

STRUCTURE AND PHYSIOLOGIC FUNCTION OF THE LOW-DENSITY LIPOPROTEIN RECEPTOR

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■ **Abstract** The low-density lipoprotein receptor (LDLR) is responsible for uptake of cholesterol-carrying lipoprotein particles into cells. The receptor binds lipoprotein particles at the cell surface and releases them in the low-pH environment of the endosome. The focus of the current review is on biochemical and structural studies of the LDLR and its ligands, emphasizing how structural features of the receptor dictate the binding of low-density lipoprotein (LDL) and beta-migrating forms of very low-density lipoprotein (β -VLDL) particles, how the receptor releases bound ligands at low pH, and how the cytoplasmic tail of the LDLR interfaces with the endocytic machinery.

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INTRODUCTION AND HISTORICAL PERSPECTIVE

The low-density lipoprotein receptor (LDLR) is a cell-surface glycoprotein that plays a critical role in the homeostatic control of blood cholesterol by mediating the removal of cholesterol-containing lipoprotein particles from circulation [reviewed in (1)]. Indeed, familial hypercholesterolemia (FH), which is one of the most common human inborn errors of metabolism, is caused by loss-of-function mutations of the gene encoding the LDLR.

Identification of the LDL Receptor

Brown & Goldstein originally identified the low-density lipoprotein (LDL) receptor in 1973 during their search for the molecular basis of FH (see References 1–4). They first observed that cultured normal fibroblasts suppressed endogenous cholesterol synthesis when cholesterol was supplied via LDL in the serum, whereas cultured fibroblasts from FH patients failed to suppress endogenous synthesis unless cholesterol was supplied in a soluble, non-LDL form (5, 6). Shortly thereafter, they showed that the normal fibroblasts had high-affinity binding sites for LDL particles on their surfaces, whereas the fibroblasts from FH patients lacked high-affinity binding sites (7, 8). A decade later, the gene encoding the receptor was cloned and sequenced (9–12).

Physiologic Ligands for the LDL Receptor

At neutral plasma pH, circulating lipoprotein particles bind to the LDL receptor. The most important physiologic ligand for the receptor is LDL (7, 8), which contains a single copy of apolipoprotein B-100 (apoB-100) and carries approximately 65% to 70% of plasma cholesterol in humans. The LDLR also binds tightly to lipoproteins that contain multiple copies of apolipoprotein E (apoE), such as beta-migrating forms of very low-density lipoprotein (β -VLDL), or certain intermediate and high-density lipoproteins (13, 14).

Endocytosis and the Receptor-Recycling Pathway

Receptor-lipoprotein complexes enter cells by endocytosis via clathrin-coated pits, where the receptor molecules cluster on the cell surface (15–18). The complexes

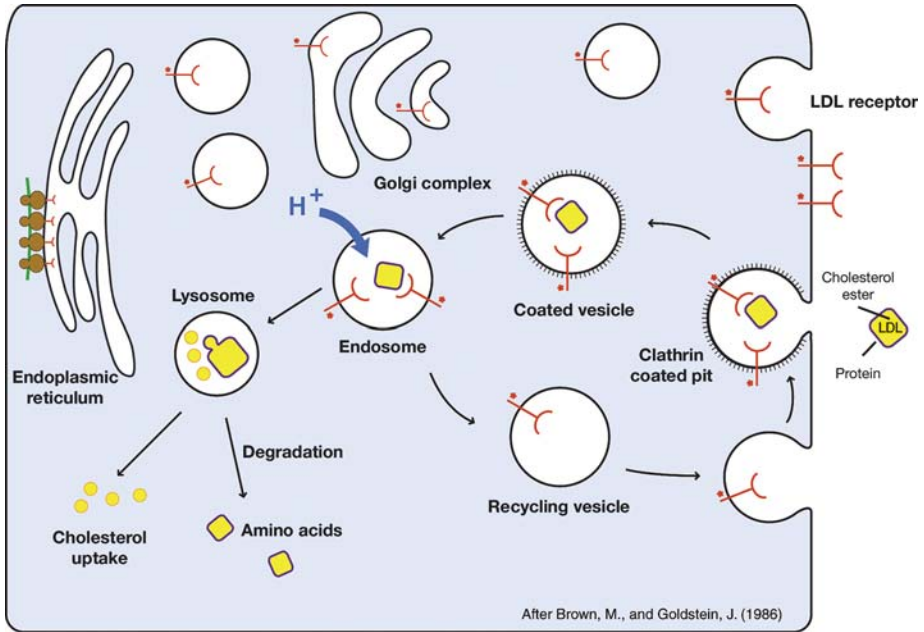


Figure 1 Schematic illustrating the uptake of lipoprotein particles by the LDLR. Entry of a receptor-ligand complex into cells occurs at clathrin-coated pits. The complex is subsequently delivered to the low-pH environment of the endosome, where dissociation of the complex takes place. The receptor is then returned to the cell surface in a process called receptor recycling. Modified and reproduced from the *Nature Encyclopedia of Life Sciences* (166) with permission.

are then delivered to endosomes, where the low-pH environment triggers release of the bound lipoprotein particles (19, 20). The receptors are subsequently returned to the cell surface in a process called receptor recycling [reviewed in (1, 3)]. The itinerary of the LDL receptor is schematically illustrated in Figure 1.

Domain Organization of the LDLR

The mature LDLR is a modular type I transmembrane protein of 839 amino acids (9–12). The receptor contains three types of extracellular protein modules (9–12), beginning with seven contiguous cysteine-rich repeats, each about 40-residues in length (Figure 2). These modules are referred to as LDL receptor type A (LA) repeats, or occasionally, complement-type repeats because they are present in the terminal components of the complement cascade (21–26), as well as in numerous other proteins in the sequence database. A 400-residue region with 35% homology to the epidermal growth factor precursor (EGFP) immediately follows the seven LA modules (11, 12). This part of the receptor consists of two epidermal growth factor-like (EGF-like) repeats, followed by a YWTD (named for the conserved

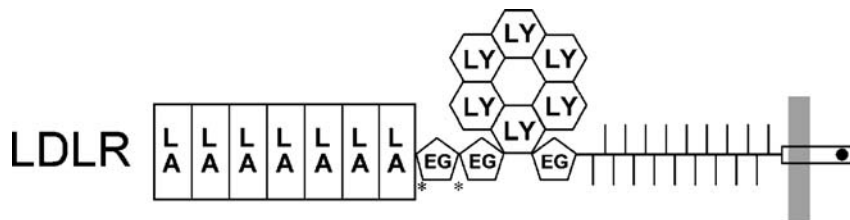


Figure 2 Cartoon illustrating the modular domain organization of the LDLR. The abbreviations are LA, LDL receptor type A (ligand-binding) modules; EG, epidermal growth factor-like modules; and LY, repeats containing a YWTD consensus motif. The ligand-binding region of the receptor spans the LA repeats, and the EGF precursor homology region, responsible for release of LDL at low pH, encompasses the EGF and YWTD repeats.

residues tyrosine, tryptophan, threonine, and aspartate) domain (27, 28) and a third EGF-like repeat. Immediately after the EGFP region is a 58-residue sequence rich in serine and threonine residues that undergoes O-linked glycosylation (10, 29), followed by the transmembrane segment and a cytoplasmic tail of 50 amino acids. The cytoplasmic tail contains an NPxY sequence that directs the receptors to clathrin-coated pits (30, 31), and it also includes the sequences required for proper sorting to the basolateral membrane in polarized cells (32, 33).

Folding of the LDLR in living cells occurs nonvectorially, with the N-terminal LA module assuming its folded structure late in the folding process (34). The receptor-associated protein (RAP) acts as an endoplasmic reticulum (ER) chaperone for proteins of the LDLR family by binding to their LA repeats [reviewed in (35)]. In transfected cells, cotransfection of RAP assists folding of the LDLR and certain transport-defective variants of the LDLR found in FH patients (36). There is also evidence that an additional ER-resident protein, called Boca in flies (37) and MESD in mice (38), appears to facilitate folding of the YWTD and/or EGF domains of LDLR-related proteins (39).

Loss of LDL Receptor Function as the Cause of Familial Hypercholesterolemia

FH results from any of a number of loss-of-function mutations in the gene encoding the LDLR, and FH is characterized clinically by an elevated concentration of plasma LDL and cholesterol [reviewed in (4, 40)]. FH heterozygotes number about 1 of every 500 persons worldwide, and more than 1000 mutant alleles that have been implicated in causing FH have now been identified [lists of known FH mutations are available at <http://www.ucl.ac.uk/fh/> (41, 41a) and http://www.umd.necker.fr/LDLR/Home_page.html (42, 42a)]. Heterozygotes, who inherit one defective or inactive allele, typically have a plasma LDL concentration roughly double that of normal individuals and a substantially increased risk

for coronary artery disease. Individuals with two defective copies of the LDLR gene develop severe premature atherosclerosis, which typically leads to death from coronary artery disease at a very early age. The relationship between FH and coronary heart disease remains the most cogent illustration of the causal relationship between high blood cholesterol levels and coronary atherosclerosis.

Studies of the effects of FH mutations have shown that the physiologic function of the LDLR can be disabled at several different steps. FH mutations have traditionally been separated into the following five classes, depending on the nature of the receptor defect: (a) null alleles, which synthesize no receptors (class 1); (b) transport-defective alleles, which are completely or partially defective in reaching the cell surface (class 2); (c) binding-defective alleles (class 3); (d) internalization-defective alleles, which fail to cluster in coated pits (class 4); and (e) recycling-defective alleles (class 5) [reviewed in (4, 40)]. To these established classes can now be added a sixth class of mutation, which exhibits a sorting defect in polarized epithelial cells as a result of a G823D mutation toward the C-terminal end of the cytoplasmic tail (33).

LDL Receptor-Related Proteins

The LDLR turns out to be the patriarch of an entire class of receptors that contain LA, EGF-like, and YWTD β -propeller modules arranged in a similar pattern (Figure 3). These LDL receptor-related proteins (LRPs) participate in a wide variety of physiologic processes, including lipoprotein transport and clearance of protease-inhibitor complexes from plasma as well as transduction of biological signals during development and differentiation [reviewed in (43–51)]. The role of two closely related LRPs, the very low-density lipoprotein receptor (VLDLR) and apoE receptor 2 (apoER2), as receptors for transducing reelin signals in the brain has been recently reviewed (52–55) as have the transport and signaling roles of LRP-1 (56), megalin (57, 58), and the more distantly related LRP-5 and LRP-6, which participate along with Frizzled proteins as coreceptors in Wnt signaling (50, 59).

This review focuses on the biochemical and structural studies of the LDLR and its ligands. We emphasize the work concerning the structural basis for binding and release of lipoprotein particles that contain apoB-100 and apoE, the mechanism for low-pH-induced release of bound ligands, and the connection between the cytoplasmic tail of the LDLR and the endocytic machinery.

LIGAND BINDING BY THE LDLR

Determining the detailed structural basis for recognition of lipoproteins by the LDLR has posed a difficult challenge because the receptor is membrane bound and the ligands are associated with lipids. Nevertheless, current knowledge about how the receptor binds ligands has advanced steadily over the last several years

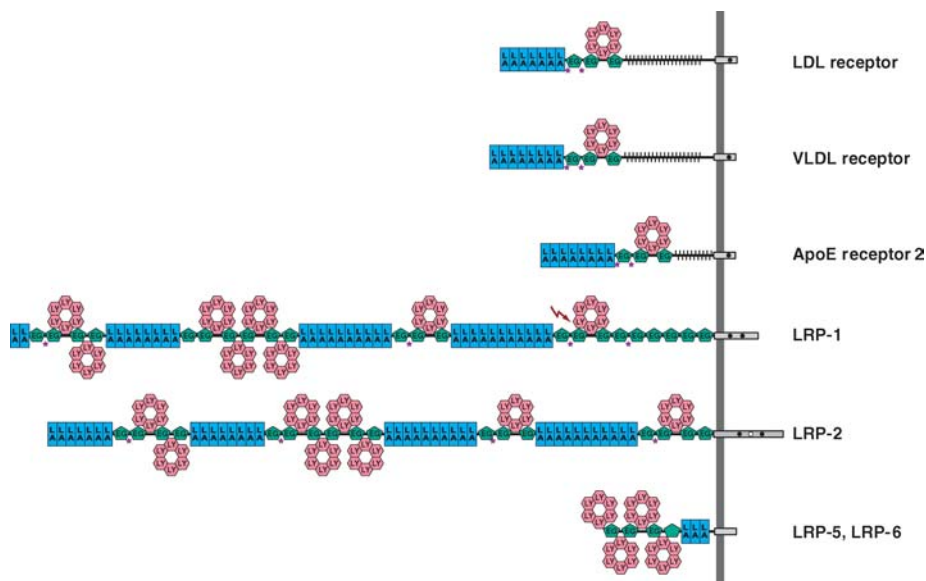


Figure 3 Core members of the LDL receptor family in humans. Abbreviations are LA, LDL receptor type A (ligand-binding) modules; EG, epidermal growth factor-like modules; and LY, repeats containing a YWTD consensus motif. Filled and open circles represent NPXY- and NPXY-like motifs, respectively. Adapted from Springer (27).

with the publication of a wealth of biochemical and structural studies of LDLR ligands, along with the publication of structures for modules and module pairs of the ligand-binding domain of the LDLR.

Biochemical and Mutational Studies Mapping the Binding of Ligands to LA Repeats

The seven contiguous LA modules located at the amino-terminal end of the receptor are responsible for binding to lipoproteins. Each LA module is encoded by its own exon, except for the central modules 3–5, which are grouped together on a single exon (12). Mutational studies of the seven LA modules examined the role of each repeat in the binding of both LDL and β -VLDL particles (60, 61). Whereas deletion of either of the first two LA modules has little effect on LDL binding in transiently transfected COS cells, deletion of any other individual LA module (LA3 to LA7), or of the first EGF-like repeat (EGF_A), results in a reduction in LDL binding of more than 50%, leading to the conclusion that LA repeats three through seven, as well as the first EGF-like repeat (EGF_A), are essential for binding of LDL at the cell surface (60, 61). In contrast, only deletion of module five

prevents high-affinity binding of β -VLDL (60), and receptors from which either the first three or last two LA modules have been deleted still retain their capacity to bind β -VLDL at near native affinity (60, 61). Point mutation of either of two conserved scaffolding residues within any individual repeat (an Ile to Asp mutation of a conserved Ile residue, or a Asp to Tyr mutation of a conserved Asp) also mimics the effect of deletion of the repeat, providing further evidence in support of these conclusions (60). Recent binding assays, performed *in vitro*, have identified the LA4-LA5 fragment of the LDLR as sufficient for binding apoE·DMPC complexes, which are believed to mimic β -VLDL with regard to receptor binding (62).

Of note, the deleterious effect upon binding of LDL that results from deletion of the EGF-like A and B repeats, or of deletion of the entire EGFP region, appears to manifest itself only at the cell surface because the deletion variant of the LDLR without the EGFP retains the ability to bind LDL on ligand blots after sodium dodecyl sulfate-polyacrylamide gel electrophoresis and subsequent transfer to nitrocellulose (19). Furthermore, recombinant receptor fragments encompassing either the seven-ligand-binding repeats plus the first EGF-like module, or the ligand-binding repeats alone, produced in secreted form by baculovirus-infected insect cells or by refolding after expression in bacteria, respectively, exhibit high-affinity binding of LDL (63–65). Taken together, these studies all support a model for LDL binding in which the energetically important contacts between LDL and the LDLR are mediated by the seven LA repeats.

Features of Physiologic Ligands Implicated in Recognition by the LDLR

RECOGNITION OF apoB-100 OF LDL PARTICLES Binding of LDL particles by the LDLR occurs with a stoichiometry of one LDL particle per receptor monomer (66). LDL particles range in size from ~ 180 – 250 Å in diameter, and these particles contain a single copy of apoB-100 as their only protein component. ApoB-100 is 4536 amino acids long (67, 68) and is insoluble in aqueous solution once deprived of its associated lipids (69). Because of the heterogeneity in size of LDL particles and the difficulty of working with apoB-100 as an isolated protein, structural studies of LDL and apoB-100 have been limited, and details of how it interacts with the LDL receptor have been slow to emerge.

The structure of apoB-100 in LDL particles has been thoroughly and carefully reviewed by Segrest et al. (70). Electron cryomicroscopy and low-resolution X-ray crystallography show LDL particles to be quasi-spherical (71) or ellipsoidal (72, 73). Each of these studies identified a knob-shaped electron-dense region on the surface of the particle. The dimensions of the knob suggest that it corresponds to the N-terminal domain, which is predicted to be a soluble, globular domain homologous to lipovitellin (74). Studies using monoclonal antibodies to identify specific apoB-100 epitopes on the surface of LDL particles by electron microscopy

suggest that apoB-100 is distributed over the surface of the LDL particle like a ribbon, with a "bow" contributed by the C-terminal end (75).

Antibody-blocking studies (76), study of naturally occurring truncation and point mutants (77, 78), and use of synthetic peptides homologous to the receptor-binding region of apoE (79) first suggested that residues ~3000–~3600 of apoB-100 are critical for binding to the LDLR. First, a panel of antibodies with epitopes that map between residue 2980 and 3569 completely block binding of LDL to the LDLR and are inaccessible to receptor-LDL complexes, whereas antibodies with epitopes outside this region fail to block binding completely (76). Second, LDL particles containing apoB-67, which terminates at residue 3040, do not bind to the LDLR (77), although LDLs with apoB-75, which terminates at residue 3386, do (78). In addition, LDL from patients with familial-defective apoB-100, which is due to an R3500Q mutation, is defective for LDLR binding (80). Third, a synthetic peptide corresponding to residues 3345–3381 of apoB-100, which encompasses a sequence from residues 3359–3367 (81) with striking similarity to the basic region of apoE implicated in receptor binding (see below), confers binding activity upon an otherwise LDL receptor-inactive, trypsinized hypertriglyceridemic VLDL particle, whereas a control peptide spanning residues 4154–4189 (predicted to lie outside the LDLR-binding site) does not (79).

To test the apoB-100 sequence requirements for binding of LDL to the LDLR, Boren et al. (82) reconstituted human apoB-100 into LDL particles in transgenic mice and combined this approach with site-directed mutagenesis. A number of important findings about the requirements for binding emerged from these studies. First, truncation of apoB-100 at residue 3497 or 3620 does not interfere with receptor binding but, rather, enhances it somewhat. Second, mutation of the arginine and lysine residues between 3359 and 3369 to serine and alanine, respectively, prevents binding to the LDLR, whereas swapping residues 141–153 from human apoE (the region implicated in receptor binding) in place of residues 3358–3370 restored binding, thus directly implicating this region as the primary determinant for binding of LDL to the receptor. Third, they showed that the R3500Q mutation found in familial defective apoB-100 interferes only with receptor binding in the context of the entire B-100 sequence but not when the C-terminal 20% of apoB-100 has been deleted, suggesting that R3500 contacts a partner site in the C-terminal region of apoB-100 to create a receptor-active conformation. In a subsequent manuscript, they showed that (a) a new patient-derived mutation, R3480W, also exhibits defective receptor binding and that (b) mutation of W4369 to tyrosine disrupts receptor binding, consistent with a model in which the active conformation is stabilized by direct contact between R3500 and W4369 (83).

RECOGNITION OF apoE-CONTAINING PARTICLES ApoE is a small two-domain (84, 85) protein of 299 amino acids (86, 87) that comprises the predominant protein component of β -VLDL particles [reviewed in (88, 89)]. ApoE is present in β -VLDL in multiple copies per particle, in contrast to apoB-100, which is present in a single copy per LDL particle. ApoE-containing lipoproteins are ligands for

all of the core members of the LDLR family with tandemly repeated LDL-A modules, including the LDLR, the VLDLR, apoER2, LRP-1, and LRP-2. Delipidation of apoE abolishes high-affinity LDLR binding, which can subsequently be reconstituted by incorporation of apoE into dimyristoylphosphatidylcholine lipid discs (90).

ApoE has three common isoforms in humans, which result from differences at residues 112 and 158. The E2 allele has cysteine at both positions, the E3 allele has cysteine at 112 and arginine at 158, and the E4 allele has arginine at both positions. The E2 isoform is defective in receptor binding (91), whereas the difference between the E3 and E4 isoforms in binding to the LDLR is negligible. The E4 allele of apoE is also an established risk factor for Alzheimer's disease (92).

LDLR binding is localized in the NH₂-terminal domain of apoE (NT-apoE), whereas the C-terminal domain is required for incorporation into lipoproteins (84, 93). The structure of NT-apoE (residues 1–191), which has been determined to 2.25 Å resolution (94), has an overall topology of an unusually elongated four-helix bundle.

Studies using chemical modification of basic residues (14), polypeptide fragments (93), antibody competition experiments (95), and site-directed mutagenesis (62, 96, 97) have shown that basic residues in the region of apoE spanning residues 136–160 participate in binding to the LDLR. Arginine and lysine residues in this region lie on one face of the fourth helix in the crystal structure, producing a single patch of positive electrostatic potential extending from the surface of the protein. Outside this region, arginine 172 also plays an important role in receptor binding of full-length apoE because the R172A mutation effectively abolishes binding to the LDLR as well (98).

An intriguing characteristic of the interaction between apoE and the LDLR is that high-affinity interaction occurs only upon complexation of apoE with lipid (89, 90, 94). Although apoE-containing lipoproteins (β -VLDL) bind to the LDLR with an apparent K_d of $\sim 1.2 \times 10^{-10}$ M (13, 99), the binding affinity of delipidated apoE is at least 500-fold lower (94, 99).

Lipid complexation has been postulated to enhance the affinity of apoE for the receptor by inducing a conformational change in apoE that permits binding to the receptor (89). Indeed, numerous studies with NT-apoE show that the four-helix bundle rearranges upon association with lipids. Early studies showed that the surface properties of NT-apoE at an air-water interface are consistent with opening of the bundle (100). Additional studies of NT-apoE using infrared spectroscopy (101), fluorescence resonance energy transfer (102, 103), NMR spectroscopy of reductively methylated protein (104), and disulfide-constrained variants (105) also indicate that the helical bundle undergoes conformational rearrangement upon incorporation into phospholipid discs.

How the conformational opening of the bundle actually increases receptor affinity is less clear. One possibility is that increased surface exposure of the basic residues on the fourth helix facilitates receptor binding. Another possibility is that

a change in helix curvature is responsible for increasing receptor affinity, an idea suggested by the bent helical structure of a binding-active 58-residue apoE peptide (encompassing residues 126–183) when complexed with the micelle-forming lipid dodecylphosphocholine (106).

Multiple copies of NT-apoE are present in each disc, and early studies pointed to the importance of avidity effects in LDLR binding (99, 107). A role for lipids in creating a multivalent ligand is indirectly supported by studies using a peptide corresponding to a linear tandem repeat of residues 141–155 of apoE (108–110), which inhibits uptake of LDL particles by the LDLR. This finding suggests (*a*) that the peptide binds directly to the receptor and (*b*) that a multivalent ligand is all that is required for apoE to bind to the LDLR. Related studies show that similar apoE-derived peptides bind to a soluble fragment from the LRP (111). Thus, the enhanced receptor affinity of apoE upon lipid association seems to result from the combined effects of a conformational rearrangement of the helical bundle and the creation of a multivalent ligand. Finally, it may be that the receptor actually requires a composite protein-lipid surface to bind apoE as a ligand with high affinity.

Folding and Structural Studies of Ligand-Binding Repeats

The seven ligand-binding repeats of the LDLR represent the founding examples of LA modules, which now (July 2004) include 1889 members listed in the Pfam Protein Families Database (<http://www.sanger.ac.uk/Software/Pfam/>). Each LA module is about 40 residues long and has six cysteines engaged in three disulfide bonds. The consensus sequence of these LA modules, illustrated in Figure 4 in sequence “logo” form (112), includes the six conserved cysteine residues, two hydrophobic residues in the N-terminal half of the module, and a cluster of conserved acidic residues with the signature sequence DCxDxSDE located just before the C-terminal cysteine residue. The six conserved cysteines form three disulfide

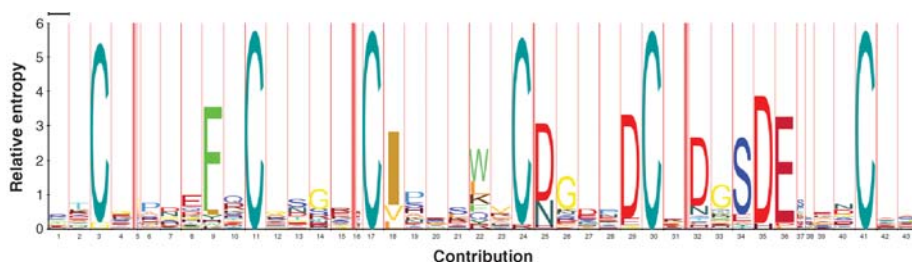


Figure 4 Sequence alignment of all LA modules from the database, illustrated using a sequence logo (112). The relative representation of each amino acid at every position in the sequence is proportional to the size of the letter at that site. Highly conserved residues include the six cysteine residues, two hydrophobic residues (Phe and Ile) in the N-terminal half of the module, and the cluster of acidic residues near the C-terminal end of the module.

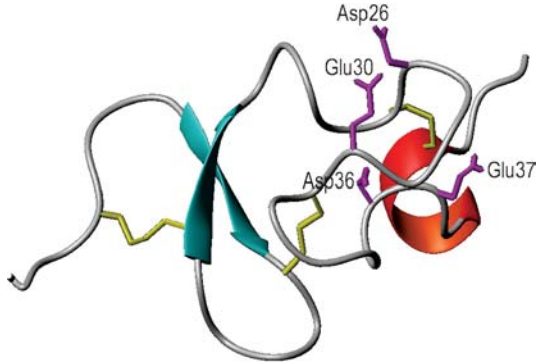


Figure 5 NMR structure of the first LA module of the LDLR (PDB accession code 1LDL). The backbone ribbon trace (*gray*) is shown with side chains of cysteines (*yellow*) and conserved acidic residues (*plum*). The structure accurately defined the topology of a representative LA module and showed that the acidic residues were situated on one face (116). However, the requirement for calcium in stabilizing the fold of the module was not yet recognized, and the calcium-binding site was not identified.

bonds with a connectivity of Cys I-Cys III, Cys II-Cys V, and Cys IV-Cys VI (113–115).

The solution structure of the first ligand-binding repeat of the LDLR was determined in 1995 by Smith and colleagues (116). The structure, determined by two-dimensional proton NMR spectroscopy, accurately defined the fold of the domain, which has an N-terminal beta-hairpin followed by a series of beta turns. The structure also showed that the conserved acidic residues clustered on one face of the repeat (Figure 5). The solution structure of the second LA module of the receptor, published shortly thereafter, also showed a similar overall fold (117).

It was recognized in early studies of ligand binding by the LDLR that binding of LDL requires calcium ions and is blocked by addition of EDTA (118). There is also a monoclonal antibody directed at the first LA repeat that recognizes only the receptor in its oxidized form in the presence of calcium (119, 120). Nevertheless, the importance of calcium in driving proper folding of LA modules was not fully appreciated prior to folding studies of LA5 and of its mutants with FH mutations (115). Using acquisition of native disulfide connectivity as a probe for tertiary structure, these studies showed that repeat 5 failed to form native disulfide bonds under conditions permitting disulfide exchange unless calcium was supplied in the folding buffer. In the presence of calcium under conditions permitting disulfide exchange, wild-type LA5 folded to a single disulfide isomer, whereas in the absence of calcium, a distribution of disulfide isomers was formed with a chromatographic profile similar to the one obtained after refolding in denaturant (115). The proton NMR spectrum of LA5 with native disulfide bonds exhibited the broad chemical shift dispersion characteristic of a folded protein in the presence of calcium but

not in its absence, indicating that the bound calcium ion is crucial for maintaining the structural integrity of the module even after formation of the native disulfide bonds.

FH mutations of LA5, six of which alter conserved acidic residues at the C-terminal end of the module, lead to module misfolding even in the presence of calcium, suggesting that the conserved acidic motif has a structural role in the coordination of calcium. Similar studies examining the consequences of an FH mutation in the first repeat, in which there is a two-residue deletion of G26 and D27, also identify a folding defect (121). The calcium requirement for folding of LA modules was subsequently confirmed not only for LA5, but also for ligand-binding repeats 1 (122), 2 (123), and 6 (124) from the LDLR, as well as for LA repeats from the LDL receptor-related protein (125) and for the LA module in the retroviral receptor Tva (126, 127). Calcium affinities of various LA modules have been measured at neutral and acidic pH, with values of K_d ranging from 40 nM to 14 μ M at pH 7.4 and 13.1 μ M for LA5 at pH 5.5 (125, 128, 129).

The crystal structure of LA5, determined to 1.7 Å resolution, showed for the first time the structural basis for calcium coordination by LA modules (130), revealing features that were not evident from the NMR structures of repeats 1 and 2 (Figure 6). The side chains of the four most highly conserved acidic residues, D196, D200, D206, and D207 directly coordinate the calcium ion via one of their backbone oxygen atoms, and the backbone carbonyls of W193 and G198 complete the coordination sphere in an octahedral geometry (130).

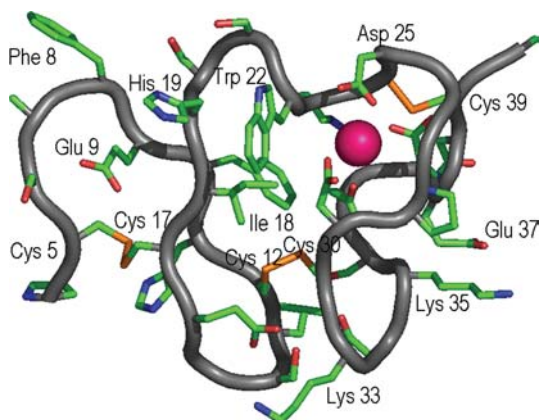


Figure 6 Model illustrating the X-ray structure of the fifth ligand-binding module of the LDLR (130), with the critical bound calcium ion illustrated as a (*red*) sphere (PDB accession code 1AJJ). Side chains are superimposed on a (*gray*) ribbon trace of the module backbone. Cysteines and additional residues are labeled as reference points. FH mutations are located at structurally significant residues.

The X-ray structure of the seventh LA repeat from cluster II of the LRP (which has four clusters of LA-type repeats) shows that the bound calcium ion is coordinated in identical geometry in that repeat as well, suggesting that this mode of calcium coordination is general for LA-type repeats (128). Structures of individual ligand-binding repeats by NMR, refined with explicit consideration of a bound calcium ion, are also consistent with this mode of calcium coordination in solution (124, 127, 131–134).

With the exception of a 12-residue linker between LA4 and LA5, short linkers of 4 or 5 residues between cysteines connect adjacent LA modules of the LDLR ligand-binding domain. The relative mobility of adjacent LA modules connected by their natural linkers has been examined for the LA1-2 and LA5-6 domain pairs using NMR spectroscopy. In each case, there is no evidence for interdomain contact, and the two modules appear to act independently in the pair (123, 129, 135). In addition, contacts between adjacent LA modules are not observed in the ectodomain structure at endosomal pH (136). The structural independence of the LA modules may reflect a functional need: Flexibility in the linker regions connecting individual modules will permit the ligand-binding repeats of the receptor to adjust their relative positions in order to bind a variety of heterogeneous apoE- and apoB-containing lipoprotein ligands of different particle shape, curvature, and diameter (135).

The high-resolution X-ray structures of LA5 from the LDLR and LA7 from cluster II of the LRP demonstrate that one central role of the conserved acidic residues is structural, because these residues coordinate the calcium ion required for the structural integrity of the module. Although the LA5 structure appeared to call into question previous models for lipoprotein binding, which had postulated that basic residues on the surface of the apolipoproteins were involved in electrostatic contacts with the conserved acidic residues, it was subsequently recognized that the bound calcium ion also serves to localize the acidic residues of the LA modules, creating an acidic patch on the surface resulting from the local excess of negative charge (124). The recent structures of the LDLR at endosomal pH (136) and of a VLDLR fragment in complex with human rhinovirus 2 (137) show that the calcium-coordinating acidic residues of LA modules can indeed form intramolecular or intermolecular contacts either within the molecule or with a nonphysiologic ligand. Thus, it seems likely that electrostatics will indeed play an important role in recognition of natural ligands by LA modules.

MECHANISM OF LOW-pH-INDUCED RELEASE OF LIGANDS BY THE LDLR

In order to fulfill its role as a transport protein, the LDLR must not only bind to its lipoprotein ligands, but let go of them once they are ferried into the cell. The trigger for release of bound lipoproteins is the change in pH that occurs once

the receptor-ligand particles reach the low-pH environment of the endosome. This section of the review discusses recent structural and biochemical studies that shed light on how the receptor interconverts between open and closed conformations, combining flexibility and rigidity of its interdomain linkers to facilitate release of bound lipoproteins upon exposure to low pH.

Regulation of Low-pH-Induced Ligand Release

Adjacent to the seven N-terminal ligand-binding modules of the LDLR is a 400-residue region of the receptor, which encompasses two EGF-like modules, followed by a series of six YWTD repeats (27) and a third EGF-like module. This part of the receptor, termed the EGFP domain, controls the related processes of lipoprotein release at low pH and recycling of the receptor to the cell surface (19).

Deletion of the entire EGFP homology domain of the LDLR [Δ EGFP, equivalent to the known mutation in FH-Osaka-2 patients (138)] reduces the ability of the receptor to bind LDL at the cell surface, but β -VLDL particles still bind to this mutant receptor with native stoichiometry and affinity (19). However, the most striking consequences of the deletion are that the mutant receptor fails to release bound β -VLDL (19) or bound LDL (139) at low pH and exhibits a receptor-recycling defect. A similar role of the EGFP domain in acid-dependent control of ligand release has also been established for the VLDLR (140). More than half (54%) of human FH point mutations lie in the EGFP domain (41, 141), further highlighting the importance of this region in contributing to proper receptor function. Deletion of the EGF-AB domain pair, which occurs in the FH *Cape Town-2* mutation, also leads to ligand-binding and receptor-recycling defects that resemble those of the Δ EGFP mutant (142). Structural studies of the EGFP region of the LDLR were thus of considerable interest.

Structural Studies of the EGF Repeats

Two different groups investigated the calcium-binding properties and the structure of the EGF-AB domain pair. Using solution NMR spectroscopy, Downing's group (143) showed that both EGF-A and EGF-B bind calcium, with coordination by the first EGF module (EGF-A) occurring in a noncanonical site. The calcium dissociation constants for the EGF-A and B modules, measured under physiologically relevant pH and ionic strength conditions using a combination of solution NMR, intrinsic protein fluorescence, and chromophoric chelator methods, are approximately 50 and 10–20 μ M, respectively (143). The structure of the EGF-AB pair, solved independently by the two groups (144, 145), shows the two EGF repeats adopt an extended, rod-like conformation (Figure 7) with the interdomain orientation defined by (a) calcium coordination at a site between the domains and (b) hydrophobic, interdomain-packing interactions akin to those in the interface between EGF modules 32 and 33 of fibrillin in the structure of that EGF pair (144, 146). Two FH mutations, G322S and R329P, near the C-terminal end of the EGF-A

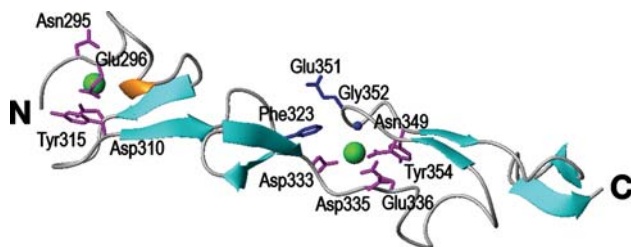


Figure 7 Structure of the EGF-AB domain pair [PDB accession code 1HJ7; (144)]. The intermodule interface is stabilized by a bound calcium ion (*green*) and hydrophobic packing of Phe 323 (*blue*) against the aliphatic portion of Glu 351 and the hole around Gly 352 (*blue*). The side chains of calcium-coordinating residues are illustrated (*purple*).

module interfere with proper folding of the EGF-AB domain pair *in vitro*, and receptors bearing these mutations exhibit a defect in cell surface expression when compared with the wild-type LDLR in stably transfected *ldl-A7* cells (139).

Identification and Crystallography of the YWTD Domain as a β -Propeller

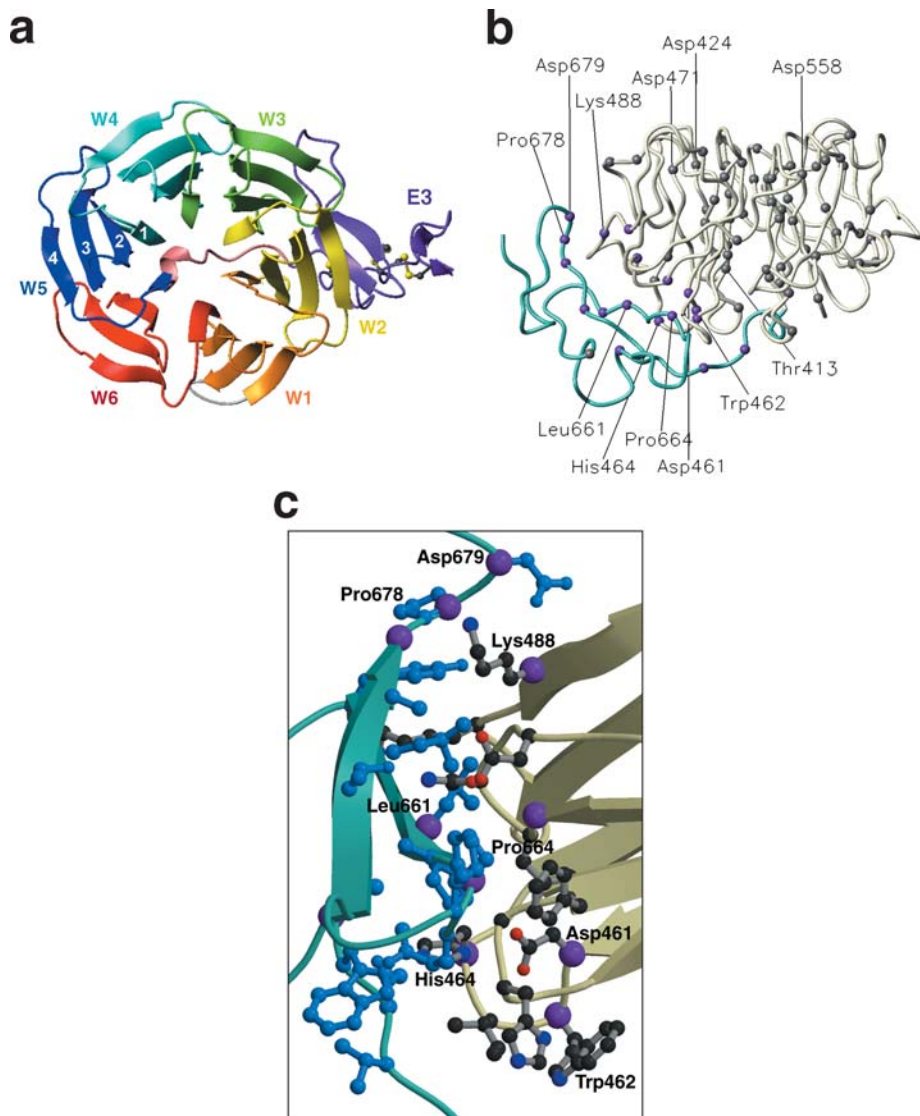
For many years, the YWTD repeat region of the LDLR was referred to as a spacer domain and was illustrated in schematics of the receptor with squiggly lines. Using computational molecular biology in combination with indirect published experimental evidence, Springer (27) was the first to deduce that the YWTD repeats combined to form a single six-bladed β -propeller domain.

Springer's prediction propelled structural studies of a fragment of the LDLR containing the YWTD domain flanked by its neighboring EGF modules (28). The structure, which was solved by X-ray crystallography to 1.5 Å resolution at neutral pH (28), confirmed the prediction that the YWTD domain is a six-bladed β -propeller, revealed an unexpected packing interface between the propeller and the third EGF module, and enabled interpretation of numerous FH mutations in their structural context (Figure 8). Although the EGF-B module was present, its position was disordered in the crystals.

In the structure of the YWTD-EGF domain pair, one subset of FH mutations alters side chains that form conserved packing and hydrogen-bonding interactions in the interior and between propeller blades. A second subset of mutations is located at the interface between the propeller and the C-terminal EGF module, suggesting a structural requirement for maintaining the integrity of the interdomain interface (28). However, the structure of the YWTD-EGF C domain pair did not immediately reveal a model of how the propeller and its adjacent EGF-like modules might trigger release of ligands at endosomal pH.

Crystal Structure of the Extracellular Portion of the Receptor at Endosomal pH

In a crystallographic tour de force, Rudenko et al. (136, 147) determined the structure of the LDLR ectodomain at endosomal pH. The final structural model includes interpretable density for all of the ligand-binding LA modules, except LA1, and for the complete EGFP region. Because the resolution of the structure



is limited to 3.7 Å, detailed analysis of side chain conformation is not possible, but the relative positions and orientations of the domains are well defined by the data.

The most remarkable feature of the ectodomain crystal structure is that the receptor adopts a closed conformation at low pH, with the key ligand-binding repeats LA4 and LA5 in direct contact with the top face of the β -propeller domain (Figure 9). In contrast, LDL receptors visualized by cryoelectron microscopy in their binding-active conformation have an elongated shape (147a,b), suggesting that the receptor adopts an open conformation at neutral pH.

Together, these findings suggest a model in which the LDLR interconverts between open and closed conformations, with the propeller domain displacing bound LDL at low pH by becoming an intramolecular ligand for the ligand-binding repeats (Figure 10). This intramolecular closure model rationalizes a number of other observations regarding release of LDL by the receptor at low pH: (a) The presence of the EGFP region (which encompasses the propeller domain) is required for release of bound ligands (19); (b) release of bound LDL is an intrinsic property of purified receptors and does not require accessory proteins (66), and (c) ultrafiltration studies suggest that the two fragments resulting from proteolytic cleavage in the linker between LA4 and LA5 remain associated at pH < 6 (136).

To determine whether the YWTD propeller domain is indeed required to trigger release of LDL at low pH, a series of propeller domain deletion studies and domain swaps were recently performed. These studies showed directly and for the first time that the propeller domain participates in mediating release of bound lipoproteins at endosomal pH (147c). Additional indirect support for the proposed intramolecular release model comes from consideration of a number of FH mutations that are predicted to affect the ability of the receptor to adopt the closed conformation, including the existence of residue mutations at the interface between the propeller and ligand-binding repeats 4 and 5 (136, 148). In addition, the X-ray structure

Figure 8 Structure of the YWTD propeller-EGF-C domain pair (PDB accession code 1IJQ). (a) Ribbon representation of the YWTD domain and adjacent C-terminal EGF-like module (E3) of the LDL receptor, colored to point out the six YWTD repeats of the six-bladed propeller. The view shown is down the central pseudosymmetry axis of the six-bladed propeller. (b) Sites of FH mutations mapped onto the structure of the YWTD-EGF domain pair. The ribbon representing the YWTD domain backbone is khaki, and the ribbon for the EGF module is light green. The C α positions of all sites implicated in FH are illustrated as spheres: α -carbon atoms of FH sites outside the interdomain interface are colored gray; α -carbon atoms of interface sites harboring FH mutations are colored purple. (c) Close-up of the YWTD-EGF interface region. Side chains of residues in the interface are shown in ball and stick representation with residues from the YWTD propeller colored according to atom type (carbon, *black*; nitrogen, *blue*; oxygen, *red*). α -carbon atoms of interface sites harboring FH mutations are colored purple. Reproduced from Reference 28 with permission.

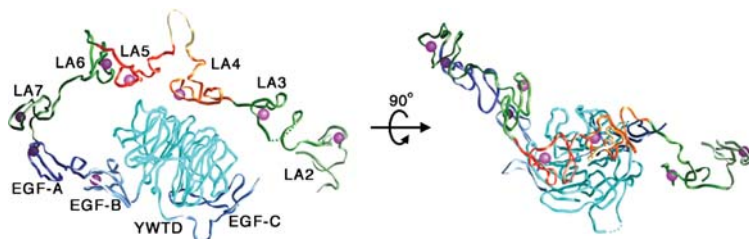


Figure 9 Backbone ribbon trace of the LDL receptor ectodomain structure (PDB accession code 1N7D). Two views of the receptor monomer are shown, related by a 90-degree rotation. The central ligand-binding modules, LA4 and LA5, form long-range contacts with the YWTD β -propeller domain and are highlighted in orange and red, respectively. The other ligand-binding modules are different shades of green. The EGF-like modules are various shades of purple, and the YWTD β -propeller is cyan. Adapted from (136) and reprinted from (167) with permission.

of the nidogen-laminin complex shows that the YWTD β -propeller domain of nidogen uses its analogous (top) face to bind a laminin fragment (149), and the structure of an LA repeat from the VLDLR in complex with human rhinovirus 2, which relies on the LDLR or VLDLR as a receptor, uses a comparable interface to bind the viral particle (137).

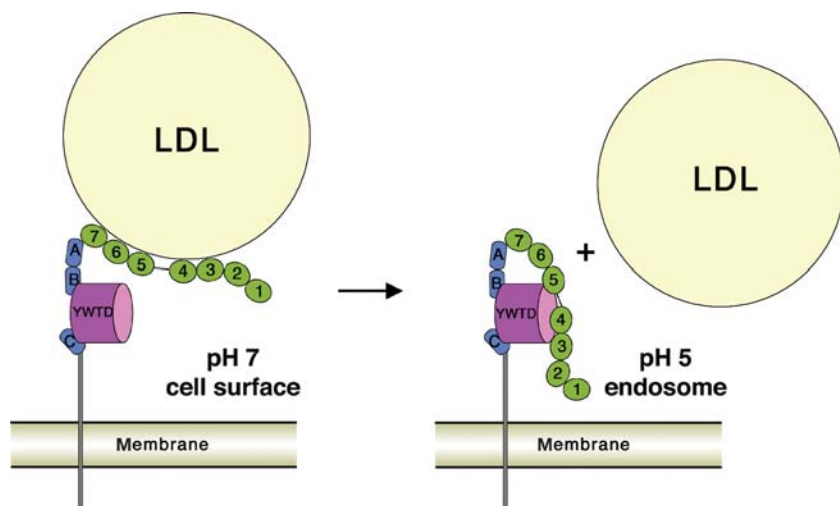


Figure 10 Model for release of bound LDL upon exposure to endosomal pH. Ligand-binding modules 3–7 participate in binding of LDL at neutral pH, whereas modules 4 and 5 are engaged in intramolecular contacts with the β -propeller domain at endosomal pH. Reproduced from Reference 167, with permission.

An Integrated View of the LDLR Ectodomain as a pH Sensor

How does the receptor sense the pH change to interconvert between an open, binding active conformation at neutral pH and a closed one to displace LDL at low pH? Three interface histidines were particularly intriguing candidates for sensors (136) because of the ability of the histidine imidazole group to titrate between neutral and endosomal pH. H562 and H586 are on the surface of the β -propeller domain, pointing at LA5 and LA4, respectively, and H190 projects from the tip of a loop on LA5. Surprisingly, mutation of any individual histidine to tyrosine (mimicking the H190Y and H562Y FH mutations and creating the additional H586Y mutant) did not interfere with the ability of the receptor to bind LDL at neutral pH on the cell surface, nor was the LDL-release activity of each mutant receptor diminished by more than about 30% when compared with the native receptor. However, when all three histidines were simultaneously replaced by either alanine or tyrosine, the mutant receptors lost the ability to release bound LDL, showing that the histidine residues participate together in the induction of the long-range intramolecular interface to trigger ligand release at low pH (147c).

As for the movement required for conformational interconversion, one proposal suggested the possibility of a hinge, or pivot point, in the linker connecting the last ligand-binding repeat with the first EGF repeat, the linker between the second EGF repeat and the propeller, or in both linkers (148). Solution NMR studies, however, revealed unanticipated findings: The linker connecting LA7 to EGF-A is not flexible, and the interface between LA7 and EGF-A is fixed and locked in virtually the same conformation at both neutral and endosomal pH (147c). Taking these data together with previous solution studies of the EGF-AB pair, the three consecutive modules LA7, EGF-A, and EGF-B all appear to adopt fixed positions with respect to one another throughout the physiologically relevant pH range.

These studies lead to a model in which the receptor uses a finely tuned balance between interdomain rigidity and flexibility to orchestrate release bound lipoproteins upon exposure to low pH. Whereas the preorganization of the LA7-EGF-A-EGF-B trio of modules acts as a rigid central scaffold to constrain the relative positions of the modules that flank them (Figure 9), the intrinsic flexibility of the intermodule linkers in the ligand-binding domain appears to impart enough flexibility to allow the receptor to achieve an open conformation at neutral pH and close subsequently at low pH.

STRUCTURE-FUNCTION CORRELATES IN THE CYTOPLASMIC TAIL

Early studies with fibroblasts of FH patients revealed the importance of the cytoplasmic tail in directing LDL receptors to clathrin-coated pits. Electron microscopic studies showed that the receptors from one particular FH patient (J.D.) failed

to localize in clathrin-coated pits (17, 150). Sequencing of this internalization-defective allele revealed the presence of a Y807C mutation in the NPVY sequence within the cytoplasmic tail (30). The importance of the FxNPxY sequence (F802-Y807) in directing receptor internalization via coated pits was confirmed by site-directed mutagenesis of the LDLR tail, which showed (a) internalization required the first 22 amino acids of the receptor tail, (b) the integrity of the NPxY sequence was critical, and (c) F802 was also important for internalization (31, 151). Bansal & Gierasch (152) showed that short peptides with the NPVY sequence had a propensity to adopt a reverse turn conformation and suggested that this conformational preference might facilitate binding to specialized adaptor proteins needed for endocytosis.

Adaptors Connecting the LDLR Cytoplasmic Tail to the Endocytosis Machinery

The link connecting the FxNPxY sequence of the LDLR to the endocytosis machinery remained unknown until Garcia et al. (153) determined the molecular basis for a rare autosomal recessive form of hypercholesterolemia that resembles FH but does not have mutations in the LDLR. All affected family members had mutations in the gene encoding a putative adaptor protein called ARH1. Importantly, Soutar's group (154) also showed that retroviral expression of ARH1 rescues LDLR internalization in transformed lymphocytes, confirming that the functional defect is caused by loss of ARH1 function.

Two characteristics of the ARH1 protein make it a good candidate as an adaptor connecting the LDLR cytoplasmic tail to the endocytosis machinery. First, ARH1 is a homologue of the Disabled (Dab) proteins Dab1 and Dab2, each of which has a PTB domain [a misnomer that stands for phosphotyrosine binding; (155, 156)] that preferentially binds unphosphorylated NPxY sequences (157, 158). Second, it includes a clathrin-binding consensus motif from residues 212–216 (LLDLE) (153).

Biochemical studies with purified, recombinant ARH1 showed direct interactions with the LDLR cytoplasmic tail and showed that binding to clathrin depended on the presence of the clathrin consensus motif (159, 160). Additional deletion mapping experiments implicated a conserved C-terminal region (residues 260–279) of ARH1 in binding to the β 2-adaptin subunit of the AP-2 clathrin adaptor (159, 160). Localization studies in HeLa cells show that ARH1 is suitably positioned to participate in LDL receptor uptake (160). The role of ARH1, as an adaptor connecting LDLR-family proteins and the endocytic machinery, also extends to *Xenopus* oocytes, in which it is required for normal uptake of vitellogenin via the vitellogenin receptor, a relative of the LDLR (161).

In certain cell types like fibroblasts, which do not exhibit an LDL uptake defect in cells from patients with ARH1 mutations, the related PTB-containing protein Dab2 may connect the LDLR to the endocytic machinery. Dab2 binds to peptides that have the FxNPxY sequence, clathrin, and the α -adaptin subunit of the clathrin

adapter protein AP-2 and also colocalizes with the LDLR transiently in clathrin-coated pits, all properties of cargo-selective clathrin adaptors (158, 162).

In addition to binding peptides with NPxY sequences, ARH1, Dab1 (an adaptor that binds to the cytoplasmic tails of the VLDLR and apoER2 and is essential for transducing Reelin signals in the brain), and Dab2 also bind phosphoinositides (PIs), with a preference for inositol 1,4,5-trisphosphate (PI-4,5P₂). Although the role of PI binding has not been firmly established for any of these proteins, it is believed that PI binding plays an important role in recruiting them to the membrane, where they carry out their functions as adaptors for endocytosis and signaling.

Structure of Tail Peptide Complexes with Disabled Proteins and Implications for the LDLR-ARH1 Complex

The structure of a complex between an LDLR cytoplasmic tail peptide and the PTB domain of ARH1 has not yet been solved. Nevertheless, recent high-resolution X-ray structures of the closely related PTB domains of Dab1 and Dab2 in binary complexes with peptides and in ternary complexes with peptides and PI-4,5P₂ provide insights into the structural basis for recognition of the LDLR by the PTB domains of endocytic adaptors at the membrane. The solved structures include a binary complex of the Dab1 PTB domain and an apoER2 peptide, a ternary complex of the Dab1 PTB domain, the apoER2 peptide and PI-4,5P₂ (163), the Dab2 PTB domain without ligands, the binary complex of the Dab2 PTB domain and an NPxY peptide from amyloid precursor protein (APP), as well as a ternary complex of the Dab1 PTB domain, the APP peptide, and PI-4,5P₂ (164).

The structures of the Dab1 and Dab2 PTB domains with bound peptides reveal how specificity for FxNPxY sequences with a nonphosphorylated tyrosine is achieved. Both PTB domains exhibit the canonical PTB fold, in which seven central β -strands form two antiparallel, near-orthogonal β -sheets, capped by a long C-terminal α -helix. Each PTB domain binds its peptide ligand in the canonical manner (Figure 11a), with the bound peptide extending the central sheet and with the NPxY sequence adopting a type I β -turn (163, 164). In the Dab1 peptide complexes, selectivity for phenylalanine at the -5 position (FxNPxY, where the "0" position corresponds to the tyrosine residue) is dictated by a hydrophobic pocket from the C-terminal helix, and selection for Tyr over pTyr is dictated by the size of the cleft created by short loops connecting β 4 with β 5 and β 6 with β 7. The loops closely approach the tyrosine side chain, situating Gly 131 within H-bonding distance of the peptide tyrosine hydroxyl (163, 164). This arrangement would sterically exclude the bulkier pTyr (Figure 11b). Similar features are observed in the binary complex of the APP peptide with the Dab2 PTB domain (164).

In the structures of the binary PTB domain-peptide complexes, there was an unanticipated clustering of basic residues on the face of the PTB domain opposite

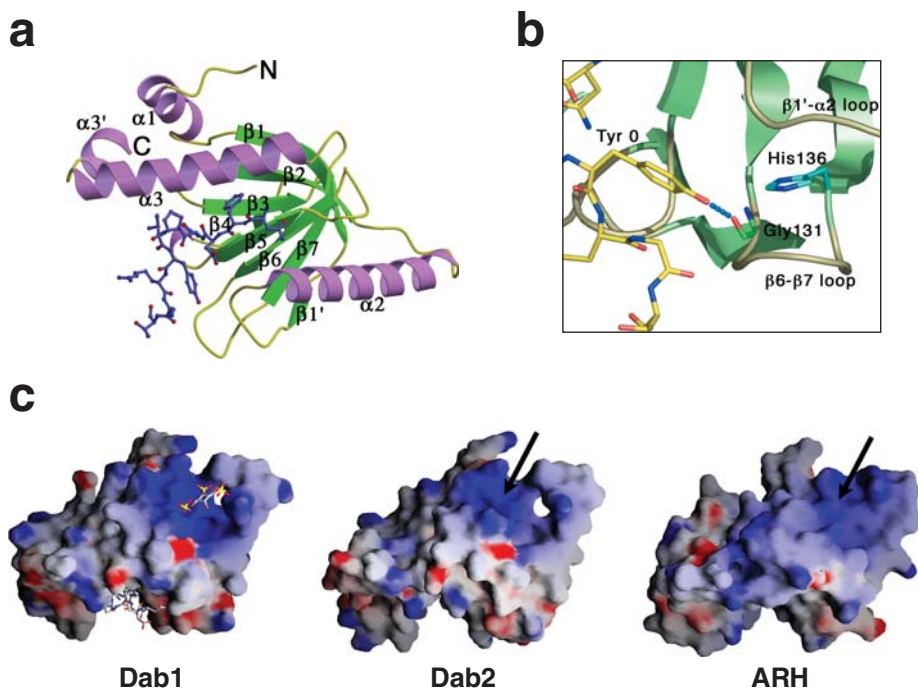


Figure 11 (a) Structure of the Dab1 PTB domain in complex with the apoER2 peptide (PDB accession code 1NTV). The Dab1 PTB domain is colored according to secondary structure, and the bound peptide is rendered in ball and stick form. (b) Close-up of the tyrosine-binding pocket. The protein backbone is shown as a ribbon, whereas selected apoER2 peptide (yellow) and protein (cyan) side chains are in ball-and-stick form. The hydrogen bond from Tyr 0 to Gly 131 is represented by a dashed purple line. (c) Surface representation of the PTB domain in ternary complex (PDB accession code 1NU2) and homology models of ARH1 (denoted ARH) and Dab2. The peptide-binding groove of the Dab1 PTB domain (left) is at the base, and the N-terminal residues of the bound peptide are projecting toward the viewer. The PI-4,5P₂-binding site is on the top face, opposite the peptide-binding groove. Dab1-based homology models of the Dab2 (center) and the ARH1 (right) PTB domains show that the location of the positively charged PI-4,5P₂-binding site is conserved (arrows). Reproduced from reference (163), with permission.

the peptide-binding groove (163, 164), suggesting that this region constituted the binding site for PIs. Structures of ternary complexes with both peptide and PI-4,5P₂ bound simultaneously to the Dab1 PTB domain confirmed this prediction; H-bonds are formed from K45 and K82 to oxygen atoms from the 4- and 5-phosphate groups of the bound PI-4,5P₂, from R124 and K142 to a 4-phosphate oxygen, from H81 to a 5-phosphate oxygen, and from R76 to a 1-phosphate oxygen. Binding of the ligands at the two sites appears to be energetically independent: Binding

affinity for one ligand is neither affected in the presence of mutations that abrogate binding of the other ligand nor altered when the other site is saturated with its ligand (165).

Sequence alignment of the PTB domains from ARH1, Dab1, and Dab2 shows that the cluster of basic residues is conserved, and a homology model of the ARH1 PTB domain predicts the existence of a similar region of positive surface electrostatic potential (Figure 11c), suggesting that the same region is responsible for PI binding and membrane recruitment by ARH1 and Dab2 as well (163, 164). Although the functional importance of the PI-binding site has yet to be examined experimentally, it is likely that PI binding will play a role in recruitment of these proteins to the membrane, where binding to the NPxY sequences of the receptor cytoplasmic tails takes place.

CONCLUSIONS

The advances over the past decade have rapidly advanced our understanding of the structural basis for lipoprotein binding and release by the LDLR. Structures of individual ligand-binding repeats explained why calcium was required for binding of lipoproteins, and the endosomal pH structure suggested a model for low-pH-induced lipoprotein release in which the receptor interconverts between open and closed conformations. The conservation of key residues in the long-range interface between the ligand-binding repeats and the propeller domain suggests that the same conformational rearrangement is also likely to occur in the VLDLR and perhaps in other more complicated LDL receptor-related proteins as well.

The remaining major gap in our understanding is the structural basis for ligand recognition at neutral pH. This question poses a substantial challenge because the ligands for the LDLR are lipoproteins and require lipid association for high-affinity receptor binding. Nevertheless, it is realistic to expect these technical hurdles to be surmounted in the future, perhaps by studies of ligand-binding fragments of the receptor with a suitable lipoprotein mimetic derived from apoE.

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