Binding of Proprotein Convertase Subtilisin/Kexin Type 9 to Epidermal Growth Factor-like Repeat A of Low Density Lipoprotein Receptor Decreases Receptor Recycling and Increases Degradation*§

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Proprotein convertase subtilisin/kexin type 9 (PCSK9) promotes degradation of hepatic low density lipoprotein receptors (LDLR), the major route of clearance of circulating cholesterol. Gain-of-function mutations in PCSK9 cause hypercholesterolemia and premature atherosclerosis, whereas loss-of-function mutations result in hypcholesterolemia and protection from heart disease. Recombinant human PCSK9 binds the LDLR on the surface of cultured hepatocytes and promotes degradation of the receptor after internalization. Here we localized the site of binding of PCSK9 within the extracellular domain of the LDLR and determined the fate of the receptor after PCSK9 binding. Recombinant human PCSK9 interacted in a sequence-specific manner with the first epidermal growth factor-like repeat (EGF-A) in the EGF homology domain of the human LDLR. Similar binding specificity was observed between PCSK9 and purified EGF-A. Binding to EGF-A was calcium-dependent and increased dramatically with reduction in pH from 7 to 5.2. The addition of PCSK9, but not heat-inactivated PCSK9, to the medium of cultured hepatocytes resulted in redistribution of the receptor from the plasma membrane to lysosomes. These data are consistent with a model in which PCSK9 binding to EGF-A interferes with an acid-dependent conformational change required for receptor recycling. As a consequence, the LDLR is rerouted from the endosome to the lysosome where it is degraded.

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§The abbreviations used are: PCSK9, proprotein convertase subtilisin/kexin type 9; EGF, epidermal growth factor; LDL, low density lipoprotein; LDLR, low density lipoprotein receptor; NCLPPS, newborn calf lipoprotein-poor serum; VLDL, very low density lipoprotein; ApoB, apolipoprotein B; ApoE, apolipoprotein E; VLDLR, very low density lipoprotein receptor; PBS, phosphate-buffered saline; DMEM, Dulbecco's modified Eagle's medium; GST, glutathione S-transferase; LRPI, LDL receptor-related protein.
is followed by the so-called epidermal growth factor (EGF) precursor homology domain (18–20). The EGF precursor domain contains two cysteine-rich EGF-like domains (EGF-A and EGF-B) separated from a third EGF repeat (EGF-C) by a β-propeller domain (21, 22). The EGF precursor homology domain is required for the LDLR to dissociate from lipoproteins in the endosome after the complex is internalized (23). Immediately downstream of the EGF precursor homology domain is a threonine- and serine-rich region, to which multiple O-linked sugars are attached, which is followed by the transmembrane domain and a relatively short cytoplasmic tail that contains all the sequences required for receptor clustering in clathrin-coated pits and for internalization of the receptor (24, 25).

In 2002, Rudenko et al. (26) crystallized the extracellular domain of the receptor at an acidic pH, providing new insights into the mechanism by which the LDLR delivers cholesterol to cells without being degraded in the process. At neutral pH, the ligand-binding repeats are predicted to be extended away from the EGF precursor homology domain and accessible to lipoproteins. When the pH falls, as occurs in the endosome, the ligand-binding domain forms a physical association with the EGF precursor homology domain. The acid-dependent conformational change in the LDLR releases the lipoprotein from the ligand-binding domain and signals the receptor to return to the cell surface.

As a first step in determining how PCSK9 binding to the extracellular domain of the LDLR results in receptor degradation, we mapped the site in the LDLR that interacts with PCSK9. We provide evidence that a direct interaction between PCSK9 and the EGF-A repeat in the LDLR interferes with the recycling of the receptor protein from the endosome to the cell surface, resulting in the receptors being rerouted to the lysosome for degradation.

EXPERIMENTAL PROCEDURES

Materials—The culture medium and fetal bovine serum were obtained from Meditech, Inc. (Herndon, VA). Complete EDTA-free protease inhibitors were purchased from Roche Applied Science; Nonidet P-40 was purchased from Calbiochem (La Jolla, CA). All other chemicals and reagents were obtained from Sigma-Aldrich unless otherwise indicated.

Site-directed Mutagenesis—A recombinant expression vector containing the full-length LDLR cDNA linked to the SV40 early promoter (pLDLR2) (19) or the coding region of the receptor cDNA driven by the human cytomegalovirus immediate early region promoter (pLDLR17) was used to generate the mutant forms of the LDLR, including deletions in the ligand-binding domain (27), the EGF precursor homology domain (23), and the clustered O-linked sugar domain (28). The VLDL receptor (VLDLR) expression construct (pVLDLR) contained one copy of a hemagglutinin epitope tag (CYPYDVPDTag) (29) at the C terminus. Mutations in EGF-A were generated using pLDLR17 or pVLDLR and the QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions. The sequences of the oligonucleotides containing the residues to be mutated (underlined) were synthesized by IDT, Inc. (Coralville, IA) and are provided in supplemental Table S1. The presence of the desired mutation and the integrity of each construct were verified by DNA sequencing.

Binding of PCSK9 to the LDLR or VLDLR—Simian COS-M cells were seeded in 60-mm dishes (1.5 × 10⁶ cells/dish) in 4 ml of DMEM (glucose, 1 g/liter) containing 10% (v/v) fetal bovine serum. After 24 h, the cells were transfected with expression plasmids for wild-type or mutant LDLR or VLDLR (8 μg/ml) using Lipofectamine 2000 (20 μl of Lipofectamine/dish) according to the manufacturer’s protocol. After 48 h, the cells were washed twice with Dulbecco’s phosphate-buffered saline (PBS; Meditech, Inc.) and then incubated in 2 ml of DMEM (glucose, 1 g/liter) containing 5% (v/v) newborn calf lipoprotein-poor serum (NCLPPS), 10 μg/ml cholesterol, 1 μg/ml 25-hydroxycholesterol, and 0.5 μg/ml purified PCSK9. The PCSK9 used in these experiments contained a FLAG tag at the C terminus and was purified using anti-FLAG M2 affinity gel chromatography (Sigma), followed by size exclusion chromatography on a Tricorn Superose 6 10/300 fast performance liquid chromatography column (GE Healthcare, Piscataway, NJ), as previously described (14).

The cells were incubated with PCSK9 for 2 h, washed three times with ice-cold PBS, and lysed in 200 μl of lysis buffer (PBS, 1.5 mM MgCl₂, 5 mM dithiothreitol, 1% (v/v) Triton X-100). The cell lysates were subjected to SDS-PAGE (8%) and transferred to nitrocellulose membranes (GE Healthcare) by electroblotting. Immunoblotting was performed using the following antibodies: an anti-LDLR polyclonal anti-serum (3143) directed against the C terminus of the LDLR (30), a monoclonal antibody (15A6) developed against full-length PCSK9 (14), and an anti-HA monoclonal antibody to detect the VLDLR (12CA5; Roche Applied Science). Antibody binding was detected using horseradish peroxidase-conjugated goat anti-mouse IgG or donkey anti-rabbit IgG (GE Healthcare), followed by enhanced chemiluminescence detection (Pierce). The membranes were then exposed to F-BX810™ Blue X-Ray films (Phoenix Research Products, Hayward, CA).

Biotinylation of Wild-type and Mutant LDLR—COS-M cells were transiently transfected with expression plasmids containing cDNAs for wild-type or mutant LDLR (1 μg/35-mm dish) using FuGENE 6 (Roche Applied Science; 6 μl of FuGENE/35-mm dish) according to the manufacturer’s protocol. After 24 h, the cell surface proteins were biotinylated exactly as previously described (14). The cells were lysed in 150 μl of lysis buffer and then subjected to centrifugation at 15,000 rpm for 5 min in a microcentrifuge to remove insoluble debris. A total of 60 μl of the cell lysate was saved. Ninety μl of lysis buffer and then subjected to centrifugation at 3,000 × g for 5 min, the pellets were washed in lysis buffer three times for 10 min at 4 °C. The cell surface proteins were eluted from the beads by adding 1× SDS loading buffer (1 mm Tris-HCl, pH 6.8, 1% SDS, 12.5% glycerol, 0.0025% bromphenol) and incubated for 5 min at 90 °C. The proteins were then analyzed by SDS-PAGE (8%) and immunoblotted using a monoclonal anti-hLDLR antibody (HL-1) directed against the linker sequence between the 40th and 50th LDLR ligand-binding repeat (31).
**PCSK9 and LDLR Degradation**

**Recombinant Adenoviral Vector Expression of LDLR in WIF-B Cells**—Recombinant adenoviral vectors containing cDNAs for wild-type and mutant forms of the LDLR were generated by *in vitro* cre-lox recombination as described previously (32). WIF-B cells that were kindly provided by Dr. Ann Hubbard (Johns Hopkins University) were cultured in 6-well plates as previously described (33). After 8 days, the cells were infected with viruses expressing β-galactosidase, the wild-type LDLR or mutant LDLR (1.4 × 10^9 particles/35-mm dish). After 48 h, the cells were washed twice with PBS and incubated in 1 ml of culture medium containing 5% (v/v) NCLPPS, 10 μg/ml cholesterol, 1 μg/ml 25-hydroxycholesterol, and 1 μg/ml purified recombinant PCSK9 for 4 h. The cells were washed thrice with ice-cold PBS and lysed in 150 μl of lysis buffer. Whole cell protein extracts were then subjected to SDS-PAGE (8%) and immunoblot analysis to detect the LDLR (HL-1) and PCSK9 (15A6). Transferrin receptor was detected using a monoclonal antibody (Invitrogen).

**Purification of GST:EGF-A Fusion Proteins**—Wild-type and mutant (D310E) EGF-A of the LDLR were expressed as recombinant GST fusion proteins using the vector pGEX-4T (GE Healthcare) in *Escherichia coli* BL-21 DE3 cells (EMD Bio-Sciences, San Diego, CA). The transformed cells were grown at 37°C in LB medium containing ampicillin (50 μg/ml) and induced with 1 mm isopropyl β-D-thiogalactopyranoside for 5 h when the A600 reached 0.8. The cells were harvested by centrifugation at 5,000 × g for 20 min at 4°C. The cell pellets were resuspended and incubated on ice for 30 min in 25 ml of PBS containing 0.1 mm phenylmethylsulfonfluoride and 1 mg/ml lysozyme. The cells were lysed using a French pressure cell operated at 1500 p.s.i. (1 p.s.i. = 6.9 kPa). The lysate was centrifuged at 16,500 × g for 30 min. The supernatants were filtered through a 0.22-μm filter, and GST:EGF-A fusion protein were concentrated using a 3-kDa molecular mass cut-off filter. Protein purity was monitored by SDS-PAGE (12%), and immunoblotting. PCSK9 was detected using a monoclonal antibody, 15A6. GST:EGF-A was detected using an anti-GST monoclonal antibody (Invitrogen).

**Ligand Blotting and pH Dependence**—Purified extracellular domain of the LDLR (amino acids 1–699) was kindly provided by J. Deisenhofer (University of Texas Southwestern Medical Center). The LDLR protein (200 ng) was size-fractionated under nonreducing conditions by 8% SDS-PAGE, transferred to nitrocellulose, and cut into individual strips. Blocking buffer (PBS plus 2.5% nonfat milk) was added to each strip. After 30 min, the strips were rinsed briefly in pH buffer (50 mM Tris-maleate, pH 7.0 to 5.2, or 50 mM sodium citrate, pH 5.0, 75 mM NaCl, 2 mM CaCl₂, and 2.5% nonfat milk) and then incubated at room temperature for 60 min with 1.0 μg/ml 125I-PCSK9 (0.5 μCi/μg) in the pH buffer in the absence or presence of 50 μg/ml nonradiolabeled PCSK9. Following three 15-min washes with pH buffer, the strips were dried and exposed to a PhosphorImager plate. The resulting signals were quantified using a Molecular Dynamics Storm 820 system (GE Healthcare). Specific binding represents total binding (absence of excess nonradiolabeled PCSK9) minus nonspecific binding (presence of excess nonradiolabeled PCSK9).

**Immunofluorescence of WIF-B Cells**—WIF-B cells were grown as previously described (33) on glass coverslips for 6–8 days. The medium was changed to F12 Coon’s modification medium (Sigma-Aldrich) plus 5% human lipoprotein-poor serum, and the cells were cultured for an additional 48 h. On the day of the experiment the cells were incubated with 5 μg/ml of either native or heat-inactivated (for 10 min at 95°C) recombinant PCSK9. The coverslips were rinsed with cold PBS and were fixed using 4% (w/v) paraformaldehyde diluted in PBS at 4°C for 5 min. The cells were permeabilized in methanol at 4°C for 10 min and then incubated in PBS plus 1% (w/v) bovine serum albumin (Buffer A) for 30 min followed by an overnight incubation at 4°C in Buffer A plus rabbit anti-bovine LDLR anti-serum (0.5 μg/ml) (4548) (34) and monoclonal antibodies to early endosome antigen 1 (1:100) (BD Transduction Laboratories, San Jose, CA), bovine mannose-6-phosphate receptor (1:100) (Affinity Bioreagents, Golden, CO), human lysosomal protein Cathepsin D (1:50) (Santa Cruz Biotechnology, Santa Cruz, CA), and human transferrin receptor (1:200) (Invitrogen). The coverslips were washed three times for 5 min with 0.1% bovine serum albumin in PBS (Buffer B) and then incubated for 1 h in buffer A with Alexa Fluor 488-conjugated goat anti-rabbit IgG or Alexa Fluor 568-conjugated goat anti-mouse IgG (1 μg/ml) (Invitrogen). The coverslips were rinsed in Buffer B prior to mounting with a 4’,6’-diamino-2-phenylindole containing mounting medium (Vecrashield, Vector Laboratories, Burlingame, CA) and imaging the sections using a 100× 1.3 objective on a confocal microscope (Zeiss LSM510 Meta 2P).

**RESULTS**

**PCSK9 Binding to the Extracellular Domain of the LDLR**—Previously, we showed that PCSK9 binds the LDLR on the surface of cells (14). To map the site of PCSK9 binding to the LDLR, we used cultured monkey kidney cells (COS-M cells) to express either wild-type LDLR or LDLRs lacking each (or all) of the ligand-binding repeats. Purified human PCSK9 (0.5 μg/ml)
was added to the medium, and after 2 h the cells were lysed and subjected to duplicate immunoblotting to detect the LDLR and PCSK9 (Fig. 1A). The antibody used to detect the LDLR recognizes both the precursor (~120 kDa) and the mature, fully glycosylated form (~160 kDa) of the receptor (35), as well as partially glycosylated forms of the receptor that are often seen when the receptor is expressed in COS-M cells and HEK-293 cells (27). Similar amounts of mature LDLR were expressed in cells transfected with each construct. No PCSK9 was found associated with the cells expressing the vector alone (lane 1), whereas PCSK9 bound to cells expressing the wild-type LDLR (lane 2) and in cells expressing LDLRs lacking one or all of the ligand-binding repeats (lanes 3–10). From these experiments we concluded that the ligand-binding repeats were not required for binding of PCSK9 to the LDLR.

Next, we examined the effect of deleting portions of the EGF precursor homology domain of the LDLR on PCSK9 binding. No PCSK9 was detected in cells expressing a LDLR missing the first EGF-like repeat, EGF-A (Fig. 1B, lane 3) or the entire EGF precursor homology domain (lane 7), whereas PCSK9 bound to cells with receptors lacking EGF-B (lane 4), the β-propeller domain (lane 5) or the clustered O-linked sugar region (lane 10). Deletion of the third EGF-like repeat (EGF-C) disrupted the intracellular trafficking of the LDLR so that no mature, fully glycosylated form of the receptor was present on the cell surface, we cannot determine whether the LDLR:PCSK9 complex was present in the total cell lysate (lane 8). From these experiments we concluded that the ligand-binding repeats were not required with a requirement for EGF-A for PCSK9 binding to the LDLR.

EGF-A is Required for PCSK9 Binding to the LDLR—Protein structures determined by solution nuclear magnetic resonance and x-ray crystallography (36–38) have indicated that the N-terminal region of the EGF-A repeat contains a calcium ion-binding site (Fig. 2). The residues that coordinate calcium binding include Asn295, Glu296, Asp310, and Tyr315 (37, 38). Substitution of a glutamate residue (Fig. 2A, lane 4) for the aspartate at position 310 prevented binding of PCSK9 to the receptor, whereas substitution of aspartate for glutamate at residue 296 (Fig. 2A, lane 5) did not interfere with binding. Substitution of two other highly conserved residues in the second half of EGF-A (Gly322 and Leu325) also did not interfere with PCSK9 binding (lanes 6 and 7, respectively).

An additional series of substitutions were made to assess the effects of changing the four calcium coordinating residues on PCSK9 binding. PCSK9 binding was either absent or markedly reduced when any of the residues were substituted with an alanine (Fig. 2B). A trace amount of binding of PCSK9 to cells expressing Y315A was seen.

To confirm that the mutant forms of LDLR that failed to bind PCSK9 reached the cell surface, we labeled the cell surface proteins with biotin, isolated the biotinylated proteins, and selectively immunoblotted the LDLR. In cells expressing wild-type LDLR, only a single band of ~160 kDa was detected in the cell surface fraction (Fig. 2C, lane 2). Both the ΔEGFA and D310E mutant receptors were also present in the biotinylated fraction (lanes 3 and 4, respectively); thus, the lack of PCSK9 binding observed in cells expressing these mutant forms of the LDLR is not due to a failure of the receptors to reach the cell surface. As expected, both the precursor (120 kDa) and the mature (160 kDa) forms of the receptor were present in the total cell lysate (Fig. 2C). No cell surface LDLR was detected in cells expressing LDLR:ΔEGF-C (lane 6). Inasmuch as the ΔEGF-C protein is not present on the cell surface, we cannot determine whether the EGF-C repeat is required for binding to PCSK9.

The EGF-A of the LDLR Is Required for PCSK9-associated Degradation of the LDLR—Next, we examined the effect of mutations in EGF-A on PCSK9-dependent degradation of the LDLR. For these experiments, we used polarized cultured hepatocytes (WIF-B cells) that were developed from a fusion of...
human fibroblasts with rat hepatoma cells (39). The cells were infected with adenoviruses encoding the wild-type LDLR, LDLR:H9004 EGF-A, or LDLR:D310E (Fig. 3). When PCSK9 was added to cells expressing wild-type LDLR, PCSK9 became associated with cells, as determined by immunoblotting, and the amount of wild-type receptor was reduced markedly within 4 h (lane 4). In cells expressing LDLR:ΔEGF-A or LDLR:D310E there was no uptake of PCSK9 and no degradation of LDLR (lanes 6 and 8). Thus, mutations that interfered with PCSK9 binding to LDLR also inhibited PCSK9-mediated degradation of the receptor.

The EGF-A of the LDLR Permits PCSK9 Binding to the VLDLR—The data in Figs. 2 and 3 demonstrated that sequences contained in EGF-A are required for PCSK9 to bind the LDLR. We next addressed whether EGF-A is sufficient to confer PCSK9 binding on a member of the LDLR family that does not bind PCSK9. For these studies, we employed the VLDLR, an LDLR family member that shows strong sequence similarity with the LDLR; the major structural difference between the LDLR and the VLDLR is the presence of an additional ligand-binding repeat in the VLDLR (40). The overall structure of the EGF precursor homology domain in the VLDLR is similar to that of the LDLR. Despite the structural similarities between the two receptors, cells expressing the VLDLR failed to bind PCSK9 (Fig. 4A). Moreover, when EGF-A in the LDLR was replaced with EGF-A from the VLDLR, the LDLR no longer bound PCSK9. Conversely, when EGF-A from the LDLR was placed in the VLDLR, the

FIGURE 2. Binding of PCSK9 to wild-type and mutant LDLR and VLDLR. The experiments shown in A and B were performed exactly as described in the legend to Fig. 1. C, biotinylation of wild-type and mutant LDLR expressed in simian COS-M cells. COS-M cells were transiently transfected with expression plasmids containing cDNAs for wild-type or mutant LDLR. After 48 h, the cells were pretreated with 2 ml of DMEM containing 5% (v/v) NCLPPS, 10 μg/ml cholesterol, and 1 μg/ml 25-hydroxycholesterol for 16 h. The cells were washed and incubated in biotinylation buffer for 20 min prior to the addition of quenching buffer, as described under “Experimental Procedures.” The cells were washed, and the cell surface proteins were isolated as described under “Experimental Procedures.” Proteins from a whole cell lysate (cell lysate) and biotinylated proteins (cell surface) were then analyzed by SDS-PAGE (8%) and immunoblotting using a monoclonal anti-human LDLR antibody (HL-1). Similar results were obtained when the experiment was repeated twice. A schematic of EGF-A is provided with the calcium-coordinating residues in green (with Asp310 designated by an asterisk), cysteine residues in gray, and the leucine that participates in PCSK9 binding in blue. A calcium ion (Ca) is shown in yellow.

FIGURE 3. PCSK9 promotes the degradation of LDLR in a sequence-specific manner in cultured hepatocytes (WIF-B cells). Recombinant adenoviruses expressing β-galactosidase (β-gal), wild-type, or mutant LDLR were used to express the LDLR in WIF-B cells as described under “Experimental Procedures.” After 48 h, the cells were washed and incubated in 1 ml of culture medium containing 5% (v/v) NCLPPS, 10 μg/ml cholesterol, 1 μg/ml 25-hydroxycholesterol, and 1 μg/ml purified PCSK9 for 4 h. The cells were washed and lysed in 150 μl of lysis buffer. Whole cell lysate protein extracts were then subjected to SDS-PAGE (8%) for immunoblot analysis. LDLR was detected using a specific anti-human LDLR monoclonal antibody (HL-1). PCSK9 was detected using a monoclonal antibody, 15A6. Transferrin receptor was detected using a monoclonal antibody (Invitrogen). Similar results were obtained from two additional independent experiments.
hybrid protein now bound to PCSK9. Thus, the EGF-A of the LDLR is sufficient to confer binding of PCSK9, even when it is placed in a cell surface protein that otherwise is incapable of binding PCSK9.

Mapping the PCSK9-binding Site in EGF-A—To define the sequence(s) within EGF-A that are required for PCSK9 binding, we compared the sequence of the human LDLR and VLDLR EGF-A repeat (Fig. 4). A total of 16 amino acids differ between the two receptors in this 40-amino acid stretch, nine of which are highly conserved in LDLRs from different species (shown in red). The residue corresponding to Asp310 that is critical for PCSK9 binding to the LDLR (Fig. 2) is also an aspartic acid in the VLDLR. Site-directed mutagenesis was used to generate VLDLR expression constructs in which each of the nine conserved amino acids in the LDLR was introduced into the corresponding position of the VLDLR. Substitution of the aspartic acid at residue 354 in VLDLR with a leucine residue (corresponding to Leu318 in LDLR) conferred PCSK9 binding to VLDLR that was comparable with that seen when the entire EGF-A of the LDLR was placed in the VLDLR (Fig. 4B, compare lanes 3 and 9). PCSK9 failed to bind cells expressing the VLDLR containing substitutions in any of the other eight amino acid residues tested.

The converse experiment was performed. An aspartic acid residue was substituted for the leucine at position 318 in the LDLR. No binding of PCSK9 was seen in cells expressing the mutant LDLR (Fig. 4C).

Although we failed to observe PCSK9-mediated degradation of the chimeric receptor when the recombinant protein was expressed in HepG2 cells (data not shown), we cannot conclude from these experiments that binding of PCSK9 does not promote receptor degradation because the recombinant protein was expressed at very high levels. PCSK9 binding alone to the LDLR does not inevitably result in receptor degradation; PCSK9 fails to degrade the LDLR in fibroblasts, Chinese hamster ovary, monkey kidney (COS-7), and McArdle RH7777 cells (2). Further studies using chimeric proteins will be necessary to determine the minimal structural requirements for PCSK9-mediated receptor degradation.

The Isolated LDLR:EGF-A Binds PCSK9—To determine whether EGF-A directly binds PCSK9, we purified recombinant
forms of the 40-amino acid peptide as fusion proteins with GST. We incubated these proteins with purified recombinant PCSK9 and precipitated the EGF-A with a polyclonal anti-GST antibody. When the incubations were performed with the wild-type EGF-A peptide, PCSK9 was co-immunoprecipitated (Fig. 5A). There was no co-precipitation when the D310E mutant of EGF-A was used. The reverse experiment was performed using an anti-PCSK9 polyclonal antibody to immunoprecipitate PCSK9. Only when PCSK9 was incubated with the wild-type peptide was GST:EGF-A co-precipitated (Fig. 5B). Thus, PCSK9 binding to EGF-A does not require any of the other sequences in the LDLR.

PCSK9 Binding to EGF-A Is Calcium-dependent—Calcium is required for LDL and β-VLDL to bind to the LDLR (41). To determine whether PCSK9 binding to EGF-A is also calcium-dependent, the co-immunoprecipitation experiment was repeated in the presence of increasing concentrations of EDTA (Fig. 6A). The buffer in which PCSK9 was resuspended contained 2 mM calcium. An EDTA concentration of 2.5 mM prevented co-immunoprecipitation of EGF-A and PCSK9. The addition of calcium (starting at 5 mM) in the presence of EDTA (10 mM) resulted in co-immunoprecipitation of the peptide with the purified PCSK9 (Fig. 6B). Thus, calcium is required for the binding of PCSK9 to EGF-A.

**DISCUSSION**

The current results demonstrate that PCSK9 binds to EGF-A in the EGF precursor homology domain of the LDLR. Muta-
A highly conserved leucine residue (Leu318) located between the corresponding residues from LDLR and VLDLR contributes to the specificity of the PCSK9-LDLR interaction. Introduction of a leucine residue into the corresponding position of VLDLR:EGF-A (residue 354) was sufficient to allow PCSK9 to bind the VLDLR. The binding of PCSK9 to the LDLR was 50-fold higher at a pH of 5.2 as compared with a pH of 7.0, suggesting that PCSK9 binds more strongly to the LDLR after internalization. Time course experiments using immunocytochemistry in cultured hepatocytes demonstrated that the addition of PCSK9 to the medium resulted in a progressive redistribution of the LDLR, first to endosomes and then to lysosomes. Taken together, these data are consistent with a model in which PCSK9 binds to the EGF-A of the LDLR at the cell surface and remains strongly associated with the receptor in the acidic environment of the endosome, disrupting the acid-dependent conformational changes that allow the LDLR to dissociate from its ligand and recycle to the cell surface (26). As a consequence, the receptor is delivered to the lysosome for degradation.

The highly localized binding of PCSK9 to EGF-A of the LDLR is striking, especially because EGF-like domains are found in a myriad of proteins (42). EGF-A in the LDLR is located adjacent to EGF-B at the N terminus of the epidermal growth factor homology domain. Similar pairs of EGF-like domains are present in other transmembrane proteins (Notch, EGF precursor protein, and thrombomodulin) as well as several members of the thrombosis cascade (protein C, protein S, factor VII, factor IX, and factor X), and extracellular matrix components (fibrillin, fibulin, and latent transforming growth factor β-binding protein) (42). These repeats all have similarly positioned cysteine residues (n = 6) that exhibit a highly conserved disulfide bonding pattern: cysteines 1 with 3, 2 with 4, and 5 with 6. Despite the ubiquitous presence of EGF-like domains, several lines of evidence suggest that PCSK9 binds selectively to the LDLR in cells. First, PCSK9 binds poorly or not at all to cells that do not express the LDLR (14). Second, the only apparent phenotype of PCSK9 transgenic mice is hypercholesterolemia secondary to LDLR-deficiency, despite more than a 100-fold increase in circulating PCSK9 levels. Third, absence of PCSK9 in mice (12) and in humans (43) is not associated with any obvious phenotypes except those related to LDLR expression. Finally, PCSK9 did not bind to the VLDLR, the LDLR family member that most closely resembles the LDLR (40).

Why does PCSK9 selectively interact with the LDLR and not with other members of the LDLR family, all of which have EGF-like domains? Replacement of conserved residues in the EGF-A domain of VLDLR with the corresponding residues from LDLR indicated that the leucine residue at position 318 contributes to PCSK9 binding. This leucine is flanked by the third and fourth cysteine residues in EGF-A, both of which are disulfide-linked to other cysteines (Fig. 3), and is conserved in all other species except the rabbit (supplemental Table S2), where the corresponding residue is histidine. None of the other human LDLR family members have leucine at this position. In the VLDLR, the corresponding residue is usually an aspartic acid; in the LDL receptor-related protein 1 (LRP-1) it is most frequently an arginine; in LRP-2 (GP330 or megalin) it is usually a serine; in LRP-4 the residue is an alanine; in LRP-5 and LRP-6 the residue is either an alanine or a glycine; and in LRP-8 the residue is a threonine. Therefore, Leu may contribute to the specificity of binding of PCSK9 to the LDLR. Because some EGF-like domains in other proteins also have a leucine at this position (for example, notch, fibrillin, perlecain, and latent transforming growth factor β-binding protein 2), this residue is not the sole
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determinant of PCSK9 binding specificity. Further studies will be required to refine the structural determinants required for PCSK9 binding.

Like many EGF-like domains, the LDLR EGF-A has a calcium-binding site at the N terminus, and another calcium ion binds at the interface with EGF-B (38). Although the N-terminal calcium does not appear to be required for proper folding of the LDLR EGF-A (36), we found that calcium was required for PCSK9 to bind EGF-A (Fig. 6). Binding of PCSK9 to EGF-A was prevented by sequestration of divalent cations using EDTA and restored when calcium was added back to the buffer (Fig. 6). Substitution of individual residues in EGF-A that are known to coordinate the N-terminal calcium, including Asn<sup>295</sup>, Glu<sup>298</sup>, and Asp<sup>310</sup>, prevented PCSK9 binding to LDLR in cultured cells (Fig. 2 and 3), and to purified EGF-A in solution (Fig. 5). From our experiments we cannot determine whether calcium facilitates PCSK9-EGF-A interaction by forming direct contacts between the two proteins or, indirectly, by altering the PCSK9-binding surface of EGF-A. In other EGF domain-containing proteins that require calcium for protein-protein interaction, solution NMR studies indicate that calcium binding induces localized changes in conformation that occur at module interfaces (44). Thus, calcium may function to stabilize the binding modules in an optimum configuration for interaction with their binding partners.

The aspartate residue (Asp<sup>310</sup>) of the LDLR that is essential for PCSK9 binding has been shown to undergo β-hydroxylation (45), a post-translation modification that appears to be restricted to EGF domains (42). The role of β-hydroxylation in the LDLR, or in any of the other 25 proteins known to undergo this post-translational modification, remains obscure. Initially, it was proposed that this modification may promote calcium binding and proper folding of the EGF-like domain, but this was shown not to be the case, at least in the coagulation factor, Factor IX (46). An alternative possibility is that β-hydroxylation promotes interactions between EGF domains and their protein partners (47). In our studies, recombinant wild-type EGF-A bound PCSK9 in a sequence-specific manner despite being produced in bacteria, which do not express aspartate β-hydroxylase (48). Whereas we cannot exclude the possibility that β-hydroxylation promotes (or inhibits) PCSK9 binding to EGF-A in vivo, the essential role of Asp<sup>310</sup> observed in this study cannot be attributed to β-hydroxylation of this residue.

The PCSK9-binding site on the LDLR is distinct from the ligand-binding region that recognizes the receptor’s lipoprotein cargo, LDL and β-VLDL. Deletion of the entire domain of the receptor that binds with high affinity to LDL and to β-VLDL (i.e. the ligand-binding domain) does not significantly affect binding to PCSK9 (Fig. 1). Thus, PCSK9 can bind to the LDLR independently of lipoproteins. However, we do not know whether the LDLR can bind lipoproteins and PCSK9 simultaneously or whether the PCSK9 that binds LDLR in vivo is lipoprotein-associated or free. Furthermore, it remains to be determined whether PCSK9 binding to EGF-A impacts lipoprotein binding to the receptor.

Our immunocytochemical data are consistent with a model in which PCSK9 binds to LDLR on the cell surface and alters the trafficking of the receptor after internalization and transit to endosomes. However, we could not determine whether PCSK9 accompanies the receptor after it enters the cell, because we were unable to visualize PCSK9 in WIF-B cells using immunocytochemistry. The epitopes recognized by our anti-PCSK9 antibodies may be masked after PCSK9 binds to the LDLR, or the protein may adopt another conformation at a lower pH.

The crystallization of the extracellular domain of the LDLR at pH 5.3 by Rudenko et al. (26) provides a possible explanation for how PCSK9 binding might interfere with receptor recycling. They showed that at acidic pH, the ligand-binding repeats R4 and R5 form extensive hydrophobic interactions and salt bridges with residues in the β-propeller domain of the LDLR (26). Based on these findings, they proposed that the ligand-binding repeats are extended at a neutral pH (open conformation), which allows them to interact on the cell surface with circulating lipoproteins. After binding lipoproteins, the receptor-ligand complex is internalized and delivered to an endosome where a major pH-dependent conformational change occurs. The ligand-binding repeats 4 and 5 form a tight association with the β-propeller region of the EGF precursor homology domain (closed conformation), resulting in release of the lipoprotein and delivery of the particle to the endosome. Recent evidence from solution NMR studies is consistent with the last ligand-binding repeat (R7) and EGF-A forming a rigid structure, stabilized by calcium and hydrophobic interactions (49, 50). The conformation of this region does not change with reduction in pH, which serves to promote the interaction between R4/R5 and the β-propeller (49, 50).

Binding of PCSK9 to EGF-A on the cell surface may interfere with the formation of the closed conformation in the acidic environment of the endosome. PCSK9 may act like a doorstop and provide steric hindrance so that the ligand-binding repeats can no longer interact with the β-propeller domain. Alternatively, PCSK9 may clamp the receptor in a “closed” conformation. The increase in binding of PCSK9 for the LDLR at lower pH (Fig. 7) would ensure that PCSK9 would not become dislodged in the endosome. As a consequence, the receptor fails to recycle to the cell surface and is delivered to the lysosome where it is degraded. Our immunocytochemical studies (Fig. 8) are compatible with this scenario. The finding that inhibitors of lysosomal degradation inhibit PCSK9-associated degradation of the LDLR (supplemental Fig. S2) (10, 51) is also consistent with this scenario.

The cellular phenotype of naturally occurring mutations in the LDLR is consistent with this model. LDLRs lacking both EGF-A and EGF-B traffic normally to the cell surface but are rapidly degraded (52). Thus, deletion of EGF-A and EGF-B appears to mimic the effect of binding of PCSK9 to EGF-A in the LDLR. The absence of these two repeats may impair the interaction between ligand-binding repeats R4 and R5 and the β-propeller domain of the LDLR in the acidic environment of the endosome that is required for efficient recycling of the receptor.

Over 25 missense or frameshift mutations in EGF-A of the
LDLR have been identified in individuals with familial hypercholesterolemia (FH) (www.ucl.ac.uk/fh). Many of the mutations involve the cysteine residues and interfere with the proper folding of the LDLR (53). Other missense mutations that involve highly conserved residues within EGF-A also interfere with trafficking to the cell membrane caused by protein misfolding (54). Selective deletion of EGF-A has been found in some patients with familial hypercholesterolemia, but the effects of the deletion on LDLR function has not been examined experimentally. None of the LDLR mutations created in this study that interfered with PCSK9 binding to EGF-A have been reported in humans. Individuals harboring such mutations would be predicted to have lower plasma LDL levels because of accelerated LDL clearance by PCSK9-bound LDLR (46). Certain individuals harboring such mutations would be predicted to have lower plasma LDL levels because of accelerated LDL clearance by PCSK9 (55). Individuals harboring such mutations would be predicted to have lower plasma LDL levels because of accelerated LDL clearance by PCSK9-bound LDLR (55), it also might be anticipated that subjects with mutations in the PCSK9-binding site of the LDLR would be more responsive to the cholesterol-lowering effects of these drugs.

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