

Functional analysis of sites within PCSK9 responsible for hypercholesterolemia^[S]

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Abstract Mutations within proprotein convertase subtilisin/kexin type 9 (PCSK9) are associated with dominant forms of familial hypercholesterolemia. PCSK9 binds the LDL receptor (LDLR), and addition of PCSK9 to cells promotes degradation of LDLR. PCSK9 mutant proteins associated with hypercholesterolemia (S127R and D374Y) are more potent in decreasing LDL uptake than is wild-type PCSK9. To better understand the mechanism by which mutations at the Ser127 and Asp374 residues of PCSK9 influence PCSK9 function, a limited vertical scanning mutagenesis was performed at both sites. S127R and S127K proteins were more potent in decreasing LDL uptake than was wild-type PCSK9, and each D374 mutant tested was more potent in reducing LDL uptake when the proteins were added exogenously to cells. The potencies of D374 mutants in lowering LDL uptake correlated with their ability to interact with LDLR in vitro. Combining S127R and D374Y was also found to have an additive effect in enhancing PCSK9's ability to reduce LDL uptake. Modeling of PCSK9 S127 and D374 mutations indicates that mutations that enhance PCSK9 function stabilize or destabilize the protein, respectively. **In conclusion**, these results suggest a model in which mutations at Ser127 and Asp374 residues modulate PCSK9's ability to regulate LDLR function through distinct mechanisms.—Pandit, S., D. Wisniewski, J. C. Santoro, S. Ha, V. Ramakrishnan, R. M. Cubbon, R. T. Cummings, S. D. Wright, C. P. Sparrow, A. Sitlani, and T. S. Fisher. **Functional analysis of sites within PCSK9 responsible for hypercholesterolemia.** *J. Lipid Res.* 2008. 49: 1333–1343.

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Familial hypercholesterolemia (FH) is characterized by elevated levels of circulating LDL cholesterol (LDL-C), resulting in atherosclerosis and an increased risk of premature coronary heart disease. The autosomal dominant form of FH (ADH) is most often associated with loss-of-function mutations within either the LDL receptor

(LDLR) or apolipoprotein B (*APOB*) gene, resulting in decreased hepatic clearance of LDL-C (1). Recently, mutations within the proprotein convertase subtilisin/kexin type 9 (*PCSK9*) gene were found to represent a third class of mutations associated with ADH (2). Several missense mutations (S127R, D129G, F216L, D374H, and D374Y) are associated with hypercholesterolemia and premature atherosclerosis (2–6). In addition, several missense (R46L, L253F, and A433T) and nonsense mutations (Y142X and C679X) are associated with low circulating LDL-C levels and a reduction in the risk of coronary heart disease (7–9). Of note, an individual with two inactivating mutations within *PCSK9* and no detectable circulating PCSK9 protein was recently identified with a significantly lower LDL-C concentration (14 mg/dl) and no observable signs of ill health due to the absence of PCSK9 (10). Together, these findings demonstrate a key role for PCSK9 in regulating circulating LDL-C levels in the human population, and point to PCSK9 as an attractive target for the treatment of hypercholesterolemia (11).

PCSK9 is a secreted protein consisting of several domains found within the proprotein convertase (PC) family of serine proteases, including an N-terminal signal peptide and prodomain, followed by a catalytic domain and Cys-rich C-terminal domain (12–14). Similar to other PC family members, PCSK9 undergoes autocatalytic processing within the endoplasmic reticulum (ER), resulting in cleavage of its prodomain following Q152, (FAQ↓SIP) (15, 16). Structural and biochemical studies of PCSK9 indicate that although other members of the PC family typically undergo a second cleavage event to release their inhibitory prodomain, the prodomain of PCSK9 remains intact following autoproteolysis, with its C terminus blocking the protease catalytic site (16–20).

PCSK9 regulates circulating LDL-C levels by reducing the number of hepatic LDLRs. Introduction of PCSK9,

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^[S] The online version of this article (available at <http://www.jlr.org>) contains supplementary data in the form of one figure.

either into the circulation of mice via parabiosis or into cultured cells, results in LDLR degradation and hence, lowering of hepatic and cellular uptake of LDL (20–22). Interestingly, although autoprocessing of PCSK9's prodomain is required for exit from the ER and secretion of the mature protein, its serine protease activity is not required for PCSK9-mediated degradation of LDLR after PCSK9 has been secreted. Coexpression of the PCSK9 prodomain and a catalytically inactive mutant (S386A) within the catalytic domain in trans facilitated the efficient secretion of the stable heterodimer and subsequent finding that this form of PCSK9 reduced the number of LDLRs on the cell surface similarly to the wild-type protein (23, 24). Although these findings do not rule out a potential role for PCSK9's catalytic activity in reducing the number of LDLRs prior to secretion, they do indicate that the protease activity of PCSK9 is not required for its effects in reducing the number of cell surface LDLRs after secretion of mature PCSK9.

PCSK9 binds directly to LDLR and requires LDLR for normal cellular trafficking and endocytosis (17, 20–22, 25). Although the region of PCSK9 responsible for binding LDLR has not been identified, the PCSK9 binding region of LDLR has been narrowed down to the epidermal growth factor-like repeat (EGF-A) within the EGF homology domain (26). LDLR requires the acidic environment of endosomes to release internalized LDL; however, the binding affinity between PCSK9 and LDLR greatly increases at an acidic endosomal pH (17, 18, 21). The gain-of-function PCSK9 mutant, D374Y, also binds LDLR with higher affinity than does wild-type protein, as measured by either Biacore or time-resolved fluorescence resonance energy transfer (TR-FRET) and is internalized into cells more efficiently than is wild-type PCSK9 (17, 20, 21). In addition, we and others have demonstrated that PCSK9 gain-of-function mutants S127R and D374Y are more potent in reducing the number of cell surface LDLRs, thereby lowering cellular LDL uptake (20, 21). Together, these findings suggest that the strength of the PCSK9-LDLR interaction is a major factor in determining the ability of PCSK9 to regulate LDLR activity and hence, serum LDL-C levels in humans.

To gain further insight into the mechanistic role of sites within PCSK9 responsible for hypercholesterolemia, we undertook a limited vertical scanning mutagenesis of Ser127 and Asp374 residues and assessed the effect of such mutations on PCSK9 function. In addition, several combinations of PCSK9 gain-of-function mutants (S127R, F216L, and D374Y) were designed to assess their relative roles in PCSK9 function. Several criteria of PCSK9 function were assayed, including: processing of the PCSK9 prodomain and secretion of mature protein; the potency of purified mutant proteins in lowering LDL uptake; and the relative binding affinities of mutant proteins to LDLR. Both energetic and molecular modeling was performed to assess the predicted effect of such mutations on PCSK9 structure. Together, our findings suggest that mutations at Asp374 of PCSK9 result in a gain-of-function phenotype predominantly due to enhancing the binding of PCSK9

to LDLR on the cell surface, whereas mutations at Ser127 may enhance PCSK9 function either at a step prior to secretion or following internalization into cells.

METHODS

Construction of wild-type and mutant PCSK9 expression vectors

The cloning of PCSK9 and construction of pcDNA3.1-F1-WT, pcDNA3.1-F1-S127R, and pcDNA3.1-F1-D374Y PCSK9 expression plasmids was carried out as described (21). Additional plasmids expressing mutant forms of human PCSK9 were developed by site-directed mutagenesis using the QuickChange II XL Site-Directed Mutagenesis kit (Stratagene, LaJolla, CA). The following primers were used for generating mutations from the pcDNA3.1-F1-WT starting plasmid. For plasmid pcDNA3.1-F1-S127A: F, 5'-CTTCCTGGTGAAGATGGCCGGC-GACCTGCTGGAGC-3' and R, 5'-GCTCCAGCAGGTGCGCCGGC-CATCTTCACCAGGAAG-3'; for plasmid pcDNA3.1-F1-S127D: F, 5'-CTTCCTGGTGAAGATGGACGGCGACCTGCTGGAGC-3' and R, 5'-GCTCCAGCAGGTGCGCCGGC-CATCTTCACCAGGAAG-3'; for plasmid pcDNA3.1-F1-S127K: F, 5'-CTTCCTGGTGAAGAT-GAAGGGCGACCTGCTGGAGC-3' and R, 5'-GCTCCAGCAGGTGCGCCCTTCATCTTCACCAGGAAG-3'; for plasmid pcDNA3.1-S127L: 5'-CTTCCTGGTGAAGATGCTGGGC-GACCTGCTGGAGC-3' and R, 5'-GCTCCAGCAGGTGCGCCAG-CATCTTCACCAGGAAG-3'; for plasmid pcDNA3.1-F1-S127T: F, 5'-CTTCCTGGTGAAGATGACCGGCGACCTGCTGGAGC-3' and R, 5'-GCTCCAGCAGGTGCGCCGGTCATCTTCACCAGGAAG-3'; for plasmid pcDNA3.1-F1-D374A: F, 5'-CATTGGTGCCTC-CAGCGCCTGCAGCACCTGCTTTG-3' and R, 5'-CAAAG-CAGGTGCTGCAGGCGCTGGAGGCACCAATG-3'; for plasmid pcDNA3.1-F1-D374E: F, 5'-ATTGGTGCCTCCAGCGAGTGCAG-CACCTGCTTTGTG-3' and R, 5'-CACAAAGCAGGTGCTGCAC-TCGCTGGAGGCACCAAT-3'; for plasmid pcDNA3.1-F1-D374F: F, 5'-CATTGGTGCCTCCAGCTTCTGCAGCACCTGCTTT-3' and R, 5'-AAAGCAGGTGCTGCAGAGCTGGAGGCACCAATG-3'; for plasmid pcDNA3.1-F1-D374K: F, 5'-CATTGGTGCCTCC-AGCAAGTGCAGCACCTGCTTTG-3' and R, 5'-CAAAG-CAGGTGCTGCACTTGCTGGAGGCACCAATG-3'; for plasmid pcDNA3.1-F1-D374L: F, 5'-CATTGGTGCCTCCAGCCTGTGCA-GCACCTGCTTTG-3' and R, 5'-CAAAGCAGGTGCTGCA-CAGGTGAGGCACCAATG-3'. The following primers were used for generating mutations from the pcDNA3.1-F1-S127R starting plasmid. For plasmid pcDNA3.1-F1-S127R D374Y: F, 5'-CATTGGTGCCTCCAGCTACTGCAGCACCTGCTT-3' and R, 5'-AAGCAGGTGCTGCAGTAGCTGGAGGCACCAATG-3'; for plasmid pcDNA3.1-F1-S127R F216L D374Y, use previous primers for F216L and D374Y. The following primers were used for generating mutations from the pcDNA3.1-F1-D374Y starting plasmid. For plasmid pcDNA3.1-F1-F216L D374Y: F, 5'-GAGGA-GGACGGGACCCGCTGCACAGACAGGCCAGCAAG-3' and R, 5'-CTTGCTGGCCTGTCTGTGCAGGCGGGTCCCGTCTCTC-3'. All PCSK9 constructs were present in a pcDNA3.1 backbone with G418 selection and carboxyl-terminal V5 and His tags (Invitrogen, Carlsbad, CA).

Stable transfection of HEK293 cells and protein purification

Stable HEK293 cell lines expressing either wild-type or mutant versions of PCSK9 were created as described (21). Cells were maintained in 1× DMEM (Mediatech, Inc. Manassas, VA) containing 1 mg/ml G418 supplemented with 10% FBS. Wild-

type and mutant PCSK9 proteins were purified from HEK293-generated media as described (21).

Western blot analysis of PCSK9

HEK293 cells (300,000/well) were plated in a 6-well plate in $1 \times$ DMEM (Mediatech) containing 100 units of penicillin and 100 μ g/ml streptomycin sulfate and supplemented with 10% FBS. The following day, cells were transfected with 1 μ g of wild-type or mutant plasmid using FuGENE 6 transfection reagent (Roche Applied Science, Indianapolis, IN) according to the manufacturer's instructions. After 3 days, the medium was changed to $1 \times$ DMEM and incubated for an additional 6 h. Media was collected, and cells were lysed in radioimmune precipitation assay buffer (Teknova, Hollister, CA) plus Complete protease inhibitor mixture (Roche Applied Science). Protein concentration was assayed using a BCA protein assay kit (Pierce, Rockford, IL). Ten micrograms of proteins from lysate or 10 μ l of medium was loaded onto 10–20% Tris/glycine gels (Invitrogen). Following transfer, membranes were incubated with anti-V5 primary antibody (1:5000; Invitrogen) and alkaline phosphatase-conjugated anti-mouse IgG (H + L) (1:3000; Promega, Madison, WI). Bands were detected using a one-step nitro blue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate kit (Pierce) according to the manufacturer's instructions.

Isolation of LDL

LDL was isolated from healthy human volunteers as previously described (21).

Labeling of LDL, LDLR, and anti-V5 antibody

LDL was labeled as described previously with either a fluorescent 3,3'-dioctadecylindocarbocyanine (DiI) particle (Molecular Probes, Carlsbad, CA) (21) or Alexa Fluor 546 as follows. Prior to labeling, LDL (0.5–2 mg/ml) was dialyzed against 200 mM sodium carbonate, pH 8.5, 100 mM NaCl, and 1 mM EDTA. Alexa Fluor 546-labeled carboxylic acid, succinimidyl ester (Invitrogen) was dissolved in Me₂SO at 10 mg/ml. LDL was then combined with a 25 \times molar excess of Alexa Fluor 546-labeled NHS and set at room temperature for 5 h protected from the light. The reaction was stopped with 15 μ l of 1 M Tris, pH 8.0. Labeled alexa fluor-LDL was dialyzed against PBS and 1 mM EDTA. Anti-V5 antibody was labeled Alexa Fluor 647 as described previously (21). LDLR was also labeled as described previously (21), except that 5 equivalents of Eu(8044)-DTA (Perkin-Elmer, Waltham, MA) were used in place of 20 equivalents of Eu(W1024)-ITC in the original labeling.

DiI- or AF-LDL uptake assay

LDL uptake was measured in HEK293 cells stably expressing the pcDNA3.1 vector alone as previously described (21). The only difference in this report is that both DiI- and AF546-labeled LDL was used to measure cellular LDL uptake. The amount of LDL uptake measured by the HEK293 cell line and the regulation of LDL uptake by added PCSK9 protein was identical with either LDL preparation (data not shown).

PCSK9-LDLR binding affinity measurements

PCSK9-LDLR interactions were measured by TR-FRET (27, 28) as described (21). Briefly, reactions took place in 50 μ l total volume in 10 mM HEPES, pH 7.4, 150 mM NaCl, 0.1 mM CaCl₂, and 0.05% (w/v) BSA. A preformed complex of Alexa Fluor 647-labeled anti-V5 antibody (40 nM) and Eu³⁺-labeled recombinant LDLR ectodomain (R and D Systems, Minneapolis, MN) (3–5 nM, or 20,000 B counts) was combined with increasing

amounts of purified wild-type, Ser127, or Asp374 mutant PCSK9 protein. The data were fit to a sigmoidal dose-response curve by nonlinear regression after normalization of the relative ratios to 100% of maximum fluorescence transfer for each protein.

Modeling of PCSK9 mutants

Molecular modeling was performed in order to compare the energetics of wild-type and mutant proteins using a recently solved structure of processed PCSK9 (17). Single point mutations of Ser127 of the prodomain and Asp374 of the catalytic domain were made in silico using the Protein Design module in the Quanta program (29). After each mutation at the Ser127 and Asp374 positions, a conformational search of the mutated side chain was made to find the optimal position of the mutated side chain residue. Using the JG protocol in the MIX modeling environment at Merck and Co., Inc. (Rahway, NJ), 100 different conformations of the mutated side chain within the protein were generated and were minimized while the other part of the protein was held fixed (30). An energy minimization routine, Batchmin, with the Merck molecular force field implemented in MIX, the modeling platform developed at Merck and Co., Inc., was used to minimize the conformers (31). The lowest energy conformer was closely examined in comparison with the crystal structure of the wild-type residue, and van der Waals' contact with other residues in the protein was checked.

RESULTS

Expression and purification of PCSK9 Ser127 and Asp374 mutant proteins

S127R and D374Y are previously described gain-of-function mutations of PCSK9 and are associated with hypercholesterolemia. To better understand how these mutations influence PCSK9 function, a vertical scanning mutagenesis was carried out at Ser127 (Arg, Ala, Asp, Lys, Leu, or Thr) and Asp374 (Tyr, Ala, Glu, Phe, Lys, or Leu) as described in Methods (Fig. 1A). To determine the effect of each mutation on PCSK9 autoprocessing and protein secretion, transient transfection of each mutant was carried out using an HEK293 cell line, followed by Western blot analysis of cell lysate and media. In agreement with previous reports, the autoprocessing of S127R was reduced, compared with wild-type, with a higher ratio of intracellular unprocessed-to-processed PCSK9 detected and relatively lower amounts secreted (Fig. 1B) (16, 32). Similarly, most other Ser127 mutations resulted in lower autoprocessing, with the S127L mutation displaying the greatest reduction. As a consequence of the reduction in autoprocessing, only a minimal amount of processed S127L PCSK9 protein was detectable in the cell media, and therefore purification of this mutant in sufficient amounts for further biochemical characterization was not feasible. In contrast, mutations made at Asp374 of PCSK9 had no effect on either autoprocessing or protein secretion (Fig. 1C).

Secreted PCSK9 proteins were purified from the media of HEK293 stable cell lines overexpressing each mutant. SDS-PAGE and Coomassie Blue staining demonstrated that all proteins were purified to homogeneity (>90%) and consisted of both mature PCSK9 fragments contain-

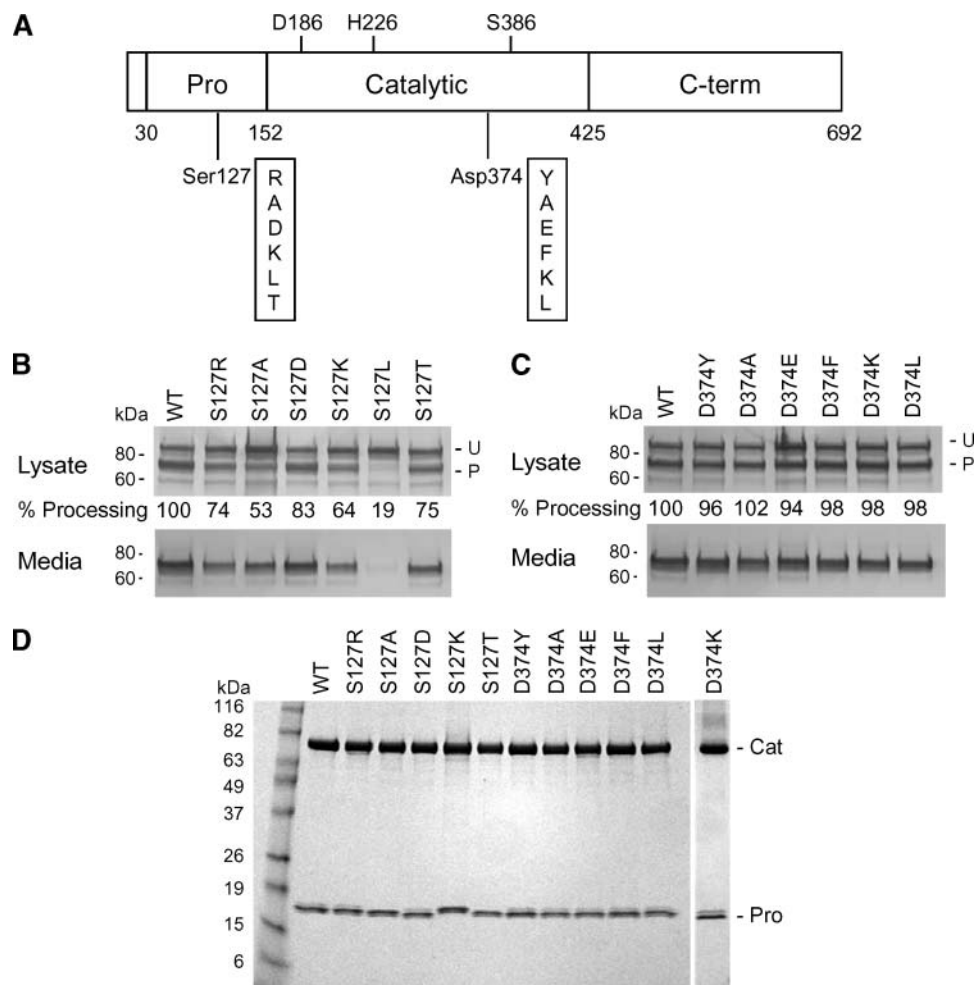


Fig. 1. Expression and purification of Ser127 and Asp374 proprotein convertase subtilisin/kexin type 9 (PCSK9) mutant proteins. **A:** Schematic of PCSK9 indicating the location of the serine protease catalytic triad (D186, H226, S386) and the Ser127 and Asp374 residues. Vertical scanning mutagenesis at Ser127 (Arg, Ala, Asp, Lys, Leu, or Thr) and Asp374 (Tyr, Ala, Glu, Phe, Lys, or Leu) of PCSK9 was performed as described in Methods. **B:** Immunoblot analysis of wild-type and Ser127 mutant PCSK9 proteins in cell lysate (Lysate) and supernatant (Media). Transient transfections of wild-type and mutant PCSK9 constructs with a C-terminal V5/His tag were carried out in an HEK293 cell line. PCSK9 was detected using an anti-V5 antibody that detects both unprocessed (U) and processed (P) forms of PCSK9. **C:** Immunoblot analysis of wild-type and Asp374 mutant PCSK9 proteins in cell lysate and supernatant. The autoprocessing of mutant PCSK9 proteins from this experiment is expressed as percentage of maturation of wild-type PCSK9 as measured in cell lysates. **D:** Coomassie Blue-stained gel of purified PCSK9 mutants. Media from stable HEK293 cell lines overexpressing wild-type, Ser127, or Asp374 mutant PCSK9 proteins was used for purification of secreted protein as described in Methods. The results shown are from 1 μ g of recombinant PCSK9 protein loaded per well. Cat and Pro indicate the mature PCSK9 fragments including the catalytic and cysteine-rich C-terminal domains and the prodomain, respectively. WT, wild-type PCSK9.

ing the prodomain and catalytic and C-terminal domains (Fig. 1D). During purification of wild-type and mutant proteins, two fractions of PCSK9 were observed with prodomains of slightly different apparent masses (see supplementary Fig. 1A). The apparent difference in mass of prodomains was not explained by differences in posttranslational modifications (e.g., sulfation of Y38) or the site of autocleavage, but was probably due to the site of signal peptide cleavage by signal peptidase (results not shown). Regardless of the difference in apparent mass of prodomains, a comparison of these fractions in a cell-based assay of PCSK9 function demonstrated that both inhib-

ited LDL uptake similarly and were therefore typically pooled for further biochemical analysis (see supplementary Fig. 1B, C).

Ser127 and Asp374 substitutions affect potencies of exogenously added PCSK9 on LDL uptake

Recently, we and others have demonstrated that transfer of media containing either PCSK9 secreted from PCSK9-expressing cell lines or purified PCSK9 protein added to fresh cells results in both the degradation of the LDLR and, as a direct consequence, inhibition of cellular LDL uptake (20–22, 33). In the current work, we compared the

relative potencies of mutant PCSK9 proteins, with mutations at Ser127 and Asp374, with that of wild-type protein in reducing LDL uptake when added to the media of an HEK293 cell line. As shown in **Fig. 2A**, addition of increasing amounts of wild-type PCSK9 protein results in a dose-dependent reduction of LDL uptake. Cellular LDL uptake was reduced by 50% at a concentration of 56 ± 10 nM for wild-type PCSK9 (**Table 1**). In comparison, both the S127R and S127K mutant proteins were significantly more effective in reducing LDL uptake (**Fig. 2A**). Similar to our previous report, S127R was 4.3-fold more effective in reducing LDL uptake ($EC_{50} = 13 \pm 5.8$ nM), whereas the similar S127K mutant was nearly 2-fold more effective ($EC_{50} = 30 \pm 14$ nM) (**Fig. 2A**). In contrast to S127R and S127K, other mutations at Ser127 (S127A, S127D, and S127T) had minimal effect on PCSK9's potency in lowering LDL uptake (**Fig. 2B** and **Table 1**).

The relative potencies of mutations at Asp374 of PCSK9 were similarly determined using a cell-based functional assay. In comparison to wild-type PCSK9, both the previously described D374Y mutant and the similar D374F mutant resulted in a 25- to 35-fold increase in reducing LDL uptake (D374Y, $EC_{50} = 2.2 \pm 0.4$ nM; D374F, $EC_{50} = 1.6 \pm 0.2$ nM) (**Fig. 2C** and **Table 1**). Of note, although both D374Y and D374F had the most profound

effect in enhancing PCSK9's ability to lower LDL uptake, each mutation made at Asp374 was more potent than wild-type PCSK9 (**Fig. 2D**). For example, D374A (6.5-fold), D374E (4.8-fold), D374K (2.5-fold), and D374L (13-fold) were each significantly more potent in reducing LDL uptake compared with wild-type PCSK9 (**Fig. 2D** and **Table 1**). Together, these data show that specific mutations at Ser127 (S127R and S127K) result in a gain of function, whereas all mutations tested thus far at Asp374 display a gain-of-function phenotype.

Combination of S127R and D374Y gain-of-function mutations additively enhance PCSK9-mediated lowering of LDL uptake

To determine whether PCSK9 missense mutations responsible for hypercholesterolemia are additive in their ability to increase PCSK9's ability to inhibit cellular LDL uptake, HEK293 cell lines overexpressing single mutations alone (S127R, F216L, and D374Y) or in combination were created. Similar to previous reports, neither the F216L nor the D374Y mutants affect PCSK9 autoprocessing or secretion, whereas the S127R mutation results in a reduction in both (**Fig. 3A** and data not shown) (16, 21, 32). Interestingly, when S127R was combined with either F216L or D374Y, the resulting double or triple mutant

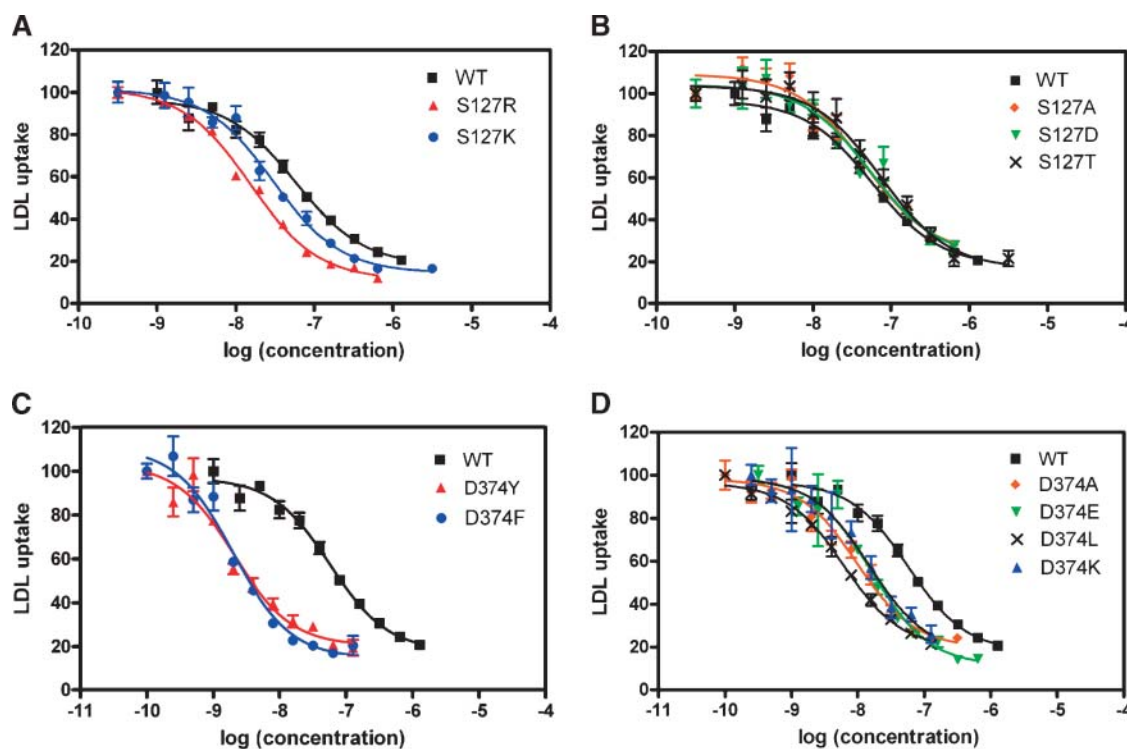


Fig. 2. Comparison of Ser127 and Asp374 PCSK9 mutant proteins in reducing cellular LDL uptake. HEK293 cells were incubated with increasing amounts of purified PCSK9 proteins and 10 μ g/ml diiodododecylindocarbocyanine- or Alexa Fluor 546-labeled LDL for 5 h. Cells were washed, and the amount of fluorescently labeled LDL uptake was measured as described in Methods. The data shown are representative of at least three independent experiments and are fit to a sigmoidal dose response curve by nonlinear regression. A: Dose response of exogenous wild-type, S127R, and S127K PCSK9 proteins reducing LDL uptake. B: Dose response of exogenous wild-type, S127A, S127D, and S127T PCSK9 proteins reducing LDL uptake. C: Dose response of exogenous wild-type, D374Y, and D374F PCSK9 proteins reducing LDL uptake. D: Dose response of exogenous wild-type, D374A, D374E, D374L, and D374K PCSK9 proteins reducing LDL uptake. Error bars represent \pm SEM. WT, wild-type PCSK9.

TABLE 1. LDL uptake results for PCSK9 Ser127 and Asp374 mutants^a

Genotype	LDL Uptake EC ₅₀ ^b	Fold-WT EC ₅₀
	nM	
WT	56 ± 10	1.0
S127R	13 ± 5.8	0.23
S127A	42 ± 20	0.75
S127D	65 ± 17	1.16
S127K	30 ± 14	0.53
S127T	54 ± 26	0.96
D374Y	2.2 ± 0.4	0.039
D374A	8.6 ± 1.6	0.15
D374E	12 ± 4	0.21
D374F	1.6 ± 0.2	0.029
D374K	22 ± 10	0.39
D374L	4.3 ± 1.9	0.08

^a The potencies of wild-type (WT) and mutant PCSK9 proteins on inhibiting LDL uptake were determined with a pcDNA3.1 cell line as described in Methods.

^b Concentration of PCSK9 protein at which 50% of LDL uptake was inhibited; values are means ± standard deviation.

PCSK9 proteins were deficient in autoprocessing and secretion to the same extent as the S127R mutant alone (Fig. 3A). PCSK9 proteins carrying either single or combinations of these mutations were purified to homogeneity for further characterization (Fig. 3B).

As shown in Fig. 3C, although both the S127R and D374Y mutants were significantly more effective in reducing the amount of LDL uptake by cells, F216L (EC₅₀ = 88 ± 26 nM) lowered LDL uptake to a similar degree as the wild-type PCSK9 protein. In agreement with this finding, combination of F216L with either S127R (S127R F216L, EC₅₀ = 10.3 ± 4 nM) or D374Y (F216L D374Y, EC₅₀ = 3.1 ± 0.9 nM) did not influence either mutation's ability to reduce LDL uptake (Fig. 3D and Table 2). In contrast, combination of S127R and D374Y resulted in a PCSK9 protein that was 70-fold more effective (EC₅₀ = 0.8 ± 0.12 nM) than wild-type PCSK9 in lowering LDL uptake and was significantly more potent than either single mutation alone (Table 2). A mutant containing all three mutations (S127R F216L D374Y) was 56-fold more potent than wild-type PCSK9 (Fig. 1D and Table 2). Therefore, although the F216L mutation had no effect on PCSK9 activity in this cell-based assay, combination of S127R and D374Y mutations resulted in a mutant with an increased potency in reducing cellular LDL uptake compared with either single mutation.

Potencies of Asp374 PCSK9 mutants in lowering LDL uptake correlate with binding affinity to LDLR

In our previous report, the binding affinity of wild-type and D374Y PCSK9 and LDLR was measured using both surface plasmon resonance (Biacore) and TR-FRET (21). Both assays demonstrated a much higher affinity between D374Y and LDLR compared with the wild-type form of PCSK9, whereas the S127R mutant bound LDLR with a 2-fold greater affinity only at an acidic pH. In this report, we measured the relative binding of wild-type, Ser127, and Asp374 mutant PCSK9 proteins and LDLR using the identical TR-FRET assay. In this assay, the specific energy transfer between PCSK9 bound to Alexa Fluor 647-labeled

anti-V5 antibody and a Eu⁺³-labeled LDLR ectodomain was measured. As shown in Fig. 4, as the amount of wild-type or mutant PCSK9 protein was increased in the reaction, the TR-FRET ratio increased until reaching a maximum level due to saturation of the anti-V5 antibody recognizing PCSK9. The relative binding efficiency of Ser127, Asp374, and wild-type PCSK9 for the LDLR was determined by measuring the relative concentrations of PCSK9 proteins required for significant PCSK9-LDLR-dependent fluorescence to be observed.

In agreement with previous results obtained using Biacore, the wild-type and the S127R mutant PCSK9 bound LDLR with relatively the same efficiency (Fig. 4A). In addition, each of the other Ser127 mutants also had a similar binding affinity to LDLR when compared with the wild-type PCSK9 protein. In contrast, each of the Asp374 mutants displayed a higher affinity for binding LDLR compared with the wild-type PCSK9 protein (Fig. 4B). Importantly, the concentration of both D374Y and D374F required for significant detection of PCSK9-LDLR-dependent fluorescence was ~25-fold lower than that of wild-type PCSK9, indicating that these mutants had a much higher affinity for binding LDLR than did wild-type protein. Both D374A and D374L mutants required ~8-fold less protein, whereas D374E and D374K mutants required ~2-fold less protein than wild-type PCSK9 for detection of significant fluorescence, indicating that each Asp374 mutant had relatively higher binding affinities for LDLR *in vitro*. In addition, as shown in Fig. 4C, when either S127R or F216L was combined with D374Y, there was no effect on the affinity of PCSK9 for LDLR beyond that observed for the D374Y mutation alone. In total, these experiments demonstrate that mutations at Ser127 resulting in a gain-of-function phenotype in the cell-based PCSK9 functional assay (S127R and S127K) do not affect binding of PCSK9 to LDLR at neutral pH, either alone or in combination with the D374Y mutation, whereas mutations at Asp374 significantly increase both the potency of PCSK9 in lowering cellular LDL uptake and the binding affinity between PCSK9 and LDLR.

Three-dimensional modeling of Ser127 and Asp374 PCSK9 substitutions

To better understand the effects of the Ser127 and Asp374 mutants on binding LDLR and PCSK9 function, we developed a model of these mutations based on the recently solved crystal structure of processed PCSK9 (17). As shown in Fig. 5A, processed PCSK9 contains a subtilisin-like catalytic site that is blocked from carrying out further proteolysis by the stably bound prodomain.

Ser127 is located within the prodomain of PCSK9 and is surrounded by two hydrophilic residues (Tyr107 and Asp129) (Fig. 5B). Mutation of Ser127 to either Arg127 or Lys127 will increase polar interactions of the mutated residue with these two amino acids via hydrogen bonding with Tyr107 and a salt bridge with Asp129 (Fig. 5B). In addition to these polar interactions, the long hydrophobic side chain of either Arg127 or Lys127 will increase hydrophobic interactions with the phenyl ring of Tyr107.

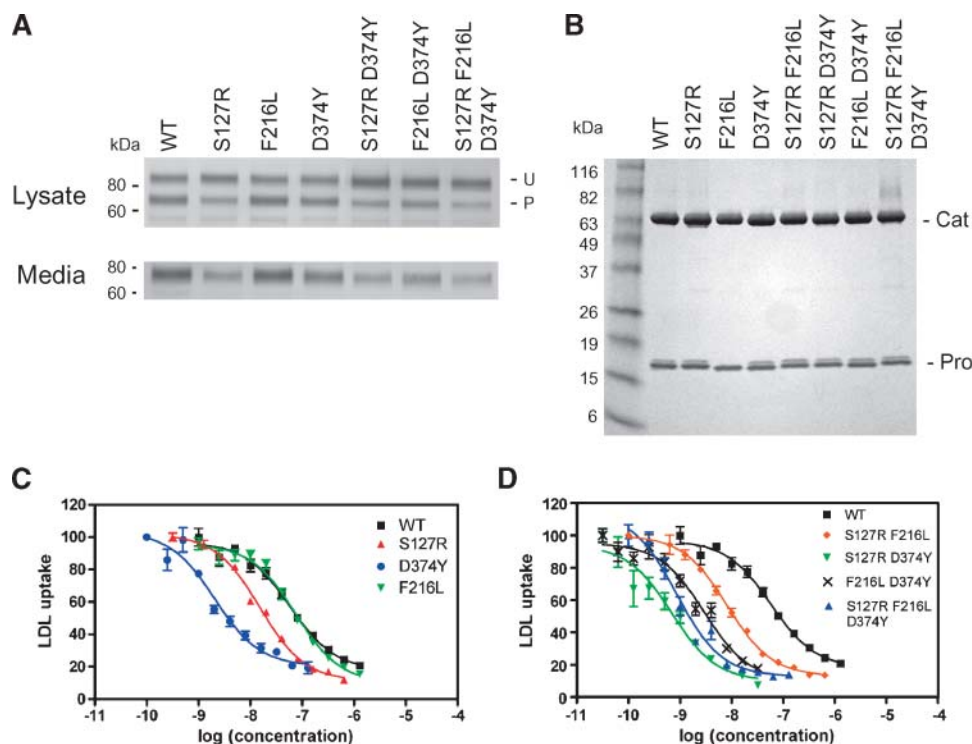


Fig. 3. Combination of PCSK9 gain-of-function mutations. **A:** Immunoblot analysis of wild-type, single, and combination PCSK9 gain-of-function mutant proteins in cell lysate (Lysate) and supernatant (Media). Transient transfections of wild-type and mutant PCSK9 constructs with a C-terminal V5/His tag were carried out in an HEK293 cell line as described in Methods. PCSK9 was detected using an anti-V5 antibody that detects both unprocessed (U) and processed (P) forms of PCSK9. **B:** Coomassie Blue-stained gel of purified PCSK9 mutants. Media from stable HEK293 cell lines overexpressing wild-type or mutant PCSK9 proteins was used for purification of secreted protein as described in Methods. The results shown are from 1 μ g of recombinant PCSK9 protein loaded per well. Cat and Pro indicate the mature PCSK9 fragments including the catalytic and cysteine-rich C-terminal domains and the prodomain, respectively. **C:** Dose response of exogenous wild-type, S127R, F216L, and D374Y PCSK9 proteins reducing LDL uptake. **D:** Dose response of exogenous wild-type, and combinations of PCSK9 gain-of-function mutations reducing LDL uptake. The data shown are representative of at least three independent experiments and are fit to a sigmoidal dose response curve by nonlinear regression. Error bars represent \pm SEM. WT, wild-type PCSK9.

Asp374 is located in the catalytic domain of PCSK9 and is proximal to the serine protease catalytic site Ser386 residue (Fig. 5). Asp374 is surrounded by polar serines (Ser221, Ser372, and Ser376) and in particular, can make a hydrogen bond contact with Ser221 (Fig. 5C) (19). Mu-

tation of Asp374 to either Phe374 or Tyr374 would introduce large hydrophobic side chains, resulting in the interruption of those polar interactions, and a destabilization of this local environment. Although these structural models predict that mutations at Ser127 resulting in gain of function will increase PCSK9 stability, and those at Asp374 will decrease PCSK9 stability, it is not clear how these changes would influence the PCSK9 and LDLR interaction.

TABLE 2. LDL uptake results for PCSK9 gain-of-function combination mutants^a

Genotype	LDL Uptake EC ₅₀ ^b	Fold-WT EC ₅₀
	<i>nM</i>	
WT	56 \pm 10	1.0
S127R	13 \pm 5.8	0.23
F216L	88 \pm 26	1.57
D374Y	2.2 \pm 0.4	0.039
S127R F216L	10.3 \pm 4	0.18
S127R D374Y	0.8 \pm 0.1	0.014
F216L D374Y	3.1 \pm 0.9	0.055
S127R F216L D374Y	1.0 \pm 0.4	0.018

^aThe potencies of wild-type and mutant PCSK9 proteins on inhibiting LDL uptake were determined with a pcDNA3.1 cell line as described in Methods.

^bConcentration of PCSK9 protein at which 50% of LDL uptake was inhibited; values are means \pm standard deviation.

DISCUSSION

Although several missense mutations within PCSK9 are associated with FH and increased degradation of LDLR, the mechanism through which they confer a gain-of-function phenotype is not well understood. In the present study, we have conducted a detailed analysis of mutations at both the Ser127 and Asp374 residues of PCSK9 associated with hypercholesterolemia. Mutant PCSK9 proteins carrying select mutations at S127, including S127R and S127K, were more potent in lowering cellular LDL

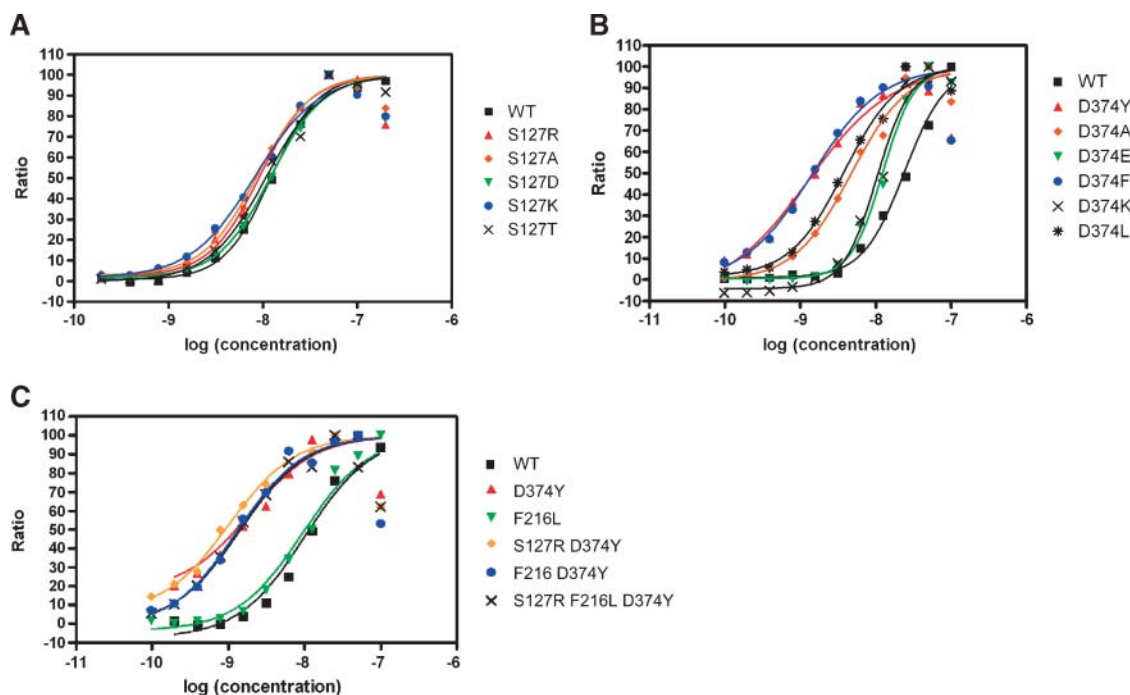


Fig. 4. Binding of Ser127 and Asp374 PCSK9 mutant proteins with the LDL receptor (LDLR). Time-resolved fluorescence resonance energy transfer (TR-FRET) analysis of wild-type and mutant PCSK9 interaction with LDLR was performed as described in Methods. Increasing amounts of purified V5-His-tagged wild-type or mutant PCSK9 protein were combined with a preformed complex of Alexa-Fluor 647-labeled anti-V5 antibody and Eu^{+3} -labeled recombinant LDLR protein. The specific fluorescence (Ratio) was detected after overnight incubation at room temperature. A: TR-FRET analysis of Ser127 PCSK9 mutant proteins binding LDLR. B: TR-FRET analysis of Asp374 PCSK9 mutant proteins binding LDLR. C: TR-FRET analysis of combination of PCSK9 gain-of-function mutations. The data shown are representative of at least three independent experiments and are fit to a sigmoidal dose response curve by nonlinear regression. WT, wild-type PCSK9.

uptake, whereas each mutation made at Asp374 resulted in a more potent PCSK9 protein. The combination of both S127R and D374Y PCSK9 mutations had an additive effect on PCSK9 function, resulting in an approximately 70-fold increase in the potency of PCSK9-mediated lowering of LDL uptake upon addition of purified protein to cells. In addition, although mutations at Asp374 strengthen the affinity between PCSK9 and LDLR, mutations at Ser127 do not significantly affect binding of PCSK9 to LDLR. Together, these results suggest that although mutations at both Ser127 and Asp374 sites within PCSK9 can result in a gain-of-function phenotype, they influence PCSK9 function through distinct mechanisms.

To determine the effects of mutations at both Ser127 and Asp374 on PCSK9 function, we began by transfecting each mutant into HEK293 cells and monitoring both the autoprocessing and secretion of mutant PCSK9 proteins. Previous work has shown that the S127R gain-of-function mutant undergoes comparably less autoprocessing when expressed in either cell lines or in mouse liver upon adenovirus-mediated overexpression (5, 16, 32). We have confirmed these results and expanded upon them by showing that several additional mutations at S127 also result in less PCSK9 autoprocessing. In our studies, S127R, S127A, S127K, S127L, and S127T resulted in less autoprocessing, with the S127L mutant displaying the greatest reduction (Fig. 1A). Based on the recently solved crystal structure

of processed PCSK9, it is not immediately evident why mutations at S127 would affect autoprocessing (17–19). However, the Ser127 residue is strictly conserved within primate, mouse, and rat PCSK9, and it is possible that prior to the autoprocessing of PCSK9, S127 plays an important role in the recognition of the cleavage site for autoprocessing (34). In contrast to Ser127 mutants, mutations at the D374 site of PCSK9 had no effect on either autoprocessing or secretion of PCSK9 protein (Fig. 1C).

Although the F216L mutation has been reported to be associated with FH, we did not observe a difference in its ability to lower cellular LDL uptake compared with wild-type PCSK9 (Fig. 3) (2). In addition, F216L binds LDLR with a similar affinity to the wild-type protein at both neutral and acidic pH (Fig. 4C) (17). However, it is possible that F216L enhances PCSK9 function *in vivo* by preventing inactivation of PCSK9 by furin/PC5/6A cleavage (35). Alternatively, F216L may also mediate its effect on PCSK9 function through another mechanism that is not recapitulated in the *in vitro* cell-based assay used in this report.

When tested in a cell-based assay measuring PCSK9-mediated inhibition of LDL uptake, both the S127R and similar S127K mutant were more potent compared with wild-type PCSK9 (Fig. 2A and Table 1). Additional Ser127 mutants (S127A, D, T) had no effect on PCSK9-mediated lowering of LDL uptake (Fig. 2B), indicating that only

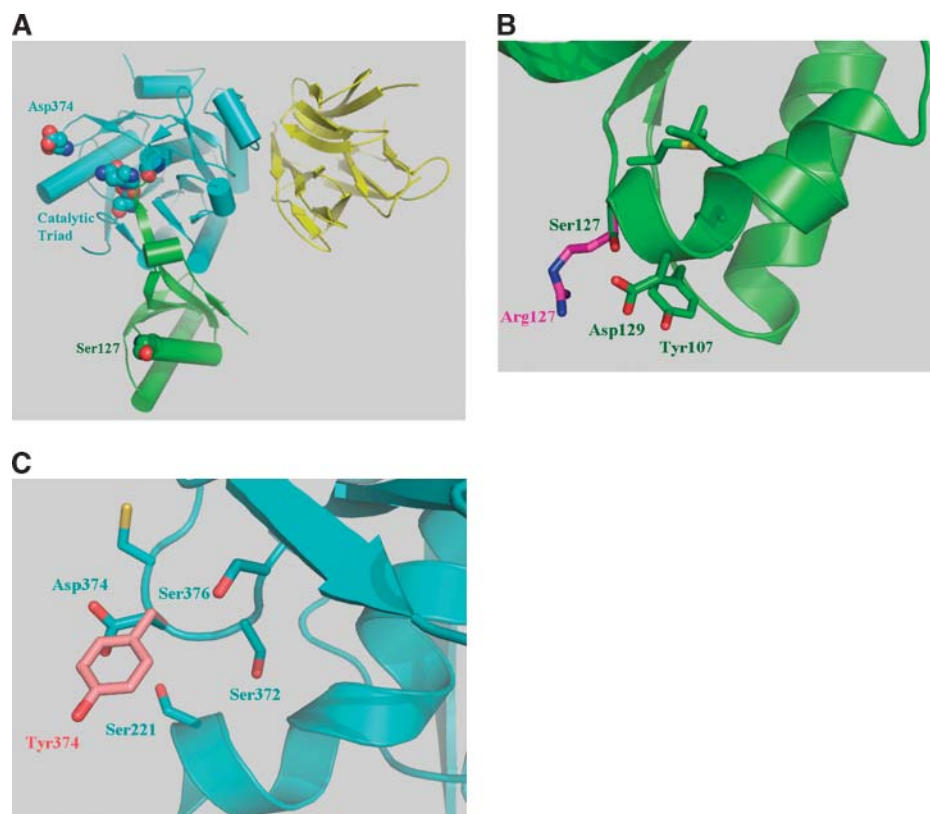


Fig. 5. Modeling the effects of Ser127 and Asp374 mutations on PCSK9 structure. A: Schematic representation of PCSK9 with prodomain in green, catalytic domain in cyan, and C-terminus domain in yellow. Ser127 of the prodomain and Asp374 of the catalytic domain are shown with space filling atoms. B: Ser127 in green and its mutant Arg127 in magenta are overlaid. Asp129 and Tyr147 are the neighboring residues and are indicated. In particular, Asp129 can make a salt bridge with the arginine mutant of S127R. C: Asp374 in magenta and its mutant Tyr374 in salmon are overlaid. Asp374 interacts with Ser221 via a hydrogen bond, and mutation D374Y would lose this interaction. In addition, introduction of a bulkier aromatic ring would destabilize the region of PCSK9.

substitution of serine for basic amino acids at this site confers a gain-of-function phenotype. In agreement with previous measurements of PCSK9-LDLR binding affinity using surface plasmon resonance, the S127R mutant bound LDLR with a similar affinity to that of the wild-type form of the protein (Fig. 4A) (21). In addition, previous work has demonstrated that fluorescently labeled S127R protein is internalized by cells with relatively the same efficiency as the wild-type protein (21). Biacore analysis of S127R binding to LDLR has been inconclusive, with the S127R mutant showing either a modestly higher affinity to LDLR when measured at an acidic pH and equivalent binding to wild-type PCSK9 at neutral pH, or with 2- to 5-fold higher affinity at neutral pH, but equivalent binding to wild-type PCSK9 at acidic pH (17, 21). When either wild-type or S127R PCSK9 was overexpressed in an HepG2 cell line, the ability of both to enhance degradation of LDLR required clathrin-mediated endocytosis, indicating that S127R may have an enhanced ability either to bind LDLR after cellular uptake or to more efficiently shuttle LDLR to the lysosomes for degradation (25). These results, in addition to the observed lower autoprocessing and secretion of S127R and S127K mutant proteins,


suggest that these mutations confer a gain-of-function phenotype by enhancing the affinity of PCSK9 for LDLR, either prior to secretion of the protein, or after cellular internalization of the PCSK9-LDLR complex into endosomes. Alternatively, it is possible that the prodomain of PCSK9 is recognized by an accessory protein or coreceptor required for PCSK9-mediated degradation of LDLR, and that S127R and S127K mutations strengthen this interaction, resulting in more efficient trafficking of the PCSK9-LDLR complex to lysosomes for degradation. It will be of particular interest to identify such accessory factors, inasmuch as they may constitute additional targets for treatment of hypercholesterolemia.

In contrast with mutations at S127, each Asp374 mutant tested in this report was more potent in lowering cellular LDL uptake (Fig. 2). In addition, the binding affinity between D374 mutants and LDLR correlated well with their relative potencies in reducing LDL uptake, with both D374Y and D374F being most potent and displaying the highest binding affinity for LDLR (Figs. 2C, 4B). In agreement with previous measurements of PCSK9-LDLR binding affinity using surface plasmon resonance and ligand blotting, the D374Y mutant bound LDLR with a signifi-

cantly higher affinity than that of the wild-type form of the protein (Fig. 4B) (20, 21). The D374Y mutant has also been shown to be internalized more efficiently than wild-type PCSK9, indicating a stronger association with LDLR on the cell surface (20, 21). Together, these results indicate that Asp374 makes a critical contact with LDLR, possibly with the EGF-A domain, thereby regulating their interaction and the efficiency by which PCSK9 directs LDLR into lysosomes for degradation. Interestingly, these studies indicate that the ability of PCSK9 to reduce the number of LDLRs is limited by the wild-type Asp374 residue and its presumed interaction with LDLR. This is evidenced by the observation that each mutation made at Asp374 results in a stronger interaction with LDLR and more-efficient removal in cell culture. One possible explanation for this role of Asp374 is that Asp374 is located where the surrounding residues of LDLR disfavor Asp374, such as negatively charged or hydrophobic residues. Therefore, Asp374 prefers to form an intramolecular hydrogen bond with Ser221 of PCSK9. Mutation of Asp374 to neutral hydrophobic residues such as tyrosine could then relieve the unfavorable charge interactions with LDLR, thereby strengthening the PCSK9-LDLR interaction. In addition, mutations at Asp374 are predicted to be less stable because of the disruption of interaction between Asp374 and Ser221, so that these mutants of PCSK9 may prefer energetic compensation by interacting with LDLR (Fig. 5). Recently, a D374H mutation resulting in a severe form of hypercholesterolemia has also been reported, further demonstrating the critical role of this site in regulating PCSK9 function (6).

While this manuscript was under review, the cocrystal structure of a complex between PCSK9 and the EGF-A repeat of LDLR at pH 4.8 was reported (36). The overall fold of PCSK9 in the cocrystal is identical to apo-PCSK9, with the exception of the C terminus, which is disordered in the cocrystal and is visible in the apo structures. Importantly, in agreement with the observed lack of effect on LDLR binding of the Ser127 mutations, despite being more potent in the cell-based assay of PCSK9 function, (Figs. 2, 4) this site is located in the prodomain and is >40 Å from the protein-protein interface of PCSK9 and EGF-A. Therefore, as discussed previously, it is possible that the S127R mutation modulates interactions between PCSK9 and cofactors or accessory proteins that are required for PCSK9 function in cell culture and in vivo. Interestingly, the Asp374 residue, shown to play an important role, in both this report and in previous reports, in modulating the interaction between PCSK9 and LDLR, is positioned 4 Å from EGF-A His306 in the cocrystal structure and presumably forms a salt bridge under low pH (20, 21, 36). Substitution of Asp374 for Tyr would be predicted to favor a hydrogen bond formed between D374Y and His306 of EGF-A, thereby increasing the affinity between PCSK9 and LDLR. However, in our studies, both D374Y and D374F mutations had a similar effect on PCSK9-mediated lowering of LDL uptake (Fig. 2C) and affinity for LDLR binding, (Fig. 4B). Therefore, it is possible that in addition to hydrogen bonding between D374Y

and His306 of EGF-A, π stacking between these large aromatic substitutions and the His306 aromatic ring of EGF-A also contributes to the observed increase in affinity between these mutants and LDLR. In addition, we observed higher affinity binding between D374L and D374A PCSK9 mutants and LDLR, which would not be predicted to form a hydrogen bond, salt bridge, or π stacking with His306 of EGF-A (Fig. 4B). Therefore, these results indicate that the increased affinity between Asp374 mutants and LDLR could be attributed to several factors, including an intermolecular salt bridge between His306 within the EGF-A repeat of LDLR and Asp374 in PCSK9, intermolecular π -stacking interactions of the aromatic rings, stabilization/destabilization around the D374 environment in PCSK9, via hydrogen bond with S221, and electrostatic interactions.

In summary, we have shown that although mutations at both Ser127 and Asp374 of PCSK9 are associated with FH, and are more potent in reducing cellular LDL uptake, they influence PCSK9 function through distinct mechanisms. The findings with mutations at Asp374 also reinforce the important role that the PCSK9-LDLR interaction plays in determining PCSK9's ability to regulate LDLR and may aid in the discovery of novel anti-PCSK9 molecules for the treatment of hypercholesterolemia. 

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