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The Role of Calcium in Lipoprotein Release by the LDL Receptor†

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Abstract

The LDL receptor (LDLR) mediates efficient endocytosis of VLDL, VLDL remnants and LDL. As part of the uptake process, the LDLR releases lipoproteins in endosomes. Released lipoproteins are subsequently trafficked to lysosomes for degradation, while the LDLR recycles back to the cell surface for further rounds of uptake. Endosomes have at least two features that can promote lipoprotein release: an acidic pH and low concentrations of free calcium. The relative contributions of acidic pH and low free calcium to lipoprotein release are not known. Here, we generated fibroblasts that express either normal LDLR or an LDLR variant that is unable to employ the acid-dependent release mechanism to determine the relative contributions of acidic pH and low free calcium on lipoprotein release. We show that endosomal concentrations of free calcium can drive lipoprotein release at rates that are similar to those of acid-dependent release and that the calcium-dependent and acid-dependent mechanisms can cooperate during lipoprotein release. Assessment of lipoprotein uptake by these two cell lines showed that LDL uptake requires the acid-dependent mechanism, while uptake of the VLDL remnant, β-VLDL, does not. We propose that endosomes use both the acid-dependent and calcium-dependent release mechanisms to drive lipoprotein release and that the acid-dependent process is only required for LDL release.

Keywords

LDL; VLDL; LDLR; calcium; endosome; recycling

The low density lipoprotein (LDL) receptor (LDLR) is the principal endocytic receptor that removes very low density lipoprotein (VLDL), VLDL-remnants and LDL from the circulation. Lipoprotein uptake involves first binding of lipoproteins to the LDLR on the cell surface, followed by internalization of the LDLR-lipoprotein complex through clathrin coated pits. 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). Internalization requires the cytoplasmic domain of the LDLR, which contains determinants that interact with components of the clathrin coated pit machinery (2–6). After internalization, the LDLR-lipoprotein complex traffics to endosomes, where the LDLR releases bound lipoproteins. Released lipoproteins are subsequently degraded in lysosomes, while the LDLR recycles back to the cell surface for further rounds of lipoprotein uptake. Loss-of-function mutations that impair any step in this pathway reduce the clearance rates of VLDL, VLDL remnants and LDL and result in familial hypercholesterolemia (FH), a common autosomal dominant disorder that promotes atherosclerosis and the development of coronary artery disease (7–10).

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Lipoproteins bind to the ligand-binding domain (11). This domain consists of seven LDLR type A (LA) repeats, which are small, irregular domains held together by calcium and cystine bridges (12;13). LDL binding requires LA repeats 3–7, which interact with apolipoprotein B100 (apoB100) on LDL (11;14–16). The binding of VLDL and VLDL-remnants involves LA repeats 4 and 5 (LA4/5), which associate with apolipoprotein E (apoE) on VLDL/VLDL remnants (11;16–19).

Lipoprotein release occurs in endosomes, whose lumen is acidic and has low concentrations of free calcium (high pCa, where pCa = −log(free[Ca^{2+}])). Acidic pH alone can drive lipoprotein release through a process that requires the EGF-homology domain (20;21). The EGF-homology domain consists of two EGF-like domains (EGF-A and EGF-B), six YWTD repeats that form a six-bladed β-propeller and a third EGF-like repeat (EGF-C) (1;22). At acidic pH, the β-propeller of the EGF-homology domain forms an intramolecular contact with LA4/5 of the ligand-binding domain (23). Recent work suggests that this contact drives an allosteric change in LA4/5 that accelerates lipoprotein dissociation (24). In addition to the acid-dependent process, low concentrations of calcium can promote release because the LDLR requires calcium to bind LDL and VLDL (25;26). Loss of calcium induces structural changes in LA repeats that have been proposed to disrupt lipoprotein-binding ability (27–31). LA repeats bind calcium with affinities in the nanomolar range at neutral pH and in the low micromolar range at acidic pH (27;30–32). Endosomes have a pH in the range of 5.5–6.5 and a free calcium concentration in the range of 2.5–10 μM (33). Thus, the endosomal lumen has the potential to trigger lipoprotein release through both a purely acid-dependent process that involves the EGF-homology domain and low-free-calcium-dependent process that directly disrupts lipoprotein-binding surfaces.

Of the two potential triggers, the acid-dependent process involving the EGF-homology domain has been viewed as the principal mechanism of lipoprotein release (34;35); however, a role for the calcium-dependent process has not been excluded. A key impediment to characterization of the calcium-dependent process has been separating the two processes, both of which are promoted by low pH. Here, we generated fibroblast cells that express an LDLR variant (LDLR-ΔBC) lacking the EGF-B, the β-propeller and EGF-C modules of the EGF homology domain. The LDLR-ΔBC expressing cells have normal lipoprotein binding, but are unable to employ the acid-dependent mechanism of lipoprotein release. Thus, the LDLR-ΔBC receptor provides a tool with which to characterize the calcium-dependent mechanism and to determine the relative contributions of the acid-dependent and calcium-dependent processes on lipoprotein release.

EXPERIMENTAL PROCEDURES

Materials

LDLR$^{-/-}$ primary human fibroblasts, human LDL and rabbit beta migrating VLDL (β-VLDL) were a gift of Michael Brown and Joseph Goldstein (Department of Molecular Genetics, UT Southwestern Medical Center, Dallas, TX). Rabbit polyclonal anti-LDLR (4548) was a gift of 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). ^{125}I-Bolton-Hunter reagent was from Perkin-Elmer (Boston, MA). Alexa546 succinimidyl ester was from Invitrogen (Carlsbad, CA).

Baculovirus mediated protein expression of WT LDLR ectodomain

The cDNA encoding residues 1-699 of the LDLR was cloned into the pFastBac plasmid (Invitrogen). Recombinant plasmids were used to produce infectious baculoviruses that

directed the synthesis of secreted LDLR ectodomains using the Bac-to-Bac system (Invitrogen).

Introduction of LDLR variants into LDLR$^{-/-}$ Fibroblasts

WT LDLR or LDLR-ΔBC was stably expressed in *LDLR*$^{-/-}$ fibroblasts (549T) using a retroviral system as previously described (24). Briefly, LDLR variants were cloned into the pMX-IRES-GFP bicistronic retroviral vector (36). Retroviral vectors were cotransfected with the pAmpho packaging vector (Clontech, Mountain View, CA) into 293T cells to produce infectious, replication-defective retroviruses. Recombinant retroviruses were used to infect 549T cells, which are derived from primary human fibroblasts from an individual that lacks LDLR expression (*LDLR*$^{-/-}$). 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). Surface LDLR expression was monitored by flow cytometry using anti-LDLR mouse monoclonal antibody, C7 (Santa Cruz Biotechnology, Santa Cruz, CA).

Cell Culture

All fibroblasts were cultured in Medium A (DMEM medium supplemented with 10% (v/v) fetal bovine serum, 20 mM HEPES pH 7.5, 100 units/ml penicillin G and 100 μg/ml streptomycin). Prior to experimentation, fibroblasts were starved of lipoproteins for 24 hrs 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 units/ml) and streptomycin (100 μg/ml)).

Gel Filtration

Gel filtration was conducted on a Superdex200 10/30 column attached to an Äkta FPLC (Amersham, Piscataway, NJ). The column was equilibrated in buffers containing 25 mM HEPES, 25 mM maleate, 150 mM NaCl, and 0.5% TritonX-100 at pH 6.0. Free calcium was buffered between 0.3 μM and 80 μM using either 25 mM EDTA (for 0.3 – 5 μM free calcium) or 25 mM EGTA (for 10 – 80 μM free calcium). Samples were equilibrated in the same buffers prior to loading. 0.5 ml fractions were collected, electrophoresed on 5–17% SDS-PAGE gels, transferred to nylon membranes and immunoblotted for the LDLR using the 4548 polyclonal antibody. Thyroglobulin (85Å), apoferritin (61Å), amylase (48Å), aldolase (45Å), bovine serum albumin (36Å) and carbonic anhydrase (20Å) were used as standards.

Lipoprotein labeling

Human LDL and rabbit β-VLDL were labeled with ^{125}I using the Bolton-Hunter method (37). LDL was labeled with Alexa-546 using the succinimidyl ester of Alexa-546 with the manufacturer's recommended protocol (Invitrogen). β-VLDL was labeled with 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) 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 hrs at 37°C in the dark. The density of the suspension was increased to 1.019 by the addition of 0.0199g KBr per ml of suspension and centrifuged at 120,000 × g for 16 hrs at 4°C. DiI-β-VLDL was removed from the top of the tube, dialyzed against PBS, and stored at 4°C in the dark until use.

Lipoprotein binding assays

^{125}I-LDL and ^{125}I-β-VLDL binding assays were performed in triplicate using established methods (38). Assays were preformed at 4°C for 90 min using concentrations of ^{125}I-LDL or ^{125}I-β-VLDL indicated in the figure legends. In all binding experiments, cells infected with

empty retrovirus (Vector control) were used as negative controls and values of lipoprotein bound by vector cells were subtracted from values of WT LDLR, LDLR-ΔBC and LDLR-ΔAC cells. Binding by vector cells was < 5% of WT LDLR cells. Results are presented as means ± standard deviation.

In Vitro Release of Cell Surface Bound Lipoprotein

Cells were pre-incubated at 37°C with Medium C, which consisted of Medium B supplemented with 0.45 M sucrose to prevent clathrin coated pit endocytosis (39). Cells were then incubated with either 10 μg/ml ^{125}I-LDL or 5 μg/ml ^{125}I-β-VLDL in Medium C for 30 min at 37°C and washed with warm Medium C. For acid-dependent release cells were incubated at 37°C at the indicated pH with Medium D (bicarbonate free MEM supplemented with 0.45 M Sucrose, 20 mM HEPES, 20 mM maleate and 10% lipoprotein poor serum). For release experiments in which the calcium concentration was varied, cells were incubated with Medium E (bicarbonate free MEM supplemented with 0.45 M Sucrose, 20 mM HEPES, 20 mM Maleate, 25 mM chelator (HEDTA, EGTA or EDTA), 10% lipoprotein poor serum and sufficient calcium chloride to bring free calcium to the indicated calcium concentration at the indicated pH). The total calcium needed to achieve the indicated free calcium concentrations was determined using WEBMAXC (http://www.stanford.edu/~cpatton/maxc.html) (40). After incubation for the indicated time in either Medium D or E, cells were washed 2 times with ice cold TBSC (50 mM Tris pH 8.0, 150 mM NaCl, 2 mM $CaCl_2$), 1 time for 10 min with ice cold TBSC supplemented with 1% BSA and 2 times with ice cold TBSC. Remaining cell-associated, ^{125}I-labeled lipoproteins were liberated by incubation with 0.1 N NaOH and counted. In all experiments, parallel trials with vector-infected fibroblasts were used as negative controls. Values for vector-infected cells were subtracted from values of WT LDLR and LDLR-ΔBC cells to obtain receptor specific values for lipoprotein binding and release. Rate constants for release were determined by plotting the $\ln(\Delta y/\Delta y_0)$ vs time where Δy is the amount of lipoprotein bound at time t and Δy_0 is the amount of lipoprotein bound at time 0. The slope of this plot provides an estimate of the rate constant assuming a two state model of release ($\Delta y = \Delta y_0 e^{-kt}$). This procedure was used with data shown in figures 5 and 6 to determine the rate constant shown in Table 1.

Cellular Lipoprotein Uptake

Cells were incubated with either 10 μg/ml Alexa546-LDL or 5 μg/ml DiI-β-VLDL in warm Medium B 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. All experiments used vector infected cells as negative controls and mean values for the vector cells were subtracted from values of WT LDLR, LDLR-ΔBC and LDLR-ΔAC cells to produce the reported values. Vector cell fluorescence means did not significantly increase with time and by one hour were < 5% of WT LDLR cells. Results shown are representative of three independent experiments.

Initial Endocytic Rates

Initial internalization rates were determined as previously described (41;42). Briefly, cells were incubated with 5 μg/ml ^{125}I-β-VLDL for 1 hr at 4°C in Medium F (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 5 μg/ml ^{125}I-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 hrs 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 ± standard deviation.

Monensin treatment and analysis of surface biotinylated proteins

Fibroblasts were treated with 10 μg/ml LDL or 5 μg/ml β-VLDL in the presence or absence of 30 μM monensin for the times indicated in the figure legends. Cells were washed with ice cold PBS and incubated with buffer B (1 mg/ml Sulfo-NHS-(LC)-biotin (Pierce) in PBS, pH 8.0) for 30 min at 4°C. Cells were then washed with ice cold PBS and incubated with Buffer C (100 mM NaCl and 20 mM Tris pH8.0) for 30 min at 4°C. Cells were lysed in 0.5 ml Buffer D (1% TritonX100, 4 mM EGTA, 10 mM Tris pH 8.0) and lysates clarified by centrifugation for 15 min at 20,000 × g at 4°C. Biotinylated proteins from 400 μl of the lysate were precipitated by addition of 40 μl of a 50% slurry of neutravidin agarose (Pierce) followed by end over end mixing for 1 hr at 4°C. Biotinylated proteins bound to the agarose were washed 3 times with Buffer E (15 mM Tris pH 8.0, 500 mM NaCl, 4 mM EGTA and 0.5% TritonX100) and one time with Buffer F (15 mM Tris pH 8.0, 4 mM EGTA and 0.5% TritonX100). Biotinylated proteins were eluted from neutravidin agarose by the addition of 100 μl of 4x SDS-PAGE sample buffer and heating to 100°C for 10 min. Samples were loaded on 5–17% SDS-PAGE gels, transferred to PVDF membranes and immunoblotted for LDLR.

RESULTS

The calcium-dependent mechanism has the potential to drive lipoprotein release from the LDLR; however, characterization of this mechanism has been hampered by the ability of the LDLR to release lipoproteins using the EGF-homology domain, which triggers release through in an acid-dependent, but calcium-independent process. To characterize the calcium-dependent mechanism of lipoprotein release, we generated fibroblasts that express either normal LDLR (WT LDLR) or an LDLR variant (LDLR-ΔBC) that lacks the EGF-B, β-propeller and EGF-C modules and thus should not be able to utilize the acid-dependent mechanism of lipoprotein release. The EGF-A module was not removed because removal of EGF-A impairs LDL binding (11;20). The parental fibroblasts we used are 549T cells, which are immortalized primary skin fibroblasts from an $LDLR^{-/-}$ individual. These fibroblasts do not express any LDLR and have exceptionally poor ability to bind either LDL or β-VLDL (24). These fibroblasts were immortalized by introduction of human telomerase, which prevents sensescence, but does not change cell morphology or cell growth rate in fibroblasts (43). Comparison of WT LDLR and LDLR-ΔBC fibroblasts showed that both fibroblasts express similar total and surface numbers of LDLRs (Fig 1A and 1B) and that the LDLR-ΔBC has normal ability to bind to LDL and the VLDL remnant, β-VLDL (Fig 1C and 1D). The normal surface expression of LDLR-ΔBC indicates that deletion of the EGF-B to EGF-C region does not impair either maturation or intracellular trafficking of the LDLR, while the normal lipoprotein binding properties indicate that the deletion does not remove regions necessary for lipoprotein binding. The normal ability of LDLR-ΔBC cells to bind LDL is consistent with a previous report showing that a secreted LDLR variant consisting of just the ligand-binding domain and the EGF-A module efficiently competes with cellular LDLRs for binding to LDL (44). The normal ability of LDLR-ΔBC cells to bind β-VLDL is consistent with the observation that a fusion protein of GST with LA4/5 retains the ability to bind apoE-containing lipoproteins (19). Together, these experiments show that deletion of the EGF-B to EGF-C region of the EGF-homology domain of the LDLR has little effect on the production of mature LDLR, on the intracellular trafficking of LDLR or on the ability of the LDLR to bind lipoproteins.

At acidic pH, the β-propeller of the EGF-homology domain forms an intramolecular contact with LA4/5 of the ligand-binding domain (23), suggesting that acid-dependent lipoprotein

release requires the interaction of the β-propeller with LA4/5 (21). We tested whether lipoprotein binding by the LDLR-ΔBC was sensitive to acidic pH using an assay system in which cells expressing either WT LDLR or LDLR-ΔBC were first incubated with ^{125}I-LDL or ^{125}I-β-VLDL to saturate surface LDLRs and then incubated with media buffered between pH 5.5 and pH 7.5 to examine acid-dependent release. Incubations at acidic pH resulted in the loss of both LDL and β-VLDL from the surface of cells expressing WT LDLR, but had little ability to drive loss of surface bound LDL or β-VLDL from cells expressing LDLR-ΔBC (Fig 2). Thus, the LDLR-ΔBC is unable to support acid-dependent lipoprotein release, consistent with the proposal that interaction of the β-propeller with LA4/5 is required for acid-dependent release.

We next compared lipoprotein release by the LDLR-ΔBC and WT-LDLR expressing cells in response to low concentrations of free calcium. Cells expressing WT LDLR or LDLR-ΔBC were loaded with ^{125}I-LDL or ^{125}I-β-VLDL and then incubated with media containing free calcium concentrations from 1.8 mM (pCa 2.7) to 100 nM (pCa 7.0). The pH was held at pH 7.0 to prevent acid-dependent release through the EGF-homology domain. In response to reduced concentrations of free calcium, both WT LDLR expressing cells and LDLR-ΔBC expressing cells released LDL and β-VLDL with similar dependence on free calcium (Fig 3). These observations show that the EGF-B, β-propeller and EGF-C modules are not required for lipoprotein release in response to low calcium, consistent with the proposal that low calcium disrupts the lipoprotein binding surfaces of the LDLR (27;29–31). These observations also indicate that the acid-dependent pathway is independent of the calcium-dependent pathway of lipoprotein release.

At neutral pH, half-maximal lipoprotein release occurred at a free calcium concentration of 0.5–2 μM (pCa 5.7–6.2) for LDL and ~0.2 μM (pCa 6.7) for β-VLDL. These values are lower than the free calcium concentration in endosomes, which ranges from 2.5–10 μM (pCa 5.0–5.6); however, the ligand-binding domain of the LDLR binds calcium with weaker affinity at acidic pH than at neutral pH (30), suggesting that lipoprotein release mediated by low free calcium may be augmented by endosomal pH. In fibroblasts, endosomal pH drops to a pH of ~6 within 10 min and to a pH of ~5.5 within 20 min (33). To test the role of pH on calcium dependent release, we compared lipoprotein release by LDLR-ΔBC expressing cells at pH 6.0 and pH 5.5 over a calcium range of 1 μM to 1.8 mM (Fig 4). At 3 μM free calcium, the majority of bound LDL was released at both pH 6.0 and pH 5.5, while ~40% of bound β-VLDL was released at pH 6.0 and 70% of bound β-VLDL was release at pH 5.5. These observations indicate that endosomal concentrations of free calcium can drive lipoprotein release in the absence of the EGF-homology domain. Low pH facilitates calcium-dependent release, presumably because the ligand-binding domain binds to calcium with weaker affinity at acidic pH than at neutral pH (30).

For this calcium-dependent mechanism to play a significant role in lipoprotein release, rates of release should be comparable to those of acid-dependent release. We used the LDLR-ΔBC expressing cells in time courses of lipoprotein release to determine rate constants for calcium-dependent lipoprotein release. The calcium-dependent rates were compared to rates of acid-dependent release by WT LDLR in the presence of millimolar calcium. Low concentrations of free calcium showed robust ability to drive lipoprotein release of both LDL and β-VLDL (Fig 5 and Table 1). Significantly, the rate constants of calcium-dependent lipoprotein release by the LDLR-ΔBC at 3 μM free calcium (pCa 5.6) were comparable to measured rate constants of acid-dependent lipoprotein release by WT LDLR at 1.8 mM free calcium (pCa 2.7) (Fig 6 and Table 1). At pH 5.5, the acid-dependent release rates were 7.1×10^{-3} sec^{-1} for LDL and 5.1×10^{-3} sec^{-1} for β-VLDL, while the calcium-dependent release rates were 1.1×10^{-2} sec^{-1} for LDL and 4.1×10^{-3} sec^{-1} for β-VLDL (Table 1). Acid-dependent and calcium-dependent release rates were also similar at pH 6.0 with acid-dependent release rates of 1.9×10^{-3} sec^{-1}

for LDL and 8.4×10^{-4} sec^{-1} for β-VLDL and calcium-dependent release rates of 4.9×10^{-3} sec^{-1} for LDL and 1.0×10^{-3} sec^{-1} for β-VLDL (Table 1). Each of these rate constants was significantly faster than the intrinsic rates of lipoprotein dissociation observed with the LDLR-ΔBC in the presence of 1.8 mM free calcium (Fig 5 and 6 and Table 1). Together these observations indicate that both the acid-dependent and calcium-dependent processes provide robust mechanisms to accelerate lipoprotein release.

We next tested whether the calcium-dependent and acid-dependent processes cooperate during lipoprotein release. To test this possibility, WT LDLR cells were used in time courses of lipoprotein release in the presence of media at a pH of 5.5 or 6.0 and at a pCa of 5.0 or 5.6. In all cases, with the exception of β-VLDL release at pH 6.0 and pCa 5.0, reduced free calcium accelerated lipoprotein release (Fig 6 and Table 1). These observations indicate that the calcium-dependent mechanism can cooperate with the acid-dependent mechanism to accelerate lipoprotein release.

The ability of low calcium to promote lipoprotein release suggested the possibility that low calcium might also disrupt the pH-dependent interaction of the β-propeller with LA4/5. We tested this possibility by following LDLR ectodomain conformation as a function of free calcium at pH 6.0, when the β-propeller normally interacts with LA4/5. The acidic conformational state, sometimes referred to as the “closed” state, has a smaller hydrodynamic (Stokes) radius than the extended, “open” conformational state, which predominates at neutral pH (23;45). Using gel filtration to follow Stokes radius, reductions in the free calcium concentration shifted the Stokes radius from ~35Å at high free calcium to a Stokes radius of ~43Å at low free calcium. The half-maximal transition was near 1 μM free calcium (Fig 7). This observation indicates that low calcium not only disrupts lipoprotein binding, but can also reset LDLRs that undergo acid-dependent lipoprotein release back into an open conformation.

Lastly we addressed whether the release mechanisms characterized in the surface release experiments play a role in cellular uptake of lipoproteins. Cellular uptake involves binding of lipoprotein to the LDLR, internalization of LDLR-lipoprotein complexes, release of lipoprotein in endosomes and recycling of the LDLR back to the cell surface. To follow lipoprotein uptake, WT LDLR cells and LDLR-ΔBC cells were incubated with Alexa546 labeled LDL or DiI labeled β-VLDL over the course of four hours. Neither fluorophor is sensitive to acidic pH and both accumulate in lysosomes as a function of lipoprotein uptake (42). LDL uptake was severely compromised in LDLR-ΔBC cells as compared to WT LDLR cells (Fig 8A), while β-VLDL uptake was similar for both LDLR-ΔBC and WT LDLR cells (Fig 8B). These observations indicate that fibroblasts require the acid-dependent release process for LDL uptake but not for β-VLDL uptake.

The ability of the LDLR-ΔBC cells to support normal β-VLDL uptake was unexpected because a previous report used transfected ldl-A7 CHO cells to show that deletion of the entire EGF homology domain (ΔAC) not only eliminated acid-dependent β-VLDL release, but also reduced β-VLDL uptake (20). To directly compare lipoprotein handling by the LDLR-ΔAC and LDLR-ΔBC receptors, we generated fibroblasts expressing the LDLR-ΔAC receptor. Consistent with the prior observations with transfected CHO cells, the LDLR-ΔAC fibroblasts were unable to bind LDL but did bind β-VLDL, though with an affinity that was approximately one third as strong as WT LDLR (Fig 9A and B). Also consistent with the prior observations, the LDLR-ΔAC cells were unable to support either LDL or β-VLDL uptake (Fig 9C and D). The EGF-A module is known to be required for LDL binding (16), and thus the inability of the LDLR-ΔAC to bind LDL can explain the inability of the LDLR-ΔAC cells to support LDL uptake. By contrast, the LDLR-ΔAC cells bound β-VLDL with reduced but still strong affinity, suggesting that the failure of the LDLR-ΔAC cells to support β-VLDL uptake occurs at a later stage in the endocytic pathway of β-VLDL uptake.

Davis et al. showed that treatment of LDLR-ΔAC CHO cells with β-VLDL caused loss of receptors from the cell surface, suggesting that the LDLR-ΔAC fails to recycle in the presence of β-VLDL (20). We tested whether lipoprotein treatment influenced the surface level of receptors by incubating WT LDLR, LDLR-ΔBC, and LDLR-ΔAC cells with either LDL or β-VLDL, biotinylating surface proteins with a non-cell-permeable biotinylation reagent, isolating biotinylated proteins with neutravidin agarose and immunoblotting for the LDLR. LDL treatment did not reduce surface receptors on any of the three cell types (Fig 10A). β-VLDL treatment had little effect on surface receptors on either WT LDLR or LDLR-ΔBC cells, but greatly reduced surface receptors on LDLR-ΔAC cells (Fig 10B). These observations indicate that β-VLDL treatment inhibits the ability of the LDLR-ΔAC to recycle, but not the recycling ability of the LDLR-ΔBC or WT LDLR.

Recycling of the WT LDLR can be inhibited by treatment with the proton ionophore, monensin, an effect that can be augmented by co-treatment with lipoproteins (46;47). We compared the effect of lipoprotein/monensin co-treatment on receptor recycling in WT LDLR, LDLR-ΔAC and LDLR-ΔBC cells. Consistent with expectation, WT LDLR cells rapidly lost surface receptors when treated with a combination of LDL and monensin or β-VLDL and monensin (Fig 10A and B). LDLR-ΔAC cells likewise lost surface receptors with either co-treatment; however, the LDLR-ΔBC cells showed little loss of surface receptors when treated with LDL and monensin and a greatly reduced rate of loss of surface receptors when treated with β-VLDL and monensin (Fig 10A and B). A reduced rate of receptor loss could result either from a reduced rate of internalization or from a faster rate of recycling. To distinguish between these possibilities, we compared the initial rates of β-VLDL internalization by LDLR-ΔBC and WT LDLR cells. As shown in Figure 10C, the LDLR-ΔBC supported a normal rate of β-VLDL internalization, suggesting that the slower rate of surface receptor loss results from an enhanced ability of the LDLR-ΔBC to recycle in the presence of monensin and β-VLDL.

DISCUSSION

The mechanisms by which the endosomal lumen triggers lipoprotein release from the LDLR are not fully understood. In *in vitro* assays, both acidic pH and low calcium are sufficient to drive lipoprotein release. The acid-dependent mechanism requires the EGF-homology domain (20), which forms an intramolecular contact with the ligand-binding domain at acidic pH (23). This association appears to drive an allosteric process that accelerates lipoprotein release (24). In cells, the acidification of endosomes also promotes the gating of an endosomal calcium channel, most likely the transient receptor potential V2 channel (33;48). Activation of the endosomal calcium channel rapidly lowers the free calcium concentration in the endosomal lumen to micromolar concentrations (33). Low free calcium can drive lipoprotein release by extracting calcium from the LA repeats and thereby inducing structural changes in the lipoprotein-binding surfaces on the LDLR (27;29;30). Here, we show that this calcium-dependent mechanism accelerates release of both LDL and β-VLDL with rates similar to those of the acid-dependent mechanism (Table 1). We propose that both the acid-dependent and calcium-dependent mechanisms operate during endosomal release of lipoproteins. In the case of acid-dependent release, low calcium may act subsequent to lipoprotein dissociation to disrupt the closed conformational state of the LDLR (Fig 7), thereby restoring the LDLR to an open conformation prior to delivery to the cell surface.

If both the acid-dependent and calcium-dependent processes provide efficient mechanisms of lipoprotein release, why have both? One potential reason for two mechanisms is to make the release process less reversible. The *in vitro* release experiments mimic three aspects of endosomal conditions: temperature (37°C), acidity (pH 5.5–6.0) and free calcium (pCa 5.0–5.6). One feature, which cannot be mimicked, is lipoprotein concentration. In the *in vitro* assays, lipoproteins release into the extracellular milieu, which has a large volume. As a consequence,

release is irreversible because the concentration of lipoprotein is very low in the release media. By contrast, the endosomal lumen has a small volume and the endosomal concentration of lipoprotein is high after release. This high concentration of lipoprotein has the potential to reverse the release process. The combined action of the acid-dependent and calcium-dependent mechanisms may prevent rebinding of lipoproteins after release.

The inability of LDLR-ΔBC cells to support LDL uptake indicates that the calcium-dependent process is not sufficient for cellular release of LDL and that LDL uptake requires the acid-dependent release process. Consistent with this observation, mutations that increase the acid requirement for acid-dependent release impair cellular uptake of LDL (24). Interestingly, mutations that increase the acid requirement by as little as 0.5 pH units can reduce LDL uptake by more than 90%, suggesting that LDL uptake is exquisitely sensitive to the acid-dependent release process.

Whether LDL uptake also requires the calcium-dependent process is not clear. The LDLR completes a cycle of LDL uptake every ~12 min (49), indicating that LDL release occurs in early endosomes, where the pH is ≥6.0 and free calcium concentrations are ≥10 μM (33). Comparison of the calcium-dependent rate constant of LDL release by LDLR-ΔBC at pH 6.0 and pCa 5.0 (9.5×10^{-4} sec^{-1}) with acid-dependent release by WT LDLR at pH 6.0 and pCa 2.7 (1.9×10^{-3} sec^{-1}) indicates that the acid-dependent process is a stronger driver of LDL release at early endosomal conditions (Table 1). However, LDL release by WT LDLR at pCa 5.0 (3.1×10^{-3} sec^{-1}) was faster than at pCa 2.7 (1.9×10^{-3} sec^{-1}), suggesting that low free calcium may facilitate LDL release. Unfortunately, inhibition of the endosomal calcium channel does not provide a means to determine whether LDL uptake requires the calcium-dependent process because fusion of primary endosomes with early endosomes requires calcium efflux from the endosomal lumen (50–52). Use of the calcium ionophore, A23187, to increase cellular calcium and collapse the endosomal calcium gradient inhibits LDL uptake by ~60% (53). This observation is consistent with the possibility that LDL release is inhibited when endosomal calcium cannot be reduced; however, calcium plays multiple roles in endocytosis and elevated levels of intracellular calcium can promote, inhibit or have little effect on endocytic uptake depending upon the ligand and system (54–57).

A key observation of this study is that β-VLDL uptake is normal in LDLR-ΔBC expressing cells. This observation indicates that the acid-dependent lipoprotein release is not required for β-VLDL uptake and suggests that the calcium-dependent process may be the principal driver of β-VLDL release. Two questions raised by this observation are first, why does β-VLDL uptake not require acid-dependent release and second, why is the process of endosomal release of β-VLDL not sufficient for LDL release?

One potential answer to the question of why β-VLDL uptake does not employ the acid-dependent lipoprotein release is the observation that acid-dependent β-VLDL release requires harsher acidic conditions than LDL release (Fig 2 and Fig 6). This difference in pH sensitivity may be caused by the higher affinity of the LDLR for β-VLDL as compared to LDL or by the ability of multiple LDLR to bind β-VLDL simultaneously as compared to the 1:1 stoichiometry between LDL and the LDLR (58;59). Consistent with the latter possibility, the number of apoE present on β-VLDL influences uptake of β-VLDL (60). Reduced sensitivity to low pH may thus preclude the acid-dependent mechanism for cellular release of β-VLDL.

A likely key to the question of why the mechanism driving β-VLDL release cannot support LDL release is differences in the intracellular trafficking of LDL and β-VLDL after internalization. Comparison of the endocytic path of LDL and β-VLDL in macrophages has shown that while internalized LDL rapidly accumulates in late endosomes and lysosomes, internalized β-VLDL accumulates first in peripheral structures, presumably an endosome-like

compartment, before trafficking to degradative compartments (60–62). This bypass suggests that β-VLDL may be held in a specialized endocytic compartment to provide the LDLR-β-VLDL complex with additional time or a more conducive environment for release. Targeting of β-VLDL to these compartments appears to involve engagement of multiple LDLR because accumulation of β-VLDL in these peripheral structures is augmented by increased apoE content of β-VLDL (60). While similar experiments have not been done in fibroblasts, fibroblasts show delayed degradation of apoE containing lipoproteins (61;63;64), consistent with the possibility that fibroblasts also have a bypass segment in the degradative pathway of β-VLDL. Residence of β-VLDL in peripheral endosome-like structures may facilitate the calcium-dependent release process.

The ability of the LDLR-ΔBC, but not LDLR-ΔAC, cells to support β-VLDL uptake suggests that the EGF-A module plays a critical role in receptor trafficking. Consistent with this possibility, natural deletions that remove either both EGF-A and EGF-B or just EGF-A result in familial hypercholesterolemia (FH) (65–69) and fibroblasts from individuals that are homozygous for the deletion removing both the EGF-A and EGF-B modules express an LDLR variant that recycles poorly in the presence of β-VLDL (70). The EGF-A module is also been implicated in LDLR trafficking because the secreted protein, PCSK9, binds to EGF-A and promotes LDLR degradation by redirecting internalized LDLR to lysosomes (71). The observation that the LDLR-ΔBC receptor was less sensitive than the LDLR-ΔAC to monensin (Fig 10) also suggests that the EGF-A promotes receptor recycling. Interestingly, the WT LDLR is sensitive to monensin and this sensitivity is heightened in the presence of lipoprotein (42;46). The reduced sensitivity of the LDLR-ΔBC to monensin/lipoprotein co-treatment indicates that the EGF-B to EGF-C (BC) region enhances the monensin sensitivity of the WT LDLR and suggests that the BC region may act to inhibit recycling prior to lipoprotein release. The process of recycling prior to lipoprotein release is termed retro-endocytosis and in fibroblasts, accounts for ~10% of the LDL that is internalized by the LDLR (72;73). Interestingly, LDLR-ΔBC cells bind LDL normally; however, these cells are unable to accumulate LDL and the surface level of receptors does not change in the presence of LDL (Fig 1C, Fig 8A and 10A). These observations suggest that the LDLR-ΔBC recycles prior to lipoprotein release. The BC region may thus not only participate in acid-dependent release, but may also prevent receptor recycling until release has occurred.

In summary, both acidic pH and low free calcium can drive lipoprotein release. The acid-dependent process is required for LDL uptake, but not for β-VLDL uptake, suggesting that the calcium-dependent process may participate in the cellular release of β-VLDL. Cellular accumulation of β-VLDL requires the EGF-A module, which appears to participate in LDLR trafficking.

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Abbreviations

ApoB100
: Apolipoprotein B100

ApoE
: Apolipoprotein E

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

EDTA
ethylenediaminetetraacetic acid

EGF
epidermal growth factor

EGTA
ethyleneglycol-O, O′-bis(2-aminoethyl)-N, N, N′, N′-tetraacetic acid

HEDTA
N-(2-hydroxyethyl)ethylenediamine-N, N′, N′ tetraacetic acid

HEPES
4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid

LA
LDLR type A repeat

LDL
low density lipoprotein

LDLR
low density lipoprotein receptor

MEM
minimal essential medium

D-MEM
Dulbecco's modified Eagle's medium

VLDL
very low density lipoprotein

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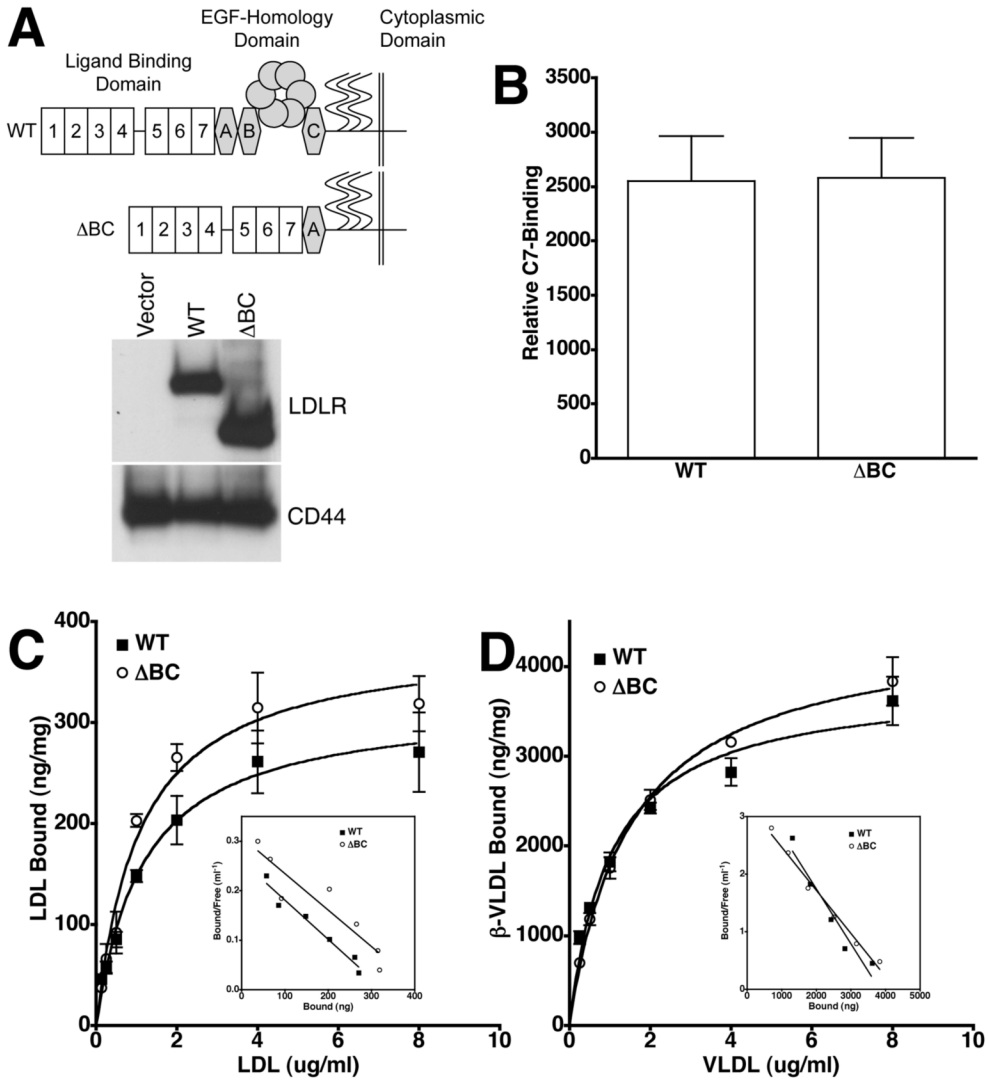

Figure 1. The LDLR-ΔBC has normal expression and ability to bind lipoproteins

Panel A shows the domains contained in WT LDLR and the LDLR-ΔBC protein. The seven LA repeats of the ligand-binding domain are labeled with numbers. Letters identify the three EGF-like repeats of the EGF-homology domain. The six circles indicate the YWTD repeats of the β-propeller. The wavy lines indicate the O-linked sugar domain. The cytoplasmic domain is labeled. Below the line drawing are immunoblots showing the relative expression of WT LDLR (WT) or LDLR-ΔBC (ΔBC). Also shown is an immunoblot of CD44, which is a plasma membrane protein that is used as a loading control. Panel B shows the relative surface expression of LDLR by cells expressing WT LDLR or LDLR-ΔBC as determined by cell cytometry using the C7 monoclonal antibody against the LDLR ectodomain. Panel C shows saturation binding of ^{125}I-LDL to cells expressing WT LDLR or LDLR-ΔBC. Panel D shows saturation binding of ^{125}I-β-VLDL. The inserts in Panels C and D are Scatchard plots of the saturation data shown in the respective panels.

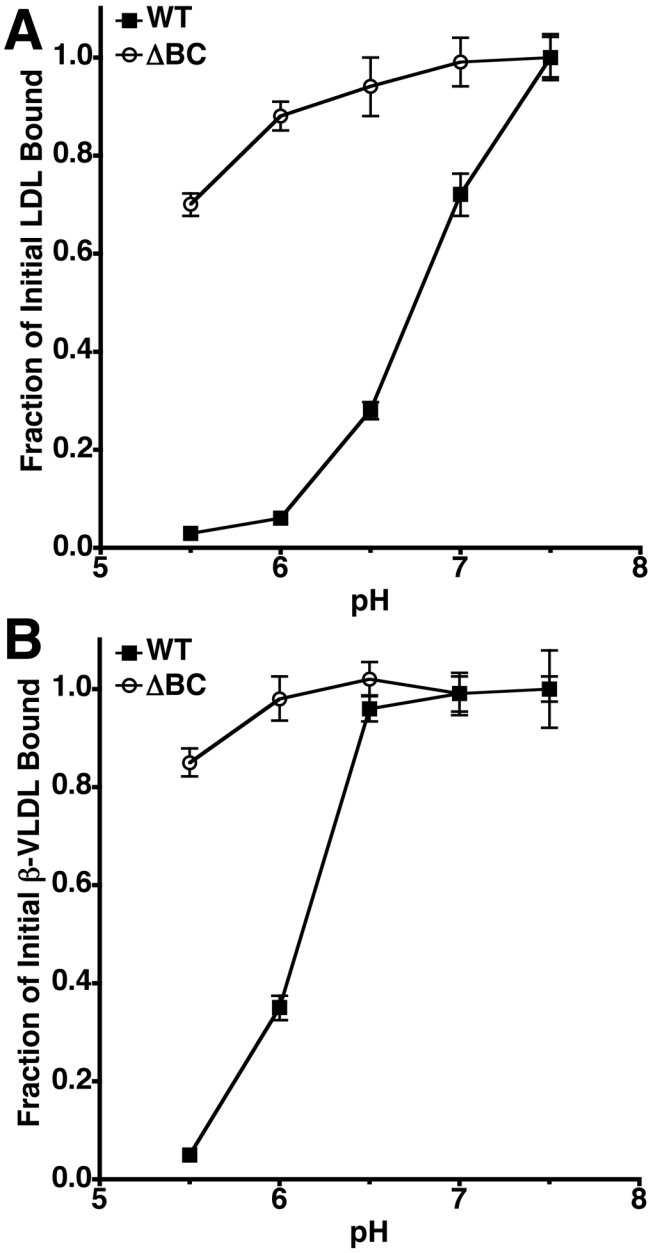

Figure 2. The LDLR-ΔBC does not support acid-dependent lipoprotein release

Cells expressing WT LDLR (WT) or LDLR-ΔBC (ΔBC) were incubated with either 10 μg/ml ^{125}I-LDL (Panel A) or 5 μg/ml ^{125}I-β-VLDL (Panel B) for 1 hr, washed and further incubated with media at pH 5.5–7.5 for 30 min. Cell associated lipoprotein remaining after treatments were determined by γ-counter. Data is presented as a fraction of cell associated lipoprotein at pH 7.5. All experiments were performed in triplicate. Error bars show standard deviation. Calcium concentration was maintained at 1.8 mM (pCa 2.7) for all experiments.

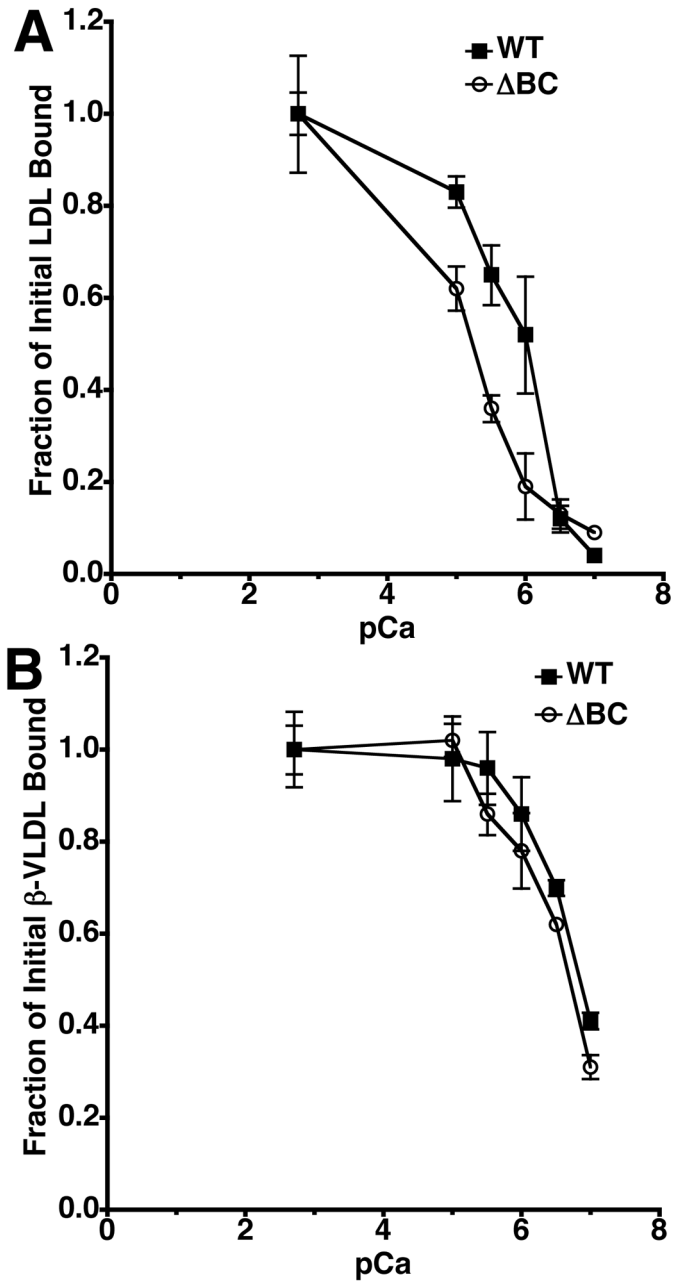

Figure 3. Low calcium drives similar lipoprotein release from LDLR-ΔBC and WT LDLR expressing cells

Cells expressing either WT LDLR (WT) or LDLR-ΔBC (ΔBC) were incubated with either 10 μg/ml ^{125}I-LDL (Panel A) or 5 μg/ml ^{125}I-β-VLDL (Panel B) for 1 hr, washed and then incubated for 30 min with media at pH 7.0 and the indicated free calcium concentrations. Free calcium concentrations are shown as pCa (−log(free [Ca^{2+}])). Cell associated lipoprotein remaining after treatments were determined by γ-counter. Data is presented as a fraction of cell associated lipoprotein at pH 7.0 and pCa 2.7. All experiments were performed in triplicate. Error bars show standard deviation.

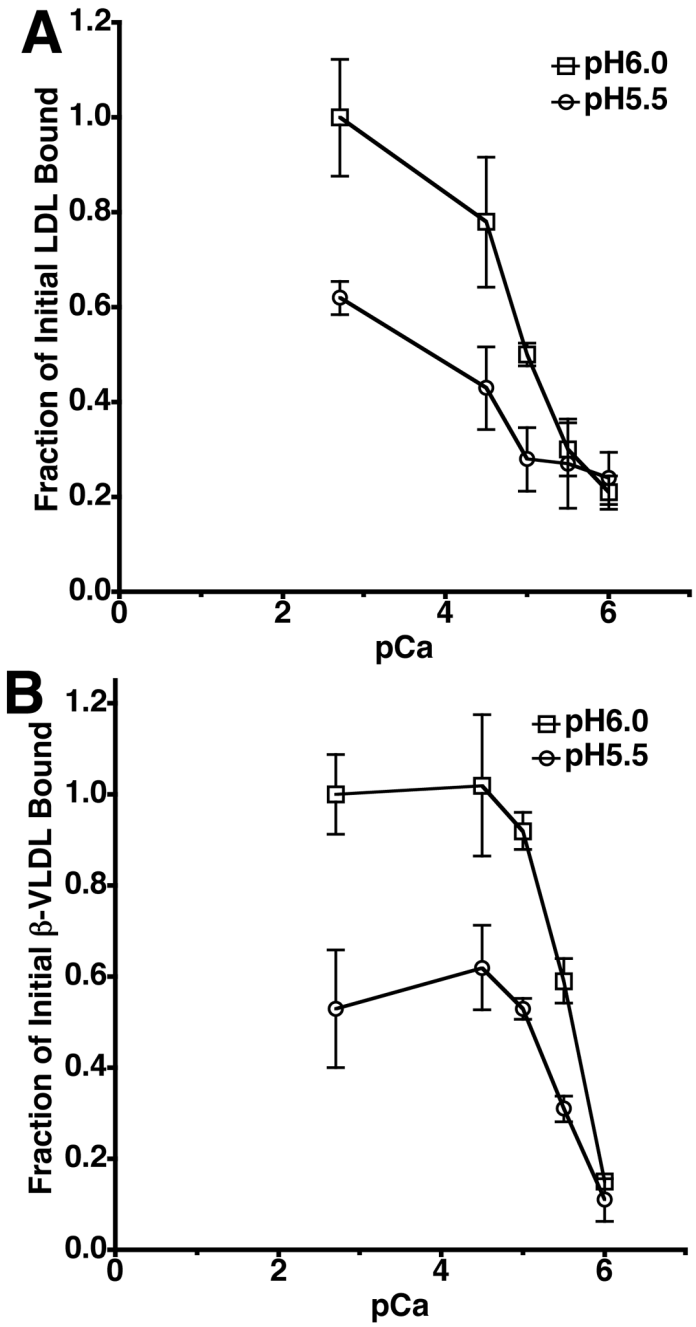

Figure 4. Acidic pH augments calcium-dependent lipoprotein release by the LDLR-ΔBC

Cells expressing LDLR-ΔBC were incubated with either 10 μg/ml ^{125}I-LDL (Panel A) or 5 μg/ml ^{125}I-β-VLDL (Panel B) for 1 hr, washed, and further incubated with for 30 min with media at pH 6.0 or pH 5.5 and the indicated free calcium concentrations. Free calcium concentrations are shown as pCa (−log(free [Ca^{2+}])). Cell associated lipoprotein remaining after treatments were determined by γ-counter. Data is presented as a fraction of cell associated lipoprotein at pH 6.0 and pCa 2.7. All experiments were performed in triplicate. Error bars show standard deviation.

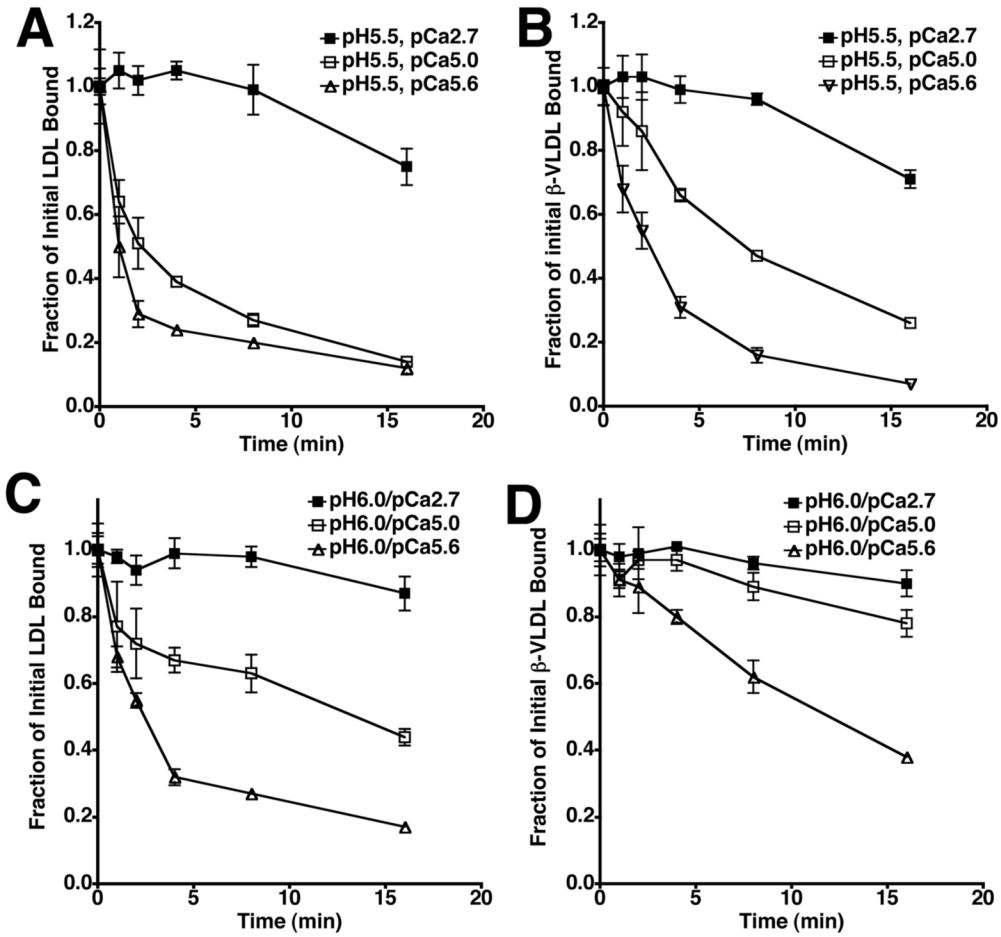

Figure 5. The calcium-dependent mechanism mediates efficient lipoprotein release at endosomal pH

Cells expressing LDLR-ΔBC were incubated with either 10 μg/ml ^{125}I-LDL (Panels A and C) or 5 μg/ml ^{125}I-β-VLDL (Panel B and D) for 1 hr, washed, and further incubated for the indicated times with media at pH 5.5 (Panels A and B) or pH 6.0 (Panels C and D) and the indicated free calcium concentrations. Free calcium concentrations are shown as pCa (−log (free [Ca^{2+}])). Cell associated lipoprotein remaining after treatments were determined by γcounter. All experiments were performed in triplicate. Error bars show standard deviation.

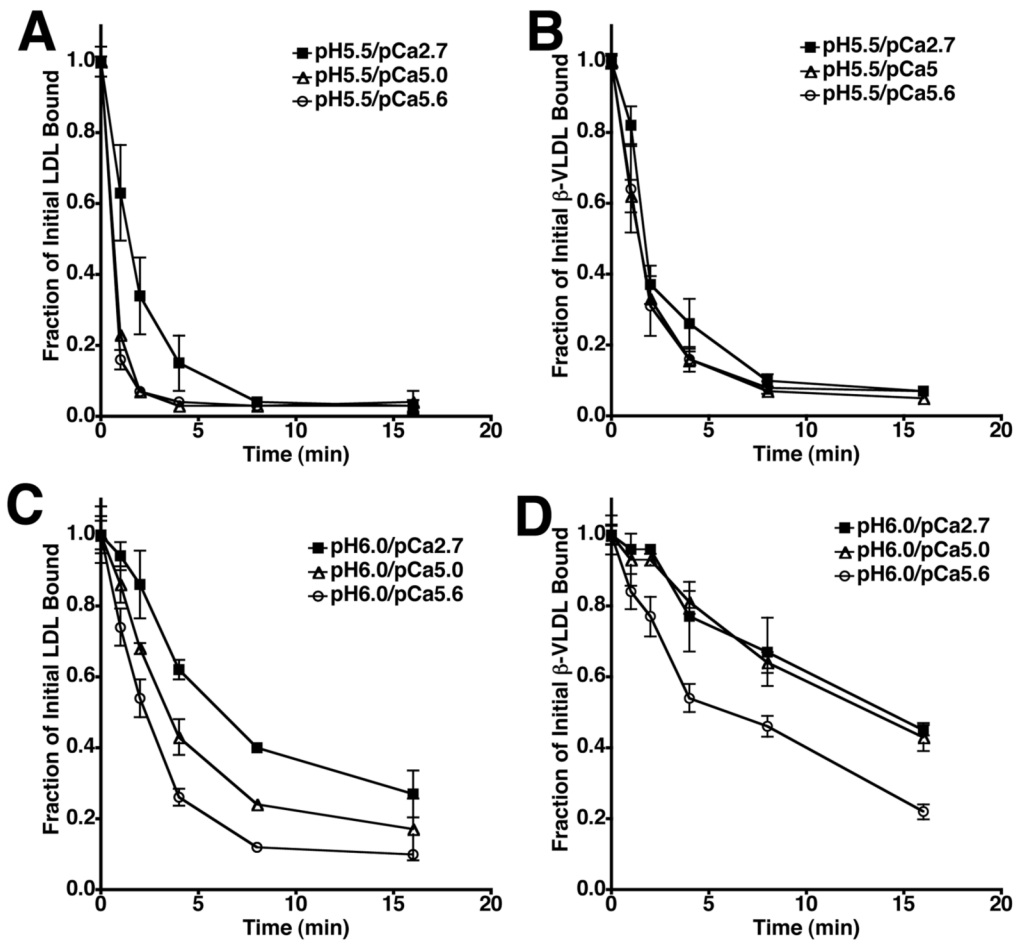

Figure 6. The calcium-dependent and acid-dependent mechanisms cooperate during lipoprotein release by WT LDLR

Cells expressing WT LDLR were incubated with either 10 μg/ml ^{125}I-LDL (Panels A and C) or 5 μg/ml ^{125}I-β-VLDL (Panel B and D) for 1 hr, washed, and further incubated for the indicated times with media at pH 5.5 (Panels A and B) or pH 6.0 (Panels C and D) and the indicated free calcium concentrations. Free calcium concentrations are shown as pCa (−log (free [Ca^{2+}])). Cell associated lipoprotein remaining after treatments were determined by γ-counter. All experiments were performed in triplicate. Error bars show standard deviation.

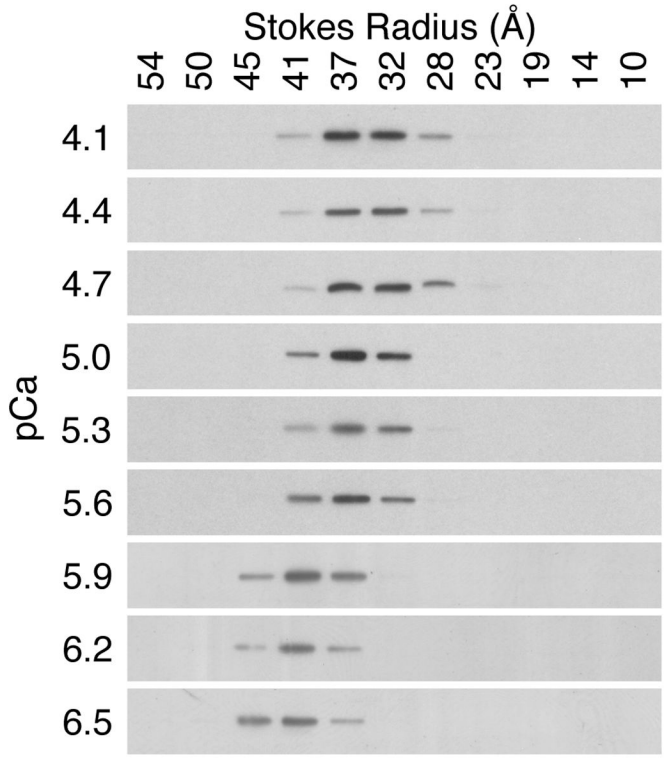

Figure 7. Low calcium disrupts the acid-dependent closed conformation of the LDLR

WT LDLR ectodomain was incubated at pH 6.0 and the indicated free calcium concentrations for 30 min and then chromatographed over a superdex200 gel filtration column equilibrated at the same pH and free calcium concentration. Free calcium concentrations are shown as pCa (−log(free [Ca^{2+}])). Fractions corresponding to the indicated Stokes radius were electrophoresed on 5–17% SDS-PAGE gels and immunoblotted for LDLR.

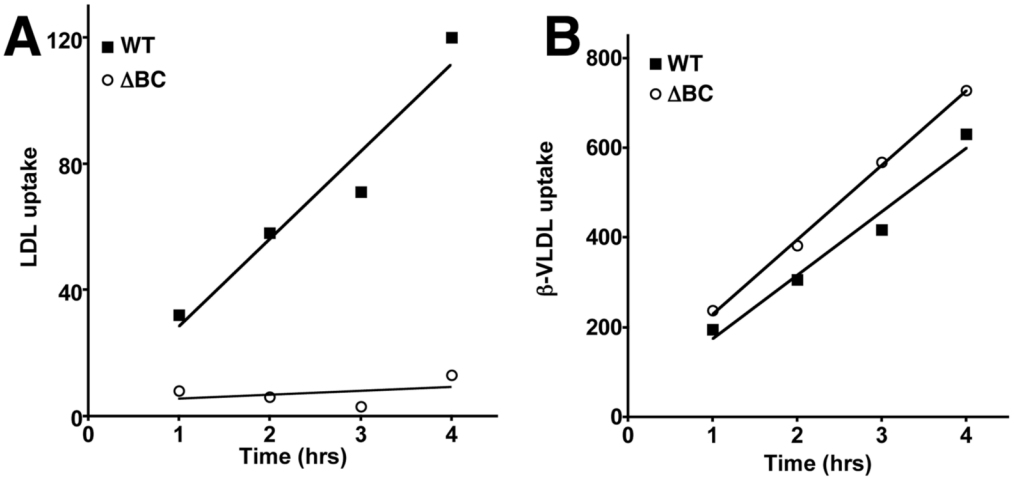

Figure 8. LDL uptake requires the acid-dependent mechanism, while β-VLDL uptake does not

Cells expressing WT LDLR (WT) or LDLR-ΔBC (ΔBC) were incubated with either 10 μg/ml Alexa546-LDL (Panel A) or 5 μg/ml DiI-β-VLDL (Panel B) for the indicated times. Cells were then washed, fixed and processed by cell cytometry. Mean fluorescence values are shown for 10,000 cells at each time point. Experiment shown is representative of three independent trials.

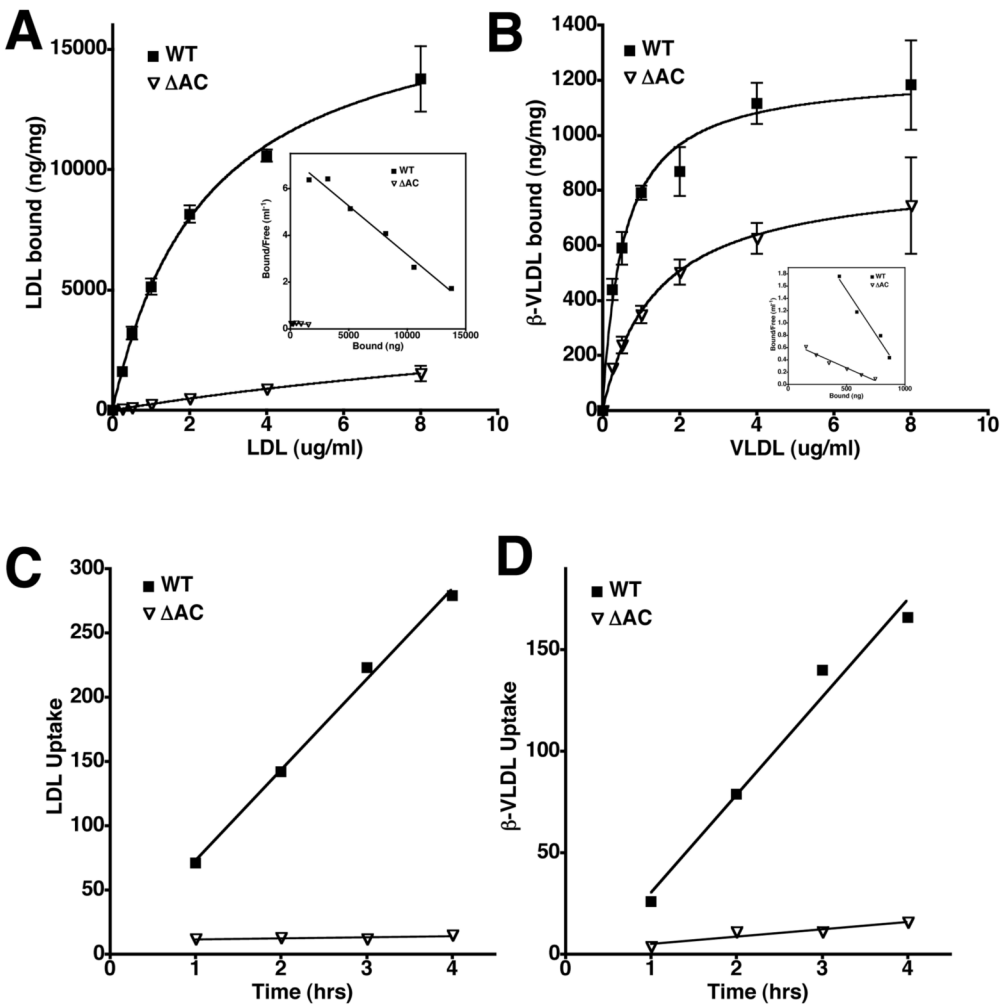

Figure 9. The LDLR-ΔAC cannot support either LDL or β-VLDL uptake

The affinity of LDL and β-VLDL for fibroblasts expressing only the LDLR-ΔAC receptor was measured using saturation binding assays with ^{125}I-LDL (Panel A) and ^{125}I-β-VLDL (Panel B). The inserts in Panels A and B are Scatchard plots of the saturation data shown in the respective panels. Panels C and D compare the ability of the LDLR-ΔAC cells to support uptake of Alexa546-labeled LDL and DiI-labeled β-VLDL with WT LDLR cells over a four hour time course. Lipoprotein uptake experiments shown are representative of three independent trials.

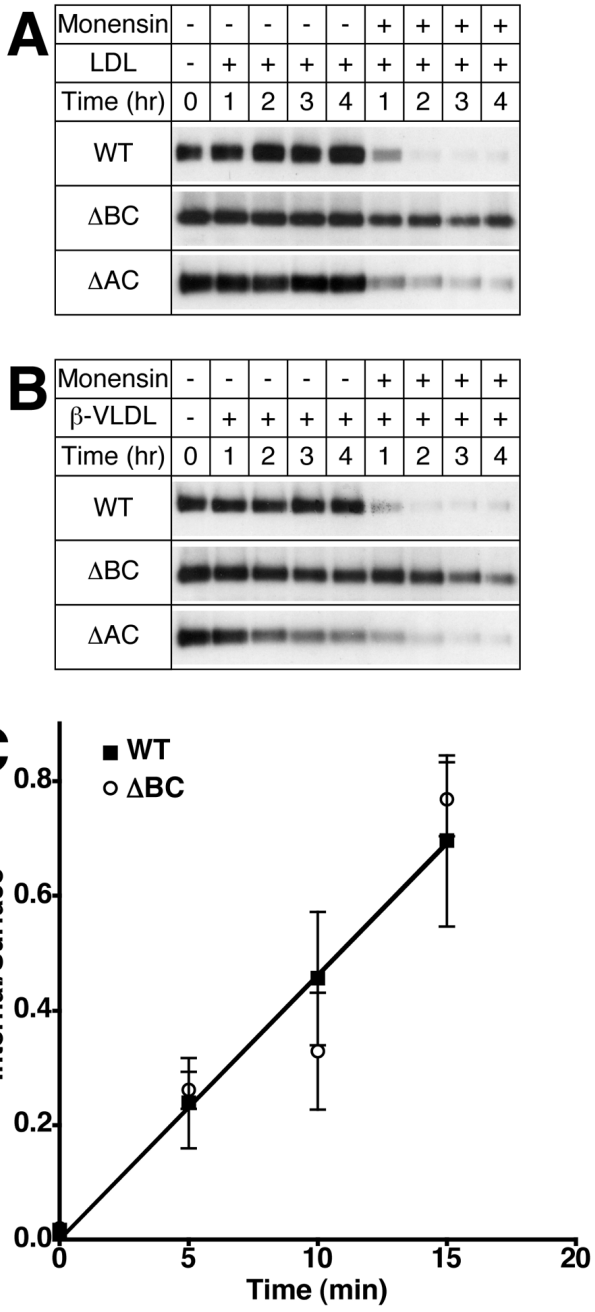

Figure 10. LDLR-ΔAC recycling is sensitive to β-VLDL treatment, while LDLR-ΔBC recycling is resistant to the effect of lipoproteins and monensin

The levels of surface receptor on fibroblasts expressing WT LDLR (WT), LDLR-ΔBC (ΔBC) or LDLR-ΔAC were compared using surface biotinylation over a four hour time course of LDL (Panel A) or β-VLDL (Panel B) treatment in the presence or absence of 30 μM monensin. Surface biotinylated proteins were isolated by neutravidin agarose and immunoblotted for the presence of LDLR. Experiments shown are representative of three independent trials. Panel C compares the initial rates of β-VLDL internalization by WT LDLR (WT) and LDLR-ΔBC (ΔBC) cells. Experiments were performed as described in Experimental Procedures and are presented as means ± one standard deviation.

Table 1

Rates of Lipoprotein Release

LDLR	pH	Lipoprotein	pCa2.7	pCa5.0	pCa5.6
ΔBC	6.0	LDL	1.3×10^{-4}	9.5×10^{-4}	4.9×10^{-3}
ΔBC	6.0	VLDL	9.84×10^{-5}	2.5×10^{-4}	1.0×10^{-3}
ΔBC	5.5	LDL	2.1×10^{-4}	4.4×10^{-3}	1.1×10^{-2}
ΔBC	5.5	VLDL	2.8×10^{-4}	1.5×10^{-3}	4.1×10^{-3}
WT	6.0	LDL	1.9×10^{-3}	3.1×10^{-3}	4.7×10^{-3}
WT	6.0	VLDL	8.4×10^{-4}	8.9×10^{-4}	1.6×10^{-3}
WT	5.5	LDL	7.1×10^{-3}	2.3×10^{-2}	2.4×10^{-2}
WT	5.5	VLDL	5.1×10^{-3}	8.0×10^{-3}	8.0×10^{-3}

All rate constants are in sec^{-1}.