

The PGC-1-related Protein PERC Is a Selective Coactivator of Estrogen Receptor α *

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Peroxisome proliferator-activated receptor γ coactivator-1 (PGC-1) is a tissue-specific coactivator that enhances the activity of many nuclear receptors and coordinates transcriptional programs important for energy metabolism. We describe here a novel PGC-1-related coactivator that is expressed in a similar tissue-specific manner as PGC-1, with the highest levels in heart and skeletal muscle. In contrast to PGC-1, the new coactivator shows high receptor specificity. It enhances potently the activity of estrogen receptor (ER) α , while having only small effects on other receptors. Because of its nuclear receptor selectivity, we have termed the new protein PERC (PGC-1 related Estrogen Receptor Coactivator). We show here that the coactivation function of PERC relies on a bipartite transcriptional activation domain and two LXXLL motifs that interact with the AF2 domain of ER α in an estrogen-dependent manner. PERC and PGC-1 are likely to have different functions in ER signaling. Whereas PERC acts selectively on ER α and not on the second estrogen receptor ER β , PGC-1 coactivates strongly both ERs. Moreover, PERC and PGC-1 show distinct preferences for enhancing ER α in different promoter contexts. Finally, PERC enhances the ER α -mediated response to the partial agonist tamoxifen, while PGC-1 modestly represses it. The two coactivators are likely to mediate distinct, tissue-specific responses to estrogens.

Nuclear receptors are ligand-regulated transcription factors with a broad range of functions in development, physiology, and behavior. They include steroid hormone receptors for glucocorticoids, mineralocorticoids, progestins, estrogens, and androgens, as well as receptors for thyroid hormone, retinoids, vitamin D, and intermediary metabolites (1). They use a conserved DNA binding domain (DBD)¹ to interact with specific

sites in the genome, termed hormone response elements (HREs). DNA-bound receptors can activate the expression of genes in the vicinity of HREs, via two transcriptional activation functions, denoted AF1 and AF2. AF1 lies in the N-terminal part of the receptors and varies significantly from one receptor to another. AF2 is located at the conserved ligand binding domain (LBD) and relies on an agonist ligand-induced protein conformation (2–5). Depending on cellular and promoter context, AF1 and AF2 act independently or synergistically to regulate gene expression.

A large number of proteins that interact with the AF2 domain and enhance the activity of nuclear receptors have been identified (reviewed in Refs. 6–8). They include the three members of the p160 steroid receptor coactivator (SRC) family (SRC-1/NcoA-1, TIF2/GRIP1/NcoA-2, AIB1/pCIP/ACTR/RAC3/SRC-3), the coregulators CBP and p300, components of the Mediator complex, individual coactivators such as PGC-1, NRIF3, ASC-2/RAP250, PELP1, and CAPEP, and the family of CITED proteins (6–12). Most of these coactivators harbor one or multiple LXXLL motifs (L being leucine and X any amino acid) within short amphipathic helices (13, 14). These LXXLL motifs, also called NR boxes, interact with a hydrophobic pocket of the ligand-activated LBD of the receptors, thereby recruiting the coactivators to target DNA sites (15–17). The diverse coactivators are then thought to regulate transcription via enzymatic modification of chromatin or other transcription proteins, and/or physical recruitment of components of the transcriptional machinery (reviewed in Refs. 6–8). The multitude of nuclear receptor coactivators suggests that at least some of them carry distinct and specific functions. They may do so by interacting with specific subsets of receptors, acting in selective cell types, directing receptor function to subsets of target genes or conferring regulation by other signals.

Of the so far identified AF2 coactivators, most interact with many, if not all, nuclear receptors. Although particular LXXLL motifs of SRC-1, TIF2, and SRC-3 display preferences for specific receptors, the three p160 coactivators can enhance the activity of most nuclear receptors (18, 19). CBP and p300 are general coactivators, not only of nuclear receptors but also of many nonreceptor transcription factors (7). AF2 coactivators that display receptor specificity include NRIF3, PELP1, CAPEP, and CITED1. NRIF3 enhances selectively the activity of the thyroid hormone receptor (TR) and retinoid X receptor (RXR), without affecting the glucocorticoid (GR), estrogen (ER) or vitamin D receptors (9). The other three receptor-selective coactivators potentiate preferentially the activity of the two ERs, ER α and ER β (10–12). None of the ER-specific AF2 coactivators described so far distinguish between ER α and ER β ,

progesterone receptor; MR, mineralocorticoid receptor; AR, androgen receptor; AD, activation domain; RRM, RNA recognition motif; β -gal, β -galactosidase; HA, hemagglutinin.

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF468496 and AF468497.

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¹ The abbreviations used are: DBD, DNA binding domain; HREs, hormone response elements; LBD, ligand binding domain; SRC, steroid receptor coactivator; TR, thyroid hormone receptor; RXR, retinoid X receptor; GR, glucocorticoid receptor; ER, estrogen receptor; PPAR, peroxisome proliferator-activated receptor; PGC-1, PPAR γ coactivator 1; HNF, hepatocyte nuclear factor; NRF, nuclear respiratory factor; PRC, PGC-1-related coactivator; PERC, PGC-1-related estrogen receptor coactivator; ERE, estrogen response element; aa, amino acid(s); PR,

receptors that bind similar ligands and carry distinct biological functions (20, 21).

Few coactivators show tissue-specific expression. One of them is PGC-1, which is expressed at high levels in tissues such as heart, skeletal muscle, kidney, and brown fat (22–24). PGC-1 expression is induced also in a tissue-specific manner, in response to particular physiological states such as exposure to cold or fasting (22, 25, 26). Induction of PGC-1 in response to signals indicating metabolic needs of an organism can then lead to the activation of pathways important for energy homeostasis, such as adaptive thermogenesis, mitochondrial biogenesis, fatty acid oxidation, and gluconeogenesis (22, 25–29). PGC-1 interacts with and enhances the activity of many nuclear receptors, like the peroxisome proliferator-activated receptors (PPAR) α and γ , TR, GR, ER α , hepatocyte nuclear factor 4 (HNF4), as well as nonreceptor transcription factors like the nuclear respiratory factor 1 (NRF1) (22, 24, 26–28, 30). A characteristic feature of PGC-1, not shared by other nuclear receptor coactivators, is its C-terminal domain. It harbors sequence motifs typical of RNA processing regulators and has been implicated in the regulation of pre-mRNA splicing (31).

The existence of sequence-related coactivators, such as the three p160 SRC proteins, or CBP and p300, may reflect the evolutionary adaptation of duplicated genes to similar but distinct biological functions. Recently, a PGC-1 related coactivator (PRC) that is ubiquitously expressed and enhances the activity of NRF1 was described (32). Here, we report the cloning and characterization of a third member of the family. PERC (PGC-1 related estrogen receptor coactivator) is expressed in a tissue-specific manner and displays a striking preference for functional interactions with ER α among the nuclear receptors.

EXPERIMENTAL PROCEDURES

Cloning of PERC—Total RNA was isolated from HeLa cells with the Trizol reagent (Invitrogen). Full-length cDNA was synthesized either by standard procedures using oligo(dT) primers or with the GeneRacer kit (Invitrogen). Oligo(dT)-primed cDNA was used to amplify sequences from exon 2 to the end of the predicted PERC open reading frame. The 5' part and first exon of the cDNA, which were absent from the published genome sequence, were amplified in a nested polymerase chain reaction (PCR), using internal exon 3-specific PERC primers, 5' GeneRacer Primers, and cDNA synthesized with the GeneRacer kit. Multiple clones were analyzed and sequenced. Two types of PERC cDNAs were found at a ratio of 1:1 (of 12 clones). They differed by a 117-bp sequence, which corresponds to exon 4 of PERC. Restriction sites were introduced by PCR at the 5' and 3' ends of the PERC coding sequences, and full-length PERC (including the 117-bp exon 4) and PERC-s (lacking exon 4) clones were constructed by standard subcloning procedures. The PERC sequences have been submitted to the GenBank™ data base under accession numbers AF468496 and AF468497. The full-length PERC is the human homolog of the recently described mouse PGC-1 β (33).

Expression Analysis—Total RNA was isolated from tissues of 6–8-week-old mice using the Trizol reagent and checked for its integrity by agarose gel electrophoresis and ethidium bromide staining. RNA (400 ng) was converted to cDNA in a 20- μ l reaction at 45 °C for 45 min using MultiScribe reverse transcriptase (Applied Biosystems) and random hexamer primers according to the manufacturer's instructions. Real-time PCR with the LightCycler system (Roche Diagnostics) was used for the amplification and quantification of PERC, PGC-1, and β -actin cDNA. LightCycler reactions were performed in a final volume of 15 μ l, using 3 μ l of cDNA, 10 pmol of specific primers, and the LC FastStart SYBRGreen kit (Roche Diagnostics) as recommended by the manufacturer (denaturation at 95 °C for 15 s, annealing at 60 °C for 5 s, extension at 72 °C for 10 s; 40 cycles, with the PCR product being monitored at 72 °C at the end of each cycle). A melting curve from 65 to 95 °C (0.05 °C/s) at the end of the reaction was used to check the purity and nature of the product. In all cases, a single PCR product was detected. Primers were chosen with the help of the OLIGO 4 program and were from different exons, so as to avoid amplification of possible DNA contamination of the RNA preparation. The sequences of the primers and the sizes of the PCR products were as follows: 5'-CAA GCT CTG ACG CTC TGA AGG-3' (exon 4) and 5'-TTG GGG AGC AGG CTT TCA

C-3' (exon 5) for PERC (product 201 bp), 5'-GGA GCC GTG ACC ACT GAC A-3' (exon 4) and 5'-TGG TTT GCT GCA TGG TTC TG-3' (exon 5) for PGC-1 (product 176 bp), 5'-GGT CAT CAC TAT TGG CAA CGA G-3' (exon 3) and 5'-GTC AGC AAT GCC TGG GTA CA-3' (exon 4) for β -actin (product 196 bp). Control reactions performed on plasmid DNA confirmed that the PGC-1 primers could not amplify PERC sequences and vice versa. For quantification, standard amounts for each template (from 400,000 to 128 plasmid copies, in 1:5 dilutions) were analyzed in parallel to the samples. The cycle numbers needed for a log-linear phase product to reach the crossing point, which was set above the background noise, were plotted against the logarithm of the input plasmid copy number and fitted to a standard curve. The cDNA copy numbers for each gene were calculated from the standard curve, and the copy numbers of PERC and PGC-1 were normalized to the number of β -actin copies in the sample. Results shown are from duplicate reactions, using the same cDNA preparation. Similar results were obtained from independent preparations of cDNAs from two female and two male mice.

Plasmid Constructs—PERC deletion and point mutants were generated by standard PCR methods and verified by sequencing. All PERC variants were subcloned into pcDNA3/HA, pcDNA3/GAL4DBD (containing Gal4 DBD as a *HindIII*/*NdeI* fragment from pGBKT7 (CLONTECH)), and pGADT7 (CLONTECH). More information on the plasmids is available on request. Expression plasmids p6RGR, p6RMR, pSVARo, pSG5/ER α , pcDNA3/HA-hPGC-1, and pSG5/SRC-1e, as well as the luciferase reporter plasmids pTAT3-Luc, pERE-tk-Luc (one copy of the vitellogenin A2 ERE fragment (–334 to –289 nucleotides, relative to transcription initiation) (vERE)), and pGK1 have been described (24). The following expression and luciferase reporter plasmids were generously provided: pSG5/hPR (34), pSG5/hER β (E. Treuter), pSV-SPORT1/mPPAR γ 2 and p3xPPRE-tk-Luc (M. Meyer), pSG5/hTR β and pSG5/mRXR α (H. Gronemeyer), pMMTV-LTR-Luc (35), pminPbLUC-neo (F. Hamy), pC3-Luc (5). For the expression of the Gal4DBD/hER α -LBD fusion in yeast, the hER α -LBD (308C) was amplified by PCR from pSG5/ER α and subcloned into pGBKT7 to yield pGBKT7/hER α (308C). To generate hER α AF2 mutant L539/540A, the LBD was amplified by PCR from pRST7/hER α -LL (30) and subcloned either into pGBKT7 to yield pGBKT7/hER α (308C)-LL or into pSG5/ER α to yield pSG5/hER α -LL. The luciferase reporter plasmids p Δ (vERE)x1-Luc and p Δ (vERE)x2-Luc were constructed by cloning the vERE-containing *HindIII* fragment from pERE-tk-Luc into the *HindIII* site upstream of the minimal alcohol dehydrogenase promoter of pALuc (35). p Δ (cERE)x1-Luc and p Δ (cERE)x2-Luc have a monomer or dimer of the sequence 5'-GAG CTC GAG AGG TCA CAG TGA CCT GTC-3' (consensus (cERE) half-sites are underlined) at the *SalI* site of p Δ -Luc. p Δ (DR4)x2-Luc has the sequence 5'-CTT AGG TCA CTT CAG GTC AGC CTC GAG GGA GGT CAC TTC AGG TCA GTC-3' (DR4 half-sites are underlined) at the *HindIII*/*SalI* sites of p Δ -Luc.

Cell Culture and Transfections—COS7 and U2OS cells were cultured in Dulbecco's modified Eagle's medium supplemented with 9% fetal bovine serum. Charcoal-stripped fetal bovine serum was used when assaying hormone responses. Media lacking phenol red were used in experiments with AR or ERs. Cells were seeded into six-well plates 24 h prior to transfection by the calcium phosphate precipitation method. All transfections included 0.2 μ g of p6RlucZ for normalization of transfection efficiency. Standard amounts of expression and reporter plasmids per transfection in coactivation assays were: 1 μ g of nuclear receptor expression plasmid, 1 μ g of luciferase reporter, 0.5 μ g of pcDNA3/HA-PERC (and its variants) or pcDNA3/HA-hPGC-1. For coactivation of AR in COS7 and coactivation of ER α in U2OS, 1 μ g of pcDNA3/HA-PERC, pcDNA3/HA-hPGC-1, and pSG5/hSRC-1e was used. When assaying the transcriptional activity of the Gal4DBD-PERC fusion proteins in COS7, 0.5 μ g of pcDNA3/GAL4DBD-PERC (or its variants) and 1 μ g of the Gal4-responsive pGK1 luciferase reporter were transfected. After overnight exposure to the DNA-calcium phosphate precipitate, cells were washed and incubated for an additional 24 h in fresh medium containing either hormone or vehicle (0.1% ethanol or Me₂SO). Assays for luciferase and β -gal activities were performed as described previously (24). Luciferase values normalized to β -gal activity are referred to as luciferase units. Data shown represent the mean \pm S.D. of four to six values from at least two independent experiments performed in duplicates.

Yeast Two-hybrid Interaction Assay—A diploid yeast strain with integrated Gal4-responsive β -gal reporters (CG1945xY187, CLONTECH) was transformed by the lithium acetate transformation method with pGBKT7/hER α (308C) or pGBKT7/hER α (308C)-LL (Gal4 DBD fused to the hER α LBD) and pGADT7/PERC constructs (Gal4 AD fused to PERC wild type or mutants). Transformants were grown to stationary phase, diluted 1:20 in selective media containing either ethanol

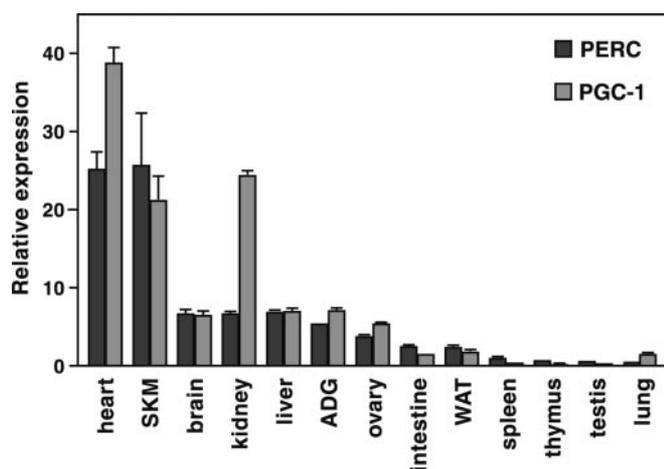


FIG. 2. PERC mRNA is expressed in a tissue-specific manner. Levels of mouse PERC and PGC-1 mRNAs in different mouse tissues were determined by real-time quantitative RT-PCR (see “Experimental Procedures”), normalized to β -actin mRNA levels, and expressed as copies of PERC or PGC-1 per 1000 β -actin copies in each sample. Data shown are from a 6–8-week-old male, except for the ovary RNA, which is from a 6–8-week-old female. Comparable results were obtained with cDNAs prepared from tissues of one more male and female. *SKM*, skeletal muscle; *ADG*, adrenal gland; *WAT*, white adipose tissue.

conserved short motifs of as yet unknown function (Fig. 1A). In contrast to PGC-1 and PRC, which have short serine/arginine-rich stretches (RS motif) N-terminal to the RRM, PERC has no RS domain. Instead, PERC has two glutamic acid-rich stretches (aa 430–450 and aa 807–824). A similar stretch of glutamic acids has been described in the nuclear receptor coactivator PELP1 (10). The second conserved region between the three proteins is the N-terminal region (Fig. 1, A and B). The first 130 aa of PERC are predominantly acidic residues, interspersed with leucines (25% aspartic and glutamic acids, 14% leucines, and just one basic residue). Alignment of this region with PGC-1 and PRC highlights the presence of a conserved leucine-rich motif (aa 92–96 of PERC), termed L1 here. In addition, PERC has two LXXLL motifs, indicated as NR1 and NR2 in Fig. 1. NR1 shows sequence conservation to the LXXLL motifs of PGC-1 and PRC, while NR2 is unique to PERC. The similarity between PERC and PGC-1 extends beyond NR1 and includes the region of a third Leu-rich motif of PGC-1; a Leu-motif is, however, not discernible in this region of PERC (Fig. 1B). Finally, consistent with the presence of nuclear localization signal sequences, PERC is a nuclear protein (Fig. 1C).

PERC Is Expressed in a Tissue-specific Manner—To determine PERC mRNA levels in different tissues in a quantitative and sensitive manner, we employed real-time RT-PCR with RNA from mouse tissues. Primers were chosen so as to detect specifically the long, exon 4+ PERC transcript. As seen in Fig. 2, PERC was detected at highest levels (>20 copies of PERC/1000 copies of β -actin) in heart and skeletal muscle. Intermediate levels (5–10 copies of PERC/1000 copies of β -actin) were seen in brain, kidney, liver, and adrenal gland. Lower PERC levels were detectable in ovary, intestine, and white adipose tissue. Expression in spleen, thymus, testis, and lung was below 1 copy/1000 copies of actin. The tissue distribution of PERC appears very similar to that of PGC-1 (22–24). Quantitation of PGC-1 mRNA in the same tissue samples demonstrated that the two genes are indeed expressed with similar profiles and at similar levels in most tissues. A notable exception is the kidney, where PGC-1 levels were significantly higher.

The PGC-1 Homolog Selectively Enhances the Activity of ER α —The similarity to PGC-1 and the presence of two LXXLL

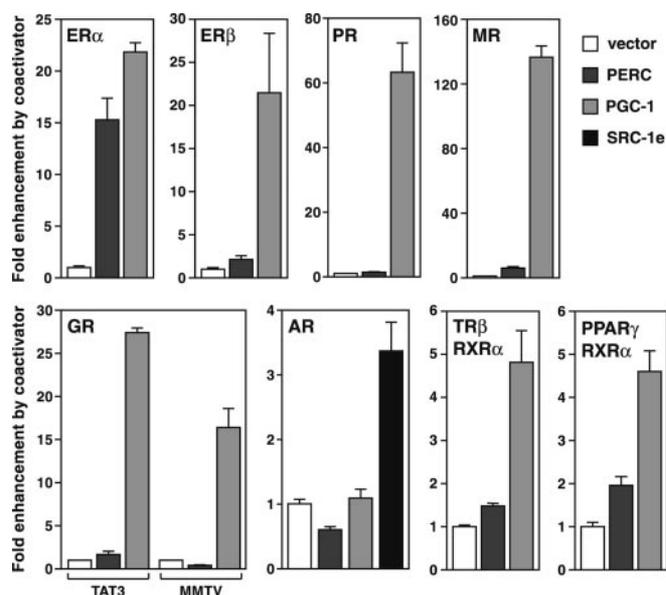


FIG. 3. PERC selectively enhances the activity of ER α . COS7 cells were cotransfected with expression plasmids for the indicated nuclear receptors, the corresponding luciferase reporter constructs (pERE-tk-Luc for ER α and ER β ; pTAT3-Luc for PR, MR, and GR; pMMTV-LTR-Luc for GR; pminPbLUCneo for AR; p Δ (DR4)x2-Luc for TR β /RXR α ; 3xPPRE-Luc for PPAR γ /RXR α), and either pcDNA3 control vector (*white bars*) or expression vectors for PERC (*dark gray bars*), PGC-1 (*light gray bars*), or SRC-1e (*black bars*). Cells were treated with 50 nM 17 β -estradiol (ER α and ER β), progesterone (PR), aldosterone (MR), or corticosterone (GR), 100 nM dihydrotestosterone (AR), T3 (TR β /RXR α), or 1 μ M rosiglitazone and 1 μ M 9-*cis*-retinoic acid (PPAR γ /RXR α) for 24 h and assayed for luciferase activity. Data are expressed as fold enhancement of nuclear receptor activity by coactivator in the presence of hormone, *i.e.* activity in the presence of hormone and absence of coactivator was set equal to 1 for all receptors.

motifs suggested that PERC could function as a coactivator of nuclear receptors. To test this, we evaluated the effect of PERC overexpression on the ligand-dependent transcriptional activity of different nuclear receptors. We introduced full-length nuclear receptors, with or without PERC, in COS7 cells and assessed their ability to induce the expression of appropriate luciferase reporters in the presence of hormone. To our surprise, PERC had either no or just marginal effects on ER β , progesterone receptor (PR), mineralocorticoid receptor (MR), GR, androgen receptor (AR), TR β /RXR α , or PPAR γ /RXR α , especially when compared with the activity of PGC-1 under the same conditions (Fig. 3). The one nuclear receptor where PERC functioned as a potent coactivator was ER α . The selective activation of ER α was not due to a special feature of the estrogen-responsive luciferase construct (single copy of vERE upstream of the thymidine kinase promoter), because ER β function at the same estrogen-responsive reporter was minimally affected by PERC. Moreover, PERC had at most a 2-fold effect on GR activity irrespective of whether this was measured with a reporter having three tyrosine aminotransferase GREs or part of the MMTV LTR. Neither PERC nor PGC-1 had any effect on AR, which was however responsive to the effects of SRC-1, a coactivator of the p160 family. We concluded that PERC shows a remarkable selectivity for ER α , while its homolog PGC-1 can activate potentially most nuclear receptors.

PERC Interacts with ER α in a LXXLL-, AF2-, and ligand-dependent Manner—To determine whether PERC and ER α interacted physically, and if so, to find out the requirements for such an interaction, we employed the yeast two-hybrid system. As shown in Fig. 4, the LBD of ER α interacted with full-length PERC in a ligand-dependent manner. Mutations in helix 12 of the ER α LBD (L539A/L540A) abolished the interaction, indi-

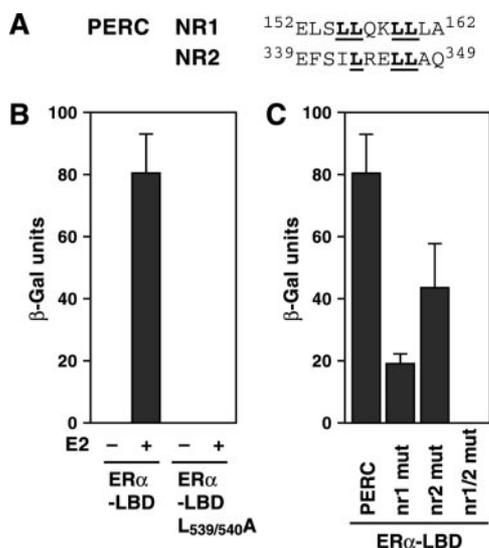


FIG. 4. PERC interacts physically with the LBD of ER α in a ligand, AF-2, and LXXLL-motif dependent manner. *A*, amino acid sequences of PERC motifs NR1 and NR2. Leucines indicated in *bold* were substituted with alanines in PERC nr1 and nr2 mutants. *B*, yeast expressing Gal4DBD-ER α -LBD (wild type or AF2 mutant L539/540A) and either Gal4AD alone (not shown) or Gal4AD-PERC were grown in the absence or presence of 10 μ M 17 β -estradiol (*E2*) and assayed for β -gal activity. No activity was detected in yeast expressing Gal4DBD-ER α -LBD and Gal4AD. *C*, yeast expressing Gal4DBD-ER α -LBD and the indicated Gal4AD-PERC variants were grown in the presence of 10 μ M 17 β -estradiol and assayed for β -gal activity.

cating that it depends on the structural integrity of the AF2 domain (4, 36). To test the involvement of the two LXXLL motifs of PERC in the interaction with ER α , we substituted the leucines in each motif by alanines (Fig. 4A). Mutations in either NR1 or NR2 alone reduced the interaction, while the double nr1/nr2 mutation abolished it (Fig. 4C). In conclusion, PERC interacts via two motifs, NR1 and NR2, with a ligand-dependent conformation of the ER α AF2 domain.

We next determined whether the requirements of the interaction detected by the two-hybrid assay were also important for the ability of PERC to enhance the activity of full-length ER α . Coexpression of PERC with the receptor in COS7 cells enhanced the activity of ER α in the presence of the agonist estradiol, but had no effect in the absence of hormone or the presence of the antagonist tamoxifen (Fig. 5A). Enhancement required an intact AF2 function, because the AF2 mutation L539A/L540A abolished responsiveness to PERC (Fig. 5A). Finally, mutations in either motif NR1 or NR2 reduced PERC activity, and the double nr1/nr2 mutation abolished coactivation (Fig. 5B). These findings demonstrated that PERC function in ER α signaling depends on an agonist ligand and intact complementing interaction surfaces: AF2 of ER α and NR1/NR2 of PERC. Interestingly, NR1 is missing in the natural isoform PERC-s, which lacks the 39 aa encoded by exon 4. Coexpression of this short isoform showed indeed that PERC-s had a reduced ability, similar to that of the PERC nr1 mutant, to enhance the hormone-dependent activity of ER α . Consequently, mechanisms that regulate the alternative splicing of exon 4 of PERC could modulate cellular responses to estrogens.

A Potent Bipartite Transcriptional Activation Domain (AD) in the N Terminus of PERC Is Required for Coactivation—The N-terminal region and in particular motif L1 of PERC is well conserved among the three members of the PGC-1 family (Fig. 1B). In PGC-1 and PRC, this region harbors a potent transcriptional AD (24, 28, 32). To test whether PERC also carries such an AD, we asked if full-length PERC tethered to DNA activates transcription. A fusion of PERC to the DBD of Gal4, which

recruits PERC to a Gal4-responsive luciferase reporter, indeed activated transcription strongly (Fig. 6A). Deletion of the first 91 aa of PERC abolished activation, indicating that the N-terminal part is essential for the activation function (Fig. 6A). The first 91 aa (N91) fused to the Gal4 DBD were sufficient to activate transcription. However, full transcriptional activity of PERC required additional sequences up to aa 128. Gal4 DBD fused to aa 1–128 (N128) was the strongest PERC activator, enhancing transcription by more than 20,000-fold in COS7 cells (Fig. 6A). Within the 91–128 region, the conserved motif L1 contributed to the activation function. Point mutations that substituted the leucines of L1 with alanines reduced PERC transcriptional activity, in the context of both full-length PERC and the N128 construct (Fig. 6A). Our findings suggest a bipartite N-terminal AD. The first part is encoded by aa 1–91 and is essential, while the second part relies on motif L1 and contributes to full activity. This bipartite AD function is crucial for the ability of PERC to enhance the activity of ER α (Fig. 6B). Deletion of the first 91 aa or mutations in motif L1 abolished or reduced, respectively, PERC coactivation (Fig. 6B), suggesting that both components of the AD are required for full function of PERC in ER signaling.

PERC and PGC-1 Confer Distinct Functional Properties to Ligand-activated ER α —To address whether PERC and PGC-1 fulfill similar functions when acting with ER α , we compared the effects of the two coactivators on estrogen signaling in different contexts. First, we evaluated PERC and PGC-1 function on ER α -activated transcription at different promoter contexts (Fig. 7A). A single consensus ERE upstream of the minimal alcohol dehydrogenase promoter was preferentially responsive to PGC-1 activity. PERC caused a small, reproducible 2–3-fold enhancement, compared with a 10-fold increase by PGC-1. ER α acting from two copies of the consensus ERE or a longer vitellogenin A2 ERE fragment (–334 to –289 nucleotides, relative to transcription initiation) upstream of the same minimal promoter was equally responsive to the two coactivators. On the other hand, two copies of the vitellogenin ERE fragment, or a 1.8-kb fragment of the estrogen-responsive complement 3 (C3) promoter, were enhanced stronger by PERC than by PGC-1 (Fig. 7A). These observations suggest that PERC and PGC-1 may selectively activate distinct ER α targets genes.

ER α signaling depends on the nature of the activating ligand, as well as the cellular and promoter context (5, 37, 38). In particular, there are classes of ER ligands that act in a tissue-selective manner. For example, tamoxifen is an antagonist in the mammary gland but an agonist in the bone, uterus, and cardiovascular system (reviewed in Ref. 39). One of the underlying molecular mechanisms for the agonist action of tamoxifen is thought to involve the cooperation of tamoxifen-bound ER α with tissue-specific cofactors. To determine how PERC and PGC-1 affect the response to tamoxifen, we employed the C3 promoter, which has been characterized for its responsiveness to this agonist (38, 40). In the osteosarcoma cells U2OS, tamoxifen activated the C3 promoter strongly, although not as well as estradiol (Fig. 7B). PERC expression further enhanced the tamoxifen response by 2-fold. In contrast, PGC-1 modestly repressed the tamoxifen-induced response (Fig. 7B). These findings suggest that the relative activities of PERC and PGC-1 may contribute to the tissue-specific action of partial agonists like tamoxifen.

DISCUSSION

We report here the cloning and characterization of PERC, a new member of the PGC-1 family of proteins and a coactivator of ER α . In contrast to PGC-1, which activates many nuclear receptors, PERC shows a unique receptor selectivity. It po-

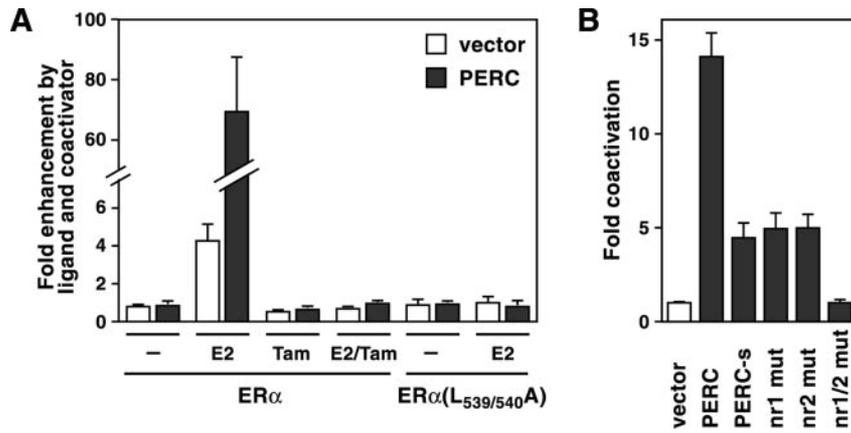


FIG. 5. Coactivation of ER α by PERC depends on an agonist ligand and the integrity of AF2 of ER α and NR1/NR2 of PERC. A, COS7 cells transfected with expression plasmids for ER α (wild type or AF2 mutant), the ER-responsive luciferase reporter pERE-tk-Luc, and either pcDNA3 control vector (white bars) or PERC expression vector (dark gray bars) were treated for 24 h with ethanol vehicle (-), 50 nM 17 β -estradiol (E2), 5 μ M tamoxifen (Tam) or both ligands (E2/Tam) and assayed for luciferase activity. Data are expressed as fold enhancement by PERC, with activity in the absence of PERC and ligand set equal to 1. B, COS7 cells transfected with an expression plasmid for ER α , the ER-responsive luciferase reporter pERE-tk-Luc, and either pcDNA3 vector control (white bars) or expression vectors for PERC and its indicated variants (dark gray bars) were treated for 24 h with 50 nM 17 β -estradiol and assayed for luciferase activity. PERC-s lacks exon 4 (aa 156–194). Data are expressed as fold enhancement of ER α activity by PERC in the presence of hormone.

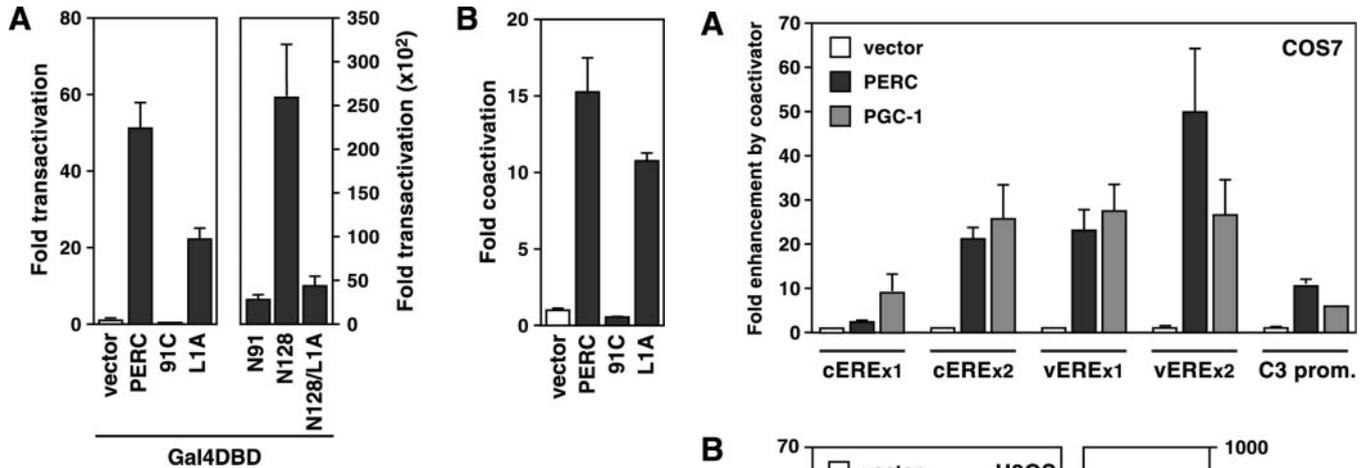


FIG. 6. A bipartite transcriptional activation domain in PERC is required for coactivation of ER α . A, transcriptional activity of PERC. COS7 cells transfected with the luciferase reporter pGK1 and either Gal4DBD control vector or the indicated Gal4DBD-PERC variants were assayed for luciferase activity. Data are expressed relative to the activity in cells expressing the Gal4DBD alone (vector), which was set equal to 1. Note that the y axis scales are different in the two panels. B, coactivation function of PERC. COS7 cells transfected with an ER α expression plasmid, the ER-responsive luciferase reporter pERE-tk-Luc, and either pcDNA3 vector control (white bars) or expression vectors for PERC and its indicated variants (dark gray bars) were treated for 24 h with 50 nM 17 β -estradiol and assayed for luciferase activity. Data are expressed as fold enhancement of ER α activity by PERC in the presence of hormone. 91C, aa 91–1023 of PERC; N91, aa 1–91 of PERC; N128 and N128/L1A, aa 1–128 of PERC wild type and PERC L1A mutant, respectively.

tently enhances the ligand-dependent activity of ER α , while having only minimal effects on the activity of the related receptor ER β or other nuclear receptors tested here. Furthermore, PERC and PGC-1 confer distinct properties to ER α signaling. Thus, the relative activities of the two coactivators may contribute to the specific profiles of estrogen responses in different tissues.

PERC, PGC-1, and the recently described PRC (32) define a new, small family of coactivators. The conserved features of the family reside primarily in the N- and C-terminal domains, which carry the effector functions of these coactivators: activation of transcription and regulation of pre-mRNA processing

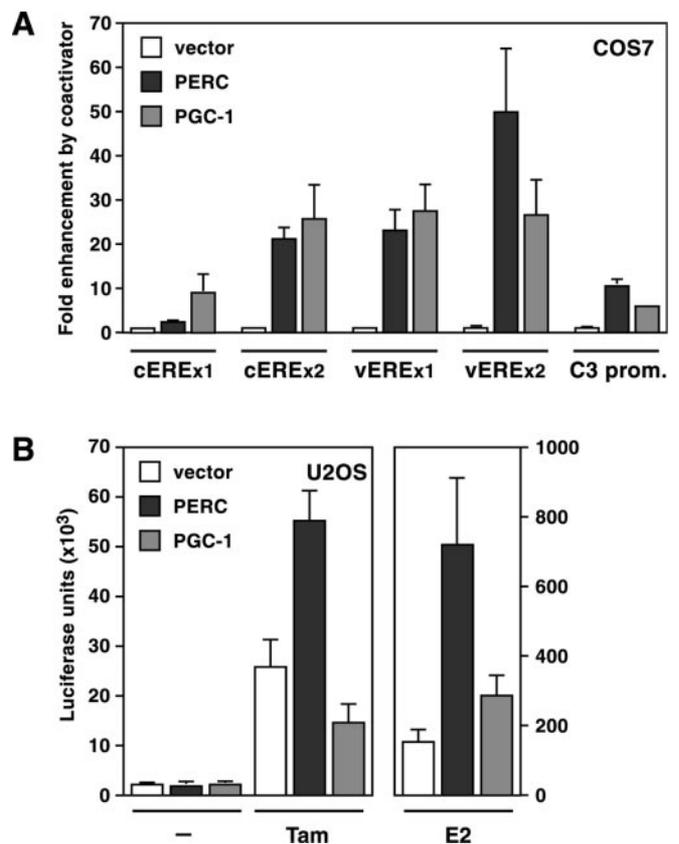


FIG. 7. PERC and PGC-1 confer differential promoter- and ligand-specific activation of ER α . A, coactivation of ER α by PERC or PGC-1 in different promoter contexts. COS7 cells transfected with an ER α expression plasmid, the different ER-responsive luciferase reporters (cERE \times 1, cERE \times 2, vERE \times 1, vERE \times 2, and C3 promoter) and either pcDNA3 control vector (white bars) or expression vectors for PERC (dark gray bars) and PGC-1 (light gray bars) were treated for 24 h with 50 nM 17 β -estradiol and assayed for luciferase activity. Data are expressed as fold enhancement of ER α activity by each coactivator in the presence of hormone. B, the activity of tamoxifen-bound ER α in U2OS osteosarcoma cells is enhanced by PERC but not by PGC-1. U2OS cells transfected with an ER α expression plasmid, the reporter pC3-Luc, and either pcDNA3 control vector (white bars) or expression vectors for PERC (dark gray bars) and PGC-1 (light gray bars) were treated for 24 h with ethanol vehicle (-), 50 nM 17 β -estradiol (E2), or 5 μ M tamoxifen (Tam) and assayed for luciferase activity.

(24, 28, 31, 41). Thus, the three coactivators are likely to employ similar mechanisms to mediate their biological functions. Whether PERC, which lacks the RS domain of PGC-1, is able to regulate RNA processing has to be addressed in future experiments. PERC, PGC-1, and PRC also share sequence similarities outside the effector domains: the LXXLL motifs that enable interactions with nuclear receptors and additional small conserved motifs that may represent interaction surfaces for other transcription factors or regulators (Fig. 1). At the same time, the significant sequence divergence, particularly in the unique central domains of the proteins, suggests that the three members of the family have acquired unique functions and roles.

The mechanism by which the N-terminal AD of PERC regulates transcription is not clear yet. The corresponding region of PGC-1 can interact with SRC-1 and CBP, suggesting that it acts as a scaffold for the recruitment of other coactivators (41). Our studies here indicate a bipartite AD that contacts more than one target. The reduced transcriptional activity of the L1A mutant points to the conserved motif L1 as one of the interaction surfaces. An additional contact must reside in the first 90 aa, which are essential and sufficient for transcriptional activation. Neither SRC-1 nor CBP overexpression enhanced PERC transcriptional activity, implicating targets other than these two coregulators. Since PGC-1 and PERC are strong activators of transcription in yeast, which do not have SRC-1 or CBP, it seems likely that the N-terminal ADs can contact evolutionary conserved components of the transcriptional machinery (24).² Delineation of the exact interaction surfaces of PGC-1, PRC, and PERC, as well as identification of the proteins they contact, will shed light on the mechanisms by which these ADs act.

An important feature of the PGC-1 family is the presence of LXXLL motifs, which mediate interactions with the LBDs of nuclear receptors. PERC has two canonical LXXLL motifs: NR1, which is conserved in PGC-1 and PRC, and NR2, which is unique to PERC (28, 30, 32, 42). Both NR boxes contribute to the physical interaction with ER α and to efficient coactivation of this receptor. Notably, the presence of NR1 depends on the inclusion of the small exon 4. The detection of two PERC isoforms, with and without this exon, and the decreased ability of the short PERC-s to activate ER α , suggest that regulation of this alternative splicing event could be used to modulate ER α signaling. Interestingly, the mouse homolog of PERC, which was recently described as PGC-1 β , harbors an additional LXXLL motif that is upstream of NR1 (aa 140–144) and not conserved in the human protein (33). We do not know yet whether this third motif functions as a nuclear receptor interaction domain, and if so, whether it enables functional interactions with ER α or other receptors. Although no data have been presented yet on the ability of the mouse protein to coactivate the different receptors we have tested here, it is possible that the mouse and human homologs may have diverged in their nuclear receptor specificity.

Our experiments demonstrate clearly that PERC is a coactivator of ER α . The fact that this coactivation function depends on a physical interaction between the LXXLL motifs of PERC and the AF2 domain of ER α raises the question of why PERC has only minor effects on many other nuclear receptors that harbor similar AF2 domains. The reason for this receptor selectivity is not clear, particularly since PERC can interact physically with other ligand-activated receptors, such as GR.² One possible explanation is that the affinity of the GR-PERC interaction is lower than that of GR with other endogenous AF2

coactivators. If so, PERC may not get recruited efficiently at GR target sites. An alternative explanation is that the physical interaction mediated by the PERC NR boxes and the receptor AF2 binding pocket is a necessary, but not sufficient, step for coactivation. Coactivators have been proposed to undergo conformational changes subsequent to docking to transcription factors. These changes may enable their enzymatic activities or the recruitment of additional regulators (41, 43). Similarly, the conformation of nuclear receptors may change upon interaction with coactivators. Thus, specificity in the functional interaction between PERC and ER α could be due to conformational changes subsequent to binding that may activate either PERC, by unmasking its AD, or ER α , by enabling its AF1 activity. Consistent with an activation step for PERC, we have observed that deletion of C-terminal and central domains of PERC result in a much more potent transcriptional regulator (Fig. 6). It seems likely that the PERC AD is masked in the context of the full-length protein, similar to what has been shown for PGC-1 (41).

Besides their differences in nuclear receptor specificity, PERC and PGC-1 display distinct preferences for the promoter context in which they enhance ER α activity. The two types of EREs we have tested, a vERE and a synthetic cERE, contain the same consensus core but differ in the flanking sequences. Such differences have been shown before to influence ER α -ERE interactions (44). Moreover, the vitellogenin fragment includes additional 5' sequences, where a second, nonconsensus ERE can be discerned (–312 to –298 nucleotides, relative to transcription initiation). Finally, due to the difference in the length of the flanking sequences, the dimerized elements vERE \times 2 and cERE \times 2 present ER α binding sites with different spacing. Thus, multiple properties, such as flanking sequences, the presence of additional nonconsensus sites, and the spacing between EREs, may account for the distinct utilization of PERC and PGC-1 at the different promoters. Notably, PERC seems to prefer promoters with multiple sites, such as the dimerized EREs, or the C3 promoter that has at least three EREs (40). Different response elements may induce distinct nuclear receptor conformations and thereby influence either the recruitment of the coactivators or the activity of the recruited coactivators (45, 46).

An additional context that reveals differences in PERC and PGC-1 function is the ability of the two coactivators to promote the agonistic effect of the partial agonist tamoxifen. In a cell and promoter context where tamoxifen is an agonist, PERC enhances this agonist activity, while PGC-1 represses it. In this respect, PERC acts like the p160 coactivators, which can enhance the agonist activity of tamoxifen (47–49). Presumably, PERC can interact, directly or indirectly, with the tamoxifen-induced conformation of ER α . PGC-1 cannot do so, at least in the context of the C3 promoter in U2OS cells. Because of its antagonist activity in the mammary gland, tamoxifen is used to treat estrogen-dependent breast tumors. Many of these tumors develop resistance to tamoxifen and some start recognizing it as an agonist (reviewed in Ref. 39). Our findings suggest that the nature, as well as the relative levels of different AF2 coactivators, may determine the cellular response to tamoxifen. Evaluation of PERC and PGC-1 levels in breast tumors will be important to test whether these two coactivators contribute to the responsiveness, or lack of, to endocrine therapy.

PERC mRNA distribution is very similar to that of PGC-1. PGC-1 function in heart, muscle, and liver may mediate physiological state signals to tissue-specific transcriptional activation of proteins that regulate energy and glucose homeostasis. For example, in response to exposure to cold, PGC-1 induces the expression of uncoupling proteins and stimulates energy

² D. Kressler and A. Kralli, unpublished observations.

expenditure in brown fat and muscle, while in response to fasting, it stimulates gluconeogenesis in liver (Refs. 26 and 29 and reviewed in Ref. 50). The similar expression profile of PERC may be indicative of a second pathway that relates energy needs to specific metabolic responses, possibly under different regulatory input and with a different outcome. This could increase specificity and flexibility of the transcriptional responses. Estrogens can have profound effects on systems other than the reproductive one. In both males and females, estrogens have protective effects on the cardiovascular and skeletal system, regulate adipose function, and affect glucose and lipid metabolism (51–54). Mice that lack a functional ER α have increased adipose mass, develop mild glucose intolerance and insulin resistance, and show decreased energy expenditure (54, 55). Similarly, humans with deficiencies in estrogen signaling show a propensity for insulin resistance and altered lipid metabolism (52). It will be interesting to test whether these estrogen effects require PGC-1, PERC, or a combination of the two coactivators.

The mechanisms by which estrogens act in a tissue- and promoter-specific manner are complex (20, 21). Mice with genetic ablations of the p160 coactivators SRC-1 or SRC-3/AIB1 show only mild defects in estrogen signaling (56–58). Thus, it seems likely that multiple coactivators can cooperate with ERs to mediate appropriate tissue-specific and physiological state-dependent responses. The molecular unraveling of estrogen activity will require an understanding of all ER α and ER β interactors as possible contributors to estrogen signaling. Here, we have described a tissue-specific coactivator, PERC, which shows a remarkable selectivity for ER α over other nuclear receptors. Future studies will define the reason for selectivity, as well as the biological role of PERC.

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