

The M2 Module of the Cys-His-rich Domain (CHRD) of PCSK9 Protein Is Needed for the Extracellular Low-density Lipoprotein Receptor (LDLR) Degradation Pathway^{*[5]}

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Background: PCSK9 regulates low-density lipoprotein receptor levels in the liver. The importance of the M1, M2, and M3 modules within the C terminus of PCSK9 is unknown.

Results: The M2 module is needed for the extracellular, but not intracellular, activity of PCSK9.

Conclusion: The integrity of the M2 module is essential for the extracellular function of PCSK9.

Significance: Targeting the M2 module should neutralize circulating PCSK9 and reduce LDL-cholesterol.

PCSK9 enhances the cellular degradation of the LDL receptor (LDLR), leading to increased plasma LDL cholesterol. This multidomain protein contains a prosegment, a catalytic domain, a hinge region, and a cysteine-histidine rich domain (CHRD) composed of three tightly packed modules named M1, M2, and M3. The CHRD is required for the activity of PCSK9, but the mechanism behind this remains obscure. To define the contribution of each module to the function of PCSK9, we dissected the CHRD structure. Six PCSK9 deletants were generated by mutagenesis, corresponding to the deletion of only one (Δ M1, Δ M2, Δ M3) or two (Δ M12, Δ M13, Δ M23) modules. Transfection of HEK293 cells showed that all deletants were well processed and expressed compared with the parent PCSK9 but that only those lacking the M2 module were secreted. HepG2 cells lacking endogenous PCSK9 (HepG2/shPCSK9) were used for the functional analysis of the extracellular or intracellular activity of PCSK9 and its deletants. To analyze the ability of the deletants to enhance the LDLR degradation by the intracellular pathway, cellular expressions revealed that only the Δ M2 deletant retains a comparable total LDLR-degrading activity to full-length PCSK9. To probe the extracellular pathway, HepG2/shPCSK9 cells were incubated with conditioned media from transfected HEK293 or HepG2/shPCSK9 cells, and cell surface LDLR levels were analyzed by FACS. The results showed no activity of any secreted deletant compared with PCSK9. Thus, although M2 is dispensable for secretion, its presence is required for the extracellular activity of PCSK9 on cell surface LDLR.

ine proprotein convertases (PC)² family (1, 2). Its high expression in liver, localization of its gene *PCSK9* on human chromosome 1p32, and characterization of two gain-of-function (GOF) mutants in families with high levels of circulating low density lipoprotein cholesterol (LDLc) led to its identification as the third locus implicated in autosomal dominant hypercholesterolemia, with the low-density lipoprotein receptor (LDLR), apolipoprotein B (3), and apolipoprotein E (4) as the other three.

Early studies demonstrated that, similar to the other PC family members, PCSK9 is first synthesized as a zymogen (proPCSK9) that undergoes an autocatalytic cleavage of its prosegment (1) at VFAQ₁₅₂↓ (5, 6) within the endoplasmic reticulum (ER). This cleavage is a prerequisite for the exit and secretion of the prosegment=PCSK9 complex from the ER, as the zymogen is not secreted (1, 5). It became apparent that, different from the other eight PC family members, PCSK9 never gets rid of its inhibitory prosegment (2) and is thus secreted as a catalytically inactive protease. This tight prosegment=PCSK9 interaction was confirmed by the crystal structure of PCSK9 that revealed multiple points of contact between the prosegment and the catalytic subunit of PCSK9 (7–9). These data further showed that following the catalytic domain, the PCSK9 structure exhibits the presence of an exposed hinge region (residues 422–439) (10) followed by a C-terminal Cys/His-rich domain (CHRD) composed of three repeat modules termed M1 (amino acids 453–531), M2 (amino acids 530–605), and M3 (amino acids 604–692) (supplemental Fig. S1) (7).

Soon after its discovery, it became clear that PCSK9 is implicated in the degradation of the LDLR itself, as its overexpression in mice (11), primary hepatocytes (12), and/or cell lines including its natural GOF mutants (5) resulted in decreased levels of this receptor. It was also shown that the degradation of the PCSK9=LDLR complex occurs in acidic compartments (5) likely to be endosomes/lysosomes (13). Cellular studies showed that PCSK9 targets the LDLR for degradation by two pathways:

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is the latest and ninth member of the mammalian subtilisin-like ser-

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² The abbreviations used are: PC, proprotein convertase; GOF, gain of function; LDLc, LDL cholesterol; LDLR, LDL receptor; ER, endoplasmic reticulum; CHRD, cysteine/histidine-rich domain.

an intracellular one from the *trans* Golgi network directly to lysosomes, implicating clathrin light chains (14), and an extracellular one (15) requiring clathrin heavy chain-mediated endocytosis of the cell surface PCSK9=LDLR complex (13).

Biochemical, cell biological, and structural studies demonstrated that the catalytic subunit of PCSK9 binds the epidermal growth factor-like repeat A domain of the LDLR (16–18). As suspected from the exclusive secretion of the catalytically inactive prosegment=PCSK9 complex, it was formally shown that the catalytic activity of PCSK9 was not required for its ability to enhance the degradation of the LDLR (19), suggesting that the latter is performed by undefined endogenous endosomal/lysosomal proteases. This was further confirmed for the other two receptors that PCSK9 also targets, namely Very Low Density Receptor and Apolipoprotein E receptor 2 (20).

The cell surface endocytosis of the PCSK9=LDLR complex is a dominant degradation pathway that shunts the typical LDLR recycling route (21, 22). As a major consequence of PCSK9 action, levels of hepatocyte cell surface LDLR decrease, as confirmed in mouse knockout models (23, 24), leading to the accumulation of LDLc in mouse and human plasma (23–25). In cases of high levels or GOF mutants of PCSK9, this can result in inflammation and the formation of atherosclerotic plaques (26), ultimately leading to cardiovascular disease (27). Accordingly, inhibition or silencing of PCSK9 function is a novel powerful therapeutic approach to lower LDLc, as attested by multiple ongoing phase II clinical trials (2, 28).

In contrast to major advances in PCSK9-based therapies, the molecular mechanisms regulating the sorting of the prosegment=PCSK9=LDLR complex to endosomes/lysosomes by the extracellular or intracellular pathways are poorly defined. Our present understanding of the subcellular trafficking of the PCSK9=LDLR complex leading to its degradation is that it requires the presence of the CHR1 (13, 29) but not the cytosolic tail of the LDLR (30). Furthermore, removal of the acidic N-terminal sequence of the prosegment significantly enhances the degradation efficacy of the complex (17, 31, 32).

To expand our understanding of the contribution of the CHR1 in the enhanced degradation of LDLR by PCSK9, we present structure-function studies in which we dissected the M1, M2, and M3 modules of the CHR1 and analyzed the activity of resulting deletants in the extracellular and intracellular pathways of LDLR degradation. The data demonstrated the critical requirement for the M2 domain in the extracellular pathway but not for the intracellular one.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Human PCSK9 and its mutant cDNAs (hPCSK9 L455X and PCSK9 CHR1) were cloned into pIRES2-EGFP (Clontech, Mountain view, CA) as described (1, 13). Human HepG2/shPCSK9 cells essentially lacking endogenous PCSK9 (14) and HEK293 cells (ATCC) were cultivated in DMEM (Invitrogen) supplemented with 10% FBS (Wisent). Puromycin (2 μ g/ml, Invitrogen) was added only to HepG2/shPCSK9 cells as a selection antibiotic. Lipoprotein-deficient serum was from Biomedical Technologies.

cDNA Constructs—The construct pIRES2-EGFP-human PCSK9-V5 (C-terminal V5-tag) (1) was used as a template to

generate cDNAs coding for human PCSK9 mutants and module deletants. The oligonucleotides used are listed in [supplemental Table S1](#). Two-step PCRs were used to introduce deletion mutants as described previously (5). All constructs contain a V5 tag at the C terminus. All constructs were confirmed by DNA sequencing.

Cell Culture and Transfections—HepG2/shPCSK9 cells were seeded at 1×10^5 cells/well in a 12-well microplate (Greiner Bio-One). After 24 h, the cells were transfected with 1 μ g of cDNAs using FuGENE HD (Roche Applied Science), and 24 h post-transfection, the cells were washed and then incubated with fresh DMEM without serum for an additional 24 h before recovering media and cells.

Preparation of Conditioned Media— 2×10^6 HEK293 or HepG2/shPCSK9 cells in a 100-mm Petri dish coated with poly-L-lysine (Invitrogen) were transfected with a total of 4 μ g of cDNA using Effectene (Qiagen). At 24 h post-transfection, the cells were washed and incubated with serum-free media. Conditioned media were recovered 72 h post-transfection. The spent media were then concentrated on an Amicon Ultra-15 centrifugal filter unit with a 10-kDa membrane cutoff (Millipore). Concentrated V5-tagged PCSK9 and its derivatives in conditioned media were quantitated by enzyme-linked immunosorbent assay detecting V5-tagged proteins (ELISA-V5, Invitrogen, catalog no. R960-25).

Media Transfer Experiments—HepG2/shPCSK9 cells were seeded in a 12-well microplate at 3×10^5 cells/well (Greiner Bio-One). After an overnight incubation, cells were washed and incubated in LPDS media (Dulbecco's phosphate-buffered saline (Invitrogen), 0.1% sodium pyruvate (Invitrogen), and 5% lipoprotein-deficient serum (Biomedical Technologies)). Following 24-h incubation, media were replaced by conditioned media containing PCSK9 or its mutants/deletants at a final concentration of 1.2 μ g/ml. After 4 h of incubation at 37 $^{\circ}$ C, cells were lysed in 1 \times radioimmune precipitation assay buffer (RIPA: 150 mM NaCl, 50 mM Tris-HCl (pH 8.0)) containing 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS supplemented with 1 \times complete protease inhibitor mixture (Roche Applied Science) and then analyzed.

Western Blot Analyses—Media were recovered 48 h post-transfection, and the cells were lysed in 1 \times radioimmune precipitation assay buffer. Proteins in the cell lysates and media were resolved by 10% Tris-glycine SDS-PAGE. The gels were blotted onto PVDF (PerkinElmer Life Sciences) membranes (GE Healthcare), blocked for 1 h in TBS-T (50 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% Tween 20) containing 5% nonfat milk, and immunoblotted with a homemade polyclonal human PCSK9 antibody (1:1000) (13), human LDLR antibody (1:1000, R&D Systems), β -actin (1:5000; Sigma), and mAb V5-HRP (1:5000, Sigma). Appropriate horseradish peroxidase-conjugated secondary antibodies (1:10000, Sigma) were used for detection with enhanced chemiluminescence using the ECL Plus kit (GE Healthcare). Quantitation of protein bands was obtained using ImageJ software.

FACS Analysis—HepG2/shPCSK9 cells were incubated at 37 $^{\circ}$ C for the indicated times (0, 30, 60 min) with various conditioned media containing WT PCSK9, single-point mutants, or module deletants. The cells were then washed three times

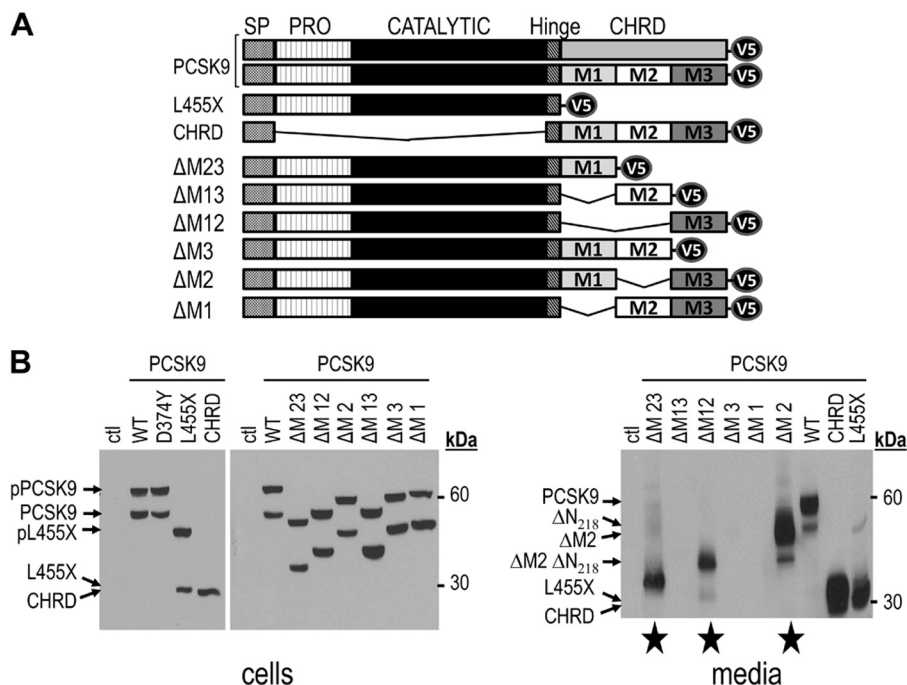


FIGURE 1. Expression, autocleavage, and secretion of CHRD module deletants. *A*, schematic representation of V5-tagged WT human PCSK9, PCSK9 domain deletants (L455X, lacking CHRD, and CHRD, lacking the prosegment (PRO) and catalytic domain but retaining the hinge region (10)), and PCSK9 CHRD module deletants (Δ M1, Δ M2, Δ M3, Δ M12, Δ M23, and Δ M13). SP: Signal Peptide. *B*, HEK293 cells were transiently transfected with cDNAs encoding the above constructs. 48 h post-transfection, proteins in cell lysates and media were resolved by SDS-PAGE and analyzed by Western blot analysis using mAb-V5. The migration positions of reference molecular weight proteins, as well as those of the precursor and mature PCSK9 products are emphasized. The secreted CHRD module deletants are labeled with a star. Ctl, control.

with solution A (calcium/magnesium-free Dulbecco's PBS (Invitrogen) containing 0.5% bovine serum albumin (Sigma) and 1g/liter glucose). The cells were then incubated for 10 min at room temperature with 1 \times Versene solution (Invitrogen) followed by the addition of 5 ml of solution A. The cells were then incubated for 40 min in solution A containing a human LDLR mAb-C7 (1:100, Santa Cruz Biotechnology). Following washes, the cells were then incubated for 20 min in solution A containing a secondary antibody (Alexa Fluor 647 donkey anti-mouse antibody, 1:250, Molecular Probes). Following suspension in PBS containing 0.2% of propidium iodide, the cells were analyzed by FACS for both propidium iodide (dead cells) and LDLR in live cells with Alexa Fluor 647 using the FACS Becton Dickinson LSR (BD Biosciences).

Immunocytochemistry—Cells were washed three times with PBS, fixed with a solution of 4% paraformaldehyde, 4% sucrose in PBS for 15 min, and permeabilized with a solution of 25% BSA, 10% fetal bovine serum, 0.3 M glycine, and 0.1% Tween/PBS for 10 min. The cells were then incubated for 30 min with 5% BSA (Fraction V, Sigma), followed by an overnight incubation at 4 °C with selected antibodies (1:100 goat polyclonal anti-hLDLR, R&D Systems; 1:500 mouse mAb-V5, Invitrogen; 1:200 human TGN46, Serotech; 1:200 human protein disulfide isomerase, Santa Cruz Biotechnology). The cells were then incubated for 60 min with the corresponding Alexa Fluor-conjugated secondary antibodies (Molecular Probes) and mounted in ProLong Gold antifade reagent (Molecular Probes, Invitrogen). Immunofluorescence analyses were performed with a Zeiss LSM 710 confocal microscope coupled with a Nikon Eclipse TE2000-U laser scanning microscope with 405-, 488-,

543-, and 633-nm laser lines. Images were processed with Zen 2009 software. For internalization experiments, HepG2/shPCSK9 cells were preincubated for 15 min at room temperature followed by 30 min of incubation with conditioned media at room temperature, and then transferred to 37 °C for 0, 60, or 180 min before preparation for immunofluorescence analysis as described above.

RESULTS

Expression, Zymogen Processing, and Secretion of CHRD Module Deletants—The reported crystal structures of PCSK9 revealed that its C-terminal CHRD contains three distinct Cys/His-rich modules named M1, M2, and M3 (7–9) (supplemental Fig. S1). The CHRD was shown to be critical for targeting the PCSK9=LDLR complex to endosomes/lysosomes for degradation (13, 29). Thus, it was of interest to define the contribution of each module in this process and whether they could equally affect the extracellular (33) and intracellular (14) pathways of PCSK9-induced LDLR degradation. Accordingly, six C-terminally V5-tagged CHRD module deletants were generated by mutagenesis, resulting in human PCSK9 forms lacking only one (Δ M1, Δ M2, or Δ M3) or two (Δ M12, Δ M23, or Δ M13) modules (Fig. 1A). To analyze the fate of each deletant and its ability to fold and be secreted, among other cell lines, we first selected HEK293 cells, which are efficiently transfected, allowing the collection of media with enough material for multiple extracellular incubations. Accordingly, HEK293 (or HepG2/shPCSK9) cells were transiently transfected with cDNAs coding for these constructs. Cell lysates and media were then analyzed by Western blot analysis using mAb-V5. The data show that, similar to

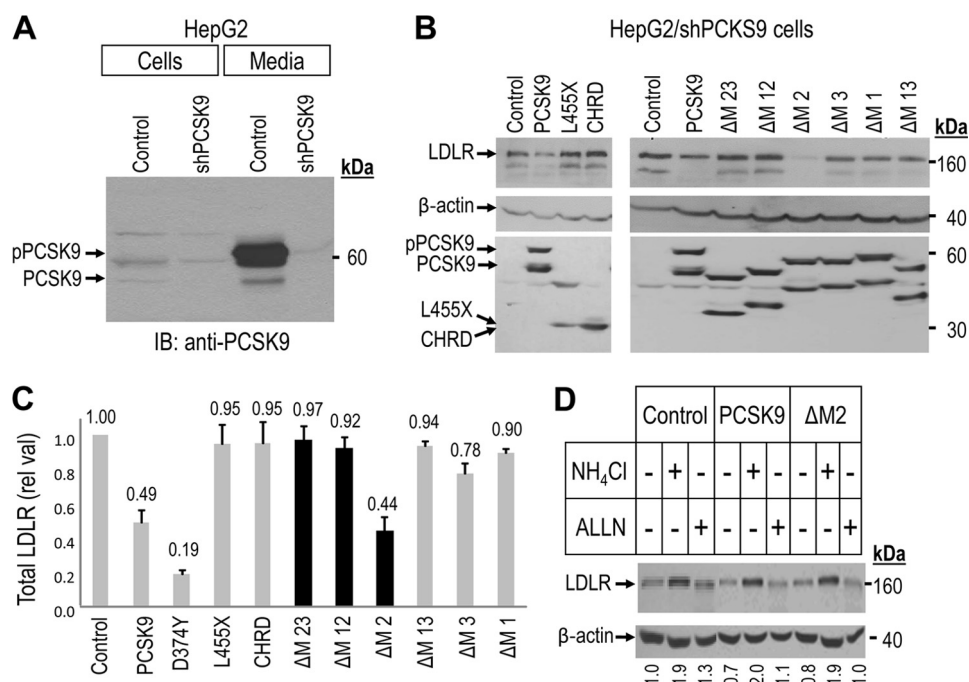


FIGURE 2. Effect of intracellular expression of CHRD module deletants on total LDLR levels in HepG2/shPCSK9 cells. Western blot analysis of total LDLR in HepG2/shPCSK9 cells transiently transfected with either an empty vector control, WT PCSK9, L455X, CHRD, and CHRD module deletants (Δ M23, Δ M12, Δ M2, Δ M3, Δ M1, and Δ M13). *A*, intracellular and secretion levels of endogenous PCSK9 in HepG2 cells expressing non-target shRNA (*Control*) compared with HepG2/shPCSK9 cells. The migration positions of proPCSK9 and PCSK9 forms are emphasized. *B*, immunoblot. *B*, total LDLR reducing activity of WT PCSK9, L455X, CHRD, and CHRD domain deletants compared with cells expressing an empty vector control (*upper panels*). The levels of cellular and secreted PCSK9 forms are shown (*center panels*) as well as those of the β -actin cellular loading controls (*lower panels*). Endogenous LDLR was detected using a polyclonal antibody recognizing its extracellular domain, and all PCSK9 constructs, except the control empty vector one, were detected with a V5 HRP-conjugated mAb. *C*, total LDLR levels normalized to β -actin. As negative controls we used either L455X (a construct lacking the CHRD) or the secreted CHRD domain, which had no effect on the LDLR (31). The *black bars* emphasize the secreted CHRD module deletants. These data are representative of at least three independent experiments. *D*, intracellular degradation of LDLR by PCSK9 and PCSK9 Δ M2 occurs in acidic compartment(s) and is blocked upon 7h incubation of HepG2/shPCSK9 cells by 10 mM NH₄Cl but not by 50 μ M acetyl-Leu-Leu-norleucinal (ALLN).

WT PCSK9 (1, 5) and its construct lacking the CHRD (L455X) (31), each CHRD module deletant is synthesized as a zymogen (pPCSK9), which is cleaved into a mature form, similar to WT PCSK9 (Fig. 1*B*, *cell lysates*, *left panel*). The CHRD only shows one protein product because it lacks both the prosegment and catalytic domain of PCSK9. However, only PCSK9, L455X, and CHRD and PCSK9 Δ M23, Δ M12, and Δ M2 are secreted (Fig. 1*B*, *media*, *right panel*). This suggested that loss of the M2 domain does not negatively affect folding and/or secretion (Fig. 1*B*, *media*, *right panel*). In contrast, in the presence of M2, the loss of M1, M3, or both (Δ M1, Δ M3, Δ M13) prevents secretion into the medium (Fig. 1*B*, *media*, *right panel*) but not zymogen cleavage (*A*, *cells*, *left panel*). In agreement, immunocytochemistry (*supplemental Fig. S2*) confirmed that Δ M1, Δ M3, and Δ M13 are retained in the ER and colocalize with the ER marker protein disulfide isomerase. In contrast, the secreted Δ M2, Δ M12, and Δ M23 colocalize with the *trans* Golgi network marker protein TGN46. We also noted that all secreted constructs containing the catalytic domain exhibit small amounts of the product of furin-cleavage at Arg₂₁₈, resulting in the loss of the N-terminal residues 152–218 (Δ N₂₁₈) (34, 35).

LDLR Degradation by Overexpressed PCSK9 and Its Domain Deletants—Because PCSK9 is mostly expressed in hepatocytes (1, 24) it was pertinent to study its ability to degrade LDLR in these cells. We previously reported the activity of PCSK9 on LDLR in the human hepatocyte-derived HepG2 cell line (5, 31). Furthermore, because of the endogenous expression of PCSK9

in naïve HepG2 cells, we engineered cells lacking its expression by stable shRNA silencing. The resultant HepG2/shPCSK9 cells showed a more robust activity of added PCSK9 (14). Accordingly, we used these cells to further study the various PCSK9 constructs. Western blot analysis of the expression of endogenous PCSK9 in HepG2 cells expressing non-functional shRNA (*Control*) versus HepG2/shPCSK9 cells revealed that in the latter, the total protein levels (cells and media) of endogenous PCSK9 are reduced by > 90% (Fig. 2*A*).

We next transiently transfected cDNAs coding for PCSK9 and its various V5-tagged deletion constructs in HepG2/shPCSK9 cells and analyzed their intracellular production and effects on the levels of LDLR 48 h post-transfection (Fig. 2*B*). All the data obtained with HepG2/shPCSK9 cells were confirmed in naïve HepG2 cells (*supplemental Fig. S3A*). The expression and intracellular processing of PCSK9 and its deletants were very similar to those observed in HEK293 cells (Fig. 1*B*, *cells*, *left panel*). Western blot analysis of total LDLR in these cells (Figs. 2*B*, *upper panel*, and *C*) showed that only PCSK9, its GOF D374Y mutant, and PCSK9 Δ M2 effectively enhance the degradation of endogenous LDLR in these HepG2/shPCSK9 cells. Furthermore, WT PCSK9 and its Δ M2 deletant are equipotent in this respect (Fig. 2*C*). Surprisingly, even though PCSK9 Δ M23 and Δ M12 are secreted (Fig. 1*B*), they do not enhance LDLR degradation, similar to the PCSK9 L455X and the CHRD (Fig. 3*C*). In accordance with the observed absence of secretion of the module deletants PCSK9 Δ M13, Δ M3, and Δ M1 (Fig. 1*B*,

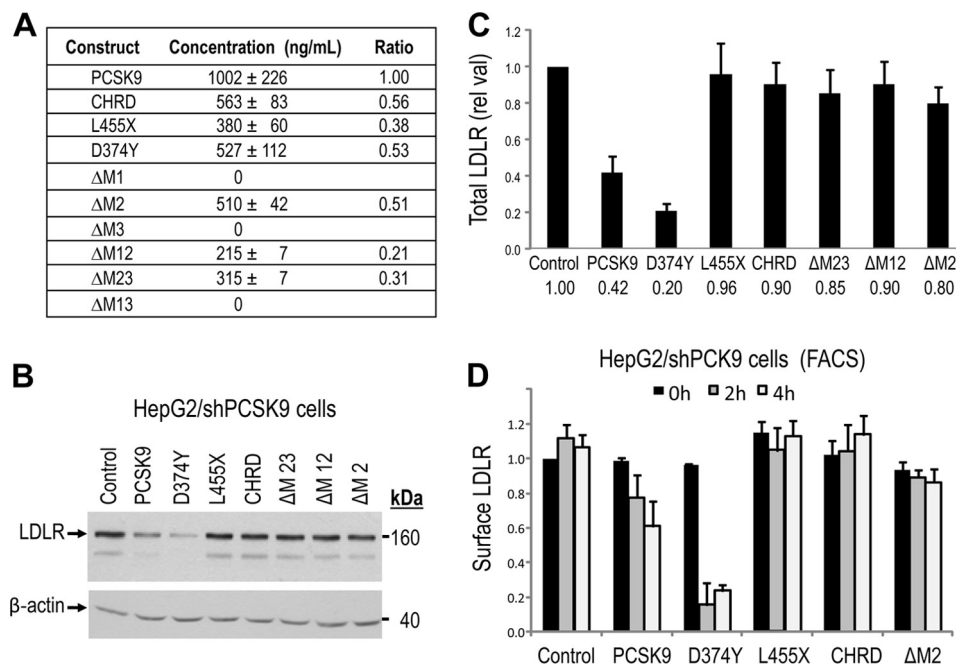


FIGURE 3. Lack of modulation of LDLR levels following extracellular incubation of HepG2/shPCSK9 cells with CHR module deletants. *A*, ELISA-V5 assay of secreted PCSK9, L455X, CHR, D374Y, and CHR deletants (Δ M2, Δ M12, and Δ M23) from transiently transfected HEK293 cells. *B* and *C*, HepG2/shPCSK9 cells were incubated with equal amounts of PCSK9 constructs (1.2 μ g/ml) for 4 h at 37 °C. Cells were then lysed, and total LDLR levels were analyzed by Western blot analysis (*B*) and normalized to β -actin (*C*). Total LDLR was detected using a polyclonal antibody recognizing its extracellular domain, and all PCSK9 constructs were detected with a V5 HRP-conjugated antibody. *D*, HepG2/shPCSK9 cells were incubated for 0, 2, and 4 h at 37 °C with equal amounts (1.2 μ g/ml) of secreted PCSK9 constructs, including Δ M2. Cells were detached as described under "Experimental Procedures," and the levels of cell surface LDLR were measured by FACS analysis. As negative controls we used either a construct lacking the CHR (L455X) or that expressing only the CHR, both of which had no effect on LDLR levels, similar to the PCSK9 Δ M2 construct. These data are representative of at least three independent experiments. Only PCSK9 and its D374Y mutant significantly ($p < 0.001$) enhance the degradation of the LDLR.

media, right panel), these constructs are completely inactive on the LDLR (Fig. 2, *B* and *C*).

The similar activity of PCSK9 and its M2 deletant begged the question of whether they both act in a similar fashion to induce LDLR degradation in acidic endosomal/lysosomal compartments. Similar to what was previously observed for WT PCSK9 in HEK293 cells (5), incubation of HepG2/shPCSK9 cells with 10 mM of the alkalinizing agent NH_4Cl completely abrogated the effects of both PCSK9 and its Δ M2 deletant (Fig. 2*D*), as also observed in control cells. Note that the proteasome inhibitor acetyl-Leu-Leu-norleucinal does not significantly affect the ability of either PCSK9 or PCSK9 Δ M2 to induce the degradation of LDLR. Thus, we can conclude that similar to WT PCSK9, the PCSK9 Δ M2 construct also enhances the degradation of the PCSK9=LDLR complex in acidic compartment(s).

LDLR Degradation in HepG2/shPCSK9 Cells by Extracellular PCSK9 and its Domain Deletants—We next proceeded to gauge the effects of PCSK9 and its domain deletants on the extracellular pathway of LDLR degradation. Accordingly, we concentrated approximately ten times the media of HEK293 cells obtained 72 h post-transfection with each cDNA construct. The concentration of each V5-tagged protein in these media was assessed by an ELISA-V5 (Fig. 3*A*). As expected, no protein is secreted from the Δ M1, Δ M3, and Δ M13 constructs. We also observed that the D374Y mutant and the deletants analyzed are all less secreted than WT PCSK9, with Δ M23 and Δ M12 being the least (five times less) secreted ones (Fig. 3*A*, *Ratio column*).

All protein constructs were adjusted to a final 1.5 μ g/ml concentration in LPDS media and then incubated for 4 h at 37 °C with HepG2/shPCSK9 cells that were preincubated for 24 h in LPDS media to stimulate LDLR expression (5). Unexpectedly, Western blot analysis of total LDLR revealed that only WT PCSK9 and its GOF D374Y are active in this assay. These data confirm the observed inactivity of extracellular L455X and CHR on LDLR (31) and extends this observation to constructs lacking M23 and M12 modules, expected from their inactivity in the intracellular pathway (Fig. 2, *B* and *C*). However, in contrast to the intracellular expression of PCSK9 Δ M2 (Fig. 2, *B* and *C*), its extracellular incubation with HepG2/shPCSK9 cells had no significant effect on LDLR levels (Fig. 3, *B* and *C*). This unexpected observation was repeated many times and further confirmed in naïve HepG2 cells (*supplemental Fig. S3B*) as well as by FACS analysis of cell surface LDLR following 0, 2-h, and 4-h incubations (Fig. 3*D*).

To test whether the lack of activity observed upon incubation of cells with 1.5 μ g/ml may be due to a lower intrinsic activity that may be detectable at higher concentrations, HepG2/shPCSK9 cells were incubated for 4 h at 37 °C with increasing concentrations (0, 0.5, 1, 3, and 5 μ g/ml) of either PCSK9 or its Δ M2 deletant (Fig. 4*A*). The data show that 5 μ g/ml PCSK9 can reduce total LDLR levels by \sim 60% but that PCSK9 Δ M2 is still completely inactive at the same concentration. Finally, to eliminate the possibility that post-translational modifications in hepatocytes would be different from HEK293 cells, we also confirmed the loss of extracellular function of PCSK9 Δ M2, even

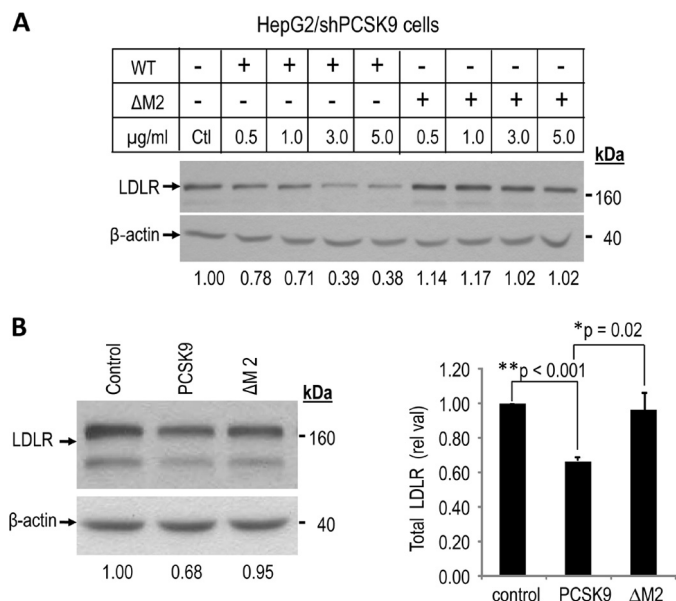


FIGURE 4. Loss of extracellular degradation activity on LDLR by PCSK9 Δ M2 produced in and/or tested on HepG2/shPCSK9 cells. Wild type PCSK9 and PCSK9 Δ M2 obtained from HEK293 or HepG2/shPCSK9 cells incubated for 4 h at (A) increasing concentrations (0 [control, Ctl], 0.5, 1.0, 3.0, and 5.0 μ g/ml, quantified by ELISA assays) with HepG2/shPCSK9 cells, or (B) 1.5 μ g/ml (quantified by ELISA assays) with HepG2/shPCSK9 cells. Cells were then lysed and total LDLR was analyzed by Western blot. Total LDLR was detected using a polyclonal antibody recognizing its extracellular domain and its levels were normalized to β -actin cellular loading controls. These data are representative of three independent experiments. Statistical values were estimated by Student's *t*-test and considered significant when *p* values are < 0.05 . The data show that PCSK9 significantly reduces LDLR (**, $p < 0.001$) and that the LOF of PCSK9 Δ M2 versus PCSK9 is significant with *, $p = 0.02$, but not versus control, supporting its complete LOF.

when it is produced in and tested on HepG2/shPCSK9 cells (Fig. 4B). Thus, we can conclude that the M2 domain is critical for the activity of PCSK9 on LDLR in the extracellular pathway but not in the intracellular one.

Internalization of Extracellular PCSK9, Δ M2, and L455X in HepG2/shPCSK9 Cells—In view of the inactivity of either PCSK9 L455X or PCSK9 Δ M2 on LDLR, it was of interest to define the critical step that these deletants cannot achieve and that is required to enhance the degradation of the PCSK9=LDLR complex. To avoid degradation in acidic compartments, HepG2/shPCSK9 cells were preincubated overnight with 5 mM NH_4Cl and maintained in these conditions until cell fixation. To slow down cellular metabolism, cells were placed for 15 min at room temperature. To allow cell surface binding, HepG2/shPCSK9 cells were incubated for 30 min with each of the three constructs. Subsequently, incubations were shifted to 37 °C for 0, 60, or 180 min to follow the possible cellular internalization of the proteins by confocal microscopy under permeabilizing conditions (31).

As expected, analysis of confocal microscopy images showed that PCSK9 colocalized with cell surface LDLR and, after 60 and 180 min, was internalized into the cell with the LDLR (Fig. 5A). Confocal images revealed that at $t = 0$ min, L455X (supplemental Fig. S4) and the module deletant Δ M2 (Fig. 5B) also colocalized with the LDLR, indicative of their binding to this receptor. Subsequently, at $t = 60$ and 180 min, both deletant proteins were also found to increasingly colocalize with intracellular

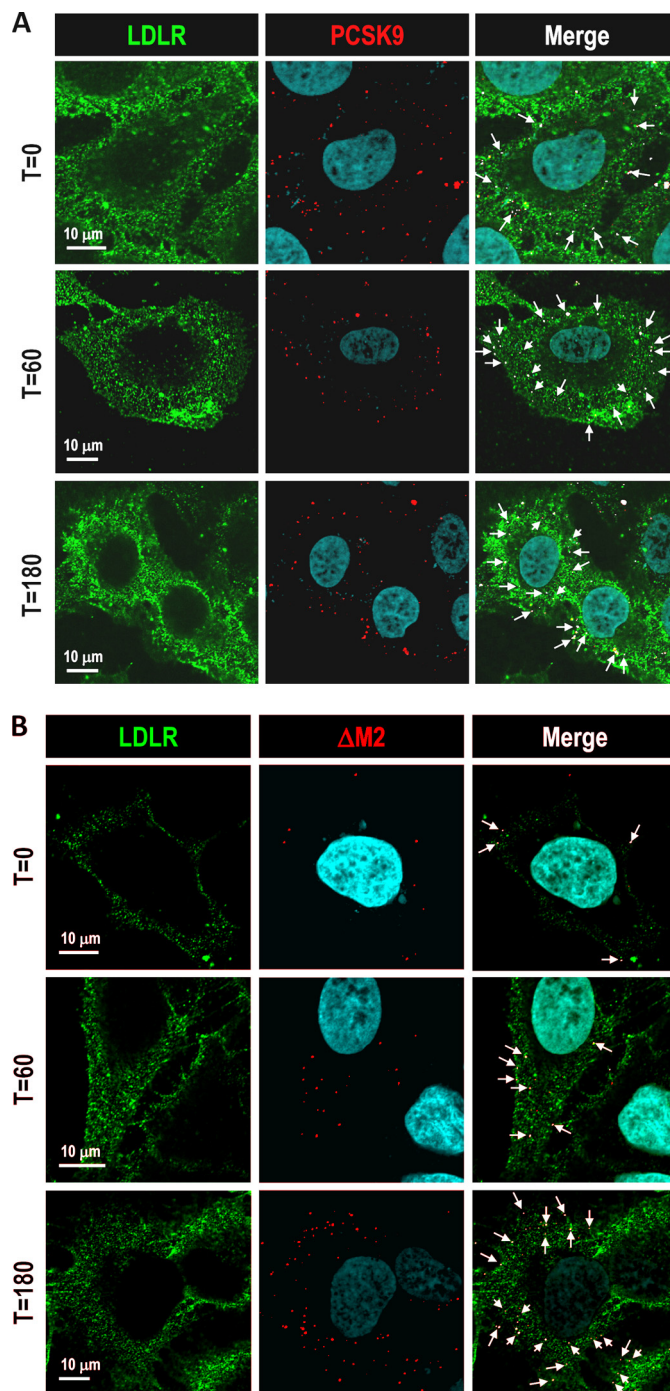


FIGURE 5. Internalization of PCSK9 Δ M2. HepG2/shPCSK9 cells were incubated at the indicated times with HEK293 media containing 1.5 μ g/ml of PCSK9 (A) and PCSK9 Δ M2 (B), both V5-tagged. After 0, 60, or 180 min of incubation, cells were prepared for immunocytochemistry under permeabilizing conditions, and the internalization of V5-tagged proteins was analyzed by confocal microscopy. Scale bar = 10 μ m.

LDLR, suggesting that their internalization also occurred (Fig. 5B and supplemental Fig. S4).

Role of the Basic Residues Containing Loops within the Hinge Region and M2—The hinge region of PCSK9, linking the catalytic subunit to the CHR1, is not a disordered sequence as it is visible in all crystal structures and contains an exposed loop, possibly representing a binding region to a partner protein. The latter could regulate the PCSK9-induced degradation of LDLR.

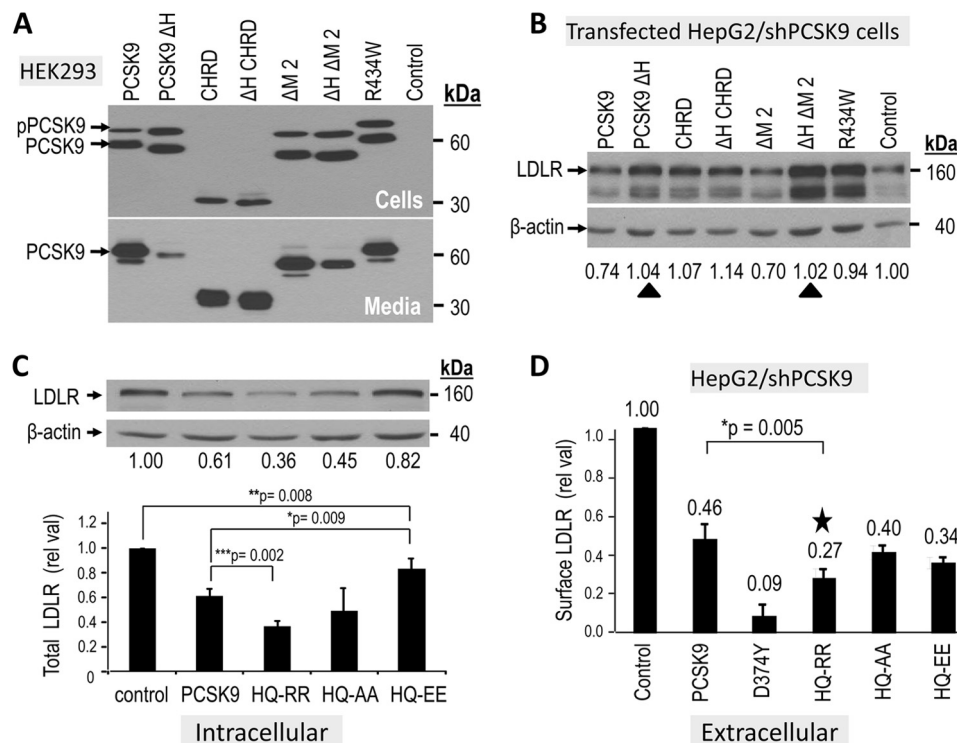


FIGURE 6. Impact of basic residues in the hinge region and M2 module on the activity of PCSK9. Hinge region: A, HepG2/shPCSK9 cells were transiently transfected with various V5-tagged constructs containing or lacking the hinge region of PCSK9 (aa 405–420) and/or lacking the M2 module. At 48 h post-transfection, the proteins in the media and cell lysates were separated by SDS-PAGE and analyzed by Western blot using mAb-V5. The migration positions of proPCSK9 (pPCSK9) and mature PCSK9 are emphasized. B, the total LDLR-degrading activity of each construct was analyzed 48 h post transfection by Western blot of cell lysates using a polyclonal LDLR antibody, and the levels of the ~160 kDa represents the immature form of the receptor. The *black triangles* point to the abrogation of the intracellular activity in WT and Δ M2 constructs upon removal of the hinge region. M2 module: C, HepG2/shPCSK9 cells were transiently transfected with various V5-tagged constructs expressing PCSK9 Δ M2, WT PCSK9 or some of its single point mutant (GOF D374Y) and the charge modified double point mutants (HQ553,554RR; HQ553,554AA; HQ553,554EE). The total LDLR-degrading activity of each construct was analyzed in B. D, cell surface LDLR levels were evaluated by FACS analysis following 4 h incubation of HepG2/shPCSK9 cells with most of the constructs in C. The *star* emphasizes the GOF of the HQ553,554RR mutant in this assay. These data are representative of at least three independent experiments. Statistical significance is emphasized (*, $p < 0.05$).

Alternatively, the hinge region could be necessary for the correct folding of PCSK9 and the spatial positioning of the CHR versus the rest of the molecule to optimize its function. Indeed, we showed previously that a natural mutation of the basic residue at Arg-434 present within an exposed loop within the hinge region (supplemental Fig. S5, A and B) into Trp (R434W) results in an ~70% reduction in the extracellular pathway function of PCSK9 (10). In this report, we extend this observation and show that following transfection of HepG2/shPCSK9 cells, the R434W mutant lacks intracellular activity on the LDLR (Fig. 6B). These data point to the critical importance of the hinge region in regulating both the intracellular and extracellular activity of PCSK9 on the LDLR. We thus analyzed the effects of constructs lacking the hinge region (PCSK9 Δ H, Δ H Δ M2, and Δ H CHR; supplemental Fig. S5) on the overall intracellular activity of PCSK9. Overexpression of these V5-tagged constructs (supplemental Fig. S6) in HEK293 cells revealed that removal of the hinge region does not affect the expression or secretion of the CHR. Similarly, zymogen processing of PCSK9 and its Δ M2 deletant are not affected, but the lack of the hinge region results in a significantly reduced secretion of PCSK9 and less so of the Δ M2 construct (Fig. 6A). Lack of the hinge region in the latter proteins results in a complete loss of their intracellular activity on LDLR (Fig. 6B), supporting the

notion that the hinge region is critical to maintain an active PCSK9 conformation.

A survey of known PCSK9 natural mutations modifying the charge around basic residues within an exposed loop of the CHR M2 module (residues 530–605, supplemental Fig. S5C) led us to concentrate on the only reported natural GOF H553R and LOF Q554E (36) mutants found within the sequence RVHCH₅₅₃QQGH. Accordingly, we doubly mutated residues 553 and 554 to Arg, Ala, or Glu (HQ-RR, HQ-AA, and HQ-EE). The resulting double mutants did not affect the zymogen processing or secretion of PCSK9 (Fig. S7) or its capacity to enhance the degradation of the LDLR via the intracellular pathway (Fig. 6C). However, we note that the HQ-RR mutant results in a 1.7-fold enhanced extracellular activity of PCSK9, as measured by quantitative FACS analysis of cell surface LDLR (Fig. 6D). This result agrees with the observation that the H553R is a GOF but also circumscribes this enhanced activity to the extracellular pathway and not the intracellular one. We conclude that the critical importance of the M2 domain in the extracellular and intracellular pathways can be enhanced by specific mutations that increase the basicity of this exposed loop, such as the selected GOF HQ-RR mutation. This finding further emphasizes the major role played by the M2 module, and specific exposed residues within, in regulating the activity of PCSK9.

DISCUSSION

Hypercholesterolemia, which is characterized by elevated blood LDLc, is one of the main risk factors for cardiovascular disease worldwide (37) and is observed in > 20% of the Western population. Among existing treatments, “statins,” which target HMG-CoA reductase, are by far the most efficient drugs. However, at least 15% of patients either do not respond to statin treatment, encounter serious side effects (38), or do not reach ideal target levels of circulating LDLc to control their dyslipidemia. Consequently, new drugs to treat elevated LDLc and tools to identify responders *versus* non-responders to LDLc-lowering treatments are needed urgently.

Studies performed over the past 9 years have indicated that the proprotein convertase PCSK9 plays a central role in regulating blood LDLc levels (2, 21). Indeed, the mature, active form of PCSK9 binds to the LDLR at the external face of the plasma membrane to enhance its degradation in endosomes/lysosomes, thus preventing internalization of circulating LDLc by liver hepatocytes. Most notably, PCSK9 has been shown to be an efficient therapeutic target in humans (2, 21, 39). Indeed, injections of PCSK9-silencing siRNAs or anti-PCSK9 mAbs, currently in phase II and III clinical trials, have been shown to efficiently reduce LDLc in the blood circulation of patients (2, 28).

Natural mutations within the hinge region and CHR domain were reported to modify the function of PCSK9 and, thus, circulating LDLc levels. For example, the R434W hinge mutation results in a LOF of PCSK9 (10). The five significant LOF mutations in the CHR are in the M1 (S462P), M2 (Q554E), and M3 (P616L, S668R, and C679X) modules, and the reported eight GOF mutations are in the M1 (R469W, E482G, R496W, A514T, F515L, and A522T), M2 (H553R), and M3 (V624M) modules. Even though the hinge region and M2 module exhibit the highest prevalence of structural mobility, the latter possibly because of the lack of disulfide stabilization (7), they are the structural units with the least reported natural mutations, with only two juxtaposed mutations (GOF H553R and LOF Q554E) within the exposed loop of the M2 module (supplemental Fig. S5).

On the basis of the above observations, the requirement of the CHR for the activity of PCSK9 (13, 29) and the demonstration of the presence of intracellular and extracellular pathways by which PCSK9 enhances the degradation of the LDLR (14, 15), we aimed to define the contribution of the hinge region and the three CHR modules in the regulation of the PCSK9 activity.

Accordingly, we generated six CHR module deletants (Δ M1, Δ M2, Δ M3, Δ M12, Δ M23, or Δ M13) (Fig. 1A). We next evaluated their role in the PCSK9 intracellular processing, secretion, subcellular localization, and the two LDLR degradation pathways. Interestingly, intracellular expression of the CHR domain module deletants in HEK293, HepG2/shPCSK9 (cells in Figs. 1B and 2B) or HepG2 cells (not shown) revealed that the lack of one or two modules in the CHR domain did not affect the ability of proPCSK9 to undergo autocatalytic processing into mature PCSK9. Surprisingly, among all six CHR module deletants, only PCSK9 Δ M2, Δ M12, and Δ M23

were secreted (media in Fig. 1A), indicating that for secretion, M2 was the only dispensable module.

Our previous data showed that except for the GOF D374Y, cellular overexpression of WT PCSK9 mostly results in lowering LDLR levels by the intracellular pathway (14). This is due to the relatively low secretion levels of PCSK9 from transfected cells (\sim 0.1 μ g/ml), *versus* the ten times higher concentration necessary to effectively enhance LDLR degradation from the extracellular pathway (31). We conclude that the intracellular overexpression of PCSK9 and its Δ M2 deletant exhibit a comparable ability to enhance the degradation of the LDLR, likely by the intracellular pathway.

We next assessed the extracellular activity of the three secreted CHR module deletants (Fig. 3, B and C, and supplemental Fig. S3). Unexpectedly, none of them showed any activity on the LDLR, even the Δ M2 at a concentration up to 5 μ g/ml, which results in an \sim 60% reduction of LDLR levels by WT PCSK9 (Fig. 4A). This conclusion was further confirmed by FACS analysis, which also revealed that similar incubations resulted in no detectable reduction of cell surface LDLR levels (Fig. 3D). We analyzed the cellular association of each construct by incubating them with HepG2/shPCSK9 cells followed by extensive washes. Western blot analysis of cell lysates demonstrated that all of them bound to cells (data not shown). Thus, the lack of activity of the PCSK9 L455X and CHR module deletants is not due to their inability to bind and likely internalize into cells.

To rationalize the inactivity of the extracellular Δ M2 protein, immunocytochemistry was used to analyze its fate. Confocal images showed that PCSK9, L455X, and even the Δ M2 deletant colocalize with LDLR at all time points, revealing their cell surface binding and internalization (Fig. 5 and supplemental Fig. S4). However, these steps were not accomplished with the same efficiency, as evidenced by the higher binding and faster internalization of WT PCSK9 *versus* L455X or Δ M2. On the basis of these observations, it is clear that the inability of PCSK9 Δ M2 to enhance LDLR degradation does not reside in its lack of LDLR binding or its cointernalization with the LDLR, suggesting that the M2 module carries structural requirements to achieve the critical and late LDLR sorting and degradation steps within endosomes/lysosomes.

The hinge region is tightly linked to the CHR domain, even if the hinge is only 17 amino acids long, its sequence seems to be important to maintain the full activity of WT PCSK9. Notably, the only natural mutation (R434W) reported (10) in the hinge region is a loss of function. This evidence led us to more closely examine the role of the hinge region in the folding, secretion, and, ultimately, in PCSK9 activity. On the basis of PCSK9 crystal structures, we selected to delete the exposed loop (residues 422–439) corresponding to the hinge region (supplemental Fig. S5). Interestingly, although autocatalytically processed, PCSK9 lacking amino acids 422–439 is very poorly secreted (PCSK9 Δ H, supplemental Fig. S6A). In contrast, removal of the same sequence from either the N terminus of the CHR or the Δ M2 construct (PCSK9 Δ H Δ M2) had much less effect. Overexpression of either construct in HepG2/shPCSK9 cells revealed that none of these constructs is active on the LDLR (Fig. 6B). Thus, although the PCSK9 Δ M2 is active intracellularly, the loss of the

hinge region abrogates its activity. Hence, the integrity of the hinge region is critical for the intracellular activity of PCSK9 (Fig. 6B). This is likely due to its structural requirement as a linker providing flexibility to the CHRDR. Amazingly, replacement of only one basic residue (Arg-434) by a Trp within the hinge region results in an almost complete intracellular loss of function (R434W in Fig. 6B) and a reduction by \sim 70% of its extracellular activity on LDLR (10).

The gain of a basic residue at neutral pH in the H553R mutant within the M2 domain does not affect the intracellular activity (data not shown) and, yet, results in a GOF (36), as also observed for the double mutant HQ₅₅₄-RR₅₅₄ (Fig. 6D). Interestingly, the intracellular activity is either not affected when HQ₅₅₄ is replaced by neutral residues (HQ₅₅₄-AA₅₅₄) or decreased if replaced by acidic residues (HQ₅₅₄-EE₅₅₄; Fig. 6C). The only natural mutations reported in the M2 module (36) are in the exposed His-rich HCHQQGH₅₅₇ loop (supplemental Fig. S5C), resulting in either an increased basicity at neutral pH (GOF, H553R) or increased acidity (relatively weak LOF, Q554E). We thus conclude that the whole M2 module is only critical for the extracellular activity of PCSK9 and that natural mutations within it affect this pathway. This is relevant, because neutralizing mAb injections are being presently tested in phases II and III of various clinical trials to reduce hypercholesterolemia by inhibiting the function of PCSK9 on LDLR (2, 28). All these mAbs target the catalytic subunit of PCSK9 and prevent its extracellular binding to cell surface LDLR. The only attempts to obtain CHRDR-directed interfering mAbs against the extracellular activity of PCSK9 targeted the M3 domain, achieving a maximum of 50% reduction in PCSK9 activity (40). According to this study, targeting the M2 module of the CHRDR could represent a novel strategy to inhibit the extracellular activity of PCSK9.

This concept of increased basicity favoring the CHRDR contribution to the extracellular activity of PCSK9 has also been proposed recently (41). In agreement with the present results, the authors show that replacements of up to 7 His, mostly located in the M2 module, do not affect the intracellular activity of PCSK9. Unfortunately, the authors have not measured the extracellular activity of their mutants, which according to our study should have been the predominantly affected pathway. Furthermore, the authors suggest that replacement of the CHRDR by an unrelated, similarly charged basic domain, results in a comparable activity to WT PCSK9 (41). Although the total basicity of the CHRDR may well be important, our data further zoom in on a specific site within the M2 domain that is a hot spot for natural mutations. We show that, indeed, the higher basicity of the HQ₅₅₄-RR₅₅₄ mutant within an exposed loop results in a robust \sim 2-fold higher extracellular activity on LDLR (Fig. 6D).

A recent report also suggested that the M2 module is dispensable for the intracellular activity of PCSK9 (42). However, the authors did not measure the extracellular function of their PCSK9 Δ M2 construct, which is abrogated according to this work (Figs. 3 and 4).

The mechanism governing the critical role of the M2 module, and possibly its exposed HCHQQGH₅₅₇ loop (supplemental Fig. S5), in regulating the extracellular activity of PCSK9 is

not yet defined. One possibility would be that the M2 module interacts with a critical, as yet undefined, protein that would result in the efficient targeting of the PCSK9=LDLR complex to lysosomes for degradation by endogenous hydrolases (13, 29). This would fit with the observation that an excess of CHRDR could compete extracellularly with PCSK9 for LDLR degradation (42). Even though it was reported that *in vitro* high concentrations of the CHRDR could bind the N-terminal R1-R7 repeat domain of the LDLR, the crystal structure of PCSK9 bound to the ectodomain (18) or full-length (PDB codes 3M0C and 3P5B) LDLR do not support this model, as the CHRDR is far away in space from the β -barrel repeat domains of this receptor. Thus, the hepatocyte CHRDR-binding protein remains to be identified.

In conclusion, the structural requirements of the hinge and M2 domains are not the same for the intracellular and extracellular pathways of PCSK9-induced LDLR degradation. Although the R434W mutation in the hinge region is absolutely deleterious for the intracellular activity of PCSK9, it reduces by \sim 70% the extracellular one. In contrast, the loss of M2 is tolerated for the intracellular activity of PCSK9 but not for the extracellular one. This information should lead to the development of more targeted approaches, possibly against the M2 domain, to inhibit the extracellular function of PCSK9 and reduce circulating LDLc.

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