Quantitative fluorescence imaging reveals point of release for lipoproteins during LDLR-dependent uptake

Shanica Pompey, Zhenze Zhao, Kate Luby-Phelps, and Peter Michaely

Department of Cell Biology, University of Texas Southwestern Medical Center, Dallas, TX

Abstract  The LDL receptor (LDLR) supports efficient uptake of both LDL and VLDL remnants by binding lipoprotein at the cell surface, internalizing lipoprotein through coated pits, and releasing lipoprotein in endocytic compartments before returning to the surface for further rounds of uptake. While many aspects of lipoprotein binding and receptor entry are well understood, it is less clear where, when, and how the LDLR releases lipoprotein. To address these questions, the current study employed quantitative fluorescence imaging to visualize the uptake and endosomal processing of LDL and the VLDL remnant \( \beta \)-VLDL. We find that lipoprotein release is rapid, with most release occurring prior to entry of lipoprotein into early endosomes. Published biochemical studies have identified two mechanisms of lipoprotein release: one that involves the \( \beta \)-propeller module of the LDLR and a second that is independent of this module. Quantitative imaging comparing uptake supported by the normal LDLR or by an LDLR variant incapable of \( \beta \)-propeller-dependent release shows that the \( \beta \)-propeller-independent process is sufficient for release for both lipoproteins but that the \( \beta \)-propeller process accelerates both LDL and \( \beta \)-VLDL release. Together these findings define where, when, and how lipoprotein release occurs and provide a generalizable methodology for visualizing endocytic handling in situ.——Pompey, S., Z. Zhao, K. Luby-Phelps, and P. Michaely. Quantitative fluorescence imaging reveals point of release for lipoproteins during LDLR-dependent uptake. J. Lipid Res. 2013. 54: 744–753.

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The LDL receptor (LDLR) protects against coronary artery disease by internalizing atherogenic lipoproteins, principally LDL and the LDL precursor, VLDL remnants. Lipoprotein uptake begins with binding of LDL or VLDL remnants to LDLRs on the cell surface, followed by internalization of LDLR-lipoprotein complexes through clathrin-coated pits into endocytic vesicles. Nascent endocytic vesicles recruit the early endosome antigen 1 (EEA1) protein (1, 2), which is a part of the molecular machinery that drives fusion of endocytic vesicles with each other and with early endosomes. Endocytic vesicles and endosomes acidify via the V-pump and lose calcium via the TRPV2 calcium channel (3–6). Both acidic pH and loss of calcium accelerate lipoprotein dissociation from the LDLR (7, 8). After release, the LDLR recycles back to the surface for additional rounds of uptake, while released lipoproteins progress through the endosomal system, eventually reaching lysosomes, where hydrolysis occurs.

The molecular details of how acidic pH and loss of calcium drive release are generally well understood; however, the importance of each process for release during endocytosis is not clear. The LDLR consists of seven LDLR type A (LA) repeats; two EGF-like repeats (EGF-A and EGF-B); six YWTD repeats that form a \( \beta \)-propeller; a third EGF-like repeat (EGF-C); a single transmembrane domain; and a short cytoplasmic tail. LDL binds to the LDLR through interaction of apolipoprotein B100 (apoB100) of LDL with LA3-7 and the EGF-A modules of the LDLR (9, 10). VLDL remnants bind to the LDLR through interaction of apolipoprotein E (apoE) of remnants with LA4 and LA5 of the LDLR (10, 11). LA repeats are calcium-binding modules, and loss of calcium results in a conformational change that accelerates release (8, 12–14). LA4, which is required for both apoB100 and apoE binding, has particularly weak calcium-binding affinity (15), suggesting that loss of calcium from LA4 initiates lipoprotein dissociation by the calcium release process. Acidic pH promotes loss of calcium by protonating the glutamates and aspartates that coordinate calcium in LA repeats. Acidic pH also accelerates lipoprotein release through a calcium-independent

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Abbreviations: \( \beta \)-VLDL, beta migrating VLDL; EEA1, early endosome antigen 1; EM, electron microscopy; LDLR, LDL receptor; MC, Manders overlap coefficient; PC, Pearson product-moment correlation coefficient; WT, wild-type.

To whom correspondence should be addressed.

E-mail: Peter.Michaely@utsouthwestern.edu

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process in which mildly acidic pH drives the β-propeller into contact with LA4 and LA5 (16, 17). This intramolecular contact accelerates lipoprotein release, most likely through an induced structural change in LA5 (18). The β-propeller-dependent release process has been proposed to be necessary for LDL release in endosomes because deletion of the EGF-B, β-propeller, and EGF-C modules (BC region) cripples cellular accumulation of LDL (8). By contrast, VLDL remnant accumulation is not affected by this deletion, suggesting that the calcium release process supports remnant release. Why different release processes might have different roles during uptake of different lipoproteins is not clear.

To address this question, we determined where, when, and how cells drive lipoprotein release during endocytosis. Previous efforts to characterize LDLR release during endocytosis have been hindered by the inability to visualize the release process. Here, we developed a quantitative 3D fluorescence imaging approach that captures the release process. We find that the majority of both LDL and VLDL remnants dissociate from the LDLR prior to entry of lipoprotein into early endosomes and that both release mechanisms operate during uptake of both lipoproteins. We also find that receptors lacking the BC region fail to support LDL accumulation not because release fails to occur but because LDL internalized by these receptors is inefficiently delivered to early endosomes and is instead resecreted.

**MATERIALS AND METHODS**

**Materials**

All cell culture reagents were purchased from Gibco/Invitrogen (Carlsbad, CA). Rabbit anti-LDLR IgG was a gift from Joachim Herz (Department of Molecular Genetics, UT Southwestern Medical Center, Dallas, TX). Formaldehyde was from Fluka (Buchs, Switzerland). All Alexa probes were from Molecular Probes/Invitrogen (Carlsbad, CA). All other chemicals were from Sigma (St. Louis, MO). LDL was prepared from freshly drawn human plasma as previously described (19). Beta migrating VLDL (β-VLDL) was prepared from the serum of cholesterol-fed rabbits as previously described (20).

**Cell culture**

Fibroblast cells were cultured in normal medium (D-MEM supplemented with 10% (v/v) fetal bovine serum (FBS), 20 mM HEPES (pH 7.5), 100 units/ml penicillin G, and 100 μg/ml streptomycin. Prior to all experiments, cells were starved of lipoprotein for 24 h using FLPPS medium (D-MEM supplemented with 10% (v/v) fetal lipoprotein poor bovine serum (FLPPS)), 20 mM HEPES (pH 7.5), 100 units/ml penicillin G, and 100 μg/ml streptomycin.

**Pulse-chase uptake of fluorescent lipoprotein**

Cells on coverslips in 24-well dishes were placed on ice and incubated with ice-cold MEM medium (MEM supplemented with 10% FLPPS) for 10 min to halt endocytosis. Cells were then incubated with either 10 μg/ml Alexa-labeled LDL or 5 μg/ml Alexa-labeled β-VLDL in MEM medium for 90 min on ice. Cells were washed with ice-cold MEM medium and shifted to 37°C by adding 39°C FLPPS medium and floating the 24-well dish in a 37°C water bath within a 37°C CO₂ incubator. Uptake was halted by rapidly replacing media with ice-cold PBS.

**Immunofluorescence**

Cells were fixed with 3% paraformaldehyde in PBS on ice for 20 min. Cells were then washed with ice-cold PBS and permeabilized with 10 μg/ml digitonin in PBS for 2 min on ice. Cells were blocked with 1% normal goat serum (NGS) in PBS for 1 h at room temperature and incubated with primary antibody in PBS + 1% NGS for 1 h at room temperature. Cells were then washed with PBS + 1% NGS and incubated with Alexa-labeled secondary antibodies (Invitrogen) in PBS + 1% NGS for 1 h at room temperature. Following secondary incubation, cells were washed, DAPI stained (3 μg/ml in PBS) for 10 min at room temperature, and then washed and mounted using Aqua poly/mount (Polysciences).

**Image acquisition and colocalization**

Four-channel fluorescence z-stacks were taken at each time point of each experiment using a Deltavision pDV microscope with a 60×/1.42NA objective (Applied Precision). Acquisition settings were identical for all datasets to allow results from different time points and conditions to be compared quantitatively. Z-stacks were deconvolved using the blind deconvolution algorithm of Autoquant X (Media Cybernetics). Colocalization was evaluated quantitatively using the coloc module of Imaris (Bitplane/Andor). Intensity thresholds were set automatically using the method of Costes (21). Colocalization statistics (MC and PC) for each two-channel pair were output as csv files and analyzed with Excel. Voxel dimensions were 0.21526 × 0.21526 × 0.2 μm. Typical experiments generated 50,000 to 500,000 doubly fluorescent voxels per colocalization channel per 3D reconstruction. All reported quantitative imaging data are means ± SEM using image datasets acquired from at least three independent experiments performed on different days. An example of primary data is presented in supplementary Fig. I.

**Lipoprotein internalization rate**

Rates of lipoprotein internalization were determined as previously described (22, 23). Briefly, cells were incubated with 10 μg/ml 125I-LDL or 5 μg/ml 125I-β-VLDL for 1 h at 4°C in MEM media. Media were changed for the times indicated with warm FLPPS media also containing 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 protease buffer (PBS + 1 mM EDTA) for 2 h at 4°C. The cell suspension was then centrifuged at 5,000 g for 10 min over a cushion of 10% sucrose in PBS. The tubes were frozen in liquid nitrogen, cut to separate the cells (internal) from the solution (surface-bound material released by protease K), and counted on a γ counter. Nonspecific activity was assessed in parallel experiments in the presence of 250 μg/ml unlabeled lipoprotein. Nonspecific activities were subtracted from mean values for each data point. Data are means ± SD from four replicate trials and are representative of three independent experiments.

**Lipoprotein accumulation assay**

Rates of lipoprotein accumulation were determined as previously described (22). Briefly, cells were incubated with 10 μg/ml Alexa546-LDL or 5 μg/ml Alexa546-β-VLDL for 1, 2, 3, or 4 h at 37°C, washed, scraped, fixed, and washed. Mean cell fluorescence was determined by flow cytometry from 10,000 gated events. Events were gated based upon side and forward scatter consistent with intact cells. Mean values were normalized to WT LDLR cell uptake at 4 h. Three separate experiments were
Lipoprotein degradation and excretion assay
Lipoproteins were incubated with cells at 4°C at a concentration of 10 μg/ml 125I-LDL, 10 μg/ml 125I-VLDL, 10 μg/ml unlabeled β-VLDL, 5 μg/ml 125Iβ-VLDL, or 1.25 μg/ml 125Iβ-VLDL ± 10 μg/ml unlabeled LDL in MEM medium for 90 min. Cells were then extensively washed with ice-cold MEM medium and either shifted to 37°C by medium replacement with warm FLPPS medium or washed, hydrolyzed, and assayed for labeled lipoprotein binding per milligram of cell protein. At the indicated times, conditioned media from 37°C-shifted cells were isolated, and intact lipoproteins were precipitated using 5% trichloroacetic acid (TCA). TCA-insoluble counts represent retro-endocytosed LDL. TCA-soluble counts represent degraded LDL. Counts were converted to picograms of LDL using the specific activity of LDL.

Lipoprotein isosurface construction and analysis
Lipoprotein-containing isosurface objects were created for each deconvolved z-stack using Imaris 3D rendering and measurement software (Bitplane/Andor). Creation of surfaces was automated with Imaris Batch Coordinator, so that the same criteria were applied to all datasets. Minimum intensity thresholds were determined interactively for each color channel, and the same thresholds were used for all datasets that were compared. The data were not smoothed, and the minimum voxel size filter was set at 1.0. Channel intensity statistics for each resulting surface set were output as csv files and analyzed with Excel. For each time point and condition, lipoprotein-containing surface objects were sorted into four classes on the basis of their mean intensity in the EEA1 and LDLR channels. Minimum intensity thresholds were determined for each channel, and the same thresholds were used for all datasets that were compared. Objects with both EEA1 and LDLR mean intensity below their respective thresholds were assigned to the LDL/VLDL-only class. Objects with EEA1 below threshold and LDLR above threshold were assigned to the LDL/VLDL + LDLR class, etc. The sum of the total lipoprotein fluorescence contained within the surface set was computed for each class and normalized by the total overall lipoprotein fluorescence contained within the unsorted surface set to obtain the fraction of lipoprotein in each class for each time point and condition. These procedures were performed separately on three independent experiments. The reported data are the means of the percentages of each class at each time point and condition ± SEM.

RESULTS
The progression of lipoprotein through the endocytic system was visualized by quantitative 3D fluorescence imaging of the LDLR, clathrin heavy chain, EEA1, LDL, and the VLDL remnant β-VLDL. Lipoproteins were directly labeled with Alexafluor dyes, whereas the LDLR, clathrin, and EEA1 were labeled by indirect immunofluorescence using Alexafluor-conjugated antibodies. Image stacks of each visualized field were acquired by wide-field epifluorescence microscopy and deconvolved to generate 3D images. The approach generated intensity information for three fluorescent components with spatial resolution of 0.2 × 0.21526 × 0.21526 μm per voxel for four million voxels per image stack. Use of pulse-chase methodology allowed visualization of lipoprotein progression through the endocytic system.

We employed two measures of colocalization to assess lipoprotein transit through the endocytic system. The first measure is the Manders overlap coefficient (MC), which is simply the number of voxels with above-threshold fluorescence in two channels divided by the number of voxels with above-threshold fluorescence for one of the two individual channels (27). For example, the MC of the LDLR with clathrin provides the fraction of LDLR-positive voxels that also contain clathrin. The second metric is the Pearson product-moment correlation coefficient (PC), which was calculated for the population of voxels that contain above-threshold fluorescence in both channels (doubly fluorescent voxels). PC is the normalized covariance in fluorescent intensities between two channels. Values range from −1 to 1, with 1 indicating a one-to-one correspondence in intensities, 0 indicating that the two intensities are uncorrelated, and −1 indicating that the intensities are inversely correlated. Because fluorescent intensity is a function of the abundance of a component within a voxel, the fluorescent intensity of two components will covary if the two components interact stoichiometrically. For this reason, PC is widely used to distinguish true association from random overlap (28). The MC and PC values are the means ± standard errors of at least 15 image stacks of randomly chosen fields of view from three independent trials (minimum of 5 image stacks per experimental condition per trial). Use of 5 images was sufficient sampling because the mean PC and MC values of different trials were not significantly different (P > 0.05). Additional method details and example images are provided in supplementary Fig. I. Additional discussion of the use of MC and PC for colocalization analysis has been detailed in recent reviews (24, 29).

Changes in MC and PC indicate relative change in association. When two components begin to associate, MC rises as the number of doubly fluorescent voxels increases. PC also increases because the new population of doubly fluorescent voxels has covariant fluorescent intensities. During dissociation, MC decreases as the number of doubly fluorescent voxels decreases. PC also declines because partial loss of interacting components occurs stochastically within the population of doubly fluorescent voxels, thereby generating variability in the ratio of fluorescent intensities. This loss of stoichiometry occurs prior to complete loss of fluorescence and provides a truer measure of dissociation than MC. We have employed MC and PC to follow LDLR interactions with lipoprotein and the transit of lipoprotein through EEA1-positive early endosomes during lipoprotein uptake.

We began our analysis of lipoprotein uptake by visualizing lipoprotein binding at the cell surface. Fibroblasts expressing normal LDLR (WT LDLR cells) were surface loaded or not with fluorescent lipoprotein at 4°C and then...
immmunostained for clathrin and the LDLR. Lipoprotein binding requires the presence of LDLRs on the surface of these cells (17). As a consequence, all surface-bound lipoprotein should have an associated LDLR, and the MC for LDL with LDLR and the MC for β-VLDL with LDLR were both exceptionally high (Fig. 1). In fibroblasts at steady state, approximately half of all LDLRs are surface exposed, with the remainder in transit through the endocytic system (30). As a consequence, MC values for LDLR with lipoprotein were lower than MC values for lipoprotein with LDLR. While PC for LDLR with LDL and PC for LDLR with β-VLDL were both strongly positive, the PC for LDLR with LDL was significantly higher than the PC for LDLR with β-VLDL. This difference may result from differences in lipoprotein binding stoichiometry in that LDL has one copy of apoB100 and binds to the LDLR with a fixed 1:1 stoichiometry, whereas β-VLDL has multiple copies of apoE and can thus bind the LDLR with a range of stoichiometries. Prior thin-section electron microscopy (EM) has shown that, although LDLRs are constitutively targeted to coated pits, only 25–50% of all surface LDLRs are present in coated pits at steady state (22, 31). The MC values of 0.4–0.5 for LDLR with clathrin in the presence and absence of lipoprotein is consistent with the EM quantification. MC values for clathrin with LDLR were lower than MC values of LDLR with clathrin because clathrin is abundant in the cytosol and on internal membrane compartments, such as the trans-Golgi network, and because not all clathrin structures at the surface contained LDLRs. Prior thin-section EM has shown that binding of β-VLDL, but not LDL, induces LDLR localization to coated pits (22). These EM observations suggest that β-VLDL binding allows LDLRs to access or to induce the de novo formation of a novel population of coated pits. Consistent with the EM observations, MC for clathrin with LDLR increased in the presence of β-VLDL but not LDL (Fig. 1B). PC for doubly fluorescent voxels containing both clathrin and LDLR were similar irrespective of lipoprotein, suggesting that coated pits with either induced or constitutively targeted LDLRs have similar LDLR density. The concordance between published EM data and the current quantitative imaging indicates that the imaging approach accurately visualizes associations between lipoprotein, the LDLR, and clathrin.

Following coated-pit targeting, LDLR-lipoprotein complexes are internalized into coated vesicles. These vesicles uncoat to form nascent endocytic vesicles and acquire early EEA1, a component of the molecular machinery that drives fusion of endocytic vesicles with each other and with early endosomes. Where in this endocytic process lipoprotein release occurs has not been formally demonstrated; however, early endosomes have been the presumed location because these endocytic compartments have luminal pH and calcium concentrations that are sufficient to drive both β-propeller-dependent and calcium-release-dependent mechanisms (5, 8). To visualize when and where release occurs, we performed pulse-chase uptake of fluorescently labeled lipoproteins in conjunction with immunostaining for the LDLR and EEA1. Surface LDLRs of WT cells were loaded with fluorescent lipoprotein at 4°C, washed to remove unbound lipoprotein, and then chased at 37°C. MC and PC were determined for LDLR with LDL and for LDLR with β-VLDL to assess release of lipoprotein from the LDLR. Entry into early endosomes was assessed using the MC and PC for EEA1 with LDL and for EEA1 with β-VLDL. We observed that both MC and PC for LDLR with lipoprotein fell rapidly during the chase (Fig. 2A, B), indicating the simultaneous release of lipoprotein and loss of LDLR from endocytic compartments bearing lipoprotein. Curve fitting of the decrease in PC to a single-site dissociation model yielded a half-life of 0.89 min for LDLR-LDL complexes and 5.1 min for LDLR-β-VLDL complexes. The rapidity of the LDL dissociation is consistent with the ability of even mildly acidic pH to efficiently drive LDL release (17). The slower release of β-VLDL is consistent with the observation that β-VLDL binding is less sensitive to both acidic pH and low calcium than is LDL binding (8). The fall in PC values for LDLR with lipoprotein coincided with increases in the MC and PC for EEA1 with lipoprotein (Fig. 2C, D). Importantly, the rises in PC and MC for EEA1 with LDL preceded the rises in PC and MC for EEA1 with β-VLDL. The correlation of slower lipoprotein release with slower lipoprotein entry into early endosomes suggested that lipoprotein release is coupled to entry into early endosomes.

To distinguish this possibility from the formal alternative that β-VLDL internalization is slower than LDL internalization, we assayed lipoprotein internalization rates. In these experiments, LDLRs on the surface of WT LDLR cells were loaded with 125I-labeled LDL or β-VLDL at 4°C followed by a 15 min chase at 37°C. At time points during the chase, internalized and surface-bound lipoprotein were assayed and used to calculate the ratio of internal versus surface lipoprotein. Rate constants derived from the

Fig. 1. Quantitative imaging of clathrin, LDLR, and lipoprotein prior to uptake. Cells were treated or not with saturating concentrations of Alexa546-labeled LDL or β-VLDL at 4°C, washed, fixed, and counterstained for LDLR and clathrin by indirect immunofluorescence. A: MC and PC for LDLR-lipoprotein colocalization. Fifteen fields from three separate experiments were processed for MC of LDL with LDLR and β-VLDL with LDLR (MC LwR); MC of LDLR with LDL and LDLR with β-VLDL (MC RwL); and PC of LDLR with LDL and LDLR with β-VLDL (PC). Data are means ± SEM, n = 15. *P < 0.05 compared with no lipoprotein. B: MC and PC for LDLR-clathrin colocalization. Fifteen fields from three experiments were processed for MC of LDLR with clathrin (MC Rwc); MC of clathrin with LDLR (MC CwR); and PC of LDLR with clathrin in the presence or absence of LDL or β-VLDL. Data are means ± SEM, n = 15. *P < 0.05 compared with no lipoprotein.
quantified the fraction of total surfaces that contained only lipoprotein during the course of lipoprotein uptake. We observed that the population of lipoprotein-only isosurfaces rose rapidly as isosurfaces with LDLR declined. The rise in lipoprotein-only surfaces preceded the rise in isosurfaces with EEA1 voxels, indicating that LDLRs leave endocytic vesicles prior to the entry of lipoprotein into early endosomes (Fig. 3E, F).

To determine how endocytic compartments drive lipoprotein release, we compared lipoprotein uptake in WT internal/surface ratios showed that LDL and β-VLDL internalized at the same rate with a receptor cycling time of 14 min (Fig. 3A, B). The endocytic rates observed in WT LDLR cells are similar to rates of LDLR-dependent uptake previously observed in human fibroblasts (32). Similarity between endocytic rates of LDL and β-VLDL internalization has also been observed previously (23). These findings indicate that a slower rate of internalization is not responsible for the slower release and entry into early endosomes of β-VLDL.

Quantitative imaging of LDLR colocalization with EEA1 provides further support for the hypothesis that lipoprotein release precedes lipoprotein entry into early endosomes. Because release of β-VLDL from the LDLR is slower than release of LDL (8), if entry into early endosomes is independent of release, then LDLR colocalization with EEA1 should increase during the chase and β-VLDL should promote more LDLR colocalization with EEA1 than LDL. By contrast, if early endosome entry is dependent upon release, then LDLR colocalization with EEA1 should remain constant during the chase irrespective of lipoprotein. Examination of MC for EEA1 with LDLR showed that at no time point with either lipoprotein did LDLR content in EEA1-positive voxels increase (Fig. 3C). PC values for EEA1 with LDLR were low and at no time rose above the 0.1 threshold for significant colocalization in the presence of either lipoprotein (Fig. 3D). Quantitative imaging also afforded the ability to track lipoprotein-containing compartments that lack both LDLR and EEA1 as a function of time. For this purpose, we generated isosurfaces from lipoprotein-positive voxels (supplementary Fig. II) and

![Fig. 2. LDL dissociates from the LDLR and enters EEA1-positive endosomes faster than β-VLDL. Surface LDLRs were saturated with Alexa488-LDL or β-VLDL at 4°C, washed, and shifted to 37°C for the indicated times. At each time point, cells were rapidly chilled to 4°C, fixed, and immunostained for LDLR and EEA1. Thirty fields from three separate experiments were processed for MC and PC of LDLR with LDL or β-VLDL (A and B) or for MC and PC of EEA1 with LDL or β-VLDL (C and D). Data are means ± SEM, n = 30.](image)

![Fig. 3. Lipoprotein release occurs prior to lipoprotein entry into early endosomes. A: LDL and β-VLDL internalize with similar kinetics. Surface LDLRs were saturated with 125I-LDL or 125I-β-VLDL at 4°C and then shifted to 37°C in the presence of 125I-lipoprotein for the indicated times. Surface-bound lipoprotein was then released by protease K, and surface and cell-associated (internal) lipoprotein was separated by centrifugation. Data are means ± SD, n = 4. B: Rates derived from linear regression of the data in (A). C and D: Lipoprotein internalization does not chase LDLRs into early endosomes. Data from Fig. 2 were processed for MC and PC of LDLR with EEA1 in the presence of LDL or β-VLDL. Data are means ± SEM, n = 30. E and F: Lipoprotein releases from the LDLR prior to entry of lipoprotein into early endosomes. Lipoprotein isosurfaces from three pulse-chase experiments were generated as described in Materials and Methods and in supplementary Fig. II. Isosurfaces were classified into four bins: surfaces that lack both LDLR and EEA1; surfaces that contain LDLR but not EEA1; surfaces that contain both LDLR and EEA1; and surfaces that contain EEA1 but not LDLR. Percentages of total lipoprotein surfaces for each bin were calculated for each experiment and are reported as means ± SEM, n = 3.](image)
LDLR cells with uptake in cells expressing the LDLR-ΔBC, which lacks the EGF-B, β-propeller, and EGF-C modules (BC region). Cell surface release assays have shown that deletion of the BC region sharply impairs acid-dependent release of both LDL and β-VLDL (8) and that PC values for LDLR with LDL and LDLR with β-VLDL decayed with slower kinetics in LDLR-ΔBC cells compared with WT LDLR cells (Fig. 4A, B). Consistent with the conclusion that release precedes entry into early endosomes, PC values for EEA1 with LDL and EEA1 with β-VLDL peaked with delayed kinetics in LDLR-ΔBC cells compared with WT cells (Fig. 4C, D), and at no time did PC for EEA1 with LDLR rise to the 0.1 significance threshold (Fig. 4E, F).

MC for EEA1 with LDLR-ΔBC also showed no change over the chase with either lipoprotein (Fig. 4G, H). These findings show that, although the β-propeller independent release mechanism is sufficient for both LDL and β-VLDL release, the β-propeller accelerates both LDL and β-VLDL release.

Deletion of the BC region reduces LDL accumulation by 70%, but it has no effect on β-VLDL accumulation (Fig. 5A, B) (8). Because the LDLR-ΔBC binds both LDL and β-VLDL with normal affinity and supports normal rates of lipoprotein uptake (8), the reduction in LDL accumulation suggests that the majority of LDL that is internalized by the LDLR-ΔBC is excreted. In support of this possibility, the total LDL fluorescence intensity associated with LDLR-ΔBC cells fell rapidly during the chase with LDLR cells with uptake in cells expressing the LDLR-ΔBC, which lacks the EGF-B, β-propeller, and EGF-C modules (BC region). Cell surface release assays have shown that deletion of the BC region sharply impairs acid-dependent release of both LDL and β-VLDL (8) and that PC values for LDLR with LDL and LDLR with β-VLDL decayed with slower kinetics in LDLR-ΔBC cells compared with WT LDLR cells (Fig. 4A, B). Consistent with the conclusion that release precedes entry into early endosomes, PC values for EEA1 with LDL and EEA1 with β-VLDL peaked with delayed kinetics in LDLR-ΔBC cells compared with WT cells (Fig. 4C, D), and at no time did PC for EEA1 with LDLR rise to the 0.1 significance threshold (Fig. 4E, F).

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kinetics that mirrored the rate of LDL dissociation from the LDLR-ΔBC (Fig. 5C, D). Published work has shown that a fraction of both LDL and β-VLDL that is internalized by the LDLR is excreted through a process termed retro-endocytosis (23, 33, 34). We tested whether the BC deletion specifically augmented retro-endocytosis of LDL by comparing LDL and β-VLDL degradation and excretion in pulse-chase experiments using 125I-labeled LDL and β-VLDL. LDLR-ΔBC cells supported less degradation and more excretion of LDL compared with WT LDLR cells, while degradation and excretion of β-VLDL was unchanged by the BC deletion (Fig. 5E, F). These observations indicate that deletion of the BC region compromises LDL uptake by facilitating loss of LDL through retro-endocytosis.

Why the BC deletion compromises transfer of LDL but not of β-VLDL into early endosomes is unclear. One possibility is that LDL release and β-VLDL release occur in distinct compartments and only transfer of lipoprotein from the LDL release compartment to early endosomes is sensitive to the BC deletion. We tested whether LDL and β-VLDL are segregated into distinct pre-early endosomal compartments using the pulse-chase uptake methodology with a combination of LDL and β-VLDL. If cells segregate LDL from β-VLDL into separate release compartments, MC and PC between LDL and β-VLDL should decline prior to entry of lipoprotein into early endosomes. Instead, we observed that the MC and PC of LDL with β-VLDL increased throughout the chase (Fig. 6A). Furthermore, while fusion between LDL-only and β-VLDL-only vesicles was observed in live cell imaging experiments, we failed to detect fission events generating LDL-only or β-VLDL-only vesicles from endocytic compartments with both lipoproteins (data not shown). These findings indicate that LDL and β-VLDL are not segregated prior to entry into early endosomes and that a common pre-early endosomal compartment accommodates both LDL and β-VLDL. This conclusion is consistent with thin-section EM experiments showing that LDL and β-VLDL cotraffic through the endocytic system (35). Compared with single lipoprotein uptake experiments, the release of lipoprotein from the LDLR was slowed and entry of lipoprotein into early endosomes was slightly delayed by the presence of a second lipoprotein (Fig. 6B, C); however, lipoprotein degradation was not affected (Fig. 6D). These findings show that cells do not segregate LDL and β-VLDL into separate release compartments but rather use a common pre-endosomal compartment for release. The presence of multiple lipoproteins slows release in this common compartment; however, this delay is small and does not influence final degradation rates.

DISCUSSION

The current work defines where, when, and how the LDLR releases LDL and the VLDL remnant β-VLDL during endocytosis using the fibroblast model system. The quantitative 3D fluorescence imaging shows that the LDLR releases both lipoproteins rapidly following internalization and that most release occurs prior to entry of lipoprotein into EEA1-positive early endosomes. Biochemical structure-function studies previously identified two release mechanisms: a β-propeller-dependent mechanism that is triggered by acidic pH and a β-propeller-independent mechanism that is triggered by loss of calcium. Quantitative imaging shows that both mechanisms participate in the release of both lipoproteins.

Key features of the quantitative imaging approach were the size of the datasets and the use of both MC and PC to follow lipoprotein progression through the endosomal system. The 3D fluorescence imaging of multiple fields across multiple experiments generated datasets composed of several million of fluorescent voxels per dataset. Use of MC with these datasets provided a robust assessment of
Exposure of fibroblasts (36, 37). Thus, the transit time for LDLRs through the endocytic system is 6–7 min. Lipoprotein uptake provided the means to quantify the kinetics of LDLR internalization and entry into early endosomes. Additionally, analysis of fluorescence intensities inside lipoprotein isosurface objects allowed us to classify subpopulations of lipoprotein-containing compartments based upon their contents and to examine the changes in these subpopulations over time.

Our finding that lipoprotein release occurs rapidly is consistent with the speed of LDLR internalization cycles. The time required for the LDLR to complete a cycle of lipoprotein internalization is 12–15 min in fibroblasts (Fig. 3A) (32). At steady state, half of all LDLR are surface exposed in fibroblasts (36, 37). Thus, the transit time for LDLRs through the endocytic system is 6–7 min. Lipoprotein release must therefore be fast; estimates of LDL and β-VLDL release from the time-dependent change in PC suggest rates of 0.78 min⁻¹ for LDL release and 0.14 min⁻¹ for β-VLDL release. Actual rates are likely faster because PC cannot distinguish between LDLR that is lipoprotein-bound and LDLR that has released lipoprotein but has not yet trafficked away from the endocytic compartment holding lipoprotein. The rapid rate of release is incompatible with release in early endosomes because lipoprotein colocalization with EEA1 does not peak until 8–15 min (Fig. 2). Consistent with this conclusion, MC and PC for EEA1 with LDLR are small and remain small throughout uptake of both LDL and β-VLDL (Fig. 3). Furthermore, quantitative imaging identified a population of lipoprotein voxels lacking both LDLR and EEA1, which appeared following the rapid loss in PC of LDLR with lipoprotein and prior to the increase in PC of EEA1 with lipoprotein. These observations indicate that release occurs prior to lipoprotein entry into early endosomes.

Rapid release satisfies the kinetics of LDLR transit through the endosomal system, but it is problematic for the mechanism of release. The two known release processes, the β-propeller-dependent process and the β-propeller-independent process, cooperate in surface release assays (8); however, the pH and calcium concentrations necessary to achieve rapid release are not thought to exist early in the endocytic process. The rate of endocytic release inferred from the change in PC equates with rates of surface release observed for pH between 5.5 and 6.0 and calcium concentrations between 2.5 and 10 μM (8). Ratios measurements of endosomal pH and calcium have shown that dextrans do not experience these conditions until 20 min after internalization (5). How then does the LDLR complete its endocytic cycle in the allotted 6–7 min? One possibility is that the pH and calcium measured in the bulk of the lumen by labeled dextrans is different from the pH and calcium experienced by the LDLR, which, as an integral membrane protein, is attached to the limiting membrane. It is at the limiting membrane where the V-pump and the TRPV2 channel inject protons and withdraw calcium ions, and this proximity may facilitate lipoprotein release from the LDLR.

In addition to lipoprotein release, LDLRs must recycle back to the cell surface, and the short transit time of LDLRs through internal compartments necessitates rapid recycling. This rapid recycling likely contributes inefficiency to the uptake process through premature commitment of LDLRs to the recycling pathway, thereby resulting in excretion (retro-endocytosis) of lipoprotein. Not surprisingly, inhibitors of endosome acidification increase retro-endocytosis (38); however, these inhibitors also hinder LDLR recycling, resulting in loss of LDLRs from the cell surface (39). This latter observation suggests that the LDLR has a mechanism that hinders entry of LDLRs into the recycling pathway prior to exposure of LDLRs to an acidified compartment. Acidic pH drives lipoprotein release and this retention mechanism likely reduces the loss of internalized lipoprotein to retro-endocytosis. Recent work has shown that the intracellular retention of LDLRs that occurs in the presence of acidification inhibitors requires the BC region (8). This region undergoes a massive conformational change in response to acidic pH (16, 40) and this change may turn off the retention activity of the BC region. Why deletion of the BC region allows retro-endocytosis of LDL but not of β-VLDL is not clear. LDL and β-VLDL do not separate following internalization (Fig. 6), indicating that the two lipoproteins traffic through common release compartments. One possibility is that an intrinsic feature of β-VLDL hinders LDLR-β-VLDL complexes from entering the recycling pathway. β-VLDL has been shown to augment intracellular retention of LDLRs when receptor recycling is impaired by endosomal acidification inhibitors or by deletions that remove the EGF-A and BC regions (7, 8, 39). Features that may hinder LDLR-β-VLDL access to the recycling pathway include size (β-VLDL is much larger than LDL) and valency (β-VLDL contains multiple binding sites for LDLRs whereas LDL contains only one LDLR binding site).

Failure of LDLRs to recycle results in eventual lysosomal degradation of receptors. Failure to recycle can result from mutations in the LDLR (class V FH mutations) (41) or from the action of two regulatory proteins, IDOL and PCSK9. IDOL is an E3 ligase that ubiquitinates the LDLR and drives LDLR trafficking to lysosomes via the ESCRT complex (42). PCSK9 is a secreted protein that inhibits LDLR recycling by binding to the EGF-A module of the LDLR, which normally promotes LDLR recycling (8, 43). Inhibitors of IDOL and PCSK9 hold promise as cholesterol-lowering therapies (44, 45). Our data show that LDLRs in fibroblasts are normally excluded from early endosomes irrespective of lipoprotein release efficiency (Fig. 4), and future characterization of this activity may identify new therapeutic targets that can be exploited to prolong LDLR half-life and thereby facilitate LDL clearance.

In summary, the present work identifies where, when, and how LDLRs release lipoprotein during endocytosis. Release is rapid and occurs prior to entry of lipoprotein.
into early endosomes. Release for both LDL and β-VLDL is triggered by both β-propeller-dependent and β-propeller-independent mechanisms.13

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