Introduction

The LDL receptor (LDLR) constitutes the founding member of a family of single-pass transmembrane proteins found in higher eukaryotes. Closely related members of this family include the LDLR, the very low-density lipoprotein receptor (VLDLR), apolipoprotein E-receptor 2 (ApoER2), and LDL receptor-related proteins 1 and 2 (LRP-1 and 2, respectively). LDLR family members share is the ability to transport lipoprotein particles into the circulation, because the cells responsible for lipoprotein packaging and secretion also express the receptors (Twisk et al., 2000). Current evidence suggests that the receptor-associated protein (RAP), a 39 kDa protein that was originally identified because it co-purified with LRP-1 (Strickland et al., 1991), facilitates the delivery of LDLR family proteins to the cell surface by preventing premature intracellular association of these receptors with their ligands (Bu et al., 1995; Willnow et al., 1995, 1996). RAP, which is normally retained intracellularly by a ER-retention sequence at its C-terminal end (Bu et al., 1995), binds to all core LDLR family proteins at neutral pH (e.g., Andersen et al., 2003), acts as a near-universal antagonist of ligand binding in biochemical assays, and enhances surface expression of LRP-1 when expressed as a transgene in RAP-deficient cells (Willnow et al., 1996).

Sequence alignment, biochemical, and biophysical studies suggest that RAP consists of three helical domains of approximately 100 amino acids each (Figure 1A; Lazic et al., 2003). The structural basis for recognition of ligand binding modules by RAP is not known, but quantitative and mutational studies examining the binding of the RAP domains to LA repeats from various LDLR-family proteins have established the following: (1) RAP is capable of binding to LA module pairs, but not individual modules; (2) the affinity of the third domain of RAP (RAP-D3) for a given LA module pair is at least an order of magnitude greater that that of the other RAP domains; (3) among the LA module pairs that bind to RAP-D3, sequence alignment of the individual LA modules shows conservation of an acidic and an aromatic residue at two specific positions of the module (Figure 1B); and (4) mutational studies confirm that the conserved acidic and aromatic residues make substantial contributions to RAP binding affinity (Andersen et al., 2000, 2001).

Results and Discussion

Because RAP binds to all LDLR family proteins and can antagonize the binding of a wide variety of ligands, we sought to uncover general principles underlying recognition of ligands by LDLR-family proteins by solving the structure of a complex between RAP-D3 and a typical LA module pair. Attempts to crystallize complexes of RAP-D3 with LA module pairs from LRP-1 were unsuccessful. From sequence alignment of LDLR module pairs with the RAP binding module pairs from LRP-1, we recognized that both modules of LA3-4 from the LDLR have the consensus acidic and aromatic residues important for RAP binding (Figure 1B), and indeed found that LA3-4 forms a stable complex with RAP-D3 that is readily purified to homogeneity. We succeeded in growing crystals of the LA3-4:RAP-D3 complex and determined its structure to 1.26 Å resolution (Figure 1C, Table 1, and see Figure S1 in the Supplemental Data available with this article online).
The overall shape of the protein complex is elongated, with the two LA modules positioned along the axis of the helical hairpin formed by RAP-D3 (Figure 1C). The binding interface is composed of two distinct docking sites on RAP-D3, one for each LA module. As a result, the interface between RAP-D3 and the LA domain pair in the complex is discontinuous. The total amount of solvent-accessible surface area of RAP-D3 buried in the complex is only 761 Å², with a roughly equivalent amount of surface area buried at each site (391 Å² and 370 Å² buried by LA3 and LA4, respectively).

In the cocrystal structure, RAP-D3 forms a three-helix bundle (Figure 1C). The bundle has a short, 12-residue N-terminal helix that buttresses the base of an elongated antiparallel helical hairpin formed by the other two helices, 41 and 38 residues (about 60 and 57 Å in length, respectively). When compared with the NMR structure of RAP domain 1 (RAP-D1), also a three-helix bundle, the length of the first helix is the same in both.
structures, but the second and third helices of RAP-D1 are much shorter, extending for only 27 and 18 residues, respectively (Nielsen et al., 1997). Structure-based sequence alignment, together with secondary structure predictions, suggests that the second domain of RAP (RAP-D2) will also form a three-helix bundle nucleated by a short N-terminal helix. The highest degree of sequence similarity among the three domains of RAP corresponds to the short N-terminal helix and the loop immediately following it, suggesting that this helix may serve as an anchor to stabilize the bundle.

Each of the two LA modules in the complex adopts a canonical LA fold, with a short $\beta$-hairpin near the N-terminal end of the module, three characteristic disulfide bonds, and a highly conserved calcium binding site (Fass et al., 1997). Coordination of a calcium ion at this site is required for folding of LA modules and for maintenance of their structural integrity (Blacklow and Kim, 1996), explaining the calcium dependence for binding of all known ligands. At each site, the calcium ion is coordinated by side chains from four conserved acidic residues and two backbone carbonyl groups in octahedral geometry (Fass et al., 1997). On both LA3 and LA4, one backbone carbonyl group is derived from an aspartate residue (D110 on LA3 and D149 on LA4), and the other is derived from an aromatic residue (F105 on LA3 and W144 on LA4). We designate the first calcium-coordinating aspartate as Asp I (D108 on LA3 and D147 on LA4); the second, participating via its backbone carbonyl, as Asp II (D110 on LA3 and D149 on LA4); and the third as Asp III (D112 on LA3 and D151 on LA4). Acidic residues are universally present at these three positions in all LRP-1 LA module pairs that exhibit binding to RAP-D3 with high affinity (Andersen et al., 2000).

**Structural Features of the LDLR-RAP Interface**

**Electrostatic Interactions Dominate the Binding Interface**

The interface between RAP-D3 and LA3-4 is dominated by electrostatic interactions between complementary charged surfaces at each docking site (Figures 2A and 2B). The strongly positive potential on the RAP-D3 binding surface is concentrated around K256 and K270, two basic residues located on the same face of helix two of RAP and separated by four helical turns. The negative surface potential on LA3-4 derives primarily from the surface-exposed aspartate residues that participate in calcium coordination.

**Details of the Recognition Interface from Two Near-Identical Docking Sites**

Each LA module combines four residues to create a specialized pocket for a lysine side chain protruding from the second helix of RAP-D3 (Figures 2C and 2D). The LA3 pocket encircles K270 of RAP (Figure 2C), and the LA4 site surrounds K256 (Figure 2D), with the mode of lysine recognition nearly identical at each site (Figures 2 and 3A). Carboxylate oxygen atoms from three aspartates (Asps I–III) at each site comprise an “acidic necklace” surrounding the C'-amino group of the lysine residue in a tripartite salt bridge. In addition, an aromatic residue (F105 of LA3 and W144 of LA4) packs its side chain against the aliphatic portion of the lysine in the pocket (Figures 2C and 2D). All four LA residues that form the binding pocket participate in calcium coordina-

tion, and, as a consequence, the relative positions of their side chains are constrained. Because calcium binding is a universal feature of all LA modules, the ligand binding pockets observed for LA3 and LA4 are paradigmatic and representative of virtually all LA modules, with the mode of lysine recognition likely to be general (schematically illustrated in Figure S2).

Additional favorable electrostatic interactions augment the core interface between the acidic necklaces and their entrapped lysine residues (Figure S3). The positively charged side chains of RAP K253 and R296 enhance the electrostatic potential created by K256, and R285 enhances the electrostatic potential created by K270. These basic residues form hydrogen bonds with residues on the LA modules, adding to the stability of the complex.

The electrostatic contacts seen in our structure illuminate the results of prior mutational studies investigating the binding of RAP to LRP-1. Remarkably, random mutagenesis of RAP-D3 identified only two residues critical for binding of RAP-D3 to LRP-1 and heparin (Migliorini et al., 2003): K256 and K270, the two lysines encircled by the acidic necklaces of LA3 and LA4 in our cocrystal structure. In contrast, mutations of lysine and/or arginine residues outside of the acidic pockets (e.g., K306A) had little impact on affinity (Migliorini et al., 2003).

**Comparison with Other LA-Containing Complexes**

Several lines of evidence argue that the binding mode used for recognition of RAP represents a general strategy for protein binding by LA modules. First, a number of known ligands for LDLR family proteins are highly basic, with clusters of basic residues on apolipoprotein E (ApoE) and apolipoprotein B implicated in binding to the LDLR (Boren et al., 1998; Knott et al., 1985; Lalazar et al., 1988; Weisgraber et al., 1983). Second, aspartates I and III of the LA modules, which form two-thirds of the acidic necklace around the lysine residues of RAP, are highly conserved because of their other structural role as calcium-coordinating residues (Figures 1–3). Third, residues of LA repeats from LRP-1 and ApoER2 identified by mutagenesis to be critical for binding RAP (Andersen et al., 2000, 2003) are analogous to the conserved aromatic and Asp II residues that complete the core of each lysine binding pocket in the structure of the complex.

The most striking evidence for the generality of the binding mode emerges from comparing the LDLR-RAP structure to other structures (Figure 3). In the structure of the LDLR at endosomal pH, the propeller domain of the LDLR closes on LA repeats 4 and 5 promoting release of bound ligands at endosomal pH (Rudenko et al., 2002). In the low pH structure, the calcium-coordinating aspartate residues of the LA modules form intramolecular pockets for binding two lysine side chains projecting from the propeller domain (K560 and K582), with analogous contacts to those in the LDLR:RAP-D3 complex (Figures 3B and 3C). In the 3.5 Å X-ray structure of human rhinovirus 2 (HRV2) with the LA2-3 pair from the VLDLR (Verdaguer et al., 2004), the acidic necklace (two aspartates and a glutamate from the VLDLR) surrounds an absolutely conserved HRV2 lysine residue required for productive infection (K1224). In all structures, the binding of the lysine in the pocket is augmented by a hydrophobic interaction between an aromatic residue...
of the LA module and the aliphatic region of the lysine (Figures 3B–3D).

**Avidity in Ligand Binding by LDLR Family Proteins**

Our structure also argues that avidity effects play a major role in the binding of ligands by LDLR-family proteins. In the cocrystal structure, the contact interface between each individual LA module and RAP is small (<400 Å²), suggesting that a single module would be insufficient to confer high-affinity binding. Indeed, studies mapping the binding of RAP to LA modules from the second repeat cluster in LRP-1 showed that it was possible to detect binding of RAP to a number of different LA module pairs, but not to individual modules (Andersen et al., 2000), nor to LA module pairs in which one of the modules lacks Asp II of the lysine binding pocket, indicating that only a single intact docking site is usually insufficient. Even in the structure of the LDLR at low pH, two LA modules participate in the long-range interface with the β-propeller domain (Rudenko et al., 2002), suggesting that contacts between the propeller domain and a single module would not be enough to stabilize the closed, binding-inactive conformation.
Modeling ApoE Binding from the RAP-D3:LA3-4 Complex

Besides RAP, all members of the LDLR family can also bind ApoE-containing lipoproteins. A cluster of basic amino acids between residues 140 and 160 (LaZar et al., 1988), together with R172 (Morrow et al., 2000), constitute the LDLR binding site. Although 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 (Wilson et al., 1991), ApoE does not bind to the LDLR with high affinity (nM) until it is associated with lipid. The recent 10 Å resolution crystal structure of a receptor binding active ApoE-DPPC particle shows that lipid induces global rearrangements of the helices that comprise the receptor binding domain and repositions R172 close to the 140–160 helix (Peters-Libeu et al., 2006).

Within the 140–160 region, residues K143 and K146 make important contributions to LDLR binding, and arginines are unable to substitute for lysines in the K143R/K146R double mutant (Zaiou et al., 2000). Because the conformation around these two lysines essential for LDLR binding remains helical in both lipid-free and lipid-associated apoE (Peters-Libeu et al., 2006; Raussens et al., 2003), and because this region retains the ability to bind monoclonal antibodies in both the presence and absence of lipid (Raffai et al., 1995), we superimposed this helix from the lipid-free ApoE structure onto the second helix of RAP-D3 to model the interface between a single LA module and the receptor binding region of ApoE. In the model, the side chain of K146 of ApoE superimposes well onto K256 of RAP-D3 in the acidic pocket of the LA module, K143 from the preceding turn of the ApoE helix superimposes on K253 of RAP-D3, and there are no steric clashes created elsewhere (Figure 4). When relocated upon complexation with lipid, R172 would then be available to make supplemental contacts with the LA module like those mediated by R296 on helix 3 of RAP-D3. Finally, lipid association undoubtedly enhances the affinity of ApoE for the receptor not only because it repositions R172 near the 140–160 helix to create a complete binding site, but also because it creates at least two docking sites per particle to increase avidity: two copies of ApoE are present in the crystalized, binding-active particle, and two LA modules are necessary to form stable complexes with both ApoE and RAP-D3 (Andersen et al., 2000, 2001; Fisher et al., 2004).

The affinity of RAP for LDLR family proteins at neutral pH also raises the question of how RAP:receptor complexes dissociate during delivery of mature receptors to the cell surface. Prior studies suggest that RAP dissociates from LRP-1 at a pH below ~6.5 (Bu et al., 1995), and we observe that RAP-D3:LA3-4 complexes are no longer completely stable to gel filtration when the pH drops below ~6.5 (Figure S4). The intrinsic reduction in affinity may result from titration of key histidine residues oriented toward the interior of the H2–H3 helical hairpin of RAP-D3, like H257, H268, and H290 (Figure S6). RAP-D3 has marginal thermodynamic stability to begin with (Lazic et al., 2003); after titration, burial of the positively charged histidine residues in the interface will become highly unfavorable, with the acquisition of positive charge promoting unfolding of the bundle and facilitating release of the receptor. The ability of RAP-D3 to dissociate from LA3-4 without participation of the LDLR β-propeller domain contrasts with the situation for bound lipoproteins, which require the propeller domain to let go of LDL at endosomal pH, and with the intramolecular binding of the propeller domain to the LA modules, which is induced by a reduction in pH.

Finally, the structure of the LDLR-RAP complex has important implications for the interplay between ligand binding by LDLR family proteins and the function of RAP as an escort protein. The participation of four calcium-coordinating residues in the binding of RAP-D3 at each docking site provides compelling structural evidence that RAP binds to lipoprotein receptor modules...
only after they have folded to their native conformation. The binding of RAP to a unique feature of the calcium-loaded LA domain in its native conformation thus supports the proposal that it functions as an escort protein, rather than as a traditional "folding" chaperone like Hsp60 and Hsp70 proteins. Because the binding sites on LA modules for RAP and plasma-derived ligands appear to be overlapping, formation of complexes between the lipoprotein receptors and RAP should preclude premature binding of ligands by directly occluding binding sites for lipoproteins and for a number of other ligands as well. Indeed, for certain LDL receptor-related proteins, it appears that the LA modules of these receptors will be occupied by RAP during maturation, by ligands upon cell-surface binding, and finally by an intramolecular propeller domain upon delivery to intracellular endosomes at low pH for ligand release and subsequent receptor recycling.

Experimental Procedures

Protein Expression and Purification
LA module pairs and RAP-D3 were expressed essentially as described previously (Andersen et al., 2000; 2001; Fisher et al., 2004). Complexes between LA module pairs and RAP-D3 were formed at 4°C, purified by size exclusion chromatography, and then concentrated to 20 mg/ml.

Crystalization, Data Collection, and Structure Determination
LA3-4/RAP-D3 complexes (20 mg/ml in 150 mM NaCl, 2.5 mM CaCl2, 20 mM Tris [pH 8.0]) crystallized from hanging drops in 40% MPD (Table 1). Heavy atom sites were found by SOLVE (Terwilliger and Berendzen, 1999) using single isomorphous replacement including anomalous scattering (SIRAS). An initial model built with ARP/WARP (Perrakis et al., 2001) contained 85% of the residues. The missing residues were built manually in Coot (Emsley and Cowtan, 2004). The resulting model was refined with SHELXL-97 (Sheldrick and Rob Meijers for help with data collection. This work was supported by R01 HL-61001 (to S.C.B.) and by NRSAs and National AHA SDG grants (to N.B.). X-ray data were measured at beamline X29A of the NSLS.

Received: December 22, 2005
Revised: February 10, 2006
Accepted: February 17, 2006
Published: April 20, 2006

References


Supplemental Data
Supplemental Data include five figures and can be found with this article online at http://www.molecule.org/cgi/content/full/22/2/277/DC1/.

Acknowledgments
We thank Michael Eck for helpful discussions and sharing resources and Rob Meijers for help with data collection. This work was supported by R01 HL-61001 (to S.C.B.) and by NRSAs and National AHA SDG grants (to N.B.). X-ray data were measured at beamline X29A of the NSLS.


Accession Numbers

Coordinates for the cocrystal structure have been deposited in the Protein Data Bank under the accession code 2FCW.