The molecular mechanism of lipoprotein binding by the low-density lipoprotein (LDL) receptor (LDLR) is poorly understood, one reason being that structures of lipoprotein–receptor complexes are not available. LDLR uses calcium-binding repeats (LRs) to interact with apolipoprotein B and apolipoprotein E (ApoB and ApoE). We have used NMR and SPR to characterize the complexes formed by LR5 and three peptides encompassing the putative binding regions of ApoB (site A and site B) and ApoE. The three peptides bind at the hydrophilic convex face of LR5, forming complexes that are weakened at low [Ca$^{2+}$] and low pH. Thus, endosomal conditions favour dissociation of LDLR/lipoprotein complexes regardless of whether active displacement of bound lipoproteins by the $\beta$-propeller in LDLR takes place. The multiple ApoE copies in very low density lipoproteins ($\beta$-VLDLs), and the presence of two competent binding sites (A and B) in LDLs, suggest that LDLR chelates lipoproteins and enhances complex affinity by using more than one LR.

Introduction

The receptor of low-density lipoproteins (LDLR) is a multi-domain transmembrane protein of 839 residues [1,2] that belongs to a family of membrane receptors that include the VLDL receptor, apoER2, MEGF7, LRP, LRP1B and the Megalin receptor [3], all of which are formed by various combinations of a few common domains. The primary ligands of LDLR are the cholesterol-rich LDL and $\beta$-VLDL lipoproteins [4,5], and its chief role is maintaining cholesterol homeostasis. Many reported mutations in the gene encoding LDLR lead to familial hypercholesterolemia, a disease that affects 1 in 500 persons worldwide [6]. The ligand-binding domain of LDLR contains seven homologous LR repeats, each comprising approximately 40 residues and including three disulfide bonds and a structural calcium ion [7,8]. The fifth LR repeat (LR5) appears to play a major role in lipoprotein recognition [4,5].

Interaction of low-density lipoprotein (LDL) particles with LDLR is mediated by a single copy of apolipoprotein B (ApoB), which, with 4536 residues, is one of the largest proteins known [9,10]. Although the size and complexity of ApoB have prevented a detailed structural characterization, its binding region has been identified as being located around residues 3356–3368 (termed site B), which are exposed on the surface of LDL.
LDL particles [11]. Additional binding sites have been proposed, such as site A (residues 3143–3155), although their relevance has been questioned [12–14]. In addition to one copy of ApoB, β-VLDLs include several copies of the smaller apolipoprotein E (ApoE) (299 residues) [15,16]. The C-terminal domain of ApoE anchors the protein to the lipid particle, while the N-terminal domain is a four-helix bundle [17] that binds LDLR through its fourth helix [18–20] and additional residues [21].

Total de-lipidation of ApoE and ApoB leads to protein aggregation [17,22], which complicates structural studies of their binding to LDLR. So far, it has been shown that positively charged peptides with sequences found in ApoE bind to LDLR [23,24] or LRP receptors [25], a docking model of the ApoE(135–151)/LR5 complex has been proposed [26], and weak interactions between LR3, LR4 and LR5 and a linear fragment of ApoE have been described [27,28]. However, detailed structural information for the complexes formed by ApoB and ApoE with LDLR is lacking.

LR5 is one of the most important repeats within the ligand-binding domain of LDLR [5,29,30]. We have described the oxidative folding pathway of wild-type LR5 repeat and mutant LR5 repeats found in familial hypercholesterolaemia [31], and have proposed that the structural instability of LR5 under endosomal conditions favours LDL release in the endosome [32,33]. Here, we determine the affinity of LR5 for peptides comprising putative binding sequences of ApoB and ApoE. At extracellular pH, peptides representing site A and site B from ApoB and the binding region of ApoE3 bind LR5 with similar affinity (K_D values of 12, 23 and 56 μM for site A, site B and ApoE3 peptides). The binding takes place at a common site in LR5 that includes some of the residues involved in calcium binding together with several conserved residues of previously unknown function.

Results and Discussion

LR5 binds apolipoprotein peptides at extracellular pH in a Ca^{2+}-dependent manner

Performing surface plasmon resonance (SPR) experiments at 25 °C allowed us to determine the association and dissociation rate constants (k_on and k_off) and the equilibrium dissociation constant (K_D) for binding of LR5 to the three apolipoprotein peptides. Although all three peptides bind to LR5 and dissociate from it fast which complicates determination of the rates, accurate values have been derived for each peptide complex by global fitting of various traces obtained at various peptide concentrations (see Experimental procedures). Previous to this analysis, we first discarded the possibility that the association observed between the apolipoprotein mimetic peptides and LR5 is non-specifically driven by electrostatic interactions by analysing a randomized peptide with the same amino acid composition as site A of ApoB. The SPR response of the randomized peptide (see binding traces at several pH values in Fig. S1) is almost negligible compared to that of the ApoB site A peptide or the two other mimetic peptides (Figs 1–4).

**Fig. 1.** ApoB site B peptide binding to LR5 under extracellular pH conditions (NaCl/Pi buffer, pH 7.4) in the presence of either 250 μM CaCl_2 (A, B) or 1 mM EDTA (C, D). (A, C) SPR association and dissociation curves of the ApoB site B peptide at 25 μM (dark green), 35 μM (blue), 50 μM (brown), 70 μM (pink), 100 μM (green) and 200 μM (black). (B, D) The initial association step, with the experimental traces indicated by black triangles and their global fitting to Eqsns (1–3) (see Experimental procedures) indicated by red lines. All experimental points in the initial 5 s are shown, but only one in ten experimental points is shown for the rest of the trace.
The importance of the structural calcium ion in the interaction of LDLR with lipoproteins was highlighted by the observation that the calcium chelator EDTA disrupts LDL and β-VLDL binding to LDLR [34]. We tested whether the site A, site B or ApoE3 mimicking peptides (Table 1) bind to LR5 under otherwise extracellular conditions lacking calcium (NaCl/Pi, pH 7.4, 1 mM EDTA). In the presence of calcium, the three apolipoprotein mimetic peptides bind LR5 with observed $K_D$ values in the micromolar range (Table 2). The binding/dissociation kinetics of the site B peptide at various peptide concentrations are shown in Fig. 1, and those of site A and ApoE3 peptides are shown in Figs 2 and 3. Calcium removal does not abolish binding of the peptides to LR5, but significantly reduces the affinity of each of these complexes (Table 2). Site A, site B and ApoE3 peptides differ in terms of binding kinetics and with regard to the influence exerted by Ca$^{2+}$ on the
The affinity of their complexes with LR5. The ApoE3 peptide binds fastest to LR5-Ca\(^{2+}\) (\(k_{\text{on}} = 0.129 \mu\text{M}^{-1}\text{s}^{-1}\)), and its rate of binding decreases tenfold when calcium is not present (\(k_{\text{on}} = 0.011 \mu\text{M}^{-1}\text{s}^{-1}\)). Similarly, the site A ApoB peptide binds to LR5-Ca\(^{2+}\) faster (\(k_{\text{on}} = 0.031 \mu\text{M}^{-1}\text{s}^{-1}\)) than it binds to ApoLR5 (\(k_{\text{on}} = 0.010 \mu\text{M}^{-1}\text{s}^{-1}\)). In contrast, there appears to be no calcium dependence regarding the binding kinetics of site B ApoB peptide. On the other hand, the complexes formed by either site A or site B peptides with LR5-Ca\(^{2+}\) show similar \(k_{\text{off}}\) values that are increased (faster dissociation) in the absence of Ca\(^{2+}\), especially for the site A complex, while the ApoE3/LR5 complex dissociates more rapidly than those of the site A or site B peptides and in a calcium-independent manner. The combined effect of the binding and dissociation rate constants determines that calcium increases the affinity of the complexes by factors of 2.5 (site B peptide), 8 (ApoE3 peptide) and 25 (site A peptide), as shown in Fig. 5A. That the peptides still bind LR5 in the absence of Ca\(^{2+}\) may be surprising in view of the reported random coil conformation of ApoLR5 [35], and may be

**Table 1.** Sequences of apolipoprotein mimetic peptides. For ApoE3, a Q156A substitution was introduced within the 141–158 ApoE sequence to increase the peptide helix content.

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Apolipoprotein sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE3</td>
<td>WGLRKLKRRLRDLDEHKR</td>
<td>WG-(141-158 ApoE3)</td>
</tr>
<tr>
<td>ApoB site B</td>
<td>WGRLTRLRGLKSLATA</td>
<td>WG-(3356-3368 ApoB)</td>
</tr>
<tr>
<td>ApoB site A</td>
<td>WGVKAYKKNKHHRS</td>
<td>WG-(3143-3155 ApoB)</td>
</tr>
<tr>
<td>Control</td>
<td>WGSQVTQELRALMDETMKEL</td>
<td>WG-(54-71 ApoE3)</td>
</tr>
<tr>
<td>Randomized ApoB site A</td>
<td>KWRNSHGKVKYQHAK</td>
<td>–</td>
</tr>
</tbody>
</table>

**Table 2.** Binding and dissociation rate constants, and dissociation equilibrium constants of complexes formed by three apolipoprotein mimetic peptides and LR5 under extracellular conditions (NaCl/Pi buffer, CaCl\(_2\) 250 \(\mu\text{M}\), pH 7.4).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Presence of Ca(^{2+})</th>
<th>(k_{\text{on}}) ((\mu\text{M}^{-1}\text{s}^{-1}))</th>
<th>(k_{\text{off}}) (s(^{-1}))</th>
<th>(K_D) ((\mu\text{M}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoE3</td>
<td>Yes</td>
<td>0.129 ± 0.0060</td>
<td>2.97 ± 0.01</td>
<td>23 ± 8</td>
</tr>
<tr>
<td>ApoE3</td>
<td>No</td>
<td>0.011 ± 0.0011</td>
<td>2.15 ± 0.09</td>
<td>190 ± 30</td>
</tr>
<tr>
<td>ApoB site B</td>
<td>Yes</td>
<td>0.011 ± 0.0011</td>
<td>0.59 ± 0.01</td>
<td>55 ± 3</td>
</tr>
<tr>
<td>ApoB site B</td>
<td>No</td>
<td>0.013 ± 0.0034</td>
<td>1.58 ± 0.05</td>
<td>126 ± 7</td>
</tr>
<tr>
<td>ApoB site A</td>
<td>Yes</td>
<td>0.031 ± 0.009</td>
<td>0.40 ± 0.06</td>
<td>13 ± 3</td>
</tr>
<tr>
<td>ApoB site A</td>
<td>No</td>
<td>0.010 ± 0.0011</td>
<td>2.90 ± 0.1</td>
<td>300 ± 20</td>
</tr>
</tbody>
</table>

Fig. 4. ApoB site B peptide binding to LR5 at pH 7.0, 6.5, 6.0 or 5.5 in the presence of 250 \(\mu\text{M}\) CaCl\(_2\) and 150 mM NaCl. (A) SPR association and dissociation curves of the ApoB site B peptide at 12.5 \(\mu\text{M}\) (red), 17.5 \(\mu\text{M}\) (orange), 25 \(\mu\text{M}\) (dark green), 35 \(\mu\text{M}\) (blue), 50 \(\mu\text{M}\) (brown), 70 \(\mu\text{M}\) (pink), 100 \(\mu\text{M}\) (green) and 150 \(\mu\text{M}\) (cyan). (B) The initial association step, with the experimental traces indicated by black triangles, and their global fitting to Eqns (1–3) (see Experimental procedures) indicated by red lines. All experimental points in the initial 5 s are shown, but only one in ten experimental points is shown for the rest of the trace.
related to a stabilizing effect of the peptides on the structure of the repeat. It should be noted that the site A, site B and ApoE3 peptides correspond to protein segments that are thought to adopt helical conformations in the apoprotein context. Because the helicities of free peptides are typically much lower than those exhibited within the corresponding proteins [36], it is expected that the affinities for ApoB and ApoE3 exhibited by LR5 are greater than those reported for the peptides in Table 2. This SPR analysis clearly demonstrates the sequence-specific, non-electrostatically driven interaction between apolipoprotein peptides and LR5. Further analysis (e.g. by a split-ubiquitin assay or cellular uptake of a fluorescent peptide in competition with an inhibitory ligand, such as the receptor-associated protein (RAP) is required to confirm the interaction and mechanisms in the cell.

The hydrophilic convex face of LR5 is the lipoprotein-binding site

Elucidation of the LDLR sequence [37] led to the initial proposal that the complex between the receptor and ApoE was stabilized by electrostatic interactions established by the negatively charged repeats of LDLR and a positively charged sequence found in ApoE. This proposal was generalized to the LDLR/LDL complex when multiple positively charged segments were found in the ApoB sequence [38,39]. However, the crystal structure of LR5 [8] showed that the conserved acidic residues of the repeat (D196, D200, D206 and E207) are essentially buried and are involved in direct binding of a structural calcium ion. Two faces were observed in the LR5 surface: a hydrophilic convex face enclosing the calcium-binding pocket, and a hydrophobic concave face that was proposed to constitute the lipoprotein-binding site [8]. However, indirect support for involvement of the hydrophilic convex face of LR5 in lipoprotein binding was later provided by the X-ray structure of the extracellular domain of LDLR at endosomal pH [29], by the structure of the complex between RAP and LR3/LR4, in which the convex faces of several repeats appear to be involved in protein binding to RAP [40], and by modelling work on binding of LR5 to an ApoE fragment [26]. More recently, there have been contradictory reports supporting a major role in lipoprotein binding for either the hydrophobic concave face [41,42] or for the hydrophilic convex face [27,43,44]. Further complicating this scenario, a possible role of the β-propeller domain of LDLR in LDL binding has also been proposed [43,45].

To determine experimentally which face of LR5 binds lipoproteins, we performed a solution NMR chemical shift perturbation analysis using 15N-labelled LR5. Initially, a perturbation analysis was performed at 25.0 °C, but only small changes in chemical shift were observed (data not shown). According to AGADIR predictions [46] and circular dichroism spectra (data not shown), the helical content of the synthesized peptides approximately doubles as the temperature decreases from 25.0 to 5.0 °C. We thus increased the peptide helical content by reducing the temperature, and performed chemical shift perturbation experiments at 5.0 °C. Binding of either site A, site B or ApoE3 peptides to LR5-Ca2+ produces significant changes in the chemical shift of several LR5 residues (Fig. 6). In contrast, the presence of a non-binding control peptide (Table 1) does not perturb LR5-Ca2+ chemical shifts (Fig. 6). Inspection of the LR5 X-ray structure [8] indicates that the perturbed residues are clustered in the three-dimensional structure. Thus, they may be identified as the binding site common to ApoB and ApoE.
The LR5 residues implicated in ApoB site B, ApoB site A and ApoE3 binding are highlighted in Fig. 7. As judged from the observed chemical shift perturbations, W193, G197, G198, D200 and D206 appear to be similarly important for binding of the three peptides, while the region around E180, F181, H182 and C183, also appears to be important in the ApoE3 and site A complexes. All these residues are located at the hydrophilic convex face of LR5, and we propose that they constitute a general lipoprotein-binding site in
LR5. This lipoprotein-binding site essentially coincides with the sites used by LR5 and by the homologous LR3/LR4 repeats to bind the LDLR β-propeller domain and RAP, respectively [29,40]. This involvement of the convex face of LR repeats in recognizing different but related proteins emphasizes the challenge of understanding how the affinity for its partners is modulated along the LDLR cellular route [47]. Other repeats of the receptor, in addition to LR5, are likely to use a similar binding site to interact with lipoproteins, as suggested by binding of LR repeats to a ubiquitinated peptide derived from ApoE [27].

**Conserved residues of LR5 repeats from vertebrates are crucial for lipoprotein binding**

Conserved residues of homologous proteins are typically involved in protein stabilization or protein function. All the conserved residues of the seven LR repeats in human LDLR (Fig. 8) play distinct roles in maintaining the stability of the modules: the six cysteines form three disulfide bonds, F181 and I189 form a small hydrophobic core, D196, D200, D206 and E207 coordinate the structural calcium ion, and D203 is important in maintaining a hydrogen bond network connecting the two lobes of the repeats [8,48]. Given the clear structural role of these residues and the fact that not all the repeats are equally important for lipoprotein binding, it is not surprising that some of the lipoprotein-binding residues identified in LR5 are not conserved among the repeats. They are nevertheless conserved among LR5 repeats from different species. Alignment of LR5 sequences for a number of vertebrates from human to fish (Fig. 8) reveals several residues (P175, E180, H182, S185, E187, H190, W193 and G197) that are conserved within vertebrate LR5 repeats but not among the seven human repeats. The reason for the conservation of P175 is not obvious. S185 and E187 play a structural role, being involved in the hydrogen bond network mentioned above [8,48]. H190 has been implicated in the mechanism of LDL release [29,49], and E180, H182, W193 and G197 are precisely located at the lipoprotein-binding site (Fig. 2). Residue W193 appears to be particularly important, playing the dual role of contributing to calcium coordination and to apoprotein binding. Trp residues are well suited to form stabilizing cation/p interactions [50,51] with the positively charged residues present in the three lipoprotein peptides analysed here. The key role played by calcium-coordinating tryptophan residues of LDLR repeats in the interaction established by the receptor with other proteins has been highlighted [47], and its importance in the binding of lipoproteins has also been demonstrated by mutational studies [42,43]. At present, it is unclear whether the interaction of W193 with LDL or other proteins influences the coordination of the calcium-binding site. It is also possible that the contribution of other residues, such as G198, to calcium coordination may be influenced by the binding of proteins to LR5.

**LR5 binding to ApoB site B peptide is weaker at endosomal pH: implications for the mechanism of LDL release**

We tested binding of the three lipoprotein peptides to LR5 under endosomal mimicking conditions (10 mM MES, 150 mM NaCl, pH 5.5) by SPR. However, two
of the three apolipoprotein peptides (ApoB site A and ApoE) could not be analysed at this pH due to non-specific interactions with the surface of the Biacore chips that could not be alleviated by increasing the ionic strength or changing the buffer. Fortunately, for the apoB site B peptide, we were able to analyse fine binding/dissociation traces at several pH values: 7.0, 6.5, 6.0 and 5.5, which evaluation of the effect of the endosomal decrease in pH on the affinity of its complex with LR5. The experimental traces and their fits to Eqns (1–3) are shown in Fig. 4, and the fitted values of $k_{\text{on}}$, $k_{\text{off}}$ and $K_D$ at the various pH values are shown in Table 3. The affinity of the complex is markedly reduced as the pH is decreased. At pH 7.4, under very similar but not identical solution conditions, the $K_D$ was 55 μM (Table 2). The data in Table 3 indicate that, at pH 7.0, the $K_D$ is 142 μM and progressively increases to 854 μM at pH 5.5 (see also Fig. 5B). The lower affinity at lower pH is mainly due to faster dissociation of the complex at low pH, with the increase in $k_{\text{off}}$ being noticeable between pH 6.5 and 6.0. The fact that the affinity of the complex is reduced as a consequence of faster dissociation rather than of slower formation is relevant because the direct effect of reducing the pH is to favour dissociation of previously formed LDL/LDLR complexes. Complementary evidence for weakening of the ApoB site B/LR5 complex at low pH has been obtained by NMR. We first tried to use chemical shift perturbation analysis to estimate the dissociation constants, but no plateau was found in the experimental range tested. Nevertheless, we were able to determine the slopes of the variation of the chemical shift with the equivalents of titrant (7.5 and 13 ppb-equiv⁻¹ at pH 5.0 and 7.5, respectively, data not shown). These numbers indicate that binding of ApoB site B to natively folded LR5-Ca²⁺ is reduced at low pH (5.0) compared with extracellular pH conditions.

The helical content of the three peptides is the same at pH 5.0 and 7.5 according to AGADIR [46]. As the weaker binding observed at low pH cannot be attributed to lower peptide helicity, it must be concluded that endosomal pH weakens the binding of native LR5-Ca²⁺ to the ApoB site B peptide. The molecular reason for this cannot be related to weakening of cation/π interactions between W193 and basic residues of the peptide because Trp/Lys and Trp/Arg interactions are similarly strong at pH 7.5 and 5.0, while Trp/His interactions are even stronger at pH 5.0 [50,51] than at pH 7.5. Thus, the most likely reasons for weakening of the complex at low pH are either weakening of charge/charge interactions due to protonation of LR5 carboxylates, or protonation of H190 and/or H182 if they are buried in the complex without forming a suitable stabilizing interaction.

The weakening of the ApoB peptide binding to LR5 at low pH has a bearing on the mechanism of LDL release in the endosome. The currently accepted model for LDL release states that the LDL particles that bind to LR repeats at the cell surface are displaced by the β-propeller of LDLR, which, under endosomal conditions, displays a higher affinity for the repeats than LDL [29,49]. It may also be argued that a role for the β-propeller in LDL release is demonstrated by mutational experiments indicating that, when this domain is removed, internalized LDLR is not recycled to the cell surface [52]. However, the mechanism of LDLR recycling is not fully understood, and a lack of recycling need not be incompatible with LDL/LDLR dissociation. More recent work implying specific involvement of the propeller in LDL release at low pH [42,43] was performed in the presence of Ca²⁺.

Our observation that only a small fraction of the LR5 population is in the native Ca²⁺-complexed form under endosomal conditions is suggestive of an alternative mechanism of LDL release based on reversible binding/folding events [33]. We proposed that LDL release may be simply triggered by LR5 destabilization at the low pH and low calcium concentrations that are found in the endosome [33]. An admitted weak point in our proposal was that the potential influence of lipoprotein binding on the stability of LR5 was not examined, and therefore the possibility that LR5 is far more stable in complex with LDL than in isolation cannot be excluded. However, our present analysis of LR5 binding to lipoprotein mimicking peptides suggests the possibility that LDL/LDLR complexes may no longer be stable at endosomal pH and Ca²⁺ concentration. Endosomal conditions appear to result in dissociation of the LDL/LDLR complex by reducing the affinity of LR5 for ApoB (this work) and unfolding the LR5 repeat [31], which displaces further the equilibrium towards dissociation. It is nevertheless unclear at present, and remains to be tested, whether

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**Table 3.** Binding and dissociation rate constants, and dissociation equilibrium constants of complexes formed by the ApoB site B peptide and LR5 at various pH values (10 mM PIPES buffer, 150 mM NaCl, 250 mM CaCl₂).

<table>
<thead>
<tr>
<th>Peptide</th>
<th>pH</th>
<th>$k_{\text{on}}$ (μM⁻¹s⁻¹)</th>
<th>$k_{\text{off}}$ (s⁻¹)</th>
<th>$K_D$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ApoB site B</td>
<td>7.0</td>
<td>0.016 ± 0.001</td>
<td>2.28 ± 0.04</td>
<td>142 ± 20</td>
</tr>
<tr>
<td>ApoB site B</td>
<td>6.5</td>
<td>0.012 ± 0.001</td>
<td>2.17 ± 0.03</td>
<td>183 ± 20</td>
</tr>
<tr>
<td>ApoB site B</td>
<td>6.0</td>
<td>0.010 ± 0.002</td>
<td>4.47 ± 0.2</td>
<td>430 ± 130</td>
</tr>
<tr>
<td>ApoB site B</td>
<td>5.5</td>
<td>0.008 ± 0.002</td>
<td>6.96 ± 0.4</td>
<td>854 ± 400</td>
</tr>
</tbody>
</table>
the complexes formed by LDLR and LDL, which are presumably stronger than those formed between LR5 and the peptides analysed here, are similarly disrupted by endosomal pH conditions.

Proposal for multivalent binding of LDLR to LDL and β-VLDL

It is generally accepted that the LDLR-binding region of ApoE is the fourth helix, although additional residues have been reported as important for binding [21]. To some extent, the observed binding of the ApoE3 peptide to LR5 was expected. In contrast, the binding of the site A peptide to LR5 was not anticipated. When the sequence of ApoB was published [9,38,39,53], several basic regions were singled out as potential binding sites for the LDLR [9,11,38,39,53,54], and two of them (site A, residues 3147–3157; site B, residues 3359–3369) were identified as the main candidates. Phylogenetic studies [14] suggested that site B was the primary region involved in interaction with the LDLR, but also attributed an important role to site A in LDL binding to LDLR. Experiments with truncated LDLs that contain either both site A and site B [55] or neither [56] confirmed that the sequence encompassing both site A and site B is required for LDLR binding, while subsequent studies with LDL lacking the site B sequence [13] indicated that site A alone is not sufficient for LDL binding. Mutational experiments [12] also indicated that site B is indeed necessary for receptor binding.

However, it has been shown here (Table 2) that, under extracellular conditions, a peptide encompassing the sequence of site A binds LR5 with higher affinity than a site B peptide. Possible explanations for the observed inability of LDLs lacking site B to effectively bind to LDLR may be that site A is not accessible in the mature lipoprotein, or that additional LDL/LDLR interactions are required to ensure effective binding. Although the affinities of sites A and B for LR5 within the context of the entire ApoB protein are probably higher than the micromolar affinities exhibited in isolation, it is uncertain whether the nanomolar affinities characteristic of high-affinity complexes are achieved. In this respect, an efficient way to increase the affinity of the LDL/LDLR complex would be to make use of the chelating effect. It seems likely that site A and site B bind LDLR by associating with LR5 plus one additional repeat (Fig. 9), greatly increasing the overall affinity of the LDL/LDLR complex. A sequential binding model may be envisaged in which site B binds first to LDLR. Then, re-organization in the LDL leads to a greater exposure of site A, allowing recognition of this site by another LR in the receptor. However, given the scarcity of kinetic data, the existence of alternative kinetic binding mechanisms cannot be excluded, and additional investigation is required.

The observed affinity of the ApoE3/LR5-Ca²⁺ complex may similarly be too low to justify efficient binding. We thus propose that β-VLDL particles use more than one copy of ApoE in order to bind simultaneously to LR5 and additional repeats of LDLR, consistent with reported observations of formation of low-affinity complexes between LR4 and LR5 and a ubiquitinated ApoE peptide [27,28].

In summary, our kinetic and structural analysis indicates that the binding of three apolipoprotein mimetic peptides (ApoB site A, ApoB site B and ApoE) to LR5 is fast, takes place at the convex hydrophilic face of LR5, and it is modulated by pH and calcium concentration. We propose that the binding of LDL and β-VLDL by LDLR is multivalent.

Experimental procedures

Cloning and purification of LR5

The fragment corresponding to the fifth repeat of the LDLR gene was amplified by PCR and cloned into pGEX-4T-3
(Amersham Biosciences, Little Chalfont, UK), and recombinant LR5 was expressed and purified as described previously [33]. Pure, reduced, unfolded LR5 was re-folded by exhaustive dialysis under conditions permitting disulfide exchange, and further purified by reverse-phase HPLC in a Symmetry C18 column (Waters, Milford, MA, USA). Silver staining after SDS/PAGE showed the LR5 was 99% pure. MALDI-TOF MS using a Bruker Ultraflex mass spectrometer confirmed the purity of the repeat and the uniqueness of the disulfide pattern [31]. A theoretical extinction coefficient (ε280) of 6050 M⁻¹·cm⁻¹ was used in determination of LR5 concentrations [57].

**Isotope labelling for NMR studies**

The plasmid containing the fragment of the gene corresponding to LR5 was transformed into competent *Escherichia coli* BL21 cells. Colonies were grown in ampicillin-containing LB agar plates for 30 h. Selected colonies were grown in LB medium (10 mL) for 5 h. The harvested cells were resuspended in minimal medium in which the only nitrogen source was ¹⁵NH₄Cl (1 g liter⁻¹), and cultured in 2 L Erlenmeyer flasks (typically containing 1 L of culture). Induction was performed after approximately 10 h by adding 1 mM isopropyl thio-β-galactoside. Purification of labelled LR5 was performed as described for the unlabelled protein.

**Design of peptides encompassing apolipoprotein sequences**

The five peptides used in this work were synthesized by Pompeu Fabra University (Spain) and ChinaPeptides (Shanghai, China), and are summarized in Table 1. The site A and site B peptides mimic the ApoB sequences traditionally known as site A (residues 3143–3155) and site B (residues 3356–3368). The ApoE3 peptide mimics ApoE (141–158), and contains a Q156A substitution to increase the N-helix propensity. The control peptide mimics residues 54–71 of ApoE, a segment of the protein that is located in the first helix of the bundle and is not expected to bind to LR5. The randomized ApoB site A peptide has the same composition as the ApoB site A peptide but with a random order of the original amino acid residues. An N-terminal Trp residue followed by a Gly was added to each peptide to allow peptide quantification [57] (ε₂₈₀ = 5500 M⁻¹·cm⁻¹ for ApoE3, site B and control peptides; ε₂₈₀ = 6990 M⁻¹·cm⁻¹ for the site A peptide and the randomized ApoB site A peptide). The five peptides are acetylated and amidated, respectively, at their N- and C-termini.

**NMR experiments**

All experiments were performed using a Bruker Avance 600 MHz spectrometer with LR5 ¹⁵N-labelled samples of 100–350 µM concentration in 20 mM phosphate buffer (pH 7.5) with 8% D₂O and 3 mM CaCl₂. At 5 °C, two-dimensional ¹H-¹⁵N-HSQC spectra were recorded in the absence and the presence of 2.5 equivalents of ApoE3, site A or site B peptides. Spectral assignment at 278 K was based on the published assignment at 5°C [35], corroborated by analysis of a 3D-HSQC-NOESY spectrum of LR5-Ca²⁺. All chemical shifts were estimated from the ¹H-¹⁵N-HSQC spectra by interpolation of the peak centre using NMRPipe [58] and scripts written in-house.

**Surface plasmon resonance experiments**

SPR experiments were performed in a Biacore T200 instrument (GE Healthcare, Fairfield, CT, USA) at 25 °C. Lyophilized LR5 was immobilized on a CMS chip by amine coupling according to the manufacturer’s instructions using HBS/EP (10mM Hepes, 150mM NaCl, 3mM EDTA, 0.005% Tween-20) as running buffer. One flow cell was activated with EDC/NHS (N-(3-dimethylaminopropyl)-N’-ethylcarbodiimide hydrochloride/N-hydroxysuccinimide), blocked with 1 M ethanolamine, and used as a control surface. Experiments were performed in NaCl/P, buffer, pH 7.4, with either 250 µM CaCl₂ or 1 mM EDTA. The peptides (ApoE3, site A and site B peptides) were injected at a flow rate of 30 µL·min⁻¹ for 40 s, and dissociation steps were followed for 10 min. Typically, four to six peptide concentrations were used for each measurement. Similar experiments were performed with ApoB site B peptide and the randomized ApoB site A peptide in 10 mM PIPES buffer, 150 mM NaCl, 250 µM CaCl₂ at pH 7.0, 6.5, 6.0 and 5.5 with 0.1 mg·mL⁻¹ BSA (Sigma-Aldrich, St. Louis, MO, USA) to avoid non-specific interactions. Data analysis was performed using software developed in our laboratory and implemented in MLAB. The SPR signal was considered to consist of the binding/dissociation signal plus the bulk-effect signals affecting each of the two steps. The association and dissociation events were analysed separately, considering their corresponding bulk effects as very fast exponential signals. Thus, the dissociation part of each trace was fitted to:

$$ S = A_{off} e^{-k_{obs}t} + A_{bulk} e^{-k_{bulk}t} + S_{end} $$

where S is the signal detected by the instrument, A_off is the signal change attributed to LR5/peptide dissociation, k_obs is the apparent dissociation rate constant, A_bulk is the amplitude of the bulk effect, k_bulk is the rate constant of the bulk effect, and S_end refers to the SPR signal at the end of the dissociation process. Because the experiments were performed using a continuous flow of running buffer, the dissociation observed is an irreversible process where k_obs = k_off. Therefore, the zero-order dissociation rate constant (k_off) may be directly obtained from the fitting.
Similarly, the association part of the curves was fitted to:

$$S = A_{on} \times e^{-k_{on}t} + A_{bulk} \times e^{-k_{bulk}t} + S_{end}$$

(2)

where $A_{on}$ is the signal change attributed to LR5/peptide binding, $A_{bulk}$ and $k_{bulk}$ are the amplitude and rate constant of the bulk effect, respectively. $S_{end}$ refers to the SPR signal at the end of the dissociation process, and $k_{obs}$ is the apparent association rate constant, which is related to the microscopic $k_{on}$ and $k_{off}$ by:

$$k_{obs} = k_{on} \times [P] + k_{off}$$

(3)

where $[P]$ represents the concentration of free peptide.

For each of the three peptides investigated, the traces obtained at various peptide concentrations were globally fitted to Eqns (1) or (2) plus (3) in order to derive common $k_{on}$, $k_{off}$ and $k_{bulk}$ values for the various traces. In the case of the randomized ApoB site A peptide, very minor binding was observed and fitting was not performed.

Acknowledgements

We acknowledge financial support from grants BFU2010-16297, BFU2010-19451, CTQ2009-10353 from Spain and Grupo Protein Targets B89 from the Diputación General de Aragón (Spain). X.A.-M. was supported by a Basque Government fellowship. We thank S. Bronsoms (Proteomics facility, Universitat Autonoma de Barcelona) for analysis of MALDI-TOF data, and S.C. Blacklow (Department of pathology, Harvard University) for the NMR assignments of the LR5 module.

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**Supporting information**

Additional supporting information may be found in the online version of this article at the publisher’s web site:

**Fig. S1.** Binding of the randomized ApoB site A peptide to LR5 in PIPES buffer at pH 7.0, 6.5, 6.0 or 5.5 in the presence of 250 μM CaCl₂ and 150 mM NaCl.