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Versatility in ligand recognition by LDL receptor family proteins: advances and frontiers

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Summary

Proteins of the low-density lipoprotein receptor family transport cholesterol-carrying particles into cells, clear protease-inhibitor complexes from the circulation, participate in biological signaling cascades, and even serve as viral receptors. These receptors utilize clusters of cysteine-rich LDL receptor type-A (LA) modules to bind many of their ligands. Recent structures show that these modules typically exhibit a characteristic binding mode to recognize their partners, relying primarily on electrostatic complementarity and avidity effects. The dominant contribution of electrostatic interactions with small interface areas in these complexes allows binding to be regulated by changes in pH *via* at least two distinct mechanisms. The structure of the subtilisin/kexin family protease PCSK9, a newly identified molecular partner of the LDLR also implicated in LDL-cholesterol homeostasis, also raises the possibility that the LDLR and its related family members may employ other strategies for pH-sensitive binding that have yet to be uncovered.

Introduction

The low-density lipoprotein receptor (LDLR) is the protein primarily responsible for uptake of cholesterol-carrying particles into cells. Most circulating plasma cholesterol in humans is found in the form of low-density lipoprotein (LDL) particles, which contain apolipoprotein B (apo B) as their primary protein component. Cell-surface receptors bind at neutral pH to LDL particles *via* interactions with apo B, and also bind with high affinity to other classes of lipoprotein particles that contain apolipoprotein E (apoE). Receptor-ligand complexes enter cells by endocytosis through clathrin-coated pits, release bound lipoprotein particles in the low pH milieu of the endosome, and are then returned to the cell surface in a process called receptor recycling [1].

Human mutations that prevent normal clearance of LDL lead to the genetic disease familial hypercholesterolemia (FH), an autosomal dominant disorder characterized by elevated plasma LDL-cholesterol levels and an increased risk of atherosclerosis. The relationship between FH and accelerated cardiovascular disease is the most cogent illustration of the causal relationship between elevated cholesterol levels and coronary atherosclerosis [2]. Most cases of FH in humans result from loss-of-function mutations in the gene encoding the LDL receptor, or by loss-of-function mutations in the gene encoding apoB, the main protein constituent of LDL. Indeed, Brown and Goldstein first identified the LDL receptor in their search for the molecular

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basis of FH [1,3]. In 2003, a new molecular culprit for FH was identified: a proprotein convertase of the subtilisin/kexin family called PCSK9 [4]. Normally, PCSK9 binds to LDL receptors and facilitates their destruction by a mechanism that remains poorly understood. Mutations that enhance the activity of PCSK9 are associated with FH, whereas mutations that diminish its activity lead to reduced levels of circulating LDL-cholesterol [5] (see [6] for a recent review).

More generally, the LDLR also constitutes the founding member of an entire class of related transmembrane receptors. These proteins combine three types of extracellular modules in different arrangements to carry out a wide range of physiologic functions, both as transport proteins and as receptors or co-receptors in signal transduction cascades. In the LDLR and its closest relatives, which include the very low-density lipoprotein receptor (VLDLR), apolipoprotein E receptor-2, LDL receptor-related protein-1 (LRP) and megalin, the three module types are cysteine-rich repeats called LDL receptor type-A (LA) modules, EGF-like modules and YWTD beta-propeller domains.

Binding-site mapping studies for the core LDLR-family proteins show that the clusters of tandemly-repeated LA modules make the dominant contribution to the binding of most known ligands. In the case of the LDLR, a recombinant minireceptor that contains only the seven LA repeats retains the ability to bind LDL *in vitro*, showing that the rest of the protein is not necessary for LDL binding [7]. In addition, as few as two LA repeats (the LA4-LA5 fragment of the LDLR) suffice to bind apoE•phospholipid complexes, which are believed to represent the minimal lipid complex that exhibits high-affinity binding to the receptor [8]. In both the LDLR and VLDLR, the propeller domain is required to facilitate release of bound lipoproteins upon exposure to endosomal pH [9–11].

This review will focus on the structural basis for recognition of partners by the LA modules of the LDLR and related proteins, and on unresolved questions about ligand recognition emerging from advances in the structural characterization of PCSK9 and lipid-associated apoE, two physiologically important ligands for the LDLR.

Structural features of isolated LA modules

Each LA module is approximately 40 residues long, contains three disulfide bonds, and binds a calcium ion that is required to maintain the structural integrity of the module. The bound calcium ion is coordinated in an octahedral geometry by side-chains from four conserved acidic residues and the oxygen atoms from two backbone carbonyl groups (Figure 1). The coordination of the calcium ion by these surface exposed carboxylate groups results in a characteristic negative surface electrostatic potential localized on one face of the module around the bound ion. In the absence of calcium, the structural integrity of the LA modules is lost and, as a result, the receptor fails to bind lipoprotein particles. The vast majority of FH-associated mutations of residues in the LA modules probably disrupt module folding by altering either the calcium coordinating residues or other key scaffolding residues in the module.

Paradigmatic LA-module binding mode from the structure of an LDLR-RAP complex

The receptor-associated protein (RAP) is an ER-resident protein that binds tightly to LRP and other LDL receptor family proteins early in the secretory pathway, but then dissociates from the receptors at the lower pH of the Golgi compartment. The modulation of affinity by pH is believed to play an important role in the function of RAP as an escort protein for LDLR family members by preventing premature intracellular binding of ligands to the receptors during the

routing stage of receptor transport, enabling delivery of mature receptors to the cell surface [12].

The recent 1.26 Å structure of a complex between a two-module region of the LDLR (LA repeats 3–4) and the third domain of RAP (RAP-D3) provides a template for understanding the structural basis underlying ligand recognition by the LA modules of LDLR-family proteins [13]. In the LA3-4/RAP-D3 complex, the RAP domain forms a three-helix bundle, and contacts the receptor fragment at two docking sites, one per module. The mode of contact between each LA module and the RAP domain is nearly identical at each of the two sites. The interface at each docking site is dominated by electrostatic interactions between conserved acidic residues on the LA module and a lysine residue projecting from helix 2 of the RAP helical bundle (Figure 2). Three carboxylate oxygens constitute an “acidic necklace” [13] encircling the lysine residue at each docking site, and the aliphatic part of the lysine side chain also packs against an aromatic residue in each case.

This overall arrangement, in which acidic residues within the calcium coordination sphere combine with the nearby tryptophan residue to form a primary interface with a basic residue, appears to constitute a paradigmatic mode for the binding of LA domains to their ligands. Other structures containing inter- and intramolecular binding interfaces between LA repeats and their partners also share the essential elements of the recognition mode seen in the LA3-4/RAP-D3 complex (Figure 3). These complexes include the long-range intramolecular interface between LA repeats four and five and the YWTD beta-propeller domain [14], and a VLDLR fragment bound to human rhinovirus serotype 2 (HRV2), for which the LDLR and VLDLR serve as the primary receptor for cell entry [15]. In the structure of the giant erythrocyte respiratory complex from the earthworm *Lumbricus terrestris*, interactions between the LA repeats of three linker chains with the hemoglobin *b* subunits of the complex also exhibit this canonical binding mode with a minor variation: the basic side chains projecting into the acidic pocket are from arginine residues, rather than from lysines [16].

Selectivity of LA modules for various ligands may then be determined by secondary contacts to nonconserved residues from adjacent loops that decorate the LA surface nearby [17]. This binding mode also appears to be tailored for a multivalent kind of recognition, because of the small area contributed by each LA repeat to the interface. Thus, two repeats are used in the LA3-4/RAP-D3 interface, multirepeat fragments exhibit much higher affinity for the HRV2 capsid than single repeats [15], and the LA4-5 pair of repeats is needed to establish the long-range interface with the propeller domain at endosomal pH [14].

Regulation of affinity by pH: histidine switches as affinity modulators

The LDLR is in an open, binding-active conformation at neutral pH, but adopts a closed, binding inactive conformation at low pH with a long-range interface between the propeller domain and LA repeats four and five [14]. Biochemical studies implicate three histidine residues at the interface between the propeller domain and LA repeats four and five in reversibly modulating the conformation of the LDLR as a function of pH [9]. The structures of the LA repeats themselves do not change dramatically, nor are there large conformational movements within the propeller domain. Rather, the available data suggests that protonation of three key histidine residues favors the transition to the closed conformation by stabilizing the long-range interface.

The stability of RAP-lipoprotein receptor complexes also exhibits sensitivity to changes in pH. These complexes rely on the histidine residues of RAP to modulate its affinity for LRP [12], yet there are no histidine residues directly in the RAP-D3/LDLR interface. Instead, several histidines are buried in the interior of the RAP-D3 helical bundle [13]. The RAP-D3 helical

bundle undergoes a pH-dependent unfolding transition at a pH ~6.3 [12], and mutation of the buried histidine residues to phenylalanine stabilizes the bundle to both thermal and low pH-induced unfolding (Carl Fisher, Kristine Estrada and SCB, unpublished data). Together these studies suggest that titration of the buried histidines promotes the unfolding of the helical bundle as the pH is reduced below ~6.3, consistent with the idea that the complexes may dissociate as an indirect consequence of bundle unfolding during passage through the mildly acidic environment of the Golgi during maturation [12,13,18], though further studies are needed to test this proposal directly.

Apolipoprotein recognition

Despite nearly 20 years of investigation, the structural basis underlying recognition of ApoE and ApoB-containing lipoprotein particles by the LDLR and related receptors has remained elusive. In the lipid-free structure of the receptor-binding domain of ApoE, which adopts a four-helix bundle structure, the basic region required for binding to the LDLR lies on the solvent-exposed face of the fourth helix of the bundle [19], potentially in position to form contacts with the calcium-coordinating acidic clusters of the LA repeats. Nevertheless, even though the basic residues in the 140–160 region are exposed on the surface of a helix in the ApoE receptor-binding domain in its lipid-free state, ApoE does not bind to the LDLR with high affinity (nM) until it is associated with lipid [19]. Association with lipid causes the bundle to open up so that the hydrophobic interiors of the helices pack against the aliphatic tails of the lipids [20], but an explanation for why lipid association enhances the affinity of ApoE for the receptor has remained elusive.

The 10 Å-resolution crystal structure of a receptor-binding active ApoE-dipalmitoyl phosphatidylcholine particle [21], together with more recent analysis of similar particles in solution by small angle X-ray scattering [22], shows that lipid induces global rearrangements of the helices that comprise the receptor-binding domain (Figure 4). Biochemical studies have shown that R172, a residue disordered in the structure of the lipid-free N-terminal domain, makes an important contribution to high-affinity receptor binding [23]. A model based on the 10 Å-resolution x-ray diffraction data, combined with this and other biochemical observations, suggest that R172 is repositioned close to the 140–160 helix to create the receptor-binding epitope [21]. Electron paramagnetic resonance studies of ApoE using site-directed spin labels incorporated into a series of single cysteine substitution variants surrounding R172, together with fluorescence studies using an environmentally sensitive probe, also argue that lipid association induces a structural transition of this region of the protein from random coil to alpha helix and relocation to a more hydrophobic environment, again supporting the notion that lipid interaction triggers a crucial structural transition around R172 that enables high-affinity receptor binding [24].

This information, in combination with the RAP-D3:LA3-4 co-crystal structure suggests an eminently testable model for recognition of ApoE by the LDLR and its family members, as discussed in ref. 13 (see Fig. 4 of ref. 13). The key features of this model are: 1) a primary interface between the conserved calcium-binding residues of the LA repeats and positively charged lysine residues on the fourth helix of the 4-helix bundle, analogous to a RAP-D3 docking site, 2) supplementary contacts provided by R172, which appears to be relocated near the basic residues of the fourth helix upon lipid association [21], and 3) avidity effects due to the recruitment of at least two copies of ApoE per binding-active particle, because two copies of ApoE are present in the crystallized, binding-active particle, and at least two LA modules are necessary to form stable complexes with ApoE [8]. Whether or not any or all features of this model are correct, of course, await the results of future structural studies.

PCSK9 structure

The newest player in the LDL-cholesterol uptake drama is the subtilisin/kexin family proprotein convertase PCSK9 (also known as NARC-1; [25]). A number of studies in cell culture and animal models have shown that secreted PCSK9 can reduce surface LDLR levels, and the presence of PCSK9 in the culture medium results in the redistribution of receptors from the plasma membrane to the lysosomes [26], perhaps by interfering with normal recycling of receptors after internalization (see [6] for a review).

Like other members of this protease family, PCSK9 contains a prodomain (residues 31–152), a catalytic domain (residues 153–452), and a C-terminal domain (residues 453–692), and undergoes autocatalytic cleavage during maturation [25]. Unlike other members of the family, however, the prodomain remains tightly bound to the catalytic and C-terminal regulatory domains after self-cleavage [27], inhibiting the catalytic activity of the protein after autoprocessing.

Recent studies show that the self-cleaved form of PCSK9 binds directly to the LDLR [28]. Biochemical studies using recombinant proteins have revealed that the first EGF-like repeat of the LDLR (EGF-A) suffices for binding of recombinant, purified PCSK9, and that binding to this repeat is calcium dependent [26]. Additionally, the affinity of PCSK9 for the receptor is enhanced at endosomal pH [26,29]. Moreover, mutations associated with PCSK9 gain of function result in enhanced LDLR clearance from the cell surface [28,30], and at least one mutant, D374Y, exhibits ~25-fold increased affinity for the receptor at neutral pH [28,29]. However, it is still not clear whether the latent proteolytic activity of the enzyme is required for receptor downmodulation.

The structure of PCSK9 at neutral pH has now been reported by two groups, and raises many intriguing new questions about PCSK9 biochemistry and function [29,31]. The two most striking features of the structure are (i) the catalytic site of the enzyme is blocked by the prodomain in a non-covalent complex (Figure 5A), and (ii) the C-terminal domain, which consists of three beta-domain modules related to one another by a pseudo three-fold axis, displays a surface rich in histidine residues along a stripe derived primarily from the second module (Figure 5B).

These recent studies, reporting the structure of PCSK9 and its binding site on the LDLR, raise a number of immediate questions. How is PCSK9 recognition achieved by the LDLR and how is the affinity of the complex regulated by pH? Does the increased affinity at endosomal pH result from the change in LDLR conformation? Does it result from changes in the structure and/or surface electrostatic potential of PCSK9 as a result of titration of clustered histidine residues on its surface, or by some other mechanism? Are there conditions under which release of the prodomain is induced, unfettering the protease, or is the protease activity not required for downregulation of receptor levels? Determining the answers to these and other questions should provide crucial insights into the mechanism by which PCSK9 reduces cell surface LDLR levels, and may ultimately lead to new approaches for treating hypercholesterolemia.

Conclusions

Recent structural advances have identified a canonical mode for formation of complexes between LA repeats and certain molecular partners. The structures, combined with biochemical studies, also provide insights into how the stability of these complexes is modulated by the differences in pH that are found in the cellular compartments through which the protein complexes pass. On the other hand, the structural basis for recognition of lipoprotein particles still remains incompletely understood, and the structure of a complex between the LDLR and

PCSK9 has not yet been solved. It seems likely, however, that these gaps in knowledge will be filled in the near future.

Acknowledgments

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Figure 1.

X-ray Structure of LA module five (LA5) from the LDL receptor [32] (PDB ID code 1AJJ). A. Ribbon representation. Side chains are superimposed on a gray ribbon trace of the module backbone, with the bound calcium ion illustrated as a red sphere. Cysteines and additional residues are labeled as reference points. B. Surface electrostatic potential. The negative surface potential (red) around the bound calcium ion is indicated with a black oval. Panel A reproduced from reference [33] with permission (www.annualreviews.org).

Figure 2.

A. Overall structure of the LA3-4/RAP-D3 complex (PDB ID code 2FCW). The LA3-4 (red) and RAP-D3 (blue) polypeptide chains are illustrated as ribbons, and a transparent molecular surface of LA3-4 is shown in light gray. The calcium ions bound by LA3 and LA4 are shown as yellow spheres. Disulfide bonds in LA3-4 are rendered as sticks, with sulfur atoms colored orange. B. Interactions between LA4 and lysine 256 of RAP-D3 in the LA3-4/RAP-D3 structure. LA module backbone traces are illustrated as crimson ribbons, and interacting side chains (carbon: salmon, oxygen: red) are rendered as sticks. The RAP-D3 polypeptide backbone is shown as a blue ribbon, with the lysine side-chain (carbon: cyan, nitrogen: blue) in stick form. The calcium ions bound by LA3 (C) and LA4 (D) are shown as yellow spheres. Hydrogen bonds are shown as dashed black lines. Adapted from reference [13], and reproduced with permission (www.molecule.org).

Figure 3.

Comparison of the LA4/RAP-D3 interface (A–D, gray) with other LA module interfaces. A. Comparison with the LA3/RAP-D3 interface (backbone: cyan ribbon). B. Comparison with the LDLR intramolecular interface [14] between LA4 and the YWTD β -propeller (backbone: magenta ribbon; PDB ID code: 1N7D). C. Comparison with the LDLR intramolecular interface between LA5 and the YWTD β -propeller (backbone: orange ribbon). (D) Comparison with the interface between VLDLR repeat three and HRV2 [15] (backbone: green ribbon; PDB ID code: 1V9U).

Figure 4.

A. Four helix bundle structure of the N-terminal domain of apoE in its lipid-free form (pdb ID code 1BZ4; [34]). Ribbons representing the four helices in the N-terminal domain are colored red (helix 1), blue (helix 2), green (helix 3) and yellow (helix 4), and ribbons of the connecting regions are colored gray. Side chains are illustrated as sticks, with K143 and K146, two lysines of the fourth helix implicated in receptor binding [35], explicitly indicated. B. Model of the α -helical hairpin conformation of apoE-DPPC HDL-like particles derived from X-ray diffraction studies. The residues implicated in receptor binding are located on helix 4 and R172 is in the cyan-colored region. Figure 4B adapted from page 450 of reference [36], and reproduced with permission (www.elsevier.com).

Figure 5. Structure of the PCSK9 protein after self-cleavage (PDB ID code 2P4E). A. Ribbon representation illustrating the three domains: prodomain (olive), catalytic domain (blue), and C-terminal domain (purple). The C-terminal end of the prodomain is occluding the catalytic site (black circle). B. Clustered histidines (labeled) lie on one face of the C-terminal domain, suggesting a potential role in enhancing the affinity of PCSK9 for the LDLR at endosomal pH [29,31].