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PCSK9 binds to multiple receptors and can be functionally inhibited by an EGF-A peptide

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ABSTRACT

Proprotein convertase subtilisin/kexin type 9 (PCSK9) binds to low density lipoprotein receptor (LDLR) and induces its internalization and degradation. PCSK9 binding to LDLR is mediated through the LDLR epidermal growth factor-like repeat A (EGF-A) domain. We show for the first time that an EGF-A peptide inhibits PCSK9-mediated degradation of LDLR in HepG2 cells. In addition to LDLR, we show that PCSK9 also binds directly to ApoER2 and mouse VLDLR. Importantly, binding of PCSK9 to either LDLR or mouse VLDLR was effectively inhibited by EGF-A while binding to ApoER2 was less affected. In contrast, LDL receptor-associated protein (RAP), which interacts with LDL receptor repeat type A (LA) domains, inhibited PCSK9 binding to ApoER2 with greater efficacy than either LDLR or mVLDLR. These data demonstrate that while PCSK9 binds several receptors via its EGF-A binding domain, additional contacts with other receptor domains are also involved.

PCSK9 was originally cloned as neural apoptosis-regulated convertase 1 [1]. Although some reports have suggested PCSK9 is involved in neuronal development [1–3], more attention has been focused on its role in regulating LDLR expression and subsequently serum LDL levels [4–9]. The ability of PCSK9 to influence serum LDL levels results from a post-translational reduction in liver LDLR protein [10–12]. The reduction of LDLR is also independent of PCSK9 enzymatic activity, though autocatalytic activity is required for secretion [13,14]. PCSK9 appears to alter the fate of LDLR, either preventing its transport to the surface or interfering with recycling of internalized receptor [15]. Poirier et al. have raised the possibility that, in addition to LDLR, PCSK9 may also interact with VLDLR and ApoER2 [16]. These authors showed that PCSK9 could associate with cells that had been transfected with either of these receptors and that PCSK9 could mediate their degradation in transfected cells. The structural basis by which PCSK9 could bind to ApoER2 and VLDLR has not been explored, nor has binding to either of these proteins been directly demonstrated.

Recently, Zhang et al. revealed that the EGF-A domain of LDLR is important for PCSK9 binding [17]. As is the case with LDLR [18], PCSK9 binding to EGF-A is pH and calcium dependent [17]. The interaction between PCSK9 and LDLR has been further illuminated by the co-crystal structure of PCSK9 and the LDLR EGF-A domain [19]. This structure shows that PCSK9 and EGF-A interact across a relatively small and flat 530 Å^2 contact patch [19].

Here we report that PCSK9 directly and specifically binds to ApoER2 and mVLDLR in addition to LDLR. We also report that a synthetic EGF-A domain peptide blocks the interaction of PCSK9 with LDLR. EGF-A also diminished PCSK9-mediated degradation of LDLR and maintained LDL uptake in HepG2 cells. This is the first demonstration that the effect of PCSK9 on LDLR can be blocked. In EGF-A inhibited PCSK9 binding to mVLDLR and to a lesser extent with ApoER2. In contrast, recombinant RAP inhibited PCSK9 binding to ApoER2 more effectively than binding to LDLR and VLDLR, suggesting the involvement of additional receptor domains in binding PCSK9.

Materials and methods

Recombinant proteins and antibodies. Polyhistidine-tagged human LDLR, human ApoER2, mouse VLDLR and mouse reelin were purchased from R&D Systems as were antibodies to LDLR and mouse VLDLR. Polyhistidine-tagged human JNK2α2 and mouse endostatin were purchased from Calbiochem Inc. Recombinant rat RAP was obtained from PROSPEC Protein Specialists and human RAP from Molecular Innovations. Polyclonal rabbit anti-LDLR used for Western blotting and in-cell Western was obtained from PROGEN Biotechnik. Mouse monoclonal anti-β-actin and M2 anti-FLAG antibodies were obtained from Sigma.

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Expression and purification of PCSK9. Human PCSK9 (GenBank NM_174936.2) was amplified from liver cDNA using a pair of primers (N-terminal primer, 5′-GCCGCCACCATGGGCACCGTCAGCTCCAG GCG-3′ and C-terminal primer, 5′-TCACTTGTCATCGTCGTCCTTGTA GTCCTGGAGCTCCTGGGAGGCCTGCGCCAG-3′) designed to insert a C-terminal FLAG epitope tag. The product was cloned into pCDNA 3.1 and sequence confirmed. Human PCSK9-FLAG was purified from supernatants of stably transfected HEK293 cells essentially as described by Lagace et al. [15].

Synthetic LDLR EGF-A domain peptide. The human LDLR EGF-A domain peptide GTNECLDNNGGCSHVCNDLKIGYECLCPDGFQLVAQ RRCEDI-NH2 (*S*:*S* = C1–C3, C2–C4, C5–C6) was synthesized by CS Bio Inc. Sequence and purity of the product were confirmed by HPLC and mass spectrophotometry. The dissociation constant (K_d) between PCSK9 and EGF-A was estimated by temperature-dependent fluorescence (TdF). Briefly, 20 μl of 1 μM of PCSK9 and 2.5–20 μM of EGF-A peptide were mixed in buffer containing Sypro Orange (5×) in a strip-sealed 96-well PCR plate. For pH 5.2 determinations, the buffer was 25 mM citric–phosphate, pH 5.2, 150 mM NaCl, ±10 mM $CaCl_2$; pH 7.4 buffer was 25 mM HEPES, pH 7.4, 150 mM NaCl, ±10 mM $CaCl_2$. The plate was placed in a real time thermocycler (Chromo4, Bio-Rad) for melting in 45 min from room temperature to 90 °C in increments of 0.5 °C. The measured fluorescent intensities were fitted to generate melting point data. The concentration-dependent melting point shifts were subjected to nonlinear regression analysis to derive TdF K_d values using custom algorithms based on previous literature [20–22] and were based on an assumed average enthalpy change of binding at 7000 cal/mol with an assigned uncertainty of approximately 50%.

Alphascreen binding assays. An Amplified Luminescent Proximity Homogeneous Assay (ALPHA, Perkin-Elmer) capable of directly determining the interaction between PCSK9-FLAG and a putative binding partner was established. This technique requires that "donor" and "acceptor" beads be brought into proximity via protein–protein interaction, resulting in increased luminescence [23]. Receptor binding to PCSK9 was determined as follows: 5 μl of recombinant receptor at the appropriate concentrations was incubated with 2.5 μl PCSK9-FLAG (1.4 μg/ml, 30 min). About 2.5 μl of biotinylated anti-Flag-M2 antibody (1.8 μg/ml) was added and the mixture incubated for 1 h. Afterward 5 μl of streptavidin donor bead and nickel chelate acceptor bead (1:1 mixture) was added and the assay incubated overnight. AlphaScreen signal (counts per second) was analyzed using an EnVision microplate reader (Perkin-Elmer). All data points were determined in triplicate. Assays were carried out at 23 °C in buffer containing 25 mM HEPES, 0.1 M NaCl, pH 7.4, 0.1% BSA.

The inhibition assays were determined similarly with slight adjustments to assay volumes and protein concentrations. Briefly, 5 μl of 1.25 μg/ml of PCSK9-Flag and 1.25 μg/ml of His-tagged receptor was incubated with 2.5 μl of inhibitor at the appropriate concentrations for 30 min followed by the addition of 2.5 μl of anti-Flag-BioM2 (1.8 μg/ml) and a 1 h incubation.

LDLR degradation assays. LDLR degradation was determined by Western blotting of whole-cell lysate from HepG2 cells (ATCC, HB-8065). HepG2 cells were treated with media or media containing PCSK9 (100 nM), EGF-A (at concentration indicated) or both for 6 and 18 h. Total cell protein (35 μg/lane) was run on a reducing 10% SDS–PAGE and protein transferred to PVDF membrane. LDLR

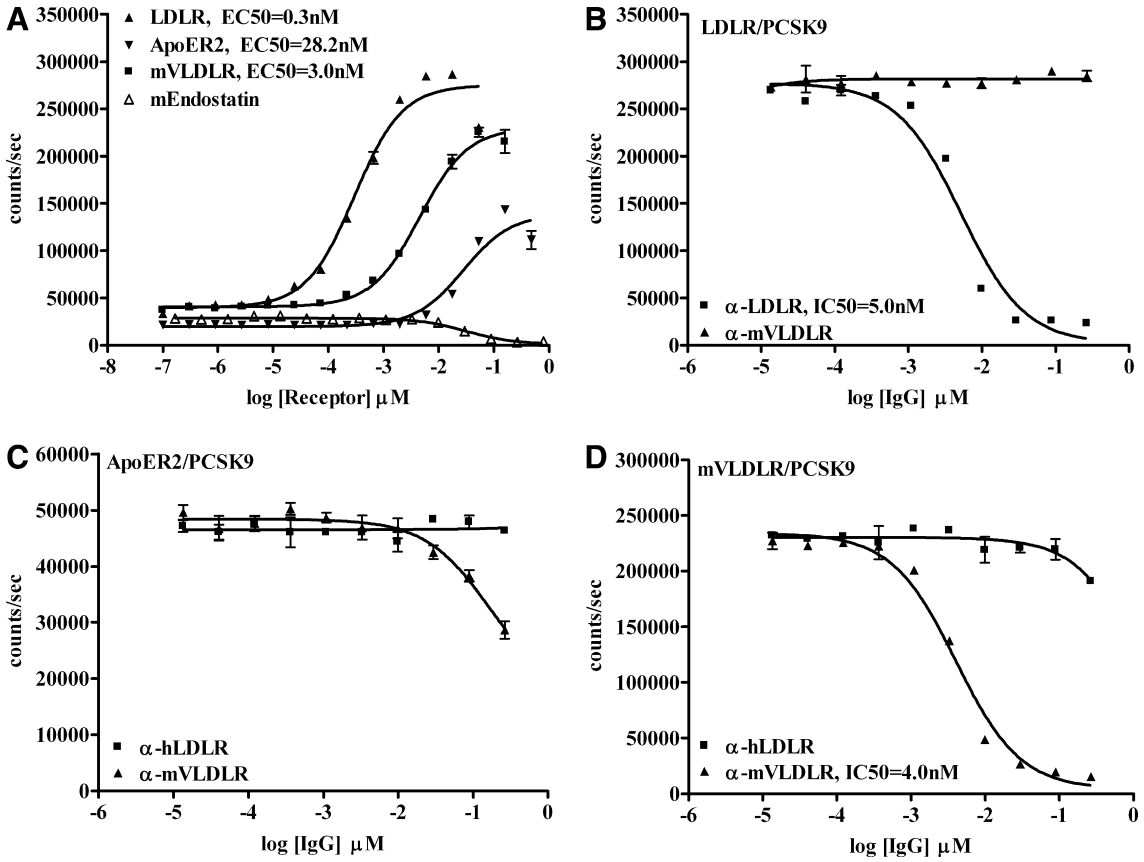

Fig. 1. PCSK9 specifically interacts with LDLR, ApoER2, and mVLDLR. (A) Serial dilutions of the His-tagged LDLR, ApoER2, mVLDLR were incubated with PCSK9-Flag and the interaction detected by Alphascreen as described in experimental procedures. His-tagged mEndostatin was used as an unrelated protein control. (B) Polyclonal antibodies to LDLR or mVLDR were used to block interaction of PCSK9 with LDLR. (C) Polyclonal antibody inhibition of PCSK9 binding to ApoER2. (D) Polyclonal antibody inhibition of PCSK9 binding to mVLDLR. All assay points were determined in triplicate and EC_{50} values were calculated using Graphpad Prism 4.0. Error bars (standard deviation) are shown. Results shown are representative of at least three independent experiments.

was detected using rabbit anti-LDLR and IRDye 800CW goat anti-rabbit (Li-Cor Biosciences). Bands were visualized with an Odyssey infrared imaging system (Li-Cor Biosciences).

In addition to Western blotting, in-cell western was performed to quantitate EGF-A inhibition of PCSK9-mediated LDLR degradation. HepG2 cells were seeded in 384-well collagen I coated plates and treated with EGF-A (at the indicated concentrations) and/or PCSK9 (100 nM) for 18 h. Detection of LDLR and β-actin was performed according to the manufacturer's protocol (Li-Cor Biosciences) using the antibodies described above in conjunction with IRDye 800CW goat anti-rabbit (Li-Cor) and IRDye 680 goat anti-mouse (Li-Cor). The assay was read on an Odyssey infrared imaging system (Li-Cor) and the signal for LDLR protein in each well normalized to β-actin content.

LDL uptake assay. For LDL uptake determinations, HepG2 cells were treated with appropriate dilutions of EGF-A made in HBSS containing 1% BSA ± 100 nM PCSK9 and the mixture incubated for 1 h, 23 °C before addition to the cells. Following an 18 h incubation, treatment buffer was removed from the cells and dil-LDL (9 μg/ml in HBSS, 1% BSA, Invitrogen) added for 90 min, 23 °C. Cells were then fixed and fluorescence intensity read out using an Analyst GT (Molecular Devices).

Results and discussion

Conflicting results have been reported in regard to the ability of PCSK9 to interact with proteins other than LDLR. Zhang et al. reported that no binding of PCSK9 to VLDLR could be detected in transfected COS-M cells [17]. In addition, these authors showed that binding to VLDLR could be conferred by introducing the EGF-A domain of LDLR into VLDLR. In contrast, Poirier et al. showed enhanced PCKS9 association with CHO-A7 cells expressing either ApoER2 or VLDLR after an overnight incubation [16]. These authors further demonstrated enhanced degradation of ApoER2 and VLDLR when coexpressed with PCSK9 in a variety of cell lines. In order to assess PCSK9's interaction with these proteins more directly we developed a cell-free binding assay as described above. We observed a concentration-dependent binding of PCSK9 to LDLR, ApoER2 and mVLDLR (Fig. 1A). The omission of either of the protein pair or substitution of His-tagged mouse endostatin resulted in no increase in signal. Substitution of His-tagged mouse reelin or JNK2a2 also resulted in no signal (data not shown).

Polyclonal antibodies raised against LDLR or mVLDLR selectively inhibited the interaction with those proteins in a concentration-dependent manner (Fig. 1B–D). A non-specific control polyclonal IgG was also unable to block any of the interactions (not shown). Commercial polyclonal antibodies capable of recognizing the extracellular domain of ApoER2 were not available. The EC_{50} values obtained for receptor binding suggest that LDLR may bind to PCSK9 with higher affinity than either mVLDLR or ApoER2.

As mentioned above, Zhang et al. have shown that the EGF-A domain of LDLR is critical for PCSK9 interaction and the recently reported crystal structure of PCSK9 complexed with the LDLR EGF-A domain has provided critical insight into the nature of this interaction [17,19]. In our analysis of the LDLR interface in the X-ray structure coordinates (RCSB code 3BPS), there was no obvious reason to why the corresponding EGF domains of ApoER2 and VLDLR would not be able to interact with PCSK9, particularly as the LDLR L318 difference with VLDLR, cited previously [17], comprises only a small portion of the interaction surface. We therefore proceeded on the hypothesis that the PCSK9 domain shown to

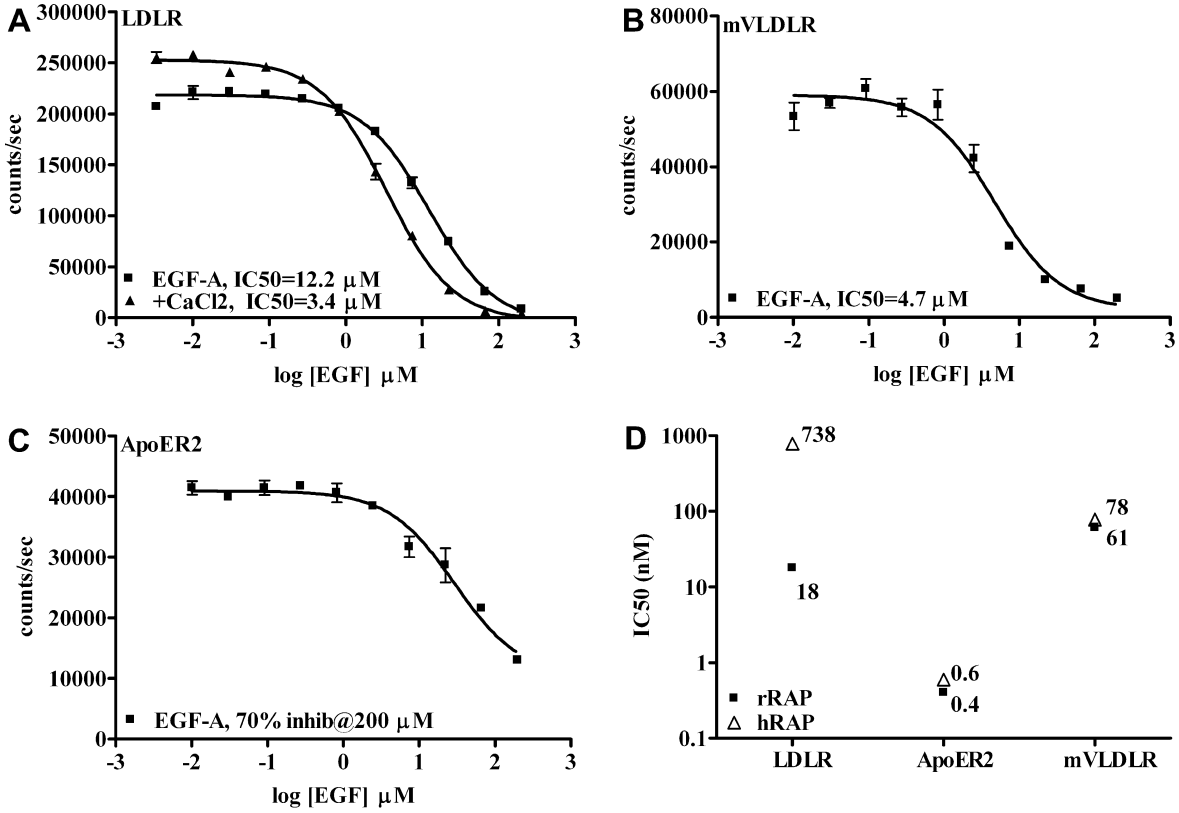

Fig. 2. Interaction of PCSK9 with LDLR, ApoER2, and mVLDLR are inhibited by EGF-A peptide and recombinant RAP. (A) Serial dilutions of EGF-A (indicated) were used to inhibit PCSK9 binding to LDLR in the presence or absence of 2 mM added calcium. (B and C) EGF-A was used at the indicated concentrations in the presence of 2 mM added calcium to inhibit PCSK9 binding to mVLDLR and ApoER2. (D) IC_{50} values for inhibition of PCSK9 binding to the indicated receptors using recombinant rat or human RAP as indicated in the legend. The numerical IC_{50} values obtained for the experiment shown are indicated. In all cases, the results shown are representative of at least two independent experiments.

interact with the LDLR EGF-A was also involved in binding ApoER2 and mVLDLR. In order to address this question we used a synthetic LDLR EGF-A domain peptide in an attempt to block the PCSK9-receptor interaction. The ability of the synthetic peptide to bind to recombinant PCSK9 was confirmed by TdF. The binding of EGF-A to PCSK9 as determined by this method was pH and calcium sensitive with the highest affinity, Kd 0.3 μM, determined at pH 5.2 in the presence of added calcium versus a Kd of 1 μM at pH 7.4 in the presence of added calcium or 10 μM with no added calcium. These findings are consistent with previous reports showing binding of PCSK9 to either LDLR [18,24] or EGF-A [17]. We could not determine a TdF dissociation constant for PCSK9 in low pH in the absence of calcium. The reason for this is unknown, but one plausible hypothesis is that the calcium is absolutely required to maintain the EGF-A fold at low pH.

As expected, the synthetic EGF-A blocked the PCSK9-LDLR interaction with an IC_{50} of 3.4 μM (12.3 μM in the absence of calcium, Fig. 2A). EGF-A also inhibited the PCSK9-mVLDLR interaction with an IC_{50} of 4.7 μM (Fig. 2B). In contrast, the PCSK9-ApoER2 interaction was poorly inhibited by the EGF-A peptide and we were unable to completely block binding even with 200 μM EGF-A (Fig. 2C). Since PCSK9 binding to all three of these proteins was inhibited by EGF-A it suggests the same PCSK9 domain is used to engage each of them, however the binding of ApoER2 appeared to be less reliant on this interaction.

In order to further explore PCSK9 interactions with these proteins, we took advantage of another protein known to interact with all three receptors, the LDL receptor-associated protein or RAP. RAP has been shown to interact with the LA domains found in LDLR and related receptors [25,26]. This interaction protects these receptors from being bound by their ligands while in the endoplasmic reticulum [27]. The presence of either human or rat RAP inhibited binding of PCSK9 to each of the receptors tested, albeit with different efficacies (Fig. 2D). This suggests that, in addition to the EGF-A domain, the LA domains of LDLR are also involved in binding PCSK9. Interestingly, RAP was much better at blocking ApoER2 (IC_{50} 0.4 nM for rRAP) than either LDLR or mVLDLR (IC_{50} of 18 and 61 nM with rRAP, respectively). Together with the data regarding EGF-A inhibition of PCSK9 binding, this suggests a model wherein PCSK9 interaction with ApoER2 may rely more on the LA domains and less on the EGF-A domain than is the case with LDLR or mVLDLR. Previous reports have shown that PCSK9 degrades LDLR in a post-ER compartment [12,28]. It is possible that RAP protects LDLR and the related receptors from being bound by PCSK9 while still in the ER, shielding them from degradation at least until they reach the Golgi.

We also examined whether the EGF-A peptide could inhibit LDLR degradation and restore LDL uptake using HepG2 liver cells as a model system. PCSK9 very effectively enhanced degradation of mature LDLR in HepG2 cells incubated with recombinant protein (Fig. 3A). The presence of EGF-A inhibited this effect and showed increased LDLR protein in HepG2 cells incubated with PCSK9 for 6 or 18 h. This effect appeared minimal at 1.5 μM EGF-A, but showed a clear increase at 15 μM. These concentrations of EGF-A had no obvious effect on LDLR protein levels in the absence of PCSK9. In order to make a quantitative determination of the effects of EGF-A, we used in-cell protein detection to determine total LDLR protein levels after 18 h exposure to PCSK9. EGF-A showed a clear concentration-dependent ability to maintain LDLR protein (Fig. 3B). In a parallel experiment EGF-A inhibition of PCSK9 function was reflected in a corresponding increase in HepG2 uptake of dil-labeled LDL (Fig. 3C). Although we have not assessed the ability

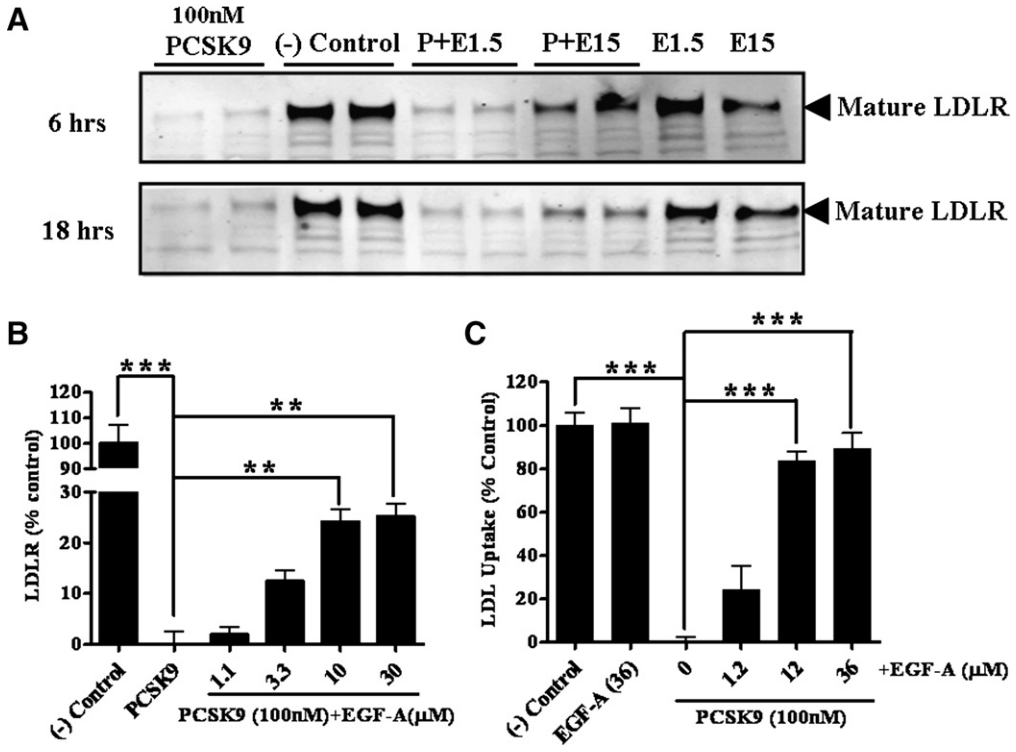

Fig. 3. EGF-A peptide inhibits PCSK9-mediated degradation of LDLR. (A) HepG2 cells were treated with culture media (control), PCSK9, PCSK9 + EGF-A at 1.5 μM or 15 μM (P+E1.5, P+E15) or EGF-A alone (1.5 or 15 μM) as indicated for 6 and 18 h. Whole cell protein lysates were prepared and duplicate samples analyzed for LDLR protein by Western blotting using an anti-LDLR polyclonal. The band corresponding to mature LDLR (~160 kD) is indicated. Results shown are from one of two experiments. (B) HepG2 cells were plated into a 384-well plate and treated with buffer, PCSK9 or PCSK9 + EGF-A as indicated for 18 h. The amount of total LDLR protein relative to the amount of β-actin in each well was determined as described in experimental procedures. Results are expressed as a percent of LDLR protein relative to the buffer control and are the average of five wells. Error bars shown are standard deviation. (C) Uptake of dil-labeled LDL was determined in HepG2 cells treated as indicated for 18 h. Where indicated, $^{**}p < 0.01$, $^{***}p < 0.001$ as determined by one-way ANOVA and Bonferroni's multiple comparison test.

of RAP to inhibit PCSK9-mediated degradation we did attempt to look at effects on LDL uptake. As might be predicted given its known function, RAP proved to be very effective in blocking dil-labeled LDL uptake on its own, thus any effect of RAP on PCSK9 function could not be observed.

In conclusion, we have provided the first direct evidence for PCSK9 binding to ApoER2 and mVLDLR as well as to LDLR. We have shown that this binding can be inhibited by a synthetic LDLR EGF-A domain peptide as well as by receptor-specific antibodies. We have also provided data to suggest that, while it certainly binds to the EGF-A domain of LDLR and likely to an EGF domain(s) of mVLDLR and ApoER2, PCSK9 may also interact with the LA domains found in LDLR, mVLDLR, and in particular ApoER2. Finally, we shown for the first time that inhibition of PCSK9 binding to LDLR can prevent LDLR degradation and maintain LDL uptake.

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