The Epidermal Growth Factor Homology Domain of the LDL Receptor Drives Lipoprotein Release through an Allosteric Mechanism Involving H190, H562, and H586*

Zhenze Zhao and Peter Michaely

From the Department of Cell Biology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75390-9039

The low density lipoprotein (LDL) receptor (LDLR) mediates efficient endocytosis of VLDL, VLDL remnants, and LDL. As part of the endocytic process, the LDLR releases lipoproteins in endosomes. The release process correlates with an acid-dependent conformational change in the receptor from an extended, “open” state to a compact, “closed” state. The closed state has an intramolecular contact involving H190, H562, and H586. The current model for lipoprotein release holds that protonation of these histidines drives the conformational change that is associated with release. We tested the roles of H190, H562, and H586 on LDLR conformation and on lipoprotein binding, uptake, and release using variants in which the three histidines were replaced with alanine (AAA variant) or in which the histidines were replaced with charged residues that can form ionic contacts at neutral pH (DRK variant). Contrary to expectation, both the AAA and DRK variants exhibited normal acid-dependent transitions from open to closed conformations. Despite this similarity, both the AAA and DRK mutations modulated lipoprotein release, indicating that H190, H562, and H586 act subsequent to the conformational transition. These observations also suggest that the intramolecular contact does not drive release through a competitive mechanism. In support of this possibility, mutagenesis experiments showed that LDL binding was inhibited by mutations at D203 and E208, which are exposed in the closed conformation of the LDLR. We propose that H190, H562, and H586 are part of an allosteric mechanism that drives lipoprotein release.

The low density lipoprotein (LDL)2 receptor (LDLR) is a type I transmembrane protein that functions as the principal endocytic receptor for very low density lipoprotein (VLDL), VLDL remnants, and LDL. Lipoproteins bind to the extracellular domain (ectodomain) of the LDLR, which contains a ligand binding domain, an epidermal growth factor (EGF) homology domain, and an O-glycosylated domain (1). The ligand binding domain provides the majority of binding surfaces for lipoproteins (2). This domain consists of seven LDLR type A (LA) repeats, which are small, irregular domains held together by calcium and cystine bridges (3, 4). The EGF homology domain participates in lipoprotein release and consists of two EGF-like domains, six YWTD repeats that form a six-bladed β-propeller, and a third EGF-like repeat (5, 6). Internalization of the LDLR requires the cytoplasmic domain (7, 8).

The LDLR binds to lipoproteins that contain either apolipoprotein B100 (apoB100) or apolipoprotein E (apoE). LDL binds to the LDLR via apoB100, while VLDL and VLDL remnants bind via apoE (9–11). LDL and VLDL compete for binding and interact with overlapping regions of the LDLR (2, 12–14). Both apoB100 and apoE contain clusters of positively charged residues that are required for binding (15–19). These positively charged residues have been proposed to contact acidic residues on the LDLR that are clustered by calcium (20, 21).

Following lipoprotein binding, LDLR-lipoprotein complexes internalize through clathrin-coated pits and traffic to sorting endosomes where lipoprotein release occurs (22, 23). Lipoprotein release requires the EGF homology domain and acidic pH (5). Release occurs at pH 6, which corresponds to the luminal pH of sorting endosomes (5, 23). Coincident with lipoprotein release, the LDLR undergoes a conformational transition from an extended, “open” conformation, which predominates at neutral pH, to a compact, “closed” conformation at acidic pH (24). The crystal structure of the LDLR ectodomain at acidic pH shows that in the closed conformation, the β-propeller region of the EGF homology domain forms an intramolecular contact with LA repeats 4 and 5 (24). At the center of this contact are three histidines (H190, H562, and H586) that form van der Waals contacts and ionic interactions between the β-propeller and the LA repeats. The ionic interactions presumably only form when histidine is positively charged. The imidazole ring of histidine has a pK_a of ~6, suggesting that the three histidines are protonated at endosomal pH.

These observations have led to the current model for acid-dependent lipoprotein release (25). This model proposes that the β-propeller of the EGF homology domain binds to the lipoprotein binding surfaces of LA4 and LA5. Release occurs through dissociation of lipoprotein from the LA repeats fol-
lowed by binding of the β-propeller to LA4/5. The model also stipulates that the binding of the β-propeller with LA4/5 requires protonation of H190, H562, and H586. Consistent with this model, mutation of all three histidines to alanine inhibits LDL release (26).

The current model makes two key predictions. First, the conformational state of the receptor should depend upon the ability of residues at 190, 562, and 586 to form ionic contacts. Second, lipoproteins should bind to the same surface as the β-propeller.

Here, we tested the current model. We examined the role of the three histidines on receptor conformation and on lipoprotein binding, uptake, and release using LDLR variants in which the three histidines were replaced with alanine or in which the histidines were replaced with charged residues that can maintain ionic interactions at neutral pH. We also used mutagenesis to test whether lipoproteins bind to the same surface on the LDLR as the β-propeller of the EGF homology domain.

EXPERIMENTAL PROCEDURES

Materials—All cell culture reagents were from Invitrogen (Carlsbad, CA). LDLR−/− primary human fibroblasts, human LDL, and rabbit β-migrating VLDL (β-VLDL) were a gift from Michael Brown and Joseph Goldstein (Department of Molecular Genetics, UT Southwestern Medical Center, Dallas, TX). Rabbit polyclonal anti-LDLR (4548) was a gift from Joachim Herz (Department of Molecular Genetics, UT Southwestern Medical Center, Dallas, TX). Mouse monoclonal anti-LDLR (C7) was from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa546 succinimidyl ester was from Invitrogen. All other chemicals were from Sigma.

Baculovirus-mediated Protein Expression—Residues 1–699 of the LDLR, LDLR-AAA, and LDLR-DRK were cloned into the pFastBac plasmid (Invitrogen). Recombinant plasmids were cotransfected with the pAmpho packaging vector (Clontech, Mountain View, CA) into 293T cells to produce infectious, replication-defective retroviruses. Retroviruses were added to LDLR−/− fibroblasts (549T) in the presence of hexadimethrine bromide to facilitate viral entry. Infection rates as assessed by GFP-positive fluorescence were ~5%. GFP-positive, LDLR-expressing fibroblasts were purified using two rounds of fluorescence-activated cell sorting (FACS) with a MoFlo High Performance Cell Sorter (Dako, Glostrup, Denmark). Purity was ~90% after the first sort and >95% after the second sort. Surface LDLR expression was monitored by flow cytometry using anti-LDLR mouse monoclonal antibody, C7 (Santa Cruz Biotechnology, Santa Cruz, CA).

Lipoprotein Binding Assays—Human 125I-LDL and rabbit 125I-β-VLDL binding assays were performed in triplicate using established methods (30). Assays were preformed at 4 °C for 90 min using concentrations of 125I-LDL or 125I-β-VLDL indicated in the figure legends. Results are presented as means ± S.D.

Initial Endocytic Rates—Initial internalization rates were determined as previously described (31, 32). Briefly, cells were incubated with 10 μg/ml 125I-LDL or 5 μg/ml 125I-β-VLDL for 1 h at 4 °C in Medium C (bicarbonate-free MEM supplemented with 20 mM HEPES pH 7.5 and 10% lipoprotein-poor serum). Medium was changed for the times indicated with warm Medium B that also containing either 10 μg/ml 125I-LDL or 5 μg/ml 125I-β-VLDL. Cells were extensively washed with ice-cold PBS and incubated with 1 mg/ml Protease K in Buffer A (PBS + 1 mM EDTA) for 2 h at 4 °C. The cell suspension was then centrifuged at 5000 × g for 10 min over a cushion of 10% sucrose in PBS. The tubes were frozen in liquid nitrogen, cut to separate the cell pellet (internal) from the solution (surface-bound material released by protease K) and counted on a gamma counter. Results are presented as means ± S.D.

Fluorescent Lipoprotein Labeling—DiI (3H-Indolium, 2-(3-(1,3-dihydro-3,3-dimethyl-1-octadecyl-2H-indol-2-ylidene)-1-propenyl)-3,3-dimethyl-1-octadecyl-, perchlorate) was used to label β-VLDL by adding 300 μl of 3 mg/ml DiI suspended in DMSO to 10 mg of β-VLDL in 10 ml of lipoprotein-poor serum with gentle mixing. The suspension was mixed end-over-end for 16 h at 37 °C in the dark. The density of the suspension was increased to 1.019 by the addition of 0.0199 g of KBr per ml of...
Roles of H190, H562, and H586

suspension and centrifuged at 120,000 \( \times g \) for 16 h at 4 °C. Dil-β-VLDL was removed from the top of the tube, dialyzed against PBS, and stored at 4 °C in the dark until use. LDL was labeled using Alexa-546 succinimidyl ester using the manufacturer’s recommended protocol (Invitrogen).

Lipoprotein Accumulation—Cells were incubated with either 10 \( \mu g/ml \) Alexa546-LDL or 5 \( \mu g/ml \) Dil-β-VLDL in Medium C for 1 h at 4 °C. The medium was then replaced with warm Medium B containing 10 \( \mu g/ml \) Alexa546-LDL or 5 \( \mu g/ml \) Dil-β-VLDL for the times indicated. Cells were washed with ice-cold PBS, suspended by gentle scraping in PBS and fixed in the presence of 3% paraformaldehyde. Cells were washed with PBS and analyzed by flow cytometry on a FACS Calibur (BD Biosciences, San Jose, CA). Mean fluorescence intensities were recorded for 10,000 events for each experiment.

Acid-dependent Release of Cell Surface-bound Lipoprotein—Assays were performed at both 4 and 37 °C. For assays at 4 °C, cells were incubated with either 10 \( \mu g/ml \) \( ^{125}I \)-LDL or 5 \( \mu g/ml \) \( ^{125}I \)-β-VLDL in Medium C for 1 h at 4 °C. Cells were was washed with ice-cold Buffer B (TBS + 1% bovine serum albumin) and incubated with Medium D (bicarbonate-free MEM supplemented with 20 \( \mu M \) HEPES, 20 \( \mu M \) maleate, and 10% lipoprotein-poor serum) at pH 5.5, 6.0, 6.5, 7.0, or 7.5 for 30 min at 4 °C. The cells were washed twice with ice-cold TBS, one time for 10 min with ice-cold Buffer B and two times with ice-cold TBS. Remaining cell-associated, \( ^{125}I \)-labeled lipoproteins were liberated by incubation with 0.1 N NaOH and counted on a gamma counter. For assays at 37 °C, cells were preincubated with Medium E, which consisted of Medium B supplemented with 0.45 M sucrose to prevent clathrin-coated pit endocytosis (33). Cells were then incubated with either 10 \( \mu g/ml \) \( ^{125}I \)-LDL or 5 \( \mu g/ml \) \( ^{125}I \)-β-VLDL in Medium E for 30 min at 37 °C, washed with warm Medium E, and incubated for 30 min at 37 °C with Medium F (bicarbonate-free MEM supplemented with 0.45 M sucrose, 20 \( \mu M \) HEPES, 20 \( \mu M \) maleate, and 10% lipoprotein-poor serum) at pH 5.5, 6.0, 6.5, 7.0, or 7.5. The cells were washed twice with ice-cold TBS, one time for 10 min with ice-cold Buffer B and two times with ice-cold TBS. Remaining cell-associated, \( ^{125}I \)-labeled lipoproteins were liberated by incubation with 0.1 N NaOH and counted. All experiments were performed in triplicate and are presented as a fraction of counts in parallel assays in which the acid release step was omitted. Rate constants for release were determined by non-linear curve fitting using a single-phase exponential decay model. Data for the curve fitting was from Fig. 5 with the exception of β-VLDL release at 4 °C, which used release data collected over 30 min (data not shown).

RESULTS

H190, H562, and H586 have been proposed to constitute a pH sensor that controls receptor conformation, thereby regulating lipoprotein binding and release (24–26). To test whether these histidines control the conformational state of the LDLR, we used a baculovirus expression system to produce proteins encompassing the LDLR ectodomain of normal LDLR, of an LDLR variant in which H190, H562, and H586 were replaced with alanine, and of an LDLR variant in which the histidines were replaced with charged residues. If the histidine contacts are required for the closed conformation, then the alanine (AAA) variant should be fixed in the open conformation. By contrast, if the ionic interactions made by the histidines are sufficient to drive adoption of the closed variant, then replacement of the histidines with charged residues can form ionic contacts at neutral pH should fix the LDLR ectodomain in the closed conformation. The charged variant (DRK) had the following mutations: H190D, H562R, and H586K mutations. These mutations were designed to form two ionic contacts: one between K586 and D149, and one between R562 and D190. In panel B, gel filtration was used to determine the hydrodynamic (Stokes) radius of ectodomains from normal (WT), LDLR-AAA (AAA), and LDLR-DRK (DRK) receptors as a function of pH.

![FIGURE 1. Mutations at H190, H562, and H586 have little effect on LDLR ectodomain conformation. H190, H562, and H586 come together at the interface between the β-propeller region of the EGF homology domain and LA repeats 4 and 5. The left panel of A shows the orientation of the three histidines in the closed conformation of the LDLR. The right panel of A shows a model of the interface with the H190D, H562R, and H586K mutations. These mutations were designed to form two ionic contacts: one between K586 and D149, and one between R562 and D190. In panel B, gel filtration was used to determine the hydrodynamic (Stokes) radius of ectodomains from normal (WT), LDLR-AAA (AAA), and LDLR-DRK (DRK) receptors as a function of pH.](image-url)
significant role in LDLR function. Familial Hypercholesterolemia (FH) is associated with tyrosine substitutions at H190 and H562 (34, 35). Furthermore, triple alanine or tyrosine mutations at H190, H562, and H586 inhibit acid-dependent LDL release from the surface of CHO cells expressing these variants (26). How might the three histidines participate in LDLR function?

To answer this question, we used fibroblasts that stably express full-length normal LDLR, LDLR-AAA or LDLR-DRK receptors in experiments measuring lipoprotein binding, uptake, and release. Full-length cDNAs for normal LDLR, LDLR-AAA, and LDLR-DRK were introduced into LDLR−/− fibroblasts by retroviral infection. LDLR expression was driven by the 5′-LTR of the retrovirus, which also produced GFP through an internal ribosomal entry site downstream of the coding sequence for the LDLR. GFP-positive cells thus express LDLR, allowing LDLR-expressing cells to be purified by FACS. Infection rates were kept to ~5% so as to ensure that infected cells had only a single integration event. Consistent with a homogeneous expression level, the variance observed in surface LDLR of infected cells was similar to that seen in normal human fibroblasts expressing the endogenous LDLR (data not shown). Immunoblots of cell lysates showed that LDLR expression was similar in each cell line and that the total LDLR expression level in the infected fibroblasts was only 2–3-fold higher than endogenous LDLR expression in normal human fibroblasts (Fig. 2A). No immature LDLR was observed, indicating that all three variants were glycosylated normally in the Golgi.

Flow cytometry using the C7 anti-LDLR antibody showed that cells expressing normal LDLR, LDLR-AAA, and LDLR-DRK had similar numbers of LDLR on their cell surfaces (Fig. 2B). No LDLR was detected in LDLR−/− fibroblasts infected with retroviruses lacking an LDLR gene (Vector). Together, these observations indicate that the AAA and DRK mutations did not impair the expression or surface delivery of the LDLR.

The role of the three histidines in lipoprotein binding was determined by comparing the affinity of 125I-LDL and 125I-β-VLDL for cells expressing normal LDLR, LDLR-AAA, and LDLR-DRK. β-VLDL is a VLDL remnant particle that is commonly used to assess the binding and uptake of VLDL/VLDL remnants by the LDLR (36). Binding was assessed in saturation experiments in which increasing concentrations of 125I-LDL or 125I-β-VLDL were incubated with cells at 4 °C. In these assays, LDLR-AAA cells bound LDL normally; however, LDLR-DRK cells showed reduced ability to bind LDL (Fig. 3A). Scatchard analysis of the binding data indicated that LDLR-AAA and LDLR-DRK cells bound LDL with lower affinity than normal LDLR expressing CHO cells (data not shown). By contrast, both the LDLR-AAA cells and LDLR-DRK cells bound β-VLDL normally (Fig. 3B). The ability of LDLR-AAA cells to bind both LDL and β-VLDL normally indicated that the three histidines were not required for lipoprotein binding.

After internalization, LDLR-lipoprotein complexes are transported to endosomes where lipoprotein release occurs. We followed lipoprotein release using two assays: 1) surface assays in which cell-surface bound lipoproteins were released in response to acidic buffers, and 2) cellular assays in which lipoprotein release was followed as a function of lipoprotein internalization and accumulation over time.

In the surface assays, normal LDLR, LDLR-AAA, and LDLR-DRK cells were incubated with 125I-labeled lipoproteins at 4 °C or 37 °C and then shifted to medium at pH 5.5–7.5 in the absence of lipoprotein for 30 min. Release was followed by measuring the amount of 125I-labeled lipoproteins that remained cell associated after the 30-min incubation.

In surface assays at 4 °C, LDLR-AAA cells showed reduced ability to release lipoproteins in response to acidic buffers. Normal LDLR-expressing cells had half-maximal LDL release at pH 6.5, while LDLR-AAA cells had half-maximal release below pH 5.5 (Fig. 4A). The observations with LDLR-AAA cells support the results of Beglova et al. (26) who used flow cytometry to show that at 4 °C, CHO cells expressing the LDLR-AAA have reduced ability to release surface-bound LDL in response to low pH. LDL release by LDLR-DRK cells is not reported because LDL binding by these cells was defective. β-VLDL release from normal LDLR-expressing cells at 4 °C showed half-maximal release at pH 5.8 (Fig. 4B), which is similar to the half-maximal β-VLDL release previously observed in CHO cells expressing normal LDLR (5). LDLR-AAA cells showed reduced ability to
release β-VLDL (half-maximal release below pH5.5), while LDLR-DRK cells showed half-maximal release of β-VLDL at pH 6.4.

Lipoprotein release was also compared in the three cells at 37 °C to test whether the differences observed at 4 °C were consistent with acid-dependent release at physiological temperature. These assays used medium containing 0.45M sucrose to prevent clathrin-coated pit internalization of lipoproteins (33). At 37 °C, normal LDLR-expressing cells had half-maximal LDL release at pH 6.5, while LDLR-AAA cells had half-maximal release at pH 5.8 (Fig. 4C). β-VLDL release experiments at 37 °C showed half-maximal release points at pH 5.8, pH 6.2, and pH 6.5 for LDLR-AAA, normal LDLR, and LDLR-DRK cells, respectively (Fig. 4D). Together the LDL and β-VLDL release experiments showed that the AAA mutation increased the acid dependence of lipoprotein release, while the DRK mutation reduced the acid dependence of lipoprotein release. These observations also showed that β-VLDL release required a more acidic environment than LDL release.

Surface assays were also used to determine rate constants for lipoprotein release at both 4 °C and 37 °C. 125I-labeled lipoprotein was bound to cells as before and then incubated with pH 5.5 media for 0–16 min (Fig. 5). As with the pH experiments, release was followed as the amount of 125I-labeled lipoprotein that remained cell associated at the end of the incubation. Rates of release were calculated from the resulting curves using a single-phase exponential decay model (Table 1). At 4 °C, the rates of LDL and β-VLDL release from cells expressing normal LDLR were 7.9 ± 10⁻³ s⁻¹ and 9.6 ± 10⁻⁴ s⁻¹, respectively. These rates are approximately two orders of magnitude faster than the corresponding lipoprotein dissociation rates at neutral pH (37), indicating that acidic pH accelerated lipoprotein dissociation from the normal LDLR. LDLR-AAA cells had slower than normal rates for both LDL and β-VLDL release (Fig. 5 and Table 1). Release by LDLR-AAA cells also plateaued at higher than normal levels for both LDL and β-VLDL. By contrast, LDLR-DRK cells had slightly faster than normal rates of β-VLDL release (Fig. 5, B and D and Table 1). Temperature also played a major role: LDL release rates doubled at 37 °C, while β-VLDL release rates increased by more than 10-fold. These kinetic experiments indicated that the AAA mutation slowed lipoprotein release.

Cellular assays for lipoprotein release combined assays measuring initial rates of lipoprotein internalization with assays measuring steady-state rates of lipoprotein accumulation. Initial rate experiments were conducted over 15 min and meas-

**FIGURE 3.** The effect of the AAA and DRK mutations on lipoprotein binding. Saturation binding of 125I-LDL (A) and 125I-β-VLDL (B) was performed using LDLR retroviruses. Experiments were performed in triplicate and data are presented as means ± S.D.

**FIGURE 4.** The AAA mutation hinders acid-dependent lipoprotein release, while the DRK mutation potentiates β-VLDL release in vitro. Release of prebound 125I-LDL (A and C) or 125I-β-VLDL (B and D) from fibroblasts expressing normal LDLR (WT), LDLR-AAA (AAA), or LDLR-DRK (DRK) retroviruses. Experiments were performed in triplicate and are reported as the mean of the fraction of cell-associated lipoprotein remaining ± S.D.
Roles of H190, H562, and H586

FIGURE 5. The AAA mutation slows release. Release of prebound 125I-LDL (A and C) or 125I-β-VLDL (B and D) from fibroblasts expressing normal LDLR (WT), LDLR-AAA (AAA), or LDLR-DRK (DRK) in response to pH 5.5 medium was determined at 4 °C (A and B) and 37 °C (C and D) over a 16-min time course. 37 °C trials had 0.45 M sucrose present to prevent coated pit internalization. All experiments were performed in triplicate and are reported as the mean of the fraction of cell-associated lipoprotein remaining ± S.D.

TABLE 1

| Lipoprotein | Temperature | WT       | AAA       | DRK       |
|-------------|-------------|----------|-----------|-----------|
|             | °C          | $r^1$    | $r^1$     | $r^1$     |
| LDL         | 4           | $7.9 \times 10^{-3}$ | $4.5 \times 10^{-3}$ | N/D$^a$  |
| β-VLDL      | 4           | $9.6 \times 10^{-4}$ | $1.1 \times 10^{-5}$ | $1.0 \times 10^{-3}$ |
| LDL         | 37          | $2.2 \times 10^{-2}$ | $9.4 \times 10^{-3}$ | N/D$^a$  |
| β-VLDL      | 37          | $1.0 \times 10^{-2}$ | $7.4 \times 10^{-3}$ | $1.2 \times 10^{-2}$ |

$^a$ N/D, not determined.

ured the ability of cells to internalize 125I-labeled lipoproteins. The lipoprotein accumulation assays used flow cytometry to follow the fluorescent intensity of cells incubated with fluorescently labeled lipoproteins (Alexa546-labeled LDL or Dil-labeled β-VLDL) over a course of 4 h. Alexa546 and Dil dyes are not degraded in lysosomes and provide an easily quantified measure of the amount of lipoprotein that reaches lysosomes (32). Because the LDLR completes ~5 endocytic cycles per hour (38), if the AAA or DRK mutations prevent release, then lipoprotein should internalize but not accumulate. In this way, the combination of assays for internalization and accumulation provided a measure of the ability of the LDLR to release lipoproteins under cellular conditions.

Cellular assays compared LDL and β-VLDL uptake in normal LDLR, LDLR-AAA and LDLR-DRK cells. LDLR-AAA cells had a normal rate of LDL internalization (Fig. 6A), but a very slow rate of LDL accumulation relative to normal (Fig. 6B). Thus, the AAA mutation disrupted cellular LDL release by the LDLR. In β-VLDL uptake assays, both the LDLR-AAA cells and LDLR-DRK cells showed reduced rates of β-VLDL internalization (Fig. 6C). This reduction likely resulted in the lower level of β-VLDL accumulation that was observed at the 1-h time point in the β-VLDL accumulation assay; however, the rates of β-VLDL accumulation by all three cells over the 4-h time course were similar (Fig. 6D). As compared with LDL, the trafficking of β-VLDL from endosomes to lysosomes is slow (39–41). The increased residence time of LDLR-β-VLDL complexes in endosomes may allow near normal endosomal release of β-VLDL from LDLR-AAA. For LDL by contrast, the LDLR-AAA may exit endosomes prior to release, leading to reduced LDL accumulation.

The reduced initial rates of β-VLDL internalization exhibited by the LDLR-AAA and LDLR-DRK cells suggested that clathrin-coated pit internalization of LDLR-β-VLDL complexes was sensitive to mutations at H190, H562 and H586. The cytoplasmic domain of the LDLR has two internalization mechanisms that can support β-VLDL internalization: a well characterized FDNPVY-dependent pathway and a less understood, FDNPVY-independent pathway that only serves for VLDL/VLDL remnant uptake (32, 42, 43). To determine whether the histidine mutations influenced the FDNPVY-dependent pathway, LDLR−/− fibroblasts were infected with retroviruses that encoded LDLR variants that combined the Y807C mutation, which inactivates the FDNPVY sequence (7), with either the AAA (LDLR-AC) or DRK (LDLR-DC) mutations. All receptors expressed normally in these fibroblasts (Fig. 7A). Comparison of the initial rates of internalization showed that LDLR-Y807C, LDLR-AC, and LDLR-DC fibroblasts had similar rates of β-VLDL internalization (Fig. 7B). These observations suggested that mutations in H190, H562, and H586 influenced FDNPVY-dependent β-VLDL internalization.

Together, the assays for lipoprotein binding, uptake and release show that H190, H562, and H586 participate in β-VLDL internalization and in lipoprotein release. To address how H190, H562, and H586 participate in release, we employed mutagenesis to identify the residues on LA5 that are required for normal lipoprotein binding with the goal of determining whether lipoproteins bind to the same surface as the EGF.
Roles of H190, H562, and H586

FIGURE 6. The effect of the AAA and DRK mutation on lipoprotein release in cellular assays. The ability of fibroblasts expressing normal LDLR (WT), LDLR-AAA (AAA), or LDLR-DRK (DRK) to internalize (A and C) and accumulate (B and D) LDL (A and B), and β-VLDL (C and D) was determined. Internalization assays determined the ratio of internal/surface 125I-LDL (A) or 125I-β-VLDL (C) over 15 min at 37 °C. The accumulation assays determined the amount of Alexa546-LDL (B) or DiI-β-VLDL (D) fluorescence associated with each cell type over 4 h. Data are presented as the percent of normal (WT) at 4 h. All experiments were performed in triplicate and are presented as means ± S.D.

FIGURE 7. The reduction in β-VLDL internalization by the AAA and DRK mutations involves the FDNPVY sequence. A shows immunoblots of cell lysates from LDLR−/− fibroblasts infected with retroviruses expressing no LDLR (Vector), normal LDLR (WT), LDLR-Y807C (YC), LDLR-Y807C + AAA (AC), or LDLR-Y807C + DRK (DC). B shows the internal/surface ratios of β-VLDL endocytosis at the indicated times at 37 °C. Experiments were performed in triplicate, and the data are presented as means ± S.D.

We tested whether cleft 2 was involved in lipoprotein binding by introducing into LDLR−/− fibroblasts LDLR variants that had lysine substitutions at E187, D203, or E208 (Fig. 9A) and measuring lipoprotein binding affinity. LDLR-E187K, LDLR-D203K, and LDLR-E208K were introduced into LDLR−/− fibroblasts using retroviral infection. Cells expressing these variants had similar levels of LDLR expression (Fig. 9B). In 125I-LDL binding assays, cells expressing LDLR-D203K or LDLR-E208K had normal LDL binding affinity, while cells expressing LDLR-E187K had a 5-fold loss in LDL binding affinity (Fig. 9C). By contrast, in 125I-β-VLDL binding assays, cells expressing LDLR-E187K, LDLR-D203K, or LDLR-E208K showed a 6-fold, 6-fold, or 10-fold reduction in β-VLDL affinity, respectively (Fig. 9D). These observations indicate that E187 is involved in both LDL and β-VLDL binding, while D203 and E208 are only involved in β-VLDL binding. The effects on β-VLDL binding by the D203K and E208K mutations implicate cleft 2 as the binding site for apoE.

DISCUSSION

The current model for lipoprotein release by the LDLR holds that H190, H562, and H586 serve as a pH sensor, which, when protonated, facilitates the association of the β-propeller of the EGF homology domain with lipoprotein binding surfaces on LA4/5 (24–26). The results of this study show that these histidines have little impact on the acid-dependent transition between the open and closed state of the LDLR (Fig. 1). Thus, H190, H562, and H586 do not constitute the pH sensor.

Our results also suggest that the EGF homology domain does not drive release though competition with lipoproteins. Previous kinetic experiments of lipoprotein dissociation at neutral
FIGURE 8. Sequence comparisons. A compares the amino acid sequence of LA5 from 12 species. E180, E187, D196, D200, D203, and E207 are conserved in all species. E208 is conserved in 8 of the 12 species.

B compares the amino acid sequence of human LA5 with the other six LA repeats of human LDLR. D206 and E207 are conserved between different repeats. Boxed residues are acidic residues that are conserved with human LA5. Residue numbering is for human LA5.

FIGURE 9. The role of E187, D203, and E208 on lipoprotein binding. A shows the structure of LA5 in ball and stick representation. Carbon is colored dark gray; nitrogen is blue; oxygen is red; and sulfur is yellow. Calcium is shown as a light-gray sphere. E187, D203, and E208 are labeled. B shows immunoblots of cell lysates from fibroblasts that were infected with retroviruses expressing no LDLR (Vector), normal LDLR (WT), LDLR-E187K, LDLR-D203K, or LDLR-E208K. The upper portion of B shows the immunoblot for LDLR, while the lower portion shows the immunoblot for CD44, which was used as a loading control. C shows the Scatchard plot of 125I-LDL binding to the cell surface of the indicated fibroblasts, while D shows the Scatchard plot of 125I-β-VLDL binding.

pH showed that the dissociation rate constants for HDLc (an apoE-containing lipoprotein) and LDL from normal LDLR are 1.7 × 10^{-5} \text{s}^{-1} and 6.3 × 10^{-5} \text{s}^{-1}, respectively (37). The dissociation rates for β-VLDL are likely similar to those for HDLc, because both lipoproteins bind to the LDLR via apoE and both have similar binding affinity for the LDLR (12, 37, 46). Under acidic conditions, the lipoprotein dissociation rates increase (Fig. 5 and Table 1); however, acidic buffers are unable to drive β-VLDL release from LDLRs lacking an EGF homology domain (5), indicating that the EGF homology domain accelerates lipoprotein release in response to acidic conditions. Competitive mechanisms of inhibition influence ligand binding but do not accelerate the rate of ligand dissociation. Thus, the kinetics of lipoprotein dissociation argue that the EGF homology domain does not act in a competitive manner and suggests that the β-propeller region forms an allosteric interaction with LA4/5.

An allosteric interaction implies that lipoproteins bind to a different site on LA4/5 than the EGF homology domain. Consistent with this possibility, the LDLR-AAA ectodomain displayed a normal acid-dependent conformational change (Fig. 1); however, cells expressing the LDLR-AAA variant had defective lipoprotein release (Figs. 4–6). These observations suggest that adoption of the closed conformation is not sufficient to drive release. If the β-propeller can form the intramolecular contact but not drive lipoprotein release, then the lipoprotein binding sites must be distinct from the binding site for the β-propeller.

Subsequent mutagenesis experiments support the possibility of distinct binding sites for lipoproteins and the EGF homology domain. Replacement of D203 or E208 with lysine impaired the ability of the LDLR to bind to β-VLDL (Fig. 9), suggesting that D203 and E208 contact basic residues of apoE. D203 and E208 are part of cleft 2, which is exposed in the closed conformation. Significantly, LDL binding affinity was not reduced, indicating that D203 and E208 are not involved in LDL binding and that the D203K and E208K mutations do not have global effects on the folding of LA5.

Allosteric interactions frequently drive conformational changes that alter protein function. Comparison of the crystal structure of LA5 alone (44) and LA5 as part of the crystal structure of the LDLR ectodomain (24) suggests that LA5 has an altered conformation in the closed conformation. In the closed conformation, H190 interacts with backbone atoms of N148 of LA4 and the imidazole ring of H562 of the EGF homology domain. These interactions appear to pull H190 toward the junction between the β-propeller and LA4, distorting the strand containing H190 in LA5. One consequence of this distortion is that the side chain of E187 points in opposite directions in the two structures. E187 is near both cleft 1, which interacts with the β-propeller, and cleft 2, which we propose binds to apoE. Thus, H562, H190, and E187 may act as part of an allosteric switch. Consistent with
Roles of H190, H562, and H586

This new allosteric model differs with the previous model (Fig. 10B) in two respects. First, the previous model proposed that the β-propeller bound to the same surface on LA4/5 as lipoproteins. This aspect of the previous model necessitated release of lipoproteins prior to engagement by the β-propeller. As discussed above, the kinetics of lipoprotein release (Table 1) do not support this idea. The new model proposes that lipoproteins and the β-propeller bind to separate surfaces on the LDLR. Second, the previous model stipulated that association of the β-propeller with LA4/5 required ionic interactions involving H190, H562, and H586. The gel filtration data (Fig. 1) shows that these three histidines do not control the transition from the open to the closed conformational state of the LDLR. The new model proposes that the closed conformational state is necessary but not sufficient for release and that H190, H562, and H586 are part of an allosteric mechanism of lipoprotein release.

Interestingly, our data also show that mutations at H190, H562, and H586 reduce the rate of β-VLDL internalization (Fig. 6). How the three histidines facilitate β-VLDL uptake is not clear; however, the effect of these mutations on β-VLDL internalization was FDNPVY-dependent (Fig. 7), suggesting that the three histidines promote the association or activity of adaptor proteins that bind to the FDNPVY sequence of the LDLR. Dab-2 and the autosomal recessive hypercholesterolemia protein (ARH) are two adaptors that bind to the FDNPVY sequence and to components of the endocytic machinery of clathrin-coated pits (50, 51). Dab-2 or ARH are required for FDNPVY-dependent LDLR internalization (52, 53). Interestingly, lipoprotein binding to the LDLR promotes the association of ARH with the plasma membrane (54), suggesting that lipoprotein binding is coupled to adaptor protein engagement. Consistent with this possibility, we have recently shown that lipoproteins promote the coated-pit targeting and internalization of the LDLR (32). The mechanism by which H190, H562, and H586 might participate in the coupling of lipoprotein binding with internalization is currently under investigation.

Acknowledgments—We thank Angela Mobley for FACS assistance, Michael Brown and Joseph Goldstein for LDL and β-VLDL, Brenda Pallares for administrative support, and Jonathan Cohen and Richard Anderson for helpful discussions.
