Loss- and Gain-of-function PCSK9 Variants

CLEAVAGE SPECIFICITY, DOMINANT NEGATIVE EFFECTS, AND LOW DENSITY LIPOPROTEIN RECEPTOR (LDLR) DEGRADATION

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Background: Autocatalytic zymogen processing of pro-PCSK9 requires cleavage at Gln152. Results: Only a few residues can replace Gln152 without loss of function. Non-favorable residues result in a dominant negative zymogen retaining intracellularly native pro-PCSK9. A wide variety of Asp374 gain-of-function mutations were characterized. Conclusion: Zymogen processing is necessary for secretion. Its inhibition results in a dominant negative protein. Significance: A novel strategy for PCSK9 inhibition is suggested.

The proprotein convertase PCSK9 is a major target in the treatment of hypercholesterolemia because of its ability bind the LDL receptor (LDLR) and enhance its degradation in endosomes/lysosomes. In the endoplasmic reticulum, the zymogen pro-PCSK9 is first autocatalytically cleaved at its internal Gln152, resulting in a secreted enzymatically inactive complex of PCSK9 with its inhibitory prosegment (prosegment/PCSK9), which is the active form of PCSK9 on the LDLR. We mutagenized the P1 cleavage site Gln152 into all other residues except Cys and analyzed the expression and secretion of the resulting mutants. The data demonstrated the following. 1) The only P1 residues recognized by PCSK9 are Gln > Met > Ala > Ser > Thr ≈ Asn, revealing an unsuspected specificity. 2) All other mutations led to the formation of an unprocessed zymogen that acted as a dominant negative retaining the native protein in the endoplasmic reticulum. Analysis of a large panoply of known natural and artificial point mutants revealed that this general dominant negative observation applies to all PCSK9 mutations that result in the inability of the protein to exit the endoplasmic reticulum. Such a tight quality control property of the endoplasmic reticulum may lead to the development of specific PCSK9 small molecule inhibitors that block its autocatalytic processing. Finally, inspired by the most active gain-of-function mutant, D374Y, we evaluated the LDLR degradation activity of 18 Asp374 variants of PCSK9. All Asp374 mutations resulted in similar gain-of-function activity on the LDLR except that D374E was as active as native PCSK9, D374G was relatively less active, and D374N and D374P were completely inactive.

Mammalian genomes encode nine secretory proprotein convertases (PCs) related to bacterial subtilisin and yeast kexin (1–3). Seven of them, PC1/3, PC2, furin, PC4, PC5/6, PACE4, and PC7, cleave precursors at single or pairs of basic amino acids, and the last two convertases, SKI-1/S1P and PCSK9, cleave at non-basic sites.

The last member, PCSK9, discovered in 2003 (4) turned out to have a major role in regulating cholesterol homeostasis by increasing the levels of circulating low density lipoprotein cholesterol (5). The underlying mechanism involves the ability of PCSK9 to bind to and enhance the degradation of the hepatic LDL receptor (LDLR) (6) in acidic subcellular compartments (7, 8), likely endosomes/lysosomes (9). Early biosynthetic analyses revealed that this enzyme is synthesized as a zymogen (pro-PCSK9) in the endoplasmic reticulum (ER) that is autocatalytically activated (4) through cleavage at the C terminus of its inhibitory prosegment, i.e. at the VFAQ152 ↓ sequence (8, 10). As is the case for most other PCs (1, 2), such zymogen cleavage allows PCSK9 to exit from the ER as a complex with its prosegment. However, different from the other PCs, the inhibitory prosegment remains permanently bound to the catalytic subunit of the secreted PCSK9 as a prosegment-PCSK9 complex (4, 8), keeping it in a catalytically inactive state (11). This unusual behavior for a PC (1) suggested that either the prosegment is removed under certain cellular conditions, thereby releasing the active enzyme, or that the enzymatic activity of PCSK9 is not necessary for its ability to promote the degradation of the LDLR. The validation of the latter hypothesis on three PCSK9 targets, namely the LDLR (12, 13), VLDL receptor, and ApoER2 (14), confirmed that PCSK9 acts non-enzymatically on these receptor targets.

The enzymatically dead form of the secreted PCSK9 precluded the study of its catalytic preference on substrates other than itself. Only a single study revealed that mutation of the P1
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cleavage site Gln\textsuperscript{152} into Ala still allowed the enzyme to exit the cell, suggesting that PCSK9 may have a relaxed specificity pocket (8), but the activity of the secreted mutant protein on LDLR was not studied. In 2011, Mayne et al. (15) published a seminal work on the analysis of the mechanism behind a hypercholesterolemia phenotype observed in a French-Canadian family. The report showed that these subjects harbored a novel Q152H mutation at the autocatalytic P1 Gln\textsuperscript{152} site of wild type (WT) PCSK9. Bioinformatic analysis of this mutant showed two unique results. 1) The presence of a His\textsuperscript{152} instead of the WT Gln\textsuperscript{152} abrogated the ability of pro-PCSK9 to autocatalytically cleave itself in the ER, and 2) the zymogen pro-PCSK9-His\textsuperscript{152} remained in the ER and acted as a dominant negative, preventing the exit of a co-expressed WT form of PCSK9. The net result was that the level of secreted WT PCSK9 was drastically reduced in the presence of this loss-of-function (LOF) natural mutation. This rationalized the low level of circulating PCSK9 in these subjects and their hypercholesterolemia phenotype (15).

In view of the clinical importance of inhibiting PCSK9 for controlling hypercholesterolemia, a number of research laboratories in collaboration with pharmaceutical companies recently reported very encouraging Phase I and II clinical trials using inhibitory monoclonal antibodies or antisense oligonucleotides (2, 16). So far, no small molecule inhibitor has been reported to block the function of PCSK9 likely because of the flat surface of interaction between the catalytic domain of PCSK9 and the EGF-A domain of the LDLR (17, 18).

Finally, biochemical and cellular analyses revealed that the gain-of-function (GOF) D374Y mutation originally described by Timms et al. (19) results from a 10–25-fold higher affinity of PCSK9 for the LDLR (11, 20, 21). This is the most damaging GOF mutation of PCSK9, leading to severe hypercholesterolemia and early death from premature coronary heart disease (22). Although the activities of a few other Asp\textsuperscript{374} artificial mutations were reported (20, 23), they did not cover the spectrum of all amino acids, and their effects on cellular LDLR were not investigated.

We herein characterized the pro-PCSK9 zymogen activation and analyzed the functional consequence on LDLR degradation of all Gln\textsuperscript{152} mutants and a wide variety of LOF mutants. Furthermore, we also present comparative data on the critical importance of Asp\textsuperscript{374} and all its possible mutants in regulating the function of PCSK9 on LDLR.

EXPERIMENTAL PROCEDURES

Plasmids and Reagents—Human PCSK9 and its various mutant cDNAs were cloned into pIRES2-EGFP (Clontech) as described (4). The DsRed2 encoding a red fluorescent protein (RFP) (Clontech) was fused in phase to the C terminus of PCSK9, resulting in the PCSK9-RFP chimera. We also used a PCSK9 construct in which a V5 tag was inserted between the signal peptide (amino acids 1–30) and the start of the prosegment (amino acids 31–152), generating a V5-pro-PCSK9 construct (14). The sequences of all constructs were confirmed by DNA sequencing.

Cell Culture and Transfections—HEK293 cells (American Type Culture Collection) were routinely cultivated in Dulbeco’s modified Eagle’s medium (DMEM; Invitrogen) supplemented with 10% fetal bovine serum (Invitrogen) and maintained at 37 °C under 5% CO\textsubscript{2}. At 60% confluence, HEK293 cells were transiently transfected in 10-cm\textsuperscript{2} dishes (0.5 μg of cDNAs) with Effectene (Qiagen) according to the manufacturer’s instructions.

Bioinformatic Analyses and Immunoprecipitations—HEK293 cells were transiently transfected as described above. At 48 h post-transfection, the cells were washed in Cys/Met-free RPMI 1640 medium containing 0.2% BSA and pulse-labeled for 3 or 4 h with 250 μCi/ml [\textsuperscript{35}S]Met/Cys (PerkinElmer Life Sciences). After the pulse, the media were recovered, and the cells were lysed as reported (24). The media and cells lysates were immunoprecipitated with the monoclonal antibodies mAb-V5, or mAb-His\textsubscript{6} or with an in house rabbit polyclonal PCSK9 antibody (9). The immunoprecipitated proteins were resolved by SDS-PAGE on 8% Tricine gels, dried, and autoradiographed as described (24).

Western Blot Analyses—Cells were lysed in ice-cold radioimmunoprecipitation assay buffer (50 mM Tris-HCL, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a mixture of protease inhibitors (Roche Applied Science). Proteins were analyzed by SDS-PAGE on an 8% Tricine gel. Following the addition of a reducing Tricine sample, solubilized proteins were separated by SDS-PAGE on an 8% Tricine gel. Proteins were visualized using V5-horseradish peroxidase (HRP), PCSK9-HRP, or RFP-HRP and revealed by enhanced chemiluminescence. Quantitation of band intensity was done with Scion Image software from the Scion Corp. (Frederick, MD).

Reverse Transcription-PCR (RT-PCR) Analysis of X-box-binding Protein 1 (Xbp-1) Splicing—HEK293 cells were transfected with empty pIRES vector, WT PCSK9, or its Q152A, Q152H, and Q152W mutants alone or together. Forty-eight hours post-transfection, the cells were treated for 4 h with buffer or 5 μg/ml tunicamycin to induce ER stress. The cells were then lysed, and total RNA was collected (TRIzol\textsuperscript{®}, Invitrogen) as recommended by the manufacturer. Typically, 250 ng of total RNA were used for cDNA synthesis in a total volume of 20 μl using SuperScript II reverse transcriptase, 25 μg/ml oligo(dT)\textsubscript{12–18}, 0.5 mM 2′-deoxynucleoside 5′-triphosphates, and 40 units of RNaseOUT, all products from Invitrogen and used according to the recommendations of the manufacturer. Primers used to amplify the Xbp-1 cDNA bearing the intron target of IRE1\textalpha ribonuclease activity and PCR conditions were described previously (25). A 289-bp amplicon was generated from unspliced Xbp-1, whereas a 263-bp amplicon was generated from spliced Xbp-1. Four-hour treatment of non-transfected cells with 5 μg/ml tunicamycin was used as a control for ER stress (26).

FACS—HuH7 cells were incubated for 4 h or overnight at 37 °C with various PCSK9 constructs and then washed three times with calcium/magnesium-free Dulbecco’s PBS containing 0.5% bovine serum albumin (Sigma) and 1 g/liter glucose (solution A). Cells were then incubated for 5 min at 37 °C with 500 μl of 1× Versene solution (Invitrogen) and layered on 4 ml of solution A. Cells were then centrifuged for 5 min at 1,200 rpm and resuspended in 1 ml of solution A containing a 1:100
RESULTS

Importance of Gln^{152} in the Zymogen Processing of Pro-PCSK9 and the Dominant Negative Effects of Its Mutants—We first wished to define the importance of the nature of the P1 residue in the autocatalytic processing of pro-PCSK9 into PCSK9 within the ER environment (4, 8). Accordingly, we first undertook a biosynthetic analysis of the WT PCSK9 (Gln^{152}) and all its possible P1 (site of autocatalytic cleavage of pro-PCSK9 into PCSK9) mutants except Cys following a 3-h \[^{35}S\]Met/Cys pulse labeling of HEK293 cells transiently expressing these C-terminally V5-tagged constructs. The results of immunoprecipitations with a mAb-V5 followed by SDS-PAGE of cell extracts and media are shown in Fig. 1A. It is evident that the P1 residue Gln^{152} is the favored residue because it allowed the most effective autocatalytic processing of pro-PCSK9 into PCSK9 following by its maximal secretion. The previously reported small amount of the inactive furin-cleaved form at Arg^{218}, namely PCSK9-ΔN^{218} (27), was also present. We also noticed that Gln^{152} could be replaced by Ala, Met, Thr, Ser, or Asn and still allow for some autoprocessing and secretion, albeit at significantly lower levels. All other P1 mutants resulted in the intracellular accumulation of pro-PCSK9, likely in the ER (4, 8, 9). To better evaluate the ratios of secreted PCSK9 versus intracellular pro-PCSK9, we performed a similar experiment but quantitated these human proteins in cells and media by an ELISA that recognizes all forms of human PCSK9 (28). The triplicate data obtained are summarized in Fig. 1B, and the percent ratios of immunoreactive PCSK9 in the media over cells are shown in Fig. 1C. These steady-state results confirm and extend the 3-h pulse labeling data of Fig. 1A and allow us to determine a rank order of preference for the P1 residue, namely Gln > Met > Ala > Ser > Asn ≈ Thr >> Tyr > Phe. All other P1 residues are essentially not cleaved, including Glu, Asp, Gly, His (a natural mutant (15)), Ile, Lys, Leu, Pro, Arg, Val, and Trp (Fig. 1C). It was repeatedly noted that the mutants Q152G, Q152S, and Q152L result in seemingly lower levels of PCSK9 expression within the 3-h pulse. However, this was not seen when the overnight media were analyzed by ELISA (Fig. 1C) or Western blotting (Fig. 2A). Interestingly, the Q152N mutant and to a lesser extent the Q152T mutant resulted in a PCSK9 that is secreted but much more sensitive to furin inactivation into PCSK9-ΔN^{218} (Fig. 2A, media), emphasizing the importance of Gln^{152} in protecting the processed WT PCSK9 from excessive cleavage inactivation by furin at the cell surface (29).
To gain more insights on the longer term effects of the P1 variants, we next undertook a steady-state analysis of the forms of PCSK9 from cells and media. Under Western blot conditions 24 h after transfection, we began to see differences in the intracellularly accumulated forms of some Gln152 mutants as compared with the 3-h pulse experiment (compare cells in Figs. 1A and 2A). Thus, although all mutants exhibited over time a variable extent of intracellular autocatalytic cleavage of pro-PCSK9 into PCSK9, the prominent mutants are Gln, Ala, Gly, His, Leu, Met, Asn, Ser, and Thr. However, only pro-PCSK9 containing a P1 Gln, Ala, Met, Asn, Ser, and Thr allow the secretion of PCSK9. This suggested that even though autocatalytic processing of pro-PCSK9 into PCSK9 in the mutants Q152G, Q152H, and Q152L (underlined above) can partially occur the proteins are not secreted as was also observed for both the LOF C679X truncated mutant (27, 30) and E498K (supplemental Fig. S1).

In a remarkable study, Mayne et al. (15) showed that the Q152H natural mutation resulted in the accumulation of pro-PCSK9 in the ER (15) as confirmed and refined here (Figs. 1A and 2A). Interestingly, when this mutant was co-expressed with a WT sequence with Gln152, it retained the WT protein intracellularly as pro-PCSK9 and prevented its secretion (15). For a quantitative approach, we co-expressed untagged PCSK9 (native) with the V5-tagged Gln152 mutants and analyzed the total levels of PCSK9 in cells and media by our sensitive ELISA (28). The data obtained (supplemental Table S1) revealed that the largest dominant negative effect was observed with the mutants that were not secreted: Q152L, Q152H, Q152F, Q152Y, Q152I, Q152P, Q152R, Q152V, Q152K, Q152E, Q152D, and Q152W (supplemental Table S1). Note also that replacement of Gln152 by Met152 did not affect the level of secretion of PCSK9, and hence, Met is the only amino acid that is as well tolerated as Gln at the zymogen processing site.

When compared with the ER stress induced upon incubation of cells with the N-glycosylation inhibitor tunicamycin, the observed dominant negative effect of the Gln152 mutants is not the result of ER stress due to their overexpression in HEK293 cells as evidenced by Xbp-1 amplicons amplified by RT-PCR (25, 31). The data show that only the ER stress inducer tunicamycin enhanced the levels of spliced Xbp-1, resulting in a 263-bp amplicon (26), whereas expression of the PCSK9 constructs did not grossly modify the ratio of the 289-bp unspliced Xbp-1 to the spliced 263-bp form (not shown).

Previously, we reported that following synthesis only the zymogen pro-PCSK9, but not the processed PCSK9, can oligomerize with itself in the ER and that this multimerization requires disulfide bonding (4). In agreement, co-expression of C-terminally His-tagged PCSK9-His₆ with V5-tagged PCSK9 revealed that pro-PCSK9-His₆ co-immunoprecipitated with the V5-tagged protein in cells (Fig. 2B), but no co-immunoprecipitation of PCSK9 was observed in the media (not shown). The same was found for the V5-tagged Q152A, Q152H, and Q152W mutants and the secretable double mutant of WT PCSK9 in which the only two free Cys67 and Cys301 (11) were mutated to Ala (Fig. 2B). We conclude that multimerization of pro-PCSK9 does not depend on Gln152 and that the absence of free Cys in pro-PCSK9-C67A/C301A does not prevent its co-immunoprecipitation with WT pro-PCSK9-His₆.

**FIGURE 2. Steady-state expression and secretion of PCSK9 Gln¹⁵² mutants and their oligomerization.** A, 24 h after transfection of HEK293 cells, the expression and secretion of PCSK9 Gln¹⁵² mutants were analyzed by Western blot (WB) using mAb-V5. B, HEK293 cells were transfected with V5-tagged PCSK9 and its C67A/C301A, Q152A, Q152H, and Q152W mutants with PCSK9-His₆ or without (control). 24 h post-transfection, cell lysates were immunoprecipitated (IP) with mAb-V5 or mAb-His₆, and the immunoprecipitates were resolved by SDS-PAGE and analyzed by Western blot using mAb-V5-HRP. The migration positions of pro-PCSK9 and PCSK9 as well as that of the furin-cleaved form PCSK9-ΔN₂₁₈ are shown. This figure is representative of at least three independent experiments.
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FIGURE 3. Inhibition of zymogen processing by Gln152 mutants. HEK293 cells were transiently co-transfected with untagged PCSK9 (native PCSK9) and WT PCSK9-V5 or its Q152A, Q152D, Q152E, and Q152F mutants. 48 h post-transfection, the cells were radiolabeled for 3 h with [35S]Met/Cys. The media and cell lysates were then immunoprecipitated with a PCSK9-specific antibody (9), the immune complexes were resolved by SDS-PAGE, and the dried gel was autoradiographed. This figure is representative of at least three independent experiments.

Biosynthetic analysis of the co-expression of native PCSK9 with the representative V5-tagged Q152A, Q152D, Q152E, and Q152F mutants is depicted in Fig. 3. The data show that co-expression of WT PCSK9 with the mutant Q152A did not significantly affect the intracellular zymogen processing of the other non-secretable mutants reduced the levels of the autocatalytic cleavage of pro-PCSK9 into PCSK9 in the cells and substantially increased the relative levels of pro-PCSK9 (Fig. 3).

Thus, it is possible at least in part that the dominant negative effect observed is due to inhibition of the zymogen processing, of WT pro-PCSK9 by mutants that cannot undergo autocatalytic processing and that oligomerize with themselves and/or the WT zymogen. These data suggest that it is likely that most forms of PCSK9 that can barely exit from the ER or not exit at all (i.e. remain endoglycosidase H-sensitive; not shown) will more or less efficaciously retain WT pro-PCSK9 within the ER in the cell. A plausible working model is presented in Fig. 4.

We next wished to gauge the activity of the secreted Gln152 mutants by their ability to enhance the degradation of the LDLR on the cell surface of HuH7 cells (32). Accordingly, WT PCSK9 and its Q152A, Q152M, and Q152S mutants were produced in HEK293 cells, and their levels in the media were analyzed by ELISA. Equal amounts of these proteins (750 ng/ml) were then incubated at 37 °C for 4 h with HuH7 cells, and then the levels of cell surface LDLR were quantitated by FACS. In this assay, the activities of the mutants Q152A, Q152M, and Q152S were similar to that of WT PCSK9 (Fig. 5). These data demonstrated that although the mutants Q152A, Q152M, and Q152S are secreted less than WT PCSK9 (Figs. 1C and 2A, media), when incubated at equal concentrations, their activities on cell surface LDLR are similar to that of WT, and all significantly reduced LDLR levels compared with control (Fig. 5). Thus, the LOF of the Q152A and Q152S mutants would mostly be due to their decreased levels in the media and not to a significant loss of intrinsic activity once secreted.

Exchangeability of the Prosegment of PCSK9—The above data suggested that inhibition of the zymogen processing of PCSK9 may be a novel approach to block the secretion of PCSK9 and hence its activity on the LDLR. Co-expression of the WT protein with a mutant that cannot undergo zymogen processing is one way to achieve this goal. Another approach would be to inhibit the catalytic site of pro-PCSK9 and prevent its zymogen processing. This method was used previously to inhibit the convertases furin, PC7 (33), and PC5/6 (34) via the expression of the inhibitory WT prosegment in trans.

Herein, we investigated the effect of overexpression of the N-terminally V5-tagged WT prosegment on the secretion of native PCSK9. Biosynthetic analysis revealed that a small per-
percentage of intracellular untagged pro-PCSK9 and secreted PCSK9 co-immunoprecipitated with the V5-tagged prosegment (Fig. 6A). By using a PCSK9-specific antibody (9), we estimated that this represents <10%, suggesting that the WT prosegment in trans can replace, albeit not efficiently, that of pro-PCSK9. These data demonstrated that the V5-prosegment can bind pro-PCSK9 before the occurrence of zymogen processing in the ER and that it can also partially replace the WT sequence of the processed and secreted PCSK9 (Fig. 6A, media). A similar low exchange efficiency has already been seen upon expression of pro-PCSK9 and PCSK9 as well as that of the furin-cleaved form, PCSK9-ΔN218, and those of the V5- and untagged prosegments are emphasized. The control consisted of replacing the native PCSK9 by the empty vector pIREs (pIR). This figure is representative of two independent experiments. Ab, antibody.

We showed previously that co-expression of WT native PCSK9 lacking the prosegment (native PCSK9-Δpro) with an N-terminally V5-tagged prosegment allows the secretion of native PCSK9 in complex with the V5-tagged prosegment (14). Upon co-expression of native PCSK9-Δpro with V5-tagged prosegment mutants ending with His152 or Trp152 instead of the WT Gln152, we noted that although all prosegments co-immunoprecipitated with native PCSK9-Δpro in cells (likely in the ER) the only V5-prosegment-native PCSK9 complex secreted was that carrying the WT Gln152 (Fig. 6B). Furthermore, co-expression of the full-length native PCSK9 with the same prosegments also revealed that, like the WT, the mutant prosegments bound the zymogen pro-PCSK9 in the cell. Here again, the WT V5-prosegment allowed secretion of the mature V5-prosegment-PCSK9 complex. Interestingly, the prosegment ending with the natural mutation Q152H resulted in a complex that is readily cleaved by furin into native PCSK9-ΔN218, whereas the prosegment ending with Trp152 did not allow any secretion of native PCSK9 bound to the V5-prosegment (Fig. 6B). However, the use of a PCSK9 antibody instead of mAb-V5 revealed that here again <10% of the total secreted native PCSK9 originating from pro-PCSK9 is bound to the V5-prosegment (see supplemental Fig. S2, arrow). Finally, the latter data show that overexpression of the WT or mutant prosegments with pro-PCSK9 did not result in inhibition of intracellular zymogen processing or in a significant reduction in the level of secreted WT PCSK9 (supplemental Fig. S2). We conclude that, contrary to other convertases (33–35), in trans overexpression of the prosegment or its Gln152 mutants does not inhibit PCSK9 processing or secretion; however, only a negligible amount of prosegment can be replaced in pro-PCSK9.

The Dominant Negative Effects of Multiple PCSK9 Mutants—We next tackled the question of whether the dominant negative effect observed with the Gln152 mutants would also be seen with other selected natural and/or artificial PCSK9 variants that occur in the prosegment (amino acids 31–152) as well as in the catalytic and CHRD domains (amino acids 153–692). Herein, we concentrated on mutants that significantly reduce or abolish the secretion of PCSK9 (8).

Analysis of the media and cell extracts by ELISA of the V5-tagged PCSK9 lacking its prosegment (Δpro) (14, 32) and...
may cause an exchange of prosegment between the monomers and that such an exchange may be occurring during autocatalytic processing. To test this hypothesis, we transiently expressed WT PCSK9 tagged at the N terminus of its prosegment with V5 or at the C terminus of the whole protein with a red fluorescent protein (RFP of ∼20 kDa) individually or both together for 24 h in HEK293 cells. The addition of the RFP domain to the C terminus of PCSK9 did not affect the autoprocessing of pro-PCSK9-RFP, the secretion of PCSK9-RFP, or its activity on the LDLR (not shown). The cell lysates and media were immunoprecipitated with mAb-V5, an RFP-specific antibody, or our PCSK9 antibody (9). The precipitates were then resolved by SDS-PAGE, and the separated proteins were analyzed by Western blot using one of the above three antibodies (supplemental Fig. S3). The data clearly show that the cross-complex V5-prosegment-PCSK9-RFP is never detected in the media or cells because immunoprecipitation with mAb-V5 never leads to co-immunoprecipitation of PCSK9-RFP and vice versa. We conclude that zymogen processing of pro-PCSK9 into PCSK9 is intramolecular for each monomer as is the case for all other mammalian PC family members (1) and related bacterial subtilases (39).

**The Effect of Various Tyr<sup>374</sup> Mutations on the Activity of PCSK9 on the LDLR**—The Anglo-Saxon mutation in which the Asp<sup>374</sup> is replaced by a Tyr (D374Y) resulted in 10–25-fold enhanced binding of PCSK9 to the EGF-A domain of the LDLR (11, 20, 40). A similarly increased LDLR degradation activity by PCSK9 was also reported for the Portuguese GOF D374H natural mutation (23). We therefore investigated whether residues other than Tyr<sup>374</sup> or His<sup>374</sup> could also result in a similar GOF. Accordingly, Asp<sup>374</sup> was replaced by all amino acids except Cys, and the biosynthesis and secretion of all such mutants were analyzed following a 3-h pulse with [<sup>35</sup>SlMet/Cys. The data show that all mutants were well processed and secreted except for D374P, which seemed to be cleaved to a much lower molecular mass form that remained in the cell (Fig. 8). We also noted a slight rise in the apparent molecular mass of the D374N possibly due to N-glycosylation at the resulting consensus Asn<sup>374</sup>-Cys-Ser site (where the underlined residues are the critical recognition motif for N-glycosylation). These data were confirmed by ELISA analysis of the levels of PCSK9 in the cells and media of these mutants expressed in HEK293 cells (Fig. 9, A and B). Note that for the non-secretable D374P mutant the cellular levels were low (Fig. 9A), suggesting that the unusually processed form of its zymogen is degraded over time as it now exhibits a Pro-Cys<sup>375</sup>-Ser-Thr-Cys<sup>378</sup> sequence that may not favor the proper disulfide bond formation of Cys<sup>375</sup> to Cys<sup>378</sup> (11).

We next co-expressed equal amounts of cDNAs coding for native PCSK9 with the above V5-tagged PCSK9 mutants and analyzed their total levels in cells and media by ELISA. The data obtained (supplemental Table S2) revealed that the largest dominant negative effect was observed with the mutants that are not well secreted or not secreted at all. Those that had the least effect were D480N, L455X, and V79A. All other mutants had a significant lowering effect on the secretion of WT PCSK9 (supplemental Table S2).

The Zymogen Processing of Pro-PCSK9—The fact that WT pro-PCSK9, but not PCSK9, oligomerizes in the ER (Fig. 2B) (4) suggested that it is possible that the dimerization of pro-PCSK9...
Asp374 is due to the loss of the negative charge at amino acid 374.

DISCUSSION

The discovery of PCSK9 and its critical role in the regulation of the hepatic LDLR (for comprehensive reviews, see Refs. 2 and 41–43) led to novel therapies to reduce the levels of circulating LDL cholesterol. The most successful therapy so far involved the injection of an inhibitory monoclonal antibody that blocks the PCSK9/LDLR interaction both in model mice and human (2, 16, 44). Indeed, Phase II and III clinical trials are ongoing using such an approach combined or not with statin, a widely prescribed orally active cholesterol-lowering drug. However, other approaches using injectable drugs are also being tested, including the use of adnectins and antisense oligonucleotides (2). Recently, a 66-amino acid variant of the LDLR’ EGF-A peptide fused to immunoglobulin Fc (EGF66-Fc) was reported to be potentially useful to compete with the LDLR for PCSK9 (45). Finally, we showed that a synthetic peptide mimicking the R1 domain of Annexin A2 inhibits the PCSK9 activity on LDLR by binding to the C-terminal PCSK9 CHRD domain and that in vivo this mostly occurs in extrahepatic tissues (46, 47). The above studies provided validation of PCSK9 as a viable drug candidate and a proof of principle of the therapeutic benefit of its inhibition. However, in the long run, it is likely that an orally active PCSK9 inhibitor may be more widely usable than injectable biologics for the treatment of hypercholesterolemia.

Among the proposed approaches for orally active compounds is the targeting of the PCSK9 EGF-A interaction using small molecule inhibitors. In part due to the relatively large flat surface of interaction (17, 18), no potent small molecule has yet been reported. Another approach would entail the disruption of the secreted prosegment/PCSK9 complex. However, this complex is very tight as it involves multiple binding sites (11) and is thus conceivably difficult to disrupt. However, it was reported that oxygen exchange can occur in this complex at the catalytic prosegment/PCSK9 binding interface (48), suggesting some flexibility that could be exploited.

Inspired from work on the other proprotein convertases (33, 35), we have thus tried to probe whether overexpression of the prosegment in trans could inhibit the autocatalytic processing of pro-PCSK9. The data show that 10% of the V5-tagged prosegment or its Q152H and Q152W mutants expressed in trans could substitute for the endogenous prosegment of pro-PCSK9 and that such an approach would not lead to any significant inhibition of zymogen processing or PCSK9 secretion (Fig. 6 and supplemental Fig. S2).

It was first reported by Cariou et al. (49) that the PCSK9 natural single allele double mutant R104C/V114A exhibits a drastically reduced autocatalytic processing of pro-PCSK9 and loss of PCSK9 secretion. This heterozygous LOF variant results in undetectable levels of circulating PCSK9. This was rationalized by the dominant negative activity of the R104C/V114A mutant over the WT allele that is associated with an increased
LDL cholesterol catabolic rate in humans (49). In a similar vein, Mayne et al. (15) showed that the heterozygous natural mutation Q152H at the autocatalytic cleavage site Gln152 results in much reduced circulating levels of PCSK9 and that pro-PCSK9-Q152H is not processed and acts as a dominant negative on the WT allele. The identification of two LOF mutations in the prosegment (R104C/V114A and Q152H) that resulted in a dominant negative phenotype prompted us to evaluate a large number of reported natural and artificial mutants that also exhibit processing and/or secretion defects. Indeed, all the mutants we analyzed also showed a correlation between the extent of loss of zymogen processing/secretion and their ability to act as dominant negatives in inhibiting the zymogen processing and/or secretion of PCSK9 (supplemental Tables S1 and S2 and Figs. 4 and 5).

The mechanism behind the observed dominant negative property of missense PCSK9 mutations that result in loss of secretion is likely related to the property of pro-PCSK9 to oligomerize in the ER (4) as also evidenced by the co-immunoprecipitation of co-expressed zymogens (Fig. 2B). Our results showed that the Q152D, Q152E, and Q152F mutants that exhibit loss of zymogen processing can also inhibit the zymogen processing of the co-expressed WT form (Fig. 3). However, so far we have not been able to identify a motif or sequence that would regulate the disulfide bond-dependent oligomerization propensity of pro-PCSK9. Indeed, all the deletion mutants of PCSK9 analyzed oligomerize, including the prosegment alone, PCSK9-pro, PCSK9-L455X lacking the C-terminal CHRD, and the various missense mutants presented in this work (not shown). This was evidenced by SDS-PAGE analysis in the absence of β-mercaptoethanol, which showed a number of higher molecular mass oligomers and the absence of monomers as reported earlier (4). The only exception is the CHRD domain itself, which when expressed as a secretory protein (9, 46) does not oligomerize (not shown).

Because PCSK9 is tightly bound to its inhibitory prosegment (4) and never seems to detach from it (8, 11), it was not surprising that its ability to enhance the degradation of LDLR (12, 13), VLDL receptor, and ApoER2 (14) is not dependent on its catalytic activity. Accordingly, the only known substrate of PCSK9 is itself, and its catalytic activity is required for the zymogen processing of pro-PCSK9 into PCSK9 (4). Early attempts to use mutagenesis of the autocatalytic cleavage site VFAQ152 to define the specificity of PCSK9 revealed that the P1 Gln can be replaced by Ala and that the aliphatic Val at P4 is critical (8). In this report, analysis of the P1 Gln152 autocleavage specificity revealed a rank order of preference for the P1 position of Gln > Met > Ala > Ser > Thr ≈ Asn. All other residues led to the formation of an unprocessed zymogen that acted as a dominant negative retaining the WT zymogen in the cell (Figs. 3 and 4 and supplemental Table S1). The loss of zymogen processing of these P1 mutants does not seem to be primarily due to the inability of their prosegment to bind to PCSK9 (Fig. 6). This suggests that the major stalling block abrogating zymogen processing in these mutants may be related to catalytic peptide bond cleavage efficacy rather than active site binding of these Gln152 mutants.

It was observed that some PCSK9 mutants can undergo autocatalytic cleavage in the ER but are not secreted (8, 27, 37). Normally, once cleaved in the ER, the prosegment remains tightly bound to PCSK9 and is secreted as a prosegment-PCSK9 complex (4). Some of the processed PCSK9 mutants lose their tight grip on their prosegments, e.g. E498K and C672X (supplemental Fig. S1), which may explain why they are not secreted.

We next turned our attention to the less prevalent GOF natural mutations of PCSK9 associated with autosomal dominant hypercholesterolaemia (5). Only a few GOF mutations have been reported in autosomal dominant hypercholesterolaemia (ADH) (for a review, see Ref. 50). Their identification was crucial in studying the role of PCSK9 in hypercholesterolaemia and its impact on the LDLR. Recently, we identified a novel prosegment L108R natural GOF mutant that enhances the interaction of the prosegment of PCSK9 with the β-barrel domain of the LDLR (51). Nevertheless, the most damaging mutation is the Anglo-Saxon-associated D374Y mutation (19) that increases by 10–25-fold the affinity of PCSK9 for the LDLR (11), enhances LDLR degradation (21), and increases apolipoprotein B secretion (52). The reported co-crystal structure of PCSK9 with the EGF-A domain of the LDLR (17) revealed that at acidic pH values Asp74 variants of PCSK9 forms a critical hydrogen bond with His327 within the EGF-A domain. Based on binding studies and co-crystal structure analysis, this hydrogen bond was shown to be strengthened at neutral pH for the H327Y mutant of the LDLR (53). It was therefore of interest to investigate whether Asp74 variants of PCSK9 might maintain, abolish, or enhance the activity of PCSK9 on LDLR. Previously, the GOF natural mutant D374H has been reported in Portuguese family members suffering from familial hypercholesterolaemia (23). Furthermore, a limited vertical scanning mutagenesis study of Asp74 revealed the following rank order of binding affinities to immobilized soluble LDLR: Tyr ≈ Phe >> Leu ≈ Ala > Lys ≈ Glu ≈ Asp74 (40).

In the present study, we systematically mutagenized Asp74 to all possible residues except Cys and analyzed their zymogen processing and secretion (Fig. 8) as well as their activity by FACS analysis of cell surface LDLR of HuH7 cells incubated for 4 h at 37 °C with an 800 ng/ml concentration of each mutant protein (Fig. 9). Although most PCSK9 mutants exhibited a stronger ability to lower the levels of cell surface LDLR as compared with WT Asp74 (Fig. 9C), we noted that D374N is inactive and D374G is less active than WT PCSK9. Although well secreted (Figs. 8 and 9B), the D374N mutant is probably N-glycosylated at the newly created consensus Asn74-Cys-Ser motif (Fig. 8), likely affecting its conformation and resulting in a LOF. The D374G is well processed and secreted (Figs. 8 and 9B) but probably does not adopt a favorable conformation for activity on LDLR. The D374P is not secreted at all and seems to be degraded intracellularly (Fig. 8). It is to be noted that our cellular incubation results revealed that WT and D374E PCSK9 have very similar activities toward the cell surface LDLR (Fig. 9), similar to what was reported based on FRET-based binding data (40). However, different from the latter study, we did not see a large difference between the activities of the D374K and D374L mutants. This may be attributable to using HuH7 cells here as opposed to in vitro binding to soluble LDLR in the earlier study.
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(40). Nevertheless, we can conclude that replacement of the negatively charged Asp374 by another acidic residue (D374E) does not influence its activity at neutral pH but that most other mutations that result in a secretable protein exhibit considerable GOF activities with Trp374, Met374, His374, Phe374, and Tyr374 being the most potent PCSK9 variants.

In conclusion, the results presented in this work reveal the critical importance of the P1 Gln152 for efficient autocatalytic processing of pro-PCSK9 and demonstrate that PCSK9 has limited enzymatic cleavage specificity on itself as a substrate. The unexpected general observation that PCSK9 mutants that cannot undergo zymogen processing or productive folding result in a dominant negative form of the protein has fundamental clinical implications. The search for small molecules that would disrupt the zymogen processing and/or folding of the processed PCSK9 represents a novel approach to inhibit the activity of PCSK9 with the aim of reducing LDL cholesterol.

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