Identification of the (Pro)renin Receptor as a Novel Regulator of Low-Density Lipoprotein Metabolism

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Rationale: **The (pro)renin receptor ([P]RR) interacts with (pro)renin at concentrations that are >1000× higher than observed under (patho)physiological conditions. Recent studies have identified renin–angiotensin system–independent functions for (P)RR related to its association with the vacuolar H^+-ATPase.**

Objective: **To uncover renin–angiotensin system–independent functions of the (P)RR.**

Methods and Results: **We used a proteomics-based approach to purify and identify (P)RR-interacting proteins. This resulted in identification of sortilin-1 (SORT1) as a high-confidence (P)RR-interacting protein, a finding which was confirmed by coimmunoprecipitation of endogenous (P)RR and SORT1. Functionally, silencing *(P)RR* expression in hepatocytes decreased SORT1 and low-density lipoprotein (LDL) receptor protein abundance and, as a consequence, resulted in severely attenuated cellular LDL uptake. In contrast to LDL, endocytosis of epidermal growth factor or transferrin remained unaffected by silencing of the (P)RR. Importantly, reduction of LDL receptor and SORT1 protein abundance occurred in the absence of changes in their corresponding transcript level. Consistent with a post-transcriptional event, degradation of the LDL receptor induced by (P)RR silencing could be reversed by lysosomotropic agents, such as bafilomycin A1.**

Conclusions: **Our study identifies a renin–angiotensin system–independent function for the (P)RR in the regulation of LDL metabolism by controlling the levels of SORT1 and LDL receptor. (*Circ Res*. 2016;118:222-229. DOI: 10.1161/CIRCRESAHA.115.306799.)**

Key Words: cholesterol homeostasis ■ endocytosis ■ LDL receptors ■ renin–angiotensin system ■ sortilin

The (pro)renin receptor [(P)RR] has been implicated as a receptor for renin/prorenin (denoted as [pro]renin)-stimulated signaling, and plays a role in local renin–angiotensin system activation by nonproteolytically activating bound prorenin.[1] In experimental assays (pro)renin-(P)RR signaling results in extracellular signal-regulated kinase 1/2 (Erk1/2) activation, and as a consequence upregulation of profibrotic factors, such as transforming growth factor β, collagen, and fibronectin.[2–6] However, the physiological relevance of the (pro)renin–(P)RR interaction is questionable because the (pro)renin concentrations required are >1000× higher than observed under (patho) physiological conditions.[7,8] Recently, (pro)renin-independent functions for (P)RR have been reported, including a function as an accessory protein of the vacuolar H^+-ATPase (V-ATPase).[9] V-ATPases are multisubunit complexes, and they are expressed virtually in all cells types. They play an important role in protein trafficking, receptor recycling, and lysosomal degradation by acidifying intracellular compartments.[10,11] Depletion of the (P)RR results in decreased protein levels of V-ATPase subunits, impaired acidification of intracellular compartments, and defects in autophagy.[12–14] V-ATPases are also found at the plasma membrane in certain cell types, such as intercalated cells of the collecting duct. Accordingly, we previously reported that the (P)RR is required for both prorenin-dependent and prorenin-independent regulation of V-ATPase activity in collecting duct cells.[15] The (P)RR has been also recently implicated in canonical Wnt and PCP signaling,[16–18] emphasizing the notion that our understanding of (P)RR function remains incomplete.

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To address this, we used an unbiased proteomics approach to discover potential novel functions of the (P)RR. We mapped the (P)RR-interactome and identified sortilin 1 (SORT1) as

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Nonstandard Abbreviations and Acronyms	
EGF	epidermal growth factor
Erk1/2	extracellular signal-regulated kinase 1 and 2
LDL	low-density lipoprotein
LDLR	low-density lipoprotein receptor
(P)RR	(pro)renin receptor
RAS	renin–angiotensin system
SORT1	sortilin 1
TRAF4	tumor necrosis factor receptor–associated factor 4
V-ATPase	vacuolar H^+-ATPase

a novel (P)RR-interacting partner. We show that the (P)RR post-transcriptionally controls protein abundance of SORT1, and unexpectedly that of the low-density lipoprotein receptor (LDLR). We demonstrate that as a consequence LDL uptake in several cell types is sensitive to (P)RR levels. Collectively, our results indicate that the (P)RR represents a previously unrecognized regulator of LDL metabolism.

Methods

A brief description of the methods is provided below. For a detailed description of the methods please refer to the Online Data Supplement.

Cell Culture and Transfections

HEK293, A431, Huh7, and HepG2 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C and 5% CO_2. LDLA7 Chinese hamster ovary (CHO) cells were kindly provided by Dr Monty Krieger (MIT, Cambridge, MA)[19] and maintained in DMEM/F12 supplemented with 5% fetal bovine serum, 100 U/mL penicillin, and 100 mg/mL streptomycin at 37°C and 5% CO_2. For both siRNA and plasmid transfection, HEK293 cells were transfected with lipofectamine 2000 according to the manufacturer's protocol. Unless indicated otherwise, cells were cultured in sterol-depleted medium (DMEM supplemented with 10% bovine lipid-deficient serum (5 μg/mL simvastatin, and 100 μmol/L mevalonate), to increase LDLR expression, for 18 hours before experiment. A431 and LDLA7 CHO cells were transfected with 40 nmol/L siRNAs by JetPrime, and HepG2 cells were transfected with 40 nmol/L siRNAs by RNAiMax after the manufacturer's protocols. For plasmid transfection, HepG2 cells were transfected with JetPrime using the manufacturer's protocol.

RNA Isolation and Quantitative Polymerase Chain Reaction

Total RNA was isolated from cells using TRIzol after the manufacturer's protocol. One milligram of total RNA was reverse transcribed with the iScript reverse transcription kit (Bio-rad). SYBR Green real-time quantitative polymerase chain reaction assays were performed on a Lightcycler 480 II apparatus (Roche) using SYBR Green master mix (Roche). Gene expression was normalized to the expression of 36B4, and expressed as mean±SEM. Primers are listed in Online Table I.

Immunoprecipitation and Immunoblotting

For coimmunoprecipitations, HEK293 cells were lysed in intraperitoneal lysis buffer. Cell lysates were cleared by centrifugation at 1000*g* for 5 minutes at 4°C, and protein content was measured with the bicinchoninic acid (BCA) assay. Of each lysate, 1 mg of protein was precleared with 50 μL prewashed ProtA Dynabeads for 1 hour at 4°C, and then nutated for 1 hour at 4°C with 9 μg antih(P)RR antibodies, anti-SORT1 antibodies, or nonspecific rabbit IgGs as control, coupled to 50 μL Protein-A Dynabeads. For protein expression studies, A431 and HepG2 cells were lysed in radioimmunoprecipitation assay (RIPA) buffer. Lysates were cleared by centrifugation at 10,000*g* at 4°C for 5 minutes, and protein contents were measured using the BCA assay. For immunoblotting, immunocomplexes, or lysates containing an equal amount of proteins (10–25 μg) were resolved by SDS-PAGE and probed using the primary antibodies listed in Online Table II. Subsequently, HRP (horseradish peroxidase)-conjugated goat antimouse or goat anti-rabbit antibodies were added and detected with ECL (enhanced chemiluminescence).

LDL Uptake Assays

LDL uptake was measured using DyLight 488-labeled LDL, as described previously.[20] Briefly, HepG2 or A431 cells were incubated in sterol-depleted medium for 16 hours before adding LDL. Cells were incubated with 5 μg/mL DyLight488-labeled LDL in DMEM supplemented with 0.5% BSA for 3 hours at 37°C or 4°C. Subsequently, cells were washed twice with ice-cold PBS supplemented with 0.5% BSA and lysed in RIPA buffer. LDL uptake was determined by quantification of the fluorescence signal on a Typhoon imager (GE Healthcare) and corrected for the protein content in the lysate as determined with the BCA assay.

Measurement of Cell Surface LDLR by FACS

Surface LDLR density after knocking down the (P)RR was measured as described before.[20] Briefly, cells were maintained on sterol-depleted medium for 16 hours before the experiment, dissociated with TrypLE Express and incubated in fluorescence-activated cell sorting (FACS) blocking buffer on ice for 30 minutes. Next, 100 000 cells were stained in 50-μL FACS buffer containing PE-conjugated anti-LDLR antibody for 1 hour on ice. After 3 washes, cells were directly analyzed on an FACS Calibur (BD Biosciences).

Statistical Analysis

Data are presented as mean±SEM. One-way ANOVA followed by the Bonferroni correction was performed for comparison of >2 groups. Student's *t* test was performed for comparison of 2 groups. $P < 0.05$ were considered significant.

Results

(P)RR-Interactome Reveals Potential Novel Functions of the (P)RR

The (P)RR has been recently implicated in cellular functions unrelated to its ability to bind (pro)renin.[21] To identify such functions, we mapped the (P)RR-interactome in HEK293 cells using a TAP-based approach (Online Figure I). We identified 40 proteins that copurified with N-terminally TAP-tagged (P)RR in 2 independent purifications (Online Table III), but not with the tag only. In this set of proteins we found several V-ATPase components, including those known to interact with (P)RR,[17] which validates our proteomics approach and suggests that the N-terminally TAP-tagged (P)RR is at least functional for interacting with the V-ATPase. To better interpret the profile, we compared it with CRAPome, a recently published database of contaminants in currently 343 affinity purification-mass spectrometry profiles (www.crapome.org).[22] Many proteins found in our profile, especially transporters, are also present at high frequency in other purifications in the CRAPome. Apart from V-ATPase subunits, we found several (P)RR-interacting partners with low frequency in CRAPome that are involved, among others, in signal transduction, lipid metabolism, mitochondrial transport, and protein folding.

From the identified proteins, SORT1 is the candidate with the highest Mascot score, and it is not found in the CRAPome. Tumor necrosis factor receptor–associated factor 4 (TRAF4), despite having a Mascot score in the lower range was another attractive candidate, as it can mediate activation of Erk1/2, Akt, and Wnt/β-catenin signaling,[23–25] all of which have been

recently suggested to be modulated by (P)RR. This screen was performed with a heterologous construct, and we therefore first wanted to validate the interactions of these proteins with endogenous (P)RR. Thus, we tested if SORT1 and TRAF4 interact with endogenous (P)RR by coimmunoprecipitation in HEK293 cells. We found that SORT1, but not TRAF4, coimmunoprecipitated with endogenous (P)RR (Figure 1A). Conversely, (P)RR also coimmunoprecipitated with endogenous SORT1 (Figure 1B), establishing the (P)RR–SORT1 interaction. To determine the function of the (P)RR–SORT1 interaction, we first tested the consequence of silencing the (P)RR on SORT1. Unexpectedly, this resulted in a 42% decrease in SORT1 protein abundance, reminiscent of that seen with other V-ATPase subunits (Figure 1C and 1D).[12] Importantly, this occurred in the absence of changes in *SORT1* transcript level (Figure 1E). In addition, in agreement with the absence of an interaction between (P)RR and TRAF4 we observed no effect of silencing (P)RR on TRAF4 levels (not shown). Our proteomic screen therefore resulted in identification of the (P)RR as a post-transcriptional regulator of SORT1.

(P)RR Regulates Cellular LDL Uptake

SORT1 is implicated in several cellular functions,[26,27] and it has recently been identified as an important determinant of LDL metabolism,[28–30] and of circulating levels of LDL in humans.[29,31] The identification of a functional interaction between (P)RR and SORT1 led us, therefore, to test the role of the (P)RR in LDL metabolism. As SORT1 can also directly contribute to LDL uptake by cells,[28,30] we first tested whether the (P)RR affects LDL uptake in A431 and HepG2 cells, 2 cell types that display high LDLR abundance after sterol depletion. In A431 cells, reducing (P)RR mRNA levels (by ≈90%) resulted in a 2-fold decrease in LDL uptake (Figure 2A–2C). To rule out the possibility that this is an off-target effect, we silenced *(P)RR* expression in these cells using 2 additional siRNAs. Both siRNAs reduced LDL uptake to the same extent (Online Figure IIA), confirming that the decrease in LDL uptake is because of reduced (P)RR expression. Similar to A431 cells, silencing *(P)RR* expression in HepG2 cells decreased uptake of LDL by 40% (Figure 2D). Silencing (P)RR also reduced LDL uptake in A431 and HepG2 cells when these were cultured in complete medium (ie, medium containing lipoproteins), even though overall uptake was much lower when compared with that in sterol-depleted cells (Online Figure IIB and IIC). Furthermore, the effect of silencing (P)RR seems to be time-dependent, as LDL uptake was further decreased when silencing was extended to 72 hours (Online Figure IID). Silencing (P)RR also reduced LDL uptake in Huh7 cells to a similar extent as in HepG2 cells (Online Figure IIE). Taken together, these data show that (P)RR depletion affects LDL metabolism in multiple cell lines, suggesting that the (P)RR is a common regulator of LDL metabolism.

Endocytosis of LDL via the LDLR pathway is a clathrin-dependent process.[32] Therefore, decreased LDL uptake in response to (P)RR depletion may reflect a gross perturbation of clathrin-dependent endocytosis. To test this possibility, we

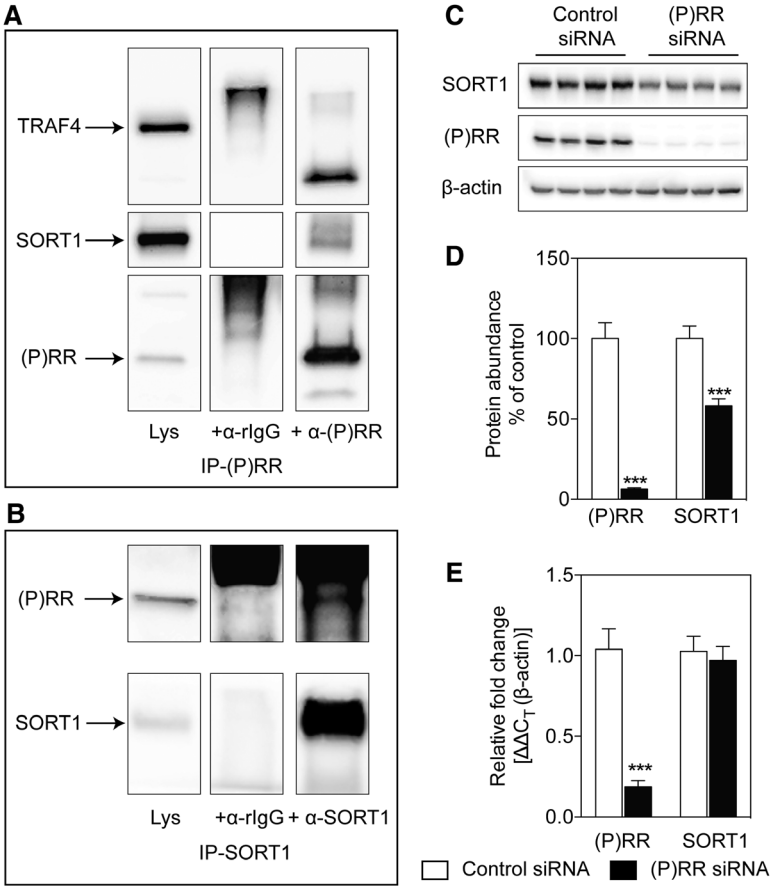

Figure 1. (Pro)renin receptor ([P]RR) interacts with sortilin-1 (SORT1) and regulates its protein abundance. **A** and **B**, Representative blot of 2 independent experiments showing coimmunoprecipitation of (P)RR with SORT1, but not with tumor necrosis factor receptor–associated factor 4 (TRAF4). Total lysates from HEK293 cells were immunoprecipitated with antibodies against (**A**) the (P)RR or (**B**), SORT1 or with rabbit IgGs (rIgG) as negative control and immunoblotted as indicated. **C**, HEK293 cells were transfected with control or (P)RR siRNA for 48 hours. Total lysates were immunoblotted and a representative of 2 independent experiments in duplicate is shown. **D**, (P)RR and SORT1 protein abundance was quantified and normalized to the level of β-actin in the same lysate. Each bar and error represent the (P)RR and SORT1 protein abundance relative to that in control siRNA transfected±SEM (n=8). ***P<0.001. **E**, (P)RR silencing in HEK293 cells successfully suppresses (P)RR mRNA levels, and does not affect SORT1 mRNA levels; n=6, ***P<0.001.

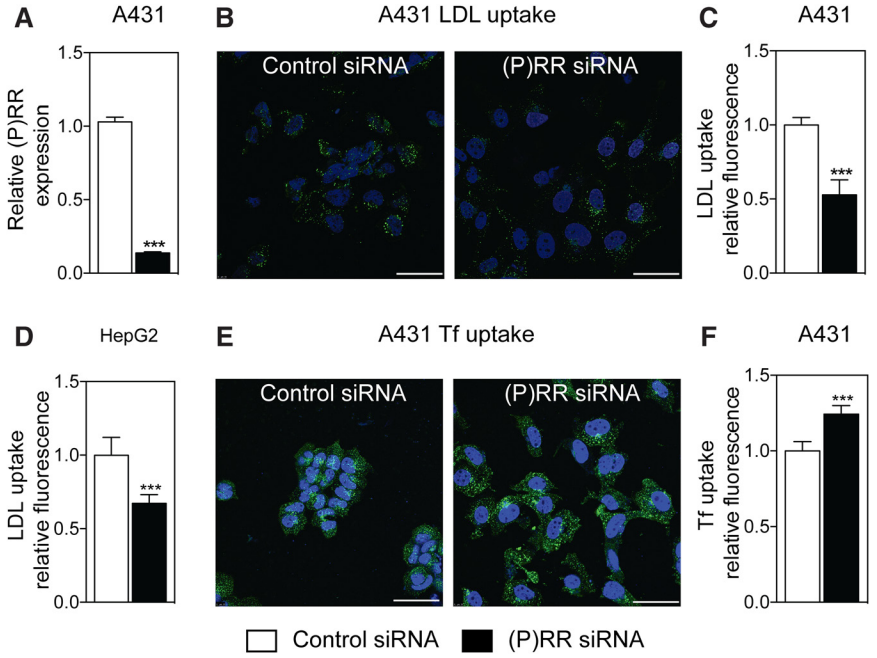

Figure 2. (Pro)renin receptor ([P]RR) silencing reduces low-density lipoprotein (LDL) uptake without grossly affecting endocytosis. A, A431 cells transfected with (P)RR or control siRNAs for 48 hours. Expression of *(P)RR* was determined by quantitative polymerase chain reaction. **B**, A431 cells were treated as in **A**. Subsequently, cells were cultured with Dylight488-labeled LDL for an additional 3 hours. Representative fluorescence images of cells are shown. Nuclei are counterstained with 4′,6-diamidino-2-phenylindole (blue). Scale bar, 10 μm. **C** and **D**, Quantitative measurement of LDL uptake in (**C**) A431 cells, and (**D**) HepG2 cells after (P)RR silencing. **A–F**, Open and closed bars represent control siRNA and (P)RR siRNA, respectively. Each bar and error are the mean±SEM (n=6). ***P<0.001. **E**, A431 cells were treated as in **A** and incubated with fluorescent-labeled transferrin (Tf). Representative fluorescence images are shown. **F**, Quantitative measurement of fluorescence-labeled Tf uptake in A431 cells; n=6, ***P<0.001.

studied the uptake of 2 ligands that are taken up via a clathrin-dependent pathway, namely transferrin and epidermal growth factor (EGF) that are ligands for the transferrin receptor and EGF receptor, respectively. Silencing of *(P)RR* expression in A431 cells did not influence abundance of either the transferrin receptor or the EGF receptor (Online Figure IIIA). Consistent with this, and in contrast to the observed effects on LDL uptake, transferrin uptake in A431 and HepG2 cells was not decreased (Figure 2E and 2F; Online Figure IIIB). In fact, transferrin uptake was slightly increased in A431 cells after (P)RR silencing (Figure 2F). A431 cells contain high levels of the EGF receptor, and incubating these cells with Alexa488-labeled EGF for 15 minutes at either low (100 ng/mL) or high (500 ng/mL) levels,[33] allowed us to evaluate both clathrin-dependent and clathrin-independent internalization pathways, respectively. Irrespective of the dose, silencing of *(P)RR* expression did not affect EGF uptake in A431 cells (Online Figure IIIC and IIID). Taken together, these results indicate that (P)RR depletion does not grossly attenuate clathrin-dependent endocytosis and that (P)RR differentially affects endocytosis of cargo, largely attenuating LDL uptake yet sparing EGF and transferrin internalization.

(P)RR Controls Stability of the LDLR Protein and LDL Uptake

Consistent with our initial observation in HEK293 cells, silencing of *(P)RR* expression in A431 and HepG2 cells also decreased the abundance of endogenous SORT1 protein (Figure 3A and 3B). Recent studies from the Rader Laboratory have shown that SORT1 can directly bind LDL and mediate LDL internalization in hepatocytes[28] and macrophages.[34] Hence, a simple explanation for the reduction of LDL uptake in (P)RR-depleted cells would be reduced SORT1 abundance. To test this we made use of LDLA7 cells, which is a CHO-derived cell line that lacks functional LDLR resulting in strongly diminished uptake of LDL.[19,35] Similar to the other cell lines, silencing *(P)RR* expression reduced SORT1 abundance in LDLA7 cells, and vice versa, implying that a functional (P)RR–SORT1 interaction does not require the presence of the LDLR (Figure 3C). Overall LDL uptake in LDLA7 cells is low, but nevertheless silencing *(P)RR* or *SORT1* significantly reduced LDL uptake in these cells. However, this reduction was minimal and attenuated in comparison with the effect that silencing these genes had in the other cells tested (Figure 3D, and compare with Figure 2C and 2D), suggesting that the LDLR is necessary for the large effect of (P)RR on LDL uptake.

Having ruled out the possibility that the effect of si(P)RR on LDL uptake is largely because of direct SORT1-mediated LDL uptake, we considered involvement of the LDLR pathway in this phenotype. In fact, under the sterol depletion regimen and the LDL concentration (5 μg/mL) we used in these experiments, it is highly likely that the primary entry portal for LDL internalization would be the LDLR, and not SORT1. In line with this, we found that next to reducing SORT1 levels, silencing of *(P)RR* also reduced total LDLR abundance (Figures 3A and 3B; Online Figure IVA and IVB) and LDLR cell-surface density (Figures 3E and 3F) without affecting *SORT1* and *LDLR* mRNA expression (Online Figure IVC and IVD).

Unexpectedly, in these experiments we found that silencing *SORT1* also effectively reduced cellular LDLR abundance to an extent comparable with that achieved by *(P)RR* silencing, and as a consequence also resulted in attenuated LDL uptake (Figure 3G; Online Figure IIB–IIE). Despite the existence of strong evidence linking SORT1 to LDL-cholesterol metabolism in humans,[28,29,31] to the best of our knowledge there is no report indicating that SORT1 can affect LDLR levels. Therefore, in view of the mutual effect (P)RR and SORT1 have on the LDLR and on each other's protein level we reasoned that the 2 may act in concert to control LDLR function. In support of

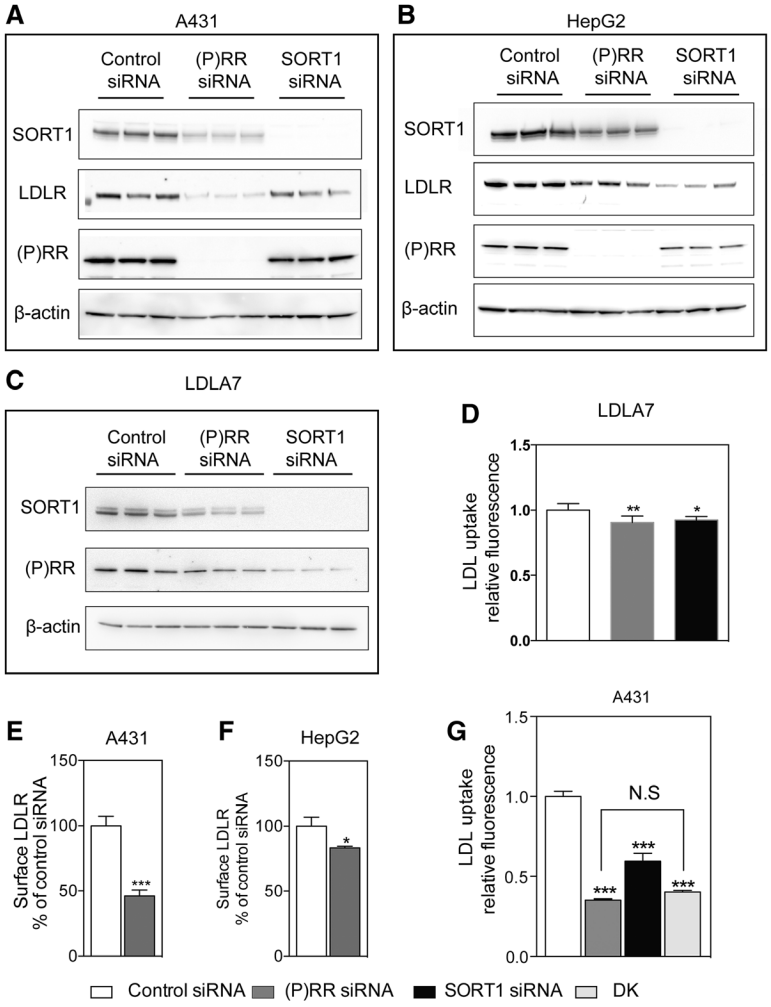

Figure 3. The (Pro)renin receptor ([P]RR)–sortilin-1 (SORT1) interaction controls low-density lipoprotein (LDL) receptor (LDLR) protein stability and LDL uptake. A and **B**, A431 (**A**) or HepG2 (**B**) cells were transfected with control, (P)RR, or SORT1 siRNAs for 48 or 72 hours, respectively. Total cell lysates were immunoblotted as indicated and a representative blot of at least 3 independent experiments is shown. **C** and **D**, LDLA7 CHO cells were transfected with control, (P)RR, or SORT1 siRNA for 48 hours. Total cell lysates were immunoblotted as indicated and a representative blot of 3 independent experiments is shown. **D**, LDLA7 CHO cells were treated as indicated above, and subsequently cultured with Dylight488-labeled LDL for an additional 3 hours. Quantitative measurement of LDL uptake is shown; n=9, **P*<0.05, ***P*<0.01. **E** and **F**, LDLR at the cell-surface was measured by FACS (fluorescence-activated cell sorting) in (**E**) A431 and (**F**) HepG2 cells. Open and closed bars represent control and (P)RR siRNA, respectively (n=6). **P*<0.05; ****P*<0.001. **G**, A431 cells were transfected with control, (P)RR, SORT1 siRNA, or both ([P]RR+SORT1 siRNA; DK) for 48 hours. Subsequently, cells were cultured with Dylight488-labeled LDL for an additional 3 hours. Quantitative measurement of LDL uptake is shown; n=9, ****P*<0.001.

this concept, we found that in both HepG2 and A431 cells combined silencing of *(P)RR* and *SORT1* did not result in an additive reduction in LDL uptake when compared with depletion of the (P)RR or SORT1 alone (Figure 3G; Online Figure IIB and IIC). Furthermore, we found that overexpression of SORT1 in HepG2 cells mildly increased protein abundance of (P)RR and LDLR (Online Figure V). However, this was not sufficient to overcome degradation of the LDLR induced by silencing (P)RR, indicating that this outcome is not solely dependent on reduced SORT1 protein. These results point toward the (P)RR acting as a post-transcriptional regulator of the LDLR and consistent with this notion 3 different lysosomotropic agents, bafilomycin A1 (BafA1), NH_4Cl, and chloroquine rescued LDLR levels after *(P)RR* silencing (Figure 4). None of these lysosomal blockers reversed the degradation of SORT1 after *(P)RR* silencing. We therefore tested alternative degradation pathways that may explain the decrease in SORT1 protein. Unexpectedly, blocking the 2 other major degradation pathways, autophagy, and the proteasome (using 3-Methyladenine or MG132, respectively), also failed to rescue SORT1 protein (Online Figure VI). Therefore, the mechanism underlying reduced SORT1 protein after silencing of (P)RR remains currently unknown.

A plausible explanation for reduced LDLR levels in *(P)RR*-depleted cells is that the receptor is subject to accelerated internalization from the plasma membrane and subsequent degradation. We tested this idea by following surface-biotinylated LDLR in control and (P)RR-depleted cells. In these experiments, the cells were initially sterol depleted to increase abundance of the LDLR, and disappearance of surface-biotinylated LDLR was followed over time by addition of medium, which either contained or lacked lipoproteins. In the absence of added lipoproteins, disappearance of labeled LDLR was negligible (not shown). Similarly, when lipoprotein-containing medium was added, even though effective *(P)RR* silencing reduced total cellular LDLR levels, disappearance of the LDLR or transferrin receptor (as control) from the plasma membrane was unchanged (Online Figure VII). In this, the effect of (P)RR seems to be distinct from that mediated by the ubiquitin ligase inducible degrader of the LDLR and proprotein convertase subtilisin/kexin type 9, which induce specific removal of the LDLR from the membrane and target the receptor for lysosomal degradation.[20,36,37] Accordingly, overexpressing (P)RR did not affect PCKS9-induced LDLR degradation (Online Figure VIII). Therefore, our results rather point toward an event controlled by the (P)RR, which is required for proper trafficking or recycling of the LDLR to the plasma membrane.

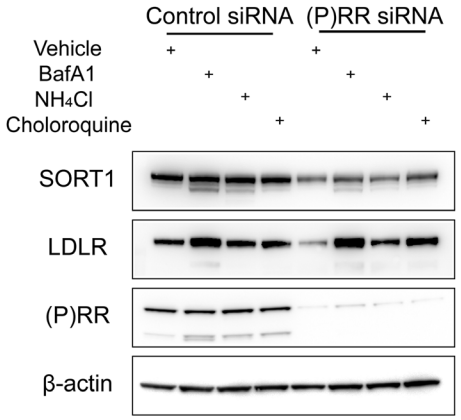

Figure 4. (Pro)renin receptor ([P]RR)–induced reduction in low-density lipoprotein receptor (LDLR) abundance is rescued by lysosomotropic agents. HepG2 cells were treated with control or (P)RR siRNA for 72 hours. Cells were incubated with vehicle control, 100 nmol/L bafilomycin A1 (BafA1), 10 mmol/L NH_4Cl, or 25 μmol/L chloroquine for 6 hours. Total cell lysates were immunoblotted as indicated, and a representative blot of 3 independent experiments is shown. SORT1 indicates sortilin-1.

Discussion

In this study, using an unbiased proteomics approach, we identify a novel role for the (P)RR in LDL metabolism. The main finding of this study is the identification of (P)RR as a post-transcriptional regulator of LDLR abundance of LDL uptake into cells, plausibly by regulating trafficking of the receptor to the plasma membrane.

Our proteomic screen identified several potential (P)RR-interacting proteins. Of these, SORT1, had the highest Mascot score and was therefore chosen for study. SORT1 was recently identified as a regulator of LDL metabolism, and its genetic locus is strongly associated with plasma LDL levels and the risk for cardiovascular disease.[29,31,38,39] SORT1 controls hepatic very low–density lipoprotein secretion, and can also bind and internalize LDL directly, thereby serving as a major regulator in determining plasma LDL levels.[28,29,34] In this study, we found that (P)RR depletion reduces SORT1 protein abundance without affecting its transcript levels, and attenuates cellular accretion of LDL. Similarly, SORT1 depletion led to a comparable reduction in cellular LDL uptake and decreased (P)RR abundance reciprocally. Because combined silencing of *SORT1* and *(P)RR* did not additively reduce LDL uptake, we speculate the 2 act through a common pathway. Our current study also reveals a previously unrecognized function of SORT1 in regulating LDLR protein levels in hepatocytes. This finding is consistent with recent reports demonstrating that SORT1 deficiency leads to increased plasma LDL cholesterol,[29,40] and provides an additional mechanism that may contribute to this outcome. In addition, our results may also provide an explanation why genetic ablation or silencing of SORT1 resulted in a less pronounced increase in plasma LDL-cholesterol on a $Ldlr^{-/-}$ background.[29,41] The ability of SORT1 to control LDLR activity hints that it may influence efficacy of statins by regulating LDLR activity. Interestingly, a recent meta-analysis study found that genetic variances in the *SORT1* locus are associated with the LDL-cholesterol response to statin therapy.[42]

Our study demonstrates that regulation of the LDLR by the (P)RR is a post-transcriptional event culminating in lysosomal degradation of the LDLR. Grossly, 2 cellular scenarios may fit this pattern of regulation. The first involving accelerated removal and degradation of plasma membrane LDLR, and the second resulting from increased lysosomal targeting of newly synthesized LDLR. Our biotinylation experiments of cell-surface LDLR support the second scenario as we found that silencing *(P)RR* expression did not accelerate the degradation of the plasma membrane LDLR pool or affected lysosomal targeting of the LDLR by exogenous proprotein convertase subtilisin/kexin type 9. Given the established role of SORT1 in intracellular trafficking of apolipoprotein B, proprotein convertase subtilisin/kexin type 9, Trk, and Glut4,[28,43–45] we speculate that SORT1 is essential for proper trafficking of the nascent LDLR protein toward the plasma membrane, and that this is dependent on (P)RR function.

This raises the question of how (P)RR influences LDLR trafficking? An attractive possibility may be that this could be because of (P)RR being an accessory component of the V-ATPase complex. This complex is implicated in protein sorting and membrane targeting,[11,46] and previous studies demonstrated that depleting the (P)RR in podocytes decreases the level of V-ATPase subunits leading to autophagosomal defects.[13,14] However, we found that lysosomotropic agents reversed (P)RR-induced reduction in LDLR protein abundance, indicating the existence of an intact endolysosomal pH gradient and functional lysosomes. A caveat of these experiments is that we used siRNA to suppress (P)RR expression, whereas the above-mentioned studies used a Cre-Lox approach to genetically ablate (P)RR. Therefore, in our experiments, despite effective silencing, minimal levels of (P)RR may still exist. Nevertheless, several additional lines of evidence suggest that loss of PRR in the studied cells did not grossly affect V-ATPase activity. Endocytosis of EGF receptor and transferrin receptor was not affected by (P)RR silencing. Because pH and V-ATPase activity are critical for overall endocytosis and receptor recycling, our findings suggest that a general acidification defect of the endolysosomal compartment does not occur after (P)RR silencing in the cells we studied, consistent with our earlier study.[15] We find it also unlikely that (P)RR depletion affects the V-ATPase only in a subset of LDLR-enriched endocytic vesicles as our kinetic analysis of plasma membrane removal of the LDLR revealed no major alterations after silencing of (P)RR. In agreement with our conclusion, Kissing et al[47] recently reported that macrophages lacking PRR do not display altered acidification and phagolysosomal defects. Therefore, an alternative explanation for our observations may be that the functional interaction of the (P)RR and SORT1 serves to ensure proper trafficking of nascent LDLR to the plasma membrane. This may be reminiscent of the role SORT1 plays in apolipoprotein B metabolism, where SORT1 has been demonstrated to promote both apolipoprotein B secretion and degradation in hepatocytes.[28,48]

Summary

In conclusion, we report that the (P)RR is a previously unrecognized regulator of LDL metabolism, which specifically

regulates cellular LDL uptake by modulating LDLR protein abundance. As such, our report warrants future studies to assess the full spectrum of proteins whose trafficking/secretion is subject to regulation by the (P)RR–SORT1 functional interaction, and to elucidate the role of (P)RR in lipoprotein metabolism in vivo.

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Disclosures

None.

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Novelty and Significance

What Is Known?

- The low affinity of the (pro)renin receptor ([P]RR) for both renin and prorenin raises doubt about its in vivo significance as a renin–angiotensin system component.
- Recent studies show that the (P)RR has functions beyond the renin–angiotensin system, including participation in Wnt signaling and modulation of vacuolar H^+-ATPase activity.

What New Information Does This Article Contribute?

- The (P)RR, by controlling the protein abundance of sortilin-1 and the low-density lipoprotein (LDL) receptor, is a novel regulator of LDL.
- Sortilin-1 not only acts as a receptor for low-density lipoprotein, but also controls LDL receptor protein abundance.

The (P)RR is now believed to largely have renin–angiotensin system–independent functions. Using a proteomics approach to identify potential novel functions of the (P)RR, we found that it interacts with sortilin-1, a recently identified receptor for low-density lipoprotein. Silencing the (P)RR led to decreased sortilin-1 and LDL receptor protein abundance, thereby reducing the cellular uptake of low-density lipoprotein. As such, our study identifies the (P)RR as a new regulator of LDL metabolism, and suggests that mutations in the (P)RR gene might associate with circulating LDL levels.