL to either receptor.

EXPERIMENTAL PROCEDURES

Materials

Plasmids pSLH16 containing the mouse metallothionein-I promoter fused to a hybrid human LDL receptor gene (7) and pLDLR4-Cys 807 (16) were kind gifts from Dr. Goldstein and Dr. Brown (University of Texas, Southwestern Medical Center, Dallas, TX). The plasmid pLDLR2 was provided by Dr. Tokuo Yamamoto (Tohoku University, Sendai, Japan) under permission of Dr. Goldstein and Dr. Brown (17). Anti-mouse IgG-rhodamine (AP-192R) and anti-rabbit IgG-DTAF (AP-182F) were purchased from Chemicon International (Temecula, CA). DiI was purchased from Molecular Probes (Eugene, OR). Fetal calf serum (FCS) was obtained from Irvine Scientific (Santa Ana, CA). DMEM/F12 was obtained from Life Technologies (Rockville, MD). Penicillin and streptomycin were from Bio Whittaker (Walkersville, MD). All other chemicals used were reagent grade.

Antibodies

RP2, a rabbit polyclonal antibody directed against the amino acid residues 164 to 173 of the rat LDLR (18), was prepared as described (14) and purified with affinity column. IgG-HL1, a mouse monoclonal antibody directed against the amino acid residues 163 to 174 of the human LDLR (14) was a kind gift from Dr. Goldstein and Dr. Brown (University of Texas, Southwestern Medical Center, Dallas, TX).

Construction of the LDL receptor expression vectors

Three LDLR expression vectors pSLH16, pHY1-Neo, and pHY2-Hygro were used in this study. The plasmid pSLH16 has been described previously and contains human wild-type LDLR (WT LDLR) minigene (7). The plasmid pHY1-Neo contains the SV40 early region promoter that drives transcription of the minigene for Y807C LDLR, an endocytosis-defective human LDLR, in which Tyr residue at position 807 was replaced by Cys (16). The plasmid pHY2-Hygro harbors the SV40 early region promoter to drive transcription of the minigene for I189D h/r LDLR, a rat–human chimeric receptor defective in ligand binding with two mutations.

The plasmids pHY1-Neo and pHY2-Hygro were constructed by engineering pLDLR2-Neo and pLDLR2-Hygro as intermediate plasmids. To prepare pLDLR2-Neo and pLDLR2-Hygro, 2.2 kb and 1.9 kb NruI-SalI fragments were excised from pREP9 and pCEP4 (both from Invitrogen, San Diego, CA) encoding neomycin- and hygromycin-resistant genes, respectively, and subcloned into the HindIII-SalI fragment of pLDLR2 encoding LDLR cDNA (17) with DNA Blunting Kit (Takara Shuzo, Kyoto, Japan).

To construct pHY1-Neo, a 1.1kb BglII-SmaI fragment encoding the membrane spanning and cytoplasmic domains of Y807C LDLR was excised from pLDLR4-Cys 807 (16) and subcloned into pSLH16. To complete construction, A 13 kb SacI-SacII fragment of this intermediate plasmid was then subcloned into a 6.2 kb SacI-SacII fragment of pLDLR2-Neo.

The plasmid pHY2-Hygro was constructed as follows. To replace the residues 164–172, a 3.3 kb BglII-BglII fragment was excised from pSLH16 (7) and subcloned into bacteriophage M13 mp18 (Pharmacia, Uppsala, Sweden) with SalI-BglII linker (19). After site-directed mutagenesis using synthesized oligonucleotide composed of 75 nucleic acids (10, 20), the BglII-BglII fragment was subcloned back to pSLH16. To this plasmid, another mutation was introduced at amino acid residue 189 (5), using Transformer Site-Directed Mutagenesis Kit (CLONTECH, Palo Alto, CA) with mutagenic primer 5′-AGTGGCGAGTGCGACCACTCCA and selection primer Trans Oligo ScaI/StuI (CLONTECH). A 13 kb SacI-ApaI fragment was obtained from this intermediate plasmid and subcloned into a 6.2 kb SacI-ApaI vector fragment of pLDLR2-Hygro, thereby engineering pHY2-Hygro. All the regions containing mutation were sequenced by ALF DNA sequencer (Pharmacia).

Isolation of stably transfected cell lines

ldlA-7 cells, a line of mutant Chinese hamster ovary cells lacking functional LDLR (21), were provided by Dr. Tokuo Yamamoto (Tohoku University, Sendai, Japan) under permission of Dr. Krieger (MIT, Boston, MA). For transfection, plasmids were introduced into *ldlA-7* cells with Lipofectin reagent (Life Technologies, Rockville, MD) (22). All cells were grown and set up for experiments as previously described (23) except that DMEM/F12 containing 24 mm bicarbonate, pH7.4, 50 units/ml penicillin, and 50 μg/ml streptomycin was used as growth medium.

To establish cell lines expressing WT LDLR stably, pSLH16 was co-transfected with pSV3-Neo as described by Davis et al. (4). On day 2, incubation with growth medium containing 10% (v/v) FCS and 700 μg/ml of GENETICIN (Life Technologies) was started. On days 10 to 12, three visualized colonies were picked after incubation with growth medium containing 10% (v/v) fetal calf lipoprotein-deficient serum (23), 10 μg/ml cholesterol, 0.1 μg/ml 25-hydroxycholesterol, and 20 μg protein/ml DiI-labeled LDL (24) for 5 h. The colonies were expanded into cell lines, out of which a cell line designated as WT cell was used for further study. To establish cell lines expressing Y807C LDLR stably, cells were transfected with pHY1-Neo and incubated in the same selective medium for 10–12 days. A colony that was visualized by DiI-labeled LDL was isolated and expanded. The cells were then transfected with pCEP4 encoding hygromycin resistance gene and placed in growth medium containing 10% (v/v) FCS plus 400 μg/ml of hygromycin B (Boehringer Mannheim, Mannheim, Germany). After expansion of isolated colonies, three cell lines were established and a cell line designated ED cell was used. To prepare cell lines stably expressing I189D h/r LDLR, cells were transfected with pHY2-Hygro and cultured in growth medium containing 10% (v/v) FCS plus 400 μg/ml hygromycin B for 10–12 days. Three colonies were selected and expanded. The cells were subjected to another transfection with pREP9 encoding neomycin resistance gene and selection was further conducted with 700 μg/ml GENETICIN. After expansion of selected colonies, a cell line designated LBD cell was used for further analysis. To make the cell lines expressing both Y807C LDLR and I189D h/r LDLR, pHY1-Neo was co-transfected with pHY2-Hygro, which was followed by selection in growth medium containing 10% (v/v) FCS plus 500 μg/ml GENETICIN and 300 μg/ml hygromycin B. The colonies visualized by DiI-labeled LDL were picked and expanded into cell lines. A total of 8 cell lines designated LBxE-1 to 8 were used in this study. To prepare a mock-transfected cell line, salmon sperm (ss) DNA was introduced together with pCEP4 and pREP9 and colonies were selected in the selective medium containing both 500 μg/ml GENETICIN and 300 μg/ml hygromycin B.

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All the transfected cells above were further subcloned with limited dilution method using 96-well plates and the expression levels of the introduced LDLR genes were assessed by immunoblot with antibody IgG-HL1, RP2, or both.

LDL receptor assays

Human LDL and ^{125}I-labeled human LDL were prepared as described (23). Degradation, internalization, and binding of ^{125}I-labeled LDL were measured in monolayers of intact cells as described (23). To evaluate affinity of LDL binding, each cell line was incubated at 4°C with 1–32 μg protein/ml of labeled LDL. Scatchard plots were analyzed with Mac Ligand.

Immunofluorescence light and confocal microscopy

Cells grown on coverslips were fixed, permeabilized, and stained as previously described (25). The cells were double-labeled by incubation with mouse monoclonal antibody IgG-HL1 and rabbit polyclonal antibody RP2 (15 μg/ml each) which was followed by incubation with both anti-mouse IgG-rhodamine (AP-192R) and anti-rabbit IgG-DTAF (AP-182F) (20 μg/ml each). The cells were examined and photographed on an Optiphot-2 microscope (Nikon, Tokyo, Japan). For high resolution analysis, the cells were visualized using an Axiovert 100 microscope (Zeiss AG, Oberkochen, Germany) equipped with LSM 410 laser scanning confocal microscope (Zeiss).

Preparation of extracts from cultured cells

To prepare cell extracts, cells were scraped from plastic tissue culture dishes with a rubber policeman, washed in PBS, and lysed at 4°C in buffer B containing 10 mm HEPES (pH 7.4), 200 mm NaCl, 2.5 mm $MgCl_2$, 2 mm $CaCl_2$, 1% (v/v) TX-100, 2 mm PMSF (phenylmethylsulfonyl fluoride), 1 mm 1,10-phenanthroline, 1 μg/ml of pepstatin A, 0.1 mm leupeptin, and 0.5 μg/ml aprotinin. Cellular debris was pelleted by centrifugation at 10,000 *g* for 10 min, and lysates (50 μg protein per lane) were subjected to non-reducing 7.5% SDS-PAGE.

Immunoblotting

Aliquots of the cell extracts were subjected to non-reducing 7.5% SDS-PAGE (26), and transferred onto a nitrocellulose filter (ECL Kit, Amersham Corp, Buckinghamshire, U.K.). Filters were blocked with buffer D (500 mm NaCl, 35 mm Tris, pH 7.4, 10% skim milk, 0.2% (v/v) Tween-20) for 2 h, incubated with 2 μg/ml of either IgG-HL1 or RP2 in buffer D for 20 min, washed with buffer E (500 mm NaCl, 35 mm Tris, pH 7.4, 0.1% SDS, 1% (v/v) Nonidet P-40, 0.5% sodium desoxycholate) for 20 min, incubated with either HRP-coupled sheep anti-mouse antibody or HRP-coupled donkey anti-rabbit antibody (both from Amersham Corp) at 1:1000 dilution in buffer D plus 1% (v/v) Nonidet P-40 for 20 min and washed with buffer E for 20 min. Blots were developed by chemiluminescence (ECL kit, Amersham Corp.). Density of each band was measured and analyzed by densitometer with NIH Image.

Other assays

Protein concentrations of the lipoproteins and the cell extracts were determined by the method of Lowry et al. (27) and by Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA), respectively. Data were analyzed with Student *t*-tests to determine statistical significance.

RESULTS

Specificity of antibodies to LDLRs

The LDLRs and antibodies used in this study are summarized in **Fig. 1** and **Table 1**. Y807C LDLR is a mutant version of the human LDLR, in which Tyr at position 807 was replaced by Cys (Fig. 1B). This mutation has been shown to cause reduction of endocytosis efficiency (4, 10). To detect Y807C LDLR, we used a mouse monoclonal antibody IgG-HL1 which was raised against a polypeptide corresponding to the amino acid residues 163–174 of the human WT LDLR (14). These residues constitute the "linker" between the cysteine-rich repeats IV and V of the ligand binding domain of the WT LDLR and Y807C LDLR (Figs. 1A, 1B) and are known to be highly divergent among species (28). I189D h/r LDLR, the other version of the mutant LDLR, contained two mutations as shown in Fig. 1C. First, LDL binding domain was disrupted by substituting residue Asp for Ile at 189 (5). As the second mutation, the residues 164–172 were replaced by the residues 164–173 from the rat LDLR. By this replacement, the "linker" of the human receptor was exchanged with that of the rat receptor. Thus, I189D h/r LDLR was regarded as a "ligand-binding-defective and human-rat (h/r) chimeric" receptor (Fig. 1C). To detect I189D h/r LDLR, we raised a rabbit polyclonal antibody RP2 directed against a polypeptide corresponding to the residues 164–173 from the rat receptor (Table 1).

We first tested the specificity of the antibodies by immunoblot in detection of the receptors expressed in the transfected cell lines listed in **Table 2**. When the extracts from WT cell and ED cell were applied to immunoblot analysis with IgG-HL1, the bands corresponding to WT LDLR and Y807C LDLR respectively, were detected (**Fig. 2A**; lanes 1 and 3). In contrast, no receptor was recognized in the extract either from LBD cell transfected with I189D h/r LDLR gene, or from the mock-transfected cell (Fig. 2A; lanes 2 and 7). On the other hand, I189D h/r LDLR expressed in LBD cell was recognized by polyclonal antibody RP2, which was raised against the "linker" sequence of the rat receptor (Fig. 2B; lane 2). In sharp contrast, no receptor band was observed by RP2 in the extract either from WT cell, from ED cell, or from the mock-transfected cell (Fig. 2B; lanes 1, 3, 7). These results demonstrate that antibodies IgG-HL1 and RP2 could recognize Y807C LDLR and I189D h/r LDLR respectively, with high specificity. As expected from these observations, in the 8 cell lines transfected with both genes for Y807C LDLR and I189D h/r LDLR, the LDLR was detected by either antibody. The representative immunoblots of the receptors expressed in LBxE-1, LBxE-2, and LBxE-3 cells are shown in Figs. 2A and 2B (lanes 4, 5, 6).

Localization of LDLRs by light and confocal microscopy

We next studied the localization of the two mutant receptors in the transfected cell lines by double immunofluorescence with antibodies IgG-HL1 and RP2. As shown in **Fig. 3a**, the LBD cell expressing solely I189D h/r LDLR was labeled with RP2 and DTAF-labeled secondary antibody. I189D h/r LDLR was mainly detected as strong green-colored staining in the perinuclear region. Also, small green dots throughout the cell image were revealed. As expected from the immunoblot experiments, no staining was observed with IgG-HL1 and

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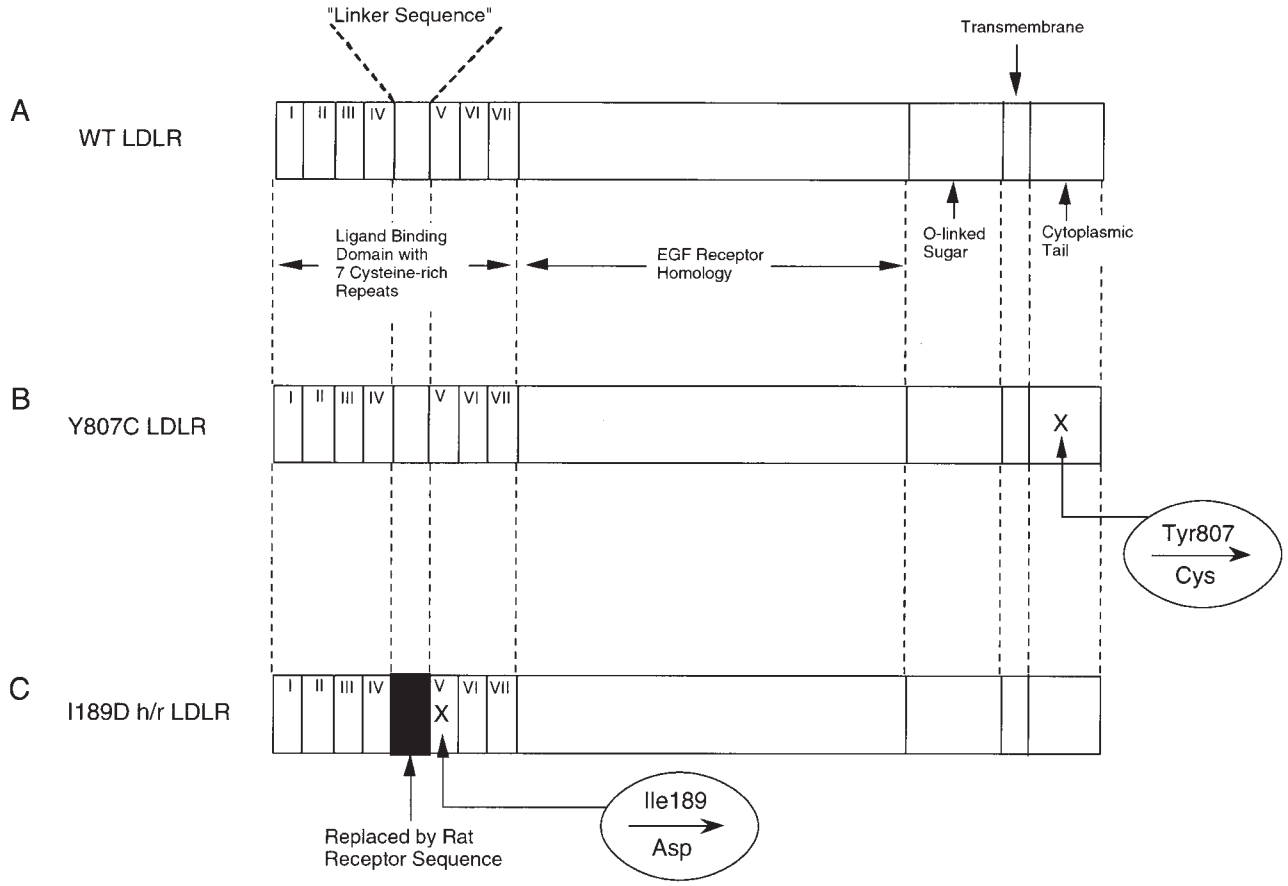

Fig. 1. Schematic diagram of the domain structure of wild-type (WT) LDLR (A), Y807C LDLR (B), and I189D h/r LDLR (C). The domains of the proteins are delimited by dotted lines. The mutations caused by single amino acid alternation are indicated by X. In the ligand binding domain of I189D h/r LDLR, the "linker" sequence between the cysteine-rich repeats IV and V (residues 164–172) was replaced by the residues 164–173 from the rat LDLR as indicated by closed box.

rhodamine-labeled secondary antibody (Fig. 3d). When LBD cell was stained without permeabilization by TX-100, part of the small dots could still be observed but the strong staining in the perinuclear region was abolished (data not shown). Therefore, it was thought that the small dots throughout the cell could represent the receptors clustering in the coated pits, endosomes and lysosomes, and that the majority of I189D h/r LDLR was located near by the nucleus. This pattern of localization is identical to that of WT LDLR (29, 30). These results indicate that I189D h/r LDLR, which has an intact cytoplasmic tail, was internalized by the cell as efficiently as WT LDLR.

We next tested the ED cell expressing solely Y807C LDLR, which is defective in clustering in the coated pit and subsequent internalization (10, 31). In contrast to LBD cell, ED cell was not stained with RP2 and DTAF-labeled secondary antibody (Fig. 3b). As shown in Fig. 3e, there was vague diffuse rhodamine-labeled staining over the cell image, whereas very little expression could be observed in the perinuclear region. This distribution pattern did not change, even when the cell was stained without permeabilization by TX-100 (data not shown). These results were in accordance with the previous observation that Y807C LDLR is dispersed on the non-coated membrane of the cell surface (31).

TABLE 1. Comparison of the LDL receptors used in this experiment

LDL Receptor	Transfected Plasmid	Mutation		Function		Recognition by Antibodies		Ref.
		Single Amino Acid Mutation	Substitution Mutation	Ligand Binding	Endocytosis	IgG-HL1	RP2	
Wild-type (human)	pSLH16	(−)	(−)	normal	normal	(+)	(−)	7
Y807C (human)	pHY1-Neo	Tyr807 → Cys	(−)	normal	very slow	(+)	(−)	16
I189D h/r (human/rat chimera)	pHY2-Hygro	Ile189 → Asp	human receptor residues 164–172 → rat receptor residues 164–173	defective	normal	(−)	(+)	10, 18

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TABLE 2. Internalization index for ^{125}I-labeled LDL in hamster cells transfected with different mutated versions of the human LDL receptor plasmids

Cell Lines	Expressing Receptors	Degraded (a)	Intracellular (b)	Surface-bound (c)	Internalization Index (a + b)/c	Internalization Index % of control	LDLR Expression Level Y807C (d)	LDLR Expression Level I189D h/r (e)	Expression Ratio of Y807C to I189D h/r d/e
		ng LDL/mg protein					*arbitrary unit*		
Mock	none	13	145	3	ND	ND	0	0	
WT	wild-type	1424	689	357	5.9	100	ND	0	
LBD	I189D h/r	26	186	3	ND	ND	0	32	
ED	Y807C	443	441	478	1.8	31	49	0	
LBxE-1	Y807C and I189D h/r	722	481	326	3.7	62	14	27.2	0.51
LBxE-2		790	524	648	2	34	57.7	34.1	1.70
LBxE-3		479	379	256	3.3	57	19.1	9.9	1.93
LBxE-4		876	517	541	2.6	44	48.4	31.4	1.54
LBxE-5		1004	603	507	3.2	54	65.9	23.6	2.79
LBxE-6		722	541	551	2.3	39	33.6	10.3	3.26
LBxE-7		191	280	102	4.6	78	6.6	23.1	0.29
LBxE-8		342	286	151	4.1	70	3	10.1	0.30

On day 5 of cell growth, each monolayer received 2 ml of medium containing lipoprotein-deficient serum and 0.01 mg protein/ml of ^{125}I-labeled LDL in the absence or presence of 1 mg protein/ml of cold LDL. After incubation for 5 h at 37°C, the high affinity values for degraded, intracellular, and surface-bound ^{125}I-labeled LDL were determined. These values represent the mean of triplicate experiments. The expression levels of LDLRs are shown in arbitrary units based on the quantitative analysis of the immunoblots with NIH Image; ND, not determined.

We then examined LBxE-3 cells expressing both I189D h/r LDLR and Y807C LDLR. In striking contrast to the ED cell expressing only Y807C LDLR, this cell was strongly stained in the perinuclear region with IgG-HL1 and rhodamine-labeled secondary antibody. Small dots and vague diffuse staining were also seen over the cell image (Fig. 3f). On the other hand, as detected with DTAF-labeled antibody, the expression pattern of I189D h/r LDLR in this LBxE-3 cell was identical to that observed in LBD cell (Figs. 3a and 3c). As I189D h/r LDLR was not detected with rhodamine-labeled antibody (Fig. 3d), the strong rhodamine-staining in the perinuclear region of the LBxE-3 cell could not result from detection of the co-expressing I189D h/r LDLR but was attributed to the change in the pattern of localization of Y807C LDLR. In the non-permeabilized LBxE-3 cells, the perinuclear region was not stained with either rhodamine- or DTAF-labeled antibody (data not shown). It is therefore suggested that in the LBxE-3 cell, while a part of Y807C LDLR still remains on the cell surface, most of the Y807C LDLR were located near by the nucleus.

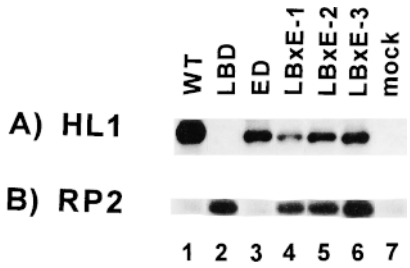

Fig. 2. Immunoblot of the LDLRs in the cell lines transfected with the genes for wild-type and two mutant LDLRs. Cell lysate was prepared from each cell line as described in Experimental Procedures. The samples (50 μg protein each) were applied to non-reducing 7.5% SDS-PAGE, transferred onto nitrocellulose filter. The filter was incubated either with mouse monoclonal antibody IgG-HL1 (A) or with rabbit polyclonal antibody RP2 (B). The bands were visualized using HRP-coupled sheep anti-mouse antibody or HRP-coupled donkey anti-rabbit antibody at 1:1000 dilution. Blots were developed by chemiluminescence. The WT (lane 1), the LBD (lane 2), and the ED cells (lane 3) were transfected with plasmids encoding WT LDLR, I189D h/r LDLR, and Y807C LDLR, respectively. The cells were transfected with both plasmids encoding I189D h/r LDLR and Y807C LDLR (lanes 4, 5, 6). Mock transfected cell line received salmon sperm DNA (lane 7).

To further localize the LDLRs in LBxE-3 cells, we conducted double immunofluorescence confocal microscopy. As shown in **Fig. 4a**, the majority of I189D h/r LDLR, as detected by DTAF-labeling, was shown in the perinuclear region of the cell. On the other hand, in the same cells, Y807C LDLR, labeled by rhodamine, was expressed both on the cell surface and in the perinuclear region (Fig. 4b). This cell surface distribution of Y807C LDLR could correspond to the vague diffuse rhodamine stainning shown in Figs. 3e and 3f. When these two images were overlapped, the perinuclear rhodamine-labeling was superimposed by DTAF-labeling and merged into yellow-colored staining, while such color merging was not observed on the cell surface (Fig. 4c). These results suggest that Y807C LDLR in the perinuclear region were co-localized with I189D h/r LDLR.

Degradation and binding of LDL

As the results above indicate that Y807C LDLR is internalized by the co-expressing cells associated with I189D h/r LDLR, we next investigated degradation, internalization, and binding of ^{125}I-labeled LDL in the transfected cell lines (Table 2). A minimal amount of LDL was shown to bind and be internalized either by LBD cell or by the mock-transfected cell in accordance with previous reports

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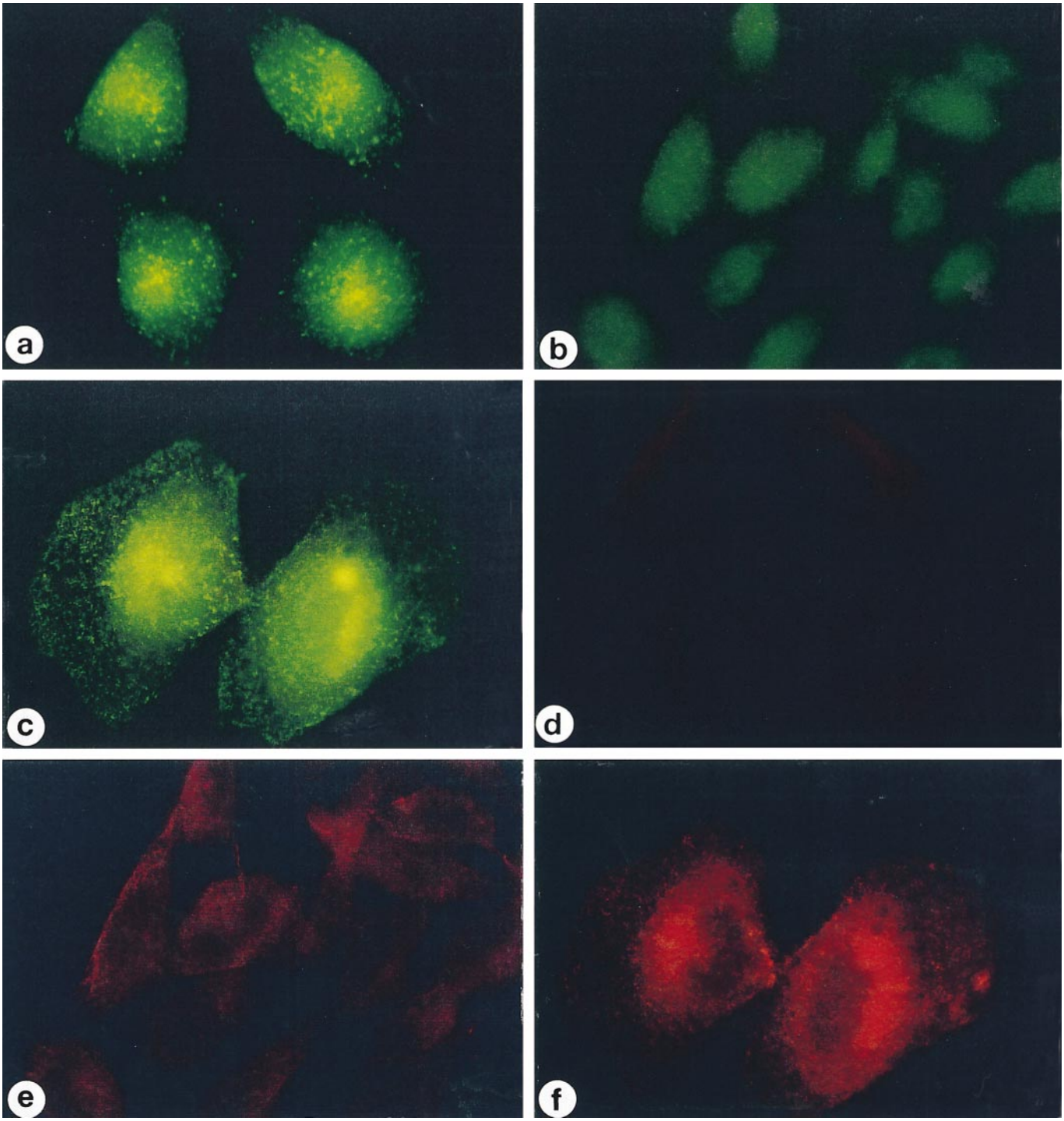

Fig. 3. Localization of I189D h/r LDLR and Y807C LDLR in the transfected *ldlA-7* cell lines by double immunofluorescence microscopy. The LBD cell expressing I189D h/r LDLR solely (a and d), the ED cell expressing Y807C LDLR solely (b and e), and the LBxE-3 cell co-expressing both mutant receptors (c and f) were grown in growth medium containing 10% (v/v) LPDS for 18 h, fixed in 4% paraformaldehyde, and then permeabilized with 0.1% Triton X-100. The cells were double-labeled by incubation with both rabbit polyclonal antibody RP2 and mouse monoclonal antibody IgG-HL1 (15 μg/ml each). Then the cells were incubated with both anti-rabbit IgG-DTAF and anti-mouse IgG-rhodamine (20 μg/ml each). I189D h/rLDLR was visualized by DTAF (green color) (a, b, c), and Y807C LDLR was visualized by rhodamine (red color) (d, e, f).

(5, 6). When WT cell expressing human WT LDLR was incubated with 5 μg protein/ml of ^{125}I-labeled LDL for 5 h, LDL was degraded and internalized efficiently: total uptake of LDL as a sum of degraded and internalized LDL was 2,113 ng/mg cell protein. On the surface of this cell line, 357 ng of LDL was bound per mg cell protein, thus the ratio between total uptake and surface binding of the ^{125}I-labeled LDL (internalization index) was 5.9. In ED

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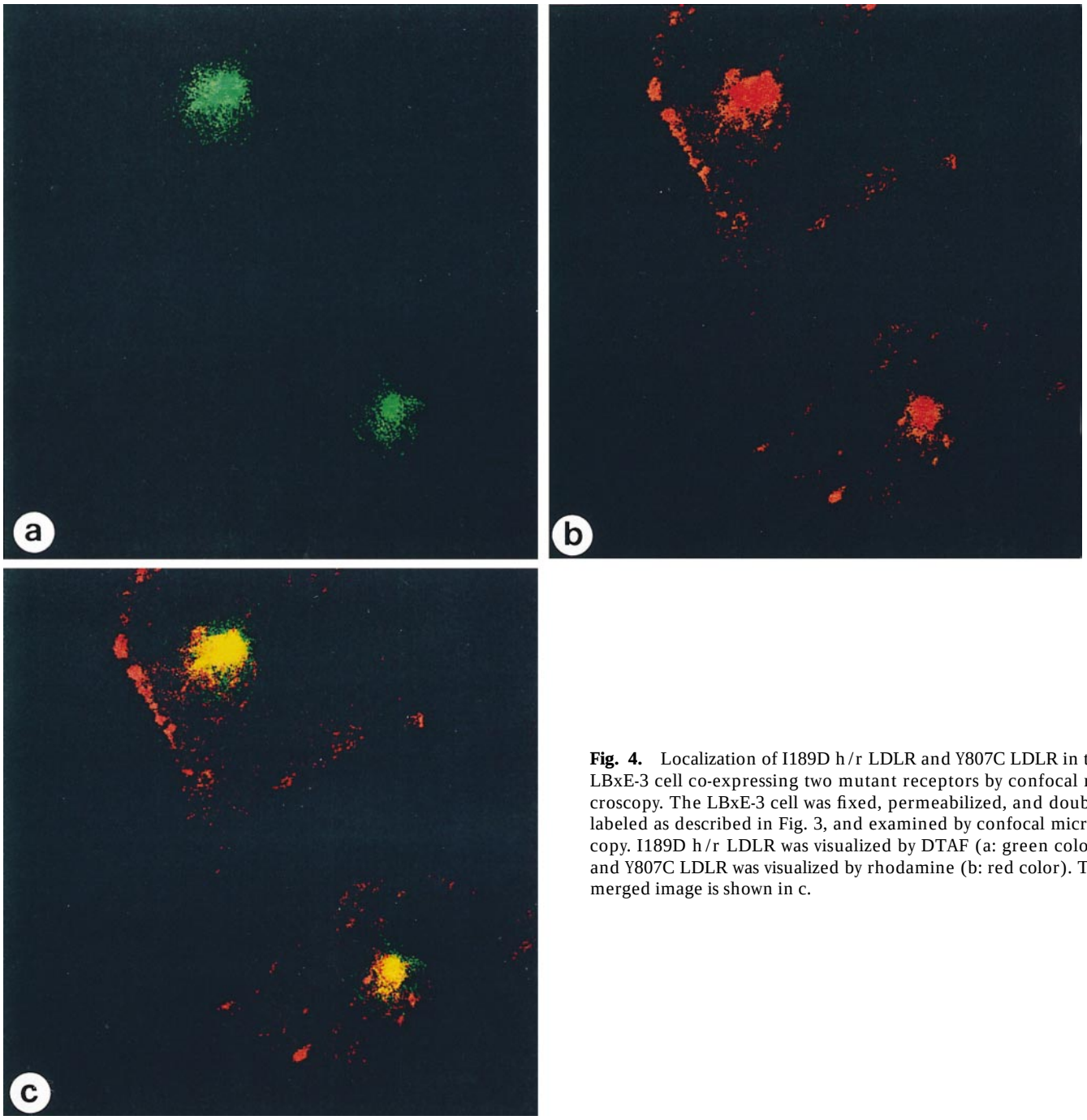

Fig. 4. Localization of I189D h/r LDLR and Y807C LDLR in the LBxE-3 cell co-expressing two mutant receptors by confocal microscopy. The LBxE-3 cell was fixed, permeabilized, and double-labeled as described in Fig. 3, and examined by confocal microscopy. I189D h/r LDLR was visualized by DTAF (a: green color), and Y807C LDLR was visualized by rhodamine (b: red color). The merged image is shown in c.

cell expressing Y807C LDLR solely, the internalization index was as low as 1.8, i.e., 31% of that of the WT cell, which was in accordance with the result described previously (16). In contrast, all of the eight cell lines co-expressing Y807C LDLR and I189D h/r LDLR internalized LDL more efficiently than ED cell did. Internalization indexes of these cells ranged from 2 to 4.6, i.e., 34 to 78% (54.8 ± 15.2 (mean ± SD)) of WT cell ($P < 0.01$ vs. ED cell). As shown in **Fig. 5,** in these co-expressing cells, the surface binding of ^{125}I-labeled LDL showed linear correlation with Y807C LDLR expression levels as presented in arbitrary units, whereas such correlation was not obtained with the expression levels of I189D h/r LDLR (Table 2). As these results suggest that binding of LDL to the co-expressing cells was mediated by Y807C LDLR but not by I189D h/r LDLR, we further studied whether co-presence of the binding-defective I189D h/r LDLR could affect the binding kinetics of LDL to the co-expressing cells. When specific binding of ^{125}I-labeled LDL was analyzed with the LBxE-5 cell by incubation with several concentrations of ^{125}I-labeled LDL at 4°C, a linear Scatchard plot with an apparent dissociation constant (k_d) of 6.8 μg/ml was obtained. This value was indistinguishable from those of WT cell and ED cell expressing solely WT LDLR and Y807C LDLR, respectively, i.e., 7.0 and 9.8 μg/ml. These results suggest that the enhanced LDL internalization in the co-expressing cells

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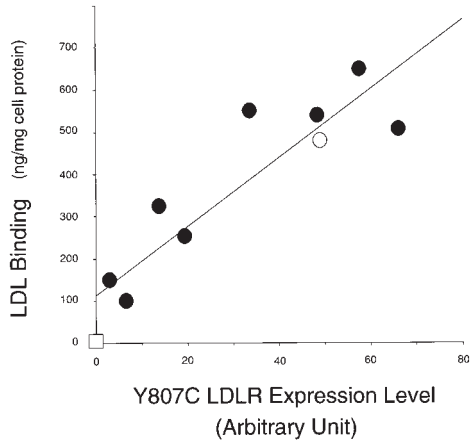

Fig. 5. Relation between the surface binding of ^{125}I-labeled LDL and the expression levels of Y807C LDLR in the ED cell (open circle), LBD cell (open square), and 8 cell lines co-expressing two mutant receptors (closed circles). Each value of surface binding of ^{125}I-labeled LDL was taken from the data in Table 2 and represents the mean of triplicate experiments. The expression levels of LDLRs are shown in arbitrary units based on the quantitative analysis of the immunoblots with NIH Image.

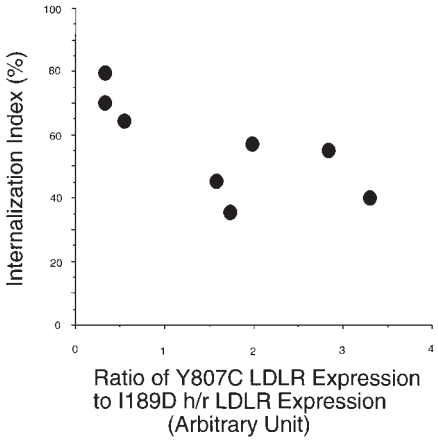

Fig. 6. Relation between internalization index for ^{125}I-labeled LDL and expression ratio of Y807C LDLR to I189D h/r LDLR in 8 cell lines co-expressing two mutant receptors. The values of internalization index and LDLR expression ratio were taken from Table 2.

was caused by the elevation of the endocytosis efficiency of Y807C LDLR and that binding of LDL to Y807C LDLR was not affected by the co-existence of I189D h/r LDLR. Among the cell lines expressing similar levels of I189D h/r LDLR (e.g., LBxE-3,LBxE-6 and LBxE-8), lower expression of Y807C LDLR was associated with higher internalization index (Table 2). Internalization index values were correlated inversely to the expression ratio of Y807C LDLR to I189D h/r LDLR as presented in arbitrary units (**Fig. 6**).

DISCUSSION

In this study, we established stably transfected hamster *ldlA-7* cell lines co-expressing two mutant LDLRs with a distinct functional defect and demonstrated that the molecular defect of Y807C LDLR in coated pit-mediated endocytosis was compensated by co-expression with I189D h/r LDLR. In LBD cell expressing I189D h/r LDLR only, the receptor was endocytosed efficiently, as the motif for endocytosis was not disrupted in I189D h/r LDLR, whereas very little amount of LDL was bound and internalized because of the defect in ligand binding (4, 5) (Fig. 3a, Table 2). In contrast, in ED cell expressing Y807C LDLR solely, very few receptors were internalized, although LDL binding was not prohibited (Fig. 3e, Table 2). This observation was compatible with a previous report that the structural motif required for recognition by adapter molecules of clathrin-coated pits such as AP-2 is disrupted in Y807C LDLR (10, 32).

The striking feature was that in the cells co-expressing both receptors, Y807C LDLR was found not only on the cell surface but also in the perinuclear region (Fig. 3f). We reasoned that this was caused by enhanced internalization of Y807C LDLR based on the following grounds. First, IgG-HL1 and rhodamine-labeled secondary antibody did not detect I189D h/r LDLR (Figs. 2A, 3d). When the LBxE-3 cell was applied to immunostaining without permeabilization, the perinuclear region could not be labeled with IgG-HL1 and rhodamine-labeled secondary antibody. Second, when examined by confocal microscopy, the labeling of Y807C LDLR in the perinuclear region was merged with that of I189D h/r LDLR (Fig. 4c). Taken together, these results suggest that a larger number of Y807C LDLR were internalized in the LBxE-3 cell than in the ED cell. When the LBxE-3 cell was examined by immunoelectronmicroscopy, Y807C LDLRs were found in coated pits, in some of which I189D h/r LDLRs were also detected. It is therefore conceivable that, in the LBxE-3 cell, an increased number of Y807C LDLRs clustered in coated pits and were then internalized efficiently as were I189D h/r LDLRs (H. Yoshida, M. Yokode, A. Yamamoto, R. Masaki, T. Murayama, H. Horiuchi, and T. Kita, unpublished results).

To further investigate the itinerary of Y807C LDLR in the co-expressing cells, we studied the binding and uptake of LDL in these cells. As assessed by ^{125}I-labeled LDL internalization index, the ratio between incorporated and surface-bound LDL, ^{125}I-labeled LDL entered into the co-expressing cells on average 1.8 times more efficiently than into the ED cell expressing solely Y807C LDLR (Table 2) (16). In the co-expressing cells, surface binding of ^{125}I-labeled LDL was correlated to the expression level of Y807C LDLR (Fig. 5) but not to that of I189D h/r LDLR (Table 2). Also, it was noted that kinetics of LDL binding to the co-expressing cell was indistinguishable from that to the ED cell or the WT cell. As I189D h/r LDLR had minimal capability to bind LDL (Table 2), it was conceivable that the increase in internalization index of the ^{125}I-labeled LDL was mediated by enhanced endocytosis of Y807C LDLR and that the kinetics of LDL binding to Y807C LDLR was not affected by co-presence of I189D h/r LDLR. As it is known that the binding of LDLR and LDL particle occurs with 1:1 molar stoichiometry (32),

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one LDL particle is suspected to bind to one Y807C LDLR with the same affinity as to wild-type LDLR. When the three cell lines were subjected to immunocytochemical studies, the cells were grown in growth medium that did not contain LDL but did contain lipoprotein-deficient serum (LPDS). It is therefore conceivable that enhanced endocytosis of Y807C LDLR in the co-expressing cells occurs independently of the presence of LDL.

It has been known that many endocytosed membrane proteins including the LDLR appear in early endosomes and are recycled back to the plasma membrane. In the co-expressing cells, a time-course study showed that internalization and degradation of LDL increased linearly up to 12 h (data not shown). It is therefore likely that in these cells Y807C LDLR could be recycled back to the plasma membrane efficiently.

There are several possibilities to be proposed as the mechanism by which endocytosis of Y807C LDLR was enhanced in the co-expressing cell. Among those possibilities would be the complex formation of the two different receptors. van Driel et al. (14) treated hamster cells with chemical cross-linking agents and showed that the LDLRs on the cell surface could be associated into oligomer, most of which were dimer, by non-covalent bondage. Also, Heuser and Anderson (15) demonstrated by scanning electronmicroscopy that many of the LDL particles appeared to be present in groups of two particles, estimating that 50–70% of the LDL particles were in dimeric form. As it is known that the binding of LDLR and LDL particle occurs with 1:1 molar stoichiometry (32), these results suggest that each LDL dimeric cluster bound to the two LDLRs associated closely with each other. In this study, the internalization index was inversely correlated to the relative expression ratio of Y807C LDLR to I189D h/r LDLR presented in arbitrary units (Fig. 6, Table 2). These observations would suggest that Y807C LDLR and I189D h/r LDLR could be associated together into oligomeric forms in the co-expressing cells and that Y807C LDLR was internalized by the cell accompanied by I189D h/r LDLR. Although the mechanism for oligomer formation of LDLR is to be studied further, it may be mediated by a non-covalent bond as was proposed by van Driel et al. (14).

In order to present direct evidence of the complex formation of Y807C LDLR and I189D h/r LDLR, we treated LBxE-2 cells co-expressing both mutant LDLRs with a cross-linking agent DTSSP (3,3′-dithiobis sulfosuccinimidylpropionate) according to the method described by van Driel et al. (14). To isolate the Y807C LDLR-I189D h/r LDLR complex, we then subjected this cross-linked cell lysate to two sequential immunoprecipitations with HL-1 and RP2, but could not obtain the complex because of difficulty in dissociating the antigen from the antibody used for the first immunoprecipitation reaction. It is therefore to be determined whether the complex formation of the two receptors is responsible for the increase in endocytotic efficiency of Y807C LDLR.

The patients with familial hypercholesterolemia (FH) have an inborn genetic defect in the LDLR and show marked elevation of the plasma LDL concentration. In this report, we demonstrated that co-expression of two distinct defective LDLRs could result in a functional compensation presumably due to the oligomeric association of the receptor molecules. Provided that such an oligomer could be formed in the body, the molecular defect of the mutant LDLR could be compensated by the wild-type receptor in some of the FH heterozygote subjects. Among FH heterozygotes, some subjects show unexpectedly low plasma LDL-cholesterol levels. Although other genetic backgrounds that could govern lipoprotein metabolism may be involved in the clinical feature, heterooligomer formation between mutant and wild-type LDLR would be one of the possible mechanisms for the failure of a marked increase in the plasma cholesterol levels of these subjects. Most of the studies on LDLR thus far have been based upon the experiments in which a single type of mutant LDLR gene was introduced into cultured cells or transgenic animals. Such experimental systems have not been able to take account of interaction between the two distinct LDLRs, the result of which might have been inconsistent with the clinical feature of some FH subjects.

In summary, we demonstrated that the co-expression of I189D h/rLDLR in *ldl-A7* CHO cell could improve the endocytosis efficiency of Y807C LDLR but had no effect on the manner of LDL binding to either receptor. Although we could not show Y807C LDLR-I189D h/r LDLR oligomer complex directly, these results strongly suggest that LDLRs could exist being associated to form an oligomer during coated pit-mediated endocytosis. As different types of LDLRs expressed in FH heterozygotes and compound heterozygotes, it would be of importance to elucidate the biological role of co-expression of two kinds of LDLRs in the pathophysiological interpretation of these patients.

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