Ligand Binding Properties of the Very Low Density Lipoprotein Receptor

ABSENCE OF THE THIRD COMPLEMENT-TYPE REPEAT ENCODED BY EXON 4 IS ASSOCIATED WITH REDUCED BINDING OF M$_r$ 40,000 RECEPTOR-ASSOCIATED PROTEIN

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The very low density lipoprotein receptor (VLDLR) binds, among other ligands, the M$_r$ 40,000 receptor-associated protein (RAP) and a variety of serine proteinase-serpin complexes, including complexes of the proteinase urokinase-type plasminogen activator (uPA) with the serpins plasminogen activator inhibitor-1 (PAI-1) and protease nexin-1 (PN-1). We have analyzed the binding of RAP, uPAPAI-1, and uPAPN-1 to two naturally occurring VLDLR variants, VLDLR-I, containing all eight complement-type repeats, and VLDLR-III, lacking the third complement-type repeat, encoded by exon 4. VLDLR-III displayed ~4-fold lower binding of RAP than VLDLR-I and ~10-fold lower binding of the most C-terminal one of the three domains of RAP. In contrast, the binding of uPAPAI-1 and uPAPN-1 to the two VLDLR variants was indistinguishable. Surprisingly, uPAPN-1, but not uPAPAI-1, competed RAP binding to both VLDLR variants. These observations show that the third complement-type repeat plays a crucial role in maintaining the contact sites needed for optimal recognition of RAP, but does not affect the proteinase-serpin complex contact sites, and that two ligands can show full cross-competition without sharing the same contacts with the receptor. These results elucidate the mechanisms of molecular recognition of ligands by receptors of the low density lipoprotein receptor family.

The low density lipoprotein receptor (LDLR) family comprises a group of multiligand endocytosis receptors with a characteristic domain structure. Besides LDLR, the known mammalian family members are α$_1$-macroglobulin receptor/low density lipoprotein receptor-related protein (α$_1$MR/LRP), gp330/megalin, and very low density lipoprotein receptor (VLDLR) (for reviews, see Refs. 1–3) and apolipoprotein E receptor-2 or LR8B (LDLR-relative with 8 binding repeats) (4, 5). All members of the family contain a C-terminal cytoplasmic domain with at least one copy of the sequence NPXY, directing internalization via clathrin-coated pits, followed by a single transmembrane domain. The N-terminal extracellular part of the receptors contains clustered complement-type repeats (CTRs) and epidermal growth factor (EGF) precursor homology domains, the latter consisting of multiple copies of cysteine-rich EGF-like repeats and spacers with the consensus sequence YWTD (for a review, see Ref. 2). The members of the family show distinct although partially overlapping binding specificities and bind with high affinity, Ca$^{2+}$-dependently, a variety of ligands like proteinases, proteinase-inhibitor complexes, apolipoproteins, extracellular matrix proteins, and viruses (for reviews, see Refs. 1, 2, 6, and 7). Ligand binding seems to be mediated by the CTR clusters (8–12).

VLDLR binds a variety of serine proteinase-serpin complexes (13–16), the proenzyme form of urokinase-type plasminogen activator (uPA) (14), apolipoprotein E-containing lipoproteins (17, 18), apo(a) (19), lipoprotein lipase (14, 20), and thrombospondin-1 (21). VLDLR contains one CTR cluster (positioned at the N terminus), two EGF-like repeats that are separated from a third one by a YWTD spacer region, an O-linked sugar domain, binds the MR/LRP and gp330/megalin (for reviews, see Refs. 1–3) and apolipoprotein E receptor-2 or LR8B (LDLR-relative with 8 binding repeats) (4, 5). All members of the family contain a C-terminal cytoplasmic domain with at least one copy of the sequence NPXY, directing internalization via clathrin-coated pits, followed by a single transmembrane domain. The N-terminal extracellular part of the receptors contains clustered complement-type repeats (CTRs) and epidermal growth factor (EGF) precursor homology domains, the latter consisting of multiple copies of cysteine-rich EGF-like repeats and spacers with the consensus sequence YWTD (for a review, see Ref. 2). The members of the family show distinct although partially overlapping binding specificities and bind with high affinity, Ca$^{2+}$-dependently, a variety of ligands like proteinases, proteinase-inhibitor complexes, apolipoproteins, extracellular matrix proteins, and viruses (for reviews, see Refs. 1, 2, 6, and 7). Ligand binding seems to be mediated by the CTR clusters (8–12).

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The M$_r$ 40,000 receptor-associated protein (RAP) binds with high affinity to α$_1$MR/LRP and gp330/megalin (for reviews, see Refs. 1 and 28), VLDLR (18, 29), and LR8B (5), but with only low affinity to LDLR (30, 31). The biological function of RAP may be that of a molecular chaperone in the endoplasmic reticulum (32–34). Mature human RAP consists of 323 amino acids.
Fig. 1. Schematic presentation of the structure of VLDLR. A, schematic showing the domain structure of VLDLR-I, VLDLR-II, VLDLR-III, and VLDLR-IV. Boxes 1-8 represent the ligand-binding complement-type repeats. Also indicated are the EGF precursor homology domain region with the EGF-like repeats A and B separated from repeat C by the YWTD spacer, the O-linked sugar domain (short bar), the transmembrane domain (long bar), and the cytoplasmic domain with the NPXY sequence (>). Exons encoding the respective regions are indicated above the presentation of the VLDLR-I domain structure. B, nucleotide and derived amino acid sequences around exon 4 of VLDLR-I, VLDLR-II, VLDLR-III, and VLDLR-IV.

acids (35). Due to internal sequence homology, a three-domain structure has been proposed (36, 37). The three-dimensional structure of the N-terminal domain was determined by NMR spectroscopy (38).

Many of the ligands binding to one and the same family member do not show any obvious structural similarity, and there is no general cross-competition between the different ligands. For example, α₂-macroglobulin-proteinase complexes and uPA-serpin complexes do not compete for binding to α₂-MR/LRP (42). RAP is able to inhibit the binding of all known ligands, whereas almost all other ligands do not inhibit RAP binding (for reviews, see Refs. 1 and 28). Equilibrium binding and competition patterns between different ligands have shown that different domains of RAP and of serine proteinase-serpin complexes make independent contacts with the receptors (15, 36, 39, 40, 41, 43–45). Thus, these receptors exhibit unique principles of molecular recognition.

To elucidate the mechanisms of molecular recognition of ligands by this receptor family, we have now compared the binding of RAP and of complexes between the serine proteinase uPA and the serpins plasminogen activator inhibitor-1 (PAI-1) and protease nexin-1 (PN-1) to the VLDLR variants VLDLR-I and VLDLR-III, differing with respect to the presence of CTR-3 (Fig. 1).

EXPERIMENTAL PROCEDURES

VLDLR cDNA Constructs—cDNA of the VLDLR types II and III was generated by PCR-based mutagenesis of phV58, the human VLDLR-I (full-length) cDNA cloned in phBluescript KS (22). VLDLR-II cDNA, lacking exon 16, was generated with primers 5'-CTG GAT CAG AGC TAG CCA CTC-3' (upstream primer), 5'-AGG AAA ATG GCC GAG ACT GTC AAA GTA ATG TGA CCA CAG CAG TAT-3' (upstream primer), and 5'-CTC TAT TGC CAT TGT CCC AAC CAT TGA A-3' (downstream primer). The NheI/HindIII fragment of phV58 was ligated into the EcoRI/HindIII sites of pBluescript KS. VLDLR-III cDNA, lacking exon 4, was generated with primers 5'-TTC TTC CTC CTT TCG GAA GGG CTG-3' (upstream primer), 5'-CAG ATG AAA GCC CAG AAC AGT GCC GCA ATA TAA CAT GAT GTC GTC CCG AGC-3' (mutagenesis primer), and 5'-GAC ACT CTT TCA GGG GCT G-3' (downstream primer). The NcoI fragment of phV58 was exchanged with the NcoI fragment of the PCR product. VLDLR-IV cDNA was generated by ligation of the VLDLR-III EcoRI/NheI fragment and the VLDLR-II NheI/HindIII fragment into the EcoRI/HindIII sites of pBluescript KS. The cDNAs of the VLDLR variants were excised from the cloning vectors by BamHI/ClaI digestion and cloned into the BglII/ClaI sites of expression vector pCMV4 (46). The intended changes were confirmed by nucleotide sequencing.

VLDLR Expression and Purification—CHO-ldla7 cells, a line of mutant Chinese hamster ovary cells lacking LDLR (47), were stably transfected with cDNA for the various VLDLR variants (13). The transfected cells were maintained in Ham's F-10 medium with 5% fetal bovine serum and 0.4 mg/ml G418. The expression of the correct VLDLR mRNA (and only the correct ones) by the transfected cells was confirmed by reverse transcription-PCR with primers spanning exons 4 and 16, respectively. The primers spanning exon 16 (VLR-I and VLR-II) were those described previously (27). PCR products of 377 and 293 base pairs (bp) were expected from mRNAs with and without exon 16, respectively. The primers spanning exon 4 (vle3 and vle6) were those described previously (27). PCR products of 390 and 266 bp were expected from mRNAs with and without exon 4, respectively. The transfected cells expressed mRNA giving the expected reverse transcription-PCR products, as shown in Fig. 2 in the case of VLDLR-I and VLDLR-III cDNA-transfected cells. The transfected cell lines expressed a VLDLR immunoreactivity on Western blots (see below) that was 10–20-fold higher than that of the parental cell line (data not shown).

Cell extracts were prepared by washing confluent cell monolayers with Tris-buffered saline (TBS; 50 mM Tris-HCl, pH 7.4, and 150 mM NaCl), scraping the cells from the culture dishes with a rubber policeman, pelleting the cells by centrifugation at 1000 × g, lysing the cells in ice-cold binding buffer (20 mM HEPS, 2.5 mM NaHPO₄, pH 7.4, 124 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl₂, and 1.2 mM MgSO₄) with 1% CHAPS and 1 mM phenylmethylsulfonyl fluoride using 0.5 ml of buffer/
skim milk powder in TBS with 0.5% Tween 20 (TBS-T), the wells were incubated with 100 μl of cell extract or solution of partially purified receptor, diluted 10-fold with TBS, for 3 h at room temperature. The wells were then incubated with radioactive and nonradioactive ligands in binding buffer with 0.5% bovine serum albumin. The cells were incubated for 16 h on ice. The media were then collected; the cells were washed with ice-cold binding buffer and solubilized with 1 M NaOH; and the radioactivity in the media and bound to the cells was determined. The cell densities were chosen in a range in which the amount of bound ligand did not exceed 15% of the amount added.

Mathematical Analysis of Receptor-Ligand Binding Data—Disassociation constants (K_d) for receptor-ligand binding were found by analyzing the concentration dependence of steady-state binding. The ratios between the concentrations of bound and free ligand (B/F) were determined with 1–10 pM radiolabeled ligand plus several concentrations of nonradioactive ligand. The B/F ratios were then plotted semilogarithmically versus the free ligand concentrations. The data were fitted by the method of least squares. (The equation B/F = (RL/[RL] + [L]) + C, derived under the assumption of a simple binding equilibrium R + L \rightleftharpoons RL. C is a constant representing non-displaceable, non-receptor binding. R represents the receptor, L the ligand, and RL the receptor-ligand complex. This procedure yields the K_d and [RL], values giving the best fit to the data.

In competition studies, a picomolar concentration of a radioactively labeled ligand was incubated with the receptors in the presence of varying concentrations of another, nonradioactive ligand. Assumptions include simple binding equilibria for both ligands, R + L \rightleftharpoons RL and R + 1 \rightleftharpoons RI.

Chemical Cross-linking—Affinity-purified VLDLR variants and 125I-RAP were dialyzed against 100 mM sodium citrate, pH 7.0, 150 mM NaCl, 2 mM CaCl_2, and 0.5% CHAPS. An ~0.5 nM concentration of each of the VLDLR variants was incubated with 4 nM 125I-RAP in a final volume of 50 μl for 16 h at 4 °C in the absence or presence of 50 μM EDTA. Then, 50 μl of 0.2% glutaraldehyde in 2 mM CaCl_2 was added, and the incubation was continued for an additional 1.5 h. After addition of 10 μl of 2 M glycine, incubation for another 1.5 h, and reduction of the volumes by vacuum centrifugation, the samples were subjected to SDS-PAGE and autoradiography.

RESULTS

Affinity Purification and Ligand Blot Analysis of VLDLR Variants—We purified each of the VLDLR variants VLDLR-I, VLDLR-II, VLDLR-III, and VLDLR-IV from CHO cells transfected with the corresponding cDNAs by affinity chromatography on a column of Sepharose 4B-immobilized RAP. The receptor yield was estimated to ~0.5–1.5% of the total CHAPs-extractable protein and was indistinguishable for the four receptor types, showing that all the variants were able to bind RAP. The purity of VLDLR-I and VLDLR-III is presented by a silver-stained gel (Fig. 3A), showing distinct bands with the same migration as the bands observed in immunoblot analysis with an antibody against the VLDLR C terminus, which is identical in the four variants (Fig. 3B).

The relative affinities of RAP and serine proteinase-serpin complexes for each of the four VLDLR variants were screened by ligand blot analysis of the affinity-purified preparations. The relative affinity of VLDLR protein of the different types was evaluated by immunoblot analysis. More than 95% of the binding of radiolabeled ligands in the position of VLDLR immunoactivity could be displaced by an excess of nonradioactive RAP (data not shown). With equal amounts of immunoreactivity of VLDLR-I and VLDLR-III (Fig. 3B), the signal
obtained with 20 pm $^{125}$I-RAP was ~5-fold stronger with VLDLR-I than with VLDLR-III (Fig. 3C). However, the signals obtained after incubation of each of the two variants with 20 pm $^{125}$I-uPA-PN-1 or 20 pm $^{125}$I-uPA-PAI-1 were indistinguishable (Fig. 3, D and E). VLDLR-II and VLDLR-IV showed binding properties identical to those of VLDLR-I and VLDLR-III, respectively (data not shown).

**Microtiter Well Binding Assays**—To confirm the above-mentioned results with receptors, which had not been exposed to the denaturing conditions used in the ligand blot analysis, we performed binding analysis with receptors immobilized in microtiter wells. A convenient immobilization procedure was coating of the wells with anti-VLDLR antibody, followed by binding of the receptors to the antibodies. Incubation of the antibody-coated wells with an excess of receptor over antibody was assumed to assure immobilization of equal amounts of receptor, irrespective of the exact concentration in the receptor-containing sample, allowing direct comparison of results obtained with the different receptor types. This assumption was shown to be true by demonstrating equal binding of $^{125}$I-RAP in two antibody-coated wells, successively incubated with the same receptor preparation (data not shown). As source of receptor, both purified preparations and crude cell extracts could be used, with indistinguishable results (data not shown). By this method, the receptor variants without CTR-3 were found to have an ~3-fold reduced $^{125}$I-RAP binding, whereas the binding of $^{125}$I-uPA-PN-1 was indistinguishable between the variants (Table I).

The same assay was used for studying the concentration dependence of steady-state binding of RAP to VLDLR-I and VLDLR-III in the concentration range 1–10$^5$ pm. The use of lower concentrations was limited by the specific activity of the radiolabeled RAP. The concentration dependence of binding did not deviate significantly from that expected from the simple binding reaction $R + L \rightleftharpoons RL$. The following $K_d$ values were found: VLDLR-I, 16.2 pm (range 8.7–34.7 pm, $n = 6$); and VLDLR-III, 79.4 pm (range 49.2–151.0 pm, $n = 3$), corresponding to an ~5-fold and statistically significant ($p < 0.001$) difference between the $K_d$ values. It was not possible to analyze whether the variants had different numbers of RAP-binding sites per receptor molecule by the whole cell binding assays, as the relative number of receptor molecules expressed on the cell surfaces of individual transfected cell lines could not be determined with sufficient accuracy by immunochemical methods.

**Table I**

| VLDLR variant | Binding of RAP | Binding of uPA/PN-1 |
|----------------|----------------|---------------------|
|                | %              | %                   |
| VLDLR-I        | 100            | 100                 |
| VLDLR-II       | 125 ± 16 ($n = 9$) | 113 ± 15 ($n = 6$) |
| VLDLR-III      | 41 ± 14 ($n = 11$) | 122 ± 19 ($n = 6$) |
| VLDLR-IV       | 39 ± 11 ($n = 11$) | 112 ± 20 ($n = 6$) |

**Binding of $^{125}$I-RAP and $^{125}$I-uPA/PN-1 to VLDLR variants**

The purified receptors were immobilized by the use of antibodies against their C terminus, coated onto the wells. The immobilized receptors were incubated for 16 h at 4°C with 20 pm $^{125}$I-RAP or 20 pm $^{125}$I-uPA/PN-1. The binding of each of the radioactively labeled ligands to each individual receptor variant is expressed as a percentage of the binding to VLDLR-I. Means ± S.D. and numbers of experiments ($n$) are indicated.
amounts of VLDLR-I and VLDLR-III loaded onto the gels were adjusted to result in equal signals with $^{125}$I-RAP as ligand. D3$^{R}$AP competed the binding of $^{125}$I-RAP to both VLDLR-I and VLDLR-III, but the competition was less efficient with VLDLR-III than with VLDLR-I, not only with 200 nM D3$^{R}$AP (Fig. 5B), but also with lower D3$^{R}$AP concentrations (data not shown). We did not find competition of RAP binding with D3$^{R}$AP, D2$^{R}$AP, or a fragment consisting of these domains together (D1D2$^{R}$AP). In contrast, a fragment consisting of the middle and C-terminal domains (D2D3$^{R}$AP) inhibited $^{125}$I-RAP binding to VLDLR-I and VLDLR-III (Fig. 6), with equal efficiency for the two receptor variants (data not shown).

We used the microtiter well assay to analyze quantitatively the competition of $^{125}$I-RAP binding by D3$^{R}$AP. We found that half-inhibition of the binding of 20 pM $^{125}$I-RAP required ~10-fold higher D3$^{R}$AP concentrations with VLDLR-III than with VLDLR-I (Fig. 7). The $K_r$ values estimated according to a simple competitive binding model were 0.3 and 3 nM for VLDLR-I and VLDLR-III, respectively.

**Cross-competition between RAP and $u$PA-Inhibitor Complexes**—Unexpectedly, 200 nM uPA-PN-1 competed the binding of 20 pm $^{125}$I-RAP efficiently, whereas 200 nM uPA-PAI-1 was unable to compete $^{125}$I-RAP binding detectably (Fig. 8).

**Chemical Cross-linking of VLDLR-RAP Complexes**—To analyze whether VLDLR contains multiple RAP-binding sites, we chemically cross-linked complexes of $^{125}$I-RAP and affinity-purified VLDLR variants. Fig. 9 shows an autoradiogram of SDS-PAGE of cross-linked complexes of $^{125}$I-RAP with VLDLR-I, VLDLR-II, and VLDLR-III, respectively. The faster migration of VLDLR-II was used as a control for identification of the complexes. As an additional control, we used the fact that EDTA could inhibit complex formation, in agreement with the $Ca^{2+}$ dependence of the binding. The $M_r$ values of the complexes, estimated on the basis of their migration on the gel, corresponded to one RAP molecule binding to each VLDLR molecule.

**DISCUSSION**

We have compared the ligand binding properties of VLDLR variants resulting from translation of an mRNA with all known exons and of an mRNA without exon 4, encoding CTR-3. We found that VLDLR variants without CTR-3 bound RAP weaker than those with CTR-3, whereas all variants displayed indistinguishable binding of the serine proteinase-serpin complexes uPA-PAI-1 and uPA-PN-1. Our results are compatible with the notion that the CTR cluster is implicated in ligand binding, as expected from results with other receptors of the family. Our work represents the first study of the influence of individual CTRs on the ligand binding properties of VLDLR. Our findings suggest that also other ligands may have different affinities for VLDLR variants with and without CTR-3. VLDLR mRNA without exon 4 has so far only been shown to occur naturally in brain (26) and in a neuroblastoma cell line (27). The following points strongly argue against the possibility that the lower RAP binding of the VLDLR variants without CTR-3 is an artifact due to abnormal folding of some of the molecules, for instance as a result of scrambling of disulfide bridges. 1) Differences in RAP binding could be demonstrated with receptors directly on the surface of intact cells. 2) The binding of the two serine proteinase-serpin complexes was indistinguishable, despite the different binding of RAP. 3) The residual RAP binding displayed by VLDLR-III had properties...
different from those of the RAP binding by VLDLR-I since it was more weakly competed by D3\textsuperscript{RAP}. 4) VLDLR-III could be purified by affinity chromatography with immobilized RAP in a high yield, excluding the possibility that the low binding was due to a large fraction of the VLDLR-III molecules being in a misfolded state unable to bind to RAP. We are therefore confident that our data allow us to conclude that the weaker RAP binding of VLDLR-III is a property of a native, properly folded protein produced by translation of the mRNA without exon 4.

The observation that VLDLR-I and VLDLR-III show different RAP binding, but indistinguishable serine proteinase-serpin complex binding, strongly suggests that different contributions by different CTRs to binding of different ligands are an important determinant of ligand binding specificity of VLDLR and other LDLR family members. An additional important, previously reported observation is that ligand binding involves multiple contacts between ligand and receptor. This observation was made by analyses of the binding of individual RAP domains to VLDLR-I and VLDLR-II by D3\textsuperscript{RAP}, and of other serine proteinase-serpin complexes to α\textsubscript{a}MR/LRP and VLDLR (15, 45). The most simple binding model accounting for both the observations of the contributions from different CTRs and the multicontact hypothesis is one in which each of the multiple contact sites is localized in a particular CTR. In the present case, this model would imply that a direct contact between VLDLR CTR-3 and RAP contributes strongly to RAP binding. An alternative binding model is one in which binding of different ligands depends in different ways on receptor conformation, the different binding properties of VLDLR-I and VLDLR III being due to a conformational effect of CTR-3, resulting in presentation of different RAP-binding surfaces in the two variants. This model would also imply that the spatial arrangement of the CTRs determining the binding surface for the proteinase-serpin complexes is unaffected by the lack of CTR-3.

Along the same line, RAP binding could result in a change in the spatial arrangement of the CTRs, with RAP stabilizing a receptor conformation preventing the exposure of binding sites for other ligands, in agreement with previous proposals for the RAP-α\textsubscript{a}MR/LRP interaction (36, 39, 51, 52). The two models are not mutually exclusive. With both, a combined contribution from several CTRs to a multicontact binding of each ligand would explain how the same receptor can exhibit high affinity binding of several, structurally unrelated ligands and how the variations in amino acid sequence in CTRs in different receptors can give rise to different, but overlapping binding specificities. This proposal is in agreement with a previous hypothesis by Russell et al. (9), working with LDLR.

A second important observation described here is the ability of uPA-PN-1 to compete RAP binding to VLDLR. RAP competes the binding of all known ligands to α\textsubscript{a}MR/LRP, gp330/megalin, and VLDLR (for a review, see Ref. 2). In contrast, the only other

**FIG. 6. Ligand blot analysis of competition of RAP binding to VLDLR variants by individual RAP domains.** Parallel samples of VLDLR-I and VLDLR-III were subjected to SDS-PAGE and transferred to PVDF filters. The amounts of VLDLR-I and VLDLR-III were adjusted to result in equal signals after incubation with 20 pm 125\textsuperscript{I}-RAP. Parallel blots were incubated with 20 pm 125\textsuperscript{I}-RAP and 200 nm nonradioactive D\textsubscript{1}R\textsuperscript{RAP}, D\textsubscript{2}R\textsuperscript{RAP}, D\textsubscript{3}R\textsuperscript{RAP}, and D\textsubscript{2}R\textsuperscript{RAP}, as indicated. Bound radioactive ligand was visualized by autoradiography.

**FIG. 7. Concentration dependence of competition of 125\textsuperscript{I}-RAP binding to VLDLR-I and VLDLR-III by D3\textsuperscript{RAP} as estimated by microtiter well assay.** Affinity-purified VLDLR-I and VLDLR-III were immobilized in microtiter wells coated with rabbit polyclonal antibodies against the VLDLR C terminus and incubated with 20 pm 125\textsuperscript{I}-RAP and the indicated concentrations of nonradioactive D3\textsuperscript{RAP}. In A, the ratio between bound and free 125\textsuperscript{I}-RAP (B/F) was plotted semilogarithmically versus the concentration of D3\textsuperscript{RAP}. In B, the B/F ratios in the absence of D3\textsuperscript{RAP} were set equal to 100%, and the B/F values at the various D3\textsuperscript{RAP} concentrations are expressed as a percentage thereof. Each data point shows the mean ± S.D. for three determinations. The lines drawn were obtained by fitting the data by the method of least squares to the equation B/F = [RL]/[L] = ([RL]/[K\textsubscript{f} + [L]]) + C, where [K\textsubscript{f}] = 9 pm (see "Results") and [L] = 20 pm. The estimated K\textsubscript{f} values for VLDLR-I and VLDLR-III were 0.3 and 3 nm, respectively. The estimated total receptor concentration ([RL]) was 3.6-fold lower with VLDLR-III than with VLDLR-I. This experiment is one out of a total of two.
RAP binding by D3RAP were in agreement with those predicted by the simple binding model. Moreover, the whole cell binding assays showed an ~5-fold difference in apparent $K_d$ for RAP binding to VLDLR-I and VLDLR-III, and the microtiter well binding assay showed an ~10-fold difference in the apparent $K_d$ for competition of RAP binding to VLDLR-I and VLDLR-III by D3RAP, indicating a 10-fold difference in $K_d$ for D3RAP binding. The latter observations were in agreement with our $a$ priori expectancy from the simple binding model of the different RAP binding resulting from differences in $K_d$. But we were unable to detect a difference in the apparent $K_d$ for RAP binding to VLDLR-I and VLDLR-III in the microtiter well binding assay, suggesting that the difference in $K_d$ was <3-fold under these conditions and thus smaller than the difference observed in the whole cell binding assay. Differences concerning $K_d$ determinations by whole cell and in vitro binding assays have also been observed with other receptors (see, for instance, Ref. 53) and are most likely caused by differences in the microenvironment rather than differences in the receptors themselves. In the present case, this would mean that the same structural difference between the two VLDLR variants would have different consequences for the difference in apparent $K_d$ in different microenvironments. The difference in $K_d$ for RAP binding to the variants was also considerably smaller than the difference in $K_d$ for D3RAP binding. This observation can be explained by an assumption of the N-terminal two and the C-terminal one of the three domains of RAP, respectively, making independent binding contacts with VLDLR and only the contact of the C-terminal domain being affected by the lack of CTR-3. This is in agreement with the observation that D2D3RAP is a better competitor of RAP binding to VLDLR-III than D3RAP. At the moment, however, we cannot explain the unexpected observation that the total number of RAP-binding sites (IR) found with equal amounts of VLDLR-I and VLDLR-III differed ~3-fold in the microtiter well binding assay. A possible explanation is that two out of a total of three RAP-binding sites per receptor molecule were inactivated by the lack of CTR-3, but although multiple RAP-binding sites per $a$MR/LRP molecule have been reported (54, 55), our chemical cross-linking experiment gave no indications of more than one RAP-binding site per VLDLR-I or VLDLR-III molecule (Fig. 9). Alternatively, the observed [IR] difference could be due to both VLDLR-I and VLDLR-III existing in two conformations with a considerable difference in $K_d$ for RAP and D3RAP binding, with the high affinity conformation being much less abundant in VLDLR-III than in VLDLR-I, but both conformations having a RAP affinity high enough to cause retention on RAP-Sepharose columns. This model is in line with the above-mentioned proposal that CTR-3 affects the binding properties by affecting receptor conformation.

Conclusively, our data help to gain insight into the mechanisms by which this receptor class can bind many different ligands without obvious structural similarity, but each of which may make several independent contacts with the receptors and which do not show general cross-competition.

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FIG. 8. Ligand blot analysis of cross-competition between RAP and uPA-inhibitor complexes. Parallel samples with equal amounts of immunoreactive VLDLR-I and VLDLR-III were subjected to SDS-PAGE, transferred to PVDF filters, and incubated with 20 pm [125I]-RAP (left), 20 pm [125I]-RAP and 200 nm uPA-PAI-1 (center), and 20 pm [125I]-RAP and 200 nm uPA-PN-1 (right). Bound radioactive ligand was visualized by autoradiography.

FIG. 9. Chemical cross-linking of VLDLR-RAP complexes. VLDLR-I, VLDLR-III, and VLDLR-II were incubated with [125I]-RAP in the absence or presence of EDTA. Following cross-linking with glutaraldehyde, the samples were subjected to SDS-PAGE. The radioactivity in the gels was visualized by autoradiography with an exposure time of 1 h.

ligand so far reported to be able to inhibit RAP binding was lipoprotein lipase, in the case of $a$MR/LRP (40). The capability of uPA-PN-1 for competition of VLDLR-RAP binding may be related to its unusually high affinity for VLDLR, the $K_f$ being ~140 pm, ~6-fold lower than the $K_f$ for binding of uPA-PAI-1 (15). Since binding of RAP and uPA-PN-1 is differently affected by the lack of CTR-3, we must contend that two ligands can compete for binding without sharing the same contact sites.

Important aspects of VLDLR-I and VLDLR-III ligand binding could be explained by the simple binding model $R + L \rightleftharpoons RL$. Thus, both the concentration dependence of steady-state RAP binding and the concentration dependence of inhibition of
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