Isolation and characterization of the circulating truncated form of PCSK9

Bomie Han, Patrick I. Eacho, Michael D. Knierman, Jason S. Troutt, Robert J. Konrad, Xiaohong Yu, and Krista M. Schroeder

Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, IN 46285

Abstract Proprotein convertase subtilisin-kexin type 9 (PCSK9) is a secreted protein which regulates serum LDL cholesterol. It circulates in human and rodent serum in an intact form and a major truncated form. Previous in vitro studies involving the expression of human PCSK9 genetic variants and in vivo studies of furin knockout mice suggest that the truncated form is a furin cleavage product. However, the circulating truncated form of PCSK9 has not been isolated and characterized. Utilizing antibodies which bind to either the catalytic domain or the C-terminal domain of PCSK9, the truncated PCSK9 was isolated from serum. MS was used to determine that this form of PCSK9 is a product of in vivo cleavage at Arg218 resulting in pyroglutamic acid formation of the nascent N terminus corresponding to Gln219 of intact PCSK9. We also determined that the truncated PCSK9 in serum lacked the N-terminal segment which contains amino acids critical for LDL receptor binding. A truncated PCSK9, expressed and purified from HEK293 cells with identical composition as the circulating truncated protein, was not active in inhibition of LDL uptake by HepG2 cells. These studies provide a definitive characterization of the composition and activity of the truncated form of PCSK9 found in human serum.—Han, B., P. I. Eacho, M. D. Knierman, J. S. Troutt, R. J. Konrad, X. Yu, and K. M. Schroeder. Isolation and characterization of the circulating truncated form of PCSK9. J. Lipid Res. 2014. 55: 1505–1514.

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Proprotein convertase subtilisin-kexin type 9 (PCSK9) is a secreted serine protease which regulates plasma LDL cholesterol (LDL-C) (1–3). Rare gain-of-function mutations cause autosomal dominant hypercholesterolemia, a disorder characterized by LDL-C >300 mg/dl and premature atherosclerosis (4, 5). Conversely, loss-of-function mutations are associated with reduced LDL-C and reduced risk of cardiovascular diseases (6–10). A large body of work has demonstrated that PCSK9 controls circulating LDL-C by modulating the levels of LDL receptor (LDLR) (11–14). PCSK9 binds to the LDLR and directs it into a lysosomal degradation pathway rather than the recycling pathway (15–18). This activity of PCSK9 on the LDLR is independent of its catalytic activity (18). The synthesis of PCSK9 and LDLR is induced by HMG-CoA reductase inhibitors, such as statins, under the control of the sterol-regulated transcription factor, SREBP2 (19–20). Treatment with statins increases both hepatic LDLR content and circulating levels of PCSK9 (21–22). Therefore, increased PCSK9 appears to limit both statin-induced increases in LDLR content and the resulting reduction of plasma LDL-C in humans.

PCSK9 is initially synthesized as a ~74 kDa proprotein, from which proteolytic cleavage of the ~14 kDa N-terminal prodomain results in the ~60 kDa mature form consisting of the N-terminal catalytic domain and the C-terminal domain (13). PCSK9 is expressed in multiple tissues, including liver, intestine, kidney, and cerebellum, of which the liver appears to be the major source of the circulating protein (23–25). The liver is also the major site of regulation of plasma LDL-C by PCSK9 (24, 26–28). Clearance of PCSK9 from the circulation is thought to be rapid, based on the 5 min half-life of the recombinant human protein injected into mice (26, 28). The clearance is presumed to be mediated primarily by the LDLR, as the half-life of recombinant PCSK9 is prolonged when injected into LDLR-deficient mice (26). Decreased half-life of a mutant form of PCSK9 (D374Y) that has higher LDLR degradation activity also supports this notion (26).

The proteolytic cleavage of PCSK9 may represent a second mechanism of its clearance. A truncated form of PCSK9 with a molecular mass of 52–55 kDa has been observed and represents up to 40% of the total circulating PCSK9 in human serum (29, 30). Studies in mice with
hepaticocyte-specific inactivation of furin indicate that truncated PCSK9 levels are reduced when hepatic furin is absent (29). The observation of reduced plasma levels of the truncated form in humans heterozygous for the R218S mutation in PCSK9 is consistent with furin-mediated cleavage, as this mutation disrupts the RXXR sequence recognized by furin (30). However, the naturally occurring F216L mutation, which does not disrupt the RXXR sequence, also reduces plasma levels of truncated PCSK9 (29). Additionally, a recent report shows in vitro hepsin cleavage of PCSK9 at 215, demonstrating the possibility for alternative sites of cleavage (31).

The truncated form of the protein is thought to be inactive (29, 30), although this conclusion was recently challenged (31). Given the controversy, it seems unlikely that the matter of truncated PCSK9’s activity will be resolved until the truncated PCSK9 in human serum is isolated and characterized. To address this question, we have developed a method to isolate the truncated form of human PCSK9 from serum. This approach has allowed the mass, sequence, and composition of endogenous truncated human PCSK9 to be definitively determined. We also generated the recombinant truncated form of human PCSK9, confirmed its sequence and composition as being the same as the truncated form found in serum, and evaluated its activity on cultured hepatocytes in vitro. The definitive identification of the native PCSK9 proteolytic cleavage site and the determination that cleaved PCSK9 is inactive on LDLR will clarify our understanding of PCSK9 biology.

**EXPERIMENTAL PROCEDURES**

**PCSK9 ELISA**

A human PCSK9 sandwich ELISA was developed using two anti-human PCSK9 polyclonal antibodies (R&G Systems, catalog numbers AF3888 and BAF3888). Briefly, PCSK9 antibody AF3888 was coated on a high binding ELISA plate (Costar, catalog number 9018) overnight at 4°C. The plate was washed with PBS containing 0.1% Tween-20 and blocked using 1% casein in PBS (blocking buffer). Serum samples were diluted in blocking buffer and added to the ELISA plate. After 2 h of incubation, the plate was washed and streptavidin-HRP (Millipore, catalog number 18-152) was added to the plate. After an additional hour of incubation, the plate was washed and biotinylated anti-human PCSK9 Ab BAF3888 (blocking buffer). Serum samples were diluted in blocking buffer or PBS, and read at 450 nm using the Molecular Devices SpectraMax Plus absorbance plate reader.

**Isolation of PCSK9 by differential immunoprecipitation**

Human PCSK9 was expressed in mice under liver-specific thyroxine binding globulin (TBG) promoter using a chimeric adeno-associated viral (AAV) vector comprised of the inverted terminal repeats of AAV2 packaged with the capsid from AAV8 (ReGenX, Washington, DC). Each C57BL/6 mouse was injected intravenously with \(1 \times 10^{10}\) genomic copies of AAV vector, and the mice showed stable expression of human PCSK9 4 weeks post injection at levels of 8–10 μg/ml, as determined by ELISA. Mice were euthanized using CO\(_2\) and serum was collected via cardiac stick. PCSK9 C-terminal domain and catalytic domain monoclonal antibodies were covalently linked to beaded agarose resin via primary amines using the Aminolink Plus immobilization kit (Pierce, catalog number 44894). Serum samples (290 μl) were then diluted 5-fold in immunoprecipitation buffer [50 mM HEPES (pH 7.40), 150 mM NaCl, 1% Triton X-100, 5 mM EDTA, and 5 mM EGTA] for a total of 1 ml volume and incubated overnight with 20 μg of PCSK9 antibody linked to 40 μl packed beads. The beads were washed with PBS + 0.1% NP-40 and either frozen for LC/MS analysis or boiled with the SDS-sample buffer (NuPage LDS sample buffer, Invitrogen, catalog number NP0007, containing NuPage sample reducing agent, Invertogen, catalog number NP0004) for Western blotting analysis. For isolation of the truncated form away from the intact PCSK9, the supernatant from the immunoprecipitation with the catalytic domain antibody was subjected to an additional overnight incubation with beads linked with the C-terminal domain PCSK9 antibody. These beads were then washed three times with PBS + 0.1% NP-40 before subsequent analysis. For analysis by MALDI, NP-40 in the washing step was replaced with octylglucoside.

For isolation of PCSK9 from human donors, serum was collected in-house from 20 normal human volunteers after an overnight fasting in compliance with the Lilly Research Blood Donation Program requirements. PCSK9 was isolated from 2 ml of serum following the protocol described above. For isolation of truncated PCSK9 from human serum, removal of the intact PCSK9 was repeated twice before incubation with the C-terminal PCSK9 antibody.

**PCSK9 Western blotting**

The PCSK9 captured on the beads from immunoprecipitation experiments were diluted into the SDS-sample buffer and boiled for 5 min before separation of proteins on 4–12% Bis-Tris mini-gel (Invitrogen, catalog number NP022 BOX) by electrophoresis. Proteins were transferred onto 0.2 μm nitrocellulose membrane (Invitrogen, catalog number LC2009) and Western blotting was performed according to standard procedure using a PCSK9 polyclonal antibody (R&D, catalog number AF3888) as a primary antibody and an anti-sheep IgG Alexa Fluor 680 as secondary antibody (Invitrogen, catalog number A-21102) for visualization using the Odyssey infrared imaging system (LI-COR).

**Size analysis of truncated PCSK9 by MALDI**

Immunoprecipitated PCSK9 was eluted twice with 50 μl of 0.1% trifluoroacetic acid in water before concentration and desalting on a C4 ZipTip (Millipore), and elution with 10 μl of 50% acetonitrile containing 0.1% trifluoroacetic acid. One microliter of the eluate was mixed with 1 μl of 33% acetonitrile, 0.1% trifluoroacetic acid saturated with sinapinic acid, spotted onto a MALDI target and dried under a stream of nitrogen. MALDI spectra were acquired on an Applied Biosystems 4800 MALDI TOF-TOF mass spectrometer. The spectra were analyzed with Data Explorer and MoverZ software. The intact PCSK9 showed a peak with an M+H value of 59,667 Da compared with predicted value of 57,230 Da, indicating the presence of posttranslational modifications (32). For this reason, we relied on difference in the measured mass between the intact and truncated PCSK9 to deduce the site of cleavage.

**LC/MS/MS analysis of PCSK9**

Immunoprecipitated PCSK9 was eluted from the beads by heating for 10 min at 55°C in 200 μl of 8M urea containing 10 mM DTT and 2.5 μg of chicken lysozyme before digestion with Arg-C.
for 3 h at 37°C according to the manufacturer’s protocol (Roche Applied Science, catalog number 11370290001). Following digestion, free cysteine residues were blocked by incubating with 40 mM iodoacetamide for 30 min at room temperature in the dark and the remaining iodoacetamide was quenched by adding DTT to 80 mM. For trypsin digestion, the eluted PCSK9 was first incubated with 40 mM iodoacetamide before incubation with 1 µg of trypsin (Trypsin-Gold, Promega, catalog number V5280) for 3 h at 37°C in 100 mM ammonium bicarbonate solution. Remaining iodoacetamide was quenched by adding DTT to 80 mM.

Samples were subjected to mass spectrometric analysis on a LTQ mass spectrometer (Thermo Fisher Scientific) coupled inline with a reversed phase HPLC system (Surveyor MS pump from Thermo Fisher Scientific) consisting of an XBridge C18 column (50 x 2.1 mm, 2.5 µm, Waters, catalog number 186003921) and two solvents system (0.1% formic acid in water as mobile phase A and 0.1% formic acid in acetonitrile as mobile phase B). The HPLC method consisted of 100% A at 200 µl/min for 0.5 min, 8–32% B at 200 µl/min for 16 min, 32% B at 200 µl/min for 1 min, 80% B at 600 µl/min for 1 min, followed by 100% A at 600 µl/min for 2 min, and the HPLC column was maintained at 50°C. Details on the multiple reaction monitoring method used for quantitative measurements of the digested peptides are described in supplementary Tables I and II.

**Purification of recombinant truncated PCSK9**

Truncated recombinant PCSK9 was generated by transient cotransfecting plasmids for both WT human PCSK9 (with C-terminal His-tag) and human furin into 293HEK cells using XtremeGene Ro1539 (Roche Applied Sciences) according to the manufacturer’s instructions. Conditioned culture medium was harvested after 48 h and PCSK9 was isolated using a nickel column followed by removal of the intact PCSK9 using a catalytic domain PCSK9 antibody affinity column. Unbound flow-through was collected in fractions and analyzed for complete removal of intact antibody affinity column. Unbound flow-through was collected in fractions and analyzed for complete removal of intact antibody affinity column. Unbound flow-through was collected in fractions and analyzed for complete removal of intact antibody affinity column. Unbound flow-through was collected in fractions and analyzed for complete removal of intact antibody affinity column. Unbound flow-through was collected in fractions and analyzed for complete removal of intact antibody affinity column.

**N-terminal amino acid sequencing of purified recombinant truncated PCSK9**

Automated Edman degradation amino acid sequence analysis was performed on an Applied Biosystems (ABI) Procise490 protein sequence system utilizing a standard manufacturer-provided gas phase (GP-PVDF/ABI) method after removal of N-terminal proglutamic acid residue (pGlu) with pyroglutamate aminopeptidase. Briefly, a dried PVDF membrane was loaded with 50–300 pmol of truncated PCSK9, blocked with 0.5% PVP-360 in 0.1% acetic acid (20 min at room temperature), washed three times with Milli-Q water before digestion with 1 mM of pyroglutamate aminopeptidase (Sigma, catalog number 19924) in 50 µl of 50 mM sodium phosphate, 10 mM DTT, and 1 mM EDTA (pH 7.0) (PGAP buffer) in an Eppendorf tube for 1 h at 90°C. The PVDF membrane was washed three times with Milli-Q water, dried, and loaded to a Prosorb PVDF membrane cartridge (ABI) for automated sequencing.

**LDL uptake assay**

HepG2 human hepatoma cells were grown on a poly-L-lysine-coated black 96-well plate and cultured in media supplemented with lipoprotein deficiency serum (Intralipid) at 37°C overnight. Cells were treated with various concentrations of intact or truncated PCSK9 for 2 h at 37°C before addition of 100 ng BODIPY-LDL (Invitrogen) and incubation at 4°C for an additional 4 h. LDL uptake in HepG2 cells was determined by fluorescence measurement using Acumen (TTP Labtech, Cambridge, UK) normalized for total cell number.

**RESULTS**

**Isolation of the truncated form of PCSK9**

PCSK9 circulates in humans as a 74 kDa protein which migrates on a reduced denaturing gel as a ~60 kDa component comprised of the catalytic and C-terminal domains and a ~14 kDa prodomain (13). In addition to this intact form of PCSK9, a truncated form with an apparent mass of approximately 52 kDa was identified in human serum by Western blot using a polyclonal antibody (30). We observed both the intact and truncated forms of PCSK9 in human serum when PCSK9 was isolated using a monoclonal antibody recognizing the C-terminal domain of PCSK9 (Fig. 1A, middle panel). Both forms were also found in the serum of mice expressing human PCSK9 from an AAV vector (Fig. 1A, left panel) and in serum of mice expressing only endogenous mouse PCSK9 (Fig. 1A, right panel). Absence of a prodomain band in the endogenous mouse PCSK9 sample is likely due to lack of cross reactivity of the polyclonal antibody with the mouse prodomain, although other possibilities cannot be excluded.

In an effort to characterize the truncated form, PCSK9 was immunoprecipitated from human serum using a PCSK9 C-terminal domain monoclonal antibody. The ~52 kDa band corresponding to the truncated form was cut from the SDS-PAGE gel for N-terminal sequencing. We were unable to obtain a sequence, suggesting that the N-terminus was modified in such a way as to block the Edman degradation reaction. This result was consistent with a report of an earlier effort to identify the truncated fragment (30).

We devised an approach to isolate the truncated form from intact PCSK9 based on our observation of differential binding of monoclonal antibodies to the two forms. Specifically, a C-terminal domain PCSK9 monoclonal antibody binds to both intact and truncated PCSK9, while a catalytic domain monoclonal antibody is expected to bind only to the intact form of PCSK9 (Fig. 1B). We used a serum from mice expressing human PCSK9 from an AAV vector because it contains both forms of PCSK9 at higher concentrations than found in human serum. Selective capture of intact PCSK9 by the catalytic domain antibody was confirmed by Western blotting (Fig. 1C, A samples). The unbound fraction from the first step was subjected to a second immunoprecipitation with the C-terminal domain antibody. Western blotting analysis demonstrated that the second precipitate contained only the truncated form of PCSK9 (Fig. 1C, B samples). When the serum samples were immunoprecipitated only with the C-terminal domain antibody, eliminating the first step, both intact and truncated PCSK9 (total PCSK9) were captured as shown by Western blotting (Fig. 1C, C samples). The ~14 kDa fragment corresponding to the prodomain was observed after isolation of both the intact and truncated forms of PCSK9, indicating that the truncation process did not result in complete dissociation of the prodomain.
Identification of the cleavage site for human PCSK9 overexpressed in mouse

The intact and truncated forms of human PCSK9 isolated from the mouse serum were subjected to MALDI analysis for protein mass measurement (Fig. 2). In addition to the common peak attributed to the serum albumin, the other major peaks corresponding to two different forms of PCSK9 showed a mass difference of 7,726 Da, consistent with the difference observed in Western blotting analysis. The observed mass difference was best explained if the truncation occurred between Arg218 and Gln219, followed by the conversion of the newly exposed N-terminal glutamine residue to pGlu, with a predicted mass difference of 7,730 Da. Conversion of the N-terminal Gln to pGlu is a thermodynamically favored reaction that can be catalyzed by either mildly alkaline or acidic pH. To confirm the site of truncation and the presence of pGlu at the N terminus of the truncated PCSK9, we digested each of the isolated PCSK9 forms with ArgC and analyzed the resulting peptides by LC/MS/MS.

Peptide Q219-R237 was readily found from digests of the intact PCSK9 (Fig. 3B, sample A) as well as the total PCSK9 (Fig. 3B, sample C), as expected. The low level of pE219-R237 peptide observed from the intact PCSK9 is attributed to spontaneous conversion from Q219-R237 during sample digestion. Consistently, digestion of purified recombinant PCSK9 by ArgC under the same condition produced a similar level of the pGlu-containing peptide (data not shown). In contrast, samples containing only the truncated PCSK9 (Fig. 3B, sample B) did not produce a detectable level of Q219-R237 peptide, but generated a robust signal for the peptide pE219-R237. Therefore, we conclude that cleavage occurs in vivo at Arg218 and the nascent N terminus of the resulting truncated PCSK9 exists mainly as pGlu corresponding to Gln219 of intact PCSK9.

In these samples, the levels of intact versus truncated PCSK9 appeared to be similar as judged by the signal intensity of a peptide derived from the common C-terminal domain of the protein (Fig. 3B, bottom lane). Consistently, the signal intensities of the Q219-R237 and pE219-R237 peptides were also comparable to each other (Fig. 3B, sample C). Quantitative comparison of multiple peptides generated by trypsin digestion of the same samples (Fig. 3C, see supplementary Table I for amino acid sequence of the tryptic peptides) showed that all peptides within the C-terminal...
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Fragment of Arg218 cleavage were present at similar levels between the intact and truncated form, whereas the two peptides within the N-terminal fragment (Ser153-Arg218) are almost absent in the truncated PCSK9.

Two conclusions can be drawn from these observations. First, we identified Arg218 as the major cleavage site for in vivo generation of the truncated PCSK9. Because we did not find any measurable level of the Q219-R237 peptide in truncated PCSK9, there cannot be other major cleavage sites N-terminal to Arg218. ArgC digestion of such protein would have generated a peptide with Gln219 at the N terminus. Hepsin was shown to be capable of cutting PCSK9 at Arg215 (31). However, such cleavage must be either a biologically minor event or it must be followed by further digestion in vivo at Arg218. Additionally, because all tryptic peptides C-terminal to Cys223 were found at similar levels, there cannot be another major cleavage site C-terminal to this residue. For example, a major cleavage at amino acid residue 260 would have significantly reduced the observed ratio in Fig. 3C for two peptides, 223-237 and 259-272, below the level of the other C-terminal peptides.

Second, the cleaved N-terminal segment (Ser153-Arg218) did not remain associated with the rest of the truncated PCSK9. Successful separation of the intact PCSK9 from truncated form in this experiment (Fig. 1C) would not have been possible otherwise. Moreover, if they remained associated, we should have observed a significant level of pE219-R237 peptide from the ArgC digest of the samples isolated by the catalytic domain antibody (Fig. 3B). This conclusion differs from the one drawn by Lipari et al. (31), who observed that the N-terminal segment stayed associated with the truncated PCSK9 after in vitro cleavage of recombinant PCSK9 at Arg218 by furin protease in a buffer solution. This discrepancy might be explained if the catalytic domain antibody required intact Arg218-Gln219 backbone for binding. To test this possibility, we prepared a recombinant protein corresponding to the cleaved N-terminal segment (Ser153-Arg218) and tested its binding to the catalytic domain antibody. The catalytic domain antibody clearly bound the recombinant peptide corresponding to the N-terminal segment of the cleaved PCSK9 with an affinity comparable to that for the intact PCSK9 (Fig. 4). We also show that the catalytic domain antibody is fully capable of binding the N-terminal segment generated by furin cleavage of the full-length PCSK9. After digestion of recombinant human PCSK9 in mouse serum by addition of furin, the catalytic domain antibody captured the remaining intact PCSK9 (60 kDa) and the N-terminal segment (7.7 kDa), but failed to capture truncated PCSK9 (52 kDa) (Fig. 5, lane 1). If the N-terminal segment stayed associated with the truncated PCSK9, we should have observed truncated PCSK9 in this preparation. It is conceivable that the catalytic domain antibody induced dissociation of the truncated PCSK9 from the N-terminal segment considering proximity of the epitope to the cleavage site. In this case, we expected to observe N-terminal segment as well as the truncated PCSK9 when C-terminal antibody was used to capture total PCSK9. However, when...
not an artifact induced by the catalytic domain antibody, but a natural phenomenon occurring in serum after cleavage of PCSK9 at Arg218.

Confirmation of cleavage at Arg218 for PCSK9 in human serum

Because our previous work was performed using human PCSK9 overexpressed in mouse serum, we ran subsequent experiments to show that the same cleavage and dissociation process occurs with PCSK9 in human serum. ArgC-digestion of the intact PCSK9, isolated from five independent human sera using the catalytic domain antibody, generated predominantly the Q219-R237 peptide (Fig. 6A). The low level of pE219-R237 peptide in these intact PCSK9 samples (13% of total ± 2%) was below the level of spontaneous conversion of the Q219-R237 peptide observed from digestion of recombinant PCSK9. In the same experiment, recombinant full-length PCSK9 samples (n = 4) processed in parallel as a control produced 20 ± 1% pE219-R237 peptide. ArgC digestion of the total PCSK9, immunoprecipitated with the C-terminal domain PCSK9 antibody (Fig. 6C), produced a significantly higher level of the pE219-R237 peptide (35% of total ± 2%), indicating a significant level of truncated PCSK9 with preexisting pGlu219 as the nascent N terminus. Therefore, we conclude that the catalytic domain antibody did not bring down any measurable level of truncated PCSK9 in human serum, replicating the results from human PCSK9 overexpressed in the mouse serum.

In contrast to the above observations, when the catalytic domain antibody was used to remove the full-length PCSK9 before isolation of the remaining PCSK9 with C-terminal domain antibody, the pE219-R237 peptide was highly...
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This in vitro-generated truncated PCSK9 and full-length recombinant PCSK9 were tested for their inhibitory activity on LDL uptake by HepG2 hepatoma cells in culture. While the intact PCSK9 inhibited LDL uptake in a concentration-dependent manner, the truncated PCSK9 had virtually enriched, as evidenced by the complete reversal of the ratio between the two peptides, Q219-R237 and pE219-R237 (Fig. 6B). The low level of Q219-R237 peptide in these samples is attributed to incomplete removal of the full-length PCSK9 by the catalytic domain antibody. Note that the level of pE219-R237 in this preparation is beyond what can be predicted from spontaneous conversion of the Q219-R237 peptide. These observations support the conclusion that the same mechanism of cleavage and dissociation observed for human PCSK9 overexpressed in mouse serum occurs with PCSK9 in human serum. Specifically, cleavage of PCSK9 in human serum occurs in vivo at Arg218 and results in pGlu formation at the nascent N terminus corresponding to Gln218 in the intact form. This process is followed by the in vivo dissociation of the N-terminal segment from the remaining PCSK9 molecule.

Recombinant truncated human PCSK9 is inactive on LDLR

Having confirmed the cleavage site and composition of truncated PCSK9, we generated the truncated PCSK9 for an evaluation of its activity. Native human PCSK9 with C-terminal His-tag and human furin were coexpressed in HEK293 cells to generate both intact and truncated PCSK9 (30). After isolation of total PCSK9 with a nickel column, the intact form was removed by affinity chromatography using a PCSK9 catalytic domain antibody (supplementary Fig. IV). ArgC digestion of the protein followed by the LC/MS/MS analysis showed that the Gln219 at the N terminus was fully converted to pGlu (data not shown). The N-terminal amino acid sequences obtained after treatment with pyroglutamate aminopeptidase also confirmed that the purified truncated PCSK9 had the same N-terminal amino acid sequence as the truncated form characterized from the serum (data not shown). The cleaved N-terminal segment was absent from this preparation as judged by both N-terminal amino acid sequencing and MS analysis of the ArgC digest. Therefore, this preparation of recombinant truncated PCSK9 has the same composition and cleavage site as the truncated PCSK9 found in human serum.

This in vitro-generated truncated PCSK9 and full-length recombinant PCSK9 were tested for their inhibitory activity on LDL uptake by HepG2 hepatoma cells in culture. While the intact PCSK9 inhibited LDL uptake in a concentration-dependent manner, the truncated PCSK9 had virtually
no effect on LDL uptake at all concentrations tested (Fig. 7). This demonstrates that the Arg218-cleaved truncated PCSK9 lacking the N-terminal segment is inactive as a regulator of LDL uptake.

**Quantitation of different forms of PCSK9 in human sera by MS**

Although PCSK9 is an important regulator of circulating level of LDL, measured concentration of PCSK9 showed only a weak correlation with the LDL-C in previous studies (21, 33–39). Because most assay methods for PCSK9 do not distinguish intact PCSK9 from the truncated form, we explored to determine whether the concentration of intact PCSK9 had a better correlation with LDL-C than total PCSK9. We used a single immunoprecipitation step with either the catalytic domain antibody, to capture intact PCSK9, or the C-terminal domain antibody, to capture total (intact + truncated) PCSK9 (supplementary Figs. II, III) and evaluated levels of each form in twenty human serum samples. The intact or total PCSK9 captured in this way was digested with either ArgC (cleavage after Arg) or trypsin (cleavage after Lys or Arg) and analyzed by LC/MS/MS (see supplementary Tables I, II for methods). Recombinant human PCSK9 was processed identically and in parallel as a control. The same serum samples were also subject to measurement of PCSK9 by ELISA for comparison.

Multiple tryptic peptides were used to estimate the serum concentration of PCSK9 by comparing the signal intensities to those from recombinant PCSK9 (supplementary Table III). The MS analysis reported a total PCSK9 concentration of 309 ± 126 ng/ml (mean ± SD), 30% of which was in the truncated form on average. With ArgC digestion of the same samples, we confirmed that the catalytic domain antibody failed to capture a detectable level of truncated PCSK9 with preexisting pGlu219 (supplementary Fig. IIA) and that the proportion of the pE219-R237 peptide to the Q219-R237 peptide was comparable to what was expected from measurements of multiple tryptic peptides (supplementary Fig. IIIB). The ELISA assay, which employed a polyclonal antibody and detected both truncated and intact PCSK9, reported 317 ± 106 ng/ml (mean ± SD). The values reported for each individual sample by the two assays were in close agreement with the correlation coefficient of 0.92 (Table 1). Total PCSK9 concentration measured by either ELISA or MS showed a weak correlation with LDL-C (Table 1, \( R = 0.45 \) or 0.55, respectively), consistent with previous reports (21, 33–39). Use of intact PCSK9 concentration or truncated PCSK9 concentration did not improve the correlation with the LDL-C (Table 1).

**DISCUSSION**

In this body of work, we have provided definitive identification of the circulating truncated form of PCSK9 which was observed previously (30). The identification was enabled by the differential binding of PCSK9 monoclonal antibodies to the intact and truncated forms of PCSK9. Using serum from mice expressing human PCSK9, the truncated form of PCSK9 was separated from the intact form of PCSK9 by a two-step immunoprecipitation. With MS analysis, we unequivocally demonstrated that the truncated PCSK9 contains a pGlu at the nascent N terminus corresponding to Gln219 of the intact form. We applied the same MS analysis tools to PCSK9 isolated from multiple human sera and confirmed the presence of the same truncated form. Based on quantitation of multiple tryptic peptides, the truncated form represented between 3 and 42% of the total PCSK9 in the 20 human serum samples evaluated, with an average of 27%.

Our definitive identification of the circulating truncated form of PCSK9 confirms previous reports in which the site of truncation was inferred based on indirect evidence. Benjannet et al. (30) reported that the naturally occurring gain-of-function PCSK9 mutant R218S, unlike native PCSK9, was resistant to truncation when expressed in HEK293 cells. The R218S mutation disrupts the RXRX furin-cleavage motif in PCSK9, suggesting the truncation was catalyzed by furin or related proteases. The proposed role of furin was further supported by the finding that truncation of PCSK9 was strongly reduced in mice with a conditional knockout of furin in hepatocytes (29).

The question of whether the truncated PCSK9 is functionally active was addressed previously in the studies of the furin knockout mice (29). These mice produced less truncated PCSK9 and displayed less hepatic LDLR than WT mice, suggesting that the truncated PCSK9 was inactive at LDLR degradation (29). However, there was also an increase in PCSK9 mRNA in the livers of the knockout mice, complicating the interpretation because the reduced level of LDLR in these mice could be simply due to increased expression of PCSK9 (29). Further evidence that the truncated PCSK9 is inactive was found in studies with a mutant form of PCSK9 with an enhanced furin-cleavage site. Overexpression of this mutant PCSK9 in the HEK293 cells resulted in complete cleavage of PCSK9 and did not
reduce LDLR levels in the cells (30). In contrast to these observations, Lipari et al. (31) concluded that a cleaved form of PCSK9 generated by in vitro cleavage of recombinant human PCSK9 with furin or hepsin retained most of its LDLR degradation activity. The PCSK9 which was generated in vitro had the same ~74 kDa mass as native PCSK9 and retained the 7.7 kDa N-terminal segment (31). We demonstrated unambiguously that the truncated PCSK9 isolated from serum of humans was largely devoid of the N-terminal segment. Accordingly, the cleaved form of PCSK9 reported by Lipari et al. (31), which retained LDLR degrading activity, is not representative of the truncated PCSK9 found in the circulation of humans. In our hands, limited retention of the N-terminal segment was observed after recombinant human PCSK9 was cleaved in vitro with furin in buffer solution (K. Schroeder, unpublished observations); however, cleavage of the PCSK9 with furin in the presence of mouse serum reproducibly resulted in complete dissociation of the N-terminal segment (Fig. 5). We evaluated an in vitro-cleaved PCSK9 whose composition closely resembled the cleaved PCSK9 isolated from human serum and found it inactive at degrading LDLR in HepG2 cells. The inactivity of truncated PCSK9 after dissociation of the N-terminal segment is consistent with the finding that the segment contains amino acids important for binding to LDLR (40–42). Based on our results and those of others (29, 30), we conclude that the circulating form of truncated PCSK9 is functionally inactive. While the reason for the discrepancy between our observations and those of Lipari et al. (31) is not clear, we speculate whether pyroglutamate formation of the nascent N terminus of the truncated PCSK9 plays a role in dissociation of the cleaved N-terminal segment.

Our confirmation that the truncated form of PCSK9 found in human serum is inactive has interesting implications for the efficacy of therapeutic antibodies targeting PCSK9. Considering that up to 42% of human PCSK9 circulates in the truncated form (supplementary Table IV), we propose that antibodies which bind both the truncated and intact forms of PCSK9, such as a C-terminal domain antibody reported previously (43), might be consumed unproductively by inactive antigen. Such antibodies may require higher doses to achieve efficacy compared with antibodies that recognize only intact PCSK9. This view is in contrast to the conclusions drawn by Lipari et al. (31) that neutralization of both the intact and truncated forms of PCSK9 is the more effective strategy for lowering LDL-C.

Our data demonstrate a modest positive correlation between PCSK9 and LDL-C in a set of 20 human serum samples, consistent with results obtained with larger sample sizes (33, 38). Our ELISA, like the others we have cited, does not discriminate intact from truncated PCSK9. Because the truncated PCSK9 is inactive as a regulator of LDLR, we asked whether the relationship with LDL-C is improved by comparison with intact PCSK9. The truncated form represented 30% of the total PCSK9 on the average in the serum samples evaluated, and the correlation with serum LDL-C was not improved by considering only the intact form of PCSK9. However, it remains to be determined if the correlation of intact PCSK9 and LDL-C would be stronger by an analysis of a larger, more diverse population including subjects with dyslipidemia.

In summary, we have characterized the composition and identified the cleavage site of the truncated form of PCSK9 found in human serum. In addition, we have shown that truncated PCSK9 does not lead to reduction in LDL-C uptake in a cell culture system. Therefore, we conclude that the truncated PCSK9 in circulation is inactive in vivo due to loss of the N-terminal segment which contains amino acids involved in LDLR binding and degradation.

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