

The Proprotein Convertase (PC) PCSK9 Is Inactivated by Furin and/or PC5/6A

FUNCTIONAL CONSEQUENCES OF NATURAL MUTATIONS AND POST-TRANSLATIONAL MODIFICATIONS*

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Suzanne Benjannet¹, David Rhainds¹, Josée Hamelin, Nasha Nassoury², and Nabil G. Seidah³

From the Laboratory of Biochemical Neuroendocrinology, Clinical Research Institute of Montreal, Montreal, Quebec H2W 1R7, Canada

PCSK9 is the ninth member of the proprotein convertase (PC) family. Some of its natural mutations have been genetically associated with the development of a dominant form of familial hyper- or hypocholesterolemia. The exact mechanism of action of PCSK9 is not clear, although it is known to enhance the intracellular degradation of the low density lipoprotein (LDL) receptor in acidic compartments, likely the endosomes/lysosomes. We analyzed the post-translational modifications of PCSK9 and show that it is sulfated within its prosegment at Tyr³⁸. We also examined the susceptibility of PCSK9 to proteolytic cleavage by the other members of the PC family. The data show that the natural gain-of-function mutations R218S, F216L, and D374Y associated with hypercholesterolemia result in total or partial loss of furin/PC5/6A processing at the motif RFHR²¹⁸↓. In contrast, the loss-of-function mutations A443T and C679X lead either to the lack of *trans*-Golgi network/recycling endosome localization and an enhanced susceptibility to furin cleavage (A443T) or to the inability of PCSK9 to exit the endoplasmic reticulum (C679X). Furthermore, we report the presence of both native and furin-like cleaved forms of PCSK9 in circulating human plasma. Thus, we propose that PCSK9 levels are finely regulated by the basic amino acid convertases furin and PC5/6A. The latter may reduce the lifetime of this proteinase and its ability to degrade the cell-surface LDL receptor, thereby regulating the levels of circulating LDL cholesterol.

The mammalian genome data base predicts the presence of 500–600 proteinase genes that cover all the potential enzymatic cleavages of a given species at all developmental stages. Of these, the most abundant serine proteinases represent about one-third of all five proteinase classes (1). The mammalian sub-

tilisin-like serine proteinases comprise the proprotein convertases (PCs)⁴ that are implicated in the limited cellular proteolysis of secretory precursor proteins, resulting in a diversity of bioactive products, *e.g.* through zymogen activation, the generation of active proteins and peptides and, in some cases, the inactivation of key proteins. The mammalian PC family comprises nine members: PC1/3, PC2, furin, PC4, PACE4, PC5/6, PC7, SKI-1 (subtilisin/kexin isozyme-1)/S1P (site 1 protease), and PCSK9/NARC-1 (2–4). The first seven proteinases are basic amino acid (aa)-specific PCs cleaving precursor proteins at single or paired basic residues within the motif (K/R)X_n(K/R)↓, in which *n* = 0, 2, 4, or 6 (2). These proteinases are phylogenetically more closely related to each other and to yeast kexin than to SKI-1/S1P and PCSK9/NARC-1, which belong to the pyrolysins (5) and proteinase K (4) subfamilies, respectively. The latter enzymes recognize the motifs RX(hydrophobic/aliphatic)X↓ (6) and (V/I)FAQ↓ (7, 8), respectively. These enzymes have been implicated in a wide variety of functions regulating cellular homeostasis and a number of pathologies, including cancer, inflammation, neurodegenerative diseases, atherosclerosis, and viral infections. It was recently realized that some of these convertases play critical roles in the regulation of lipids and/or sterols (6) through the inactivation of lipases, *e.g.* by PC5/6, PACE4, and furin (9); through the activation of specific membrane-bound transcription factors (SREBP-1 and SREBP-2) by SKI-1/S1P (10); or by enhancement of the degradation of the low density lipoprotein (LDL) receptor (LDLR) by PCSK9 (7, 11–13). Indeed, PCSK9 is expressed mostly in adult liver hepatocytes and small intestinal enterocytes together with the LDLR (4). It was discovered recently that point mutations in the *PCSK9* gene within its coding exons (14) are associated with either familial hypercholesterolemia (15–19) or hypocholesterolemia (20–22) phenotypes. This led to the identification of the *PCSK9* gene as the third chromosomal locus associated with autosomal dominant familial hypercholesterolemia, with the LDLR and apoB comprising the other two loci (15).

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³ To whom correspondence should be addressed: Laboratory of Biochemical Neuroendocrinology, Clinical Research Inst. of Montreal, 110 Pine Ave. West, Montreal, Quebec H2W 1R7, Canada. Tel.: 514-987-5609; Fax: 514-987-5542; E-mail: seidah@ircm.qc.ca.

⁴ The abbreviations used are: PC, proprotein convertase; aa, amino acid(s); LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; mAb, monoclonal antibody; pAb, polyclonal antibody; Ab1-hPC9, rabbit anti-human PCSK9 polyclonal antibody; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; TGN, *trans*-Golgi network; WT, wild-type; ER, endoplasmic reticulum; endo H, endoglycosidase H; CHRD, Cys/His-rich domain.

The Level of PCSK9 Is Regulated by Other Convertases

It has been suggested that some PCSK9 single point mutations result in a gain or enhanced activity of PCSK9 in the degradation of the LDLR in acidic compartments, likely endosomes (7, 13), whereas others would cause a loss of function (20, 21), and that these mutations would be associated with the development of hypercholesterolemia or hypocholesterolemia, respectively (23). It was thus hypothesized that high levels of active PCSK9 are associated with a faster rate of degradation of the cell-surface LDLR, resulting in increased amounts of circulating LDL cholesterol, as the uptake of the latter in liver hepatocytes by the LDLR will be diminished accordingly and vice versa. Thus, the level of cell-surface LDLR is indirectly proportional to the level of PCSK9 in the liver and presumably small intestine. This hypothesis is reinforced by the *in vivo* observations that, in mice lacking a functional *Pcsk9* gene (*Pcsk9* knockout mice), the level of hepatocyte cell-surface LDLR is greatly enhanced, resulting in an ~50% drop in total circulating cholesterol (24), whereas higher levels of circulating LDL cholesterol are observed in mice overexpressing PCSK9 following recombinant adenoviral infections (7, 11, 12, 25).

Regulation of the levels of active PCSK9 could be achieved by various mechanisms, which among others could implicate (i) its transcription, where its mRNA levels are up-regulated by SREBP-2 (26) and hence by hydroxymethylglutaryl-CoA inhibitors known as “statins” (27) and down-regulated by cholesterol (28) via a reduced level of activated nuclear SREBP-2 (26, 27); (ii) the translational efficiency of the PCSK9 mRNA, which may be controlled by specific factors; (iii) post-translational modifications of PCSK9, including its zymogen cleavage and/or activation, glycosylation, and sulfation (4, 29), or possibly other modifications and processing events that modulate its half-life (4); (iv) regulation of the cellular localization and/or sorting of mature PCSK9; and (v) the cell-surface effect of secreted PCSK9 (30) and its possible uptake into endosomes. It was thus plausible that some of the single point mutations of *PCSK9* associated with autosomal dominant familial dyslipidemia could enhance or abrogate one or more of these regulatory events (23).

In this study, we present evidence that the level of mature PCSK9 is indeed under the control of proteolysis by some members of the basic aa-specific PC family, including furin and/or PC5/6A, and that the cleavage by these PCs may provide a rationale behind the hypercholesterolemia phenotype associated with the French (F216L and R218S) (15, 18) and Anglo-Saxon (D374Y) (16, 17, 19) mutations and those associated with hypocholesterolemia in African Americans (C679X and A443T) (21).

EXPERIMENTAL PROCEDURES

cDNAs and Cells—PCSK9, its mutant cDNAs, and the different PCs were cloned into pIRES2-EGFP with a C-terminal V5 tag as described (4). HEK293, LoVo-C5, and hepatic HepG2 cells (American Type Culture Collection, Manassas, VA) and HuH7 cells (a gift from François Jean, University of British Columbia) were routinely cultivated in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum.

Anti-PCSK9 Antibodies—The antibodies used were as follows: anti-V5 epitope monoclonal antibody (mAb) (Invitro-

gen), rabbit anti-human LDLR-(184–196) polyclonal antibody (pAb) (Research Diagnostics), rabbit anti- α -actin pAb (Sigma), purified rabbit anti-human PCSK9-(490–502) pAb (Cayman Chemical, catalog no. 10007185), and goat anti-human PCSK9-(679–692) pAb (Imgenex, catalog no. IMX-3786). An in-house pAb directed against human PCSK9 (Ab1-hPC9) was obtained from rabbits injected with affinity-purified pro-PCSK9 (aa 31–454) expressed in *Escherichia coli* BL21 (see Fig. 3).⁵

Transfections and Biosynthetic Analyses—Transfections were done with 3×10^5 HEK293 cells using Effectene (Qiagen Inc.) and a total of 0.5 μ g of cDNAs. Alternatively, 5×10^5 HuH7 or 6×10^5 HepG2 cells were transfected with Lipofectamine 2000 (Invitrogen) and a total of 4 μ g of cDNAs. Two days post-transfection, the cells were washed and then incubated for various times with either 250 μ Ci/ml [³⁵S]Met/Cys or 400 μ Ci/ml Na₂³⁵SO₄ (PerkinElmer Life Sciences). Some experiments were done in the presence of A23187 (2 μ M), tunicamycin (5 μ g/ml), or NaClO₃ (30 mM). The cells were lysed in modified radioimmune precipitation assay buffer (150 mM NaCl and 50 mM Tris-HCl, pH 7.5), 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and proteinase inhibitor mixture (Roche Applied Science), after which the lysates and media were prepared for immunoprecipitation (31). The antibodies used were anti-V5 mAb (1:500), Ab1-hPC9 (1:200), rabbit anti-human PCSK9-(490–502) pAb (1:200), and goat anti-human PCSK9-(679–692) pAb (1:200). Immunoprecipitates were resolved by SDS-PAGE on 8% Tricine gels and autoradiographed. Most of these experiments were repeated at least three times. Quantitation was performed on a Storm imager (Amersham Biosciences) using ImageQuant Version 5.2 software.

Protein Solubilization, Western Blotting, and Immunodetection—Cells in 35-mm plates were incubated for 24 h in Dulbecco's modified Eagle's medium plus 10% fetal bovine serum. The cells were washed, incubated for 30 min with radioimmune precipitation assay buffer with proteinase inhibitors on ice, and homogenized, and the lysates were clarified (32). Protein content was assayed with Bradford reagent. Fifty micrograms of proteins (or 10 μ l of medium) were analyzed on 8% Tricine gels, followed by Western blotting for LDLR (160 kDa), α -actin (42 kDa), and PCSK9-V5. The membranes were successively incubated with primary antibodies (anti-human LDLR-(184–196), 1:2000; anti-V5-horse radish peroxidase and anti- α -actin, 1:5000; and Ab1-hPC9, 1:3000) and an appropriate secondary antibody if required and revealed using ECL Plus (Amersham Biosciences). For experiments with human plasma, 30 μ l of the latter were immunoprecipitated with Ab1-hPC9 (1:200) and then analyzed by SDS-PAGE on 8% glycine gels. Following transfer to a polyvinylidene difluoride membrane, PCSK9 was detected with the same antibody (1:3000), followed by rabbit TrueBlot (eBioscience) as a secondary antibody. Affinity removal of IgGs and albumin from plasma was performed using a ProteoSeek removal kit (Pierce).

Immunofluorescence and Confocal Microscopy—HuH7 cells were seeded on chamber slides (Nunc, Rochester, NY) and transfected the following day. Forty-eight hours post-transfec-

⁵ N. Nassoury, D. Blasiole, A. T. Oler, J. Hamelin, A. Prat, A. Attie, and N. G. Seidah, submitted for publication.

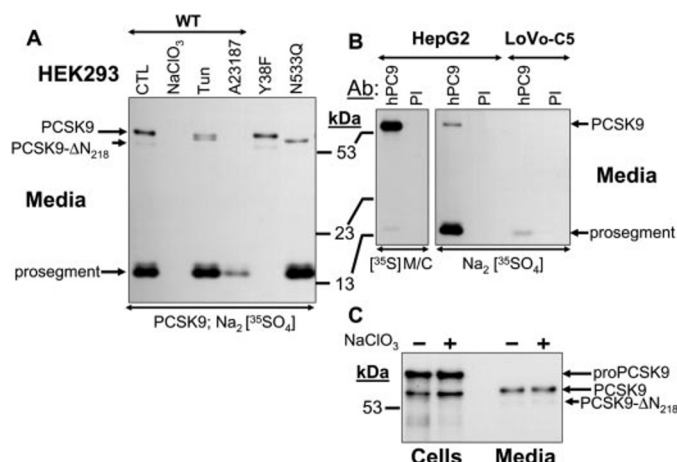


FIGURE 1. Post-translational sulfation of secreted PCSK9 influences its sorting index. HEK293 cells were transiently cotransfected with cDNA encoding V5-tagged WT PCSK9 or the Y38F tyrosine sulfation or N533Q *N*-glycosylation mutant. Forty hours post-transfection, the cells were pulsed with Na₂³⁵SO₄ for 2 h or with [³⁵S]Met/Cys for 3 h in the presence of NaClO₃ (30 mM), tunicamycin (*Tun*; 5 μg/ml), or the ionophore A23187 (2 μM) as indicated. Cell media were immunoprecipitated with anti-V5 mAb (A and C) or in-house Ab1-hPC9 (C) and analyzed on 8% Tricine gels. A, the PCSK9 Y38F mutant prevented sulfation of the prosegment, which was also blocked by NaClO₃ treatment, but not by inhibition of glycosylation by tunicamycin. CTL, control. B, Ab1-hPC9 was used to immunoprecipitate endogenous PCSK9 in HepG2 and furin-negative LoVo-C5 cell culture media. PI, preimmune rabbit serum. C, inhibition of sulfation did not impair PCSK9 secretion.

tion, the cells were fixed with 3% paraformaldehyde for 30 min at 4 °C and incubated for 1 h at 4 °C with 5% normal goat serum containing 0.1% Triton X-100 (blocking buffer), followed by overnight incubation at 4 °C with anti-V5 mAb (1:1000), rabbit anti-V5 pAb (1:1000) (Sigma), or Ab1-hPC9 in blocking buffer with or without the endosomal marker antibody (anti-MPR300, Abcam). The cells were then incubated for 60 min with Alexa Fluor 647-conjugated goat anti-rabbit IgG and with Alexa Fluor 555-conjugated goat anti-mouse IgG (10 μg/ml; Molecular Probes) and mounted in glycerol and 1,4-diazabicyclo[2.2.2]octane (Sigma). Immunofluorescence analyses were performed with a Zeiss LSM 510 confocal microscope. Images were processed with Adobe Photoshop CS2 (Version 9.0).

RESULTS

Post-translational Modifications of PCSK9, trans-Golgi Network (TGN)/Recycling Endosome Localization, and Activity toward the LDLR—One of the major aims of this work was to unravel the mechanism(s) behind the gain- or loss-of-function mutations of PCSK9 (23). Accordingly, we first concentrated on the post-translational modifications of PCSK9 and their relationship to the ability of PCSK9 to enhance the degradation of the LDLR. We had reported previously that human PCSK9 is *N*-glycosylated at Asn⁵³³ (4) and Tyr-sulfated at an unspecified site(s) (7). We now present data on the exact sites of these modifications. Biosynthetic labeling of HEK293 cells overexpressing human PCSK9 using Na₂³⁵SO₄ revealed that two Tyr sulfation sites exist, a major one in the prosegment and a minor one in the catalytic subunit (Fig. 1A). In agreement, treatment of the cells with tunicamycin, which inhibits *N*-glycosylation, or using the unglycosylated N533Q mutant (4) did not affect such labeling

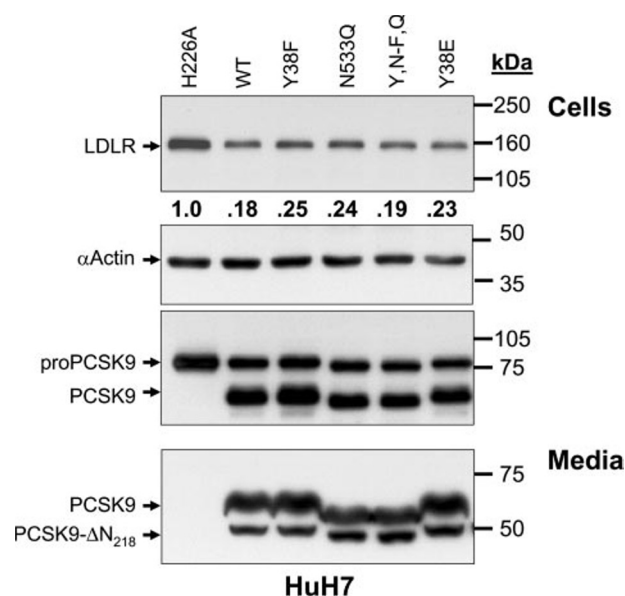


FIGURE 2. PCSK9 post-translational modification mutants retain activity toward the LDLR. HuH7 cells were transiently transfected with cDNA encoding V5-tagged WT PCSK9, the sulfation mutants Y38F and Y38E, the glycosylation mutant N533Q, the double mutant Y38F/N533Q (Y,N-F,Q), or the catalytically inactive mutant H226A. Forty hours post-transfection, cells were lysed, and proteins were resolved by SDS-PAGE on 8% glycine gels. Immunoblotting was performed for PCSK9 (anti-V5 mAb) in cells and media and the LDLR (rabbit pAb); α-actin (rabbit pAb) was used as a loading control. The ratio of LDLR to α-actin levels was calculated by densitometry with ImageQuant Version 5.2.

compared with sodium chlorate, which completely inhibited it (Fig. 1A). Thus, sulfation does not occur on the carbohydrate chain within the catalytic subunit. Interestingly, the calcium ionophore A23187, which depletes luminal calcium but does not affect the processing or secretion of PCSK9 (7), markedly diminished Tyr sulfation, suggesting that the Golgi-associated sulfotransferase(s) is calcium-dependent. Finally, our data show that Tyr³⁸ is the sulfated amino acid in the prosegment because the Y38F mutant is no longer sulfated and yet the catalytic subunit still is (Fig. 1A). We believe that Tyr sulfation is not an artifact of overexpression of PCSK9 because we could detect sulfated PCSK9 endogenously in HepG2 cells and in the furin-negative LoVo-C5 cells using Ab1-hPC9 (an in-house pAb directed against human pro-PCSK9 (aa 31–454)) (Fig. 1B).

What would be the function of Tyr sulfation and Asn glycosylation? Removal of Tyr sulfation or Asn glycosylation by chlorate or tunicamycin treatment, respectively, or using the N533Q mutant revealed no effect on the processing or secretion of PCSK9 (Fig. 1, A and C). We next tested whether sulfation or glycosylation alters the ability of PCSK9 to enhance the degradation of the endogenous LDLR found in the human hepatocyte cell line HuH7. Transient expression of wild-type (WT) PCSK9 or its sulfation (Y38F and Y38E), glycosylation (N533Q), or double (Y38F/N533Q) mutants did not substantially alter the ability of PCSK9 to enhance the degradation of the endogenous LDLR (~70–80% loss of total LDLR immunoreactivity as quantified by Storm imaging, representative of three independent experiments) (Fig. 2), suggesting that, in HuH7 cells endogenously expressing substantial amounts of LDLR, these modifications are not critical for overexpressed PCSK9 activity in this receptor.

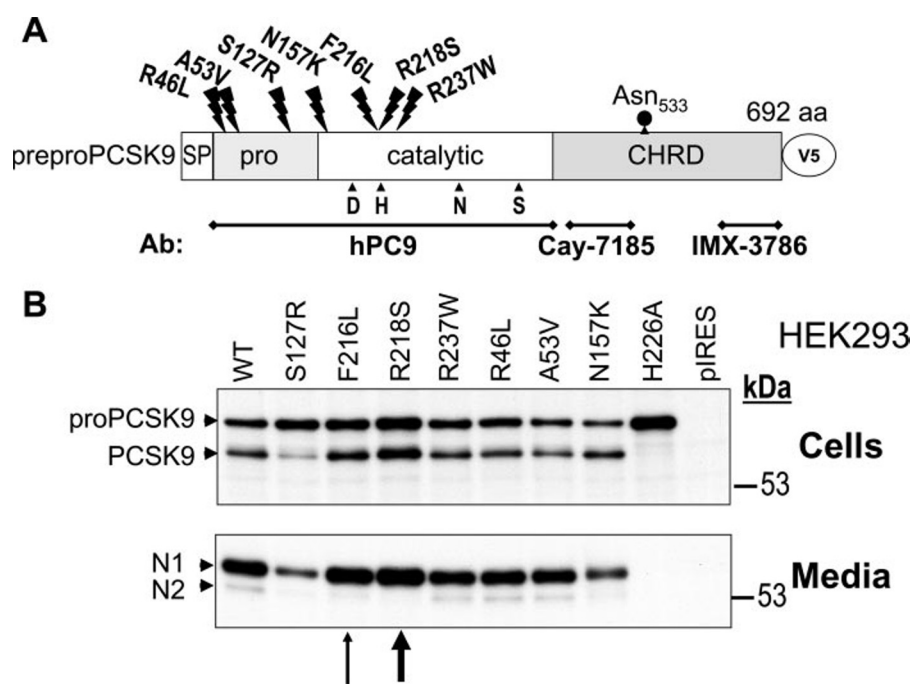


FIGURE 3. Processing of secreted PCSK9 is abrogated in the R218S mutant and reduced in the F216L mutant. A, shown is a schematic view of prepro-PCSK9 domains and natural mutants. SP, signal peptide; pro, prosegment. The active-site Asp, His, and Ser residues and the oxyanion hole Asn residue in the catalytic domain as well as the sole N-glycosylation site at Asn⁵³³ in the CHRD are emphasized. The locations of the antigens used to generate the antibodies used in this study are shown, i.e. Ab1-hPC9, rabbit anti-human PCSK9-(490–502) pAb (Cay-7185), and goat anti-human PCSK9-(679–692) pAb (IMX-3786). B, HEK293 cells were transiently transfected with cDNA encoding V5-tagged WT PCSK9, the indicated natural mutants, or the catalytically inactive mutant H226A or with the empty vector pIRES. Forty hours post-transfection, the cells were pulsed with [³⁵S]Met/Cys for 4 h. Cell lysates and media were immunoprecipitated with anti-V5 mAb. Immunoprecipitates were resolved by SDS-PAGE on 8% Tricine gels and autoradiographed. N1 is full-length secreted PCSK9, and N2 is the cleaved form of secreted PCSK9. The arrows point to the lanes in which a substantially lower level of N2 was observed (partial loss for F216L and complete loss for R218S).

segment (aa 31–152, ~15 kDa) (4). The latter complex then exits the ER and is secreted constitutively as a mature ~60-kDa protein, originally called N1 (Fig. 3B, WT lane) (4). However, in many cell lines, we also observed the presence of an N-terminally truncated form of N1 of ~53 kDa, called N2 (Fig. 3B) (4, 7). The loss of ~7 kDa from the N1 product also occurs in a number of natural mutants (Fig. 3, A and B), including S127R (15), R237W (7), R46L and A53V (15), and N157K (22), but not in the active-site mutant H226A, which remains in the ER as pro-PCSK9 (7) (Fig. 3B). Interestingly, either this N2 form is absent or its levels are substantially reduced in two natural mutants (repeated at least in three independent experiments), i.e. the French mutants R218S (18) and F216L (15), respectively (Fig. 3B, arrows). This suggested that Arg²¹⁸ may be critical for the production of the N2 form. Sequence alignment of vertebrate PCSK9 showed a complete conservation of Arg²¹⁸, which, in most cases, is found within an RXXXR or a KXXXXR sequence (Fig. 4), typical of a basic aa-specific PC recognition motif recognized by furin and/or PC5/6-like enzymes (2). The mutation R218S would completely disrupt this motif, as it eliminates the P1 Arg, and the mutation F216L would affect the P3 position of this motif (Fig. 4, lower). These observations fit with our repeated inability to obtain an N-terminal sequence of N2 using Edman degradation, as the N-terminal Gln²¹⁹ of human PCSK9 would be expected to cyclize on the sequencer into pyroglutamic acid and block the sequencing reaction (data not shown), as was observed for the N-terminal sequence of pro-PCSK9 that starts at Gln³¹ (4).

Accordingly, we tested the hypothesis that cleavage at Arg²¹⁸ is performed by one or more basic aa-specific PCs. Preliminary pulse-chase experiments revealed that (i) the intracellular processing of the N1 form of PCSK9 into N2 occurs only on the form that reaches the Golgi (endoglycosidase H (endo H)-resistant form), (ii) such processing occurs late along the secretory pathway, and (iii) the products are rapidly secreted and not stored in the HEK293 cells (data not shown). To define the enzymes implicated in this cleavage, we coexpressed WT PCSK9 (tagged with V5 at the C terminus) with all the convertases in HEK293 cells. Biosynthetic labeling of these cells with [³⁵S]Met/Cys for 4 h followed by immunoprecipitation and SDS-PAGE analysis revealed that only membrane-bound furin (not soluble furin, lacking the transmembrane cytosolic tail (33)) and, to a lesser extent, soluble PC5/6A are capable of processing the N1 form of PCSK9 into the N2 form, with the concomitant loss of the

Substrate	P8	P6	P4	P2	P1	P1'	P4'
(h,m,r,xl) PCSK9	E-D-G-T-R-F-H-R	Q	A-S-K				
(zf) PCSK9	E-D-G-T-R-V-H-R	Q	A-S-Q				
(ck) PCSK9	E-D-S-S-R-F-H-R	Q	A-S-K				
(tn) PCSK9	V-G-E-A-G-G-H-R	E	A-S-R				
(fr) PCSK9	V-E-K-G-G-G-H-R	E	A-S-R				
(h) (F216L) PCSK9	E-D-G-T-R-F-H-R	Q	A-S-K				
(h) (R218S) PCSK9	E-D-G-T-R-F-H-R	Q	A-S-K				

FIGURE 4. Sequence conservation of PCSK9 Arg²¹⁸ in multiple species. Sequence alignment of amino acids around Arg²¹⁸ in PCSK9 revealed conserved residues at the P1 (Arg²¹⁸) and P2 (His²¹⁷) positions for all species shown and the presence of consensus sequences for cleavage by basic aa-specific PCs of the (K/R)X_nR type (where n = 0, 2, or 4) for human (h), mouse (m), rat (r), *Xenopus laevis* (xl), zebrafish (zf), chicken (ck), *Tetraodon nigroviridis* (tn), and *Fugu rubripes* (fr). Reduction of cleavage at Arg²¹⁸ by the F216L mutant (thin arrow) and absence of cleavage for the R218S mutant (boldface dash) are shown. Mutated residues are underlined.

Processing of PCSK9 and Its Natural Mutants F216L, R218S, and D374Y Associated with Hypercholesterolemia—Originally, we observed that, following signal peptidase cleavage, the endoplasmic reticulum (ER)-resident zymogen pro-PCSK9 (~75 kDa) autocatalytically cleaves its N-terminal prosegment, resulting in a 1:1 tight binding complex of PCSK9 and the pro-

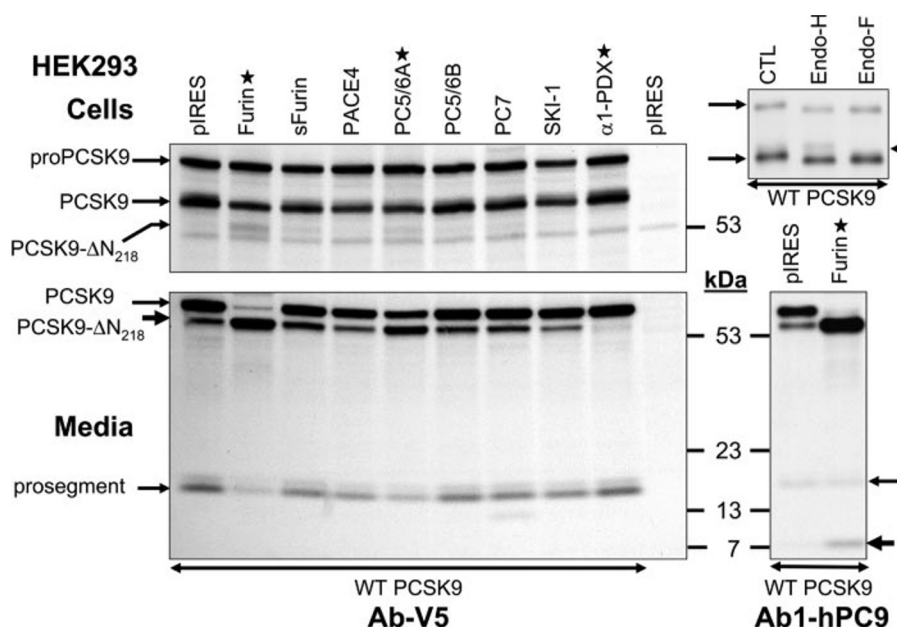


FIGURE 5. Specific cleavage of PCSK9 by the proprotein convertases furin and PC5/6A. HEK293 cells were transiently cotransfected with cDNA encoding V5-tagged WT PCSK9 and different PCs (furin, PACE4, PC5/6A, PC5/6B, PC7, and SKI-1) or α_1 -PDX. Forty hours post-transfection, the cells were pulsed with [35 S]Met/Cys for 3 h. Cell lysates and media were immunoprecipitated with either anti-V5 mAb (left and upper right panels) or in-house anti-human PCSK9 pAb (Ab1-hPC9; lower right panel). Immunoprecipitates were analyzed on 8% Tricine gels. The upper right panel depicts the sensitivity of the cellular PCSK9 forms (control (CTL)) to digestion with either endo H or F. The arrowhead points to the endo H-resistant form of cellular PCSK9, whereas the arrows point to endo H- and endo F-sensitive pro-PCSK9 and PCSK9. The stars emphasize the enhanced cleavage of PCSK9 at Arg²¹⁸ by furin and PC5/6A and the absence of cleavage in the presence of the furin/PC5/6 inhibitor α_1 -PDX (left panels). An additional band was seen with Ab1-hPC9 at ~7 kDa in the presence of furin (bottom right panel). sFurin, soluble furin extracellular domain.

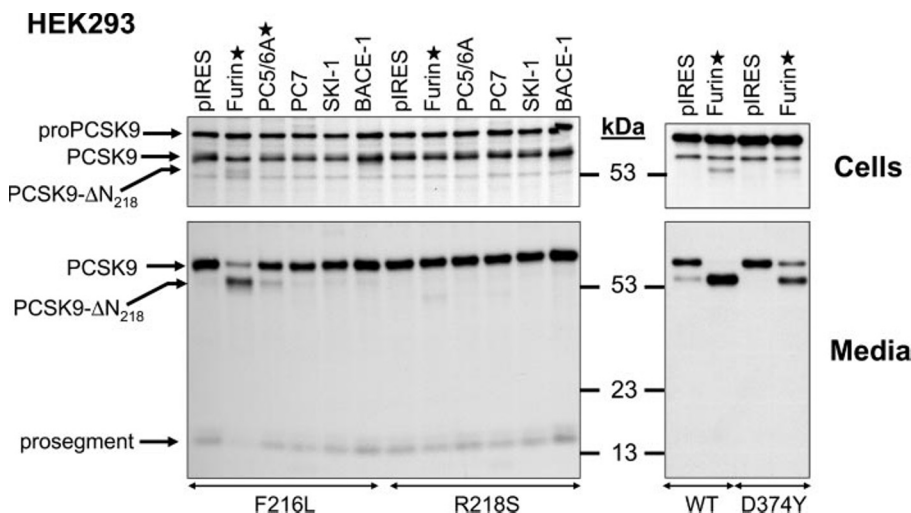


FIGURE 6. The PCSK9 R218S mutant impairs cleavage by the proprotein convertases furin and PC5/6A, whereas the F216L and D374Y mutants show partial cleavage. HEK293 cells were transiently cotransfected with cDNA encoding V5-tagged F216L, R218S, or D374Y and a panel of PCs (furin, PC5/6A, PC7, and SKI-1) or β -secretase (BACE-1). Forty hours post-transfection, the cells were pulsed with [35 S]Met/Cys for 5 h. Cell lysates and media were immunoprecipitated with anti-V5 mAb. Immunoprecipitates were analyzed on 8% Tricine gels. The stars emphasize the complete processing of WT PCSK9 by furin, the absence of processing in the R218S mutant, and partial cleavage of D374Y.

co-immunoprecipitated ~15-kDa PCSK9 prosegment (Fig. 5, lower left panel) (4). Notice that the form of PCSK9 that is competent for this processing is visible in small amounts in cell extracts as an endo H-resistant protein just above the major ER-localized endo H-sensitive PCSK9 form (Fig. 5, upper right panel, arrowhead). This intracellular form was lost upon furin digestion,

with the concomitant appearance of secreted PCSK9- Δ N218 (Fig. 5, Furin lane). Because PCs act only on substrates that reach the TGN or beyond (usually endo H-resistant forms) and because the major intracellular PCSK9 forms observed are endo H-sensitive (as evidenced by the smaller size of both pro-PCSK9 and PCSK9 by ~1.5 kDa) (Fig. 5, upper right panel, arrows), hence residing mostly in the ER, these would be expected to be resistant to furin processing (29). Such a cleavage of mature PCSK9 would be expected to release two fragments, a 66-aa N-terminal V5-negative peptide (aa 153–218) and a 474-aa C-terminal V5-immunoreactive ~53-kDa V5-positive N2 form (aa 219–692). Using Ab1-hPC9, we detected an additional ~7-kDa immunoreactive protein in the medium. This fragment possibly corresponds to the N-terminal segment of the catalytic subunit (aa 153–218) produced by furin cleavage. Because Ab1-hPC9 recognized all PCSK9 fragments in the medium, the data also show that furin did not affect the level of the ~15-kDa prosegment in the medium (Fig. 5, lower right panel). Aside from its molecular mass, the tentative identity of this ~7-kDa fragment was further tested by its [35 S]Met labeling (likely due to Met²⁰¹) but absence of its 35 SO₄ or [35 S]Cys labeling (data not shown). Thus, from now on, we will refer to the N-terminally truncated N2 product as PCSK9- Δ N218 (PCSK9 lacking the N-terminal 218 aa) (Fig. 5). The data also show that coexpression of the serpin α_1 -PDX (α_1 -antitrypsin Portland), which inhibits most of the basic aa-specific PCs (34, 35), completely blocked the formation of PCSK9- Δ N218 (Fig. 5). To further confirm that Arg²¹⁸ is the furin/PC5A-processing site, we coexpressed the R218S and F216L mutants with all the PCs and, as a control, with membrane-bound

β -secretase (BACE-1) (31). The data show that furin and PC5/6A can no longer process the R218S mutant and that both cleave the F216L mutant to a lesser extent (compare Figs. 5 and 6).

Although we originally reported that the D374Y mutant is poorly secreted (7), upon resequencing its cDNA, we observed two mutations that must have prevented its secretion. Accord-

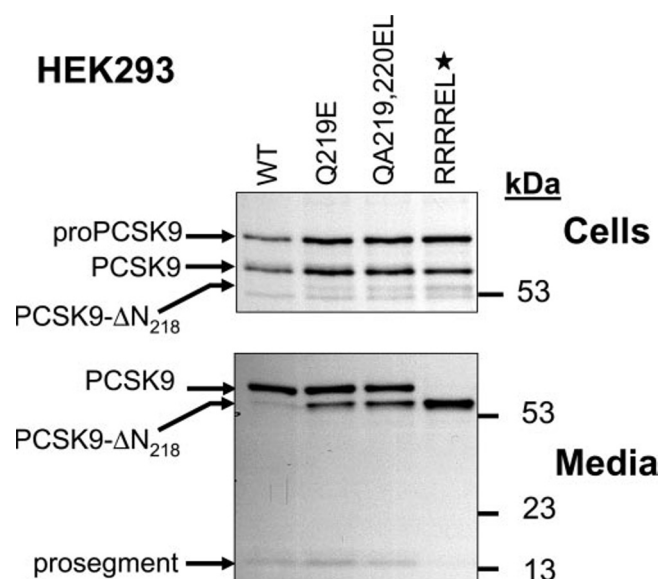


FIGURE 7. Enhanced furin cleavage of PCSK9. HEK293 cells were transiently cotransfected with cDNA encoding V5-tagged WT PCSK9 or mutants with enhanced furin cleavage sites. Forty hours post-transfection, the cells were pulsed with [³⁵S]Met/Cys for 4 h. Cell lysates and media were immunoprecipitated with anti-V5 mAb. Immunoprecipitates were analyzed on 8% Tricine gels. The Q219E and Q219E/A220L (QA219,220EL) mutants enhanced processing by endogenous furin at Arg²¹⁸ in HEK293 cells, whereas replacement of the RFHR²¹⁸QA sequence with an ideal furin cleavage sequence (RRRR²¹⁸EL) resulted in complete processing of secreted PCSK9 (star).

ingly, with this sequence-corrected cDNA, we found that, similar to the F216L mutant, the natural mutant D374Y does not show the presence of substantial amounts of secreted PCSK9-ΔN218 and is partially resistant to cleavage by endogenous convertases (Fig. 6, right panels, compare *PIRES* lanes) or by over-expressed furin (compare *Furin* lanes).

Based on the crystal structure of furin (36) and analysis of its many substrates (2), the best recognition motif of furin would be RX(R/K)R ↓ (E/D)L. This led us to design mutants of PCSK9 that should greatly enhance the ability of furin to process this molecule. Thus, upon replacement of the WT RFHR²¹⁸ ↓ QA sequence with an optimal furin recognition sequence (RRRR²¹⁸ ↓ EL), we noted that endogenous furin in HEK293 cells could completely process PCSK9 at Arg²¹⁸, whereas the motifs RFHR²¹⁸ ↓ EA and RFHR²¹⁸ ↓ EL resulted in intermediate furin cleavability (Fig. 7). Notice the virtual absence of prosegment co-immunoprecipitation with PCSK9-ΔN218 produced (Fig. 7), which would be predicted because such a cleavage would remove PCSK9 segment 153–218, which contains the active-site Asp¹⁸⁶. This also suggests that PCSK9-ΔN218 is inactive and hence unable to tightly bind the prosegment, which, in all PCs, binds only to the catalytically active convertase (37–39).

Effect of PCSK9 Cleavage by Furin/PC5/6A on Its Ability to Enhance the Degradation of the LDLR and Its Subcellular Localization—Previous studies revealed that overexpressed PCSK9 co-localizes with the LDLR⁵ and results in an enhanced degradation of the LDLR in a number of cell lines and *in vivo* (7, 12, 13). Accordingly, we analyzed the activity of WT PCSK9, mutant R218S, the RRRR²¹⁸EL variant, the active-site mutant H226A, and the Cys/His-rich domain (CHRD) of PCSK9 (aa

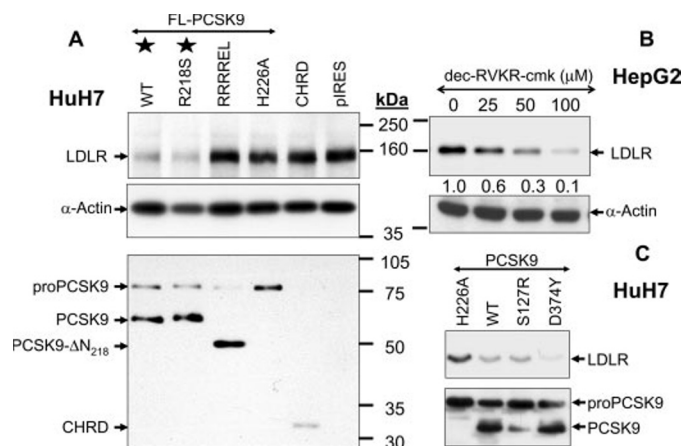


FIGURE 8. Functional consequence of PCSK9 processing by furin. A, HuH7 hepatoma cells were transiently transfected with cDNA encoding V5-tagged full-length WT PCSK9 (FL-PCSK9); mutant R218S, RRRREL, or H226A (inactive); or the CHRD alone. Forty hours post-transfection, cells were lysed, and proteins were resolved by SDS-PAGE on 8% glycine gels. After transfer onto polyvinylidene difluoride membrane, immunoblotting was performed for PCSK9 (anti-V5 mAb) and LDLR (rabbit pAb). α -Actin (rabbit pAb) was used as a loading control. Stars indicate the decrease in LDLR levels in the presence of WT PCSK9 and the R218S mutant, whereas the furin-cleaved RRRR²¹⁸EL variant was inactive. B, HepG2 cells were treated with increasing doses of decanoyl-RVVR-chloromethyl ketone (*dec-RVVR-cmk*) for 20 h. Fifty micrograms of protein were separated on 8% glycine gels, followed by immunoblotting for LDLR and α -actin as a loading control. Decanoyl-RVVR-chloromethyl ketone blocked furin-dependent PCSK9 cleavage and reduced endogenous LDLR levels. Quantification is shown as LDLR/ α -actin ratios obtained by densitometry. C, HuH7 cells were transiently transfected with cDNA encoding V5-tagged WT PCSK9 or mutant H226A, S127R, or D374Y and processed for immunoblotting with anti-V5 mAb or anti-human LDLR (184–196) antibody. D374Y resulted in enhanced LDLR degradation compared with WT PCSK9 and the S127R mutant.

405–692) on the degradation of the LDLR in HuH7 cells (Fig. 8A). The results revealed that only WT PCSK9 and its R218S mutant are active in enhancing the degradation of the LDLR compared with the *PIRES* control empty vector, whereas the RRRR²¹⁸EL variant and the CHRD are inactive, similar to the active-site mutant H226A (Fig. 8A). Therefore, PCSK9-ΔN218 is an inactive form of PCSK9 that is secreted from cells compared with the active-site mutant H226A, which results in uncleaved zymogen pro-PCSK9 that remains in the ER (7). Thus, the RRRR²¹⁸EL variant of PCSK9 provides an ideal control for the activity of PCSK9 toward the LDLR within the secretory pathway, as opposed to active-site mutants that do not exit from the ER and hence do not co-traffic with the LDLR to the cell surface/endosomes (4, 7).

As a further proof that furin and PC5/6A cleave and inactivate PCSK9, we tested the effect of the dose-dependent inhibition of the endogenous convertases in HepG2 cells, which have limiting amounts of furin and no PC5/6A (39), by the membrane-permeable convertase inhibitor decanoyl-RVVR-chloromethyl ketone (40). The data show that incubation of HepG2 cells for 20 h with increasing doses of this inhibitor resulted in a progressive lowering of the levels of LDLR and that ~90% of the LDLR was lost at 100 μ M (Fig. 8B). Under these conditions, we did not observe loss of cellular viability (data not shown). This result independently confirms the critical role played by furin in HepG2 cells in regulating the level of the LDLR.

Finally, we also compared the ability of the S127R and D374Y mutants to enhance the degradation of the LDLR. Western

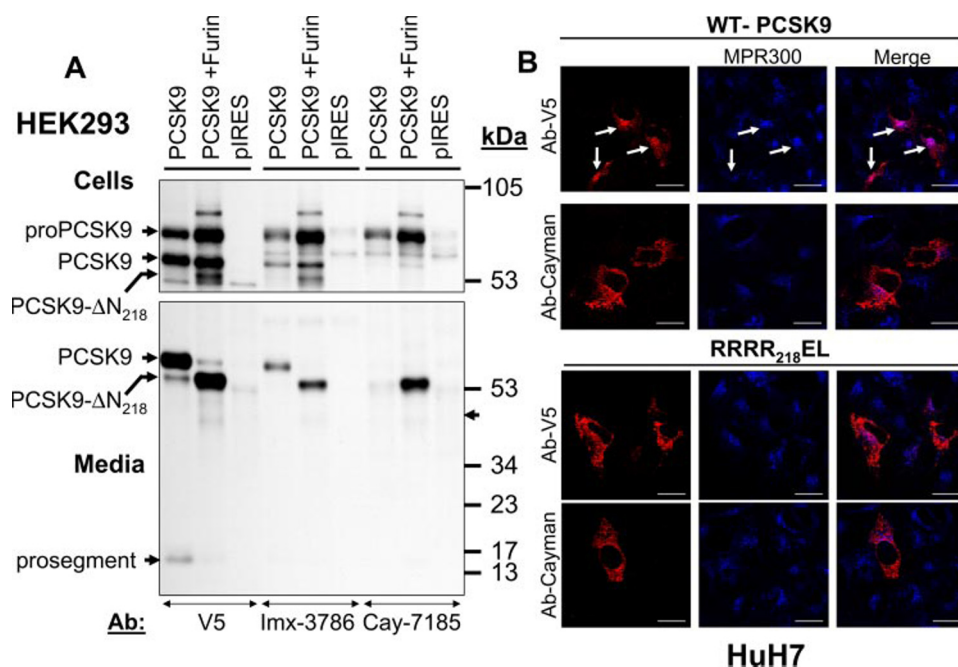


FIGURE 9. An antibody against pro-PCSK9 and its furin-cleaved form (PCSK9-ΔN₂₁₈) does not label PCSK9 in TGN/recycling endosomes. A, immunoprecipitation of different PCSK9 forms with commercial antibodies. HEK293 cells were transiently cotransfected with cDNA encoding V5-tagged WT PCSK9 and either furin or an empty vector. Cell lysates and media were immunoprecipitated with anti-V5 mAb, goat anti-human PCSK9-(679–692) pAb (*Imx-3786*), or rabbit anti-human PCSK9-(490–502) pAb (*Cay-7185*). Goat anti-human PCSK9-(679–692) pAb and anti-V5 mAb recognized pro-PCSK9, mature PCSK9, and its furin-cleaved form in both cells and media. Rabbit anti-human PCSK9-(490–502) pAb did not recognize mature PCSK9 in cells and media. B, immunofluorescence localization of PCSK9 and its RRRR²¹⁸EL (furin-cleaved) mutant in HuH7 cells. HuH7 cells were transiently transfected with cDNA encoding WT PCSK9 or its RRRR²¹⁸EL mutant. Anti-V5 mAb showed mature PCSK9 in the TGN/recycling endosomes, whereas rabbit anti-human PCSK9-(490–502) pAb (*Ab-Cayman*) did not. The RRRR²¹⁸EL mutant does not reside in endosomes and accordingly was detected only in the ER by rabbit anti-human PCSK9-(490–502) pAb and anti-V5 mAb. Magnification was $\times 1000$; scale bars = 10 μm .

blots of HuH7 cells showed that, although the autocatalytic processing of D374Y is similar to that of WT PCSK9, the former is more active in enhancing the degradation of the LDLR compared with either S127R or WT PCSK9 (Fig. 8C). This result agrees with the observed reduced susceptibility of the D374Y mutant to furin processing (Fig. 6, right panels).

To provide a rationale for the inability of the RRRR²¹⁸EL variant to enhance the degradation of the LDLR, we analyzed the biosynthesis of V5-tagged PCSK9 in the absence and presence of excess furin using three different antibodies: anti-V5 mAb and two commercially available anti-human PCSK9 antibodies, goat anti-human PCSK9-(679–692) and rabbit anti-human PCSK9-(490–502). Biosynthetic analysis of PCSK9 alone or in the presence of overexpressed furin in HEK293 cells revealed that, upon immunoprecipitation, both anti-V5 and goat anti-human PCSK9-(679–692) antibodies recognized all the C terminus-containing entities of PCSK9, *i.e.* in cells, pro-PCSK9, PCSK9, and its furin-cleaved form PCSK9-ΔN₂₁₈; and in media, PCSK9 and PCSK9-ΔN₂₁₈. In contrast, rabbit anti-human PCSK9-(490–502) antibody recognized only pro-PCSK9 and PCSK9-ΔN₂₁₈ but not PCSK9 (Fig. 9A). This unexpected observation could be rationalized by the hypothesis that, in the native conformation of PCSK9, segment 490–502 may interact with some amino acids in N-terminal sequence 153–218 of PCSK9 or its prosegment (aa 31–152), which remains noncovalently complexed with the catalytic subunit (Figs. 5 and

9A), hence masking its recognition by rabbit anti-human PCSK9-(490–502) pAb. This is further supported by the fact that this antibody recognized all denatured PCSK9 forms on Western blots (data not shown). Interestingly, the Italian mutation R496W (which results in a hypercholesterolemia phenotype) was reported in segment 490–502 (recognized by rabbit anti-human PCSK9-(490–502) pAb) (18, 41).

Evidence is now available to suggest that the PCSK9-enhanced degradation of the LDLR occurs in acidic endosomes/lysosomes (7, 13). We therefore tested the ability of PCSK9 and its RRRR²¹⁸EL variant to enter the TGN/recycling endosome pathway of HuH7 cells using anti-V5 or rabbit anti-human PCSK9-(490–502) antibody (Fig. 9B). Indeed, although anti-V5 mAb showed the presence of PCSK9 in the TGN/recycling endosome pathway co-localizing with the cation-independent mannose 6-phosphate receptor (measured with anti-MPR300 antibody), rabbit anti-human PCSK9-(490–502) antibody did not, as expected, because the latter could recognize only inactive

pro-PCSK9 (ER-localized) and PCSK9-ΔN₂₁₈ (Fig. 9, A and B). In agreement, neither antibody detected the presence of the PCSK9 RRRR²¹⁸EL variant in endosomes (Fig. 9B). Because the latter is expected to generate PCSK9-ΔN₂₁₈ via furin/PC5/6A cleavage, this result suggests that the inability of PCSK9-ΔN₂₁₈ to enhance the degradation of the LDLR (Fig. 8) may be due to its the combined loss of catalytic potential and absence from endosomes/lysosomes, the compartment where such degradation is believed to take place (7, 13). We conclude that the natural mutations R218S, F216L, and D374Y associated with hypercholesterolemia are correlated with the complete or partial loss of convertase degradation by furin and/or PC5/6A. This would result in an enhanced function of PCSK9, likely due to an increased level of the convertase within endosomes co-localizing with the LDLR.

Circulating Forms of PCSK9 in Human Plasma—To substantiate the physiological relevance of the *ex vivo* observation of the cleavage of human PCSK9 by furin/PC5/6A into secretable PCSK9-ΔN₂₁₈, we characterized the forms of PCSK9 that are found in the normal plasma of two individuals, one female and one male, as well as in lipoprotein-deficient serum prepared from a commercial pool of normal human plasma (Bioreclamation Inc.). After multiple trials with various antibodies, we selected our in-house human antibody (Ab1-hPC9) for immunoprecipitation, followed by Western blotting. We used, as a secondary antibody, rabbit TrueBlot anti-IgG, which does not

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recognize denatured IgGs and hence would not interfere with the immunodetection of PCSK9 on Western blots. The data show that, in all cases, PCSK9 and its PCSK9- Δ N218 product are clearly the circulating forms in both male and female plasma, the ratio of which could vary between individuals (Fig. 10). Furthermore, a similar result was obtained with pooled human sera. We used preimmune rabbit serum as a control. Taking into account the slightly higher molecular mass of

V5-tagged PCSK9 compared with untagged PCSK9 (Fig. 10, *left panel*), we also show that the plasma forms comigrated with markers obtained from the medium of HEK293 cells overexpressing the uncleavable R218S or fully processed RRRR²¹⁸EL variant. Interestingly, PCSK9 immunoreactivity was lost when the lipoprotein-deficient serum was subjected to preclearing using an affinity column that removes IgGs and albumin (Fig. 10), and hence, special care must be taken when sera are fractionated. We conclude that furin/PC5/6A processing of PCSK9 is physiological and that, because some full-length protein circulates, it is likely that PCSK9 could enhance the degradation of the LDLR at sites distal from the liver.

Analysis of the PCSK9 Mutants Associated with Hypocholesterolemia—In two recent studies, Hobbs and co-workers reported a number of mutations associated with hypocholesterolemia (20, 21), possibly resulting from loss of function (23). Hypocholesterolemia was reported in patients harboring a heterozygous C679X mutation or only a homozygous A443T mutation (20, 21). We now present the biosynthetic analysis of two of these mutants, *viz.* the mutant C679X with a stop codon replacing Cys⁶⁷⁹ and the point mutant A443T. The data show that, although pro-PCSK9 C679X is processed into PCSK9 C679X, it remains in the ER (endo H-sensitive and co-localized with calnexin) (data not shown) and is not secreted (Fig. 11). In view of the loss of Cys⁶⁷⁹, it is predicted that a disulfide bond is no longer formed, likely resulting in a partially unproductively folded protein that is still active in *cis* for autocatalysis. This

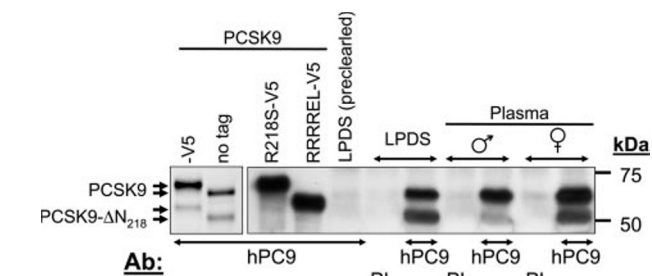


FIGURE 10. Presence of the furin-cleaved PCSK9 form in human plasma. Human plasma was obtained from two healthy volunteers, one male (R. E.) and one female (J. H.). Human lipoprotein-deficient serum (LPDS) was prepared from a commercial pool of plasma. One-hundred microliters of plasma were immunoprecipitated with Ab1-hPC9 or preimmune rabbit serum (PI). Immunoprecipitates were separated on 8% glycine gels, and PCSK9 forms were detected with rabbit TrueBlot according to the manufacturer's instructions. Media from HEK293 cells transfected with R218S or RRRR²¹⁸EL were immunoprecipitated and loaded as markers of PCSK9 forms. Note the migration difference between V5-tagged and untagged PCSK9 (*left panel*). The cleared serum was obtained as described under "Experimental Procedures."

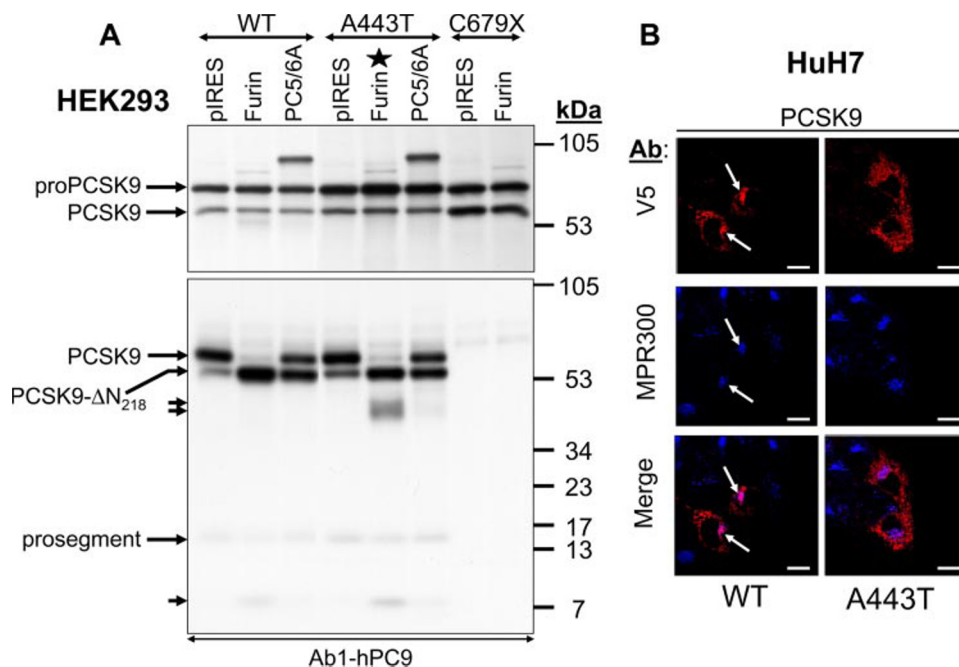


FIGURE 11. Differential processing and immunofluorescence of hypocholesterolemia-associated PCSK9 mutants. A, HEK293 cells were transiently cotransfected with cDNA encoding V5-tagged WT PCSK9 or the natural mutant A443T or C679X and an empty vector (pIRES), furin, or PC5/6A. Forty hours post-transfection, the cells were pulsed with [³⁵S]Met/Cys for 3 h. Cell lysates and media were immunoprecipitated with Ab1-hPC9 and analyzed on 8% Tricine gels. The C679X mutant underwent autocatalytic processing but was not secreted from cells, whereas the A443T mutant was normally processed and secreted, but furin further processed it into an ~10-kDa smaller fragment (*arrow*). In the interest of conciseness, we did not show the PC5/6A coexpression data with the C679X mutant because it was blocked in the ER and was not processed (data not shown). B, shown are the results from immunofluorescence analysis by confocal microscopy of the expression of transiently transfected WT PCSK9 and its A443T mutant in HuH7 cells using anti-V5 pAb. The localization of PCSK9 was compared with that of a TGN/recycling endosome marker, the cation-independent mannose 6-phosphate receptor (anti-MPR300 antibody). Magnification was $\times 1000$; scale bars = 20 μ m.

suggests that a specific retention mechanism of this mutant exists, which does not allow it to leave the ER, likely implicating chaperones such as calnexin, calreticulin, and ERp57 (42) or even a reticulocalbin-like protein (43). Notably, an R682X mutant ending at Ser⁶⁸¹, *i.e.* extended by only three amino acids, behaves like WT PCSK9 (7). Therefore, the C679X mutant would be expected to have no activity toward the LDLR because of its inability to leave the ER and traffic toward the cell surface and endosomes, where the LDLR is degraded (data not shown). Finally, because of its retention in the ER (Fig. 11A), C679X is no longer accessible to furin processing, which usually takes place in the TGN, on the cell surface, or in endosomes (44).

Fig. 11 shows the results from the analysis of the biosynthesis of the A443T mutant, which is normally processed and secreted. However, upon coexpression with furin (but much less so with PC5/6A), a new cleavage product appeared, migrating as a fuzzy doublet ~10 kDa smaller than PCSK9- Δ N218. Thus,

the A443T mutation results in a higher susceptibility of PCSK9 to furin cleavage into a new protein that would lose a larger segment of the catalytic chain and hence would likely be inactive. Finally, immunofluorescence analysis by confocal microscopy in HuH7 cells transiently transfected with either WT PCSK9 or its A443T mutant revealed that the latter did not sort to TGN/recycling endosomes compared with WT PCSK9, which co-localized with the cation-independent mannose 6-phosphate receptor (anti-MPR300-positive) (Fig. 11B). In conclusion, the two hypocholesterolemia-associated mutations result in loss of function due either to the mislocalization of PCSK9 in the ER (C679X) or at least in part to a combination of higher degradation by furin and an inability to enter the recycling endosome pathway (A443T).

DISCUSSION

Up until recently, the natural mutations of the LDLR and its ligand apoB were the only ones known to be associated with the autosomal dominant form of familial hypercholesterolemia. These mutations result in high circulating levels of LDL cholesterol, leading to the development of xanthomas and premature atherosclerosis. In 2003, it became clear that a third gene, *viz.* PCSK9, is also genetically associated with autosomal dominant hypercholesterolemia. Thus, it was shown that the heterozygous single point mutations S127R and F216L in the coding sequence of PCSK9, the ninth member of the PC family (4), are responsible for the high levels of circulating LDL cholesterol in French families (15). This conclusion was quickly confirmed by other groups that also reported a number of new heterozygous missense mutations associated with autosomal dominant hypercholesterolemia, including D374Y (16, 17, 19), R218S (18), R496W (45), R237W (7), N157K (22), and many others that were thought to result in a gain of function of PCSK9 (23), although the underlying mechanism is still not well understood. In contrast, other heterozygous mutations such as Y142X, C679X, and L253F and the homozygous mutation A443T are instead associated with hypocholesterolemia, resulting in low levels of circulating LDL cholesterol (20, 21). Interestingly, the mutations R46L and A53V, which were originally reported to be polymorphic variants of PCSK9 (15), as well as R237W (7) and N157K (22) turned out to be associated, in some cases, with the presence of either low or high circulating LDL levels (21, 22), suggesting the presence of PCSK9 modifier genes.

In this study, we have presented data to support the novel notion that PCSK9 is under the control of proteolysis by other members of the PC family (2, 3). Up until now, it was thought that, with the exception of PC2, all other PC family members were autonomous in that their zymogen processing and activation were not dependent on other convertases or other proteins. In the case of PC2, although its zymogen processing is autocatalytic (46), its productive folding and hence activation depend on a protein called 7B2, which acts both as an inhibitor and a partner protein (47) and which is processed by furin (48). When we first reported the biosynthesis of WT PCSK9 (originally called NARC-1), we had noticed the presence of two secreted forms: a major one of ~60 kDa (called N1) and a minor one of ~53 kDa (called N2). Because we could not sequence the

latter form, we concluded that its N terminus was blocked and could possibly be a Gln that cyclized in the Edman degradation reaction. Careful analysis of the forms secreted by some of the mutants revealed that, in these, N2 was either absent (R218S) or substantially reduced (F216L and D374Y) (Figs. 3 and 6). The presence of R218S in the motif RXXR²¹⁸ (Fig. 4) suggested that processing at Arg²¹⁸ could take place by a furin-like convertase (2). Biosynthetic analyses supported this hypothesis and showed that only membrane-bound furin and, to a lesser extent, PC5/6A are capable of processing PCSK9 into the N2 product, herein called PCSK9-ΔN218 (Fig. 5). Because soluble furin is inefficient in performing such cleavage (Fig. 5) and because soluble furin does not bind to the cell surface (38), it is believed that such late intracellular processing occurs in proximity to membranes either at the cell surface or in a recycling compartment. Notably, PC5/6A binds to the cell surface via its complex formation with tissue inhibitors of metalloproteinases, *e.g.* TIMP2 and heparan sulfate proteoglycans (38), and accordingly inactivates heparan sulfate proteoglycan-bound proteins such as lipases (9). However, membrane binding or association is not sufficient to ensure processing because the membrane-bound convertases PC5/6B and PC7 (49) and SKI-1/S1P (5) do not process WT PCSK9 (Fig. 5). It is intriguing that membrane-bound furin, but not PC5/6B, can process PCSK9. The difference may be in the subcellular localization of these two enzymes and their recycling pathways, which are not identical. Thus, it has been shown that PC5/6B is localized to a paranuclear, brefeldin A-dispersible, BaCl₂-responsive, post-Golgi network (TGN) compartment distinct from furin and TGN38 (50). Interestingly, R218S completely abrogated this processing by furin, whereas F216L and D374Y severely impaired it (Figs. 5 and 6). The bulkier aromatic Phe²¹⁶ in PCSK9 may be optimal for folding and stabilization of the enzyme. The F216L mutation of the P3 residue in the motif RFHR²¹⁸ also suggests that this position may be important for furin recognition. Although the P3 requirements have not been systematically analyzed, it has been reported that furin tolerates variations at this position and that the presence of Leu at P3 has a small negative impact on k_{cat}/K_m (51). Furthermore, the crystal structure of furin in complex with the inhibitor decanoyl-RVKK-chloromethyl ketone containing an aliphatic Val at P3 suggests that this residue extends into the bulk solvent and that positive charges at this position would be better, as they would make favorable contacts with the carboxylate of Glu²⁵⁷ in furin (36). We have exploited this information in our design of the RRRR²¹⁸EL mutant (Fig. 7). The unexpected partial resistance of D374Y to furin digestion (Fig. 6) led us to speculate that Asp³⁷⁴ plays an important role in the final conformation adopted by PCSK9, possibly via salt bridges to positively charged amino acids. The observed higher activity of the D374Y mutant in the enhancement of the degradation of the LDLR (Fig. 8) may be related in part to the structural properties of this mutant that could modulate the activation of PCSK9 and its resistance to furin digestion. However, we cannot presently exclude that, aside from the relative resistance to furin processing, the hypercholesterolemia phenotype associated with the F216L and D374Y mutations could also be caused by other as yet undefined properties of these particular mutants.

The Level of PCSK9 Is Regulated by Other Convertases

The RRRR²¹⁸EL variant of PCSK9 is completely processed by endogenous furin, resulting in a well secreted product (PCSK9-ΔN218) that has lost the N-terminal segment of its catalytic structure and is no longer sorted to endosomes (Figs. 7 and 9). This provided an ideal mutant to test whether the enzyme activity of PCSK9 may be needed for its enhancement of the degradation of the LDLR (Fig. 8), as active-site mutants such as H226A do not exit the ER (7). The fact that overexpression of RRRR²¹⁸EL does not affect the levels of LDLR, whereas that of WT PCSK9 and the R218S mutant does, suggests but does not prove that PCSK9 activity requires an intact catalytic subunit. However, the role of the complexed prosegment in WT PCSK9 has yet to be clarified. So far, we know very little about the zymogen activation of PCSK9. Thus, although it is autocatalytically processed at the VFAQ¹⁵² ↓ site, it is secreted as a complex with its prosegment (7). It is still a matter of speculation whether, upon re-uptake by the cells, the PCSK9-prosegment complex could dissociate at the acidic pH of endosomes and, similar to other PCs, lead to enzyme activation, possibly following a second cleavage event of the prosegment. Definitive proof of this mechanism is still lacking, as is the finding of other PCSK9 target proteins.

The physiological relevance of the furin processing would have been best demonstrated by the analysis of plasma either from dyslipidemic patients or from conditional knock-out mice lacking liver furin (52). However, the plasma of such mice is not available, and the colony is being currently expanded in our group (in collaboration with Dr. John Creemers, Catholic University of Leuven, Belgium). Until these data become available, the best physiological criterion used in this work is the detection in human plasma of both PCSK9 and PCSK9-ΔN218 (Fig. 10). This suggests that PCSK9 may act on the LDLR at sites away from hepatocytes and intestinal enterocytes. An example would be the adrenal gland, where it was reported that adenoviral expression of PCSK9 can lower the levels of the endogenous adrenal LDLR (12). The detection of plasma PCSK9 opens the door to the future measurement of the circulating levels of this enzyme in dyslipidemia patients with either gain- or loss-of-function PCSK9 mutations and those treated with statins or other cholesterol-lowering drugs as well as in obese and type II diabetic patients.

PCSK9 is Tyr-sulfated in its prosegment (Tyr³⁸) and contains one N-glycosylation site (Asn⁵³³) (Fig. 1) (4, 7). These modifications do not affect the folding and secretion of PCSK9 (Fig. 1) or its activity toward the LDLR (Fig. 2). Targeting of the enzyme to both early and late endosomes depends on the presence of the LDLR because, in its absence, PCSK9 cannot enter endosomes.⁵ Whether this fine-tuning would mean that the secreted complex between the Tyr-sulfated prosegment and N-glycosylated PCSK9 is needed for internalization and endosome sorting will require further studies. Preliminary studies suggested that the presence of a clathrin coat is required for the sorting of PCSK9 into endosomes.⁶ Interestingly, a similar paired interaction was recently reported to explain the mechanism of endosome/lysosome sorting of the membrane-bound

CIC-7 chloride channel, which requires the membrane-bound gray lethal protein (also known as Ostm1) (53) to enter lysosomes. Here, in the absence of the dominant partner Ostm1, the protein CIC-7 is stuck in the ER (54). In the case of PCSK9, in the absence of the LDLR, it is secreted, and whatever is left in the cell is still in the ER. In the presence of the LDLR, both proteins co-localize at the cell surface and in endosomes.⁵

With respect to the mutations associated with hypocholesterolemia, the Y142X mutation (20) results in a truncated protein ending in the prosegment (data not shown). Our data also show that, although the C679X mutant is stuck in the ER and hence cannot degrade the LDLR in endosomes, the A443T mutant is oversensitive to furin digestion. Thus, not only can furin process A443T into its derivative PCSK9-ΔN218, but it can also further digest it into a smaller protein migrating on SDS-polyacrylamide gel as a fuzzy doublet at ~40 kDa (Fig. 11). The fact that this mutation transforms Ala into Thr (PNLVAA⁴⁴³LPP to PNLVAI⁴⁴³LPP) and that the latter is surrounded by three Pro residues in the sequence suggests that it might create an O-glycosylation site, which often occurs at Ser/Thr in Pro-rich regions (55), possibly rationalizing the fuzziness of the second furin product. In the homozygous state, this human mutation is associated with hypocholesterolemia. Our data suggest that this loss-of-function mutation will create an O-glycosylation site that makes PCSK9 a better target for furin degradation (Fig. 11). In this context, it is interesting to note Ala⁴⁴³ in human PCSK9 is Thr in both mouse and rat PCSK9. However, the susceptibility of these rodent PCSK9 proteins to furin digestion has yet to be defined.

So far, our studies on selected natural mutants of PCSK9 have revealed that those mutants that are resistant to furin digestion (R218S, F216L, and D374Y) are associated with hypercholesterolemia, whereas those that are missorted (C679X) or more extensively degraded by furin (A443T) are associated with hypocholesterolemia. This suggests that the level of PCSK9 is finely regulated by the basic aa-specific convertases furin and PC5/6A and those mutations that affect the processing of this enzyme and hence its half-life would tilt the balance toward a dyslipidemia phenotype either up or down.

A mutation in another hepatic protein that creates a *de novo* furin site that causes a loss of function has been reported for fibrinogen Canterbury (56). However, different from the beneficial A443T mutation of PCSK9, the creation of such a furin site in the prosegment of fibrinogen results in a loss of function associated with pathology involving prolonged blood coagulation time. In contrast, loss of furin processing has also been reported in the development of Marfan syndrome and diabetes because of mutations in the processing sites of fibrillin (57) and the insulin receptor (58), respectively. We can now add PCSK9 to this list, whereupon mutations leading to the loss of susceptibility to furin digestion could be associated with the development of hypercholesterolemia.

PCSK9 is the only proprotein convertase for which genetic variants are associated with a dominant disease. Except for the C679X mutations, all other mutations studied here affect the processing of PCSK9 by furin and, to a lesser extent, by PC5/6A. Interestingly, the Italian mutation R496W occurs in a basic aa-rich region within the CHRD (aa 405–692), which, under non-

⁶ D. Rhainds and N. G. Seidah, manuscript in preparation.

denaturing conditions, is detectable by an anti-C terminus antibody, but is not recognized by rabbit anti-human PCSK9-(490–502) antibody unless it is processed by furin (Fig. 9), suggesting that it may be masked by another N-terminal sequence. However, many other mutations have been reported, some of which may also be associated with increased response to statin therapy (21, 22). Therefore, this study is just the start of their systematic categorization in terms of their processing, subcellular trafficking, and localization, as well as ability to enhance the degradation of the LDLR. The existence of modifier genes that affect the phenotype associated with each mutation is likely, as some mutations can cause either hypercholesterolemia or hypocholesterolemia in different individuals (21, 22). Because the loss of one functional allele of PCSK9 has long-term benefits in protection against cardiovascular related mortality (21), it may be advantageous to have low levels of circulating PCSK9, which is now amenable to quantification using specific enzyme-linked immunosorbent assays. This conclusion should pave the way toward the identification of selective pharmacological PCSK9-lowering agents that could be used in conjunction with statins or other cholesterol-lowering drugs.

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