39-kDa receptor-associated protein (RAP) facilitates secretion and ligand binding of extracellular region of very-low-density-lipoprotein receptor: implications for a distinct pathway from low-density-lipoprotein receptor

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We have expressed the extracellular regions of the low-density-lipoprotein (LDL) receptor and the very-low-density-lipoprotein (VLDL) receptor, along with the full-length forms of the receptors, in insect cells in a baculovirus system. The extracellular region of the LDL receptor has been secreted successfully into the culture medium, and it retained the capacities of binding ¹²⁵I-labelled LDL and β -VLDL. In contrast, the extracellular region of the VLDL receptor remained intracellular and it did not bind ¹²⁵I- β -VLDL. This difference in expression behaviour between the homologous regions of the two receptors suggests that the two receptor systems are different in receptor-protein maturation or protein targeting. Next we developed the co-expression system with 39-kDa receptor-associated protein (RAP). This co-expression facilitated the secretion of the extracellular region of the

VLDL receptor into the culture medium and the secreted receptor bound ¹²⁵I- β -VLDL. The VLDL receptor remaining intracellular that was co-expressed with RAP also showed binding capacity to ¹²⁵I- β -VLDL, implying that the existence of RAP prevented receptor-protein aggregation or improved protein-folding status of the truncated VLDL receptor. On the other hand, expression of the extracellular region of the LDL receptor was not facilitated by RAP co-expression. Thus RAP plays an essential role in maintenance of the active conformation and secretion of the extracellular region of the VLDL receptor.

Key words: chaperone, co-expression, LDL receptor, VLDL receptor.

INTRODUCTION

Plasma lipoprotein particles are circulated in the bloodstream to target tissues, where they are recognized by specific receptors and incorporated into cells via receptor-mediated endocytosis. The low-density-lipoprotein (LDL) receptor is the best-characterized receptor [1]. In the last decade, several members of the LDL-receptor gene superfamily have been discovered. These include LDL-receptor-related protein (LRP) [2], glycoprotein 330 (gp330) [3], very-low-density-lipoprotein (VLDL) receptor [4] and apolipoprotein (apo) E receptor 2 [5], or its chicken homologue, LR8 [6]. Although the biological functions of these homologues may not be so clear as those of the LDL receptor, the VLDL receptor and LRP can bind and internalize diverse ligands, such as urokinase complexed with plasminogen activator-inhibitor type I [7], thrombospondin [8] and lipoprotein lipase [9] (for review, see [10,11]). In addition, 39-kDa receptor-associated protein (RAP), which was discovered during purification of LRP [12], also binds to LRP and the VLDL receptor. RAP competes for the binding of all known ligands to LRP. RAP has been proposed to prevent the aggregation and subsequent degradation of LRP [13,14] and/or to facilitate the receptor-protein folding [14,15]. RAP is also used as a new experimental ligand for the LDL-receptor superfamily [16,17].

Among the members of the LDL-receptor gene superfamily, we have selected the VLDL receptor and the LDL receptor [10,18] as model systems. These two receptors share five domain structures: a ligand-binding domain, an epidermal-growth-factor (EGF) precursor homology domain, an O-linked sugar domain, a transmembrane domain and a cytoplasmic tail. Despite this

similarity in the domain structure, they show distinctive characteristics, as follows. The LDL receptor binds apoB- and/or apoE-containing lipoproteins, LDL and β -VLDL, whereas the VLDL receptor binds only the apoE-containing lipoprotein, β -VLDL. It is known that the two receptors are different in tissue distribution and regulation of gene expression. In addition, the VLDL receptor binds RAP with high affinity ($K_d = 0.7$ nM) [16], whereas the LDL receptor binds RAP with low affinity ($K_d = 250$ nM) [17].

In this study, we have tried to express the extracellular domains of the LDL receptor and the VLDL receptor in soluble forms in a baculovirus system. The two homologous domains of the receptor proteins were different in their expression manners: the extracellular domain of the LDL receptor was secreted into the culture medium, whereas that of the VLDL receptor remained intracellular. This phenomenon has led us to the co-expression experiment of the VLDL receptor with RAP. Here, we have succeeded in producing a soluble and active form of the VLDL receptor with RAP co-expression and we discuss a role of RAP for the expression of the VLDL receptor.

EXPERIMENTAL

Construction of baculovirus transfer vectors

Full-length LDL receptor (pLDLR-F)

The 2.9-kb *Xba*I/*Sma*I fragment of pLDLR4 [19] was subcloned into the same sites in pUC19 (pUCLDLR). A cassette formed by annealing two oligonucleotides (5'-TCGAGACAGATGGTCA-GTCTGGAGGATGACGTGGCGCATCACCATCACCATC-

Abbreviations used: RAP, receptor-associated protein; LDL, low-density lipoprotein; LRP, LDL-receptor-related protein; VLDL, very-low-density lipoprotein; EGF, epidermal growth factor; GST, glutathione S-transferase; apo, apolipoprotein; mAb, monoclonal antibody.

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ACTAATAGCGGCCGCCCC-3' and 5'-GGGGCGGCCGC-TATTAGTGATGGTGATGGTGATGCGCCACGTCATCC-TCCAGACTGACCATCTGTC-3') was inserted into the *XhoI*/*SmaI* sites in pUCLDLR (pUCLDLRHis). Then the 2.6-kb *XbaI*/*SmaI* fragment of pUCLDLRHis was subcloned into the same sites in the baculovirus expression vector pVL1392 (PharMingen, San Diego, CA, U.S.A.).

Truncated LDL receptor (pLDLR-S1H)

The 2.9-kb *XbaI*/*SmaI* fragment of pLDLR4 was subcloned into the same sites in pBluescript II KS(−) (pBSLDLR). The following primers were used for PCR with pUCLDLR as a template: 5'-CCCACTCGCCCAAGTTTACCTGCGC-3' for the upstream primer and 5'-TCCCCGGGCGGCCGCTATTAGTGATGGTGATGGTGATGGGCGCACTGCAGCCTCAGCCTCTGTGA-3' for the downstream primer of pLDLR-S1H. pLDLR-S1H was generated by a triple ligation of *SphI*/*NotI*-digested PCR fragment and a 2.2-kb *XbaI*/*SphI* fragment from pBSLDLR with an *XbaI*/*NotI*-digested pVL1393 vector. This truncated LDL-receptor cDNA sequence encoded the first 720 amino acids (including the signal sequence) [19].

Full-length VLDL receptor (pVLDLR-F)

A 70-bp *PstI*/*SmaI* fragment and a 3.2-kb *SmaI*/*BamHI* fragment isolated from pHVR2 α [4] were subcloned into *PstI*/*BamHI*-digested pVL1392 to generate pVLhVR. To add the (His)₆ tag at the C-terminus, the following primers were used to generate PCR products with pVLhVR as a template: 5'-GCAGTATCAGAG-GTCAGTGTTCCCCC-3' for the upstream primer and 5'-TCCCCGGGTCTAGACTATCAATGATGATGATGATGATGAGCTAGATCATCATCTGTGC-3' for the downstream primer of pVLDLR-F. Amplified products were digested with *BglII* and *XbaI*, and ligated into the corresponding sites of pVLhVR.

Truncated VLDL receptor (pVLDLR-S)

PCR was carried out with the following primers using pVLhVR as a template: 5'-CAGTCTATGGTGCCAATAAATTCAC-3' for the upstream primer and 5'-TCCCCGGGTCTAGACTATCAATGATGATGATGATGATGTTGACAGTCTCGGCC-ATTTTCCT-3' for the downstream primer of pVLDLR-S. Amplified products were ligated into pCRII (Invitrogen, Carlsbad, CA, U.S.A.) to generate pVLDLPCRII. An *NheI*/*BamHI* fragment of pVLDLPCRII was ligated into the corresponding sites of pVLhVR. This truncated VLDL-receptor cDNA sequence encoded the first 750 amino acids (including the signal sequence) [4].

Full-length RAP (pVL1392RAP)

Rat RAP cDNA containing the complete coding sequence [20] was digested with *XhoI* and filled in using Klenow fragment. Following the digestion with *XbaI*, a 1.5-kb fragment containing the complete coding sequence of RAP cDNA was isolated and subcloned into the *XbaI*/*SmaI* sites in the pVL1392 vector.

Cell culture and isolation of recombinant baculoviruses

Spodoptera frugiperda cells (Sf9 cells) were propagated either as suspension or monolayer cultures in TNM-FH (Grace's powder medium/0.4% yeastolate/0.4% lactalbumin hydrolysate/0.1% pluronic F-68; Life Technologies, Rockville, MD, U.S.A.), supplemented with 10% (v/v) heat-inactivated fetal calf serum at 27 °C. *Trichoplusia ni* cells (Tn5 cells) were cultured as

monolayers at 27 °C in serum-free medium SF900 II (Life Technologies). Recombinant baculoviruses were generated by co-transfecting Sf9 insect cells with 1 μ g of plasmid DNA and 0.1 μ g of linearized AcNPV baculovirus DNA (PharMingen) with lipofectin reagent (Life Technologies). After 6 days of incubation, the virus in the supernatant was titred and individual plaques were isolated by plaque purification. High-titre virus stocks were prepared in suspension cultures of Sf9 cells infected with 0.1 plaque-forming units per cell.

Expression of recombinant baculoviruses

Sf9 or Tn5 cells were grown in a monolayer culture to a density of (1–1.5) $\times 10^6$ cells/ml. These Sf9 or Tn5 cells were infected with recombinant virus at a multiplicity of infection of 10. Wild-type virus (AcNPVC2) was used as a control. At various intervals post-infection, cells were harvested. The media and the cell lysates were subjected to Western-blot analyses. Cells were solubilized by the addition of the ice-cold lysis buffer [20 mM Tris/HCl (pH 7.5)/100 mM NaCl/2 mM CaCl₂/1% (v/v) Triton X100/1 mM PMSF/10 μ g/ml leupeptin/10 μ g/ml benzamidine/10 μ g/ml trypsin inhibitor] and incubated on ice for 30 min. The cell extracts were centrifuged at 17000 g for 10 min and the supernatants were used as cell lysate.

Preparation of cell membranes

Sf9 cells were washed with PBS and suspended in 5 ml of buffer M (PBS containing 1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml benzamidine and 10 μ g/ml trypsin inhibitor). Cells were disrupted by 20 strokes of a Teflon homogenizer and centrifuged at 1000 g for 5 min. The supernatant was centrifuged at 100000 g for 1 h. The pellet was resuspended in buffer M and quick-frozen with liquid N₂ and stored at −70 °C until used.

Western-blot analyses

Media, cell lysate and cell membranes were separated by SDS/PAGE (7.5% gel). The loading samples did not contain reducing agents. The samples were not heated prior to application to gel. Separated proteins were electrotransferred on to a nitrocellulose membrane (Schleicher and Schuell, Keene, NH, U.S.A.), and the membrane was blocked with blocking buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/2 mM CaCl₂/1% (w/v) BSA/0.05% (v/v) Tween 20]. The membranes were incubated with the monoclonal antibodies (mAbs; 0.5 μ g/ml). Membranes were then washed three times with washing buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/0.05% (v/v) Tween 20] and probed with alkaline phosphatase-conjugated anti-mouse IgG (Promega). Colour development was carried out with a commercial immunological detection kit (Promega). The mAb C7 binds the first repeat in the ligand-binding domain of the LDL receptor [21]. The mAb 6B6 was raised against a 14 amino acid linker sequence flanked by the cysteine-rich repeats 5 and 6 in the ligand-binding domain of the VLDL receptor. Immunoblot analysis using polyclonal anti-RAP antibodies was performed as described previously [20], except that alkaline phosphatase-conjugated anti-rabbit IgG (Promega) was used as secondary antibody instead of ¹²⁵I-labelled goat anti-rabbit IgG.

Labelling of lipoproteins

Rabbit β -VLDL and human LDL were prepared as described [22]. Labelling of lipoproteins with ¹²⁵I was performed using the iodine monochloride method, as described in [22]. ¹²⁵I-Labelled lipoproteins were dialysed against 150 mM NaCl containing 0.25 mM EDTA (pH 7.4). Ligand blotting was carried out as

described in [23] except that ^{125}I -labelled lipoproteins were used as ligands.

Ligand blotting with glutathione S-transferase (GST)–RAP

GST–RAP fusion protein purified with glutathione agarose [20] was dialysed against PBS. For ligand-blotting experiments using GST–RAP, blotting was performed as described in the Western-blot analyses section except that GST–RAP, anti-GST antibodies and horseradish peroxidase-labelled anti-goat IgG were used. Briefly, following transfer of separated proteins on to a nitrocellulose membrane, the membrane was blocked with blocking buffer [50 mM Tris/HCl (pH 7.5)/150 mM NaCl/1% (w/v) BSA/0.05% (v/v) Tween 20] in the presence of 2 mM CaCl_2 . Then the nitrocellulose membrane was incubated for 1 h in blocking buffer containing 2 mM CaCl_2 and either 18 nM GST–RAP or GST only (control). After being washed, the membranes were incubated for 1 h in blocking buffer containing 1000-fold-diluted polyclonal anti-GST antibodies (Amersham Pharmacia Biotech). Following the wash, the membranes were incubated with horseradish peroxidase-conjugated anti-goat IgG.

RESULTS

Expression of the LDL receptor in insect cells

Figure 1 shows a schematic drawing of the transfer vectors used in the baculovirus-infection experiments. pLDLR-S1H contains the human LDL receptor cDNA corresponding to the ligand-binding domain and the EGF precursor homology domain. pLDLR-F encoded all domains. To the 3'-ends of LDLR-S1H and LDLR-F, six consecutive histidine codons are added. The resulting baculoviruses are termed LDLR-S1H and LDLR-F, respectively.

Immunoblotting analyses and ligand-blotting experiments of the LDL receptors

The mAb C7 was used to monitor the expression of receptor proteins. Sf9 cells infected with LDLR-F produced a 110-kDa band, as shown in lane 3 of Figure 2(A). This band was also detected in the membrane fraction, as shown in lane 1 of Figure 2(B). The full-length receptor showed the doublet bands in lane 3 of Figure 2(A) and lane 1 of Figure 2(B). The two bands seen in Figures 2A and 2B may represent differently glycosylated forms of the receptors. As a baculovirus for the full-length LDL

Figure 1 Construction of expression plasmids

A schematic diagram showing the constructs used in this study. Full-length (pLDLR-F or pVLDLR-F) and truncated receptors that encoded the ligand-binding and EGF precursor homology domains (pLDLR-S1H or pVLDLR-S) were expressed under the control of polyhedrin promoter in insect cells. (His)₆ tags were added to the C-termini of the expressed receptors.

Figure 2 Expression of recombinant LDL receptors in insect cells

(A) Western-blotting analysis of full-length and truncated LDL receptors. Sf9 cells were infected at 2.4×10^6 cells/ml with a multiplicity of 10 plaque-forming units/cell for each virus. Cells were harvested 72 h post-infection, and then cell lysates (c) and medium (m) were loaded on to a 7.5% SDS/polyacrylamide gel and analysed with mAb C7. Lanes 1 and 2, medium and cell lysate from cells infected with LDLR-S1H (S1H); lane 3, cell lysate from cells infected with LDLR-F (F); lanes 4 and 5, medium and cell lysate from cells infected with wild-type baculovirus AcNPVC2 (WT). Samples of lysates and medium represented 8.0×10^5 and 4.8×10^4 cells, respectively. (B) Western-blotting analysis of crude membrane fractions of cells infected with the recombinant virus for the full-length LDL receptor. The crude membrane fraction from Sf9 cells infected either with LDLR-F (lane 1) or AcNPVC2 (lane 2) was loaded on to a 7.5% SDS/polyacrylamide gel and analysed with mAb C7. Each protein was used at $80 \mu\text{g/lane}$. (C) Ligand-blotting analysis of the recombinant LDL receptors with ^{125}I - β -VLDL. Medium or cell lysates from Sf9 cells infected with LDLR-S1H (lanes 1 and 2), LDLR-F (lane 3) and wild-type baculovirus AcNPVC2 (lanes 4 and 5) were subjected to SDS/PAGE (7.5% gel) under non-reducing conditions and transferred on to nitrocellulose. Nitrocellulose filters were incubated with ^{125}I - β -VLDL (1.0×10^3 c.p.m./ng). Autoradiography was performed for 3 h. Samples of lysates and medium represented 8.0×10^5 and 4.8×10^4 cells, respectively. (D) Ligand-blotting analysis of the recombinant LDL receptors with ^{125}I -LDL. Medium or cell lysate from Sf9 cells infected with LDLR-S1H (lane 1), LDLR-F (lane 2) and wild-type baculovirus AcNPVC2 (lanes 3 and 4) were subjected to SDS/PAGE (7.5% gel) under non-reducing conditions and transferred on to nitrocellulose filters. The filters were incubated with ^{125}I - β -LDL (2.0×10^2 c.p.m./ng). Autoradiography was performed for 56 h. Samples of lysates and medium represented 8.0×10^5 and 4.8×10^4 cells, respectively.

receptor without a histidine tag produced the recombinant protein in similar amounts (results not shown), the expression of the receptor protein was not affected by the existence of a C-terminal histidine tag.

We analysed the culture medium of Sf9 cells infected with LDLR-S1H by immunoblotting with mAb C7, as shown in Figure 2(A). The truncated form of the LDL receptors was detected as an 80-kDa band in the culture medium (lane 1 in Figure 2A). The medium of Sf9 cells infected with the wild-type

Figure 3 Expression of recombinant VLDL receptors in insect cells

(A) Western-blotting analysis of full-length and truncated VLDL receptors. Sf9 cells were infected at 2.4×10^6 cells/ml with a multiplicity of 10 plaque-forming units/cell. Cells were harvested 72 h post-infection, and medium (m) or cell lysates (c) were subjected to SDS/PAGE (7.5% gel) and analysed with mAb 6B6. Lanes 1 and 2, medium and cell lysate from cells infected with VLDLR-S (S); lane 3, cell lysate from cells infected with VLDLR-F (F); lanes 4 and 5, medium and cell lysate from cells infected with wild-type baculovirus AcNPVC2 (WT). (B) Western-blotting analysis of crude membrane fractions of full-length and truncated VLDL receptors: crude membrane fractions from (lane 1) Sf9 cells infected with VLDLR-S, (lane 2) Sf9 cells infected with VLDLR-F and (lane 3) Sf9 cells with wild-type AcNPVC2. Samples were loaded on to a 7.5% SDS/polyacrylamide gel and analysed with mAb 6B6. Samples of lysates and medium represented 8.0×10^5 and 4.8×10^4 cells, respectively. Each membrane protein was used at $80 \mu\text{g}/\text{lane}$.

virus (lane 4 in Figure 2A) as well as non-infected cells (results not shown) did not show any significant signals. The receptor proteins that remained within the cells exhibited broad and faster-migrating bands (lane 2 in Figure 2A). This pattern of electrophoresis is probably due to partial glycosylation.

To examine whether secreted LDL receptors maintain the capacity of binding ligands, ligand-blot analyses were performed using both ^{125}I -labelled β -VLDL (Figure 2C) and ^{125}I -labelled LDL (Figure 2D). The secreted form of the truncated LDL receptor (LDLR-S1H) bound both ^{125}I -labelled β -VLDL (lane 1 in Figure 2C) and ^{125}I -labelled LDL (lane 1 in Figure 2D) as well as the full-length LDL receptor (lane 3 in Figure 2C and lane 2 in Figure 2D). No significant signal was observed in the medium, nor in the lysate of Sf9 cells infected with a wild-type baculovirus (lanes 4 and 5 in Figure 2C, lanes 3 and 4 in Figure 2D). In cells infected with LDLR-S1H, the receptor protein remaining within the cells also showed the ability to bind ^{125}I -labelled β -VLDL (lane 2 in Figure 2C), although a difference in mobility on SDS/PAGE between the secreted and non-secreted forms was observed.

Expression of the VLDL receptor in insect cells

Since a truncated form of the LDL receptor was successfully secreted into the medium as an active protein, we prepared two expression vectors, pVLDLR-S and pVLDLR-F. The former encoded ligand-binding and EGF precursor homology domains and the latter encoded all domains, as shown in Figure 1. The resulting recombinant baculoviruses were termed VLDLR-S and VLDLR-F, respectively. Sf9 cells infected with VLDLR-F expressed polypeptides of 110 kDa, which reacted with mAb 6B6 (lane 3 in Figure 3A). The full-length form of the recombinant VLDL receptor was detected in the membrane fraction of Sf9 infected with VLDLR-F (lane 2 in Figure 3B). Unlike the LDL

Figure 4 Ligand-blotting analysis of recombinant LDL and VLDL receptors with GST-RAP

(A) GST-RAP blotting analysis. Media from Sf9 cells infected with LDLR-S1H (S1H, lane 1) or the crude membrane fraction from Sf9 cells infected with LDLR-F (F, lane 2), VLDLR-F (F, lane 3) or VLDLR-S (S, lane 4) were analysed by SDS/PAGE (7.5% gel) and transferred on to nitrocellulose. The filters were incubated with GST-RAP (18 nM) in the presence of 2 mM Ca^{2+} . After washing, filters were incubated with goat anti-GST antibodies followed by incubation with anti-goat horseradish peroxidase-conjugated antibodies. Each membrane protein was used at $80 \mu\text{g}/\text{lane}$. Samples of medium represented 4.8×10^4 cells. For comparison, Western-blotting analyses with mAbs C7 (B) and 6B6 (C) are shown. (B) Western-blotting analysis with mAb C7. Media from Sf9 cells infected with LDLR-S1H (lane 5) or the crude membrane fraction from Sf9 cells infected with LDLR-F (lane 6). (C) Western blotting analysis with mAb 6B6. The crude membrane fraction from Sf9 cells infected with either VLDLR-F (lane 7) or VLDLR-S (lane 8).

receptor, a truncated form of the VLDL receptor was not detected by mAb 6B6 in the culture medium of insect cells infected with VLDLR-S (lane 1 in Figure 3A), it remained within the cells (lane 2 in Figure 3A). Furthermore, the crude membrane fraction of Sf9 cells infected with VLDLR-S showed a reactivity with mAb 6B6 (lane 1 in Figure 3B). Because of the possible effect of overexpression in insect cells, the disorder of the translocation system may have resulted in the localization of this receptor in the crude membrane fraction, although this receptor did not contain the membrane-spanning domain. Thus the truncated form of the VLDL receptor was not secreted into the medium but remained intracellularly. We next examined whether this intracellular receptor could bind ^{125}I - β -VLDL, and found that could not (see below, lane 7 in Figure 6). Furthermore, the full-length VLDL receptor did not bind ^{125}I - β -VLDL (results not shown).

Ligand-binding experiments of GST-RAP to the LDL receptor and the VLDL receptor

Since the truncated VLDL receptor was expressed intracellularly and the full-length VLDL receptor did not show any reactivity with ^{125}I - β -VLDL, we next examined whether these receptors could bind GST-RAP. One membrane was blotted by GST-RAP, and an identical one was immunoblotted by mAbs specific to each receptor (Figures 4B and 4C). The truncated LDL receptor (LDLR-S1H, lane 1 in Figure 4A) showed a signal strength similar to that of the full-length LDL receptor (LDLR-F, lane 2 in Figure 4A). In the case of the VLDL receptor, the full-length receptor could bind GST-RAP, although it did not show any reactivity with β -VLDL. In contrast, the signal strength of the truncated VLDL receptor expressed intracellularly (lane 4 in Figure 4A) was much weaker than that of the full-length receptor (lane 3 in Figure 4A). These signals all disappeared in the presence of 10 mM EDTA (results not shown). A control experiment with the incubation of GST alone did not show any

Figure 5 Co-expression of RAP facilitates secretion of the truncated VLDL receptor

(A) Western-blotting analysis of co-expression of RAP with the truncated VLDL receptor (left-hand and centre panels). Sf9 cells were infected at 2.4×10^6 cells/ml with a multiplicity of 10 plaque-forming units/cell. Cells were harvested 72 h post-infection and medium or cell lysates were subjected to SDS/PAGE (7.5% gel) and analysed with mAb 6B6. Media were from cells infected with VLDLR-S alone (—, lane 1), co-infected with both RAP recombinant virus and VLDLR-S (+, lane 2) and infected with wild-type baculovirus AcNPVC2 (WT, lane 3). Cell lysates were from cells infected with VLDLR-S alone (lane 4), co-infected with both RAP recombinant virus and VLDLR-S (lane 5) and infected with AcNPVC2 (lane 6). Right-hand panel: Western-blotting analysis for expression of RAP. Cell lysates were analysed by SDS/PAGE (10% gel) under reducing conditions and Western blotting with anti-RAP antibodies. Cell lysates were from Sf9 cells infected with VLDLR-S alone (lane 7), co-infected with both RAP recombinant virus and VLDLR-S (lane 8) and infected with AcNPVC2 (lane 9). (B) Binding of GST-RAP and ^{125}I - β -VLDL to the secreted VLDL receptor. Media were from cells co-infected with both RAP recombinant virus and VLDLR-S (+RAP, lanes 1 and 3) and infected with AcNPVC2 (lanes 2 and 4). GST-RAP (left-hand panel) and ^{125}I - β -VLDL (right-hand panel) blotting are shown. Samples of lysates and medium represented 8.0×10^5 and 4.8×10^4 cells, respectively.

signal (results not shown). These results suggest that, although the binding status of the VLDL receptor with GST-RAP appeared to be different from that with β -VLDL, the truncated VLDL receptor, which remained intracellular, was produced as an inactive form.

Co-expression of the truncated VLDL receptor with RAP

To analyse whether co-expression of RAP can induce the secretion of the truncated VLDL receptor, we performed co-infection of RAP recombinant virus with VLDLR-S. Expression of RAP was confirmed by its antibody (lane 8 in Figure 5A). Sf9 cells co-infected with RAP recombinant virus produced the secreted VLDL receptor in the culture medium (lane 2 in Figure 5A). The similar effects of RAP co-expression on the secretion of the truncated VLDL receptor were observed in another cell line, Tn5 (results not shown). The intracellular receptor that was expressed with or without RAP was shown (lanes 4 and 5 in Figure 5A). No difference in signal strength was observed between them. We next examined the ligand-binding ability of this secreted VLDL receptor. Both GST-RAP (lane 1 in Figure 5B) and ^{125}I - β -VLDL (lane 3 in Figure 5B) bound to the secreted VLDL receptor. Ligand-binding ability of the truncated VLDL receptor that remained within the cell was also analysed by GST-RAP and ^{125}I - β -VLDL blots, as shown in Figure 6. For comparison, the immunoblots probed with mAb 6B6 are shown (lanes 1–3 in

Figure 6 Co-expression of RAP facilitates ligand binding of the intracellular truncated VLDL receptor

The crude membrane fractions from Sf9 cells infected with VLDLR-S alone (—, lanes 1, 4 and 7), co-infected with both RAP recombinant virus and VLDLR-S (+, lanes 2, 5 and 8) or infected with wild-type baculovirus AcNPVC2 (WT, lanes 3, 6 and 9) were analysed by SDS/PAGE (7.5% gel) and transferred on to nitrocellulose. Western blotting (left-hand panel), GST-RAP blotting (centre panel) and ^{125}I - β -VLDL blotting (right-hand panel) analyses are shown. Each membrane protein was used at $80 \mu\text{g}/\text{lane}$.

Figure 7 No enhancing effect of RAP co-expression on the expression of secreted and intracellular truncated LDL receptor

Western-blotting analysis for co-expression of RAP with the truncated LDL receptor (left-hand and centre panels). Sf9 cells were infected at 2.4×10^6 cells/ml with a multiplicity of 10 plaque-forming units/cell. Cells were harvested 72 h post-infection and medium or cell lysates were subjected to SDS/PAGE (7.5% gel) and analysed with mAb C7. Media were from cells infected with LDLR-S1H alone (—, lane 1), co-infected with both RAP recombinant virus and LDLR-S1H (+, lane 2) and infected with wild-type baculovirus AcNPVC2 (WT, lane 3). Cell lysates were from cells infected with LDLR-S1H alone (lane 4), co-infected with both RAP recombinant virus and LDLR-S1H (lane 5) and infected with AcNPVC2 (lane 6). Right-hand panel: Western-blotting analysis for expression of RAP. Cell lysates were subjected to SDS/PAGE (10% gel) under reducing conditions and analysed by Western blotting with anti-RAP antibodies. Cell lysates from cells infected with LDLR-S1H alone (lane 7), co-infected with both RAP recombinant virus and LDLR-S1H (lane 8) and infected with wild-type baculovirus AcNPVC2 (lane 9).

Figure 6). In GST-RAP blots, the signal strength of the truncated VLDL receptor produced by RAP co-expression was much stronger than that of the one without RAP co-expression (compare lanes 4 and 5 in Figure 6). Furthermore, ^{125}I - β -VLDL bound only to the truncated VLDL receptor that was co-expressed with RAP (lanes 7 and 8 in Figure 6).

Next we examined the effect of RAP co-expression on the expression of the truncated LDL receptor, as shown in Figure 7. Unlike the truncated VLDL receptor, RAP co-expression did not facilitate the expression of the truncated LDL receptor, either in the medium (lanes 1 and 2 in Figure 7) or in the cell-lysate preparation (lanes 4 and 5 in Figure 7).

DISCUSSION

LDL-receptor homologues have the ability to bind various biological molecules. There is a possibility that these LDL-

receptor homologues could be used as tools for modifying the biological pathways that are altered in pathological conditions such as inflammatory and neurological diseases. However, precise binding sites of these receptors for the ligands are still unknown. For the LDL receptor, extensive analyses of the N-terminal domain have indicated that the cysteine-rich repeats are responsible for the ligand binding [24,25]. The ligand-binding domain of the LDL receptor was reported to be secreted from transfected COS cells [26]. Recently, Simmons et al. have succeeded in the functional refolding of the ligand-binding domain of the LDL receptor produced in *Escherichia coli*. [27]. Their fragment binds apoE3. As an initial approach to understanding more about the ligand selectivity among lipoprotein receptors, we began by expressing the extracellular portions of the LDL receptor and the VLDL receptor, which included the EGF precursor homology domain in soluble forms. In the course of expression experiments, we have realized that these two receptors are different in their expression and secretion behaviours.

The extracellular region of the LDL receptor without the O-linked sugar domain was secreted successfully into the culture medium. This soluble LDL receptor bound both ^{125}I -labelled LDL and β -VLDL. On the other hand, no secretion of the truncated VLDL receptor was observed. One possible reason for this non-secretion is inefficient recognition of the truncated VLDL-receptor protein by protein-translocation machinery in insect cells. We therefore replaced the original signal sequence of the VLDL receptor with that of the LDL receptor. However, the truncated VLDL receptor carrying the LDL-receptor-derived signal peptide was not expressed (results not shown). Although the precise localization of intracellularly expressed receptor has not been determined, the VLDL receptor may be different from the LDL receptor in secretion pathways to the plasma membrane. There is another possibility, namely that the truncated VLDL receptor does not take an active conformation. Both the truncated and the full-length LDL receptors showed positive signals in RAP ligand blots. The full-length form of the VLDL receptor bound GST-RAP, although it failed to bind β -VLDL. A possible explanation for this observation might be that the full-length VLDL receptor was partially unfolded. In other words, RAP acting as a chaperone molecule may recognize the full-length VLDL receptor as being partially unfolded and bind it. The full-length form of the VLDL receptor showed a stronger signal than that of the LDL receptor, consistent with the report of Battey et al. [16]. However, the signal strength of the truncated form of the VLDL receptor expressed intracellularly was much weaker than that of the full-length form. Moreover, the truncated VLDL receptor did not bind ^{125}I - β -VLDL. Therefore, the truncated VLDL receptor that remained intracellular was inactive. By the co-expression with RAP, the extracellular region of the VLDL receptor was secreted efficiently into the medium and the secreted receptor acquired its ligand-binding capacity. However, RAP did not facilitate the LDL-receptor expression. Bu and Rennke [14] have reported that co-expression of RAP with unfolded anchor-free mini-receptors of LRP led to secretion of the soluble forms of the mini-receptors, consistent with our results. Thus similar to the case with LRP, it is suggested that RAP plays an important role in the expression of the VLDL-receptor extracellular region as a chaperone, i.e. prevention of premature ligand binding and/or direct promotion of correct folding. Consequently, the extracellular region of the VLDL receptor can be secreted as a soluble receptor.

Willnow et al. [13] have reported that VLDL-receptor processing was incomplete and the receptor remained in the endoplasmic reticulum of cardiac myocytes in homozygous RAP-

deficient mice. Notably, however, the VLDL-receptor processing in other organs was complete and the VLDL receptor expressed on the plasma membrane. Furthermore, the processing of the VLDL receptor was not affected by RAP overexpression in the heart of transgenic mice [13]. Thus there may be another factor that determines whether RAP is needed or not. In the case of LRP, overexpressed apoE accelerated LRP degradation. RAP presumably protects newly synthesized receptor molecules in endoplasmic reticulum from aggregation and subsequent degradation induced by ligand binding. It is possible that RAP or a RAP-like molecule is required for efficient functional expression of the VLDL receptor under some conditions in which apoE or unknown ligand(s) are prone to preoccupy newly synthesized receptor protein. We must also consider the difference between the expression systems. Although a simple interaction of RAP with the extracellular region of the VLDL receptor is thought to be observed in insect cells because they are expected to have few endogenous factors that affect the interaction, it should be determined whether intracellular retention of the truncated VLDL receptor is observed in other expression systems, such as Chinese hamster ovary cells or fibroblasts. We do not have any data on how RAP is involved in the biosynthetic pathway of the VLDL receptor. The association of RAP and intracellular VLDL receptor by immunoprecipitation or the precipitation using the Ni^{2+} -chelating agarose beads has not been probed, probably owing to the inefficiency of our antibody or to the extremely short period of existence of the complex. It remains to be elucidated whether other LDL-receptor homologues are controlled by an analogous mechanism. A preliminary experiment suggests that an extracellular region of apoE receptor 2, closely related to the VLDL receptor and expressed predominantly in the brain, cannot be secreted into culture medium itself (results not shown).

Recently, the molecular structure of the cysteine-rich repeat 5 in the ligand-binding domain of the LDL receptor was determined. Most of the negatively charged residues are co-ordinated with Ca^{2+} and are unavailable to interact with lipoproteins [28]. If each repeat can fold independently *in vivo*, the spatial arrangement or combination of cysteine-rich repeats possibly determines the ligand-binding specificity. Thus the overall conformation of extracellular regions, including the ligand-binding and the EGF precursor homology domains, or the tertiary structure of the ligand-receptor complex, should be determined further. In this context, the soluble LDL and VLDL receptors reported here would be valuable tools for structural studies.

In conclusion, RAP co-expression confers the efficient secretion and the ligand-binding capacity upon the truncated VLDL receptor. We speculate that the VLDL receptor is different from the LDL receptor in requirements of cellular component(s) that govern the receptor-protein maturation process or cellular trafficking.

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REFERENCES

- 1 Brown, M. S. and Goldstein, J. L. (1986) *Science* **232**, 34–47
- 2 Herz, J., Hamann, U., Rogne, S., Myklebost, O., Gausepohl, H. and Stanley, K. K. (1988) *EMBO J.* **7**, 4119–4127
- 3 Saito, A., Pietromonaco, S., Loo, A. K.-C. and Farquhar, M. G. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 9725–9729
- 4 Sakai, J., Hoshino, A., Takahashi, S., Miura, Y., Ishii, H., Suzuki, H., Kawarabayashi, Y. and Yamamoto, T. (1994) *J. Biol. Chem.* **269**, 2173–2182
- 5 Kim, D. H., Iijima, H., Goto, K., Sakai, J., Ishii, H., Kim, H. J., Suzuki, H., Kondo, H., Saeki, S. and Yamamoto, T. (1996) *J. Biol. Chem.* **271**, 8373–8380

- 6 Novak, S., Hiesberger, T., Schneider, W. J. and Nimpf, J. (1996) *J. Biol. Chem.* **271**, 11732–11736
- 7 Argraves, K. M., Battey, F. D., MacCalman, C. D., McCrae, K. R., Gåfvels, M., Kozarsky, K. F., Chappell, D. A., Strauss, III, J. F. and Strickland, D. K. (1995) *J. Biol. Chem.* **270**, 26550–26557
- 8 Mikhailenko, I., Krylov, D., Argraves, K. M., Roberts, D. D., Liao, G. and Strickland, D. K. (1997) *J. Biol. Chem.* **272**, 6784–6791
- 9 Takahashi, S., Suzuki, J., Kohno, M., Oida, K., Tamai, T., Miyabo, S., Yamamoto, T. and Nakai, T. (1995) *J. Biol. Chem.* **270**, 15747–15754
- 10 Krieger, M. and Herz, J. (1994) *Annu. Rev. Biochem.* **63**, 601–637
- 11 Strickland, D. K., Kounnas, M. Z. and Argraves, W. S. (1995) *FASEB J.* **9**, 890–898
- 12 Strickland, D. K., Ashcom, J. D., Williams, S., Battey, F., Behre, E., McTigue, K., Battey, J. F. and Argraves, W. S. (1991) *J. Biol. Chem.* **266**, 13364–13369
- 13 Willnow, T. E., Rohlmann, A., Horton, J., Otani, H., Braun, J. R., Hammer, R. E. and Herz, J. (1996) *EMBO J.* **15**, 2632–2639
- 14 Bu, G. and Rennke, S. (1996) *J. Biol. Chem.* **271**, 22218–22224
- 15 Bu, G. and Schwartz, A. L. (1998) *Trends Cell Biol.* **8**, 272–276
- 16 Battey, F. D., Gåfvels, M. E., Fitzgerald, D. J., Argraves, W. S., Chappell, D. A., Strauss, III, J. F. and Strickland, D. K. (1994) *J. Biol. Chem.* **269**, 23268–23273
- 17 Medh, J. D., Fry, G. L., Bowen, S. L., Pladet, M. W., Strickland, D. K. and Chappell, D. A. (1995) *J. Biol. Chem.* **270**, 536–540
- 18 Jingami, H. and Yamamoto, T. (1995) *Curr. Opin. Lipidol.* **6**, 104–108
- 19 Yamamoto, T., Davis, C. G., Brown, M. S., Schneider, W. J., Casey, M. L., Goldstein, J. L. and Russell, D. W. (1984) *Cell* **39**, 27–38
- 20 Herz, J., Goldstein, J. L., Strickland, D. K., Ho, Y. K. and Brown, M. S. (1991) *J. Biol. Chem.* **266**, 21232–21238
- 21 Beisiegel, U., Schneider, W. J., Goldstein, J. L., Anderson, R. G. W. and Brown, M. S. (1981) *J. Biol. Chem.* **256**, 11923–11931
- 22 Goldstein, J. L., Basu, S. K. and Brown, M. S. (1983) *Methods Enzymol.* **98**, 241–260
- 23 Daniel, T. O., Schneider, W. J., Goldstein, J. L. and Brown, M. S. (1983) *J. Biol. Chem.* **258**, 4606–4611
- 24 Esser, V., Limbird, L. E., Brown, M. S., Goldstein, J. L. and Russell, D. W. (1988) *J. Biol. Chem.* **263**, 13282–13290
- 25 Russell, D. W., Brown, M. S. and Goldstein, J. L. (1989) *J. Biol. Chem.* **264**, 21682–21688
- 26 Driel, I. R. V., Brown, M. S. and Goldstein, J. L. (1989) *J. Biol. Chem.* **264**, 9533–9538
- 27 Simmons, T., Newhouse, Y. M., Arnold, K. S., Innerarity, T. L. and Weisgraber, K. H. (1997) *J. Biol. Chem.* **272**, 25531–25536
- 28 Faas, D., Blacklow, S., Kim, P. S. and Berger, J. M. (1997) *Nature (London)* **388**, 691–693

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