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Interaction of Fibrin with the VLDL Receptor: Further Characterization and Localization of the Fibrin-Binding Site

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Abstract

Our recent study revealed that fibrin interacts with the VLDL receptor (VLDLR) on endothelial cells through its β N-domains and this interaction promotes transendothelial migration of leukocytes and thereby inflammation. The major aims of the present study were to further characterize this interaction and localize fibrin-binding site in the VLDL receptor. To localize the fibrin-binding site, we expressed a soluble extracellular portion of this receptor, sVLDLR_{HT}, its N- and C-terminal regions, VLDLR(1–8)_{HT} and des(1–8)VLDLR_{HT}, respectively, and a number of VLDLR fragments containing various combinations of CR-domains, and confirmed their proper folding by fluorescence spectroscopy. Interaction of these fragments with the $(\beta 15-66)_2$ fragment corresponding to a pair of VLDLR-binding β N-domains of fibrin was tested by different methods. Our experiments performed by ELISA and surface plasmon resonance revealed that the VLDLR(1–8)_{HT} fragment containing eight CR-domain of VLDLR and its sub-fragments, VLDLR(1–4)_{HT} and VLDLR(2–4)_{HT}, interact with $(\beta 15-66)_2$ with practically the same affinity as sVLDLR_{HT} while the affinity of VLDLR(2–3)_{HT} was about 2-fold lower. In contrast, des(1–8)VLDLR_{HT} exhibited no binding. Complex formation in solution between the fibrin-binding fragments of VLDLR and $(\beta 15-66)_2$ was detected by fluorescence spectroscopy. In addition, formation of a complex between VLDLR(2–4)_{HT} and $(\beta 15-66)_2$ in solution was confirmed by size-exclusion chromatography. Thus, the results obtained indicate that minimal fibrin-binding structures are located within the second and third CR-domains of the VLDL receptor and the presence of the fourth CR-domain is required for the high affinity binding. They also indicate that tryptophan residues of CR-domains are involved in this binding.

Fibrinogen is a complex multifunctional plasma protein that has been identified as an independent risk factor for cardiovascular diseases. Besides its prominent role in hemostasis, fibrinogen participates in various physiological and pathological processes including inflammation. Numerous data suggest that fibrin(ogen) plays a prominent role in transendothelial migration of leukocytes, which is a key step in recruitment of leukocytes from the circulation to sites of inflammation. It was suggested more than two decades ago that fibrinogen binding to vascular cell receptors mediates a specific pathway of cell-to-cell adhesion by bridging together leukocytes and endothelial cells¹. Further, it was hypothesized

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that such bridging occurs through the interaction of fibrinogen with the leukocyte receptor Mac-1 and endothelial cell receptor ICAM-1 and may contribute to leukocyte transmigration^{1,2}. Another hypothesis proposed later suggests that fibrin degradation products promote leukocyte transmigration by bridging leukocytes to the endothelium through the interaction with the leukocyte integrin CD11c and endothelial cell receptor VE-cadherin^{3,4}. We have recently discovered that fibrin interacts with another endothelial cell receptor, very low density lipoprotein receptor (VLDLR), and this interaction also promotes leukocyte transmigration⁵. This discovery suggests a novel fibrin-VLDLR-dependent pathway of leukocyte transmigration in which fibrin-VLDLR interaction plays a key role.

Plasma protein fibrinogen is a chemical dimer consisting of two identical subunits each of which is formed by three non-identical polypeptide chains, A α , B β , and γ^6 (Figure 1A). These chains are folded into a number of structural and/or functional domains^{7,8} that are involved in multiple fibrin(ogen) interactions. Conversion of fibrinogen into fibrin occurs after thrombin-mediated sequential cleavage of fibrinopeptides A and B from the NH₂-terminal portions of the A α and B β chains, respectively, that are located in the central region of the fibrinogen molecule. Fibrin molecules polymerize spontaneously to form a fibrin clot, which prevents blood loss after vascular injury and serves as a provisional matrix on which different cell types adhere, migrate, and proliferate during subsequent wound healing process.

In contrast to fibrinogen, which is rather inert in the circulation, polymeric fibrin is highly reactive towards various proteins and cell types due to the exposure/activation of its multiple binding sites upon polymer formation. Such reactivity of fibrin provides its participation in various physiological and pathological processes including wound healing, which at the early stage includes migration of leukocytes to places of injury, i.e. inflammation. Removal of fibrinopeptides B results in the activation of fibrin β N-domains including β chain residues 15–64^{7,9}. It was shown that these domains interact with endothelial receptor VE-cadherin¹⁰ and this interaction promotes fibrin-dependent angiogenesis¹¹. It was also shown that fibrin degradation products containing these domains promote leukocyte transmigration and thereby inflammation^{3,4}. Our study revealed that interaction of fibrin with the endothelial VLDL receptor also occurs through these domains⁵. Furthermore, we found that the recombinant (β 15–66)₂ fragment, which mimics the dimeric arrangement of these domains in fibrin, interacts with VE-cadherin and the VLDL receptor with practically the same affinity as fibrin^{5,9,12}. Thus, this dimeric fragment preserves functional properties of fibrin β N-domains.

The VLDL receptor is a member of the low-density lipoprotein (LDL) receptor family. It is found in different tissues including vascular endothelium^{13,14}. VLDLR consists of a number of extracellular domains that are involved in ligand binding, transmembrane domain, and cytoplasmic domain^{14,15} (Figure 1B). Originally, VLDLR was proposed to function as a peripheral lipoprotein receptor involved in the delivery of triglyceride-rich lipoproteins to peripheral tissue^{16,17}. Later, it was shown that this receptor plays an important role in reelin signaling^{18,19}, angiogenesis, and tumor growth^{14,20}. Finally, our recent study has revealed a novel function for the VLDL receptor. Namely, we found that this receptor promotes transendothelial migration of leukocytes through the interaction with fibrin⁵. We also found

that the VLDLR-fibrin interaction occurs with a very high affinity and involves the β N-domains of fibrin⁵. Besides these, nothing is known about this novel interaction. The major objectives of the present study were to further characterize the interaction between fibrin and the VLDL receptor and localize fibrin-binding site within the extracellular portion of this receptor.

EXPERIMENTAL PROCEDURES

Proteins, Peptides, and Reagents

The recombinant (B β 1–66)₂ fragment was produced in *E. coli* and purified as previously described⁹. This fragment was treated with thrombin to produce fibrin-related (β 15–66)₂ fragment using the previously described procedure²¹. Human recombinant receptor-associated protein (RAP) was expressed in *E. coli* and purified as previously described²². Mouse anti-hVLDLR monoclonal antibodies 1H5, 5F3, and 1H10²³ were kindly provided by Dr. D. Strickland (University of Maryland Baltimore). Anti-His(C-term) antibody (anti-His tag mAb) conjugated with HRP was from Invitrogen (Carlsbad, CA). Goat secondary anti-mouse polyclonal antibodies conjugated with HRP and HRP substrate SureBlue TMB were from KPL (Gaithersburg, MD). Cyanogen bromide-activated Sepharose 4B and Thrombin CleanCleave kit were obtained from Sigma (St. Louis, MO).

Preparation of Recombinant VLDLR Fragments in the Drosophila Expression System

The soluble form of human VLDL receptor, sVLDLR_{HT}, which contains the entire extracellular portion of the receptor including amino acid residues 1–770, and its fragment des(1–8)VLDLR_{HT} lacking eight CR-repeats (residues 329–770), both tagged with six His residues (His-tag) at C-termini, were prepared using the Drosophila Expression System (Invitrogen). The expression and purification of these fragments were performed utilizing previously described protocols^{23,24,25} with some modifications. Namely, the cDNAs encoding both fragments were amplified by PCR using a plasmid carrying the full-length human VLDLR sequence. The following oligonucleotides were used as primers in which restriction-recognition sites are underlined and stop codon is shown in bold: 5'-GGCGGCCGCTCGGGGGGAGAAAAGCCAAATG-3' (forward primer for sVLDLR_{HT}); 5'-GGCGGCCGCTCGGGCATATAAACGAATGCTTG-3' [forward primer for des(1–8)VLDLR_{HT}]; 5'-CCGCGCCCGTTTAAACTCAATGGTGATGGTGATGATGAGAAGTCCCTTTTGGGGG-3' (reverse primer for both fragments). The PCR products were subcloned into the pMT/BiP/V5-HisC vector for secreted expression in *Drosophila melanogaster* Schneider 2 (S2) cells using *Ava*I and *Pme*I restriction sites and then transformed into DH5 α *E. coli* host cells (Invitrogen). All resultant clones were sequenced to confirm the integrity of the coding sequences. S2 cells were co-transfected with the expression plasmids encoding the fragments and pCoBlas vector, and stable transfectant resistant to blasticidin were selected. For large-scale expression, stable S2 cells were grown in shaker flasks at 28 °C in *Drosophila* serum-free medium (HyClone SFX) supplemented with 20 mM L-glutamine, penicillin/streptomycin, Fungizone, and blasticidin at 25 μ g/ml (Invitrogen). Expression of the fragments was induced by adding 0.5 mM CuSO₄ and conditioned media was collected on day 5 of induction. The amount of the fragments in the conditioned serum free media

(HyClone SFX) was determined by Western blot analysis with the anti-His (C-terminal) antibody conjugated with HRP. Both sVLDLR_{HT} and des(1–8)VLDLR_{HT} fragments were purified from conditioned media, depleted in Cu²⁺ with chelating resin Chelex 100 (Bio-Rad, Hercules, California), using affinity chromatography on Talon Metal Affinity Resin (Clontech Laboratories, Mountain View, CA), and further purified by size-exclusion chromatography on Superdex 200 (GE Healthcare, Piscataway, NJ).

Preparation of Recombinant VLDLR Fragments in *E. coli* Expression System

Recombinant VLDLR fragments, VLDLR(1–8)_{HT}, VLDLR(1–4)_{HT}, VLDLR(5–8)_{HT}, VLDLR(1–2)_{HT}, VLDLR(2–3)_{HT}, VLDLR(3–4)_{HT}, and VLDLR(2–4)_{HT}, including amino acid residues 1–328, 1–163, 164–328, 1–82, 42–124, 84–163, and 42–163, respectively, were produced in *E. coli* strain BL21(DE3)pLysS using the pET-20b expression vector (Novagen). All fragments were tagged with six His residues. The cDNA fragments encoding these regions were produced by PCR using as a template the full-length cDNA encoding human VLDLR; the primers are presented in Table 1. The PCR products were subcloned into the pET20b expression vector using *Nde*I and *Xho*I restriction sites and then transformed into DH5 α *E. coli* host cells (Invitrogen). All resulting clones were sequenced to confirm the integrity of the coding sequences. For preparation of the recombinant fragments, the BL21(DE3)pLysS *E. coli* host cells were transformed with the resulting plasmids and all fragments were produced and refolded following the procedures described earlier²⁶. The refolded fragments were purified by size-exclusion chromatography on Superdex 200 followed by affinity chromatography on RAP-Sepharose. The purity of all fragments was verified by SDS-polyacrylamide gel electrophoresis (Figure 1E).

Preparation of the Recombinant VLDLR(1–8)_{HT} Fragment Mutant

The Trp105Ala mutant of the VLDLR(1–8)_{HT} fragment was produced by site-directed mutagenesis using QuikChange Multi Site-Directed Mutagenesis kit (Stratagene, La Jolla, CA). The pET-20b construct containing DNA encoding the VLDLR(1–328) fragment tagged with six His residues was modified by using the following mutagenic primer: 5'-TCAGTGTATCCCAGTGTCCGCGAGATGTGATGGTGAAAAT-3'. The mutation was confirmed by sequencing throughout the entire VLDLR1–328 coding region of mutant plasmid. The mutant fragment was produced in *E. coli* strain BL21(DE3)pLysS and refolded and purified by the procedures described above for the wild-type VLDLR(1–8)_{HT} fragment.

Protein Concentration Determination

Concentration of the expressed VLDLR fragments were determined spectrophotometrically using theoretical extinction coefficients ($E_{280,1\%}$) estimated from fragments' sequences by the ProtParam online tool (<http://www.expasy.ch/tools/protparam.html>). Molecular masses of these fragments were also estimated using ProtParam. The following molecular masses and $E_{280,1\%}$ values were obtained: sVLDLR_{HT}, 85.6 kDa and 12.9; des(1–8)VLDLR_{HT}, 49.7 kDa and 15.0; VLDLR(1–8)_{HT}, 36.8 kDa and 9.8; VLDLR(1–4)_{HT}, 18.7 kDa and 9.7; VLDLR(5–8)_{HT}, 19.0 kDa and 9.5; VLDLR(1–2)_{HT}, 9.9 kDa and 11.9; VLDLR(2–3)_{HT}, 9.9 kDa and 11.9; VLDLR(3–4)_{HT}, 9.5 kDa and 6.6; VLDLR(2–4)_{HT}, 14.0 kDa and 8.7. Concentration of the (β 15–66)₂ fragment was determined as previously described⁹.

Fluorescence spectroscopy

Fluorescence spectra were recorded in an SLM 8000-C fluorometer. Fluorescence measurements of thermal unfolding were performed by monitoring the ratio of the intensity at 370 nm to that at 330 nm with excitation at 280 nm in the same fluorometer. Temperature was controlled with a circulating water bath. Protein/fragment concentrations were 0.02–0.04 mg/ml. All experiments were performed in a 1-cm-pathlength quartz cuvette in 20 mM Tris buffer, pH 7.4, 150 mM NaCl (TBS) containing 1 mM CaCl₂.

Size-Exclusion Chromatography

Analytical size-exclusion chromatography was used to analyze formation of a complex between the (β15–66)₂ and VLDLR(2–4)_{HT} fragments. The experiments were performed with a fast protein liquid chromatography system (FPLC, Pharmacia) on a Superdex 75 column at flow rate of 0.5 mL/min. Typically, 500 μl of individual fragments or their mixture were loaded onto the column equilibrated with TBS containing 1 mM Ca²⁺ and followed by elution with the same buffer. Protein elution was monitored by measuring absorbance at 280 nm.

Solid-Phase Binding Assay

Wells of Immulon 2HB microtiter plates were coated overnight with the (β15–66)₂ fragment at 2 μg/ml in 0.1 M Na₂CO₃, pH 9.5 (coating buffer) at 4 °C. The wells were then blocked with Blocker BSA in TBS (Thermo Scientific, Rockford, IL) for 1 hour at room temperature. Following washing with TBS containing 0.05% Tween 20 and 1 mM CaCl₂ (ELISA-binding buffer), the indicated concentrations of VLDLR fragments in this buffer were added to the wells and also to control wells coated just with Blocker BSA in TBS and incubated for 1 hour at 37 °C. Bound fragments were detected by reaction with anti-His monoclonal antibody conjugated with HRP (1 hour at 37 °C). Alternatively, bound VLDLR fragments were detected by reaction with a mixture of anti-VLDLR mAb 1H5, 5F3, and 1H10 (1 hour at 37 °C) and the HRP-conjugated donkey anti-mouse polyclonal antibodies (1 hour at 37 °C). The peroxidase substrate, SureBlue TMB (KPL, Gaithersburg, MD), was added to the wells and the amount of bound ligand was measured spectrophotometrically at 450 nm. Data were analyzed by nonlinear regression analysis using equation 1:

$$A = A_{\max} / (1 + K_d / [L])$$

where A represents the absorbance of the oxidized substrate, which is assumed to be proportional to the amount of ligand bound, $A_{\max}$ is the absorbance at saturation, $[L]$ is the molar concentration of the ligand, and K_d is the equilibrium dissociation constant.

Surface Plasmon Resonance Analysis

Interaction of the (β15–66)₂ fragment with VLDLR fragments was studied by surface plasmon resonance (SPR) using the BIAcore 3000 biosensor (GE Healthcare), which measures the association/dissociation of proteins in real time. Immobilization of VLDLR fragments to the activated surface of CM5 sensor chip was performed using the amine coupling kit (GE Healthcare), as specified by the manufacturer. Binding experiments were

performed in HBS-P (10 mM HEPES buffer, pH 7.4, 150 mM NaCl, and 0.005% Surfactant P20) containing 1 mM CaCl₂ at 20 µl/min flow rate. The (β15–66)₂ fragment was injected at increasing concentrations and the association/dissociation between it and immobilized VLDLR fragments was monitored as the change in the SPR response. To regenerate the chip surface, complete dissociation of the complex was achieved by adding 100 mM H₃PO₄ for 30 seconds followed by re-equilibration with the binding buffer. Experimental data were analyzed using BIAevaluation 4.1 software supplied with the instrument. The equilibrium dissociation constant, K_d , was calculated as $K_d = k_{\text{diss}}/k_{\text{ass}}$, where k_{ass} and k_{diss} represent kinetic constants that were estimated by global analysis of the association/dissociation data using the 1:1 Langmuir interaction model (kinetic analysis). To confirm the kinetic analysis, K_d was also estimated by analysis of the association data using the steady-state affinity model (equilibrium analysis).

RESULTS

Preparation and Characterization of Extracellular Portion of the VLDL Receptor and its Fragments

The extracellular portion of the VLDL receptor is composed of eight complement-type repeats (CR-repeats or domains), three EGF-like domains, β-propeller and O-linked sugar domains (Figure 1B). Among these domains, CR-domains (about 42 residues each) compose the ligand binding region²⁷ and are the most probable candidates for binding to fibrin. To test this suggestion, we prepared soluble extracellular portion of the VLDL receptor (sVLDLR_{HT}) and its sub-fragments, VLDLR(1–8)_{HT} and des(1–8)VLDLR_{HT}, containing all eight CR-domains and the rest of the extracellular portion, respectively (Figure 1 C and D).

The sVLDLR_{HT} and (des1–8)VLDLR_{HT} fragments, tagged with six His residues at the C-termini to facilitate their purification and detection, were prepared using the *Drosophila* expression system. To test folding status of these fragments, we used fluorescence spectroscopy. At 4 °C, the sVLDLR_{HT} and (des1–8)VLDLR_{HT} fragments exhibited fluorescence spectra with maximum at 343 and 338 nm, respectively (Figure 2A, left and right insets, and Table 2), indicating that they both contain compact structures. When the fragments were heated in the fluorometer while the ratio of fluorescence intensity was monitored at 370 nm to that at 330 nm as the measure of the spectral shift that accompanies unfolding, both fragments exhibited a sigmoidal denaturation transition with a midpoint (T_m) at about 63 °C (Figure 2A), further confirming that they are folded into compact structures. It should be noted that at 90 °C the maximum of sVLDLR_{HT} and (des1–8)VLDLR_{HT} spectra (λ_{max}) was shifted to 347 and 345 nm, respectively (Figure 2A, left and right insets, and Table 2), suggesting that upon heat-induced denaturation some of their Trp residues were only partially exposed.

Our attempts to express the His-tagged VLDLR(1–8) fragment in the *Drosophila* expression system were not successful. Although immunoblot analysis revealed the presence of this fragment in the cells, no such fragment in the media was detected, most probably due to poor or no secretion to the media. Therefore, we expressed this fragment in the *E. coli* expression system that was previously used for preparation of GST-VLDLR(1–8) fusion protein²⁶. Since this fragment contains multiple disulphide bonds, it was refolded and

purified using protocols described in Experimental Procedures. Heat-denaturation study of the VLDLR(1–8)_{HT} fragment revealed no well-expressed sigmoidal transition (Figure 2B). However, the spectral shift from 349 to 352 nm upon heating this fragment from 4 to 90 °C (Figure 2B, left inset, and Table 2), and the downward change in fluorescence ratio, which starts at about the same temperature as denaturation of sVLDLR_{HT} and (des1–8)VLDLR_{HT} and may reflect aggregation upon denaturation, suggest that this fragment is folded into a compact structure. Since it was shown that properly folded CR-domains of the LDL receptor bind Ca²⁺ and this binding results in a significant increase in fluorescence intensity of their Trp residues²⁸, we next tested the effect of Ca²⁺ on fluorescence intensity of the VLDLR(1–8)_{HT} fragment. The experiments revealed about 80% increase in fluorescence intensity of VLDLR(1–8)_{HT} upon addition of 1 mM Ca²⁺ (Figure 2B, right inset). No such increase upon addition of Ca²⁺ was observed in the presence of 4 M GdmCl and 1 mM DTT, which were added to denature the VLDLR(1–8)_{HT} fragment (not shown). Altogether, these results clearly indicate that VLDLR(1–8)_{HT} was properly folded. It should be noted that the final yield of the properly folded His-tagged VLDLR(1–8)_{HT} fragment was about 10-fold higher than that of VLDLR(1–8) prepared from the GST-VLDLR(1–8) fusion protein using previously described protocols²⁶.

Localization of the Fibrin-Binding Site in the CR-domain Region of the VLDL Receptor

Our previous study revealed that the VLDL receptor interacts with fibrin exclusively through the β N-domains⁵. Furthermore, this study also revealed that the (β 15–66)₂ fragment, which mimics the dimeric arrangement of these domains in fibrin (Figure 1A), exhibits practically the same affinity towards sVLDLR as fibrin⁵ but, in contrast to the latter, it is highly soluble. Therefore, in the present study we used the (β 15–66)₂ fragment as the simplest soluble mimetic of fibrin. In ELISA, sVLDLR_{HT} and VLDLR(1–8)_{HT} both bound to immobilized (β 15–66)₂ while the des(1–8)VLDLR_{HT} fragment exhibited no binding (Figure 3A). This finding was confirmed in SPR experiments (Figure 3B). The observed binding occurred in the presence of 1 mM Ca²⁺; when the experiments were performed in the presence of 100 mM EDTA, practically no binding was detected (Figure 3A, inset). Altogether, these results indicate that the fibrin-VLDLR interaction is Ca²⁺-dependent, the fibrin-binding site is located within eight CR-domains of the VLDL receptor, and the remaining extracellular domains of this receptor are not involved in fibrin binding.

It should be noted that in ELISA experiments, in which bound sVLDLR_{HT} and sVLDLR(1–8)_{HT} were detected using the anti-His tag monoclonal antibody (mAb), the affinities of their binding to the (β 15–66)₂ fragment were very similar (K_d = 16.4 and 16.3 nM, respectively, Table 3) further confirming that sVLDLR(1–8)_{HT} is properly folded. At the same time, these affinities were lower than that we determined earlier for the interaction of non-His tagged sVLDLR with fibrin and (β 15–66)₂ using a mixture of anti-VLDLR(1–8) mAbs (K_d = 5.7 nM, Table 3). To clarify the reason for such a discrepancy, we performed ELISA experiments in which bound His-tagged sVLDLR_{HT} and VLDLR(1–8)_{HT} fragments were detected with the same mixture of mAbs. The experiments revealed that the affinities of interaction of these fragments with (β 15–66)₂ are higher than those determined with anti-His mAb and are very close to the affinity determined earlier for non-His-tagged sVLDLR (Table 3). Furthermore, the values of equilibrium dissociation constants determined by SPR

turned to be very similar to those determined in these ELISA experiments (Figure 3B, insets, and Table 3). These results suggest that His tag may be partially unavailable in the sVLDLR_{HT} and VLDLR(1–8)_{HT} fragments and that K_d values determined by SPR are more reliable than those determined by ELISA using anti-His tag mAb. They also indicate that His-tagged VLDLR(1–8)_{HT} expressed in *E. coli* after refolding and purification adopts physiologically active conformation similar to that of sVLDLR and His-tagged sVLDLR_{HT} prepared using the *Drosophila* expression system.

Further Localization of the Fibrin-binding Site in the VLDL Receptor

To further localize the fibrin-binding site within the CR-domains of the VLDL receptor, we first prepared two fragments, VLDLR(1–4)_{HT} and VLDLR(5–8)_{HT} including CR-domains 1–4 and 5–8, respectively, both containing His tag at their C-termini (Figure 1D). As in the case with the VLDLR(1–8)_{HT} fragment, our attempts to expressed these fragments in the *Drosophila* expression system were not successful, most probably due to the same reason mentioned above for the VLDLR(1–8)_{HT}. Therefore, we expressed them in the *E. coli* expression system and refolded using the same refolding protocol as that for VLDLR(1–8)_{HT}. After refolding, both fragments were fractionated on Superdex-75 column to separate their monomeric fractions and folding status of these fractions was tested by fluorescence spectroscopy. At 4 °C, the VLDLR(5–8)_{HT} fragment exhibited fluorescence spectrum with λ_{max} at 351 nm while at 90 °C the maximum was shifted to 354 nm (Table 2), confirming that this fragment is folded into a compact structure. The VLDLR(1–4)_{HT} fragment also exhibited a spectrum with λ_{max} at 351 nm that was practically unchanged upon heating to 90 °C (not shown). However, the maximum was shifted to 354 nm upon heating to 90 °C in the presence of 4 M urea (Table 2), indicating that this fragment is also folded into a compact structure, which is more thermostable than that of VLDLR(5–8)_{HT}. In addition, both fragments exhibited a significant increase in fluorescence intensity upon addition of Ca^{2+} while no such increase was observed upon addition of Ca^{2+} in the presence of denaturing agents, 4 M GdmCl and 1 mM DTT (Table 2), further confirming that they are properly folded.

In ELISA experiments, the VLDLR(1–4)_{HT} fragment exhibited binding to immobilized $(\beta 15\text{--}66)_2$ while VLDLR(5–8)_{HT} failed to bind (Figure 4A). This finding was confirmed in SPR experiments (Figure 4B). These results indicate that the fibrin-binding site is located within the first four CR-domains of the VLDL receptor. The value of K_d for the interaction of VLDLR(1–4)_{HT} with $(\beta 15\text{--}66)_2$ determined by ELISA using for detection anti-His tag mAb was found to be 60 nM, about 4-fold higher than that determined for sVLDLR_{HT} and VLDLR(1–8)_{HT}. However, K_d value of 3.7 nM determined for this interaction by SPR, which provides a more reliable K_d estimate as mentioned above, was practically the same as those for sVLDLR_{HT} and VLDLR(1–8)_{HT} (Table 3). This indicates that the VLDLR(1–4)_{HT} fragment preserves physiologically active conformation of the corresponding region of the VLDL receptor.

To test which of the first four CR-domains of VLDLR are involved in binding of fibrin, we next expressed three overlapping fragments, VLDLR(1–2)_{HT}, VLDLR(2–3)_{HT}, and VLDLR(3–4)_{HT}, containing CR-domains 1–2, 2–3, and 3–4, respectively (Figure 1D), in the

E. coli expression system. Again, as in the case with the other fragments described above, folding status of each of these fragments was tested by fluorescence spectroscopy. At 4 °C, these fragments exhibited fluorescence spectra with λ_{max} at 351–353 nm whose positions did not change upon heating to 90 °C (Table 2). However, upon addition of Ca^{2+} fluorescence intensity of all three fragments significantly increased and such increase was not observed when these fragments were under denaturing conditions (Table 2). In addition, λ_{max} of VLDLR(1–2)_{HT} spectrum shifted from 351 to 353 nm when this fragment was heated in the presence of 4 M urea, a situation similar to that observed with the VLDLR(1–4)_{HT} fragment. Altogether, these results confirmed that all three fragments were properly folded.

In ELISA experiments, in which bound fragments were detected with anti-His tag mAb, only the VLDLR(2–3)_{HT} fragment bound to immobilized $(\beta 15\text{--}66)_2$ while VLDLR(1–2)_{HT} and VLDLR(3–4)_{HT} failed to bind (Table 3). The K_d value determined for this binding was found to be 78 nM, i.e. much higher than those determined for larger VLDLR fragments. However, SPR experiments revealed that this binding occurs with much higher affinity. Namely, the K_d value determined by SPR was found to be 9.5 nM (Figure 5A and Table 3), i.e. only about 2.6-fold higher than that determined by this technique for the interaction of sVLDLR_{HT}, VLDLR(1–8)_{HT}, and VLDLR(1–4)_{HT} with $(\beta 15\text{--}66)_2$. Altogether, these results indicate that a pair of domains, CR-domains 2 and 3, is the minimal structure composing the fibrin binding site.

Since the results described above revealed that the affinity of VLDLR(2–3)_{HT} to $(\beta 15\text{--}66)_2$ is lower than that of VLDLR(1–4)_{HT}, this finding raised a question about a possible involvement of neighboring domain(s) in formation of the high affinity fibrin-binding site. To address this question, we prepared a recombinant VLDLR(2–4)_{HT} fragment containing CR-domains 2, 3 and 4 (Figure 1D) and confirmed its proper folding by the procedures described above for other recombinant fragments expressed in the *E. coli* expression system. In ELISA and SPR experiments, the VLDLR(2–4)_{HT} fragment interacted with $(\beta 15\text{--}66)_2$ and the affinity of its interaction determined by SPR ($K_d = 4.6$ nM) was higher than that of VLDLR(2–3)_{HT} and comparable with that of VLDLR(1–8)_{HT} and VLDLR(1–4)_{HT} (Figure 5B and Table 3). These results indicate that the presence of the fourth CR-domain increases the affinity of CR-domains 2 and 3 to fibrin by about 2-fold. This finding suggests that the fourth CR-domain may contribute to the formation of high affinity fibrin-binding site in the VLDL receptor.

Fluorescence-Detected Interaction of VLDLR Fragments with $(\beta 15\text{--}66)_2$ and RAP

Receptor associated protein (RAP) is known to be an antagonist of ligand binding of some LDL receptor family members including VLDLR to which it binds with high affinity²⁹. As mentioned in Experimental Procedures, after refolding all recombinant VLDLR fragments were subjected to size-exclusion chromatography to separate monomeric fractions and then passed through a RAP-Sepharose column. Our subsequent experiments revealed that only those fragments that bound to RAP-Sepharose interacted with $(\beta 15\text{--}66)_2$ (Table 3). This is in agreement with our previous finding that RAP inhibits interaction of fibrin and $(\beta 15\text{--}66)_2$ with the VLDL receptor⁵. Since interaction of some CR-domain containing fragments with

their ligands or RAP was shown to be accompanied by a short-wave shift of their fluorescence spectra^{28,30}, we used fluorescence spectroscopy to detect interaction of recombinant VLDLR fragments with $(\beta 15-66)_2$ and RAP in solution.

In a typical experiment, fluorescence spectra of a VLDLR fragment at 1 μ M were recorded before and after addition of RAP or $(\beta 15-66)_2$ to final molar ratio of 1:1 or 1:10, respectively. Figure 6 shows a representative example of short-wave spectral shifts upon addition of RAP or $(\beta 15-66)_2$ to the VLDLR(2-4)_{HT} fragment; the shifts for the remaining fragments are presented in Table 2. The observed spectral shifts of VLDLR(1-8)_{HT} spectra upon addition of RAP or $(\beta 15-66)_2$ were about 2 nm only, most probably due to the presence of Trp residues that do not participate in formation of the RAP-binding or fibrin-binding sites. At the same time, the spectral shifts for smaller fragments, VLDLR(1-4)_{HT}, VLDLR(2-3)_{HT}, and VLDLR(2-4)_{HT}, upon addition of RAP or $(\beta 15-66)_2$ were found to be more significant, 4-6 or 3-5 nm, respectively. No such shifts were detected for those fragments that exhibited no interaction with the $(\beta 15-66)_2$ fragment and did not bind to RAP-Sepharose. Altogether, these experiments revealed a short-wave shift in fluorescence spectra of all VLDLR fragments that exhibited affinity to RAP and $(\beta 15-66)_2$, indicating formation of a complex between these fragments and RAP or $(\beta 15-66)_2$ in solution.

Complex Formation Between VLDLR(2-4)_{HT} and $(\beta 15-66)_2$ Detected by Size-Exclusion Chromatography

To further test the interaction of VLDLR fragments with the $(\beta 15-66)_2$ fragment in solution, we used size-exclusion chromatography. VLDLR(2-4)_{HT}, the smallest fragment with the highest affinity to $(\beta 15-66)_2$ was selected for these experiments. When loaded separately to a Superdex 75 column, the $(\beta 15-66)_2$ fragment and the VLDLR(2-4)_{HT} fragment were eluted at 12.1 and 12.5 ml, respectively (Figure 7). The higher mobility of $(\beta 15-66)_2$, which has lower molecular mass than VLDLR(2-4)_{HT} (10.8 kDa vs 14.0 kDa) was unexpected. However, it can be easily explained by the fact that $(\beta 15-66)_2$ has unordered conformation, as was reported earlier⁹, and, therefore, may have higher hydrodynamic radius than compact VLDLR(2-4)_{HT} and thus is eluted earlier. When the VLDLR(2-4)_{HT} and $(\beta 15-66)_2$ fragments were incubated together for 30 min and then loaded onto the same column, the mixture was eluted at 11.8 ml, i. e. ahead of the individual components. These results further confirm formation of the complex between VLDLR(2-4)_{HT} and $(\beta 15-66)_2$ in solution.

Effect of Trp¹⁰⁵ to Ala mutation on the Interaction of VLDLR(1-8)_{HT} with $(\beta 15-66)_2$

Our fluorescence studies described above, which revealed a short-wave shift of the fluorescence spectra upon formation of the complexes, suggested that Trp residues of VLDLR CR-domains contribute to the interaction with the $(\beta 15-66)_2$ fragment. To test this suggestion, we prepared a mutant of the VLDLR(1-8)_{HT} fragment in which Trp¹⁰⁵ residue of its third CR-domain was replaced with Ala and confirmed its proper folding by the procedures described above for other recombinant fragments. Next, we compared binding of this mutant to the $(\beta 15-66)_2$ fragment with that of wild-type VLDLR(1-8)_{HT}. In ELISA experiments, when increasing concentrations of both VLDLR fragments (up to 100 nM) were added to immobilized $(\beta 15-66)_2$, the wild-type fragment exhibited significant binding

while the mutant fragment failed to bind (Figure 8). These results directly confirm the involvement of Trp residue(s) of VLDLR in the interaction with the $(\beta 15-66)_2$ fragment.

DISCUSSION

Our recent study identified VLDLR as a novel endothelial cell receptor for fibrin and revealed that interaction between fibrin and the VLDL receptor promotes leukocyte transmigration and thereby inflammation⁵. The major goals of our current studies are to establish the molecular mechanism underlying this interaction and develop its specific inhibitors that may control fibrin-dependent inflammation. We have already established that fibrin interacts with the VLDL receptor with a very high affinity and this interaction occurs exclusively through the fibrin β N-domains⁵. In the present study, we have made the next step towards these goals by identifying domains in the VLDL receptor that are involved in the interaction with fibrin and demonstrating formation of a complex between the recombinant fragments containing fibrin-binding domains of VLDLR and VLDLR-binding domains of fibrin.

To localize the fibrin-binding site, we expressed a number of VLDLR fragments and tested their interaction with the $(\beta 15-66)_2$ fragment mimicking a pair of the VLDLR-binding β N-domains of fibrin. Since most of the fragments except sVLDLR_{HT} and des(1-8)VLDLR_{HT} were expressed in the bacterial expression system and each of their CR-domains contains 6 Cys residues involved in the formation of 3 disulfide bonds, the major challenge was to refold these fragments and confirm that they form proper conformation after refolding. Our attempts to test folding status of the refolded fragments by circular dichroism (CD) were unsuccessful since their CD spectra turned to be similar to those of unfolded proteins (results not shown). This is in agreement with the results obtained by others with CR-domain containing fragments of the LDL receptor, which were folded but their CD spectra indicated the absence of ordered secondary structure^{31,32}. Since 5 out of 8 CR-domains of VLDLR contain Trp residues that may be reporters of a folded conformation, we used fluorescence spectroscopy to characterize folding status of the expressed and refolded fragments. The results obtained by this technique clearly indicate that all VLDLR fragments prepared for the present study were properly folded.

Our binding studies revealed that VLDLR(2-3)_{HT} containing the second and third CR-domains of VLDLR is the smallest recombinant fragment preserving high affinity to $(\beta 15-66)_2$. This finding is not surprising since a number of previous studies revealed that at least a pair of CR-domains is required for the interaction of some members of LDL receptor family with their ligands. For example, it was shown that CR-domains 5 and 6 of LRP cluster II compose a minimal RAP-binding unit of this receptor and no single CR-domain of this cluster shows high affinity binding to RAP^{33,34}. It was also shown that three CR-domains in the same cluster II of LRP, domains 5, 6 and 7, constitute the complete binding site for α_2 -macroglobulin²⁸. The third domain of RAP also binds to a pair of CR-domains of LDL receptor, domains 3 and 4³⁵. Finally, it was shown that CR-domains 2 and 3 of the VLDL receptor are involved in binding with human rhinovirus type 2 (HRV2)^{36,37}. Thus, our data indicate that fibrin-binding site of the VLDL receptor is formed by its second and third CR-domains. In addition, the fourth CR-domain of VLDLR seems to also be involved in fibrin

binding since the affinity of the VLDLR(2–4)_{HT} fragment to (β15–66)₂ was found to be about twice higher than that of VLDLR(2–3)_{HT}.

It should be noted that the fibrin-binding CR-domains 2 and 3 of the VLDL receptor are the same domains that are involved in the interaction with HRV2. The crystal structure of a complex between this rinovirus and a fragment containing these two domains has been reported³⁸. In this structure, individual CR-domain 3 is bound to HRV2 through a well-defined interacting surface that includes acidic calcium-chelating residues and, in particular, an exposed Trp residue that is highly conserved in most CR-domains³⁸. Our fluorescence experiments (Table 2) indicate the involvement of Trp residue of the CR-domains of VLDLR in the interaction with fibrin. Such involvement was directly confirmed by mutation of Trp residue in the third CR-domain of VLDLR, which resulted in a significant loss of the affinity of VLDLR(1–8)_{HT} to (β15–66)₂ (Figure 8). These findings suggest that interaction of fibrin with the VLDLR CR-domains may occur through a mechanism similar to that reported for HRV2-VLDLR interaction³⁸. They may also provide an explanation for the increased affinity of VLDLR(2–4)_{HT} fragment to (β15–66)₂ in comparison with that of VLDLR(2–3)_{HT}. Namely, the highly conserved Trp in the fourth CR-domain of VLDLR is replaced with Phe and this replacement may significantly decrease the affinity of this domain to fibrin. Alternatively, this replacement may eliminate fibrin-binding and the role of the fourth CR-domain may be to stabilize the structure of the second and third CR-domains in a conformation that is most favorable for the high affinity binding. Structural studies are required to select between these alternatives. In this respect, demonstration in the present study of complex formation between free (β15–66)₂ and VLDLR fragments in solution confirms the feasibility of studying such complexes by NMR.

In summary, the results of the present study revealed that the fibrin-binding site of the VLDL receptor is formed by the structures located in its second and third CR-domains and its fourth CR-domain is required for the maximal affinity of this receptor to fibrin. These results provide important information for the development of specific antagonists of fibrin-VLDLR interaction that may represent novel anti-inflammatory agents for treatment of inflammation-related cardiovascular diseases. The results also indicate the involvement of highly conserved Trp residues of VLDLR CR-domains in the interaction with fibrin. The detailed molecular mechanism underlying this interaction and the exact role of the fourth CR-domain in fibrin binding remain to be established.

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ABBREVIATIONS AND TEXTUAL FOOTNOTES

VLDLR	very low density lipoprotein (VLDL) receptor
RAP	receptor associated protein
mAb	monoclonal antibody
CR-domain	complement-type repeat domain

ELISA	enzyme-linked immunosorbent assay
SPR	surface plasmon resonance
TBS	Tris buffered saline containing 20 mM Tris, pH 7.4, and 150 mM NaCl.

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Figure 1.

Schematic representation of fibrin, the VLDL receptor, and recombinant fragments prepared for the present study. Panel A: Ribon diagram of fibrinogen molecule based on its crystal structure⁸; the individual fibrinogen chains, A α , B β , and γ , are colored in blue, green and red, respectively. The β N-domains of fibrin, whose structure have not been identified, are shown schematically as two curved green lines and the recombinant $(\beta 15-66)_2$ fragment corresponding to these domains is shown on the right. Panel B: A diagram of the VLDL receptor consisting of CR-repeats (domains), EGF-like repeats (domains), β -propeller

domain, O-linked sugar domain (O-lsd), transmembrane (TM), and cytoplasmic (Cyt) domains. Panels C and D: Diagrams of the recombinant sVLDLR_{HT} and des(1–8)VLDLR_{HT} fragments expressed in the *Drosophila* Expression System and VLDLR fragments expressed in *E.coli*, respectively. Panel E: SDS-polyacrylamide gel electrophoresis analysis of the recombinant VLDLR fragments presented in Panels C and D; the left outer lane contains Mark 12 protein markers of the indicated molecular masses.

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Figure 2.

Fluorescence-detected thermal unfolding of extracellular portion of the VLDL receptor (sVLDLR_{HT}) and its sub-fragments, des(1-8)VLDLR_{HT} and VLDLR(1-8)_{HT}. Panel A: Melting curves obtained upon heating of the sVLDLR_{HT} and des(1-8)VLDLR_{HT} fragments. Fluorescence spectra of sVLDLR_{HT} and des(1-8)VLDLR_{HT} at 4 °C (solid lines) and at 90 °C (dashed lines) are shown in the left and right insets, respectively. All experiments were performed in TBS containing 1 mM CaCl₂. Panel B: Melting curve obtained upon heating of the VLDLR(1-8)_{HT} fragment; the dashed line represents linear extrapolation of

fluorescence ratio values to highlight a downturn of this parameter. Fluorescence spectra of VLDLR(1–8)_{HT} at 4 °C (solid line) and at 90 °C (dashed line) are shown in the left inset; fluorescence spectra of VLDLR(1–8)_{HT} in the presence of 1 mM CaCl₂ (solid line) and 0.5 mM EDTA (dashed line) are in the right inset.

Figure 3.

Analysis of interaction of the sVLDLR_{HT}, des(1-8)VLDLR_{HT}, and VLDLR(1-8)_{HT} fragments with the (β15-66)₂ fragment. Panel A: ELISA-detected interaction between the (β15-66)₂ and VLDLR fragments. Increasing concentrations of sVLDLR_{HT} (filled circles), VLDLR(1-8)_{HT} (empty circles), or des(1-8)VLDLR_{HT} (filled squares) were incubated with microtiter wells coated with (β15-66)₂, and the bound fragments were detected with anti-His tag mAb, as described in Experimental Procedures. The curves for sVLDLR_{HT} and VLDLR(1-8)_{HT} represent the best fit of the data to eq. 1, the determined K_d values are

presented in Table 3; error bars represent the standard deviation of triplicate determinations. Binding of sVLDLR_{HT} at 10 nM in the presence of 1 mM Ca²⁺ or 100 mM EDTA is shown in the inset. All experiments were performed in ELISA-binding buffer. Panel B: SPR-detected interaction between the (β15–66)₂ and VLDLR fragments. The (β15–66)₂ fragment at 10 or 100 nM in HBS-P buffer with 1 mM CaCl₂ was added to immobilized sVLDLR_{HT} (broken curve), VLDLR(1–8)_{HT}, (solid curve), or des(1–8)VLDLR_{HT} (dotted curve), respectively, and its association/dissociation was monitored in real time by registering the resonance signal (response). The insets show analysis of interaction of (β15–66)₂ with sVLDLR_{HT} (inset A) or VLDLR(1–8)_{HT} (inset B) by SPR. (β15–66)₂ at increasing concentrations, 0.25, 0.5, 1, 2.5, 5, and 10 nM, was added to the immobilized VLDLR fragments and its association/dissociation was monitored in real time.

Figure 4. Analysis of interaction of the VLDLR(1–4)_{HT} and VLDLR(5–8)_{HT} fragments with the (β15–66)₂ fragment. Panel A: ELISA-detected interaction between the (β15–66)₂ and VLDLR fragments. Increasing concentrations of VLDLR(1–4)_{HT} (filled circles), or VLDLR(5–8)_{HT} (empty circles) were incubated with microtiter wells coated with (β15–66)₂, and the bound fragments were detected with anti-His tag mAb, as described in Experimental Procedures. The curve for VLDLR(1–4)_{HT} represents the best fit of the data to eq. 1, the determined K_d value is presented in Table 3; error bars represent the standard deviation of

triplicate determinations. All experiments were performed in ELISA-binding buffer. Panel B: SPR-detected interaction between the $(\beta 15-66)_2$ and VLDLR fragments. The $(\beta 15-66)_2$ fragment at 10 nM or 100 nM in HBS-P buffer with 1 mM CaCl_2 was added to the immobilized VLDLR(1-4)_{HT} (solid line) or VLDLR(5-8)_{HT} (broken line) fragments, respectively, and its association/dissociation was monitored in real time. The insets shows analysis of interaction of the $(\beta 15-66)_2$ fragment with VLDLR(1-4)_{HT} by SPR. $(\beta 15-66)_2$ at increasing concentrations, 0.25, 0.5, 1, 2.5, 5, and 10 nM, was added to immobilized VLDLR(1-4)_{HT} and its association/dissociation was monitored in real time.

Figure 5.

Analysis of interaction of the $(\beta 15-66)_2$ fragment with the VLDLR(2-3)_{HT} (panel A) and VLDLR(2-4)_{HT} (panel B) fragments by surface plasmon resonance. $(\beta 15-66)_2$ at increasing concentrations, 0.25, 0.5, 1.0, 2.5, 5.0, and 10 nM, was added to the immobilized VLDLR fragments and its association/dissociation was monitored in real time. All experiments were performed in HBS-P buffer containing 1 mM CaCl_2 .

Figure 6. Fluorescence-detected interaction of the VLDLR(2-4)_{HT} fragment with RAP and (β15-66)₂. Panels A: Fluorescence spectra of VLDLR(2-4)_{HT} at 1 μM alone (solid line) or in the mixture with 1 μM RAP (dashed line). Panel B: fluorescence spectra of VLDLR(2-4)_{HT} at 1 μM alone (solid line) or in the mixture with 10 μM (β15-66)₂ (dashed line). The spectra were normalized to the intensity of VLDLR(2-4)_{HT} alone taken as 1.0 in both cases. All experiments were performed at room temperature in TBS containing 1 mM CaCl₂.

Figure 7.

Size-exclusion chromatography of the VLDLR(2-4)_{HT} and (β15-66)₂ fragments and their mixture. Elution profiles of VLDLR(2-4)_{HT} loaded onto a Superdex 75 column at 2 μM, (β15-66)₂ loaded at 20 μM, and their mixture are shown by filled circles, open circles, and filled triangles, respectively; the absorbance at 280 nm is presented in relative units (r.u.). The mixture containing 2 μM VLDLR(2-4)_{HT} and 20 μM (β15-66)₂ was incubated for 30 min at 37 °C prior to loading onto the column. All experiments were performed at room temperature in TBS containing 1 mM Ca²⁺.

Figure 8.

ELISA-detected interaction of the wild-type and mutant VLDLR(1–8)_{HT} fragments with the (β15–66)₂ fragment. Increasing concentrations of wild-type VLDLR(1–8)_{HT} (open circles), or VLDLR(1–8)_{HT} Trp105Ala mutant (filled circles) were incubated with microtiter wells coated with (β15–66)₂, and the bound fragments were detected with anti-His tag mAb, as described in Experimental Procedures. The curve for VLDLR(1–8)_{HT} represents the best fit of the data to eq. 1, the determined K_d value was 21 ± 3 nM; error bars represent the standard deviation of triplicate determinations. The experiments were performed in ELISA-binding buffer.

Table 1

Primers Used to Produce Recombinant VLDLR Fragments.

Fragments	Sequence	Primers used	
		Forward	Reverse
VLDLR(1–8) _{HT}	Gly ¹ -Cys ³²⁸ (His) ₆	1	2
VLDLR(1–4) _{HT}	Gly ¹ -Pro ¹⁶³ (His) ₆	1	3
VLDLR(5–8) _{HT}	Pro ¹⁶⁴ -Cys ³²⁸ (His) ₆	4	2
VLDLR(1–2) _{HT}	Gly ¹ -His ⁸² (His) ₆	1	5
VLDLR(2–3) _{HT}	Lys ⁴² -Asn ¹²⁴ (His) ₆	6	7
VLDLR(3–4) _{HT}	Arg ⁸⁴ -Pro ¹⁶³ (His) ₆	8	3
VLDLR(2–4) _{HT}	Lys ⁴² -Pro ¹⁶³ (His) ₆	6	3

Primer number	Primer sequence
1	5'-TGGCGCCTCATATG GGGAGAAAAGCCAAATGTGAACCC-3'
2	5'-GCTGCTCGAGT C AGTGGTGGTGGTGGTGGTGACACTCTTTCAGGG GCTCATC-3'
3	5'-TTTAAACTCGAGT C AGTGGTGGTGGTGGTGGTGC GGGGCACAGT CCAGCTC-3'
4	5'-GATCGCCAACATATG CCAACCTGTGGCGCCCATG-3'
5	5'-GCTGCTCGAGT C AGTGGTGGTGGTGGTGGTGGCACTGTTCTGGGC TTTC-3'
6	5'-TGGCGCCTCATATGAAGACGTGTGCTGAATCTGACTTCG-3'
7	5'-TTTAAACTCGAGT C AGTGGTGGTGGTGGTGGTGATTGCCACAGTT TTCTTC-3'
8	5'-TGGCGCCTCATATGAGAACATGCCGCATACATG-3'

Restrictase-recognition sequences are underlined; stop codons are shown in bold.

Table 2

Fluorescence parameters for denaturation of the VLDLR fragments and its interaction with Ca^{2+} , RAP, and the $(\beta 15-66)_2$ fragment.

VLDLR fragments	Denaturation ^a λ_{max} shift (nm)	+ Ca^{2+} I_0 increase (%)	+ Ca^{2+} (denatured) ^b I_0 increase (%)	+ RAP λ_{max} shift (nm)	+ $(\beta 15-66)_2$ λ_{max} shift (nm)
sVLDLR _{HT}	343→347	---	---	---	---
VLDLR(1-8) _{HT}	349→352	80	no	349→347	349→347
des(1-8)VLDLR _{HT}	338→345	---	---	---	---
VLDLR(1-4) _{HT}	351→354 ^c	75	no	351→347	351→348
VLDLR(5-8) _{HT}	351→354	60	no	351→351	351→351
VLDLR(1-2) _{HT}	351→353 ^c	89	no	351→351	351→351
VLDLR(2-3) _{HT}	352→352	142	no	352→346	352→349
VLDLR(3-4) _{HT}	353→353	122	no	353→353	353→353
VLDLR(2-4) _{HT}	353→353	236	no	353→347	353→348

^aDenaturation was performed by heating up to 90 °C.

^bDenaturation was performed by addition of 4 M GdmCl and 1 mM DTT.

^cDenaturation was performed by heating up to 90 °C in the presence of 4 M urea.

Table 3

Equilibrium dissociation constants (K_d) for the interaction of the VLDLR fragments with the $(\beta 15-66)_2$ fragment.

VLDLR fragments	ELISA ^a (K_d , nM)	ELISA ^b (K_d , nM)	SPR (K_d , nM)	Binding to RAP-Sepharose
sVLDLR	-----	5.7 ± 0.4^c	3.6 ± 0.9^c	yes
sVLDLR _{HT}	16.4 ± 6.4	2.5 ± 1.2	3.6 ± 0.2	yes
VLDLR(1-8) _{HT}	16.3 ± 7.8	3.4 ± 1.5	3.1 ± 1.1	yes
des(1-8)VLDLR _{HT}	n.b. ^d	-----	n.b.	n.b.
VLDLR(1-4) _{HT}	60 ± 16	-----	3.7 ± 0.1	yes
VLDLR(5-8) _{HT}	n.b.	-----	n.b.	n.b.
VLDLR(1-2) _{HT}	n.b.	-----	n.b.	n.b.
VLDLR(2-3) _{HT}	78 ± 8	-----	9.5 ± 0.4	yes
VLDLR(3-4) _{HT}	n.b.	-----	n.b.	n.b.
VLDLR(2-4) _{HT}	40 ± 2	-----	4.6 ± 0.1	yes

^aDetermined using anti-His tag mAb (see text).

^bDetermined using anti-VLDLR(1-8) mAbs (see text).

^cDetermined earlier⁵.

^dn.b., no binding.