Secreted PCSK9 downregulates low density lipoprotein receptor through receptor-mediated endocytosis

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Abstract Proprotein convertase subtilisin/kexin type 9 (PCSK9) is a protease that regulates low density lipoprotein receptor (LDLR) protein levels. The mechanisms of this action, however, remain to be defined. We show here that recombinant human PCSK9 expressed in HEK293 cells was readily secreted into the medium, with the prosegment associated with the C-terminal domain. Secreted PCSK9 mediated cell surface LDLR degradation in a concentration- and time-dependent manner when added to HEK293 cells. Accordingly, cellular LDL uptake was significantly reduced as well. When infused directly into C57B6 mice, purified human PCSK9 substantially reduced hepatic LDLR protein levels and resulted in increased plasma LDL cholesterol. When added to culture medium, fluorescently labeled PCSK9 was endocytosed and displayed endosomal-lysosomal intracellular localization in HepG2 cells, as was demonstrated by colocalization with DiI-LDL. PCSK9 endocytosis was mediated by LDLR as LDLR deficiency (hepatocytes from LDLR null mice), or RNA interference-mediated knockdown of LDLR markedly reduced PCSK9 endocytosis. In addition, RNA interference knockdown of the autosomal recessive hypercholesterolemia (ARH) gene product also significantly reduced PCSK9 endocytosis. Biochemical analysis revealed that the LDLR extracellular domain interacted directly with secreted PCSK9; thus, overexpression of the LDLR extracellular domain was able to attenuate the reduction of cell surface LDLR levels by secreted PCSK9.**JLR** Together, these results reveal that secreted PCSK9 retains biological activity, is able to bind directly to the LDLR extracellular domain, and undergoes LDLR-ARH-mediated endocytosis, leading to accelerated intracellular degradation of the LDLR.—Qian, Y-W., R. J. Schmidt, Y. Zhang, S. Chu, A. Lin, H. Wang, X. Wang, T. P. Beyer, W. R. Bensch, W. Li, M. E. Ehsani, D. Lu, R. J. Konrad, P. I. Eacho, D. E. Moller, S. K. Karathanasis, and G. Cao. **Secreted PCSK9 downregulates low density lipoprotein receptor through receptor-mediated endocytosis.** *J. Lipid Res.* 2007. **48:** 1488–1498.

Supplementary key words proprotein convertase subtilisin/kexin type 9

Increased plasma LDL cholesterol is a major risk factor for atherosclerotic cardiovascular disease. Importantly, recent studies have suggested further benefits of very aggressive LDL cholesterol lowering compared with the typical clinical targets in place today (1, 2). Plasma LDL cholesterol is controlled primarily by hepatic cholesterol biosynthesis and hepatic low density lipoprotein receptor (LDLR) levels (3). The master transcription factor controlling mRNA levels that encode key enzymes involved in cholesterol biosynthesis and LDLR is sterol-responsive element binding protein 2 (SREBP2). When cellular cholesterol levels are low, SREBP2 is activated through sequential proteolytic cleavage by two proteases. The activated N terminus of the protein then enters the cell nucleus to mediate the transcription of genes that contain sterol response element(s) in their promoter or enhancer region (4). Cellular cholesterol levels are thus tightly regulated through this feedback mechanism.

Recent human genetic studies have revealed that proprotein convertase subtilisin/kexin type 9 (PCSK9) is a critically important additional mechanism that regulates cellular LDLR levels. Although the molecular basis has yet to be determined, multiple mutations in the PCSK9 gene have been described to result in reduced cellular LDLR levels and thus significantly increased plasma LDL cholesterol (5–8). More importantly, opposite to these "gain-of-function" mutations, Cohen et al. (9–13) found apparent loss-of-function mutations that are presumed to lead to increased cellular LDLR protein levels. In humans carrying these mutations, plasma LDL cholesterol is reduced by 30–40% compared with controls. More strikingly, an apparently healthy human subject with mutations affecting both alleles of the PCSK9 gene was described with an LDL cholesterol level of 14 mg/dl. This finding further indicates a critical role for PCSK9 in modulating LDLR

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and plasma LDL cholesterol levels and provides evidence that loss of PCSK9 function in humans is not associated with apparent deleterious effects (12).

PCSK9 belongs to the proprotein convertase family and was only recently cloned from brain tissue as a secreted protein (14). The closest homolog is site 1 protease, another protein intimately involved in cholesterol homeostasis (15). Beyond the function implied by human genetic studies, the physiological function of PCSK9 was not clear until the recent demonstration that hepatic overexpression in mice greatly reduced hepatic LDLR levels and led directly to increased plasma LDL cholesterol (16, 17). Consistent with these observations, PCSK9 deficiency in mice resulted in significantly increased hepatic LDLR levels (18). Furthermore, PCSK9 deficiency augmented statin-induced increase of LDLR protein levels and thus strongly suggested the value of PCSK9 as a pharmacological target for LDL cholesterol lowering (18).

Although the function of PCSK9 in reducing hepatic LDLR protein levels is firmly established, the precise molecular basis for this effect has remained elusive. To date, the direct substrates and the active form of the enzyme are not known. In this report, we have investigated the potential molecular mechanisms of PCSK9 activity.

METHODS

Expression and purification of human recombinant PCSK9 protein

Human PCSK9 (accession number NM_174936) was cloned by PCR from human liver cDNA (BD Biosciences Co.) using Turbo pfu polymerase (Stratagene) and the following primers: 5′ primer, 5′-GCTCCTGAACTTCAGCTCCTGCACA-3′; 3′ primer, 5′-CTGAGAGAGGGACAAGTCGGAACCATTT-3′. The nucleotide sequences encoding full-length PCSK9 were inserted into a modified pJB02 vector with a C-terminal histidine (HIS) tag. The resulting construct was used to generate a HEK293 stable cell line overexpressing PCSK9. PCSK9 was purified from conditioned medium derived from this cell line by nickel-nitrilotriacetic acid agarose according to the manufacturer's instructions (Qiagen), followed by size-exclusion chromatography on a HiLoad 16/60 Superdex 200 column (Amersham) in the storage buffer (50 mM Tris, 150 mM NaCl, and 10% glycerol, pH 8.0). The final protein concentration was determined by Bradford assay using BSA as the standard. The identity of the purified PCSK9 protein was confirmed by N-terminal sequencing. The purified protein was stored at −80°C in small aliquots.

Expression and purification of the LDLR extracellular domain

Human LDLR (accession number g15680298) was cloned by PCR from human lung cDNA (BD Biosciences Co.) using Turbo pfu polymerase (Stratagene) and the following primers: 5′ primer, 5′-GCCTGGCAGAGGCTGCGAGCATG-3′; 3′ primer, 5′-TCACGCCACGTCATCCTCCAGACT-3′. The nucleotide sequences encoding the LDLR extracellular domain (amino acids 1–788) were inserted into modified pJB02 vector with a C-terminal Flag tag. The resulting construct was used to transfect 15 liters HEK293E suspension cells. Four days after the transfection, conditioned medium was collected and concentrated 10-fold for purification. The LDLR extracellular domain

was purified by anti-Flag M2 affinity chromatography according to the manufacturer's instructions (Sigma), followed by size-exclusion chromatography on a HiLoad 16/60 Superdex 200 column (Amersham) in the storage buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, and 0.01% Brij-35, pH 7.5). The final protein concentration was determined by Bradford assay using BSA as the standard. The identity of the purified protein was confirmed by N-terminal sequencing, which starts at amino acid 25. The purified LDLR (25–788)-Flag protein was stored at −80°C in small aliquots.

Western blot analysis

HEK293 cells were seeded at 1×10^6 cells/35 mm well and grown overnight in 3:1 DMEM/F12 + 10% FBS. After 24 h of growth, cells were washed in PBS, the medium was replaced with 3:1 DMEM/F12 + 5% lipoprotein deficient serum (LPDS), and PCSK9 was added at the indicated concentrations. After 18 h, the cells were washed twice in PBS without Mg/Ca^{2+}. The cells were scraped into buffer A (10 mM HEPES, pH 7.6, 1.5 mM MgCl, 10 mM KCl, 1 mM EDTA, 1 mM EGTA, and Complete protease inhibitor; Roche) and passed through a 221/2 gauge needle 15 times. The nuclei were pelleted by centrifugation at 1,000 g for 15 min at 4°C. The supernatant was then centrifuged at 55,000 rpm for 30 min at 4°C in a TL100 rotor to pellet cellular membranes. The membrane pellet was solubilized in 100 μl of buffer l (10 mM Tris, pH 7.6, 100 mM NaCl, and 1.0% SDS). Protein concentrations were determined by a BCA protein assay kit (Pierce). Ten micrograms of membrane proteins was denatured at 85°C for 4 min and separated on a Tris/Gly SDS gel (4–20%). Proteins were transferred to a 0.45 μm nitrocellulose membrane (Invitrogen) and blocked for 1 h at room temperature in Odyssey blocking buffer. The blot was then transferred to blocking buffer containing 0.1% Tween 20 + 0.1 μg/ml rabbit anti-human LDLR antibody (RDI-PRO-61099; Fitzgerald International) at 4°C overnight. The blot was washed in PBS + 0.1% Tween 20 (3 × 10 min) and placed in fresh blocking buffer containing Alexa Fluor 680 goat anti-rabbit IgG (0.3 ng/ml; Molecular Probes, Inc.). After 1 h, the blot was washed in PBS + 0.1% Tween 20 (3 × 10 min) and imaged using the Li-Cor infrared imaging system. For tissue samples, 50–100 mg of liver was homogenized in 1.0 ml of RIPA buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1.0% Nonidet P-40, 0.5% Na-deoxycholate, 0.1% SDS, and Complete protease inhibitors; Roche). The homogenate was rocked at 4°C for 20 min to solubilize membrane proteins. After centrifugation at 14,000 rpm to clear cellular debris, protein concentrations were determined using a Pierce BCA protein assay kit. After denaturation at 85°C for 4 min, 50 μg of total protein was separated on a 4–20% Tris/Gly SDS gel. Western blot analysis was performed as described above with chicken polyclonal antibody to LDLR (14056; Abcam) or rabbit anti-cyclophilin A (catalog No. 07-313; Upstate Laboratories) as primary antibodies and Alexa Fluor 680 goat anti-rabbit/chicken IgG (Molecular Probes) as secondary antibody.

Animals

Seven week old male C57B6 mice were purchased from Harlan (Indianapolis, IN) and acclimated for 1 week before the start of the study. The mice were provided Purina 5001 chow ad libitum throughout the experiment. Recombinant human PCSK9 was dosed daily by tail vein injection into restrained animals at various doses per animal for 3 or 7 consecutive days. The animals were euthanized at 6 h after the final injection by CO_2 asphyxiation. Blood samples for serum preparation were collected by cardiac puncture, and livers were collected and frozen in liquid nitrogen.

Mouse primary hepatocyte isolation

C57B6 or C57B6 LDLR KO (Taconic) mice were anesthetized with Na-pentobarbital (275 mg/kg). After dissection, a cannula was placed in the portal vein superior to the liver. A complete cut was made through the vena cava inferior to the liver for perfusate drainage. Liver perfusion medium (17701; Invitrogen) was infused into the liver for 10 min at a rate of 6.5 ml/min. Liver digestion medium containing collagenase (17703; Invitrogen) was then infused into the liver at a rate of 8.0 ml/min for 10 min. The liver was then removed from the mouse and placed in a 100 mm tissue culture dish containing liver digestion medium. The capsule surrounding the liver was dissected, and liver cells were removed. The hepatocytes were triturated to reduce clumping and filtered through a nylon mesh into a 50 ml centrifuge tube. After a 10 min spin at 50 g, the cells were resuspended in Williams E + 10% FBS (Invitrogen). Centrifugation was repeated, and the cells were again resuspended in 40 ml of Williams E. Cells were counted using trypan blue dye to determine viability. The hepatocytes were plated to 12-well collagen-coated plates (356500; Biocoat) at a density of 10,000 viable cells/well in Williams E + 10% FBS. After overnight incubation, the cells were used for uptake and imaging studies.

Imaging of LDL/PCSK9 uptake

Purified PCSK9 was labeled using the Alexa Fluor 488 Protein Labeling kit (catalog No. A-10235; Molecular Probes) according to the manufacturer's recommendations. LDL or PCSK9 endocytosis was studied with confocal imaging of fluorescently labeled LDL (DiI-LDL or BODIPY-LDL; Molecular Probes) or PCSK9-Alexa 488. Imaging experiments were performed on cells grown on 96-well plates. For live cell imaging, we changed culture medium with Ringer's buffer (containing in mM: 130 NaCl, 5 KCl, 2 CaCl$_2$, 1 MgSO$_4$, 20 HEPES, and 5.5 D-glucose, pH 7.4). For fixed cells, we used 3.7% formaldehyde in PBS for 30 min at 25°C, followed by three washes with PBS, and kept the cells in PBS at 100 μl/well. Cells were imaged with the Zeiss LSM 510 confocal imaging system outfitted with a 40× lens (numerical aperture 0.6). Laser excitation was at 543 nm and emission at 565–615 nm for LDL-DiI, or excitation was at 488 nm and emission at 505–550 nm for LDL-BODIPY or PCSK9-Alexa 488. Laser power, detector gain/offset, and pinhole size were fixed for all samples. To image the colocalization of LDL and PCSK9 uptake, we incubated cells to LDL-DiI/PCSK9-Alexa 488. The confocal settings were as mentioned above. To avoid fluorescent signal spillover between DiI and Alexa 488, we used the multi-track function in line scan mode of the LSM 510 with alternating 488 nm/505–550 nm (excitation/emission) and 543 nm/565–615 nm. Transmitted light images were collected simultaneously to identify cells during all confocal imaging experiments.

Quantification of LDL uptake with Acumen Explorer

Cells on 96-well plates with or without PCSK9 treatment were incubated with LDL-BODIPY. Nuclei were stained with propidium iodide. Plates were scanned with the Acumen Explorer (LabTech Limited), excited by 488 nm laser and measuring emission at 500–530 nm (LDL-BODIPY uptake) or 575–640 nm (nucleus). Total fluorescence of LDL-BODIPY per objector or per well was obtained for quantification.

RNA interference knockdown of LDLR and autosomal recessive hypercholesterolemia

Lentivirus short hairpin RNA (shRNA) transduction particles were obtained from Sigma-Aldrich Corp. Knockdown for both LDLR and autosomal recessive hypercholesterolemia (ARH) genes was performed using three different lentiviral transduction particles. The sequences for LDLR and ARH are as follows: for LDLR, 5′-CCGGCCAGCGAAGATGCGAAGATATCTCGAGATATCTTCG-CATCTTCGCTGGTTTTTG-3′, 5′-CCGGGCCGTCTTTGAGGAC-AAAGTACTCGAGTACTTTGTCCTCAAAGACGGCTTTTTG-3′, and 5′-CCGGCGGGAAATGCATCTCCTACAACTCG-AGTTGTAGGAGATGCATTTCCCGTTTTTG-3′; for ARH, 5′-CCGGCGACAAGGTGTTTGCATACATCTCGAGATGTATGCAA-ACACCTTGTCGTTTTTG-3′, 5′-CCGGGTCCATATACAGGAT-CTCCTACTCGAGTAGGAGATCCTGTATATGGACTTTTTG-3′, and 5′-CCGGGAGAAAGAGAAGAGGGACAAACTCGAGTTT-GTCCCTCTTCTCTTTCTCTTTTTG-3′. A 96-well dish was seeded at 3,000–5,000 cells/well and incubated at 37°C overnight in complete medium. At the time of transfections, hexadimethrine was added to a final concentration of 8 μg/ml. Virus particles were added to each well at a multiplicity of infection of 10 plaque-forming units/cell. The cells were exposed to virus for 6 h and washed with PBS. Fresh growth medium containing 10% FBS was then added, and incubation was continued for 72 h. Cells were then used for uptake studies, or RNA was prepared to determine the effect of lentiviral expression on LDLR or ARH levels. RNA was isolated for Taqman analysis in an ABI-6100 Nucleic Acid PrepStation. The effect of lentivirus RNA interference on LDLR and ARH levels was monitored by Taqman analysis with primer pairs for LDLR and ARH commercially supplied by ABI (HS00181192-ml and HS00296701-ml). For uptake studies, the medium was changed to 100 μl of fresh 5% LPDS in DMEM/F12 (3:1) with or without 10 μg/ml PCSK9, and cells were incubated at 37°C and 5% CO$_2$ overnight. Alexa Fluor 488-labeled PCSK9 (100 ng/ml) and DiI-LDL (1 μg/ml) were added to the cells, and after a 4 h incubation at 5% CO$_2$ and 37°C, cells were washed with PBS. Uptake of Alexa Fluor 488-labeled PCSK9 and/or DiI-LDL was analyzed using confocal fluorescence microscopy.

Coimmunoprecipitation of PCSK9 and the LDLR extracellular domain

Ten micrograms of LDLR (25–788)-Flag was mixed with either 10 μg of PCSK9-HIS or control protein glutathione-S-transferase (GST)-HIS in 50 μl of binding buffer (50 mM Tris, 150 mM NaCl, 10% glycerol, and 0.01% Brij-35, pH 7.5) on ice for 1 h. The mixture was then incubated with 50 μl of anti-Flag M2 resin in 0.5 ml of binding buffer containing 1 mg/ml BSA at 4°C for 1 h. The resin was washed with 1 ml of binding buffer six times. The bound proteins were eluted with 100 μl of binding buffer containing 0.1 mg/ml Flag peptide and analyzed by immunoblot with either anti-PCSK9 antibody (Cayman Chemical Co.) or anti-GST antibody (Santa Cruz Biotechnology).

LDLR detection by flow cytometry

HepG2 cells (1×10^6) were added into 5 ml polystyrene round-bottom tubes with 3 ml of 0.1% BSA/PBS. Cells were pelleted and resuspended with 100 μl of 0.1% BSA/PBS containing 500 ng of mouse anti-bovine LDLR monoclonal antibody (clone IgG-C7; Progen Biotechnik GmbH) and incubated on ice for 1 h. Three milliliters of 0.1% BSA/PBS was then added, and the cells were pelleted again. This was followed by resuspending the cells with 100 μl of 0.1% BSA/PBS containing 200 ng of Alexa Fluor 488 goat anti-mouse IgG antibody (Molecular Probes). After incubation on ice for 1 h, cells were washed again with 3 ml of 0.1% BSA/PBS. Cells were then resuspended with 600 μl of 0.1% BSA/PBS and analyzed using flow cytometry (FACSCalibur, CellQuest Pro; Becton

Dickinson). Mouse IgG2b isotype (catalog No. 02-6300; Invitrogen) was used as the control.

RESULTS

Secreted PCSK9 reduces cellular LDLR protein levels

As a first step toward understanding how PCSK9 functions to reduce LDLR levels, we generated a HEK293 stable cell line that overexpressed human PCSK9. We engineered a six-HIS tag at the C terminus of the protein for purification purposes. Secreted PCSK9 in the medium was readily purified, and the protein was assessed to be >95% pure as judged by Coomassie blue staining (**Fig. 1**). N-terminal sequencing revealed the zymogen cleavage of the prosegment at the junction of glutamine and serine, consistent with the report by Benjannet et al. (19). The prosegment was copurified with the C-terminal domain, as revealed by the 14 kDa band on the denaturing gel (Fig. 1). To address whether secreted PCSK9 reduces cellular LDLR protein levels, we transferred the medium from PCSK9-overexpressing cells to control HEK293 cells expressing endogenous LDLR. As depicted in **Fig. 2A**, Western blot analysis of cellular LDLR revealed that LDLR protein levels (160 kDa mature form) were reduced dramatically after an overnight incubation, indicating that secreted PCSK9 was functional in degrading LDLR (lane 3). To confirm our hypothesis, purified PCSK9 was added in the medium to control cells at 5 or 10 μg/ml, and a similar level of LDLR reduction was observed (Fig. 2A, lanes 4–6). Detailed titration of PCSK9 suggested an estimated EC_{50} of ∼2 μg/ml for this effect of purified PCSK9 protein to reduce LDLR protein (Fig. 2B).
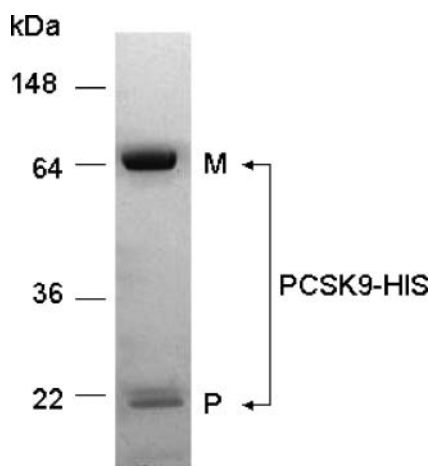

Fig. 1. Expression and purification of recombinant human proprotein convertase subtilisin/kexin type 9 (PCSK9). Full-length human PCSK9 was expressed in HEK293 cells, and secreted proteins were purified as described in Methods. Two micrograms of purified PCSK9-HIS (for histidine) was analyzed by SDS-PAGE and stained with SimplyBlue SafeStain (Invitrogen). M and P denote the mature PCSK9 (amino acids 153–692) and the prodomain (amino acids 29–152), respectively.

The reduction of LDLR by purified PCSK9 was time-dependent and required at least 6 h, and the overnight incubation of 2 μg/ml purified PCSK9 appeared sufficient to generate maximum LDLR reduction (Fig. 2C). However, using HepG2 cells, 10 μg/ml PCSK9 significantly reduced cell surface LDLR within 1 h, as assessed by fluorescent cell sorting (data not shown). As expected, the uptake of fluorescently labeled DiI-LDL was also reduced significantly (Fig. 2D) after PCSK9 treatment of the control HEK293 cells. Concentration titration experiments indicated that ∼10 μg/ml PCSK9 virtually completely abolished DiI-LDL uptake, with a calculated EC_{50} of 0.8 μg/ml in this experimental paradigm (Fig. 2E), consistent with the LDLR Western blot data. These results indicated that secreted PCSK9 was functional in degrading LDLR in cultured cells, even though its prosegment was tightly associated with the C-terminal domain.

Secreted PCSK9 reduces hepatic LDLR protein and increases plasma LDL cholesterol

To examine whether the secreted PCSK9 was functional in vivo, we administered 30 μg of protein daily by intravenous infusion to individual C57B6 mice for 7 days and examined their hepatic LDLR and plasma LDL levels. As shown in **Fig. 3A**, LDLR protein levels were reduced significantly after PCSK9 infusion (45%), and accordingly, mean plasma LDL was increased by 52% as assessed by fast-protein liquid chromatography analysis (Fig. 3B). Fasting versus feeding did not appear to have a significant impact on LDLR levels (Fig. 3A). The extent of LDL increase at 4 days after the initiation of daily PCSK9 infusion was similar to that of LDL cholesterol increase at day 7 (data not shown). Thus, in subsequent experiments, we treated the mice for 3 days to determine the in vivo PCSK9 dose-response relationship using doses of 3–300 μg/mouse. Observed dose-dependent increases in plasma LDL were accompanied by concomitant reductions in hepatic LDLR levels (Fig. 3C, D). Together, these data indicated that secreted PCSK9 reduced hepatic LDLR in vivo and that this effect was tightly coupled to increased plasma LDL cholesterol.

Secreted PCSK9 undergoes endocytosis into endosome/lysosome cellular compartments

Our results suggested that secreted recombinant PCSK9 has potent biological activities to reduce LDLR protein levels in vitro and in vivo. Because of the possibility that PCSK9 could associate with both LDL and the LDLR, we reasoned that PCSK9 might traffic together with LDLR into cellular endosome/lysosome compartments, where LDLR may be degraded. Recombinant PCSK9 was fluorescently labeled and added to HepG2 cells. DiI-LDL was also added to the same cultured cells. Two and 4 h later, endocytosis of fluorescently labeled PCSK9 and DiI-LDL was visualized using a confocal microscope. Punctate vesicle patterns were clearly observed with labeled PCSK9, indicating that secreted PCSK9 had been endocytosed into endosome/lysosome compartments. It was further
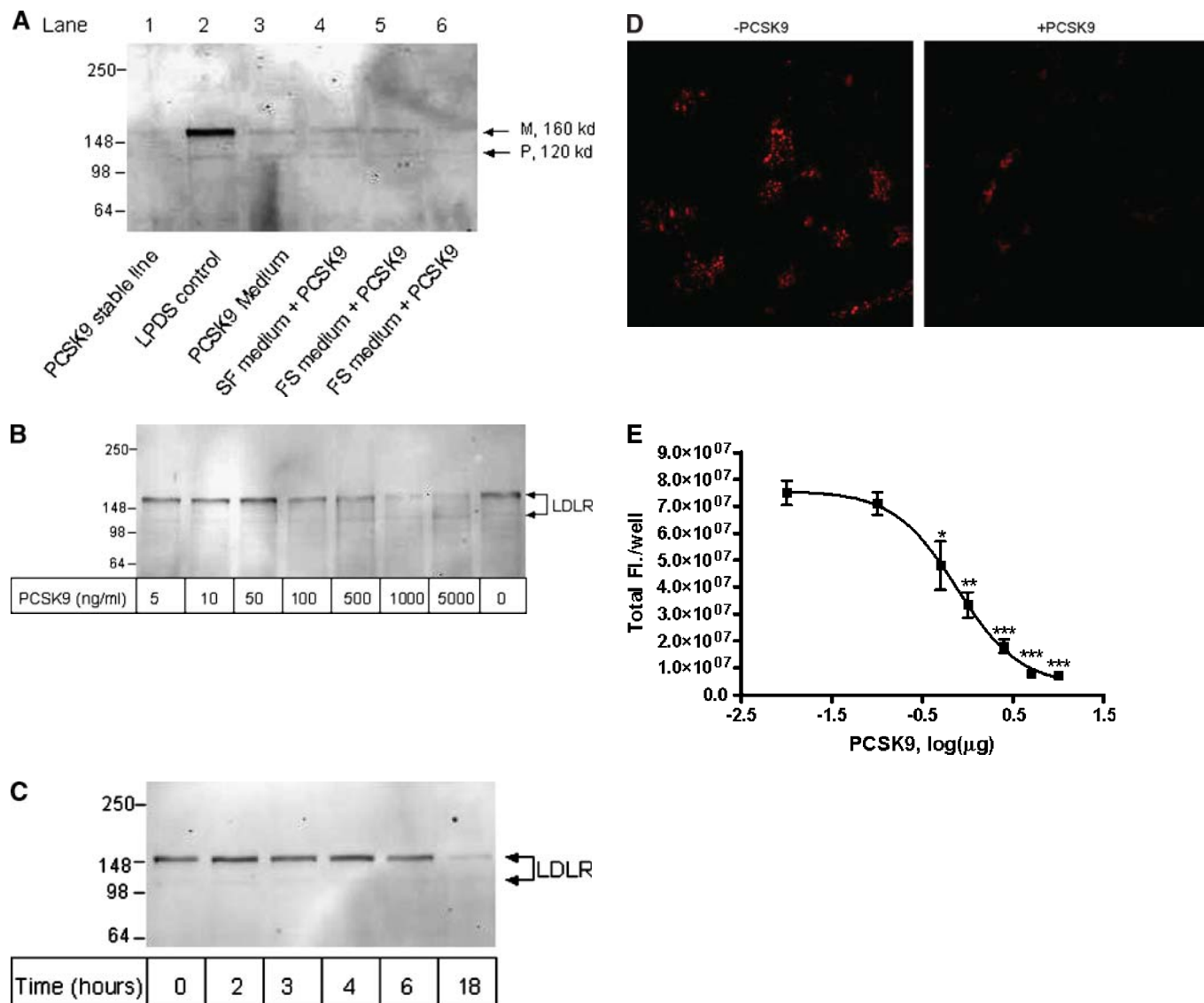
Fig. 2. Reduction of cellular low density lipoprotein receptor (LDLR) by secreted recombinant PCSK9. A: Secreted PCSK9 is fully active in reducing cellular LDLR levels. Extracts from HEK293 cells that were treated overnight as described below were obtained, and cellular LDLR levels were monitored by Western blot analysis as described in Methods. Cells cultured in LPDS medium overnight were used as a control (lane 2). Lane 1 depicts LDLR protein levels in the HEK293 stable line overexpressing PCSK9, and lane 3 shows LDLR levels from cells exposed to the medium from these PCSK9-overexpressing cells after washing. Purified PCSK9 (10 μg/ml) was added to serum-free (SF) medium (lane 4), or 5 or 10 μg/ml PCSK9 was added to full-serum (FS) medium (lanes 5, 6) for overnight incubation with HEK293 control cells. B: Concentration-dependent reduction of cellular LDLR in HEK293 cells. HEK293 cells were cultured in lipoprotein deficient serum (LPDS) as a control, and various amounts of PCSK9 were added to cells. After overnight incubation, cellular LDLR levels were analyzed by Western blot as described in Methods. C: Time-dependent reduction of cellular LDLR in HEK293 cells. HEK293 cells were incubated with 2 μg/ml purified PCSK9 for the indicated times, and cellular LDLR levels were assessed by Western blot analysis as described. D: PCSK9 reduces cellular LDL uptake. DiI-LDL was added to HEK293 cells after overnight incubation with 10 μg/ml PCSK9 followed by washing, and uptake was analyzed by confocal microscopy as described in Methods. E: Concentration-dependent reduction in cellular LDL uptake. HEK293 cells were incubated with various amounts of purified PCSK9 overnight. DiI-LDL was then added to the medium after washing, and LDL uptake was quantified as described in Methods. *** $P < 0.001$, ** $P < 0.01$, * $P < 0.05$ versus no-PCSK9 control.

confirmed that endocytosed PCSK9 was colocalized with DiI-LDL (**Fig. 4**).

PCSK9 endocytosis requires LDLR and ARH

To examine the molecular components required for cellular PCSK9 endocytosis, we first examined whether LDLR was required for this to occur using primary hepatocytes from LDLR-deficient mice. Alexa 488-labeled PCSK9 readily underwent endocytosis into cellular endosome compartments in wild-type primary mouse hepatocytes, whereas LDLR-deficient primary hepatocytes did not display any significant PCSK9 uptake (**Fig. 5A**). We
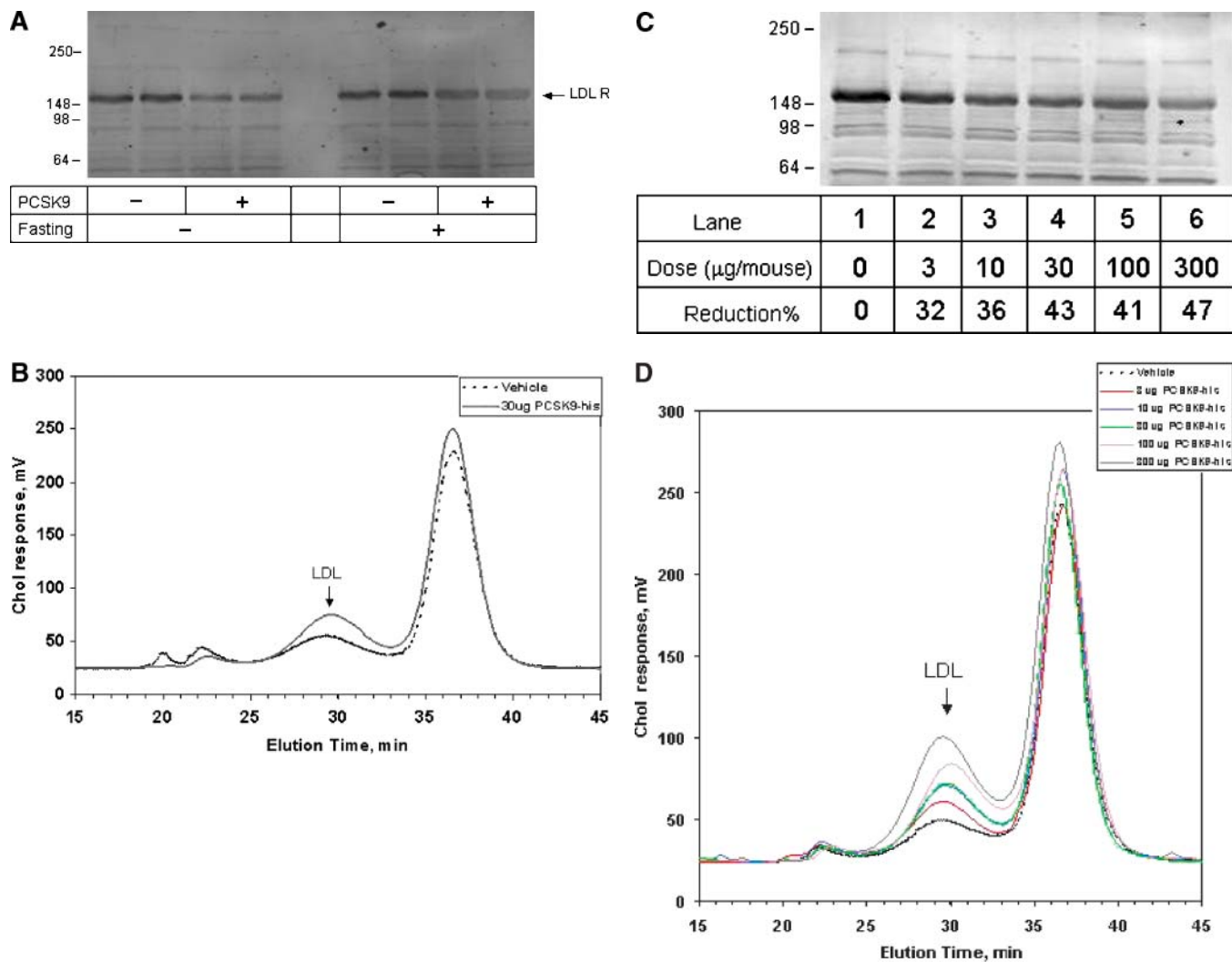
Fig. 3. PCSK9 recombinant protein reduces hepatic LDLR and increases plasma LDL cholesterol in vivo. A: Hepatic LDLR reduction by purified PCSK9 in C57B6 mice. Purified PCSK9 or vehicle was infused into C57B6 mice (10 animals each) once daily for 6 days. Five animals each were then separated into nonfasting and fasting groups for 18 h, followed by the last injection of PCSK9 or vehicle. Six hours later, liver samples (two each from each group) were processed, and LDLR protein levels were analyzed by Western blot analysis as described in Methods. B: PCSK9 increased plasma LDL cholesterol in C57B6 mice. Plasma samples from each group were pooled and analyzed by fast-protein liquid chromatography, and the increase in LDL cholesterol was quantified as described (22). C: Dose-dependent reduction of liver LDLR in mice by recombinant PCSK9. Various doses ranging from 3 to 300 μg/mouse were administered daily for 3 days to C57B6 mice (six animals per group), and hepatic LDLR proteins from pooled liver samples of each group were assessed as in A. LDLc, LDL cholesterol. D: Dose-dependent increase of plasma LDL cholesterol in mice. Pooled plasma samples from mice treated as described for C were analyzed to determine plasma LDL as described for B.

then used interfering RNA technology to reduce mRNA levels of either LDLR or the ARH protein. Lentivirus vectors designed to express interfering RNA for either LDLR or ARH resulted in reductions of ∼80% for each mRNA when measured at 72 h after the virus was added to cultured HepG2 cells (data not shown). Fluorescently labeled PCSK9 or DiI-LDL was added, and 2 h later, PCSK9 and DiI-LDL endocytosis was examined. With significantly reduced LDLR or ARH gene expression, both PCSK9 and DiI-LDL endocytosis were reduced dramatically, indicating that both LDLR and ARH are essential components of the PCSK9 endocytotic pathway (Fig. 5B).

PCSK9 interacts with the LDLR extracellular domain

The fact that PCSK9 undergoes receptor-mediated endocytosis suggests the possibility that the two proteins may interact directly with each other. To explore this possibility, we expressed the LDLR extracellular domain in a secreted form (without the transmembrane domain and the C terminus) and purified the protein (**Fig. 6A**). After incubation with recombinant PCSK9, LDLR was immunoprecipitated with an antibody against the Flag tag, which was engineered at the C terminus of the protein. Western blot analysis using a PCSK9 antibody clearly indicated that PCSK9 was coimmunoprecipitated, suggest-
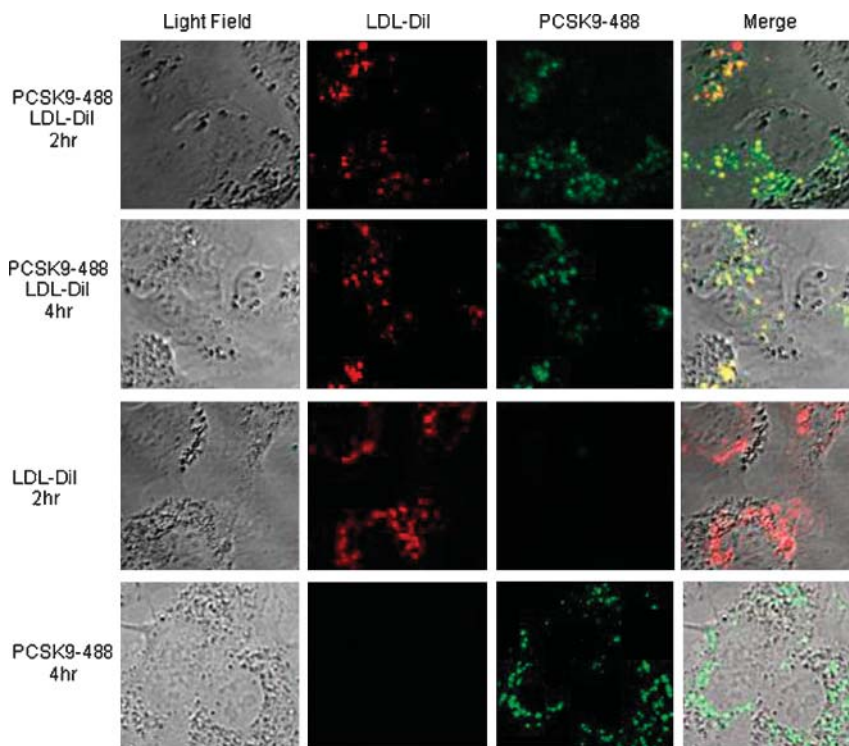
Fig. 4. Endocytosis of secreted PCSK9 into endosomal/lysosomal compartments colocalized with DiI-LDL. HepG2 cells were seeded onto 96-well plates on day 0, and on day 1, the medium was changed to LPDS followed by overnight incubation. On day 2, the cells were washed, and labeled PCSK9 and/or DiI-LDL was added either simultaneously or separately. Two or 4 h later, labeled PCSK9 and/or DiI-LDL uptake was examined with a confocal microscope and documented as described in Methods.

ing that PCSK9 can bind directly to the LDLR extracellular domain (Fig. 6B). No PCSK9 was detected when the LDLR extracellular domain protein was omitted in the incubation (Fig. 6B, lane 2). To further confirm the specificity of this observed binding, we added GST protein containing a six-HIS tag as a control; after immunoprecipitation with the Flag antibody, a GST antibody was used for Western blot analysis. No signal was detected, whereas the protein was clearly detected in the lanes to which GST was loaded directly. These findings support the concept that PCSK9 binds to the LDLR extracellular domain specifically (Fig. 6B, lanes 3–5).

Overexpression of the LDLR extracellular domain attenuates PCSK9 function

Based upon the apparent ability of PCSK9 to bind directly to the LDLR extracellular domain described above, we reasoned that cellular overexpression of the LDLR extracellular domain protein, which is readily secreted into cell culture medium, should compromise the function of PCSK9 by binding to it and preventing its access to endogenous LDLR and its ability to undergo endocytosis to mediate enhanced intracellular LDLR degradation. In HepG2 cells, human recombinant PCSK9 reduced cell surface LDLR protein to almost background levels, as assessed by fluorescent cell sorting. Expression of the LDLR extracellular domain resulted in an ∼50% increase

of cell surface LDLR, suggesting that PCSK9 function was substantially attenuated (Fig. 6C). The presence of excessive LDLR extracellular domain protein increased endogenous LDLR levels at multiple PCSK9 concentrations, further indicating that the interaction of PCSK9 with cell surface LDLR is critical for its function in reducing LDLR protein levels.

DISCUSSION

In this report, we have demonstrated that *1*) secreted recombinant PCSK9 is fully functional in reducing LDLR protein levels both in cultured cells and in vivo; *2*) PCSK9 undergoes LDLR-mediated endocytosis; and *3*) PCSK9 binding to LDLR is critical for PCSK9 function.

Although it has been amply demonstrated that PCSK9 mutations are associated with altered plasma LDL cholesterol levels, the molecular mechanism of PCSK9's action has not been fully defined. The direct substrates have yet to be identified, and the active enzymatic form of the protein has yet to be delineated. To date, the only apparent enzymatic substrate is PCSK9 itself. Thus, site-directed mutations of active site amino acids abolished its catalytic activity; accordingly, the prodomain cannot be cleaved. Failure to cleave the prodomain results in a protein that cannot exit the endoplasmic reticulum and thus
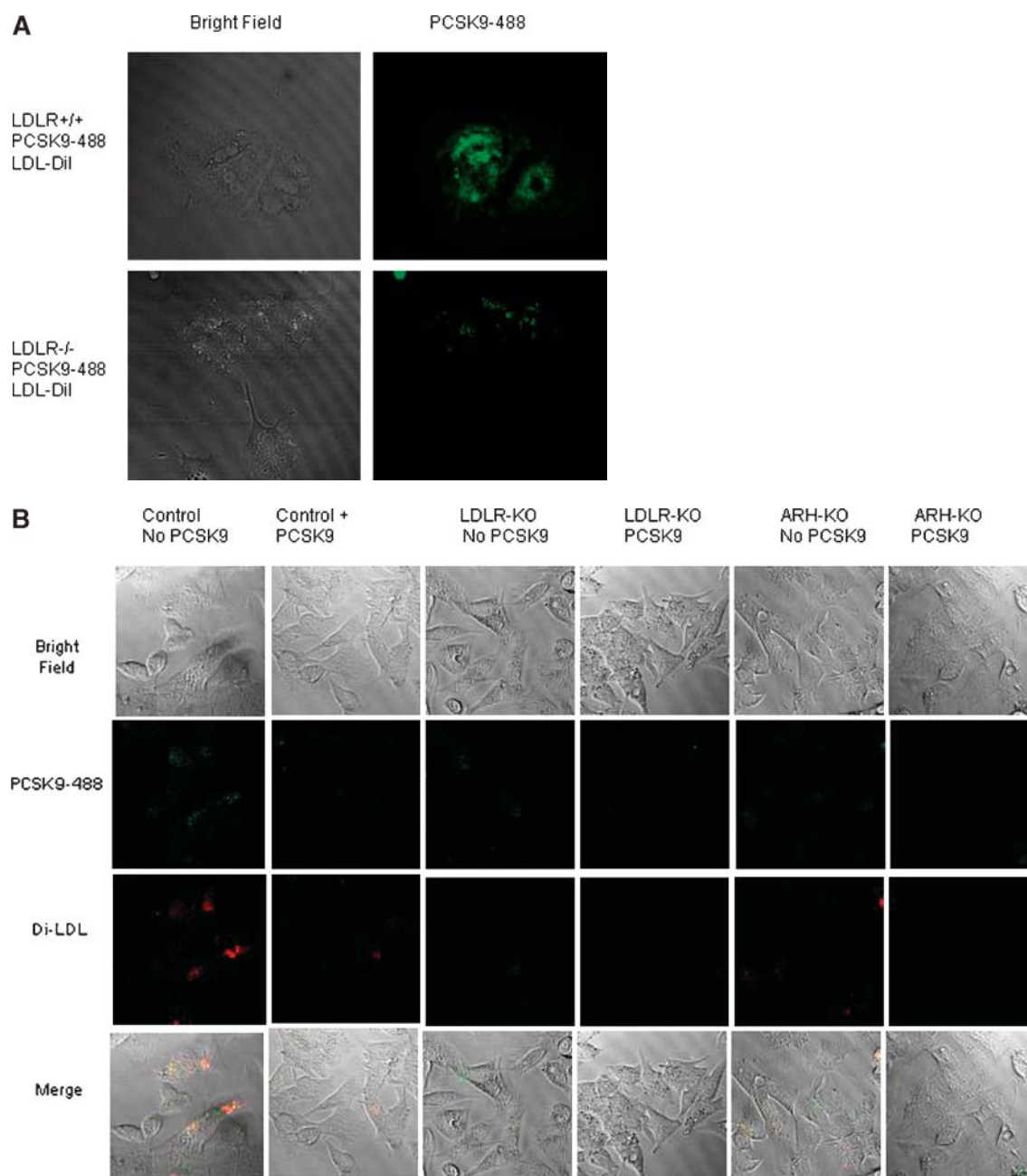
Fig. 5. LDLR-dependent endocytosis of PCSK9. A: PCSK9 endocytosis requires LDLR. Mouse primary hepatocytes were prepared from either wild-type (LDLR$^{+/+}$) or LDLR-deficient (LDLR$^{-/-}$) mice as described in Methods and allowed to grow in medium containing LPDS overnight. Alexa 488-labeled PCSK9 was then added to the culture medium, and its uptake was monitored at 2 h after PCSK9 addition. B: Reduction of LDLR and autosomal recessive hypercholesterolemia (ARH) gene expression through RNA interference knockdown (KD) reduces PCSK9 and LDL endocytosis. HepG2 cells were seeded onto 96-well plates, lentivirus encoding RNA interference for the LDLR or ARH or control lentivirus was then added, and RNA interference knockdown was allowed to continue for 72 h. This was followed by an overnight culture in LPDS with or without PCSK9 incubation in the medium. Cells were washed, and labeled PCSK9 or DiI-LDL uptake was assessed using a confocal microscope.

is unable to reduce cellular LDLR (19). Although the prodomain is cleaved, it remains associated with the rest of the catalytic domain. We initially showed that recombinant human PCSK9 protein expressed in HEK293 cells can effectively degrade LDLR in both HEK293 cells and HepG2 cells. The amount of protein required to maximally degrade LDLR was 5–10 μg/ml, and the EC$_{50}$ was ∼1–2 μg/ml.

The time course for effective LDLR reduction appeared to be >6 h in HEK293 cells, and overnight incubation achieved maximal effects. However, the effect of PCSK9 appeared to be much faster in HepG2 cells. The reduction of LDLR is limited to the mature form of LDLR, as revealed by Western blot analysis and cell surface LDLR analysis by fluorescent cell sorting as well. The reduction of mature
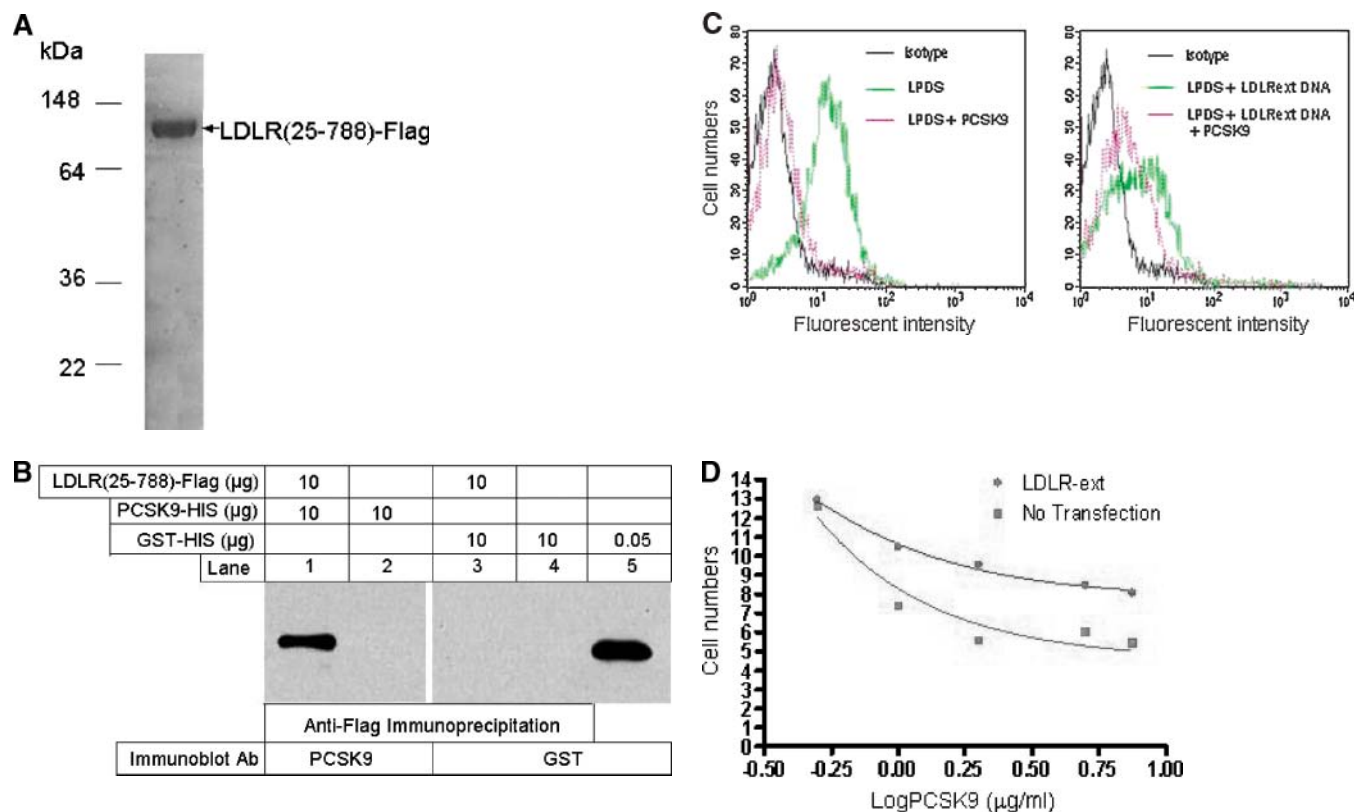
Fig. 6. Direct interaction of PCSK9 with LDLR. A: Purified human LDLR extracellular domain. The human LDLR extracellular domain was expressed in HEK293 cells and purified as described in Methods. Two micrograms of purified recombinant LDLR (25–788)-Flag was analyzed by SDS-PAGE and stained with SimplyBlue SafeStain (Invitrogen). B: PCSK9 binds to the LDLR extracellular domain. Ten micrograms each of purified PCSK9 and the Flag-tagged LDLR extracellular domain were coincubated and immunoprecipitated with a Flag tag antibody (Ab). The eluate was analyzed by Western blot using a PCSK9 antibody (lanes 1, 2). Glutathione-*S*-transferase (GST)-HIS was used as a negative control (lanes 3, 4), and GST-HIS recombinant protein was loaded onto lane 5 as a control for GST antibody. C: Cellular overexpression of the LDLR extracellular domain attenuates PCSK9 function. HEK293 cells were seeded onto a 24-well plate on day 0, and plasmids containing cDNA encoding the LDLR extracellular domain under the control of the cytomegalovirus promoter or empty vector control plasmids were transfected into the cells on day 1. PCSK9 protein was then added in medium containing LPDS at 4 h after transfection. Cell surface LDLR was then assessed on day 2 by fluorescent cell sorting as described in Methods. The left panel shows a marked PCSK9-dependent shift in LDLR-positive cells; in cells overexpressing the LDLR extracellular domain, the PCSK9-dependent shift is attenuated. D: Concentration-dependent effects of PCSK9 with or without LDLR extracellular domain overexpression.

LDLR is also consistent with our subsequent discovery that PCSK9 function requires LDLR-mediated endocytosis, because only mature LDLR is accessible to secreted PCSK9. These findings are consistent with the most recent reports by Cameron et al. (8) and Lagace et al. (20). Secreted PCSK9 was also fully functional in vivo; when infused directly into mice, it reduced hepatic LDLR protein levels and increased plasma LDL cholesterol levels, as expected. However, in attempting to measure the proteolytic activity of secreted PCSK9, we were unable to detect any enzymatic activity under a variety of conditions (data not shown). Therefore, we focused our subsequent studies on an alternative hypothesis involving a role for PCSK9 in mediating accelerated LDLR endocytosis and/or subsequently accelerated intracellular degradation.

The amount of PCSK9 in human plasma ranges from 50 to 600 ng/ml (20), and Zhao et al. (12) detected secreted PCSK9 in plasma through immunoprecipitation. It is not known at present whether PCSK9 is associated with plasma LDL particles or exists in a free form in the plasma environment. We reasoned that PCSK9, either in its free form or associated with LDL, may interact with cell surface LDLRs, resulting in the degradation of LDLRs either at the cell surface or in a different cellular compartment. Indeed, when fluorescently labeled PCSK9 was added into the medium, PCSK9 readily entered the cells and displayed an endosome-lysosome localization, which colocalized with DiI-LDL. Further assessment of this process with hepatocytes from LDLR-deficient mice or with knockdown of either LDLR or ARH strongly suggested that cellular internalization and intracellular targeting of PCSK9 were mediated through the LDLR-ARH-mediated endocytosis pathway. These results are entirely consistent with a recent report that PCSK9-mediated LDLR degradation occurs in a post-endoplasmic reticulum compartment that is pH-dependent (21).
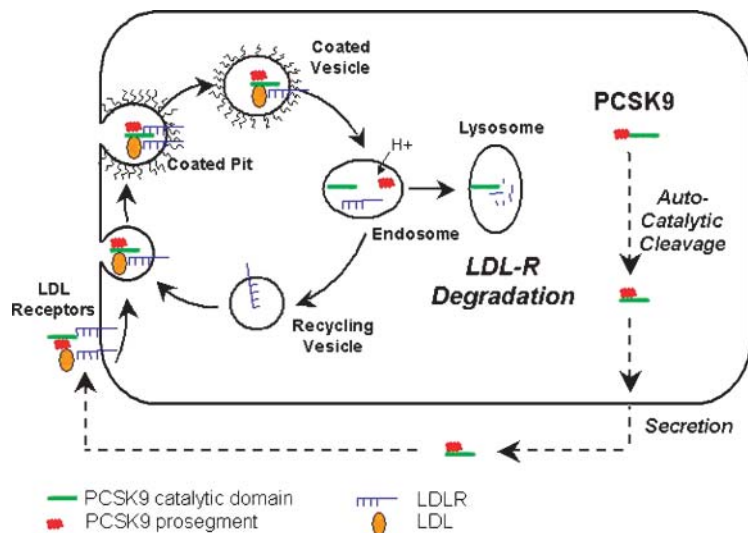
Fig. 7. A working model of PCSK9 action.

Because the initial contact of secreted PCSK9 with the LDLR is presumed to occur via the LDLR extracellular domain, we further explored this potential interaction of the two proteins. Through coimmunoprecipation experiments, specific binding of secreted PCSK9 to the LDLR extracellular domain was confirmed. Elucidation of the exact protein structural domains involved in this interaction will require further study. In additional experiments, we were able to characterize the critical role of this direct interaction by showing that disrupting PCSK9 binding to cell surface LDLR through overexpression of the LDLR extracellular domain was sufficient to markedly attenuate PCSK9 function. Thus, PCSK9 binding to the LDLR extracellular domain is an important step in the process by which PCSK9 acts to downregulate LDLR protein levels.

Based on our data, we propose a model (**Fig. 7**) in which prodomain-associated PCSK9 is secreted through the endoplasmic reticulum and other cellular compartments into the plasma environment, where it either exists in free form or may become associated with plasma LDL. PCSK9 binding to LDLR and the ensuing LDLR- and ARH-mediated endocytosis deliver both LDLR and PCSK9 to endosomal/lysosomal compartments. In this environment, we speculate that the prodomain may dissociate from the catalytic domain, potentially resulting in direct cleavage of the LDLR. Alternatively, PCSK9 may facilitate the accelerated degradation of LDLR within endosomes/lysosomes via other processes. Our data, along with the most recent findings reported by Horton and colleagues (20), which were published during the preparation of this article, strongly support such a hypothesis.

In summary, our results have shed additional light on the molecular pathway through which PCSK9 exerts its function to reduce cell surface LDLR levels and thus to control plasma LDL cholesterol levels. Further exploration of these mechanisms may provide insights to the active form and the direct substrates of PCSK9. Moreover, these findings provide a framework for the development of novel assays that could be used as a means to pursue potential therapeutic approaches. Drugs that act to impede the specific functions of PCSK9 could be developed as a likely way to further decrease LDL and to reduce atherosclerotic cardiovascular disease risk.█

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