A Novel Signal Transduction Cascade Involving Direct Physical Interaction of the Renin/Prorenin Receptor With the Transcription Factor Promyelocytic Zinc Finger Protein

Jan H. Schefe, Mario Menk, Jana Reinemund, Karin Effertz, Robin M. Hobbs, Pier Paolo Pandolfi, Patricia Ruiz, Thomas Unger,* Heiko Funke-Kaiser*

Abstract—A human renin/prorenin receptor (RER) has recently been cloned. To gain insight into the molecular function of the RER, we studied its signal transduction mechanisms. Initially, we found a ubiquitous and intracellular expression pattern of the human RER. Consistently, we observed several transcriptional start sites and a high promoter activity of the human RER. We could identify the transcription factor promyelocytic zinc finger (PLZF) protein as a direct protein interaction partner of the C-terminal domain of the RER by yeast 2-hybrid screening and coimmunoprecipitation. Coimmunoprecipitation experiments also indicated homodimerization of the RER. On activation of the RER by renin, PLZF is translocated into the nucleus and represses transcription of the RER itself, thereby creating a very short negative feedback loop, but activates transcription of the p85α subunit of the phosphatidylinositol-3 kinase (PI3K-p85α). Small interfering RNA against the RER abolished these effects. A PLZF cis-element in the RER promoter was identified by site-directed mutagenesis and electrophoretic mobility-shift assay. Renin stimulation caused a 6-fold recruitment of PLZF to this promoter region as shown by chromatin immunoprecipitation. Moreover, renin stimulation of rat H9c2 cardiomyoblasts induced an increase of cell number and a decrease of apoptosis. These effects were partly abolished by PI3K inhibition and completely abrogated by small interfering RNA against PLZF. Finally, experiments in PLZF knockout mice confirmed the role of PLZF as an upstream regulator of RER and PI3K-p85α. Our data demonstrate the existence of a novel signal transduction pathway involving the ligand renin, RER, and the transcription factor PLZF, which is of physiological and putative pathophysiological relevance. (Circ Res. 2006;99:1355-1366.)

Key Words: renin receptor ■ PLZF ■ ChIP ■ signal transduction

Renin and prorenin are classically thought of as (pro)enzymes of the renin/angiotensin system (RAS), but recent evidence suggests that they also act as hormones because of their ability to bind cellular targets.1 In 2002 a human renin/prorenin receptor (RER) has been cloned, which consists of 350 amino acids with a single transmembrane domain and specifically binds prorenin and renin. Interestingly, this receptor exerts a dual molecular function2 3 (1) Binding of renin to its receptor increases the catalytic activity of renin approximately 4- to 5-fold. Furthermore, prorenin, which does not exhibit significant ability to generate angiotensin I in solution, gains enzymatic activity comparable to renin by binding to the RER, ie, the receptor is able to unmask the catalytic activity of prorenin. (2) The RER is also able to induce a signal transduction cascade on ligand binding. Binding of renin and also prorenin causes phosphorylation of the receptor and activation of the mitogen-activated protein kinases ERK 1 and 2 (extracellular signal-regulated kinases 1 and 2), whereas intracellular calcium or cAMP levels are not altered. Remarkably, even deglycosylated renin is able to induce extracellular signal-regulated 1/2 phosphorylation.4 The mRNA of the receptor is highly expressed in brain, heart, and placenta with highest levels in the brain. In contrast, kidney, liver and pancreas show low mRNA expression levels.2 3 Via immunofluorescence, the receptor has been detected in mesangial and vascular smooth muscle cells of human heart and kidney.2 3 In addition, mRNA and protein expression have been demonstrated in macrophages, T cells, and granulocytes.4 The receptor is expressed on the cell surface in transfected mesangial cells,3 but there are also indications of an (additional) intracellular receptor localization.2 4 The cloned RER probably corresponds to the previously identified renin binding site on mesangial cells implicated in...
regulation of the plasminogen activator inhibitor-1 and hypertrophic effects. Very recently, it was shown that transgenic overexpression of the RER in smooth muscle cells causes a blood pressure elevation and an increase in heart rate.

Concerning proteins encoded by the RER gene, it is of interest that the C-terminal part of the RER is identical to the vacuolar proton-translocating ATPase (V-ATPase) membrane sector–associated protein M8-9 (APT6M8-9, also known as ATP6AP2). V-ATPases, in general, exert several cellular functions such as neurotransmitter uptake and storage, endocytosis, and receptor recycling. Furthermore, RER and CAPER (homo sapiens endoplasmic reticulum [ER]-localized type I transmembrane adaptor precursor) are identical transcripts (GenBank accession no. AY038990). CAPER was identified in a yeast 2-hybrid screen using the ubiquitous tyrosine phosphatase PRL-1 (Phosphatase of Regenerating Liver-1) as bait (personal communication, R. Herbst, Palo Alto, LA). PRL-1 is involved in the regulation of cellular proliferation and transformation and exhibits a cell cycle–dependent subcellular localization, being localized to the ER in resting cells and to centrosomes and the spindle apparatus in mitotic cells.

No direct protein interaction partner of the RER has been described so far. Therefore, the aim of this study was to analyze the signal transduction cascade of the RER based on protein–protein and downstream protein-DNA interactions, as well as on its subcellular localization. Furthermore, we studied the promoter architecture of the RER to mechanistically explain its cellular expression pattern.

Materials and Methods

Yeast Two-Hybrid Screening

The complete coding sequence of the human RER, based on GenBank accession no. GI:21325928, was cloned into the yeast 2-hybrid bait-vector pBTM117c (kind gift from Erich Wanker, MDC, Berlin, Germany) expressing a LexA DNA-binding domain using the sense primer 5'-CATGCGTGTGGTTCTCGTCGCCCT-3' and the antisense primer 5'-TCAATCCATTCGAATCTTCTGG-3'. This construct was cotransformed into L40 yeast with a GAL4 activation domain fusion library of adult human heart cDNA in pACT2 vector (Clontech, Heidelberg, Germany). Yeast 2-hybrid screening was performed according to the cDNA library guidelines of the manufacturer. Positive clones were identified and checked after retransformation on minimal medium lacking tryptophan, leucine, and histidine and an additional β-galactosidase assay.

All other methods are described in the online data supplement, available at http://circres.ahajournals.org.

Results

mRNA Expression and Basal Promoter Analysis of Human RER

To analyze the expression pattern of the RER and to find appropriate cell lines for studying RER function, we performed a RT-PCR expression analysis of several different human neuronal, glial, epithelial, and endothelial cell lines as well as human kidney and human heart (as positive controls). This analysis showed a ubiquitous mRNA expression pattern of the RER in contrast to other components of the RAS (Figure 1A). In addition, we performed a Northern blot to assess the level of RER mRNA expression. RER mRNA can be easily detected by this method indicating strong expression of this gene (Figure 1B).

To analyze the gene regulatory mechanisms responsible for the observed ubiquitous expression of the RER, we determined its transcriptional start sites and examined its promoter function. A RNA ligase–mediated 5' rapid amplification of cDNA ends (RACE) experiment, which only amplified capped mRNA, indicated multiple transcriptional start sites at positions 76, 86, 96, 102, 105, 112, and 125 bp upstream of the translational start codon (Figure 2A), which is consistent with the TATA box–less architecture of this promoter. In addition, 3 serial-deletion mutants of the human RER promoter (spanning 126, 500, and 1100 bp upstream of the translational start codon) were subcloned for luciferase reporter gene assay. The RER promoter is characterized by a very high promoter activity in the examined neuronal, endothelial, and epithelial cell lines (Figure 2B). Its activity is even higher than the activity of the human ECE-1c promoter, which was shown to be a strong, ubiquitous housekeeping
promoter (Figure 2B). Additionally, our results indicate that 500 bp of the human RER promoter are sufficient to drive maximal promoter activity in these cell types (Figure 2B).

Subcellular Localization of the RER

Because the subcellular localization of the RER may be a clue for the understanding of its function, we addressed this question using different methods. Initially, the localization of the RER within cellular membranes (ie, plasma membrane and/or membranes of organelles) was demonstrated by fractionated protein isolation followed by Western blotting of HeLa-S3 cells (Figure 3A). The observed molecular mass of approximately 38 to 39 kDa in the membrane fraction is consistent with the size of full-length RER described by Nguyen et al, whereas the lower band of approximately 35 to 36 kDa seen in cytosolic and membrane fractions (Figure 3A) likely corresponds to the 3-splice variant reported by Ramser et al, in which the 96 bp-sized exon 4 is missing.

To further analyze the cellular membrane compartment in which the RER is localized, we performed a set of fluorescence microscopic experiments. Immunocytology of c-myc- and FLAG-tagged RER in HeLa-S3 cells indicated a perinuclear localization (Figure 3B). We next generated 3 different enhanced green fluorescence protein (EGFP) fusion proteins of the RER (each as N- and C-terminal fusion): (1) a full-length wild-type RER (RER full); (2) a full-length RER in which its atypical ER-retention motif KXXXX was mutated to RXXXX (RER K/R mut); and (3) the V-ATPase segment of the RER (RER ATPase). The full-length RER construct showed again a perinuclear localization and colocalized with a marker of the ER (Figure 3C). Mutagenesis of the ER retention motif resulted in a loss of perinuclear/ER localization (Figure 3C). Interestingly, the V-ATPase segment of the RER showed a different localization pattern compared with the full-length RER, as it was localized primarily to the lysosomal compartment (Figure 3C). EGFP N-terminally fused to these constructs yielded similar results (data not shown).

Protein-Protein Interaction Partners of the RER

No direct molecular interactions of the RER have been described so far. Therefore, a major objective of our study was to identify protein interaction partners of this ubiquitously expressed receptor to gain insight into its signal transduction cascade. For this purpose, we performed a yeast 2-hybrid screening using a human adult heart cDNA library (prey) and the full-length human RER (bait). The C-terminal third of the transcription factor PLZF (Promyelocytic Zinc Finger protein) was identified as RER-interacting protein in 4 clones, 3 of which were independent. To confirm this RER-PLZF interaction, we performed a coimmunoprecipitation (CoIP) using transient transfections of full-length human RER and full-length human PLZF. Figure 4A demonstrates the ability of the RER to interact with PLZF in a system using tagged proteins. This finding was further confirmed in an endogenous context (Figure 4B).

In an additional CoIP experiment using C- and N-terminal deletion mutants of the RER, we identified the C-terminal, cytoplasmic region of the RER as the interaction domain for PLZF (Figure 4C).

Because dimerization is a common feature of several receptors, we investigated whether homodimerization may...
also be a characteristic of the RER. For this purpose, human RER constructs with 2 different tags were transiently transfected. CoIP experiments indicated that the RER is able to form homodimers (Figure 4D).

**Functional Analysis of the Renin-RER-PLZF Signal Transduction Pathway**

In initial experiments involving PLZF overexpression in HEK293 cells (which endogenously express RER and PLZF mRNA [data not shown]), we observed that RER mRNA is reduced by renin stimulation (to 80.6±3.3%) and PLZF transfection (to 72.6±5.2%), respectively (Figure 5A). A combination of both repressed RER mRNA to 45.4±4.7% (Figure 5A). We could substantiate these findings by using serial-deletion mutants of the human RER promoter as described above. Only a promoter construct comprising the region [−501; −1100] can be repressed by PLZF cotransfection (Figure 5B).

Bioinformatic analysis of the RER promoter using MatInspector (http://www.genomatix.de) indicated the presence of a PLZF consensus sequence (5′-AACTACAGTTTTCAC-3′) with a high core and matrix similarity located in the region [1097; 1083] relative to the translational start codon of the promoter.

To investigate functional downstream effects of the RER-PLZF interaction and to analyze whether these are influenced by stimulation of the RER with its ligand, renin, we performed a set of experiments in which the different components of the putative renin-RER-PLZF pathway and the promoter of the human RER were experimentally modulated in HEK293 cells.
RER stimulation was performed by renin incubation; dependence on RER was analyzed by small interfering RNA (siRNA) against this receptor; the influence of PLZF was examined by cotransfection experiments with a PLZF expression vector (overexpression was confirmed by real-time PCR [data not shown]); and the functional importance of the PLZF consensus sequence within the human RER promoter was evaluated by site-directed mutagenesis of luciferase reporter constructs. siRNA against RER repressed RER mRNA to 29.8 ± 5.2%, as shown by real-time PCR; a similar reduction was observed by Western blotting (data not shown). Readouts comprised RER promoter activity and mRNA level. The known PLZF target gene, the p85α subunit of the phosphatidylinositol-3 kinase (PI3K-p85α), served as an additional downstream candidate gene of a RER/PLZF pathway activation.17

Stimulation of the RER with renin resulted in a decrease in the activity of a wild-type RER promoter by approximately 30% compared with all controls (Figure 5C). Importantly, the presence of the RER was necessary for this effect (Figure 5C).

Consistent with this repressive effect of PLZF on the RER promoter, site-directed mutagenesis of the PLZF consensus sequence [−1097; −1083] caused a derepression (to

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### Figure 4

A, Confirmation of the RER-PLZF interaction by CoIP. HEK293 cells were transiently transfected with FLAG-tagged RER and/or c-myc-tagged PLZF. Immunoprecipitation and Western blotting were performed using anti-FLAG and anti-c-myc antibodies. Several controls were used as indicated. B, Endogenous interaction between RER and PLZF. Total lysate of HEK293 cells was subjected to immunoprecipitation using an anti-PLZF antibody or protein A agarose as control. Subsequent Western blotting of total lysate and immunoprecipitation eluates were performed as indicated. C, The C-terminal domain of the RER interacts with PLZF. HEK293 cells were cotransfected with pCEP4-PLZF-myc and deletion mutants of the RER fused to EGFP. RER full indicates full-length coding sequence; RER n-term del, deletion of the first N-terminal 16 aa; RER c-term del, deletion of the C-terminal (cytoplasmic) 18 aa; EGFP alone. CoIP was performed with an agarose-coupled anti-myc-antibody. Total lysates and immunoprecipitation eluates were subjected to Western blotting using anti-c-myc and anti-EGFP antibodies. D, The RER is able to form homodimers. The human RER was tagged with 2 different tags and transiently transfected into HEK293 cells. The ability of the RER to form homodimers was shown by CoIP.

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of promoter activity. The effect of renin stimulation was also abolished by this mutagenesis (Figure 5C).

Similar but inverse effects were observed with respect to the PI3K-p85α promoter, which is known to be positively regulated by PLZF7. RER stimulation with renin caused an increase of 45% in PI3K-p85α promoter activity compared with control; this effect was abolished by downregulation of the RER using siRNA (Figure 5D).

To verify these findings, we analyzed the effect of RER stimulation on PI3K-p85α mRNA by real-time PCR analysis. Consistent with our promoter data, stimulation with renin increased PI3K-p85α mRNA in systems with and without PLZF overexpression by 105.2±6.74% or 30.2±7.9% (relative to vehicle control), respectively (Figure 5E). Again, downregulation of RER by siRNA abolished this induction regardless the expression level of PLZF (Figure 5E).
Translocation and Promoter Recruitment of PLZF on Renin Stimulation

Additionally, we examined whether activation of the RER by renin is able to cause a translocation of PLZF from the cytoplasm to the nucleus. Subcellular localization of c-myc–tagged PLZF was evaluated by Western blotting after fractionated isolation of cytosolic and nuclear proteins. These proteins were subjected to SDS-PAGE and Western blotting. A Western blot directed against TATA box–binding protein (TBP) served as control for subcellular fractionation and loading. Incubation of HEK293 cells with renin caused a clear increase in nuclear PLZF, whereas cytoplasmic PLZF almost disappeared (Figure 6A). Importantly, this translocation of PLZF again required the presence of the RER as indicated by our siRNA experiments (Figure 6A). Similar results were obtained in untransfected HEK293 cells using Western blotting against endogenous PLZF (data not shown).

To evaluate the recruitment of endogenous PLZF to the human RER promoter within the chromatin context, we performed a chromatin-immunoprecipitation (ChIP) experiment. To ensure valid quantification of transcription factor recruitment, immunoprecipitated DNA was quantified applying real-time PCR analysis and multiple positive and negative controls. Renin stimulation of HEK293 cells did increase the PLZF recruitment to the RER promoter region encompassing the PLZF cis-element at position [−1097; −1083] approximately 6-fold (Figure 6B).

The binding of PLZF to this cis element of the human RER promoter (and not to a mutated form) was further confirmed by electrophoretic mobility-shift assay (Figure 6C), because we found a super-shifted band using a PLZF antibody. Furthermore, the observed pattern was identical compared with the positive control oligonucleotide derived from the PI3K-p85α promoter.17

Cellular Effects of the Renin-RER-PLZF Signal Transduction Pathway

To analyze the cellular effects of renin stimulation and to confirm the importance of PLZF and PI3Ks within the signal transduction cascade of the RER, we investigated the effects of renin, siRNA against PLZF, and pharmacological inhibition of PI3Ks by wortmannin in rat H9c2 cardiomyoblasts. Catalytic effects of renin incubation were excluded by blockade of the angiotensin AT receptor with 10 μmol/L losartan; mRNA of the AT1 receptor was not detectable in these cells using real-time PCR analysis (data not shown).
Renin stimulation increased the number of viable cells to 133.9% compared with vehicle control. Preincubation with wortmannin partly abolished this effect, whereas siRNA against PLZF completely abrogated proproliferative effects of renin stimulation (Figure 7A). Consistent results were observed regarding apoptosis using caspase 3 and 7 activity as readout. Renin stimulation decreased caspase activity to 45.9% (in comparison to vehicle). Wortmannin partly and PLZF siRNA completely abolished this effect (Figure 7B).

To confirm the gene regulatory downstream effects of RER activation in cardiomyoblasts, we also measured RER and PI3K-p85α mRNA in this experimental setting. RER mRNA decreased to 53.7% under renin stimulation compared with vehicle control. siRNA against PLZF (which repressed PLZF mRNA to 15.23±9.8% as shown by real-time PCR) reconstituted RER mRNA to 86.5% (Figure 7C). Renin stimulation increased PI3K-p85α mRNA to 224.2% compared with vehicle control. siRNA against PLZF abolished this effect (Figure 7D). Wortmannin did not affect RER or PI3K-p85α mRNA levels, respectively (Figure 7C and 7D).

**RER and PI3K-p85α mRNA Expression in PLZF Knockout Mice**

To substantiate our findings concerning the transcriptional regulation of RER and PI3K-p85α by PLZF in vivo, we quantified the respective transcript levels in PLZF knockout (PLZF<sup>−/−</sup>) and wild-type (WT) control mice, respectively. PLZF was not detectable in kidney and liver of WT and PLZF<sup>−/−</sup> mice. Within these organs, we did not observe any difference in mRNA levels of RER and PI3K-p85α.
contrast, a significant upregulation of RER mRNA (to 221.9% and 229.5% in heart and brain, respectively) and a significant downregulation of PI3K-p85α mRNA (to 71.8% and 48.6% in heart and brain, respectively) were detected in heart and brain of PLZF−/−/− mice compared with WT mice, which endogenously express PLZF in these organs (Figure 8A).

Discussion

In this work, we identified the transcription factor PLZF as the first direct protein-protein interaction partner of the human renin/prorenin receptor (RER). Furthermore, we describe a novel, short-loop signal transduction pathway involving renin, RER, the transcription factor PLZF, and 2 direct downstream targets, PI3K-p85α and the RER itself (Figure 8B). This pathway has been confirmed in human epithelial and rat cardiomyoblasts.

Stimulation of the RER with its ligand renin causes a translocation of PLZF from the cytoplasm to the nucleus and a recruitment of PLZF to promoter regions of PI3K-p85α and the RER. Depending on the promoter context, PLZF is able to activate the transcription of PI3K-p85α and to repress the gene expression of the RER. This dual role of PLZF as activator and also repressor has been described before but, to our knowledge, not in a single cellular context. The fascinating and uncommon observation that a receptor directly interacts with a transcription factor speaks for an extraordinary short signal transduction cascade. Similar mechanisms, by which a transcription factor or nuclear factor is directly activated at a receptor site or at an extranuclear membrane, have been described for the SMAD, JAK-STAT, Notch, and SREBP pathways. Remarkably, PLZF itself represses the promoter of its own direct interaction partner RER, thereby establishing a very short negative feedback loop. The observation that a RER promoter with a mutated PLZF cis element is refractory to effects of renin stimulation (Figure 5C) indicates that this DNA motif is necessary for this feedback mechanism. Binding of PLZF to this motif was further confirmed by our electrophoretic mobility-shift assay experiments (Figure 6C). Most importantly, the recruitment of native PLZF to the corresponding cis-element region of the RER promoter was demonstrated in the chromatin context by ChIP, with a significant increase under renin stimulation (Figure 6B). The ability of renin to decrease the gene expression of RER and to increase the gene expression of PI3K-p85α depends on the presence of the RER. Therefore, our data prove the existence of the RER as a functional renin receptor, thereby confirming the work of Nguyen et al.3

Furthermore, we were able to demonstrate cellular effects of this novel renin-RER-PLZF signal transduction pathway. RER activation caused a significant increase in cell number and a concomitant decrease in apoptosis of cardiomyoblasts. These effects are mainly mediated by PLZF as shown by siRNA experiments. PI3K inhibition did affect these cellular effect to a minor degree, consistent with PI3K-p85α being a downstream target of PLZF. These effects are in accordance with the known physiological role of PLZF as discussed below. Additionally, several studies have shown that PI3Ks contribute to cardiac hypertrophy involving increase in protein synthesis and activation of the Akt pathway.

Finally, we could demonstrate that the RER-PLZF pathway also acts in vivo. Experiments in tissues derived from PLZF knockout mice clearly show that absence of PLZF causes a derepression of RER mRNA and a decrease in PI3K-p85α mRNA in organs endogenously expressing PLZF.

The primary perinuclear subcellular localization of the RER observed in our work conflicts with the plasma membrane localization described by Nguyen et al.3 Nevertheless, all of our experiments, including use of different constructs, mutagenesis, and colocalization studies, indicated an intra-
cellular localization of the RER. Furthermore, the total cellular membrane lysates used for their kinetic studies may still contain intracellular membrane proteins and, therefore, do not exclude an intracellular localization of the RER. The possibility that our results concerning the perinuclear localization are caused by an artifact related to tags or overexpression is unlikely because we used several different C-terminal and also N-terminal tags. In addition, mutagenesis of the atypical C-terminal ER-retention signal strongly reduced the perinuclear localization of the RER. The fact that CAPER, which is identical to the RER as discussed above, can directly bind to PRL-1 (a protein observed in the ER in nonmitotic cells) also supports our results.

On the other hand, our experiments indicate that an extracellular signal (renin) can affect the signal transduction of the RER despite its mainly intracellular localization. Several mechanisms might account for this observation. Firstly, other renin-binding receptors, such as the mannose-6-phosphate receptor, could internalize renin and prorenin.\textsuperscript{25} Secondly, a nonsecreted (ie, intracellular) renin isoform, which could directly interact with an intracellular RER, has been described containing an alternative first exon termed (albeit identical) “exon 1b”\textsuperscript{26,27} and “exon 1A,”\textsuperscript{28,29} respectively. Intracellular renin administration was reported to increase inward calcium current in cardiomyocytes.\textsuperscript{30} In addition, transgenic overexpression of the nonsecreted, intracellular form of renin and angiotensinogen in the brain causes an increase in drinking volume and mean arterial pressure.\textsuperscript{31}

Thirdly, it could be possible that very small amounts of the RER within the plasma membrane are sufficient for the initiation of a RER signal transduction cascade. In this context, the observed homodimerization of the RER might also be of importance, because dimerization can affect subcellular localization.\textsuperscript{32}

In this study, we were able to demonstrate a ubiquitous expression of the human RER, a high promoter activity in different cell types and multiple transcriptional start sites in a TATA box–less promoter. These features are consistent with housekeeping properties of the RER gene,\textsuperscript{13} suggesting basal cellular functions of this protein. In this context, it is of interest that the C-terminal part of the RER is, as discussed above, identical to the V-ATPase membrane sector-associated protein M8-9, because V-ATPase have functions in almost every eukaryotic cell.\textsuperscript{10} Nevertheless, APT6M8-9 only constitutes 69 to 100 aa (GenBank accession no. GL:5031590) of the 350 aa\textsuperscript{3} of the full-length renin receptor. Therefore, the functions of the RER exceed those of a pure V-ATPase subunit, which is also supported by the data on the signal transduction and the different subcellular localizations of full-length RER and APT6M8-9 provided here. The mechanism by which the APT6M8-9 protein is generated, remains to be clarified. Alternative promoters and posttranslational cleavage by an unidentified protease are putative explanations.

The transcription factor PLZF contains multiple zinc-finger domains and is disrupted in patients with acute promyelocytic leukemia (APL) caused by t(11;17)(q23;q21) chromosomal translocation.\textsuperscript{33,34} This APL subform is characterized by PLZF-RARα (PLZF-retinoic acid receptor-α) fusion proteins, which recruit histone deacetylase 1 (HDAC1) and do not respond to retinoic acid any more, explaining the missing response of these patients to retinoic acid treatment.\textsuperscript{34,36}

Wild-type PLZF can act as growth repressor and exerts proapoptotic functions during development.\textsuperscript{33,37} Concerning the RAS, it seems important to note that PLZF was recently described as an adaptor protein of the AT1 receptor in the heart.\textsuperscript{17} This direct PLZF-AT1 receptor interaction was associated with stimulation of protein synthesis and putative cardiac hypertrophy.\textsuperscript{17}

Data obtained from PLZF knockout mice indicate that this transcription factor is involved in limb and axial skeletal patterning,\textsuperscript{37} whereas the brain phenotype of these mice was not described by the authors. Consistent with this observation, PLZF target genes include hox genes,\textsuperscript{37–39} besides the p53α subunit of PI3K\textsuperscript{17} and cyclin A2.\textsuperscript{20}

Concerning the biological significance of the RER beyond its possible role in the RAS, it was recently shown by Ramser et al that a mutation in the renin receptor gene is a cause of X-linked mental retardation (XLMR) and epilepsy (XMRE) syndrome in humans.\textsuperscript{8} Consistent with this observation are the results of a zebrafish mutagenesis screen, in which a mutation in the ATP6AP2 (which is identical to RER) gene caused a reduction in head size and necrosis of the central nervous system.\textsuperscript{40} Remarkably, the human RER mutation observed by Ramser et al altered neither binding affinity for renin nor the RER-mediated augmentation of the catalytic efficiency of renin for angiotensinogen cleavage.\textsuperscript{8} In addition, the effect of this mutation (and also the effect of wild-type RER stimulation by renin)\textsuperscript{10} on mitogen-activated protein kinase signaling are only modest,\textsuperscript{8} if non-significant. Therefore, it could be possible that the human RER mutation exerts its effect by an altered PLZF-mediated signal transduction. The complex, dynamic expression of PLZF in the developing central nervous system, and its likely role in hindbrain and also forebrain segmentation supports this notion.\textsuperscript{41,42}

Related to the role of the RER in central nervous system development is the observation that RER mRNA can be detected in human glioblastomas as well as in glioblastoma cell lines and that renin inhibitors can reduce the cell number in glioblastoma cell lines. This is probably caused by modulation of RER function, because this effect is independent of AT1 and AT2 receptor activity.\textsuperscript{43}

The clinical relevance of the RER is underlined by the interesting observation that a decoy deca-peptide corresponding to the handle region of prorenin, which competitively inhibits prorenin binding to its receptor, attenuated the development and progression of cardiac fibrosis\textsuperscript{44} and also inhibited the development of diabetic nephropathy in rat models.\textsuperscript{45} Remarkably, a recent study demonstrated the ability of RER activation to upregulate transforming growth factor-β1 in mesangial cells independent of angiotensin II actions.\textsuperscript{46}

Knowledge regarding the signal transduction of the RER is of importance to evaluate the efficacy and safety of renin inhibitors, such as aliskiren, which is currently in phase III clinical trials.\textsuperscript{47} As expected, renin inhibitors reduce plasma renin activity (ie, enzyme activity with respect to angiotensin
I generation). Nevertheless, they increase total amount of plasma renin protein, the RER ligand, dramatically (up to 34-fold). Therefore, it is crucial to examine whether renin inhibitors change the intrinsic activity of renin with respect to the RER. In this context, it is important to note that overactivation of the RER might be deleterious (eg, with respect to end-organ damage) considering the activation of mitogen-activated protein kinases and PLZF downstream of the RER as well as the effects of the handle region decay peptide mentioned above. On the other hand, a blockade of the RER signal transduction might be harmful, at least in pregnant women, because of the developmental importance of the RER and its direct interaction partner PLZF.

To conclude, our results demonstrate the existence of a novel signal transduction pathway downstream of the human renin/prorenin receptor, which involves direct binding of the transcription factor PLZF to the receptor, its translocation to the nucleus, and the positive and negative regulation of target genes. Based on the already described biomedical relevance of the RER and PLZF, respectively, this pathway, connecting both molecules, might be of importance in human physiology and pathophysiology.

Sources of Funding
This project was supported by a Forschungsförderung of the Charité–Universitätsmedizin Berlin.

Disclosures
T.U. is member of the Aliskiren International Advisory Board of Novartis Pharma AG (Basel, Suisse).

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*Circ Res.* 2006;99:1355-1366; originally published online November 2, 2006; doi: 10.1161/01.RES.0000251700.00994.0d

*Circulation Research* is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0009-7330. Online ISSN: 1524-4571

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Materials and Methods

Cell culture and tissue samples

SH-SY5Y (human neuronal) and SK-N-AS (human neuronal) were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ ml penicillin and 100 µg/ ml streptomycin. HEK293 (human epithelial), HeLa-S3 (human epithelial), EA.hy926 (human endothelial), T98G (human glial), U-87 MG (human glial), U-373 MG (human glial), and H9c2 (rat cardiomyoblast) were cultured in DMEM with 4,5 g/ l glucose supplemented with 10% FBS, 100 U/ ml penicillin and 100 µg/ ml streptomycin. All cell culture products were obtained from PAN Biotech, Aidenbach, Germany. Cells were grown in a humidified incubator at 5% CO₂ and 37 °C.

RNA of human heart and tissue of human kidney were provided by the DHZB (Berlin) and the Department of Urology (Charité – Universitätsmedizin Berlin), respectively.

The generation of the PLZF knockout mice was described previously¹ (zitat). Indicated organs of PLZF knockout mice and corresponding wild type control mice (C57BL6) were removed and immediately subjected to RNAlater solution (Qiagen, Hilden, Germany).

Constructs and site-directed mutagenesis

The complete coding sequence (CDS) of the human RER, based on GenBank GI:21325928, and human PLZF, based on GenBank GI:31543978, were subcloned into the mammalian expression vector pCEP4 (Invitrogen, Karlsruhe, Germany) with different C-terminal tags using the following primers: 5’-GCCACCATGGCTGTGTTTGTCGTGCT (sense for pCEP4-RER-FLAG/-myc/-HA); 5’-

TCACTTGTCGTCATCGTCTTTGTAGTCATCCATCCATCGAATCTTCTTCTGGT (antisense for pCEP4-RER-FLAG); 5’-
TCACAGATCTTCTTCAGAAATAAGTTTTTGTTCATCCATTCGAATCTTCTGGT  
(antisense for pCEP4-RER-myc); 5’-
TCAAGCGTAGTCTGGGACGTCGATGGGAATCCATTCGAATCTTCTGGT  
(antisense for pCEP4-RER-HA); 5’- GCCACCATGGATCTGACAAAAATGGG (sense  
for pCEP4-PLZF-myc); 5’-
TCACAGATCTTCTTCAGAAATAAGTTTTTGTTCACATAGCACAGGTAGAGGT  
(antisense for pCEP4-PLZF-myc).

Additionally, the CDS of the human RER was cloned into pEGFP-N1 and pEGFP-C3  
(Clontech, Mountain View, CA, USA) to create N- or C-terminal EGFP fusion constructs  
using the following primers: 5’- GGCACCATGGCTGTGTTTGT (sense for RER-full); 5’-
ATTCGAATCTTCTGGTTTG (antisense RER-full). Furthermore, two protein mutants of  
the RER were generated: (1) The pEGFP-N1/C3-RER-ATPase construct, which comprises  
only the vacuolar proton-translocating ATPase (V-ATPase) membrane sector-associated  
protein M8-9 (APT6M8-9) part of the RER (C-terminal 69 aa), was subcloned using the  
sense primer 5’- ATGGAGGCAAAACAAGCGAAGAACC and the antisense primer 5’-  
ATTCGAATCTTCTGGTTTG; (2) The pEGFP-N1/C3-RER-K/R-mut construct, in which  
the atypical C-terminal ER-retention signal (KXXXX) was replaced by RXXXX, was  
subcloned using the sense primer 5’- GGCACCATGGCTGTGTTTGT and the antisense  
primer 5’-ATCCATTCGAATCCTCTGGTTTG.

For mapping of interaction domains, N- and C-terminal deletion mutants of the RER were  
subcloned into pEGFP-N1 (Clontech) using following primers: 5’-  
ATGAACGAGTTAGTATATTAAA (sense RER n-term del) and 5’-  
AATCCATTCGAATCTTCTGG (antisense RER n-term del); 5’-  
GGCACCATGGCTGTGTTTGT (sense RER c-term del) and 5’-  
GATCCATGTTCAAAATATTGT (antisense RER c-term del).
The promoter of the human RER, based on GenBank GI:37546587, and serial deletion mutants were subcloned into the luciferase reporter vector pGL3-basic (Promega, Mannheim) using a common antisense primer located directly upstream of the translational start site (5’- GGTGCCGCGGCGGCAGCAGC) and following sense primers: [-1100;-1]: 5’-CTTAACATCACAGTTTTCACGGAA; [-1100;-1]-mut(PLZF): 5’-CTTTACATCTGTTTTTCAGGA; [-500;-1]: 5’-TCACAGCTGGCCGTCCGTAGCCCGC; and [-165;-1]: 5’-GTGATTGCTGGAGAAACGCGGCAGCT. Numbers indicate positions relative to the start codon.

A pGL3-basic plasmid encoding 968 bp of the promoter of human endothelin-converting enzyme-1c (ECE-1c) was used as control.

**RT-PCR**

Total RNA was isolated using NucleoSpin RNA II (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol including a DNAase digest. cDNA synthesis was performed using random hexamer primers and M-MLV reverse transcriptase (RNase H minus; Promega, Mannheim, Germany); no template controls (NTCs) and reactions without addition of reverse transcriptase (RT-) served as negative controls. PCRs were carried out using the following human-specific primer pairs: RER: 5’-ATTGGCCTATAACCAGGAG (sense), 5’-TTCCCATAACGCTTTCCCAA (antisense); angiotensin AT1 receptor (AT1R): 5’-CATATTTGTCATGATTTCTACTT (sense), 5’-GCACAAACTGTATATTGCTGT (antisense); angiotensin AT2 receptor (AT2R): 5’-ACATCTTTACCTTGCTGTG (sense), 5’-CCATACACAAACAAGGGGA (antisense); angiotensinogen: 5’-CTGTGATGAAAAGGCCCTTA (sense), 5’-ATTGCTGTAGCCTGTGCAGC
Signal transduction of the renin/prorenin receptor

(antisense); ACE: 5’-GCTGCAGCCCGCAACTTTT (sense), 5’-
CGGTGGAGTAGATCCTGCTC (antisense); HPRT: 5’-
TGCTCGAGATGTGAAGG (sense), 5’-TCCCCCTGTTGACTGTCATT (antisense);
GAPDH: 5’-TGAAGGTCGGAGTCAACGGATTTGGT (sense); 5’-
CATGTGGGCCATGAGGTCCACCAC (antisense); β-actin: 5’-
TCCCTGGAGAAGAGCTACGA (sense), 5’- AGCACTGTGTTGCGGTACAG
(antisense).

**Northern blotting**

Northern blotting was performed as described previously3, 4. The probe against the human
RER and human β-actin (for standardization) corresponds to the PCR products described
above.

**RNA ligase-mediated (RLM)- 5’-RACE**

Transcriptional start sites of human RER were determined using the GeneRacer Kit
(Invitrogen, Karlsruhe, Germany), which only amplifies capped mRNA. Reverse
transcription was performed with random hexamer primers. Nested PCR utilized
HotStarTaq (Qiagen), 5’-CTCTCCTGGTATAGGCAAT (antisense primer in first PCR)
and 5’-TCGGAAAAACAGACCGT (antisense primer in second PCR). Reaction
products were subcloned and sequenced.
Luciferase assays

The indicated cell types were seeded on day 1 in 12-well plates. 100 ng of indicated pGL3-basic constructs (encoding firefly luciferase) and 20 ng of phRL-null plasmid (encoding humanized renilla luciferase for standardization of promoter activity; Promega) per well were transfected on day 2 at 60-80% confluency using GeneJuice (Merck Biosciences, Bad Soden, Germany) according to the manufacturer’s protocol. Cells were harvested 48 hours after transfection using Passive Lysis Buffer (Promega). Reporter activities were measured in a Pharmingen Monolight 3010 luminometer (BD Biosciences, Erembodegem, Belgium) using the Dual-Luciferase Reporter Assay System (Promega). Promoter activity is expressed as relative luciferase activity (RLA)$^4$. RLA data represent the mean ± standard deviation of at least three single, parallel transfection experiments.

Subcellular protein extraction and Western blotting

Cytosolic and membrane proteins were isolated using the ProteoExtract Subcellular Proteome Extraction Kit (Calbiochem, Darmstadt, Germany). A fractionated extraction of cytosolic and nuclear proteins was performed as described previously$^4$. Protein concentrations were determined using the DC Protein Assay (Bio-Rad, München, Germany). Cytosolic, membrane and nuclear fractions were controlled by Western blot using antibodies against Akt (mouse monoclonal, 5G3; 1:1,000; Cell Signalling, Danvers, MA, USA), angiotensin AT1 receptor (rabbit polyclonal, sc-579; 1:2,000; Santa Cruz Biotechnology, Heidelberg, Germany) and TBP (rabbit polyclonal, sc-204; 1:1,000; Santa Cruz Biotechnology), respectively.

For analysis of subcellular localization of RER, 9 µg (membrane) and 12 µg (cytosolic) protein were loaded per lane, separated in a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Western blot analysis was performed with an antibody against
human RER (corresponding ATP6AP2; goat polyclonal, ab5959, Abcam, Cambridge, UK) at a dilution of 0.5 µg/ml. Horseradish peroxidase-conjugated anti-goat antibody (rabbit polyclonal; 1:2,500; DAKO, Hamburg, Germany) was used as secondary antibody.

For PLZF translocation experiments HEK293 cells were seeded on day 1 in 6-well plates. 250 ng of pCEP4-PLZF-myc expression vector per well were transfected on day 2 at 40-60% confluency using GeneJuice (Merck Biosciences, Bad Soden, Germany) according to the manufacturer’s protocol. On day 3 the cells were transfected with 50 nM siRNA against the RER [5’- GCUCCGUAAUCGCCUGUUU (sense strand)] or scrambled control siRNA [5’- UUUACCGUCGCCUUGAGCU (sense strand)] (Eurogentec, Seraing, Belgium) using jetSI-ENDO (Eurogentec) following the standard procedure. On day 5, after 24 hours of starving in serum-free medium, cells were stimulated with 10 nM human recombinant renin (mammalian expression; kind gift from Dominik N. Müller, MDC Berlin) or vehicle (0.1% DMSO in PBS) for 2 hours. Western blot was performed with anti-c-myc antibody (mouse monoclonal, 9B11; 1:2,000; Cell Signalling) and horseradish peroxidase-conjugated anti-mouse antibody (rabbit polyclonal; 1:2,500; DAKO). Chemoluminescence was detected with ECL (Amersham, München, Germany), and subsequent exposition to Hyperfilm (Amersham).

Fluorescence microscopy

HeLa-S3 cells were seeded in 8-well chamber slides (BD Biosciences, Erembodegem, Belgium) at day 1 and transfected the following day at 30-50% confluency with 50 ng of the indicated plasmid using GeneJuice (Merck Biosciences) following the standard protocol.

For immunofluorescence microscopy, 48 h after transfection cells were fixed and permeabilized in methanol for 10 minutes at −20 °C. Blocking was performed in 1x TBS
with 0.1% Tween 20, 5% skimmed milk powder and 1% BSA (Sigma-Aldrich, Taufkirchen, Germany) for 1 hour at room temperature. The primary antibodies anti-FLAG-M2 (mouse monoclonal; 1 µg/ml; Sigma-Aldrich, Taufkirchen, Germany) and anti-c-myc-tag (mouse monoclonal, 9B11; 1:2,000; Cell Signalling) were incubated overnight in blocking buffer, followed by 1 hour of incubation at room temperature with Cy3- or Cy5-conjugated anti-mouse antibody (rabbit polyclonal; 1:250; DAKO) in blocking buffer. DAPI (Invitrogen, Karlsruhe, Germany) was used for nuclear staining according to the manufacturer’s protocol.

For microscopy of the EGFP constructs, cells were stained with 1 µM ER-Tracker Red or 0.1 µM LysoTracker Red (both Invitrogen) in OPTIMEM (Gibco, Karlsruhe, Germany) for 30 minutes at 37 ºC 48 hours after transfection and subjected to imaging.

All images were obtained with a Leica DM-IRE2 (Leica, Wetzlar, Germany) fluorescence microscope with a 63x lens.

Coimmunoprecipitation (coIP)

HEK293 cells were seeded on day 1 in 175 cm² flasks. Transfection was performed on day 2 using polyethylenimine at 60-80% confluency. 20 µg of each indicated plasmid were diluted in 500 µl PBS; 120 µl of a polyethylenimine solution (0.9 mg/ml in ddH₂O; average MW 750,000; Sigma-Aldrich) were diluted in 500 µl 0.1 M NaCl. Both solutions were vortexed and incubated separately for 10 min at room temperature. The solutions were then mixed and again incubated for 10 min at room temperature before addition to the serum- and antibiotics-free growth medium, which was replaced with complete growth medium after 4 hours.

48 hours post-transfection the cells were washed twice with ice-cold PBS and lysed in ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1%
Nonidet P40, 15% glycerol and Complete protease inhibitor cocktail (Roche, Mannheim, Germany). Lysate containing 2 mg of total protein [determined by the BCA method (Bio-Rad)] was treated with anti-FLAG-M2-agarose affinity beads, EZview Red anti-c-Myc Affinity Gel or protein A agarose only (all Sigma-Aldrich), respectively, according to the manufacturer’s recommendations. 25% of eluate were subjected to a 10% SDS-PAGE and transferred to a nitrocellulose membrane. Western blot was performed with anti-FLAG-M2 antibody (mouse monoclonal; 0.5 µg/ml; Sigma-Aldrich), anti-c-myc-tag antibody (mouse monoclonal, 9B11; 1:2,000; Cell Signalling) and horseradish peroxidase-conjugated anti-mouse antibody (rabbit polyclonal; 1:2,500; DAKO, Hamburg). Detection was performed as described above.

For endogenous coIP total lysates were prepared as described above and subjected to immunoprecipitation using an anti-PLZF antibody (rabbit polyclonal, sc-22839; Santa Cruz Biotechnology) non-covalently bound to protein A agarose or protein A agarose only. Western blot was performed as described above with anti-PLZF antibody (rabbit polyclonal, sc-22839; Santa Cruz Biotechnology) and anti-RER antibody (goat polyclonal, ab5959, Abcam, Cambridge, UK), respectively. For mapping of the interacting domain, transfection (with the indicated plasmids) and lysis were performed as described above. Immunoprecipitation was carried out using EZview Red anti-c-Myc Affinity Gel (Sigma). Western blotting utilized anti-c-myc-tag (Cell Signalling) and anti-GFP antibodies (Santa Cruz Biotechnology).

**Luciferase assay and real-time RT-PCR under siRNA and renin stimulation conditions**

HEK293 cells were seeded in 24-well plates and transfected with either 100 ng of pCEP4-PLZF-myc expression vector, 50 ng of indicated pGL3-basic construct and 10 ng of phRL-null plasmid (for luciferase assay), or 100 ng of pCEP4-PLZF-myc alone (for PCR
experiments) per well as described above. siRNA transfection, renin stimulation and determination of reporter activity were performed as described above, except for an exceeded renin stimulation period of 3 hours.

Quantitative real-time RT-PCR (qPCR)

RNA isolation and cDNA synthesis were performed as described above. qPCR was performed applying a SYBR Green I reaction mix and run on a Stratagene Mx3000P (Stratagene, La Jolla, CA, USA) using the following primers: human RER: 5’-AAACAAGCGAAGAACCAGC (sense), 5’-GGTGATAATCACAGCCAAGGC (antisense); rat RER: 5’-CTTGCTGTGGCAACCTATT (sense), 5’-GGAACTGCATTCTCAAAGG (antisense); mouse RER: 5’-GGAACTGCTATTCTCAAAGG (antisense); human PI3K-p85α: 5’-CGGATCTTGCAGAGCAGTTT (sense), 5’-AGGTTGCTGGAGCTCTGTGT (antisense); rat PI3K-p85α: 5’-CCTGGAAGCCATTGAGAAGA (sense), 5’-TCGAACACATCCAAGTCCAC (antisense); mouse PI3K-p85α: 5’-CCAGCAGGATCAAGTTGTCA (sense), 5’-TCCTGGGAAGTACGGGTGTA (antisense); human PLZF: 5’-TAGGGTGCACACAGGTGAGA (sense), 5’-GTGCAGATGGTGCACTGGTA (antisense); rat and mouse PLZF: 5’-GGAGCATCATGGATCTGACA (sense), 5’-GATGACCACATCGCACAAAG (antisense); human, rat and mouse 18S rRNA: 5’-CCGCAGCTAGGAATAATGGAATA (sense), 5’-TCTAGCGCGCAATACGAAT (antisense).

Data represent the mean expression level ± standard deviation (standardized to 18S rRNA expression) calculated according to the ddC_{T} method of at least three independent
measurements per cDNA (technical triplicates). Equality of PCR efficiencies has been verified applying the program LinRegPCR and ANOVA testing5, 6.

Chromatin-immunoprecipitation (ChIP)

HEK293 cells cultured in 75 cm² flasks were starved and stimulated with renin for 2 hours as described above. Fixation at a confluence of about 90% was performed using 1% formaldehyde in 1x PBS for 7 min at room temperature. Cells were then rinsed twice with ice-cold 1x PBS, abraded in 1x PBS, and centrifuged for 5 min at 440 x g. Each pellet was resuspended in 1 ml lysis buffer (1% SDS, 50 mM Tris-HCl, 1x Complete protease inhibitor cocktail (Roche), 5 mM EDTA (final concentration); pH 8.1), followed by a 20 min incubation on ice. Sonification, immunoprecipitation and reversal of crosslink were performed according to Bryant and Ptashne7, using 3 µg of following antibodies (all from Santa Cruz Biotechnology): PLZF (sc-22839), TBP (sc-204) and MMP-2 (sc-10736).

Sonification itself was performed using the Sonoplus HD 2070/ UW 2070 sonifier with the tip MS 72 (Bandelin Electronic, Berlin, Germany; output control (power %) = 100; time = 20 sec (once); constant duty cycle).

qPCR was performed as described above applying the following primers: human RER promoter around the PLZF consensus sequence (RER-PLZF): 5’-
GCTCTGTGCCTCCTCTCTCA (sense), 5’-CCCAGCTGATGACCTTGAA (antisense); human RER around the transcriptional start site (outside the PLZF consensus sequence; RER-TSS): 5’-ACGTCCCTTCCGTAGGCCG (sense), 5’-
ACTGCGGGGCGACACGGAA (antisense); human β-actin promoter: 5’-
AATGCTGCACTGTGCGGCGA (sense), 5’-GGCGGATCGGCAAAGGCGA (antisense); human intergenic region: 5’-AAATGAAGTGAGACCCCTCC (sense), 5’-
TCAAAGGACACACAGCACCT (antisense). Input (total) and genomic DNA served as
positive controls. DNA fragments immunoprecipitated with the anti-MMP-2 antibody, as well as PCRs on an intergenic region and a no template control (NTC) served as negative controls.

Calculation of transcription factor (PLZF) recruitment (to human RER promoter) standardized to the recruitment of TBP to the TATA box region of the human beta-actin promoter (X) was calculated according to

\[
X = 2^{-\frac{[C_t[IP(PLZF), PCR(RER)] - C_t[input, PCR(RER)]]}{2\left[C_t[IP(TBP), PCR(\beta-actin)] - C_t[input, PCR(\beta-actin)]\right]}}
\]

X was calculated separately for renin stimulation \([X(\text{renin})]\) and vehicle control \([X(\text{vehicle})]\). \(X(\text{renin})\) divided by \(X(\text{vehicle})\) served as a measure for relative PLZF recruitment. Indicated standard deviation is based on three independent measurements on each template.

Concerning the negative controls, following criteria were fulfilled ("\(\geq\)" indicates a \(C_T\) difference of at least 3): (1) \(C_T[IP(MMP-2), PCR(\text{RER-PLZF})] > C_T[IP(\text{PLZF}), PCR(\text{RER-PLZF})]\); (2) \(C_T[IP(MMP-2), PCR(\beta-\text{actin})] > C_T[IP(TBP), PCR(\beta-\text{actin})]\); (3) \(C_T[IP(\text{PLZF}), PCR(\text{intergenic})] - C_T[\text{total, PCR(\text{intergenic})}] > C_T[IP(\text{PLZF}), PCR(\text{RER-PLZF})] - C_T[\text{total, PCR(\text{RER-PLZF})}]\); (4) \(C_T[IP(TBP), PCR(\text{intergenic})] - C_T[\text{total, PCR(\text{intergenic})}] > C_T[IP(TBP), PCR(\text{RER-TSS})] - C_T[\text{total, PCR(\text{RER-TSS})}] > C_T[IP(\text{PLZF}), PCR(\text{RER-PLZF})] - C_T[\text{total, PCR(\text{RER-PLZF})}]\); (6) no template controls (NTCs) yielded no amplicons after 40 cycles.

**Electromobility shift assay (EMSA)**

EMSA experiments were performed as described previously\(^9\) using the oligonucleotides

5’- CTAACTACAGTTTTCACTGG (RER native), 5’- CTTTACATCTGTTTTCACTGG
(RER mutated) and 5'-TTACATGTACTAGTGTTGTGG (PI3K-p85α); the binding reaction was performed according to Senbonmatsu et al.9. For super-shift analysis, an anti-PLZF antibody (rabbit polyclonal, sc-22839; Santa Cruz Biotechnology) was used; an anti-MMP2 antibody (rabbit polyclonal, sc-10736; Santa Cruz Biotechnology) served as control.

Cell viability and caspase 3/7 activity assay

H9c2 were seeded in 96-well plates on day 0 and transfected with 50 nM siRNA against rat PLZF [(5'-CCAGCAAGAUGUUUGAGAU (sense strand)] or scrambled control siRNA [5’-UCUCGCAGUGACUAUACAU (sense strand)] on day 1 as described above. After 48 h and 24 h of incubation in serum-free starving medium, cells were pretreated with 10 µM of losartan and, additionally, 1 µM of wortmannin or vehicle as indicated. After 30 min cells were stimulated with 10 nM renin or vehicle. 24 h later the CellTiter-Glo Luminescent Cell Viability Assay and Caspase-Glo 3/7 Assay (both Promega) have been performed following the manufacturer’s standard protocol.

Statistical analysis

A two-tailed t-test has been applied and statistical significance was assumed at p < 0.05.
References


