Identification of roles for H264, H306, H439, and H635 in acid-dependent lipoprotein release by the LDL receptor

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Abstract Lipoproteins internalized by the LDL receptor (LDLR) are released from this receptor in endosomes through a process that involves acid-dependent conformational changes in the receptor ectodomain. How acidic pH promotes this release process is not well understood. Here, we assessed roles for six histidine residues for which either genetic or structural data suggested a possible role in the acid-responsiveness of the LDLR. Using assays that measured conformational change, acid-dependent lipoprotein release, LDLR recycling, and net lipoprotein uptake, we show that H635 plays important roles in acid-dependent conformational change and lipoprotein release, while H264, H306, and H439 play ancillary roles in the response of the LDLR to acidic pH.

Supplementary key words dyllipidemias • endocytosis • low density lipoprotein • lipoproteins/metabolism • lipoproteins/receptors

The LDL receptor (LDLR) supports uptake of lipoproteins that contain either apoE or apoB100 (1, 2). The LDLR is principally responsible for the uptake of two lipoproteins: LDL, which the LDLR binds in an apoB100-dependent manner, and VLDL remnants, which the LDLR binds in an apoE-dependent manner (3, 4). Peripheral cells use the LDLR to take up LDL to supply the cholesterol needed for membrane and steroid hormone synthesis (5, 6). Liver hepatocytes use the LDLR to internalize both LDL and VLDL remnants for the purpose of reducing the circulating level of LDL (4). Uptake of VLDL remnants suppresses circulating LDL levels because VLDL remnants that are not internalized by the LDLR are converted into LDL (7, 8).

The LDLR uptake cycle consists of four steps: lipoprotein binding at the cell surface, internalization through clathrin-coated pits, release of lipoprotein in endocytic compartments, and return of LDLRs to the cell surface for further rounds of uptake (9–12). Naturally occurring mutations that hinder any step in the cycle compromise LDLR function and increase the circulating level of LDL, resulting in familial hypercholesterolemia (FH) (13). These mutations have been divided into five categories (13, 14). Class I mutations are nulls and include most insertions, deletions, and premature stop codons. Class II mutations hinder folding, resulting in loss of LDLRs to endoplasmic reticulum-associated degradation (ERAD). Class III mutations disrupt lipoprotein binding. Class IV mutations inhibit LDLR internalization, thereby trapping LDLRs on the cell surface. Class V mutations disrupt endosomal handling (lipoprotein release and LDLR recycling), resulting in loss of surface LDLRs due to retention of LDLRs in endosomal compartments.

The LDLR is a type I transmembrane protein that consists of seven LDLR type A repeats (LA repeats), two epidermal growth factor (EGF)-like repeats (EGF-A and EGF-B), six YWTD repeats that form a β-propeller, a third EGF-like repeat (EGF-C), a region that is highly O-glycosylated, a single transmembrane helix, and a short, relatively unstructured cytoplasmic domain (Fig. 1A) (15). Class II (folding) mutations can be found throughout the ectodomain and are the most common type of FH mutation. Most class III (binding) mutations are in the LA repeats. Class IV (internalization) mutations are all within the cytosolic domain. Class V (release and recycling) mutations are found in the EGF-A, EGF-B, and β-propeller domains (13).

Biochemical and cellular experiments have shown that lipoprotein release can proceed through two distinct mechanisms. The first mechanism involves loss of calcium from the

Abbreviations: EGF, epidermal growth factor; ERAD, endoplasmic reticulum-associated degradation; FACS, fluorescence-activated cell sorting; FH, familial hypercholesterolemia; GFP, green fluorescent protein; LA repeat, LDLR type A repeat; LDLR, LDL receptor; PCSK9, proprotein convertase subtilisin/kexin type 9.

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Materials and Methods

LDLR+/− human fibroblasts are 549T cells that were generated previously (32). Human LDL and rabbit β migrating VLDL (β-VLDL) were a gift of Michael Brown and Joseph Goldstein.
(Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX) and were prepared as previously described (43, 44). Rabbit polyclonal anti-LDLR (4548) was a gift of Joachim Herz (Department of Molecular Genetics, University of Texas Southwestern Medical Center, Dallas, TX). Mouse monoclonal anti-LDLR (C7) was from Santa Cruz Biotechnology (Santa Cruz, CA). Alexa546 succinimidyl ester was from Invitrogen/Thermo-Fisher. COSmic calf serum was from Gibco/GE Life Sciences (Marlborough, MA). Lipoprotein-deficient serum was generated from cosmic calf serum by potassium bromide density fractionation (45). All other chemicals and reagents were from Sigma (St. Louis, MO).

Labeling of lipoproteins
Human LDL and β-VLDL were labeled with 125I using the Bolton-Hunter method (46). LDL and β-VLDL were labeled with Alexa 546 using the succinimidyl ester of this dye according to the manufacturer’s recommended protocol (Invitrogen/Thermo-Fisher).

Baculovirus-mediated protein expression
Residues 1-699 of WT and variant LDLRs were cloned into the pFastBac plasmid (Invitrogen/Thermo-Fisher). Recombinant plasmids were used to produce infectious baculoviruses that directed the synthesis of secreted LDLR ectodomains using the Bac-to-Bac system (Invitrogen/Thermo-Fisher).

Cell culture
All fibroblasts were cultured in medium A [DMEM medium supplemented with 10% (v/v) cosmetic calf serum, 20 mM HEPES pH 7.5, 100 µg/ml penicillin G, and 100 µg/ml streptomycin]. Prior to experimentation, fibroblasts were starved of lipoproteins for 24 h by replacing the culture medium with Medium B [DMEM medium supplemented with 10% (v/v) lipoprotein poor serum, 20 mM HEPES pH 7.5, penicillin G (100 µg/ml), and streptomycin (100 µg/ml)].

Gel filtration
Gel filtration was performed as described previously (32). Briefly, gel filtration was performed using a Superdex200 10/30 column attached to an Äkta FPLC (GE/Amersham). The column was equilibrated in buffers containing 25 mM HEPES, 25 mM maleate, 150 mM NaCl, 1 mM CaCl2, and 0.5% TritonX100 at pH 6.2, 6.6, or 7.0. TritonX100 was required to maintain the LDLR ectodomains in solution at acidic pH. Samples were equilibrated in the same buffers prior to loading. 0.5 ml fractions were collected. 125I-LDL or 125I-β-VLDL in Medium C were incubated with 0.45 M sucrose, 20 mM HEPES, 20 mM maleate, and 10% lipoprotein poor serum, pH 5.5. The cells were washed four times with ice-cold TBS (pH 8.0). Remaining cell-associated, 125I-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 from the 0 time point. Rate constants for release were determined by nonlinear curve fitting using a single-phase exponential decay model (Prism, GraphPad Software Inc.). Three independent experiments were performed for each cell line and rates presented are means from the three experiments ± SD.

Fractional surface level of LDLR expression
Total LDLR expression levels relative to WT LDLR level were determined by densitometry with immunoblots performed using the 4548 polyclonal antibody to the LDLR. Surface LDLR expression levels relative to WT LDLR level were determined by flow cytometry using the C7 monoclonal antibody to the LDLR (Santa Cruz Biotechnology, Santa Cruz, CA). Fractional surface level was determined by dividing surface levels by total levels. Data were then normalized to a WT level of 0.5, based on published experiments showing that half of all cellular LDLRs are exposed on the cell surface in normal fibroblasts (48).

Acid-dependent release of cell surface bound lipoprotein
Assays were performed as previously described (21, 32). Briefly, cells were preincubated with Medium C, which consisted of Medium B supplemented with 0.45 M sucrose to prevent clathrin-coated pit endocytosis (49). Cells were then incubated with either 10 µg/ml 125I-LDL or 5 µg/ml 125I-β-VLDL in Medium C for 30 min at 37°C, washed with warm Medium C, and incubated for 0, 1, 2, 4, 8, or 16 min at 37°C with Medium D (bicarbonate free MEM supplemented with 0.45 M sucrose, 20 mM HEPES, 20 mM maleate, and 10% lipoprotein poor serum, pH 5.5). The cells were washed four times with ice-cold TBS (pH 8.0). Remaining cell-associated, 125I-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 from the 0 time point. Rate constants for release were determined by nonlinear curve fitting using a single-phase exponential decay model (Prism, GraphPad Software Inc.). Three independent experiments were performed for each cell line and rates presented are means from the three experiments ± SD.

Lipoprotein binding assays
125I-LDL and 125I-β-VLDL binding assays were performed in triplicate using established methods (43). Assays were performed at 4°C for 90 min using concentrations of 125I-LDL and 125I-β-VLDL indicated in the figures. Data are presented as means ± 1 SD.

Lipoprotein accumulation assay
Rates of lipoprotein accumulation were determined as previously described (50). Briefly, cells were incubated with 10 µg/ml Alexa546-LDL or 5 µg/ml Alexa546-β-VLDL in Medium B for 1, 2, 3, or 4 h at 37°C and then washed, scraped, fixed, and washed. Mean cell fluorescence was determined by flow cytometry (FACS-Calibur instrument, BD Scientific) from 10,000 gated events. Events were gated based on side and forward scatter consistent with intact cells. Mean values were normalized to surface LDLR level as assessed by C7 antibody FACS (FACS-Calibur instrument) and presented as a fraction of WT LDLR cell uptake at 4 h. Three separate experiments were performed, and reported data are the means of the mean values from each of the three experiments ± SD.

Sequence alignments
The 50 nearest orthologs of the human LDLR were aligned using blastp (NCBI/NLM). Amino acid residues at the indicated positions were tabulated using Excel (Microsoft). FH-associated variants were taken from the Leiden Open source Variant Database for the LDLR (http://www.ucl.ac.uk/ldlr/Current/) (51), which tabulated the previously published data of the histidine variants (13, 34-36, 39, 40).

Flow cytometry
Flow cytometry used to quantify GFP levels was performed using a FACS-Calibur instrument. All cell lines were tested in duplicate within a common experiment. Four separate experiments
were performed and reported data are the mean values from the four experiments ± SEM.

RESULTS

Before testing roles for individual histidines, we first decided to use domain deletions to assess maximal effect size in our assay systems. Deletions that eliminate the EGF-A, the EGF-B, or both modules were among the first class V FH mutations that were identified (52–54), and the degree to which these deletions compromise LDLR function provides an expectation of maximum effect size for mutations of histidines with individual domains. To compare acid-dependent conformational change, we expressed LDLR ectodomains bearing deletions of the EGF-A, the EGF-B, or both using baculovirus-infected SF9 cells and assayed conformational state as a function of pH using gel filtration. We compared the hydrodynamic (Stokes) radii of these ectodomains at three pH points: pH 7.0, when the WT ectodomain is fully in the extended state; pH 6.2, when the WT ectodomain is fully in the compact state; and pH 6.6, when the WT ectodomain is transitioning between the extended and compact states and exhibits a Stokes radius intermediate between the two states. Deletion of the EGF-A module, the EGF-B module, or both inhibited acid-dependent conformational change over this pH range with deletion of EGF-B having a greater effect than deletion of EGF-A (Fig. 2).

To compare acid-dependent release, we used recombinant retroviruses to stably express full-length LDLR variants lacking the EGF-A module (LDLR-ΔA), the EGF-B module (LDLR-ΔB), or both (LDLR-ΔAB) in LDLR−/− fibroblasts (Fig. 3A). We then compared lipoprotein release rates by binding 125I-labeled LDL or β-VLDL to the cells’ surfaces and comparing rates of release upon removal of lipoprotein and a shift to acidic medium (pH 5.5). Loss of surface lipoprotein to endocytosis was prevented using hypotonic sucrose (49). As an additional control, we used LDLR−/− fibroblasts stably expressing the LDLR−BC receptor (21, 32), which lacks the EGF-B, β-propeller, and EGF-C modules and cannot support acid-dependent release. Consistent with the gel filtration results (Fig. 2), the LDLR-ΔB receptor released LDL more slowly than WT LDLR, though not to the level seen for the LDLR-ΔBC receptor (Fig. 3B). Cells expressing the LDLR-ΔA or LDLR-ΔAB variants were not assayed for LDL release kinetics because the EGF-A module is necessary for normal LDL binding (supplemental Fig. S2) (24, 25). To compare
acid-dependent release of the LDLRΔA and LDLRΔΔB receptors, we used 125I-labeled β-VLDL. β-VLDL is a VLDL remnant particle that interacts with the LDLR via apoE (2). Its binding requires only the LA4 and LA5 modules (23, 25), and deletion of the EGF-A or EGF-B modules does not reduce the binding affinity of the LDLR for β-VLDL (supplemental Fig. S2). Release assays showed that LDLRΔA, LDLRΔB, and LDLRΔAB receptors all displayed a slower rate of β-VLDL release than WT LDLR (Fig. 3B). Release from LDLRΔΔ had similar kinetics as release from LDLRΔB, indicating that both domains participate in acid-dependent release. Release from the LDLRΔAB was slower than that of either LDLRΔA or LDLRΔB, suggesting that each domain makes independent contributions to acid-dependent release. The release rate for LDLRΔAB was not as slow as that of the LDLRΔBC, indicating that elements outside EGF-A and EGF-B also participate in acid-dependent release.

Prior work has shown that endosomal lipoprotein release normally occurs rapidly in fibroblasts (30). In these cells, the calcium release mechanism is sufficient for β-VLDL release, though the acid-dependent mechanism accelerates the rate of release (30). The calcium release mechanism also drives LDL release in fibroblasts; however, the acceleration imparted by the acid-dependent mechanism is necessary for efficient delivery of LDL to lysosomes because inhibition of acid-dependent release increases the fraction of internalized LDL that is lost through the process of retro-endocytosis (30, 55, 56). Retro-endocytosis occurs when LDLRs commit to the recycling pathway prior to releasing lipoprotein, thereby allowing internalized lipoprotein to return to the cell surface. In LDLRΔBC cells, whose LDLR cannot utilize the acid-dependent release mechanism, 70% of the internalized LDL is lost to retro-endocytosis (30). By labeling LDL or β-VLDL with a pH-insensitive dye, the net accumulation (internalization minus retro-endocytosis) of these lipoproteins can easily be measured by flow cytometry. Because the rate of LDLR-lipoprotein internalization is determined solely by the cytosolic domain (21, 57), changes in the LDL accumulation rate provide a measure of retro-endocytosis and, hence, intracellular acid-dependent LDL release. It is not understood why LDL, but not β-VLDL, is sensitive to retro-endocytosis, though the much larger size of β-VLDL may hinder its ability to enter the tubular processes that mediate vesicular export of receptors from endosomes.

We compared LDL accumulation rates in the fibroblasts stably expressing different deletions. Cells were incubated with Alexa546-labeled LDL and assayed by flow cytometry hourly over a 4 h time course. To eliminate expression differences, LDL-fluorescence values were normalized to surface LDLR levels as measured by antibody FACS. Consistent with the differences observed in the surface release assays (Fig. 3B), loss of the EGF-B module (LDLRΔB cells) slowed LDL accumulation to a rate that was intermediate between that of normal LDLR cells and LDLRΔBC cells (Fig. 3C). As expected, no defect in β-VLDL accumulation rate was observed with either LDLRΔA or LDLRΔAB cells (Fig. 3C).

Together, the assays with the domain deletions illustrate the extent to which the EGF-A and EGF-B modules contribute to acid-dependent responsiveness of the LDLR. To examine the role of histidines in the LA7-β-propeller region, we decided to make variants that would either prevent acid-dependent acquisition of a positive charge or place a permanent positive charge at histidine positions. At positions for which there were no known FH mutations, we used alanine and lysine as replacement residues. At positions for which there was an FH mutation, we used the FH mutation as one of the two substitutions. A total of 12 mutations were generated at residue amino acid positions 264, 306, 367, 439, 464, and 635 (Table 1). These mutations were introduced into baculovirus-encoded LDLR ectodomains for expression in SF9 cells and into retrovirus-encoded full-length LDLR constructs for stable expression in LDLRΔ/Δ fibroblasts (Table 1).

To examine the roles of the six histidines on acid-dependent conformational change, we compared gel filtration elution behavior of the SF9-expressed ectodomains. Because deletions of the EGF-A and EGF-B modules have a substantial effect on the molecular mass of the LDLR ectodomain, we generated two additional controls: a C304Y variant, which disrupts the disulfide-bonding pattern of the EGF-A module; and a C343Y variant, which disrupts the disulfide-bonding pattern of the EGF-B. Both mutations are associated with FH (13, 58, 59), and the disruption caused by loss of normal disulfide-bonding was expected to ablate function of the module containing the

| Mutation | LDLR Module | Associated with FH | Gel Filtration Behavior of Ectodomain | Receptor-to-GFP ratio | % Surface Receptor | Rate of Accumulation | Acid-Dependent LDL Release |
|----------|-------------|-------------------|-------------------------------------|-----------------------|--------------------|---------------------|--------------------------|
| H264K    | LA7         |                   |                                     |                       |                    |                     |                          |
| H264Q    | LA7         | Yes               | Decreased                           |                       | Decreased          | Decreased           |                          |
| H306K    | EGF-A       |                   |                                     |                       |                    |                     |                          |
| H306Y    | EGF-A       | Yes               | Decreased                           |                       | Decreased          | Decreased           |                          |
| H367A    | EGF-B       |                   |                                     |                       |                    |                     |                          |
| H367K    | EGF-B       |                   |                                     |                       |                    |                     |                          |
| H439A    | β-Propeller |                   |                                     |                       |                    |                     |                          |
| H439K    | β-Propeller | Impaired          |                                     |                       |                    |                     |                          |
| H464R    | β-Propeller | Yes               | Increased                           |                       | Decreased          |                     |                          |
| H635A    | β-Propeller | Impaired          |                                     |                       |                    |                     |                          |
| H635K    | β-Propeller | Impaired          |                                     |                       |                    |                     |                          |
| H635N    | β-Propeller | Yes               | Decreased                           |                       | Decreased          |                     |                          |

TABLE 1. Summary of results
tyrosine mutation. Consistent with this expectation, the gel filtration assay showed that both the C304Y and C343Y ectodomains exhibited reduced ability to undergo acid-dependent conformational change with an effect size that was similar to that of the EGF-A and EGF-B deletions, respectively (Fig. 2 and Fig. 4). With these controls in place, we then turned to ectodomains bearing mutations at the six histidines. None of the 12 variants tested exhibited an effect size on the order of the C304Y or C343Y variants; however, five variants (H264Q, H439A, H635A, H635N, and H635K) showed a shift at pH 6.6 toward higher Stokes radius as evidenced by an increase in the relative band intensity in the 41Å fraction over the 45Å fraction as compared with WT LDLR at this pH (Fig. 4). These results indicate that H264, H439, and H635 participate in acid-dependent conformational change.

As a first test of the cellular consequences of histidine mutations, we assessed whether any of the variants exhibited a class II defect in fibroblasts stably expressing full-length receptors. Class II defects reduce levels of receptors through ERAD, and highly damaging class II mutations cause receptors to build up in the ER (14). Receptors that fail to reach the Golgi lack O-glycosylation and run on SDS-PAGE gels as a smaller 120 kDa molecular mass species (60). Of the 12 variants, only the H635K variant exhibited a 120 kDa species. Loss of receptors to ERAD reduces the fraction of synthesized LDLR proteins that acquire O-glycosylation (mature protein). To assess the efficiency with which LDLR mRNA produces mature LDLR protein, we took advantage of the retroviral system that was used to generate stable expression of LDLR variants. The retroviral system generates a bicistronic mRNA that directs expression of both an LDLR variant and GFP. The two genes are separated by an internal ribosomal entry sequence element. This coupling was used to facilitate isolation of LDLR expressing cells; however, the level of GFP expression also provides a robust measure of the relative message level (47). We used this feature to query the efficiency with which LDLR message generated LDLR protein by taking the ratio of mature LDLR protein to GFP expression (Fig. 5D). Using this approach, we found that the H306Y, H464R, and H635K variants had a low LDLR-to-GFP ratio, while the H439K variant had a high ratio. The low ratio of H306Y, H464R, and H635K suggested that these mutations cause a class II defect. The H635K variant had the most severe phenotype, consistent with it being a more damaging type II mutation as evidenced by the presence of the lower molecular mass band in the immunoblot (Fig. 5A).

To test whether any of the variants displayed a class V defect, we compared the level of surface receptors with total receptors. A low surface-to-total ratio corresponds to a class V phenotype and is indicative of a reduced ability of the receptor to recycle from endosomes to the cell surface. Surface levels were determined by antibody FACS (Fig. 5E). Total levels were determined by immunoblot (Fig. 5B). Of the 12 variants, only two variants (H439K and H635A) had a significantly reduced surface-to-total level, consistent with a class V defect (Fig. 5F, Table 1). Primary fibroblasts from an FH individual bearing the LDLR-H464R were previously used to classify H464R as a class V variant (13); however, the fractional surface level for our LDLR-H464R expressing fibroblasts was the same as WT (Fig. 5F).

We next tested whether any of the mutations compromised the ability of the receptor to undergo acid-dependent lipoprotein release. Of the 12 variants, only the H635K showed a significant reduction in the rate of acid-dependent LDL release in the surface release assay (Fig. 6A). Using the LDL accumulation assay as a measure of cellular lipoprotein release, four variants (H264Q, H306K, H306Y, and H635K) exhibited a slower rate of LDL accumulation (Fig. 5C).

Together, these findings show that H635 plays an important role in acid-dependent conformational change and lipoprotein release, while H264, H306, and H439 make lesser contributions.

Fig. 4. Acid-dependent conformational change of LDLR variants. Ectodomains of the indicated LDLR variants were expressed in SF9 cells and run by gel filtration at the indicated pH. Shown are LDLR immunoblots from the gel filtration runs.
processes leads to either retention of the receptor in endosomes or loss of internalized LDL to premature recycling (retro-endocytosis). Protein function is tied to protein structure and changes in function commonly involve conformational change. The LDLR undergoes multiple conformational changes in response to acidic pH, and the data presented in this study suggest that H635 plays an important role in the conformational changes required for endosomal handling of the LDLR with lesser contributions made by H264, H306, and H439.

H635 is located at the hinge connecting the EGF-B to the β-propeller, and this residue adopts a different conformation at acidic and neutral pH (Fig. 7A). At neutral pH, the imidazole ring of H635 forms a hydrogen bond with G375 of the linker, likely helping to direct the N-terminal domains of the LDLR outward into an extended conformation (31). Protonation of H635 may disrupt this hydrogen bond, leading to a shift in the orientation of H635 such that at acidic pH, the H635 side chain occludes the path through which the linker normally passes at neutral pH (Fig. 7A). By blocking the neutral path, the linker is displaced to a path that directs the N-terminal domains into a position that facilitates interaction of the LA4/5 modules with the β-propeller (Figs. 1B, 7A). The displacement of the linker from the neutral path may involve specific contacts of the protonated histidine with surrounding residues because we observed effects in gel filtration behavior with all three variants at H635 (Fig. 4). Consistent with the
of histidines in lipoprotein release by the LDLR

Role of histidines in lipoprotein release by the LDLR

well conserved at position 264. 13 of 50 species have a different residue at position 264 with glutamine being the most common replacement (Fig. 8).

By contrast, the H306Y mutation has previously been associated with LDLR recycling in the context of proprotein convertase subtilisin/kexin type 9 (PCSK9) binding (63). PCSK9 is a serum factor that competes with LDL for binding to the LDLR but does not readily dissociate from the LDLR in endosomes (63–65). As a result, PCSK9 inhibits LDLR recycling, leading to lysosomal degradation of importation of this residue, mutations of H635 slowed acid-dependent release and reduced LDL accumulation (Fig. 6, Table 1). Both asparagine and lysine replacements generated a class II defect as evidenced by a reduced LDLR-to-GFP ratio (Fig. 5D) and the presence of a faster migrating species for H635K (Fig. 5A), suggesting that these mutations slowed folding of the LDLR. Histidine is the only residue found at position 635 across a panel of LDLRs from 50 species (Fig. 8), suggesting that no other residue can fully replace the functionality of histidine at this position.

The H264Q, H306K, and H306Y mutations had an interesting phenotype in that these mutations exhibited substantially reduced rates of LDL accumulation but did not show defects in acid-dependent LDL release (Fig. 6). This combination suggested that the reduced LDL accumulation rate resulted from a defect in receptor recycling; however, the surface-to-total ratio of receptor was also normal, indicating that the rate of receptor recycling was unchanged by the mutations. One possibility is that these mutations promote interaction of the LDLR with the recycling machinery. We have previously shown that fibroblasts have a mechanism that can stall endosomal progression of LDLR-lipoprotein complexes to facilitate release (30). This mechanism appears to involve the EGF-A module because the mechanism is active in cells expressing the LDLR-ΔBC variant, which lacks the EGF-B, β-propeller, and EGF-C modules, but is not apparent in cells expressing the LDLR-ΔAC variant, which additionally lacks the EGF-A module (21, 30, 61, 62).

LA7 has a rigid-body connection to LA7 (33), but it is not clear how the H264Q mutation might alter LDLR function. H264 is well exposed on the surface of LA7 and makes equivalent local contacts at neutral and acidic pH (Fig. 7B). Furthermore, no defects were observed with the H264K variant (Table 1) and histidine is not particularly

Fig. 7. H635 and H306 have different conformations at neutral and acidic pH. The orientations of H635 (A), H264 (B), H306 (C), and H464 (D) are shown at acidic pH (red) and neutral pH (green). Coordinates for the acidic and neutral structures were taken from pdb files 1N7D and 3P5C, respectively. The black dotted line in C indicates a hydrogen bond that connects S305 with H306 at neutral pH. Secondary structure in the main chain is indicated as a thin cylinder for coil and a broad ribbon for β-sheet. There is no helical structure.

Fig. 8. Histidine conservation in the LDLR. The 50 species with LDLR amino acid sequences most similar to the human sequence were aligned and residues at positions with histidine in the human sequence are displayed. Species are listed from most similar to human (top) to least similar to human (bottom). Gray indicates identity with human sequence. Single letters indicate amino acid substitutions. Above the interspecies sequence comparisons are a list of FH-associated substitutions at each position.

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By contrast, the H306Y mutation has previously been associated with LDLR recycling in the context of proprotein convertase subtilisin/kexin type 9 (PCSK9) binding (63). PCSK9 is a serum factor that competes with LDL for binding to the LDLR but does not readily dissociate from the LDLR in endosomes (63–65). As a result, PCSK9 inhibits LDLR recycling, leading to lysosomal degradation of
LDLRs (64). In essence, PCSK9 binding induces a severe class V-like phenotype. H306 resides within the PCSK9 binding surface on the EGF-A module and the H306Y mutation increases PCSK9 binding affinity (31, 63, 66, 67). PCSK9 can influence LDLR recycling in fibroblasts albeit less to a lesser extent than in hepatocytes (64, 65, 68), and the presence of a small amount of PCSK9 in serum may explain the reduced LDLR-to-GFP ratio observed for the H306Y variant (Fig. 5D). PCSK9 is not, however, responsible for the reduced LDL accumulation rate because accumulation rates were normalized to LDLR level, saturating concentrations of LDL were used in the LDL accumulation assays, and the H306K variant had similar effects as the H306Y variant in both LDL accumulation and acid-dependent release assays (Fig. 6). These observations suggest that H306 may regulate interaction of the LDLR with the recycling machinery, and mutations at this position may compromise this regulation.

Structurally, the conformation of H306 changes as a function of pH (Fig. 7C) (63). At neutral pH, the imidazole ring of H306 packs against a loop made by the C-terminal half of the EGF-A module and makes a hydrogen bond with the side chain of S305. At acidic pH, this imidazole ring does not make the hydrogen bond and is directed out into solvent. The acid-dependent displacement of the H306 side chain coincides with a small shift in the main chain of the C-terminal loop of EGF-A. Whether or how this conformational change influences LDLR function is not clear. As with H635, histidine is the only amino acid at position 306 across 50 species (Fig. 8), suggesting that no other residue can fully replace the function of histidine at this position.

The H439K mutation was also interesting in that it exhibited both an increased LDLR-to-GFP ratio and a reduced surface-to-total ratio of LDLRs. The former feature implied that the H439K mutation increased the efficiency by which the LDLR mRNA generates mature LDLR beyond that of normal LDLR, while the latter feature indicated that a population of H439K receptors was not recycling normally. One mechanism that might explain both features is a reduction in chaperone-dependent quality control in the ER. Folding of the LDLR is a complex process that involves many chaperones, some of which are specific to the LDLR family (69–73). The H439K mutation may reduce LDLR interaction with ER chaperones, resulting in inappropriate trafficking of poorly folded LDLRs to the Golgi. LDLRs with misfolding in their β-propeller would be expected to affect LDLR function in endosomes either by slowing acid-dependent release or by affecting receptor recycling. H439K did not show a significant reduction in the rate of acid-dependent release (Fig. 6A) but did show a reduced ratio of surface-to-total receptors, indicating a defect in receptor recycling (Fig. 5F). The effect may be relatively specific for a lysine substitution because the H439A variant did not show this behavior, and 31 of 50 species do not have histidine at this position with proline being most common (Fig. 8).

Of surprise was the lack of class V behavior of the H464R mutation. We did not observe a defect in acid-dependent conformational change, fractional surface receptor level, LDL accumulation, or acid-dependent release with this variant (Fig. 4–6, Table 1). Consistent with this lack of functional difference, the conformation of the histidine side chain changes little between neutral and acidic pH (Fig. 7D). The principal effect of the arginine substitution was a reduced LDLR-to-GFP level (Fig. 5A), indicative of a class II folding defect. The side chain of H464 is mostly buried within the β-propeller (Fig. 7D), and arginine may hinder the normal packing of the propeller blades.

We observed functional defects for all four FH mutations; however, the effect size of these mutations on LDLR function was small. Indeed, even complete deletion of the EGF-A or the EGF-B modules individually or collectively had only a partial effect on acid-dependent release and lipoprotein uptake (Fig. 3). In vivo, both the FH point mutations and the domain deletions are associated with substantial elevations of circulating LDL-cholesterol (13, 34–36, 39, 40, 52–54), suggesting that even small effects on LDLR function are sufficient to cause hypercholesterolemia in vivo.

From an energetics point of view, a small effect size is not surprising. The $K_D$ for LDL binding to the LDLR at pH 7.0 is 3 nM (3, 74), giving the interaction a free energy of $-10 \text{ kcal/mol}$. Protonation of a single histidine supplies 1.2 kcal/mol per pH unit below the imidazole pKa. In cellular release assays, reduction in pH from 7.2 to 6.5 accelerates lipoprotein dissociation rates 75-fold (21, 74), suggesting that protonation of multiple residues is necessary for pH responsiveness. The interface between the β-propeller and LA4/5 uses three histidines (H190, H562, and H586) to promote the allosteric changes in LA5 that accelerate lipoprotein dissociation (32). Protonation of multiple histidines may likewise be necessary to restrict the conformational freedom of the N-terminal domains of the LDLR to favor formation of the intramolecular contact between the β-propeller and LA4/5. Future work will compare LDLR variants that lack multiple histidines. This goal is not expected to be trivially achieved because, as was exemplified by the H464R and H635K mutations, many histidines play important roles in folding and preliminary efforts to express LDLR ectodomains or full-length LDLRs with large numbers of histidine replacements have failed for lack of expression.

In conclusion, we present data suggesting that H635 participates in acid-dependent conformational change and lipoprotein release with ancillary contributions made by H264, H306, and H439.

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