Role of the C-terminal domain of PCSK9 in degradation of the LDL receptors

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Abstract Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to the low density lipoprotein receptor (LDLR) at the cell surface and disrupts the normal recycling of the LDLR. In this study, we investigated the role of the C-terminal domain for the activity of PCSK9. Experiments in which conserved residues and histidines on the surface of the C-terminal domain were mutated indicated that no specific residues of the C-terminal domain, apart from those responsible for maintaining the overall structure, are required for the activity of PCSK9. Rather, the net charge of the C-terminal domain is important. The more positively charged the C-terminal domain, the higher the activity toward the LDLR. Moreover, replacement of the C-terminal domain with an unrelated protein of comparable size led to significant activity of the chimeric protein.

We conclude that the role of the evolutionary, poorly conserved C-terminal domain for the activity of PCSK9 reflects its overall positive charge and size and not the presence of specific residues involved in protein-protein interactions.—Holla, O. L., J. Cameron, K. Tveten, T. B. Strøm, K. E. Berge, J. K. Laerdahl, and T. P. Leren. Role of the C-terminal domain of PCSK9 in degradation of the LDL receptors. J. Lipid Res. 2011. 52: 1787–1794.

Supplementary key words proprotein convertase subtilisin/kexin type 9 • endosomes • recycling • sorting • low density lipoprotein

The cell-surface low density lipoprotein (LDL) receptor (LDLR) plays a key role in cholesterol metabolism (1). The number of LDLRs is regulated both at the transcriptional and posttranscriptional levels. One mechanism for posttranscriptional regulation of the number of LDLRs involves intracellular degradation of the LDLR secondary to the binding of proprotein convertase subtilisin/kexin type 9 (PCSK9) on the cell surface (2, 3).

PCSK9 is a zymogen that undergoes autocatalytic cleavage in the endoplasmic reticulum (3). It is secreted as a complex consisting of the cleaved prodomain noncovalently bound to the mature form (3). At the cell surface, secreted PCSK9 binds to the epidermal growth factor (EGF) repeat A of the extracellular part of the LDLR and is subsequently internalized as a complex with the LDLR (4). At the acidic pH of the endosomes, the affinity of PCSK9 for the LDLR increases (5, 6), and bound PCSK9 appears to act as a tag to disrupt the normal recycling of the LDLR (4). As a consequence, the LDLR-PCSK9 complex is directed to the lysosomes for degradation (4).

PCSK9 consists of 692 amino acids, of which residues 1-30 constitute the signal peptide (3). The remainder of the protein is commonly divided into three domains. Residues 31-152 constitute the prodomain, residues 153-454 constitute the catalytic domain, and residues 455-692 constitute the C-terminal domain (5, 6). Binding of PCSK9 to the EGF-A repeat of the LDLR is mediated by a patch of residues on the catalytic domain of PCSK9 (7, 8). Thus, the catalytic domain is responsible both for autocatalytic cleavage and for binding of PCSK9 to the LDLR.

Even though the C-terminal domain of PCSK9 does not interact directly with the EGF-A repeat of the LDLR (7), the C-terminal domain is nevertheless required for PCSK9-mediated degradation of the LDLR. This notion is based on the observation that a truncated form of PCSK9 lacking the C-terminal domain is able to bind to the LDLR but has no or markedly reduced ability to mediate degradation of the LDLR (5, 9, 10). The underlying mechanism for the role of the C-terminal domain, however, remains to be determined.

The C-terminal domain of PCSK9 is a three-time tandem repeat where each repeat is built from six β-strands connected with three disulfide bridges (5, 6). Apart from the 18 cysteines involved in forming disulfide bridges, few of the remaining residues of the C-terminal domain are highly conserved (supplementary Fig. 1). Thus, the

Abbreviations: EGF, epidermal growth factor; LDLR, LDL receptor; PCSK9, proprotein convertase subtilisin/kexin type 9; WT, wild-type.

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C-terminal domain is clearly less evolutionarily conserved than the catalytic domain (8). Another characteristic of the C-terminal domain of human PCSK9 is the large number of histidines, which has been proposed to underlie PCSK9’s increased affinity for the LDLR at low pH (5, 6). Changing the pH from pH 7.5 (cell surface) to pH 5.4 (endosomes), the net charge of this domain will be roughly 8-10 units more positive. Thus, it is possible that the C-terminal domain may interact with negatively charged structures within the LDLR at endosomal pH. This notion is supported by the observation that the C-terminal domain has a novel protein fold, which has been proposed to mediate protein-protein interactions (5, 6). However, whether the C-terminal domain actually interacts with the LDLR is somewhat controversial. Although several studies have failed to observe an interaction between the C-terminal domain and the extracellular part of the LDLR at pH 7.4 or at pH 6.0 (5, 9, 10), Yamamoto et al. (11) recently reported that the C-terminal domain binds to the ligand-binding domain of the LDLR, particularly at low pH.

If the C-terminal domain of PCSK9 is involved in biologically important protein-protein interactions with the LDLR, it is conceivable that this interaction involves evolutionarily conserved residues on the surface of the C-terminal domain. To study the role of individual residues of the C-terminal domain for the activity of PCSK9 toward the LDLR, we mutated conserved residues and histidines and studied how this affects degradation of the LDLR.

MATERIALS AND METHODS

Cell culture

HepG2 cells (European Collection of Cell Cultures, Wiltshire, UK) were cultured in modified Eagle’s medium (Gibco, Carlsbad, CA) as previously described (12). For experiments, HepG2 cells were seeded out in collagen-coated 12-well plates (for flow cytometry) or 6-well plates (for Western blot analysis) (BD Biosciences, San Diego, CA) and transiently transfected with the different PCSK9-containing plasmids using Fugene HD (Roche Diagnostics GmbH, Mannheim, Germany) according to the manufacturer’s instructions.

Mutagenesis

A pCMV-PCSK9-FLAG plasmid kindly provided by Jay D. Horton (University of Texas Southwestern Medical Center, Dallas, TX) was used as a template for mutagenesis to generate mutant PCSK9 constructs. This plasmid contains the coding sequence of PCSK9 followed by the sequence for the FLAG epitope tag. To purify wild-type (WT) or mutant PCSK9s, a pcDNA3.1-PCSK9-V5-his plasmid (13) was used as a template for mutagenesis. The WT-PCSK9-DsRed plasmid contains the sequence for the 225 amino acid DsRed-Express fluorescent protein as previously described (13). This plasmid was used as a template to create the L455X-PCSK9-DsRed plasmid, which encodes a truncated PCSK9 lacking the C-terminal domain fused to the DsRed fluorescent protein. A pcDNA3.1-EC-LDLR-myc-his (EC-LDLR) plasmid (13), which encodes the extracellular part of the LDLR with myc and his tags, was used as a template for mutagenesis to generate a construct encoding amino acids 299-767 of the LDLR (Δ1-7-EC-LDLR), which consists of the extracellular part of the LDLR without the seven ligand-binding repeats. The plasmid pCDNA4-LDLR (14) encoding the full-length LDLR and the primers used to generate Δ1-7-EC-LDLR were used to generate the plasmid Δ1-7-EC-LDLR, which encodes an LDLR without the ligand-binding domain. Mutageneses were carried out using QuickChange XL Mutagenesis Kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The primer sequences are listed in supplementary Table I. The integrity of the plasmids was confirmed by DNA sequencing. An empty plasmid, pcDNA3.1/myc-his (Invitrogen, Carlsbad, CA), and the two mutant PCSK9 plasmids, containing loss-of-function mutation S386A or gain-of-function mutation D374Y (15), were used as controls.

Protein purification

Wild-type or mutant PCSK9s with a V5-his tag were isolated from media of stably transfected HEK293 cells as previously described (16). When indicated, conditioned PCSK9-containing medium was concentrated by the use of a 30 kDa Amicon Ultra Centrifugal Filter Tubes according to the manufacturer’s instructions (Millipore Corp., Billerica, MA).

EC-LDLR or Δ1-7-EC-LDLR was purified from media of CHO T-Rex cells (Invitrogen) stably transfected with the relevant plasmid. The cells were cultured in 1,720 cm2 Corning Hyperflasks (Sigma-Aldrich Corp., St. Louis, MO) in the presence of tetracycline (1 µg/µl) to induce expression of the transgenes. EC-LDLR or Δ1-7-EC-LDLR in the media was purified by the use of nickel-NTA agarose resin according to manufacturer’s instructions (Qiagen GmbH, Hilden, Germany). After binding proteins to the resin, the resin was washed with 15 ml wash buffer containing 20 mM imidazole. Proteins were eluted using an elution buffer containing 250 mM imidazole. Fractions were collected and analyzed by gel electrophoresis using a Criterion Precast 4-20% Tris-HCl gel (Bio-Rad, Hercules, CA) followed by staining with ECL blue gel staining reagent (Sigma-Aldrich Corp.). Fractions containing EC-LDLR or Δ1-7-EC-LDLR were pooled and dialyzed against a buffer containing 20 mM HEPEs, 150 mM NaCl, and 2 mM CaCl2. After dialysis, the solutions were concentrated using a 50 kDa Amicon Ultra-15 concentration column (Millipore Corp.).

Western blot analyses

Western blot analyses of cell lysates or culture media of HepG2 cells transfected with PCSK9-containing plasmids were carried out as previously described (15). Briefly, 24 h after transfection, cell lysates were obtained and run on 4-20% Tris-HCl Criterion Precast Gels (Bio-Rad) and blotted onto Immuno-Blot PVDF Membranes (Bio-Rad). The membranes were immunostained with a mouse IgG anti-FLAG antibody (Sigma-Aldrich Corp.). For quantitation of purified PCSK9s with or without a normal C-terminal domain, a custom-made rabbit anti-PCSK9 antibody raised against residues 46-62 of the prodomain (Bethyl Laboratories, Inc., Montgomery, TX) was employed for Western blot analysis. A rabbit polyclonal anti-β-tubulin antibody (Nordic Biosite AB, Täby, Sweden) was used to detect β-tubulin, which was used as a loading control.

Far Western blot analysis

Purified EC-LDLR or Δ1-7-EC-LDLR (both 400 ng/lane) were run on 4-20% Tris-HCl Criterion Precast Gels (Bio-Rad) and blotted onto Immuno-Blot PVDF membranes as described above. The membranes were incubated in phosphate-buffered saline with 2.5% Blottling-Grade Blocker Nonfat Dry Milk (Bio-Rad) at 4°C overnight to reduce nonspecific binding. The membranes were washed twice in Far Western blot buffer (50 mM Tris-Maleate, 75 mM NaCl, 2 mM CaCl2, and 2.5% dry milk), and then incubated...
at room temperature for 1 h in Far Western blot buffer containing 5 µg/ml purified PCSK9. Visualization of PCSK9 bound to EC-LDLR or Δ1-7-EC-LDLR was performed using an anti-V5 antibody conjugated to horseradish peroxidase (Invitrogen).

**Quantitation of the amount of LDL internalized**

Quantitation of the amount of LDL internalized in HepG2 cells transiently transfected with PCSK9-containing plasmids was performed by flow cytometry as previously described (15). Briefly, 24 h after transfection, the cells were washed with phosphate-buffered saline and the medium was replaced with modified Eagle’s medium containing 5 mg/ml lipoprotein-deficient serum for a 24 h incubation to increase the expression of the LDLR. The cells were then incubated with 10 µg/ml of 1,1′-dioctadecyl-3,3,3′,3′-tetramethylindodicarbocyanine perchlorate-labeled LDL for 2 h at 37°C before the amount of LDL internalized was determined by flow cytometry.

**RESULTS**

**Role of conserved residues of the C-terminal domain**

The 18 cysteine residues involved in forming disulfide bridges in the C-terminal domain of human PCSK9 are absolutely conserved in all chordates, the hemichordate *Saccoglossus kowalevskii* (acorn worm), and in the corresponding domain in mollusc proteins predicted to be involved in binding and/or digestion of polysaccharides (e.g., NCBI protein sequence BAH84829 from the clam *Corbicula japonica*), but otherwise there is not a high degree of sequence conservation in this domain (supplementary Fig. 1). To identify residues of the C-terminal domain that are candidates for being involved in protein-protein interactions, 14 highly conserved residues (supplementary Fig. 1), mainly located on the domain surface (Fig. 1), were individually mutated to alanines. The effect of these PCSK9 mutants on degradation of the LDLR was determined by analysis of the amount of LDL internalized in HepG2 cells transiently transfected with the various mutant PCSK9 plasmids (Fig. 2).

Four of the mutants (R458A-PCSK9, T459A-PCSK9, W461A-PCSK9, and E481A-PCSK9) were loss-of-function mutants because their secretion was abolished or markedly reduced (Fig. 2). Secretion of R680A-PCSK9 was also severely reduced, but it only presented as a mild loss-of-function mutant. F515A-PCSK9 could appear to be a mild gain-of-function mutant, whereas none of the other mutations had any major impact on the amounts of LDL internalized. Similar results were found when HepG2 cells were incubated with PCSK9-conditioned medium from transiently transfected HepG2 cells (data not shown). Moreover, in separate experiments in which Phe515 was mutated to uncharged residues (Ala, Leu, Ser, or Glu), a positively charged residue (Arg), or a negatively charged residue (Glu), the effect of these mutations on PCSK9 activity was similar to that of WT-PCSK9 (supplementary Fig. II). Thus, Phe515, a conserved residue conspicuously sticking its hydrophobic side-chain out into the solvent in the available

![Fig. 1.](image)
Role of histidines of the C-terminal domain

The C-terminal domain of human PCSK9 contains 14 histidine residues that may potentially obtain a positive charge following a reduction in pH. The pKₐ values predicted with the PROPKA method (17) suggest that seven of the histidine residues have pKₐ values in the range 6.2-7.0. These residues are located on the surface of the domain (Fig. 1). They are likely to become protonated upon transfer of the protein from the extracellular compartment (pH ~7.4) to the sorting endosomes (pH ~6.2), resulting in an increased positive charge for this domain.

To study whether histidines of the C-terminal domain were important for the PCSK9-mediated degradation of the LDLR, the seven surface-exposed histidines (His537, His551, His553, His557, His574, His602, and His613) (Fig. 1) were selected for mutagenesis. Whereas mutating individual histidines or mutating fewer than six histidines to uncharged alanines had little impact on the activity of PCSK9, mutating six or seven histidines markedly reduced the activity (Fig. 3). This effect was not due to the failure of the mutants to be synthesized or secreted, as determined by Western blot analysis (Fig. 3).

These data are compatible with the notion that histidines of the C-terminal domain are important for the activity of PCSK9. The underlying mechanism could involve specific protein-protein interactions between histidines and residues of the LDLR or could be due to the positive charge of these histidines at low pH per se. To discriminate between these alternatives, the seven histidines were mutated not only to uncharged alanines but also to positively charged lysines (7HK-PCSK9) and to negatively charged glutamic acid (7HE-PCSK9). As Fig. 4 shows, the relative activities of the different PCSK9s on the LDLR were 7HK-PCSK9 > WT-PCSK9 > 7HA-PCSK9 > 7HE-PCSK9, which reflects the ranking of their net positive charge of the C-terminal domain. Therefore, these data indicate that it is the positive charge of the C-terminal domain that is important for the activity of PCSK9 to mediate degradation of the LDLR.

A positively charged C-terminal domain could attract negatively charged structures of the LDLR. One such structure could be the negatively charged ligand-binding domain that contains clusters of acidic residues. To determine whether WT-PCSK9 or the three mutants (7HA-PCSK9, 7HK-PCSK9, and 7HE-PCSK9) bound differently to the extracellular part of the LDLR with or without a normal ligand-binding domain, Far Western blot analyses were performed at pH 7, pH 6, and pH 5 (Fig. 5). The double bands of the extracellular part of the WT-LDLR on the blots reflect the LDLR with or without a natural deletion of ligand-binding repeats 1-4, as determined by protein sequencing of a commercially available extracellular LDLR (http://www.rndsystems.com/pdf/2148-LD_CF.pdf).

X-ray structures of PCSK9 (Fig. 1), does not appear to have an inhibitory effect on the activity of PCSK9.

Fig. 2. Effect of mutating conserved residues in the C-terminal domain of PCSK9 on autocatalytic cleavage and secretion and on internalization of LDL. HepG2 cells were transiently transfected with plasmids encoding WT-PCSK9 or mutant PCSK9 in which various conserved residues of the C-terminal domain had been mutated to alanines. The catalytically inactive mutant S386A-PCSK9 and the gain-of-function mutant D374Y-PCSK9 were used as controls. The amount of LDL internalized was analyzed by flow cytometry. Results represent means (± SEM) of three separate experiments. Autocatalytic cleavage and secretion of PCSK9 was determined by Western blot analysis of cell lysates and media using an anti-FLAG antibody. A representative Western blot is shown.
Role of the C-terminal domain for the activity of PCSK9

Media from HEK293 cells transfected with plasmids L455X-PCSK9 or WT-PCSK9 with or without the sequence for the DsRed-Express fluorescent protein were collected, and the amounts of PCSK9 of the media were adjusted by concentration/dilution to obtain equal concentrations as determined by Western blot analysis (Fig. 6B). The concentration-adjusted media were added to HepG2 cells, and the amounts of LDL internalized were determined by flow cytometry. As Fig. 6A shows, WT-PCSK9 fused to the DsRed-Express fluorescent protein had a higher activity toward the LDLR than did WT-PCSK9. The truncated L455X-PCSK9 had only a marginal activity toward the LDLR, which is in agreement with previous reports (5, 9, 10), but this activity was markedly increased when L455X-PCSK9 was fused to the DsRed-Express fluorescent protein (Fig. 6A). In fact, the activity of L455X-PCSK9 fused to the DsRed-Express fluorescent protein was similar to that of WT-PCSK9. These findings obtained by flow cytometry were confirmed by Western blot analysis of HepG2 cells incubated with the different media (Fig. 6B).

DISCUSSION

In this study, we investigated the role of individual residues of the C-terminal domain of PCSK9 for PCSK9-mediated degradation of the LDLR. A total of 14 conserved residues on the surface of the C-terminal domain were mutated to alanines. Five of the mutants (R458A-PCSK9, T459A-PCSK9, W461A-PCSK9, E481A-PCSK9, and R680A-PCSK9)
Histidines in the C-terminal domain of PCSK9 have been suggested to underlie PCSK9’s increased affinity for the LDLR at the acidic pH of endosomes (5, 6). A total of 14 histidines are found in the C-terminal domain, of which 7 are located on the surface. Our findings from mutating these 7 histidines to negatively charged glutamic acid, neutral alanines, or positively charged lysines were that the activity of the mutant PCSK9s correlated with the net charge of the C-terminal domain. The more positively charged the C-terminal domain, the stronger the binding between PCSK9 and the LDLR and the higher the activity of PCSK9. Therefore, these findings suggest that it is the positive charge of the histidines that is important for the activity of PCSK9. This notion is also supported by the observation that at least 6 histidines had to be mutated to alanines before a reduced activity could be observed in our assay.

Replacement of the C-terminal domain of PCSK9 with a similarly sized fluorescent protein containing seven histidines maintained the activity of PCSK9 toward the LDLR. Moreover, the activity of WT-PCSK9 fused to the DsRed-Express protein was higher than that of WT-PCSK9. This strongly suggests that the role of the C-terminal domain does not involve specific key-and-lock-like protein-protein interactions between conserved residues on the surface of the C-terminal domain and a matching segment of the LDLR. This type of specific interaction seems to be limited to the docking of the catalytic domain of PCSK9 to the EGF-A repeat of the LDLR (7, 8).

Complete or partial failure of secretion. Thus, it is likely that Arg458, Thr459, Trp461, Phe481, and Arg680 are highly conserved because they are required for normal folding of PCSK9 in the endoplasmic reticulum. The first 4 residues are all located on the surface of the C-terminal domain that are interacting with the catalytic domain (Fig. 1) and appear to be important for maintaining this interaction. The other 9 secreted mutants affected the amount of LDL internalized similarly to that of WT-PCSK9. Thus, it is unlikely that the role of the C-terminal domain for the activity of PCSK9 involves specific key-and-lock-like protein-protein interactions between conserved residues on the surface of the C-terminal domain and a matching segment of the LDLR. This type of specific interaction seems to be limited to the docking of the catalytic domain of PCSK9 to the EGF-A repeat of the LDLR (7, 8).

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This notion seems to be in agreement with the findings of Ni et al. (10). They found that an antigen-binding fragment bound to the C-terminal domain reduced the activity of PCSK9 by 50%. However, substituting the six residues recognized by the antigen-binding fragment with alanines had only little effect on the activity of PCSK9 (10). Thus, the mechanism for the reduced activity of PCSK9 by the bound antigen-binding fragment

**Fig. 4.** Effect of mutating seven histidines in the C-terminal domain on autocatalytic cleavage and secretion of PCSK9 and on internalization of LDL. HepG2 cells were transiently transfected with plasmids encoding mutant PCSK9s in which the seven histidine residues on the surface of the C-terminal domain (see Fig. 3 legend) had been mutated to alanine (7HA), lysine (7HK), or glutamic acid (7HE). The catalytically inactive mutant S386A-PCSK9 and the gain-of-function mutant D374Y-PCSK9 were used as controls. The amount of LDL internalized by the cells was analyzed by flow cytometry. Results represent means (± SEM) of five separate experiments. Autocatalytic cleavage and secretion of PCSK9 were determined by Western blot analysis of cell lysates and media, respectively, using an anti-FLAG antibody. Reduced electrophoretic migration of the 7HE-PCSK9 mutant was probably caused by its negative charge and resulting low degree of SDS binding (23).
Role of the C-terminal domain for the activity of PCSK9

The underlying mechanism for the role of a positively charged C-terminal domain may involve an electrostatic attraction with negatively charged structures of the LDLR. This attraction may be of a weak nature to explain the failure to observe an interaction between the C-terminal domain and the LDLR by methods such as immunoprecipitation (9) or studies of surface plasmon resonance (5, 11). However, under conditions favoring protein binding, the C-terminal domain has been shown to bind to the ligand-binding domain of the LDLR, particularly at acidic pH (11).

Previous studies have shown that negatively charged residues of the unstructured part of the prodomain of PCSK9 (residues 31-60) have an inhibitory effect on the activity of PCSK9 (7, 12, 19). One may therefore speculate that the negatively charged unstructured part of the prodomain exerts its negative effect on the activity of PCSK9 by partly neutralizing an electrostatic attraction between the positively charged C-terminal domain of PCSK9 and negatively charged structures within the LDLR.

Our results from Far Western blot analysis showed that the more positively charged the C-terminal domain, the more PCSK9 bound to the WT-LDLR and to an LDLR lacking the ligand-binding domain. Therefore, these data suggest that an interaction between the C-terminal domain does not appear to involve masking of specific residues of the C-terminal domain. The lack of a role of specific residues of the C-terminal domain (other than for those involved in maintaining its overall structure) may also explain the poor evolutionary conservation of residues of the C-terminal domain.

Fig. 5. Binding of PCSK9 mutants to extracellular LDLR and to extracellular LDLR lacking the seven ligand-binding repeats. Purified extracellular LDLR (EC) and extracellular LDLR lacking the seven ligand-binding repeats (Δ1-7EC) (both 400 ng/lane) were run on nonreducing 4-20% Tris-HCl gels and blotted onto PVDF membranes. The membranes were incubated in buffers with pH 7, 6, or 5 supplemented with purified WT-PCSK9 (5 µg/ml) or mutant PCSK9 (5 µg/ml) in which seven histidine residues on the surface of the C-terminal domain had been replaced with alanine (7HA), lysine (7HK), or glutamic acid (7HE). PCSK9 bound to the immobilized LDLR was visualized using an anti-V5 antibody. Three experiments were performed, of which one representative is shown.

Fig. 6. Effect of replacing the C-terminal domain of PCSK9 with DsRed-Express fluorescent protein. Cultured HepG2 cells were incubated with equal amounts of WT-PCSK9 (WT), WT-PCSK9 fused to the DsRed-Express fluorescent protein (WT-DsRed), truncated L455X-PCSK9 (L455X), or L455X-PCSK9 fused to the DsRed-Express fluorescent protein (L455X-DsRed) (37°C, 3 h). A: After additional 2 h incubation with 10 µg/ml Dil-LDL, the amounts of LDL internalized were determined by flow cytometry. Mean (± SEM) values from three experiments are shown. The amount of LDL internalized by cells incubated with WT-PCSK9 was assigned a value of 1. B: Quantitation of the amounts of the different PCSK9s added to the cells was performed by Western blot analysis using an anti-PCSK9 antibody against the prodomain of PCSK9. The effect of the different PCSK9s on the LDLR of HepG2 cells (37°C, 5 h) was determined by Western blot analysis using an anti-LDLR antibody. An anti-β-tubulin antibody was used to determine the amount of β-tubulin, which was used as a loading control. Representative Western blots are shown.
and the LDLR may not exclusively involve binding of the C-terminal domain to the ligand-binding domain of the LDLR. Moreover, the observation that a reduction in pH had a similar effect on the binding of PCSK9 to LDLR, with or without a ligand-binding domain, could suggest that the ligand-binding domain is not involved in mediating the increased affinity of PCSK9 for the LDLR at low pH (5, 6). However, these data from Far Western blot analysis should be interpreted with care, as the ligand-binding domain of an LDLR immobilized on a membrane may not be available for binding to the C-terminal domain of PCSK9. The data, however, may indicate in more general terms that the more positively charged the C-terminal domain, the higher its attraction to structures of the LDLR.

Because the LDLR may need to adopt a conformational change at acidic pH to recycle (20) and this conformational change is disrupted by bound PCSK9 (9), one may speculate that a C-terminal, positively charged structure in PCSK9 is required to disrupt the conformational change of the LDLR through a weak binding to the negatively charged ligand-binding domain of the LDLR. However, other data by Zhang et al. (9) indicate that PCSK9 interfering with the conformational change in the LDLR is not the sole mechanism by which PCSK9 disrupts the normal recycling of the LDLR. Thus, more studies are needed to address the role of the ligand-binding domain of the LDLR in PCSK9-mediated degradation.

In conclusion, our data suggest that the role of the evolutionary, poorly conserved C-terminal domain for the activity of PCSK9 reflects its overall positive charge and size and not the presence of specific residues involved in protein-protein interactions. However, further studies are needed to determine the exact mechanism by which an interaction between the C-terminal domain of PCSK9 and putative negative structures of the LDLR results in the LDLR being rerouted to the lysosomes instead of being recycled.

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