PCSK9-mediated degradation of the LDL receptor generates a 17 kDa C-terminal LDL receptor fragment

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Abstract Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to the LDL receptor (LDLR) at the cell surface and reroutes the internalized LDLR to intracellular degradation. In this study, we have shown that PCSK9-mediated degradation of the full-length 160 kDa LDLR generates a 17 kDa C-terminal LDLR fragment. This fragment was not generated from mutant LDLRs resistant to PCSK9-mediated degradation or when degradation was prevented by chemicals such as ammonium chloride or the cysteine cathepsin inhibitor E64d. The observation that the 17 kDa fragment was only detected when the cells were cultured in the presence of the γ-secretase inhibitor DAPT indicates that this 17 kDa fragment undergoes γ-secretase cleavage within the transmembrane domain. The failure to detect the complementary 143 kDa ectodomain fragment is likely to be due to its rapid degradation in the endosomal lumen. The 17 kDa C-terminal LDLR fragment was also generated from a Class 5 mutant LDLR undergoing intracellular degradation.

Supplementary key words cathepsin • cleavage • degradation • endosome • low density lipoprotein

The cell-surface LDL receptor (LDLR) plays a key role in the regulation of plasma levels of LDL cholesterol (1). The role of the LDLR in this respect is illustrated by the high levels of LDL cholesterol in familial hypercholesterolemia homozygotes that lack functioning LDLRs (1). At the cell surface, the LDLR binds and internalizes LDL by receptor-mediated endocytosis (1). At the mildly acidic pH of the sorting endosome, LDL is released from the LDLR and the LDLR folds back on itself to adopt a closed conformation (2, 3). Whereas released LDL is predominantly confined to the vesicular part of the sorting endosome, which eventually matures to become a late endosome, the membrane-bound LDLR recycles back to the cell membrane (4, 5). Each LDLR makes approximately 100 cycles during its lifespan of approximately 24 h, irrespective of whether it has bound LDL (5).

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a proprotein convertase that binds to the LDLR at the cell surface and reroutes the internalized LDLR to intracellular degradation (6–8). PCSK9 is synthesized as a 692 amino acid protein that undergoes autocatalytic cleavage in the endoplasmic reticulum and is secreted as a complex consisting of the cleaved prodomain noncovalently bound to the catalytic domain (6). At the cell surface, a patch of residues in the catalytic domain of PCSK9 binds to the epidermal growth factor (EGF)-A repeat of the LDLR, and the LDLR:PCSK9 complex is internalized by clathrin-mediated endocytosis (7, 9, 10). However, in contrast to LDL, which is released from the LDLR at the acidic milieu of the sorting endosome, the affinity of PCSK9 toward the LDLR increases at acidic pH (11, 12). An additional interaction between the cysteine-rich domain of PCSK9 and the ligand-binding domain of the LDLR is required for PCSK9 to remain bound to the LDLR at acidic pH (13, 14).

The mechanism by which PCSK9 disrupts the normal recycling of the LDLR could involve inhibition of active recycling, or it could involve activation of machinery that identifies an LDLR with bound PCSK9 and reroutes this LDLR to intracellular degradation. One mechanism for rerouting receptors with bound ligands to intracellular degradation is ubiquitination of their cytoplasmic domains, which subsequently activates the endosomal sorting complex required for transport (ESCRT) (15). Such a mechanism is common for signaling receptors like the EGF receptor (15). Moreover, ubiquitination of the cytoplasmic domain of the LDLR is required for Idol to mediate degradation of the LDLR in the endoplasmic reticulum (16). However, because an LDLR lacking the cytoplasmic...
domain is rerouted to intracellular degradation after binding PCSK9 (17), neither ubiquitination nor other modification of the cytoplasmic domain of the LDLR is required for the LDLR to be rerouted to intracellular degradation. One could therefore speculate that a coreceptor may recognize the LDLR:PCSK9 complex within the lumen of the sorting endosome and redirect the LDLR:PCSK9 complex to intracellular degradation.

However, an alternative mechanism for the activity of PCSK9 toward the LDLR could involve cleavage of the ectodomain of the LDLR in the sorting endosome. If the ectodomain of the LDLR is cleaved and released into the lumen of the sorting endosome, it will be located predominantly in the vesicular part of the endosome, which matures to become a late endosome that subsequently fuses with a lysosome. In this study, we addressed the question of whether an LDLR with bound PCSK9 could undergo ectodomain cleavage following internalization of the LDLR:PCSK9 complex.

### MATERIALS AND METHODS

#### Reagents

- N-(N-(3,5-difluorophenacetyl)-L-alanyl)-S-phenylglycine t-butyl ester (DAPT) and L-685,458 were purchased from Sigma-Aldrich Corp. (St. Louis, MO).
- Leupeptin was from Peptanova GmbH (Sanshausen, Germany) and E64d was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA).
- All other reagents were standard laboratory reagents.

#### Plasmids and transfections

Generation of the plasmids pcDNA4-LDLR encoding the wild-type (WT) LDLR with a HA-tag (18); Δ-EGF-A-LDLR, which encodes an LDLR lacking the EGF-A repeat (19); G544V-LDLR, which encodes the Class 2a mutant LDLR caused by mutation G544V (18); Δ-β-propeller, which encodes an LDLR lacking the β-propeller (17); and E387K-LDLR, which encodes the Class 5 mutant LDLR caused by mutation E387K (19), have been previously described. The plasmid pcDNA4-LDLR was used as a template to generate the plasmid ΔOLSD-LDLR, which encodes an LDLR lacking the O-linked sugar domain (residues 693–750). Mutagenesis was performed using QuickChange XL Mutagenesis Technology, Inc. (Santa Cruz, CA) according to the manufacturer’s instructions. The primer sequences used for mutagenesis were 5′-ATGAGGAGCCTGCTCAACACTGGCGACGTGGCTGGC3′ and 5′-GCCGCCAAGGTGGCCACGTGGAGCTCTTCA-3′.

The integrity of the plasmid was confirmed by DNA sequencing. CHO T-REx cells (Invitrogen, Carlsbad, CA) were stably transfected and cultured as previously described (20). These cells are stably transfected with a tetracycline repressor that enables regulated intramembrane proteolysis, the cytoplasmic domain remains membrane-bound, which increases its stability (22, 23). As determined by Western blot analysis using an antibody against the cytoplasmic domain of the LDLR, a rabbit polyclonal anti-LDLR antibody (Fitzgerald Industries International, Acton, MA), which recognizes the cytoplasmic domain of the LDLR, a rabbit polyclonal anti-LDLR antibody (Fitzgerald Industries International, Acton, MA), which recognizes the cytoplasmic domain of the LDLR, a rabbit polyclonal anti-LDLR antibody (Fitzgerald Industries International, Acton, MA), which recognizes the cytoplasmic domain of the LDLR.

#### Purification of PCSK9

The gain-of-function mutant D374Y-PCSK9, caused by mutation D374Y in the PCSK9 gene, was purified from media of stably transfected HEK293 cells as previously described (19), except that the gel filtration step was omitted. The purified D374Y-PCSK9 contained a C-terminal V5-his tag. There was no indication of the presence of multimers as determined by SDS-PAGE (supplementary Fig. 1).

#### Western blot analyses

Western blot analyses of cell lysates were carried out as previously described (21). Briefly, cell lysates were obtained and run on 4–20% Tris-HCl Criterion Precast Gels (Bio-Rad, Hercules, CA) and blotted onto Immuno-Blot PVDF Membranes (Bio-Rad). To detect PCSK9, the membranes were immunostained with a mouse anti-V5 antibody (Invitrogen, Carlsbad, CA). To detect the LDLR, the membranes were immunostained with a rabbit anti-LDLR antibody EP1553Y (Abcam, Cambridge, UK), which recognizes the cytoplasmic domain of the LDLR, a rabbit polyclonal anti-LDLR antibody (Fitzgerald Industries International, Acton, MA), which recognizes the link region between ligand-binding repeats 4 and 5, a rabbit anti-HA antibody (Invitrogen), or a mouse anti-Flag antibody (Sigma-Aldrich Corp., St. Louis, MO).

### RESULTS

#### PCSK9-mediated degradation of the LDLR generates a 17 kDa C-terminal LDLR fragment

The gain-of-function mutant D374Y-PCSK9 was added to cultured HepG2 cells in the presence or absence of the γ-secretase inhibitor DAPT. By inhibiting γ-secretase cleavage within the transmembrane domain of proteins undergoing regulated intramembrane proteolysis, the cytoplasmic domain remains membrane-bound, which increases its stability (22, 23). As determined by Western blot analysis using an antibody against the cytoplasmic domain of the LDLR, the amount of the mature 160 kDa LDLR decreased with time as expected, both in the presence and absence of DAPT (Fig. 1). Thus, DAPT does not affect PCSK9-mediated degradation of the LDLR. However, in the presence of DAPT, a 17 kDa band was also detected. Moreover, as the amounts of the mature 160 kDa LDLR started to decrease 2 h after the addition of D374Y-PCSK9, the amount of the 17 kDa band started to increase, and there was a significant negative correlation between the amounts of the 160 kDa band and the 17 kDa band (r = −0.93, P < 0.001) (Fig. 1). Similar data were observed with the γ-secretase inhibitor L-685,458 (data not shown). The 17 kDa band was not detected when an N-terminal antibody was used for Western blot analysis (data not shown). To confirm that the 17 kDa fragment was a part of the LDLR, Western blot analysis of lysates from HepG2 cells expressing a C-terminal HA-tagged LDLR was performed using an antibody against the HA-tag (supplementary Fig. II). Together these data indicate that in the presence of a

PCSK9 generates a 17 kDa C-terminal LDL receptor fragment

1561
G544V-LDLR, which is retained in the endoplasmic reticulum (18) (Fig. 2). Thus, the 17 kDa C-terminal fragment was only generated from an LDLR that reaches the cell surface and undergoes PCSK9-mediated degradation.

Effect of ammonium chloride on the generation of the 17 kDa fragment

Ammonium chloride prevents PCSK9-mediated degradation of the LDLR by increasing the pH of acidic organelles (26, 27). With increasing concentrations of ammonium chloride, the activity of PCSK9 toward the LDLR gradually decreased as demonstrated by the increased amount of the mature 160 kDa LDLR (Fig. 3). As the amount of the 160 kDa band increased, the amount of the 17 kDa fragment decreased proportionally ($r = -0.91, P < 0.0001$) (Fig. 3). This finding is consistent with the notion that the 17 kDa LDLR fragment is generated in an acidic organelle as a result of PCSK9-mediated degradation.

Effect of cathepsin inhibitors on the amount of the 17 kDa fragment

Leupeptin is a reversible inhibitor of serine, threonine, and cysteine cathepsins, and E64d is an irreversible inhibitor of cysteine cathepsins. Leupeptin did not prevent PCSK9-mediated degradation of the LDLR in HepG2 cells, but it almost completely prevented generation of the 17 kDa C-terminal fragment (Fig. 4). However, in the presence of leupeptin, a 40 kDa C-terminal fragment was observed (Fig. 4). These findings indicate that leupeptin inhibits the enzyme involved in the PCSK9-induced ectodomain cleavage to generate the 17 kDa C-terminal fragment. As determined by the presence of the 40 kDa C-terminal fragment, the ectodomain must have undergone a second cleavage by an enzyme insensitive to leupeptin. However, the resulting 120 kDa N-terminal fragment was not observed.

**Fig. 1.** PCSK9-mediated degradation of the LDLR in the presence of DAPT. HepG2 cells were cultured in the presence or absence of DAPT (10 µM) overnight before D374Y-PCSK9 (1 µg/ml) was added for a further incubation for various time periods as indicated. Western blot analysis of cell lysates was performed using an antibody against the cytoplasmic domain of the LDLR. The identified bands represent the 160 kDa mature LDLR, a truncated LDLR lacking ligand-binding repeats 1–4 (Δ1-4-LDLR) (24), the 120 kDa precursor LDLR, a 17 kDa C-terminal LDLR fragment as well as two nonspecific bands (*). Three separate Western blot analyses were performed, of which one representative Western blot is shown.
PCSK9 generates a 17 kDa C-terminal LDL receptor fragment

by Western blot analysis using an antibody against the ligand-binding domain (data not shown). E64d prevented the PCSK9-mediated degradation of the LDLR, and no increased amounts of the 17 kDa C-terminal fragment and no 40 kDa C-terminal fragment were observed (Fig. 4). Thus, E64d prevented both cleavages of the ectodomain of the LDLR. The metalloproteinase inhibitors batimastat, TAPI-1, and ilomastat did not prevent the PCSK9-induced generation of the 17 kDa fragment (data not shown).

Intracellular degradation of a Class 5 mutant LDLR

Class 5 mutant LDLRs that have bound LDL on the cell surface do not recycle but instead undergo intracellular degradation after being internalized (1). Western blot analysis of lysates from CHO T-REx cells stably transfected with the Class 5 mutant E387K-LDLR plasmid and cultured in the presence of LDL was performed to determine whether the 17 kDa C-terminal LDLR fragment was generated as a result of degradation of E387K-LDLR. In the presence of LDL and DAPT in the culture medium, the amount of the full-length LDLR decreased with time, and the amount of the 17 kDa fragment increased proportionally ($r = -0.98, P < 0.001$) (Fig. 5). Thus, intracellular degradation of a Class 5 mutant LDLR with bound LDL generates the 17 kDa C-terminal LDLR fragment.

DISCUSSION

We performed studies to shed light on the mechanism for sorting an LDLR with bound PCSK9 to intracellular degradation. Our key finding was that a 17 kDa C-terminal fragment of the LDLR was generated when PCSK9 was added to cells in the presence of the $\beta$-secretase inhibitor DAPT. Because the predicted molecular weight of the 77 amino acids of the cytoplasmic and transmembrane
domains of the LDLR is only ~8 kDa, cleavage of the LDLR to generate the 17 kDa C-terminal fragment must have occurred within the ectodomain.

As the amount of the 160 kDa mature LDLR decreased with time after the addition of PCSK9, the amount of the 17 kDa fragment increased proportionately. The 17 kDa fragment was not generated when PCSK9 was added to the media of cells expressing an LDLR that does not undergo PCSK9-mediated degradation. Moreover, the 17 kDa fragment was not generated in the presence of chemical reagents, such as ammonium chloride (50 mM) or E64d, which prevent PCSK9-mediated degradation of the LDLR. Thus, the 17 kDa C-terminal fragment of the LDLR appeared to be generated in an acidic intracellular compartment from an LDLR undergoing PCSK9-mediated degradation.

The observation that the PCSK9-induced 17 kDa C-terminal LDLR fragment was only detected when the cells were cultured in the presence of DAPT indicates that this 17 kDa fragment is normally cleaved by a γ-secretase within the membrane-spanning domain of the LDLR and that the cytoplasmic domain is rapidly degraded after release into the cytosol. Cytoplasmic domains released into the cytosol by γ-secretases are labile and have very short half-lives (22, 23). When γ-secretase cleavage is inhibited, these cytoplasmic domains remain membrane-bound, which makes them more stable. Support for the ability of the LDLR to undergo γ-secretase cleavage has come from a proteomics screen (28).

γ-secretase cleavage as part of regulated intramembrane proteolysis of transmembrane proteins is preceded by β-secretase cleavage of the ectodomain less than 30 residues from the membrane (22). However, neither we nor others have identified the 143 kDa N-terminal fragment complementary to the 17 kDa C-terminal fragment by Western blot analysis using antibodies against the N-terminal domain of the LDLR. A possible explanation for this failure is that the cleaved 143 kDa ectodomain fragment is rapidly acted upon by endosomal proteases. As a consequence, the ectodomain may be rapidly degraded or the antigenic epitopes may be destroyed. One factor that could contribute to rapid proteolytic degradation of the ectodomain in the endosome is that the ligand-binding repeats will unfold during prolonged residence at low calcium concentration in an acidic milieu (29, 30). It has previously been shown that rapid proteolysis of internalized LDLR is a truncated LDLR lacking ligand-binding repeats 1–4 that is degraded by PCSK9 (24). Three separate Western blot analyses were performed, of which one representative Western blot is shown.

![Fig. 4](image-url)

**Fig. 4.** Effect of leupeptin or E64d on PCSK9-mediated degradation of the LDLR. HepG2 cells were cultured in the presence or absence of DAPT (10 μM) for 1 h prior to a further 4 h incubation with or without D374Y-PCSK9 (1 µg/ml), leupeptin (300 μM), or E64d (200 µg/ml). Western blot analysis was performed using an antibody against the cytoplasmic domain of the LDLR or with an antibody against the V5 tag of D374Y-PCSK9. Δ1-4-LDLR is a truncated LDLR lacking ligand-binding repeats 1–4 that is degraded by PCSK9 (24). Three separate Western blot analyses were performed, of which one representative Western blot is shown.

![Fig. 5](image-url)

**Fig. 5.** Degradation of the Class 5 mutant E387K-LDLR in the presence of DAPT. CHO T-REx cells stably transfected with WT-LDLR plasmid or E387K-LDLR plasmid were cultured with DAPT (10 μM) and cycloheximide (100 µg/ml) for 1 h and in the presence or absence of LDL (200 µg/ml) for additional 1–5 h. Western blot analysis of cell lysates was performed using an antibody against the cytoplasmic domain of the LDLR. Δ1-4-LDLR is a truncated LDLR lacking ligand-binding repeats 1–4 that is degraded by PCSK9 (24). Three separate Western blot analyses were performed, of which one representative Western blot is shown.
ligands (reviewed in Ref. 31) and receptors (32–35) may well take place in prelysosomal compartments of the endosomal/lysosomal tract (36).

Generation of the 17 kDa C-terminal fragment could have been caused by a metalloproteinase or a disintegrin and metalloproteinase, which are commonly involved in shedding of ectodomains of transmembrane proteins at the cell surface (37), or by endosomal proteases such as cathepsins (38). However, metalloproteinase inhibitors did not prevent generation of the 17 kDa fragment. Leupeptin, which is a reversible inhibitor of cysteine, serine, and threonine proteases, did not prevent the activity of PCSK9 toward the LDLR but did prevent the increase in the amount of the 17 kDa C-terminal fragment. However, a 40 kDa C-terminal fragment was observed in the presence of leupeptin. These findings indicate that leupeptin prevents the PCSK9-induced cleavage of the ectodomain within the first 30 residues from the membrane and that the ectodomain is cleaved by another protease insensitive to leupeptin farther away from the membrane-spanning domain to generate the 40 kDa C-terminal fragment. The observation that only small amounts of the 40 kDa fragment could be detected is probably because it is rapidly being degraded. These data therefore indicate that the ectodomain of the LDLR is cleaved at least twice as a result of PCSK9-mediated degradation. If these cleavages occur in the sorting endosome, both will prevent the LDLR from recycling. The irreversible cysteine cathepsin inhibitor E64d prevented PCSK9-mediated degradation of the LDLR, which is in agreement with the findings of Wang et al. (39). No increased amount of the 17 kDa fragment was observed in the presence of E64d, and no 40 kDa fragment was observed. The explanation for the different effects of leupeptin and E64d on ectodomain cleavage of the LDLR remains to be determined, but our data indicate that generation of the 17 kDa fragment is caused by cysteine cathepsins, which predominantly act as endopeptidases (38). Even though most cathepsins in the endosomal/lysosomal tract become more active as the pH decreases, many cathepsins are active at pH of 6.0–6.2, which is found in the early endosome (31).

Our studies have shown that the 17 kDa fragment is generated during intracellular degradation of both an LDLR that has bound PCSK9 and a Class 5 mutant LDLR that has bound LDL. Previously, we have shown that the cytoplasmic domain does not play a role in rerouting any of these receptors to intracellular degradation (17, 40). Because the LDLR in both cases fails to adopt a closed conformation at acidic pH (1, 25), it is tempting to speculate that the failure of the LDLR to fold back on itself in the sorting endosome makes structures of the LDLR close to the membrane accessible to protease cleavage. A pH-induced conformational change at acidic pH is also required for the EGF receptor and asialoglycoprotein receptor to be resistant to proteolysis by the cysteine peptidase papain (41).

However, because the complementary 143 kDa ectodomain fragment has not been identified, further studies are needed to prove that the ectodomain of an LDLR with bound PCSK9 is cleaved in the sorting endosome and that this is the mechanism for sorting an LDLR with bound PCSK9 to intracellular degradation. Moreover, the notion that the cleaved ectodomain escapes detection because it is rapidly degraded appears to be at variance with studies where LDLRs subjected to PCSK9-mediated degradation have been found to colocalize with markers of late endosomes and lysosomes by confocal laser-scanning microscopy or subcellular fractionation. In these studies the mannose-6-phosphate receptor was used as marker for late endosomes (7, 42, 43), and cathepsin D was used as a marker for lysosomes (7, 27, 39). However, the mannose-6-phosphate receptor is not specific for late endosomes and cathepsin D is not specific for lysosomes. Rather, they are found in various amounts also in other organelles of the endosomal/lysosomal tract, including early endosomes (38, 44–46). Endosomal proteases may in fact themselves undergo proteolytic maturation down the endosomal/lysosomal tract as has been found for cathepsin D (47). Endosomal proteins are therefore of limited use as markers for specific organelles in the endosomal/lysosomal tract because they may be associated with an organelle through several maturation steps. Thus, further studies are needed to determine whether the ectodomain of an LDLR with bound PCSK9 is cleaved and rapidly degraded in the endosome.

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PCSK9 generates a 17 kDa C-terminal LDL receptor fragment
