9-cis-Retinoic Acid Up-regulates Expression of Transcriptional Coregulator PELP1, a Novel Coactivator of the Retinoid X Receptor α Pathway*

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Retinoid X receptor α (RXRα), functioning as either a homodimer or a heterodimer with peroxisome proliferator receptors, is known to be involved in manifesting antiproliferative effects in cells. Consequently, studies of RXRα functions and its coregulators have been in the focus for therapeutic approaches against cancer. Here we have discovered that 9-cis-retinoic acid (9-cis-RA), a RXRα-specific ligand, up-regulated the expression of transcriptional coregulatory protein PELP1 (proline-, glutamic acid-, and leucine-rich protein 1). PELP1 functioned as a coactivator of RXRα, increasing its transactivation function in response to 9-cis-RA as evident by the retinoid X receptor response element-luciferase assays. PELP1 was found to be a binding partner of RXRα, and the binding interactions were confirmed both in vitro and in vivo. An electrophoretic mobility shift assay showed greater formation and stability of RXRα homodimers on consensus oligonucleotides in PELP1-overexpressing clones in comparison to the pcDNA clones. The presence of PELP1 in these oligonucleotide-bound RXRα homodimers was proved by the supershift of the complex when incubated with PELP1-specific antibody. PELP1-overexpressing stable MCF-7 cells exhibited a significantly higher extent of 9-cis-RA-induced apoptosis than the control pcDNA clones. Silencing of PELP1 expression in parental MCF-7 cells and PELP1-overexpressing clones using PELP1-specific RNA-mediated interference compromised the susceptibility to 9-cis-RA-induced apoptosis. PELP1 could also function as a coactivator of the RXRα-peroxisome proliferator-activated receptor (PPARγ) heterodimer as evident by the peroxisome proliferator-activated receptor response element-luciferase assay in response to both 9-cis-RA and PPARγ-specific ligands. This was reinforced by the higher propensity of PELP1-overexpressing clones to undergo differentiation in response to PPARγ-specific ligands. This study has revealed a novel facet of PELP1 functions and identified it to be an important potentiator of the antiproliferative effects of 9-cis-RA and PPARγ-specific ligands.

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‡¶1 The abbreviations used are: NR, nuclear receptor(s); PELP1, proline-, glutamic acid-, and leucine-rich protein 1; RXRα, retinoid X receptor α; PPARγ, peroxisome proliferator-activated receptor γ; 9-cis-RA, 9-cis-retinoic acid; RNAi, RNA-mediated interference; PIC, preinitiation complex; ER, endoplasmic reticulum; CBP, CAMP-response element-binding protein (CREB)-binding protein; GST, glutathione sulfur-transferase; RT, reverse transcription; CRBP II, cellular retinol-binding protein; EMSA, electrophoretic mobility shift assay; WT, wild type; RXRE, retinoid X receptor response element; PPRE, peroxisome proliferator-activated receptor response element.

Nuclear receptors (NR) are ligand-activated transcription factors that on binding to small lipophilic signal molecules facilitate gene transcription and regulate diverse vital biological processes such as cell survival, proliferation, differentiation, and apoptosis (1). On activation, the NR in their homodimeric or heterodimeric complexes with other NR bind to specific DNA sequences referred to as “response elements,” which are present in the regulatory elements of target genes. On DNA binding, the NR orchestrate gene transcription by interacting with the basal transcriptional machinery through bridging factors, referred to broadly as coactivator proteins. One of the potential functions of the coactivator proteins is to directly or indirectly remodel the local chromatin structure through covalent modification of histones (acetylation, phosphorylation, or methylation) resulting in opening of chromatin, greater accessibility of the target gene promoter to transcriptional machinery, and ultimately, gene transcription. Numerous families of coactivators of different NR have been identified. Their mode of interaction with the receptors and the molecular mechanism by which they function has been established. The most well studied coactivators include p300/CBP, p300/CBP-associated factor, and members of the p160 families, which include SRC-1, GRIP1/TIF2/SRC-2, and ACTR/AIB1/RAC3/SRC-3 (for review, see Ref. 2). These coactivator functions by facilitating the initial penetration of the chromatin by histone acetyltransferase. Coactivators, which utilize histone methylation for the same purpose like CARM1 and PRMT, have also been identified (3). In addition to these families, several other coactivator proteins have been identified that function directly at the level of the preinitiation complex (PIC), enhancing its activity. This group includes TATA box-binding protein-associated factors (4), positive cofactors PC1, PC2, PC3, PCS, and PC52 (5), and several multiprotein complexes that are related to thyroid hormone receptor-associated protein-mediator complex (6).

Proline-, glutamic acid-, and leucine-rich protein 1 (PELP1) was a recent addition to the family of transcriptional coregulatory proteins and was identified as a novel estrogen receptor (ER) coactivator (7). It is a protein of 1273 amino acids that is unusually rich in prolines (13.2%), glutamic acid (12.4%), and leucine (12.9%) and, hence, was named PELP1. It has nine LXXL motifs (NR box motifs), seven toward the N-terminal region, and two in the central region. It interacts with and significantly enhances the transcriptional function of ERs, indicating that it functions as an ER coactivator. PELP1 did not have the same effect on progesterone receptor and glucocorticoid receptor, indicating that it may be an ER-specific coactivator. Even though it has no molecular mass of 160 kDa, sequence analysis showed it was distinct from the p160 family of coactivators. The conserved domains of p160 proteins such as bHLH, PER, ARNT, and SIM homology domains are absent in PELP1. It could interact with transcriptional activators like CBP and p300 in vivo, which suggest that PELP1 activates ER transcription by recruiting general coactivators such as CBP and p300. PELP1 also interacts with pRB (retinoblas-
tomato protein), a cell cycle switch protein, inducing its hyperphosphorylation in an estrogen-dependent manner, increasing estrogen-stimulated cell proliferation (8). Upon stimulation of cells with estrogen, there was an enhanced recruitment of PELP1 to the estrogen-responsive promoters and colocalization with the acetylated histone H3. Increased levels of PELP1-associated acetyltransferase activity were observed on estrogen stimulation. PELP1 also interacts with histones H1 and H3 and increases transcription by chromatin modification involving the displacement of H1 (9). In another study, PELP1 was confirmed to act as an ER coactivator but as a corepressor of glucocorticoid receptor and of non-NR sequence-specific transcription factors tested, including activating protein 1, nuclear factor κB (NF-κB), and ternary complex factor/serum response factor (10). The noted repressor activity of PELP1 was due to its ability to recruit HDAC2, which in turn might mask the acetylation of histones H3 and H4 and prevent them from acting as substrates for histone acetyltransferases. Ligand binding to the ER reverses the repressor role of PELP1, with a parallel increase in the status of histone hyperacetylation. More recently it was also shown that PELP1 functions as a coactivator of signal transducers and activator 3 (STAT3), stimulating its transcriptional activity (11). This positive regulation is brought about by the ability of PELP1 to augment growth factor-induced phosphorylation of serine 727 of STAT3 via activation of the Src-mitogen-activated protein kinase pathway. Collectively, emerging evidence suggests that PELP1 can function as a positive and a negative regulator of transcription in a transcription factor-specific manner.

Retinoids (natural retinoic acids and their synthetic derivatives) are derivatives of vitamin A. They are non-steroidal hormones that play a vital role in the development and homeostasis of almost every vertebrate tissue by regulating embryogenesis, cell differentiation, proliferation, and apoptosis (12, 13). Retinoids transduce their signals through two well-studied classes of receptors namely, retinoic acid receptor (RAR) and retinoid X receptor (RXR). RXRs are encoded by three different genes, each isotype being encoded by a separate gene (1, 14). RXRα, RXRβ, and RXRγ are the three isotypes of RXR and each isotype is encoded by a separate gene (1, 14). RXRs represent a unique class of NR as they function as obligate members of majority of NR heterodimers reported. They heterodimerize not only with RARs but also with several other receptors such as thyroid hormone receptors, vitamin D receptors (15), and peroxisome-proliferator-activated receptors (PPARs) (16). The RXRs bind with stereoselectivity to the retinoid 9-cis-retinoic acid (9-cis-RA). In addition to forming heterodimers, RXRs bind to 9-cis-RA and form functionally active homodimers that bind to the target DNA sequences (RXRE) and activate gene transcription (17).

In the present investigation we discovered that 9-cis-RA transcriptionally up-regulates expression of the PELP1 gene. PELP1, in turn physically binds with RXRs and functions as a coactivator, activating the transcriptional functions of RXRα homodimer and also of its permissive heterodimer with PPARγ. Consequently, PELP1 potentiates the apoptotic effects of 9-cis-RA and the differentiation-inducing effects of PPARγ-activating ligands.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Reagents**—MCF-7 cells were purchased from American Tissue Culture Collection (ATCC) and were maintained in Dulbecco’s modified Eagle’s medium and F-12 medium (1:1) (Media-tech, Herndon, VA) supplemented with 10% fetal calf serum. Antibodies for RXRs and PPARγ were from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-T7 tag antibody was from Bethyl Laboratories (Montgomery, TX), and anti-vinculin antibody was from Sigma. Anti-PELP1 antibody was used as reported earlier (7). Anti-mouse and anti-rabbit horseradish peroxidase or alkaline phosphatase-conjugated antibodies were from Amersham Biosciences. 9-cis-Retinoic acid, ciglitazone, and troglitazone were from Biomol (Plymouth Meeting, PA). The making and characterization of pcDNA, T7-PELP1, and T7-PELP1 H1-mutant protein—overexpressing cell lines were described earlier (9). These cells were cultured in RPMI medium (Mediatech) with 5% fetal calf serum and G418 (1 mg/ml).

**Reporter Luciferase Assays**—Cells were seeded in 6-well tissue culture plates 24 h before transfection. Subconfluent cells were transfected with PPRE-luciferase (17) or RXRE-tk-luciferase (18) using FuGENE 6 transfection reagent (Roche Applied Science). After 24 h the cells were treated with RXR- or PPAR-specific ligands as appropriate. Luciferase assay was done after 24 h using the luciferase assay kit (Promega). Each experiment was repeated a minimum of three times. The PELP1 promoter luciferase constructs were used as reported earlier (19).

**Glutathione S-transferase (GST) Pulldown Assay**—In vitro transcription and translation of RXRs was done using a T7-TNT kit (Promega), where 1 μg of cDNA in pcDNA 3.1 vector was translated in the presence of [35S]methionine in a reaction volume of 50 μl. The reaction mixture was diluted to 1 ml with Nonidet P-40 lysis buffer (25 mM Tris, 50 mM NaCl, and 1% Nonidet P-40). An equal aliquot was used for each GST pulldown assay. Translation and product size were verified by subjecting 2 μl of the reaction mixture to SDS-PAGE and autoradiography. The GST pulldown assays were performed by incubating equal amounts of GST and GST-tagged PELP1 deletion constructs, immobilized on glutathione-Sepharose beads (Amersham Biosciences) with in vitro translated [35S]labeled RXRs to which binding was being tested. Bound proteins were isolated by incubating the mixture for 3 h at 4 °C, washing 5 times with Nonidet P-40 lysis buffer, eluting the proteins with 2× SDS buffer, and separating them by SDS-PAGE. The bound proteins were then visualized by autoradiography.

**RT-PCR Analysis**—Total RNA from the cells was extracted using TRIzol reagent (Invitrogen) and treated with DNase for 15 min after which DNase was inactivated by heating the samples at 65 °C for 10 min. The cellular retinol-binding protein (CRBP II) mRNA levels were analyzed by RT-PCR with specific primers 5′-ctacagtacatccggac-3′ and 5′-aggctcagcttacggac-3′. Primers used for acyl-CoA synthase were 5′-tratgacgggaatagc-3′ and 5′-ctgcaagaggccattg-3′.

**Immunofluorescence and Confocal Microscopy**—Cells grown on glass coverslips were fixed in 4% phosphate-buffered paraformaldehyde for 15 min. Cells were permeabilized in methanol at −20 °C for 4 min. After permeabilization, cells were incubated with primary antibodies for 2 h at room temperature, washed 3 times in phosphate-buffered saline, and then incubated with secondary antibodies conjugated with 546-Alexa (red) or 488-Alexa (green) from Molecular Probes (Invitrogen). The DNA dye Topro-3 (Molecular Probes) was used for nuclear localization (blue). Confocal scanning analysis was performed using an Olympus FV300 laser scanning confocal microscope in accordance with established methods, utilizing sequential laser excitation to minimize the possibility of fluorescence emission bleed-through. Each image is a three-dimensional reconstructed stack of serial Z sections at the same cellular level and magnification. Co-localization of two proteins is shown yellow for red and green fluorescence.

**Lipid Droplet Staining with Oil Red O**—Cells were seeded into 6-well plates containing a glass coverslip in each well and cultured for 5 days in the absence or presence of 5 μM troglitazone. Subsequently, cells were
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**FIGURE 1. 9-cis-RA transcriptionally up-regulates expression of PELP1.** A, MCF-7 cells were treated with 9-cis-RA (2.5 μM) for 6 h after which total RNA was extracted. RT-PCR was done with equal amounts of RNA from 9-cis-RA- and Me2SO (DMSO) control-treated cells with PELP1 mRNA-specific primers. A simultaneous RT-PCR was also done for actin in the samples and used as control for comparison. B, the levels of PELP1 protein in MCF-7 and HeLa cells after 24 h of treatment with the vehicle (Me2SO) and 9-cis-RA (5 and 10 μM) treatment was analyzed by Western blotting (WB) of total cell lysates with PELP1-specific antibody. Vinculin expression was used as a control and is shown below. C, 9-cis-RA-induced PELP1 levels in MCF-7 cells was blocked by actinomycin D, a transcription inhibitor, and not by cyclohexamide, a translation inhibitor, showing that the up-regulation occurs at the transcriptional level. D, luciferase assay with different PELP1 promoter deletion luciferase constructs in MCF-7 cells after 24 h of treatment with 9-cis-RA. The 2-kilobase PELP1 promoter luciferase construct with 15 possible RXRα heterodimer binding sites and different deletion luciferase constructs of the promoter are schematically represented below. Data of all the luciferase assays are averages of a minimum of three independent experiments.

9-cis-Retinoic Acid Transcriptionally Up-regulates PELP1 Expression—The promoter region of PELP1 has been cloned and characterized with regard to the estrogen-induced transcriptional up-regulation (19). Analysis of the 2-kilobase PELP1 promoter sequence for putative transcription factor binding elements employing the program MatInspector Professional Version 7.2 (20) showed at least 15 possible RXRα heterodimer binding sites. This prompted us to check whether 9-cis-RA, an RXR-specific ligand, would transcriptionally up-regulate PELP1 expression. Treatment of MCF-7 cells with 9-cis-RA (2.5 μM) led to enhancement of the PELP1 mRNA expression within 6 h of treatment as evident by the RT-PCR analysis (Fig. 1A). Treatment of MCF-7 cells with 9-cis-RA for 24 h and subsequent Western blotting analysis of the total cell proteins with PELP1-specific antibody showed a clear increase in the level of PELP1 protein when compared with the control cells treated with Me2SO in a dose-dependent manner (Fig. 1B, upper panel). A similar up-regulation of PELP1 was observed in HeLa cells on treatment with 9-cis-RA (Fig. 1B, lower panel). Simultaneous treatment of cells with 9-cis-RA and actinomycin D, an inhibitor of transcription, blocked this up-regulation of PELP1 expression (Fig. 1C). On the other hand, similar treatment of cells with cyclohexamide, a protein synthesis inhibitor, did not affect the 9-cis-RA-induced up-regulation of PELP1 expression (Fig. 1C). This suggests that the increase in the level of PELP1 induced by 9-cis-RA was at the transcriptional level. To further validate
the transcription stimulating effect of 9-cis-RA on the PELP1 promoter and identify the possible 9-cis-RA-responsive region, we next carried out luc-reporter assays with PELP1 promoter and its deletion luciferase constructs (Fig. 1D). In response to 9-cis-RA treatment, a 2-fold increase in the luciferase activity with construct A, which has the complete 2-kilobase PELP1 promoter, was observed. The activity of PELP1 promoter construct C, encompassing the 700-bp region upstream of the putative start site, exhibited more than a 3-fold induction of luciferase activity. This region has four putative RXRα binding sites including one RXR/LXR, one PXR/RXR half-site, and two CAR/RXR binding consensus sequence, suggesting that this region might be responsible for the responsiveness of 9-cis-RA. Together, these results presented in Fig. 1 established that the PELP1 gene is a target of 9-cis-RA signaling.

PELP1 Functions as a Coactivator of RXRα—In addition to serving as a coactivator of ERα, PELP1 is a broad-range coregulator of several transcription factors (7, 9–11). Because 9-cis-RA up-regulates PELP1 expression (Fig. 1), we next examined the possibility of PELP1 in regulating retinoid signaling. To investigate this, we examined the effect of PELP1 on the RXRE-tk-luc-reporter assay in MCF-7 cells with transient overexpression of PELP1. Stimulation of cells with 9-cis-RA resulted in about a 2.5-fold induction of reporter activity by PELP1 as compared with about a 1.3-fold stimulation by control pcDNA, suggesting that PELP1 acts as a coactivator of RXRα (Fig. 2A). To gain further insights into PELP1 regulation of the RXRα pathway, we next used MCF-7 cells that stably overexpress PELP1. Results showed that 9-cis-RA was a potent stimulator of RXRα transactivation activity in cells with overexpression of PELP1 (Fig. 2B). In addition to recruiting chromatin modifiers such as CBP/p300, the coactivator function of PELP1 also requires binding and displacement of histone H1, which binds to DNA flanking the nucleosome core and represses the basal transcriptional activity of a variety of DNA binding factors including NRs. To show the significance of the histone H1 binding region of PELP1, we also conducted the RXRE-luc assay in MCF-7 cells stably expressing PELP1 histone H1-binding mutant protein lacking the C-terminal 253 amino acid stretch and incapable of binding to histone H1 (reported in Nair et al. Ref. 9). We found a significant reduction in the ability of PELP1-H1 mutant to transactivate RXRα, as there was only a 2-fold induction of RXRE-luc-reporter by 9-cis-RA in MCF-7/PELP1-H1 mutant cells as compared with a 4-fold induction in the case of MCF-7/PELP1 cells (Fig. 2B). These results confirmed that PELP1 could function as coactivator of RXRα and, thus, potentiate the effect of 9-cis-RA signaling in cells. The expression of T7-tagged PELP1-WT and PELP1 H1- mutant form in the respective stable clones has been shown (Fig. 2C, upper panel). The expression of similar levels of RXRα and PPARγ proteins in MCF-7/pcDNA, MCF-7/PELP1, and MCF-7/PELP1-H1 mutant cells was demonstrated by the Western blot analysis (Fig. 2C, lower panel). This ensured that the differential up-regulation of RXRE-luc observed in cells was due primarily to up-regulation of a fully functional PELP1 and not to alteration of the levels of the RXRα itself. To show the physiological relevance of the role of PELP1 as RXRα coactivator, RT-PCR analysis was done in the stable clones for the expression of CRBP II, an RXRα target gene. In response to 9-cis-RA stimulation, the expression of CRBP II in PELP WT clones was severalfold higher when compared with the pcDNA clones (Fig. 2D), which provides important proof of the potentiation of the RXRα transactivation function by PELP1.

PELP1 Physically Interacts with RXRα—Because PELP1 is a coactivator of RXRα, we next investigated whether PELP1 interacts with RXRα. Results from the GST pulldown assays, utilizing T7-tagged PELP1 deletion constructs and 35S-labeled RXRα, indicated that the N-terminal 1–400 amino acid region of PELP1, which has seven of the nine NR boxes in PELP1, has the highest binding affinity to RXRα (Fig. 3A, cont-
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FIGURE 3. PELP1 is a binding partner of RXRα. A, binding of GST-tagged deletion constructs of PELP1 to in vitro translated [35S]-labeled full-length RXRα was tested. Construct A, which encompasses the N-terminal 400 amino acids, NR-box rich region of PELP1 showed binding to RXRα. Construct B (region from 400–600 amino acids from the N-terminal) showed appreciable binding. No binding was seen with constructs C and D. B, immunoprecipitation (IP) of T7-PELP1 from PELP-WT stable cell lysate was done, and immunoblotting showed the presence of RXRα in co-immunoprecipitated proteins. No binding of RXRα to PELP1 was detected with similar immunoprecipitation of cell lysates without 9-cis-RA treatment. DMSO, Me2SO. C, 9-cis-RA-treated PELP1-WT stable cell lysate was subjected to immunoprecipitation with RXRα-specific antibody, and Western blotting (WB) of co-immunoprecipitated proteins with anti-T7 antibody detects the presence of PELP1, providing evidence for the in vivo interaction of PELP1 and RXRα. 10% of the total cell lysate was also used as input for immunoblotting. No detection of T7-PELP on immunoprecipitation with control IgG showed the specificity of the immunoprecipitation reaction. D, localization of PELP1 (red fluorescence) and RXRα (green fluorescence) in 9-cis-RA-treated PELP1-WT clones was visualized by confocal microscopy. The binding and colocalization of these two proteins was evident by the prominent yellow spots in the nuclear compartment. E, EMSA was done with 32P-labeled synthetic double-stranded oligonucleotides with consensus RXRE sequence. Nuclear extracts were prepared from pcDNA and PELP1 WT stable cells with and without treatment with 9-cis-RA. Labeled oligos were incubated with the nuclear extracts and separated on a 5% acrylamide gel. Binding of RXRα homodimer complexes to the oligonucleotides was evident by the appearance of the shifted bands. The binding of PELP1 to the oligo-bound RXRα was evident by the clear super shift of the band in the presence of PELP1-specific antibody (Ab).

A comparatively lower binding affinity was also shown by PELP1 construct B, which encompasses the region of amino acids 400–600 from the N-terminal region. No binding was observed by the deletion constructs C and D (Fig. 3A). The NR boxes are structural motifs that form an amphipathic helix employed by the coactivators to dock with the ligand activated NRs. Results from the above in vitro binding studies suggested that PELP1 may utilize its NR box-rich region to physically interact with RXRα. To validate the noted interaction of PELP1 with RXRα in the physiologic setting, we next performed co-immunoprecipitation followed by Western blot analysis from cellular extracts. Because the PELP1 antibody does not work well in immunoprecipitation assays, we used MCF-7/PELP1 cells, which allowed us to immunoprecipitate the T7-PELP1 by anti-T7 antibody. MCF-7/PELP1 and control cells were stimulated with 9-cis-RA or with the vehicle Me2SO, T7-PELP1 was immunoprecipitated from the cell lysates using an anti-T7-antibody, and immunoprecipitated proteins were separated by SDS-PAGE and immunoblotted with RXRα antibody. We found that RXRα indeed interacted with T7-PELP1, and such interaction was further enhanced by 9-cis-RA stimulation (Fig. 3B). Conversely, we immunoprecipitated the cell lysates with anti-RXRα antibody and showed coprecipitation of PELP1 (Fig. 3C). No PELP1 was detected in the lane where proteins were immunoprecipitated with control IgG. As an additional validation of in vivo PELP1-RXRα interaction, we used scanning confocal microscopy and co-localization of PELP1, and RXRα was visualized by immunofluorescence. Localization of PELP1 and RXRα was detected by red and green fluorescence, respectively. The majority of PELP1 was localized in the nucleus along with RXRα, and their colocalization was evident by yellow spots due to the overlapping of the two colors (Fig. 3D). To demonstrate the binding of PELP1 to RXRα homodimers bound to their consensus DNA sequences, EMSA was done with nuclear extract from pcDNA and PELP1 WT clones treated with 9-cis-RA using labeled synthetic double-stranded oligos with RXRE sequence (Fig. 3E). Incubation with the nuclear extracts resulted in the appearance of shifted bands, which represented the homodimer bound to the oligos. Increased binding of RXRα to the oligos was evident in both pcDNA and PELP WT clones on 9-cis-RA treatment (Fig. 3E, lanes 3 and 5, in comparison to lanes 2 and 4, respectively). Relatively, there was a higher level of formation of RXRα-bound oligos in PELP WT sample in comparison to pcDNA when treated with 9-cis-RA (lane 5 in comparison to lane 3). The levels of RXRα in these clones were the same (as shown in Fig. 2C, lower panel); this shows a greater formation and stability of the DNA bound RXRα homodimers in the presence of...
PELP1. Previous studies have shown that co-presence of coactivators such as SRC1 further increased the stability of DNA-bound RXRα homodimers in response to 9-cis-RA (21). These results suggest that increased levels of PELP1 protein may be leading to a similar enhancement of the formation and stabilization of RXRα homodimers on the RXRE element, which is a reflection of the coactivator function of PELP1 in RXRE-driven transcription. Further proof of the binding of PELP1 to the oligo-bound homodimers was provided by the clear super-shift of the band in the presence of PELP1 specific antibody (Fig. 3 E, lane 6). These results on the whole suggest that PELP1 and RXRα interact under physiologic conditions.

PELP1 Potentiates Apoptotic Action of 9-cis-RA—Retinoids are potent antiproliferative and pro-apoptotic agents, making them potential anticancer drugs (22). 9-cis-RA, a specific ligand of RXRα, is an important retinoid that has been actively investigated for its anticancer properties (23). In the context of findings that PELP1 was an interacting coactivator of RXRα, we next investigated whether PELP1 could affect the pro-apoptotic properties of 9-cis-RA. MCF-7/pcDNA, MCF-7/PELP1 WT, and MCF-7/PELP1-H1 mutant were treated with 9-cis-RA or solvent vehicle (2.5 μM for 5 days), and cell growth was documented. Results showed that 9-cis-RA induced cell death in all the cell lines tested here, as evinced by the reductions in cell numbers. Interestingly, cells overexpressing PELP1 exhibited significantly greater cell death than MCF-7/pcDNA or MCF-7/PELP1-H1 mutant-expressing cells in response to 9-cis-RA (Fig. 4A). This was evident by the fact that, after 5 days of 9-cis-RA treatment, there was 70% reduction in cell number in PELP1 clones in comparison to 50% reduction in the case of both pcDNA and PELP1-H1 mutant clones (Fig. 4A). A higher tendency of cell death of the PELP1 clones in response to 9-cis-RA was evident in three similar experiments conducted separately, and the data presented are an average of the three experiments. To further document the extent of cell death in these cells, we also collected the cells floating in the medium in the above-mentioned experiment and quantified the cell death by trypan blue staining (24). Results indicated that 9-cis-RA stimulates about 5-fold increased apoptosis in MCF-7/PELP1 cells in comparison to the control of MCF-7/PELP1-H1 mutant cells (Fig. 4B). Interestingly, MCF-7/PELP1-H1 mutant and MCF-7/pcDNA showed a comparable level of apoptosis in response to stimulation with 9-cis-RA (Fig. 4). Furthermore, a higher degree of cell death in the PELP1-expressing cells also could be observed in the photomicrographs of the cells taken after 5 days of 9-cis-RA treatment (Fig. 4C).

To further validate the propensity of the cells with deregulated PELP1 expression to undergo apoptosis, we analyzed the effect of 9-cis-RA (2.5 μM for 3 days) on the ratio of the sub-G1 cells using fluorescence-assisted cell sorting analysis. The sub-G1 population is represented by cells undergoing death, which have degraded DNA because of the shedding of apoptotic bodies and, hence, are referred to as "sub-G1 (25). Results from fluorescence-assisted cell sorting studies showed significantly greater proportions of the sub-G1 population in both basal and 9-cis-RA-treated MCF-7/PELP1 cells than in MCF-7/pcDNA or MCF-7/PELP1-H1 mutant cells (Fig. 5A). As an additional proof, annexin V staining was done for early apoptosis in these stable cell lines. Cells were treated with 9-cis-RA (5 μM) for...
16 h and then stained with fluorescein isothiocyanate-annexin V. Under a fluorescence microscope, apoptotic cells were identified by the green fluorescence due to the binding of fluorescein isothiocyanate-annexin V to phosphatidylserine, which is translocated to the external portion of the plasma membrane in apoptotic cells. In the case of each stable clone, five different fields were examined with both phase contrast and fluorescence microscopy, and the average number of apoptotic cells among total number of cells was quantitated. On 9-cis-RA treatment the MCF7/PELP1-WT cells showed a higher degree of early apoptosis (8% of cells) in comparison with pcDNA (2%) and PELP1-H1-mutant cells (1%) (Fig. 5B, right panel). A representative picture of the same field under phase contrast and fluorescence microscopy of the cells is shown for comparison (Fig. 5B, left panel). This confirmed that PELP1-overexpressing clones are more responsive to 9-cis-RA-induced apoptosis. Taken together, these results showed that the enhanced apoptosis by PELP1 was closely linked with
PELP1 Potentiates PPARγ-induced Differentiation—Both natural and synthetic PPARγ ligands possess the capacity to inhibit cell proliferation and induce cellular differentiation, and thus, are being explored as potential anticancer agents (27). Because the present study revealed a coactivator function of PELP1 for PPARγ, we next wished to determine whether PELP1 could also enhance the differentiation-inducing property of PPARγ. MCF-7/pcDNA, MCF-7/PELP1, and MCF-7/PELP1-H1 mutant cells were treated with a 5 μM concentration of troglitazone for 5 days, and the cells were stained with oil red O stain to measure the extent of neutral lipid droplets accumulation in cells. One of the physiological effects of the differentiation stimulating signals in breast cancer cells is to induce the accumulation of neutral lipid droplets. Oil red O staining is a widely used method to assess the extent of cellular differentiation where a lipophilic red dye is used to stain the neutral lipid droplets. Results showed that stimulation with troglitazone resulted in a dramatic increase of the neutral droplet accumulation in MCF-7/PELP1 cells (Fig. 8A). As expected, a detectable increase of the red droplets was also observed in the troglitazone-treated MCF-7/pcDNA cells. No staining for the lipid droplets was observed in the MCF-7/PELP1-H1 mutant cells either in the presence or absence of troglitazone. Interestingly, MCF-7/PELP1 cells exhibited an overall higher differentiating tendency even in the presence of vehicle (Me2SO), probably in response to the presence of residual PPARγ ligands. These findings raise the possibility that PELP1 could function as a coactivator of the RXRα-PPARγ heterodimer. To validate this notion, PPRE-luciferase assay was conducted in MCF-7/PELP1 clones. We found a significant induction of PPRE-luc activity in MCF-7/PELP1 cells as compared with the levels in MCF-7/pcDNA or MCF-7/PELP1-H1 mutant cells in response to treatment with troglitazone and ciglitazone (Fig. 7B). Surprisingly, there was a robust induction of PPRE-luc activity in MCF-7/PELP1 cells stimulated with 9-cis-RA. There was a 50-, 70-, and 90-fold induction of the PPRE-Luc activity upon treatment with 1, 5, and 10 μM 9-cis-RA, respectively (Fig. 7C). Comparatively low levels of induction of PPRE-luc activity were observed in the control pcDNA and PELP-H1 mutant cells (Fig. 7C), suggesting the involvement of PELP1 in the noted robust up-regulation of PPRE-luc activity. Accordingly, on citalon treatment, the expression levels of acyl-CoA synthase, which is a PPARγ target gene, was severalfold higher in MCF-7/PELP1-WT cells in comparison to the levels in MCF7/pcDNA cells (Fig. 7D). These results suggested that PELP1 could function as a coactivator of RXRα-PPARγ heterodimer in response to both 9-cis-RA- and PPARγ-specific ligands.

**PELP1 Promotes PPARγ Response Element-driven Transcription—RXRα has the distinction of being the NR that promiscuously forms heterodimers with an increasing number of other NRs. After establishing the capability of PELP1 to bind and function as a coactivator of RXRs, we next tested the possibility of whether PELP1 also modulates transcription function of a RXRa heterodimer. A large body of the previous work suggests that RXRα-PPARγ remains the best-studied heterodimer in terms of activating ligands, participating response elements, and the resulting physiological effects (26). The RXRs-PPARγ heterodimer specifically binds to the PPRE sequence motifs and brings about transcription in response to both PPARγ and RXRa ligands. We examined the effect of PELP1 overexpression on the PPRE-luc-reporter system. We found a clear increase of the luciferase activity in cells with transient increased expression of PELP1 in response to two different PPARγ ligands, namely troglitazone and ciglitazone (Fig. 7A). These findings raise the possibility that PELP1 could function as a coactivator of the RXRα-PPARγ heterodimer. To validate this notion, PPRE-luciferase assay was conducted in MCF-7/PELP1 clones. We found a significant stimulation of PPRE-luc activity in MCF-7/PELP1 cells as compared with the levels in MCF-7/pcDNA or MCF-7/PELP1-H1 mutant cells in response to treatment with troglitazone and ciglitazone (Fig. 7B). Surprisingly, there was a robust induction of PPRE-luc activity in MCF-7/PELP1 cells stimulated with 9-cis-RA. There was a 50-, 70-, and 90-fold induction of the PPRE-Luc activity upon treatment with 1, 5, and 10 μM 9-cis-RA, respectively (Fig. 7C). Comparatively low levels of induction of PPRE-luc activity were observed in the control pcDNA and PELP-H1 mutant cells (Fig. 7C), suggesting the involvement of PELP1 in the noted robust up-regulation of PPRE-luc activity. Accordingly, on citalon treatment, the expression levels of acyl-CoA synthase, which is a PPARγ target gene, was severalfold higher in MCF-7/PELP1-WT cells in comparison to the levels in MCF7/pcDNA cells (Fig. 7D). These results suggested that PELP1 could function as a coactivator of RXRα-PPARγ heterodimer in response to both 9-cis-RA- and PPARγ-specific ligands.

**PELP1 Silencing Lowers Susceptibility of Cells to 9-cis-RA-induced Cell Death—**After discovering that increased expression of PELP1 promotes 9-cis-RA-induced apoptosis, we next examined the effect of silencing PELP1 expression using specific RNAi on the ability of 9-cis-RA to induce cell death. MCF-7/PELP1 cells were transfected with PELP1-specific RNAi or control siRNA. After 48 h the cells were treated with 9-cis-RA (2.5 μM for 4 days) or with Me2SO, and cell numbers were recorded. We found a modest but reproducible reduction (about 20%) in the extent of cell death in cells with silenced PELP1 expression (Fig. 6A). This reinforced the finding that PELP1 is an efficient activator of 9-cis-RA-induced apoptosis, and silencing of its expression compromised the susceptibility to effects of 9-cis-RA. A similar silencing of the endogenous PELP1 expression in parental MCF-7 breast cancer cells also resulted in an identical lower sensitivity of cells to undergo cell death (Fig. 6B). These findings indicated that PELP1 is an essential component of the 9-cis-RA-induced cell death, presumably due to coactivator function of PELP1 for the RXRa pathway. An effective knocking down of PELP1 expression in PELP1-WT stable cell lines and in MCF-7 parental cell lines is evident by the Western blotting analysis for PELP1 in total cell lysates (Fig. 6, A and B, right panel).
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nototypes (28), and a decrease in the levels of HER2 is one of the hallmarks of differentiating cells. Consistent with this, we found that troglitazone treatment reduces the level of HER-2 protein in MCF-7/pcDNA as well as in MCF-7/PELP1 cells (Fig. 8A, lower panel). However, the extent of HER2 down-regulation was more prominent in MCF-7/PELP1 cells, indicating a higher propensity of PELP1-overexpressing cells to undergo differentiation in response to PPARγ ligands.

DISCUSSION

The binding of the hormone to the NR and the subsequent binding of the NR homodimer or heterodimer to specific DNA sequences represent the initial switch by which the NR activates the signaling cascade. The further amplification of the signal is brought about by the ability of the NRs to recruit coactivator proteins, which by their chromatin remodeling activities make the chromatin more accessible to the transcription machinery, thus activating gene transcription. In this context it is not surprising that the level of expression, subcellular localization, stability, and activity of the coactivator proteins profoundly affects the NR signaling functions. In addition to deciphering the mechanism of coactivator functions, the identification of novel coactivators is also equally important, as the overall effect of the NR signaling in the cell is the cumulative effect of several critical coactivators in hormone-stimulated cells.

RXRα is a retinoid receptor that is activated by the specific ligand 9-cis-RA and related retinoids. It functions as a homodimer and, more often, as an obligate heterodimeric partner of several other NRs. With PPARγ, for example, RXRα forms what is referred to as the “permissive” heterodimer, which is activated by either RXRα- or PPARγ-specific ligands (29). In the present investigation we found that 9-cis-RA transcriptionally up-regulates the expression of PELP1 gene and protein (Fig. 1). This appears to be a direct effect of 9-cis-RA on PELP1 gene transcription, as transcription inhibitor actinomycin D and not the translational inhibitor cyclohexamide blocked 9-cis-RA-induced PELP1 gene expression (Fig. 1C). PELP1 was identified as a coactivator of ERα (7), and several recent publications revealed its function as an important transcriptional coregulatory protein (9–11). While exploring the ultimate purpose of 9-cis-RA-induced PELP1 expression, we found that PELP1 functions as a coactivator protein of RXRα, a receptor for 9-cis-RA (Fig. 2). RXRE-luciferase assays conducted in MCF-7 cells showed that PELP1 functioned as a coactivator of RXRα (Fig. 2A). This was further confirmed by the RXRE-luciferase assay conducted in PELP1-overexpressing clones, where 3–4-fold higher activation of the luciferase in comparison to the pcDNA and PELP1-H1 mutant clones (Fig. 2B) was observed. Similar levels of expression of RXRα across all the clones showed that the effect was due to PELP1 overexpression and not due to discrepancy in the levels of RXRα in the cells (Fig. 2C). Severalfold higher expression levels of CRBP II, a RXRα target gene in MCF-7/
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PELP1 WT cells, in comparison to the MCF-7/pDNA cells (Fig. 2D) on treatment with 9-cis-RA reinforced this notion.

One of the basic prerequisites of a typical coactivator protein is that it physically interacts with the hormone-bound NR. This facilitates recruitment of the coactivator to DNA-bound NR and subsequent stimulation of target gene expression. Consistent with this notion was our finding that PELP1 binds to RXRα via its N-terminal region rich in NR-box motifs (Fig. 3) and that 9-cis-RA stimulation of cells further augmented such interactions in the physiologic setting. Co-immunoprecipitation of PELP1 with RXRα (Fig. 3, B and C) supported by the colocalization of these two proteins in the nuclear compartment (Fig. 3D) reinforced PELP1 and RXRα as interacting partners in vivo. EMSA studies showed convincingly that PELP1 could interact with DNA-bound RXRα protein. These experiments provided the necessary evidence for identifying PELP1 as a novel binding partner of RXRα, contributing to coactivator function of PELP1.

Retinoids are powerful inducers of cellular apoptosis and have been a subject of extensive research as cancer preventive and therapeutic agents (23, 30). The function of PELP1 as a coactivator of RXRα prompted us to propose a potential role for PELP1 in modulating the pro-apoptotic effects of 9-cis-RA. Indeed, it was discovered that deregulation of PELP1 enhances the susceptibility of cells to undergo apoptosis in response to 9-cis-RA (Fig. 4), whereas PELP1 knockdown confers partial resistance to 9-cis-RA-induced apoptosis (Fig. 6), suggesting that PELP1 may be an important component of the molecular machinery involved in manifestation of physiological effects of 9-cis-RA.

RXRα, in addition to modulating gene expression as a homodimer, also functions as an obligate heterodimer of several other NRs and, hence, is involved in the signaling of those NRs and their respective ligands. The RXRα-PPARγ heterodimer remains one of the most well characterized RXRα-involved NR systems in terms of the physiological effects, genes regulated, molecular mechanism, and coactivator recruitment. PPARγ is also considered an important molecular target in cancer therapy, as the PPARγ-activating ligands have anti-proliferative effects because of their ability to induce cellular differentiation (17). RXRα and PPARγ form permissive heterodimers that can be activated by ligands specific to either of the NR. In this context this study also discovered that PELP1 also acts as a coactivator of the RXRα-PPARγ heterodimer (Fig. 7). PPRE-luciferase assays conducted in MCF-7 cells with transient overexpression of PELP1 (Fig. 7A) showed that PELP1 increased the luciferase activity in response to two synthetic PPARγ ligands, troglitazone and ciglitazone. Similar luciferase assays conducted in stable cell lines exhibited greater induction of luciferase activity in PELP1-WT clones in comparison to the pDNA and the PELP-H1 mutant clones, further proving that PELP1 was a coactivator of RXRα-PPARγ heterodimer (Fig. 7B). Interestingly, in PELP1-WT clones in response to 1, 5, and 10 μM 9-cis-RA, a very high, 50-, 70-, and 90-fold induction of PPRE-luciferase was observed, respectively (Fig. 7C). This was drastic in comparison to a 2–3-fold induction observed in response to the PPARγ-specific ligands (Fig. 7B). This may be attributed to the fact that the RXRα-PPARγ heterodimer, a permissive heterodimer, is activated in response to 9-cis-RA and can stimulate expression at PPRE consensus sequences. In addition to that, 9-cis-RA can activate RXRα homodimers, that are also capable of binding and inducing transcription on PPREs (22). This cumulative effect would be responsible for the drastic induction of PPRE in response to 9-cis-RA.

Functioning as a coactivator of RXRα-PPARγ heterodimer, PELP1 was also able to potentiate the differentiation-inducing effects of PPARγ ligand as evident by the red oil O staining in the three stable cell lines. PELP1-WT clones showed a high degree of differentiation as evident by a high degree of formation of neutral lipid droplets in response to troglitazone (Fig. 8). On the whole, in this study we have shown that PELP1 potentiates the effects of 9-cis-RA and PPARγ-specific ligands by functioning as a coactivator of RXRα homodimers and RXRα-PPARγ heterodimer. 9-cis-RA also manifests its apoptotic effects by acting as a ligand for RARα, which functions as a heterodimer with RXRα and binds to retinoic acid receptor response element (RARE) elements. In addition to binding RXRα, it is possible that PELP1 may also interact with RARα and potentiates the apoptotic effects of 9-cis-RA. It is also possible that PELP1 might act as coactivator of transcription at RARE by binding to RXRα if not RARα in the heterodimer. This possibility warrants a separate study and is not included in the present investigation.

PELP1 was cloned and identified as a coactivator of ERα-mediated transcription (7). Subsequently, it was also found to function as a repressor of glucocorticoid receptor and non-NR transcription factors such as activating protein 1, NF-κB, and ternary complex factor/serum response factor (10). In the present investigation we have shown the capability of PELP1 to bind to another member of the NR family, namely RXRα, and function as a transcriptional coactivator of RXRα.
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homodimers and RXRα-PPARγ heterodimers, which are known to be involved in antiproliferative, proapoptotic and differentiation-inducing functions. In response to estrogen, PELP1 is recruited to ERα to potentiate its proliferative signal, whereas in response to RXRα- or PPARγ-specific ligands, PELP1 functions as a coactivator of RXRα homodimers and RXRα-PPARγ heterodimers to potentiate their antiproliferative effects. Overall, these findings strengthen the hypothesis that cells utilize a common pool of coactivators to facilitate signal transduction leading to distinct phenotypic changes depending upon the nature of the hormonal signal. As a parallel example, PGC-1 was identified and cloned as a coactivator of PPARγ (31), but subsequently it was found to function as a coactivator of ERα transcriptional activity (32). The ability of transcriptional coregulatory proteins such as PELP1 to interact and modulate the functions of several transcription factors may help the cell manifest myriad functions by employing relatively few common coregulators and, thus, effectively avoid the expenditure of having specialized coregulators for specific transcription factors.

To summarize, we have revealed a novel facet of PELP1 function as a potentiator of antiproliferative effects of RXRα and PPARγ-specific ligands, identifying it as a potential target for the anti-cancer therapy. This study has added one more signaling pathway to a rapidly growing list of pathways regulated by PELP1, helping to further establish it as an important transcriptional coregulatory protein in the cell.

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