Annexin A2 Is a C-terminal PCSK9-binding Protein That Regulates Endogenous Low Density Lipoprotein Receptor Levels*\[S\]

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The proprotein convertase subtilisin/kexin-type 9 (PCSK9), which promotes degradation of the hepatic low density lipoprotein receptor (LDLR), is now recognized as a major player in plasma cholesterol metabolism. Several gain-of-function mutations in PCSK9 cause hypercholesterolemia and premature atherosclerosis, and thus, inhibition of PCSK9-induced degradation of the LDLR may be used to treat this deadly disease. Herein, we discovered an endogenous PCSK9 binding partner by Far Western blotting, co-immunoprecipitation, and pull-down assays. Following two-dimensional gel electrophoresis and mass spectrometry analysis, we demonstrated that PCSK9 binds to a ~33-kDa protein identified as annexin A2 (AnxA2) but not to the closely related annexin A1. Furthermore, our functional LDLR assays and small hairpin RNA studies show that AnxA2 and the AnxA2-p11 complex could prevent PCSK9-directed LDLR degradation in HuH7, HepG2, and Chinese hamster ovary cells. Immunocytochemistry revealed that PCSK9 and AnxA2 co-localize at the cell surface, indicating a possible competition with the LDLR. Structure-function analyses demonstrated that the C-terminal cysteine-histidine-rich domain of PCSK9 interacts specifically with the N-terminal repeat R1 of AnxA2. Mutational analysis of this 70-amino acid-long repeat indicated that the RRTKK 81 sequence of AnxA2 is implicated in this binding because its mutation to AATAA 81 prevents its interaction with PCSK9. To our knowledge, this work constitutes the first to show that PCSK9 activity on LDLR can be regulated by an endogenous inhibitor. The identification of the minimal inhibitory sequence of AnxA2 should pave the way toward the development of PCSK9 inhibitory lead molecules for the treatment of hypercholesterolemia.

The proprotein convertase subtilisin kexin-like 9 (PCSK9) is the ninth member of a family of secretory serine proteinases known as the proprotein convertases (PCs) (1, 2). It is now recognized as a major candidate gene for the development of pharmacologically relevant inhibitors or silencers, because it induces an enhanced cellular degradation of the low density lipoprotein receptor (LDLR) in endosomes-lysosomes (3, 4). An increased activity of PCSK9 would thus result in an up-regulation of the level of circulating LDL cholesterol, one of the major causes leading to atherosclerosis and coronary heart disease (5, 6). Indeed, the PCSK9 gene represents the third chromosomal locus of dominant familial hypercholesterolemia (7), as was recently reconfirmed in two genome wide screens (8, 9) and a liver-specific screen (10). Both gain and loss of function mutations have been reported for PCSK9 resulting in hyper- and hypocholesterolemia, respectively (11). PCSK9 transgenic and knock-out mice recapitulated these phenotypes (12, 13).

Following autocatalytic cleavage, PCSK9 exits the endoplasmic reticulum complexed with its prosegment and is efficiently secreted (1). This secreted form can be internalized into endosomes via cell surface binding in an LDLR-dependent manner (14) and is able to degrade the cell surface LDLR (15). Alternatively, PCSK9 may enter endosomes directly from the Golgi (16, 17). PCSK9 co-localizes with LDLR in early and late endosomes (14). It was also demonstrated that the C-terminal Cys-His-rich domain (CHRD) and the prosegment of PCSK9 are critical for the co-localization of PCSK9 and the LDLR (14).

The zymogen proPCSK9 is autocatalytically converted into an inactive prosegment-PCSK9 complex in the endoplasmic reticulum (1, 3), and so far the only PCSK9 substrate known is itself. PCSK9 is the sole PC that is secreted as a catalytically inhibited prosegment-PC complex (1, 3, 18). Accordingly, the enhanced degradation of the LDLR (3, 4, 16), very low density lipoprotein receptor (VLDLR), and ApoER2 (19) in endosomes/lysosomes (14) induced by PCSK9 does not seem to require its catalytic activity (18, 20). This intriguing twist in the function of this convertase is supported by the crystal structure of PCSK9, 4

The abbreviations used are: PCSK9, proprotein convertase subtilisin kexin-like 9; aa, amino acid(s); mAb, monoclonal antibody; AnxA2; annexin A2; AnxA1, annexin A1; CHRD, cysteine-histidine-rich domain; PC, proprotein convertase; LDLR, low density lipoprotein receptor; VLDLR, very low density lipoprotein receptor; shRNA, small hairpin RNA; CHO, Chinese hamster ovary; EGF, enhanced green fluorescent protein; HA, hemagglutinin; HRP, horseradish peroxidase; CHAPS, 3-[N-(cholamidopropl)dimethylammonio]-1-propanesulfonic acid; IPG, immobilized pH gradient; MS/MS, tandem mass spectrometry; PBS, phosphate-buffered saline.

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The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1 and S2.

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which revealed an extended tight binding complex of the enzyme and its inhibitory prosegment (21). This complex binds the epidermal growth factor-like domain A of the LDLR (22, 23), with increasing strength at acidic pHs similar to those of endosomes/lysosomes (21), leading to its degradation by resident hydrolases.

The wide interest in developing a specific PCSK9 inhibitor/silencer led to the proposal of multiple approaches. One of these depends on the identification of inhibitors/modulators of the PCSK9-LDLR interaction (23). However, focusing only on the site of the PCSK9-LDLR interaction may be too restrictive. Indeed, natural point mutations of PCSK9 in remote domains also result in either hyper- or hypo-cholesterolemia (11), even though they are not implicated in the direct interaction of the catalytic domain with the LDLR/epidermal growth factor-like domain A (23). These include the hypercholesterolemic mutants S127R (prosegment) and H553R (CHRD) as well as the hypocholesterolemic variants R46L (prosegment) and Q554E (CHRD), resulting in gain or loss of function of PCSK9, respectively (7, 11, 24, 25). It was therefore possible that endogenous modulators of PCSK9 function on LDLR may exist, which could specifically interact with the prosegment, the catalytic domain, or the CHRD.

Accordingly, we set up a Far Western screen to identify such a modulator(s) in cell line extracts. Our extended analysis revealed that such a protein does exist in certain cells and that it interacts specifically with the CHRD, resulting in a loss of function, i.e. decreased ability of PCSK9 to enhance the degradation of LDLR. Herein, we describe the identification and properties of this endogenous PCSK9 modulator, as well as its domain that interacts with the CHRD.

**EXPERIMENTAL PROCEDURES**

**Expression Constructs**—Human PCSK9 and mutant cDNAs and domains thereof were cloned, with or without a C-terminal V5 tag, into pIRSE2-EGFP vector (Clontech) as previously described (3, 26). The cDNAs encoding for mouse PC5A-V5 (27) and pC-hLDLR (3) were previously reported. The cDNA encoding for p11-YFP was kindly provided by Dr. Volker Gerke (Institute of Medical Biochemistry, University of Muenster, Germany). Wild type human annexin A2 (AnxA2) (ATCC MGC-2257) and annexin A1 (AnxA1) (ATCC MGC-5095) were purchased from ATCC and subcloned into NheI/SacI-digested pIRSE2-EGFP vector. An HA epitope (YPYDVPDYA) was fused by PCR mutagenesis at the C terminus of both AnxA1 and AnxA2. All of the oligonucleotides used in the various AnxA2 constructs are listed in supplemental Table S1. Two-steps PCR were performed on AnxA2 cDNA to introduce point mutations (77RRTK→AATAK; 77RRTK→AATAA; 77RRTKKEASALK→77AATAKELASAL; and 80KKELAGKPLD) or amino acid (aa) deletions (Δ2–24, aa 2–24; ΔR1, aa 37–108; ΔR2, aa 109–192; ΔR3, aa 193–268; and ΔR4, aa 269–339) into pIRSE2-AnxA2-EGFP vector (supplemental Table S1). In addition, using PCR, the AnxA2 segment aa 49–75 was swapped with the corresponding AnxA1 segment aa 58–84 (AnxA2 (aa 49–75) > AnxA1 (aa 58–84)). Purified PCR fragments were digested with the appropriate restriction enzymes and subcloned into the corresponding digested pIRSE2-AnxA2-HA-EGFP vector. All of the final cDNA constructs were verified by DNA sequencing.

**RNA Interference**—To silence human AnxA2, a 29-mer pRS-shRNA was used: (sh3, GCATACGACTGAACTCGCCTTATCTGG) (Origene). The control pRS-shGFP vector (shCtl) contained a noneffective 29-mer shGFP cassette.

**Quantitative Real Time PCR**—Quantitative real time PCR analysis of RNA preparations was performed as previously described (13, 28). Briefly, each cDNA sample was submitted to two PCR amplifications: one for normalizing the ribosomal-S protein gene (S14 for human and S16 for mouse cDNAs) and the other for the gene of interest, each in triplicate. The Mx3500P system from Stratagene was used to perform and analyze the quantitative real time PCRs, using S14 or S16 amplifications as normalizers (28).

**Cell Culture and Transfections**—HepG2, HuH7, COS-1, BSC40, and HEK293 cell lines were grown in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum (Invitrogen), whereas CHO-K1 and CHO-K1 mutant Pgsd-677 cells that lack heparan sulfate proteoglycans (29) were grown in Ham’s F-12 medium/Dulbecco’s modified Eagle’s medium (50:50) with 10% fetal bovine serum. Y1 mouse adrenal cells were grown in F12K medium with 15% horse serum and 2.5% fetal bovine serum. All of the cells were maintained at 37 °C under 5% CO2. At 80~90% confluence, HuH7 and CHO-K1 cells were transiently transfected with Lipofectamine 2000 (Invitrogen), HEK293 cells were transfected with Effectene (Qiagen), and HepG2 cells were transfected with FuGENE HD (Roche Applied Science). Twenty-four hours after transfection, the cells were washed and incubated in serum-free medium, containing or not exogenous conditioned medium and/or purified proteins, as indicated in the figure legends, for an additional 20 h before medium collection and cell lysis. For analysis of the various AnxA2 mutants in HEK293 cells, 24 h post-transfection the cells were washed and then incubated for another 24 h in complete medium containing 50 μg of the proteasome inhibitor acetyl-Leu-Leu-Norleucinal (Calbiochem). Stable transfectants of shRNA-AnxA2 were obtained in HuH7 cells following puromycin selection.

**Antibodies and Purified Proteins**—The rabbit polyclonal antibody against PCSK9 was raised in-house as described (14). Other antibodies used were a rabbit polyclonal V5-antibody (Sigma), an unconjugated or horseradish peroxidase (HRP)-conjugated mouse monoclonal anti-V5 (mAb V5 or mAb V5-HRP; Invitrogen), goat anti-LDLR (human) (R & D Systems), HRP-conjugated mouse anti-His (Qiagen), anti-HA (Roche Applied Science), monoclonal anti-HA-Alexa Fluor 488 (Invitrogen), and mouse anti-AnxA2 (human) (BD Biosciences). Purified CHRD-His was produced in-house, purified PCSK9-His, a kind gift from Bristol-Myers Squibb, and purified AnxA2-His and AnxA1-His were purchased from EMD Biosciences.

**Cell Lysis and Subcellular Fractionation**—Mouse tissues and cells were lysed in ice cold radiolmmune precipitation assay buffer (50 mM Tris-HCl, pH 7.8, 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) containing a mixture of protease inhibitors (Roche Applied Science). For crude membrane preparations and subcellular fractionation, COS-1

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cells were homogenized in 10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 200 mM sucrose, and a protease inhibitor mixture. The homogenate was centrifuged at 720 × g for 10 min at 4 °C to remove nuclei and cell debris. The resulting supernatant S1 was centrifuged at 15,000 × g for 10 min at 4 °C. The pellet P1, containing organelles such as lysosomes and mitochondria, was solubilized in radioimmuno precipitation assay buffer, and the supernatant S2 was centrifuged at 100,000 × g for 75 min at 4 °C (SW40 rotor and Beckman ultracentrifuge). The resulting crude P2 cell membrane pellet was solubilized in radioimmuno precipitation assay, and the soluble supernatant S3 was kept for Far Western blot analysis. Quantitation of protein concentration was effected by the Bradford protein assay. The supernatant S3 (3 µg of protein) was analyzed by SDS-PAGE and compared with 30-µg protein loads from other subcellular fractions.

Far Western Blot Assays—Lysates (20–30 µg of protein), media, or purified AnxA2-His6 were heated in reducing or non-reducing Laemmli sample buffer, resolved by SDS-PAGE on 8% glycine gels, and electrotransferred onto nitrocellulose membranes (GE Healthcare). Following 1 h of incubation in 5% skim milk in Tris-buffered saline, 0.1% Tween (TBST), the membranes where incubated with conditioned medium of CHO-K1 cells overexpressing either an empty vector (pIRES-V5), PC5A-V5, CHRD-V5, pIRES-D374Y, PCSK9-V5, or its V5-tagged mutants or incubated with purified AnxA2-His6 for 3 h at room temperature. The membranes were then washed in TBST and incubated with the HRP-conjugated anti-V5 or anti-His antibodies and revealed by enhanced chemiluminescence (GE Healthcare). For competition experiments, 10 µg of His-tagged PCSK9 or CHRD were added to the PCSK9-V5 medium before Far Western blotting. For PCSK9 binding requirements with the ~33-kDa protein, 1 mM NaCl, 10 mg/ml heparin, 1 mM NaCl + 10 mg/ml heparin, or 100 mM EDTA were added to the PCSK9-V5 conditioned medium used for Far Western blotting.

Immunoprecipitation and Western Blot Assays—For immunoprecipitation cell lysates were incubated overnight at 4 °C with anti-V5-agarose beads (Sigma) and washed five times with cold lysis buffer. Following addition of reducing Laemmli sample, solubilized proteins were revealed by Western blot or separated by SDS-PAGE (8%) and stained by Coomassie Blue for band excision and mass spectrometry. As control for the immunoprecipitation, antigens complexed with the anti-V5-agarose beads were eluted with the V5 peptide (50 µm, Sigma), separated by SDS-PAGE (8%), and revealed by Western blotting with the anti-V5-HRP antibody.

Western blotting experiments were made on samples that were reduced in Laemmli buffer, heated, and resolved on 8% glycine SDS-PAGE gels. Separated proteins were then electrotransferred onto nitrocellulose and probed with HRP-conjugated anti-V5 or anti-HA tags or with primary antibodies. Bound primary antibodies were detected with corresponding species-specific HRP-labeled secondary antibodies and revealed by enhanced chemiluminescence. Quantitation of band intensity was done with the ImageJ software version 1.37 (Wayne Rasband, National Institutes of Health, Bethesda, MD).

Two-dimensional Gel Electrophoresis and Mass Spectrometry—Two-dimensional gel electrophoresis was performed according to protocols from Ref. 30. COS-1 cells were lysed in 7 M urea, 2 M thiourea, 2% CHAPS, 0.5% immobilized pH gradient (IPG) buffer (carrier ampholyte mixture; GE Healthcare), and 0.002% bromphenol blue. The protein concentration was estimated by the Bradford assay and adjusted to 0.6 µg/ml with the lysis buffer. 40 mM dithiothreitol was then added, and the cell lysates were kept rotating at 4 °C for 60 min. The samples (200 µl) were loaded onto broad pH range (pH 3–10) IPG gel strips (GE Healthcare), and the first dimension isoelectric focusing separation was achieved using an Ettan IPGphor II system (GE Healthcare). For the second dimension SDS-PAGE separation, IPG strips were equilibrated for 15 min in the SDS equilibrium buffer (6 M urea, 75 mM Tris-HCl, pH 8.8, 29.3% glycerol, 2% SDS, 0.002% bromphenol blue) containing 10 mg/ml dithiothreitol and an additional 15 min in the SDS equilibrium buffer containing 25 mg/ml iodoacetamide and applied to 12% SDS gels. The gels were then either stained in Coomassie Blue or transferred on nitrocellulose and processed for Far Western blotting with PCSK9-V5. The signal obtained at ~33 kDa in the Far Western blot was used to establish the position of the band to be excised for mass spectrometry analysis.

For protein identification by liquid chromatography-MS/MS, the bands or spots of interest were cut out from the gel and digested with trypsin (0.1 µg) for 60 min at 58 °C. The peptides were extracted from the gel at room temperature, and the supernatants were transferred into a 96-well plate and then completely dried in a vacuum centrifuge. Before the analysis, the peptides were dissolved under agitation for 15 min in 13 µl of formic acid 0.1%, then sonicated for 5 min, and centrifuged at 2,000 rpm for 1 min. Analysis of the peptide mixture was done by liquid chromatography-MS/MS using a LTQ Orbitrap mass spectrometer configured with an on-line NanoLC-two-dimensional high performance liquid chromatography system (Eksigent, Dublin, CA). Protein identification was obtained from the MS/MS spectra using Mascot analysis software (Matrix Science).

Hexa-His Pulldown Assay—20 µg of purified AnxA2-His6 or AnxA1-His6 or no proteins (for negative control) were immobilized onto a cobalt chelate resin (Thermo Scientific). The resin was then washed several times with 40 mM imidazole and incubated overnight at 4 °C with 800 µl of conditioned medium from PCSK9-V5-transfected CHO-K1 cells containing 40 mM imidazole. The resin was then washed several times with imidazole (40 mM), heated in reducing Laemmli sample buffer, and centrifuged. The supernatants were analyzed by Western blot as described above using the anti-V5-HRP or anti-His-HRP antibodies.

Immunocytochemistry—For immunocytochemistry, the cells were plated on glass-bottomed culture dishes (MatTek) and then transfected the following day. Twenty-four hours post-transfection, the cells were washed with Dulbecco’s modified Eagle’s medium, incubated for an additional 20 h without serum, and then washed three times with PBS. Cell surface labelings were made under nonpermeabilizing conditions by fixation with 3.7% formaldehyde for 10 min at room temperature. The cells were then washed in PBS, incubated for 5 min in 150 mM glycine, washed once in PBS, and incubated for 30 min in 1% bovine serum albumin in PBS. The cells were incubated.
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RESULTS

PCSK9 Binds to a ~33-kDa Protein—Fig. 1A schematically summarizes the various constructs (14) used below. Nitrocellulose blots of various cell line extracts were incubated with the conditioned medium of CHO-K1 cells overexpressing PCSK9-V5, especially in COS-1, BSC40, and HuH7 cell lysates (Fig. 1B, middle panels). As controls, we show that the conditioned medium of CHO-K1 cells transfected with either an empty pIRE-S-V5-EGFP vector or another secreted proprotein convertase PC5A-V5 (29) do not reveal any interacting protein (Fig. 1B, left panels). This suggests that the denatured ~33-kDa protein specifically binds native PCSK9. Subcellular fractionation of COS-1 cells revealed that although the ~33-kDa binding protein is associated with membranes (15,000 × g and 100,000 × g pellets), it is ~10 times more abundant in the soluble 100,000 × g supernatant (Fig. 1B, middle right panel and legend). Using the same approach, mouse tissue extracts revealed that a similar ~33-kDa PCSK9 interactor is found mostly in the small intestine, including ileum and jejunum, but is less abundant in liver, adrenals, and kidney (see large arrow in Fig. 1B, right panel). Note that in liver, the major interacting protein migrates with an apparent molecular mass of ~45 kDa (Fig. 1B, right panel, small arrow).

To identify the PCSK9-interacting domain, we incubated COS-1 cell extracts with various PCSK9 constructs (Fig. 1, A and C). The data showed that the interacting domain of PCSK9 is its C-terminal CHRD, because a construct lacking it (PCSK9-L455X-V5) does not bind to the ~33-kDa protein, whereas the CHRD alone binds at least 3-fold better than the full-length PCSK9 (Fig. 1C, left panels) and effectively competes for this interaction with the full-length protein (Fig. 1D). In agreement, constructs still containing the CHRD, such as those resulting in the furin-cleaved PCSK9 form (aa 219–692), or the uncleavable R218S (26), the non-Tyr sulfated form (Y38F) (1) or the mutant D374Y that strongly binds the LDLR (21), still interact with the ~33-kDa protein (Fig. 1C). Although 100 mM EDTA and 10 mg/ml heparin do not affect the PCSK9’s ability to bind the ~33-kDa protein, incubation in the presence of 1 mM NaCl decreased the binding by ~70%, suggesting that charges are not the only determinants (Fig. 1E). Finally, we note that heparin actually enhances the binding, and its presence largely prevents the effect of NaCl (Fig. 1E).

The ~33-kDa PCSK9 Partner Is Annexin A2—To identify the PCSK9 interactor, we overexpressed either an empty vector (pIRE-S-V5) or PCSK9-V5 in COS-1 cells. Cell extracts were then immunoprecipitated with anti V5-coupled agarose beads, followed by SDS-PAGE separation and mass spectrometry analysis of the protein(s) migrating at ~33 kDa (Fig. 2A, boxed areas). The efficacy of the immunoprecipitation control is shown in Fig. 2B. The mass spectrometric data (not shown) revealed that the ~33-kDa protein is monkey annexin A2 (AnxA2; XP_001155637), which exhibits 99% protein sequence identity to human AnxA2 (AAH09564). Furthermore, this interacting protein is found in the PCSK9-V5-overexpressing cells but not in control cells, suggesting that AnxA2 is the sought PCSK9 interactor.

To further substantiate the nature of the ~33-kDa interactor, we resolved 120 μg of proteins obtained from 1 × 106 COS-1 cells by two-dimensional SDS-PAGE-isoelectric focusing. Proteins separated by two-dimensional gels were either stained with Coomassie Blue (Fig. 2C) or transferred to nitrocellulose and processed for Far Western blotting with PCSK9-V5 (Fig. 2D). The signal obtained coinciding with a ~33-kDa/pl 7.5 protein in the far Western blot (Fig. 2D) was used to localize the exact position of the band to be excised for mass spectrometric analysis (Fig. 2C, boxed area) using tandem MS/MS. Protein data base search using Mascot analysis (Matrix Science) revealed ~77% tryptic peptide coverage with a total Mascot score of 2540, corresponding to human AnxA2. The mass spectral data (Fig. 2E) clearly confirmed that AnxA2 is the major PCSK9-interactor identified following co-immunoprecipitation with PCSK9-V5 in COS-1 cell lysates (Fig. 2A).

Specificity of the Interaction of Native PCSK9 with AnxA2—To confirm that the interaction seen in COS-1 cell extracts can be reproduced by overexpression of AnxA2 in cells that do not
interaction. These data agree with the interaction observed in that no critical disulfide bond(s) in AnxA2 is needed for this and nonreducing conditions (Fig. 3A). Native PCSK9 binds in a dose-dependent manner purified AnxA2 (AnxA2-HA), but not AnxA1, was demonstrated following function of PCSK9 (11, 34) was first tested by overexpressing PCSK9, with or without AnxA2, in CHO-K1 cells. Although the LDLR level was reduced by ~30% in PCSK9-transfected cells, its level returned to that of the control pIRES-PCSK9, with or without AnxA2, in CHO-K1 cells. To determine whether the interaction of PCSK9 with AnxA2 occurs at the external layer of the cell surface, we hypothesized that the Q554E mutation would bind more strongly to PCSK9 toward LDLR degradation. Because the CHRD binds AnxA2, substantially express this protein, we overexpressed human AnxA2 or as control human AnxA1 in CHO cells. Far Western blot analysis confirmed that PCSK9 specifically binds AnxA2 but not the closely related (~33% protein sequence identity) family member AnxA1 (Fig. 3A). This is further confirmed by pulldown assays wherein native AnxA2-His6 bound PCSK9 and its furin cleaved form (PCSK9-A218; not shown) (26), whereas AnxA1-His6 does not (Fig. 3B).

Co-immunoprecipitation of PCSK9-V5 and HA-tagged AnxA2 (AnxA2-HA), but not AnxA1, was demonstrated following their co-expression in CHO-K1 cells (Fig. 3C). Interestingly, the cellular partner of AnxA2, known as p11 (31), did not interfere with the PCSK9-AnxA2 co-immunoprecipitation (Fig. 3C), suggesting that PCSK9 could bind the physiologically observed tetrameric cell surface complex of (p11)2·(AnxA2)2 (31). Native PCSK9 binds in a dose-dependent manner purified AnxA2 and its dimer (Fig. 3D, right panel) under both reducing and nonreducing conditions (Fig. 3D, left panel), suggesting that no critical disulfide bond(s) in AnxA2 is needed for this interaction. These data agree with the interaction observed in the two-dimensional-Far Western analysis performed following iodoacetamide treatment of cell extracts (Fig. 2D). Finally, AnxA2 binds only native PCSK9 or its CHRD, but not their SDS-PAGE denatured forms (Fig. 3E). Thus, the structural integrity of PCSK9 and its CHRD is important for the interaction, whereas that of AnxA2 is not. This suggests that PCSK9 binds to a linear aa stretch of AnxA2.

The CHRD mutation Q554E in PCSK9 leads to lower levels of low density lipoprotein cholesterol (25), indicating a loss-of-function of PCSK9 toward LDLR degradation. AnxA2 is known to be a cytosolic and a membrane-associated protein through phospholipid binding. It is also known to translocate to the cell surface and to associate with diverse extracellular proteins (32, 33). To determine whether the interaction of PCSK9 with AnxA2 occurs at the plasma membrane of CHO-K1 cells (Fig. 5), supporting their co-immunoprecipitation and interaction in a cellular context.

AnxA2 Inhibits the PCSK9-enhanced LDLR Degradation—The effect of the PCSK9-AnxA2 interaction on the LDLR-lowering function of PCSK9 (11, 34) was first tested by overexpressing PCSK9, with or without AnxA2, in CHO-K1 cells. Although the LDLR level was reduced by ~30% in PCSK9-transfected cells, its level returned to that of the control pIRES-transfected cells when PCSK9 was co-transfected with AnxA2 (Fig. 6A). Furthermore, the transfection of HepG2 cells (endogenously expressing PCSK9, but not AnxA2) with AnxA2 alone increased the LDLR level by ~40% and by ~90% when AnxA2 was co-transfected with its accessory protein p11 (Fig. 6B).

To determine whether the PCSK9-AnxA2 interaction can inhibit the PCSK9-induced LDLR degradation from the extra-
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FIGURE 3. Characterization of the interaction of PCSK9 with AnxA2. A, PCSK9-V5 Far Western blot (WB) of lysates of COS-1 cells, wild type (WT) CHO-K1 cells, CHO-K1 cells overexpressing an empty vector (pIRES), AnxA1, or AnxA2. B, polyhistidine pulldown assay. Purified native AnxA2-His₆, or AnxA1-His₆, were immobilized on cobalt chelate beads and incubated with PCSK9-V5. The bound proteins were released from beads in Laemmli sample buffer and analyzed by Western blotting with the anti-V5-HRP or anti-HA-HRP antibodies. Expression of the transfected constructs was analyzed by Western blotting of cell lysates using the anti-V5-HRP or anti-HA-HRP antibodies. C, co-immunoprecipitation experiments of CHO-K1 cells co-transfected with PCSK9-V5 and either AnxA1-HA, AnxA2-HA, or AnxA2-HA and p11. Proteins immunoprecipitated with the anti-V5 mAb were revealed by Western blotting with anti-HA-HRP or anti-V5-HRP antibodies. Expression of the transfected constructs was analyzed by Western blotting of cell lysates using the anti-V5-HRP or anti-HA-HRP antibodies. D, left panels, PCSK9-V5 Far Western blots of purified AnxA2-His₆ in the absence or presence of reducing agents. Note that in nonreducing conditions, PCSK9-V5 binds both the dimer and monomer forms of AnxA2. Right panels, Western blots of the purified AnxA2-His₆ using the anti-AnxA2 mab in nonreducing or reducing conditions. E, upper panel, Media from CHO-K1 cells expressing PCSK9-V5 or the CHRD-V5 were separated by SDSPAGE (8%), transferred onto nitrocellulose, incubated with purified AnxA2-His₆, and probed with the anti-His-HRP antibody. Lower panel, The presence of overexpressed PCSK9 and CHRD in CHO-K1 cell media was verified by Western blotting using anti-V5-HRP.

FIGURE 4. The PCSK9 natural loss of function mutant Q554E strongly binds AnxA2. A, Western blot (WB) analysis of conditioned medium from CHO-K1 cells overexpressing PCSK9-V5 or its natural mutant Q554E-V5 used for Far Western blotting (FWB). B, Far Western blots of extracts from COS-1, CHO-K1, or AnxA2-transfected CHO-K1 cells with the PCSK9-V5 or Q554E-V5 conditioned media of CHO-K1 cells analyzed in A. The relative intensity of the binding of PCSK9-V5 (taken as 1 ×) or of the Q554E-V5 mutant (three times higher) to AnxA2 was calculated and normalized with respect to β-actin.

The addition of exogenous AnxA2 reduced the ability of PCSK9 to enhance LDLR degradation (from ~30 to ~10%; Fig. 6C). Note that the addition of exogenous AnxA2 to nontransfected cells also increased the level of LDLR, likely because of its effect on endogenous PCSK9 in HepG2 cells.

Finally, the addition of 1 μg of purified PCSK9 to CHO-K1 cells overexpressing the LDLR at 4 °C (preventing internalization) resulted in a visible cell surface localization of PCSK9 (Fig. 6D), and the latter was reduced by the further addition of either 5 μg or even more so 20 μg of purified AnxA2 (Fig. 6, E and F). Therefore, we deduce that because of its interaction with the CHRD, AnxA2 reduces the level of cell surface PCSK9 likely bound to the LDLR.

The effect of the PCSK9-AnxA2 interaction on the LDLR-lowering function of PCSK9 was also analyzed by immunofluorescence under nonpermeabilizing conditions to overcome problems associated with the low transfection efficiency of HepG2 cells and subsequent detection of small changes in total protein levels by Western blot. Expression of AnxA2 alone or together with p11 led to a strong increase in labeling intensity of cell surface LDLR (Fig. 7, B and C) compared with control pIRES-transfected cells (Fig. 7A). Overexpression of PCSK9 in HepG2 cells appreciably reduces the levels of cell surface LDLR, as compared with control (Fig. 7, A and D). Finally, expression of AnxA2 with either wild type PCSK9 or the gain of function mutant PCSK9-D374Y (Fig. 7F, inset) prevented their LDLR-lowering effect (Fig. 7, D–F).

shRNA Knockdown of AnxA2 Enhances LDLR Degradation in HuH7 Cells—Stable (Fig. 8A) or transient (Fig. 8B) transfection of HuH7 cells with either an AnxA2-shRNA or a control one resulted in a ~60–70% knockdown of AnxA2, as compared with control, and a reduction of ~40–70% of the LDLR levels (Fig. 8, A and B). These data support the notion that in HuH7 cells endogenous AnxA2 can inhibit the PCSK9-enhanced degradation of the LDLR. HEK293 cells (expressing negligible amounts of PCSK9 mRNA, as compared with either HepG2 or HuH7 cells (supplemental Fig. S1)) were transfected with AnxA2 or AnxA1 cDNAs with or without p11 (Fig. 8C) or with shRNAs (Fig. 8D). None of these treatments affected the levels of endogenous LDLR in HEK293 cells. This result substantiates the specific relation between AnxA2 and PCSK9 in LDLR regulation.
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Far Western blots of these cell lysates using PCSK9-V5 revealed that deletion of the N-terminal segment aa 2–24 of AnxA2, which is known to be necessary for binding to p11 and tissue plasminogen activator and to have other functions such as membrane bridging (31, 35), does not affect binding to PCSK9 (Fig. 9B). However, deletion of the first annexin-like repeat R1 of AnxA2 completely abrogated the interaction with PCSK9-V5 (Fig. 9, A and B) or CHRD-V5 (not shown), whereas deletions of the three other repeats (R2, R3, and R4) had no major effect (Fig. 9, A and B). Thus, the R1 repeat seems to be the major domain of AnxA2 implicated in PCSK9 binding. Comparison of the primary sequences of the R1 domain of AnxA2 and AnxA1 (which does not bind PCSK9; Fig. 3) indicated areas of divergent sequences (Fig. 9D). In initial screens, replacement of the AnxA2 second loop sequence \textsuperscript{80}KE\textsubscript{84} with the corresponding AnxA1 loop sequence \textsuperscript{80}GKPL\textsubscript{84} had no appreciable effect on PCSK9 binding (Fig. 9C). However, when the \textsuperscript{77}RTT\textsubscript{78}K sequence was mutated to \textsuperscript{77}AAT\textsubscript{78}K or \textsuperscript{77}AA\textsubscript{78}AAA, Far Western blots using PCSK9-V5 showed that the integrity of the positively charged sequence \textsuperscript{77}RTT\textsubscript{78}K is critical for the AnxA2-PCSK9 binding (Fig. 9C). Replacing the \textsuperscript{77}RTT\textsubscript{78}KE\textsubscript{84} sequence by \textsuperscript{77}AA\textsubscript{78}AT\textsubscript{79}A\textsubscript{80}E\textsubscript{81}L\textsubscript{82}A\textsubscript{83}A\textsubscript{84} also abolished binding of AnxA2 to PCSK9 (Fig. 9C). Because the mutation of Lys\textsubscript{80} by Gly\textsubscript{80} did not affect binding, this may mean that the critical motif is \textsuperscript{77}RRXX\textsubscript{81}. Finally, replacement of the relatively conserved segment 49–75 (Fig. 9, C and D) of AnxA2 by the corresponding one of AnxA1 (aa 58–84) also abolished binding to PCSK9 (Fig. 9C). Thus, the interaction of PCSK9 with the R1 domain of AnxA2 may be complex, requiring more than one structural characteristic for optimal binding.

**DISCUSSION**

The mechanism of LDLR trafficking, endocytosis, and recycling has been widely studied and recently reviewed (36–38). However, since the discovery of PCSK9 (1), the mechanism of LDLR degradation (3, 4, 23, 34) is being increasingly revised. The present prevailing hypothesis is that the in trans interac-
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Like domain A complex, the interaction involves the catalytic subunit of PCSK9, and the critical Ser\(^{153}\) (23), which becomes the N terminus of PCSK9 upon autocatalytic cleavage of proPCSK9 at the \(\text{H}^{149}\text{FAQ} \downarrow \text{I}^{155}\) sequence (3, 39). However, natural mutations in the prosegment or the CHRD of PCSK9 also result in modifications of the plasma cholesterol profiles in humans (11, 24, 25), indicating the presence of other determinants within these domains that can modulate circulating LDL cholesterol levels.

Annexins are part of a family of \(\text{Ca}^{2+}\)-dependent, anionic phospholipid-binding proteins, which in vertebrates is composed of 12 members (40). Annexins contain a highly conserved protein core composed of four repeats harboring calcium-binding sites necessary for their membrane association capability. The N terminus of AnxA2 (aa 1–24) mediates heterotetramer formation with the S100 protein p11 (S100A10) that enhances membrane phospholipid binding affinity resulting in the association of the protein complex with the plasma membrane (41). AnxA2 lacks a signal peptide but has nevertheless been identified on the extracellular surface of various cell types. The mechanism regulating this translocation is not known but is dependent on both expression of p11 and tyrosine phosphorylation of AnxA2 and might involve localization to caveolae (42–44). The presence of AnxA2 on the extracellular face of the plasma membrane has been demonstrated in endothelia (45), skin keratinocytes (46), epithelial cells (47, 48), and many tumor cells (49). Extracellular AnxA2 has been described as a membrane-associated receptor for a number of different proteins. Its interaction with tissue-type plasminogen activator on endothelial cell surfaces is critical (33), as demonstrated by AnxA2 knock-out mice, which are deficient in plasminogen processing into plasmin and neo-angiogenesis (50).

The data presented in this work revealed that the activity of PCSK9 on LDLR can be inhibited by AnxA2 via its binding to...
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CHRD. The CHRD and the prosession provide structural integrity to the whole PCSK9 molecule, allowing it to interact with the LDLR through its catalytic domain (21, 23). Thus, we surmise that binding of AnxA2 to the CHRD must somehow cause allosteric structural changes in the catalytic subunit of PCSK9, hampering its ability to enhance the degradation of the LDLR. Our data revealed that at 4 °C soluble AnxA2 reduces the level of cell surface PCSK9 (Fig. 6, D–F), suggesting a reduced ability to bind LDLR. In agreement, overexpression of AnxA2 increases the cell surface expression of the LDLR in HepG2 cells (Fig. 7B). Whether the PCSK9-AnxA2 complex is internalized in endosomes/lysosomes and/or the CHRD-AnxA2 interaction prevents the internalization of the PCSK9-LDLR complex is not known. Furthermore, it is yet to be determined whether PCSK9 remains associated with its prosesson upon binding to AnxA2, as it does when it binds the LDLR (23).

Analysis of the mRNA expression of AnxA2 in human cell lines by quantitative real time PCR revealed that it is highly expressed in most cells, with endothelial cells (e.g. HUVEC) being one of the richest source (supplemental Fig. S1). Although PCSK9 mRNA levels in HuH7 cells are similar to those in HepG2 cells, those of AnxA2 are ~2-fold higher in HuH7 cells. However, AnxA2 protein is much more expressed in HuH7 cells (not shown), in agreement with the Far Western data (Fig. 1B, middle panel). A similar analysis performed on mouse tissues revealed that PCSK9 is richest in liver, colon, ileum, duodenum, and kidney (13). Although not forming a perfect mirror image, AnxA2 mRNA levels peak in jejunum, the region of the small intestine that exhibits the lowest PCSK9 levels, and reach very low levels in liver, the richest source of PCSK9 (supplemental Fig. S2). This agrees with the weak interaction of PCSK9 with a ~33-kDa AnxA2-like protein in liver extracts (Fig. 1B, right panel). AnxA2 is very abundant at the surface of endothelial cells, including those of lungs, small intestine, and adrenals. This may explain the inability of high concentrations of injected/infused PCSK9 to degrade the LDLR in adrenals (51), as well as the rapid turnover of PCSK9, which is only in part dependent on the presence of the LDLR (51). On the other hand, exogenous PCSK9 is efficient in lowering the LDLR levels in liver. However, this does not exclude the possibility that other liver proteins may interact with PCSK9, as evidenced by the ~45-kDa protein detected by Far Western (Fig. 1B, right panel). The inability of PCSK9 to degrade the LDLR in fibroblasts and COS-7 cells (22) may also be due to their high expression of AnxA2 (52).

Our study is the first report on the presence of an endogenous inhibitor of PCSK9. We demonstrated the association of the CHRD of PCSK9 with AnxA2, providing evidence for a new regulatory pathway that inhibits the PCSK9-enhanced LDLR degradation. To our knowledge, the specific binding of a protein to the AnxA2 R1 domain has not been reported before. Furthermore, the identification of the R1 domain of AnxA2 that mediates binding to PCSK9 provides a potential new peptide-derived tool to regulate PCSK9 function. Derivatives or mimics of this segment could be used to inhibit PCSK9 function on LDLR and possibly on other targets, such as VLDLR or ApoER2 (19). The peptide could also be used to derive a co-crystal structure to better define the CHRD residues, including Gln554, seemingly implicated in this interaction (Fig. 4B). The CHRD is composed of six β-strand structures repeated three times and hence forming three subdomain modules: M1, M2, and M3 (21). The Gln554 residue is in an exposed loop within the CHRD and is unique to the second subdomain module M2 (21). It has been suggested that the CHRD forms a possible receptor-like structure that shares similarity to the homotrimeric hormone resistin (24), which regulates insulin secretion. However, resistin did not interact with AnxA2 as assessed by Far Western, nor did it compete with PCSK9 for binding to AnxA2 (not shown). The interaction of the R1 domain of AnxA2 with the predicted groove-like structure of the CHRD (24) or a neighboring site(s) needs further investigations.

The impact of AnxA2 on LDL cholesterol metabolism remains to be assessed in vivo. Future studies using AnxA2
knock-out mice (50) would be valuable in this respect. In conclusion, our study provided evidence for a new way to fine tune the activity of PCSK9 on the LDLR via AnxA2 interaction. This opens the way to the identification of other PCSK9-binding proteins that may further regulate its function in a temporal and/or tissue-specific manner. These may provide new therapeutic strategies to regulate the level of circulating LDL cholesterol and hence slow down the development of coronary heart disease.

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