PGC-1 Functions as a Transcriptional Coactivator for the Retinoid X Receptors*

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Ligand-dependent gene transcription mediated by the nuclear receptors involves the recruitment of transcriptional coactivators to the ligand-binding domain (LBD), which leads to interaction with the basal transcription machinery, and ultimately with RNA polymerase II. Although most of these coactivators are ubiquitously expressed, a tissue-selective coactivator, PGC-1, has recently been characterized. Because PGC-1 and the retinoid X receptors (RXRs) possess an overlapping tissue distribution, we investigated whether PGC-1 is a coactivator for the retinoid X receptors. In a transient transfection assay, PGC-1 augments ligand-stimulated RXR transcription. Furthermore, PGC-1 efficiently enhances the RXR element-driven reporter gene transcription by all three RXR isoforms. An immunoprecipitation assay reveals that PGC-1 and RXRs interact in vivo. In addition, a glutathione S-transferase pull-down assay showed that this interaction requires the presence of the LXXLL motif of PGC-1. We demonstrate further, in a mammalian two-hybrid assay, that this physical interaction also requires the presence of the AF-2 region of RXR to interact with the LXXLL motif of PGC-1, which is consistent with our protein-protein interaction results. A time-resolved fluorescence assay shows that a peptide within the NR box of PGC-1 is efficiently recruited by a ligand-bound RXRa in vitro. Finally, PGC-1 and TIF2 synergistically enhance ligand-activated RXRa transcriptional activity. Taken together, these results indicate that PGC-1 is a bona fide coactivator for RXRα.

Nuclear receptors are ligand-activated transcription factors, which regulate a large number of developmental and physiological processes in response to small lipophilic molecules (see Ref. 1 for review). Upon the binding to their cognate DNA response elements, ligand-bound nuclear receptors activate gene transcription by interacting with the basal transcription machinery via bridging factors called coactivators. One of the potential functions of these proteins is to remodel the chromatin structure locally through acetylation of the histone tails within the nucleosome, resulting in greater accessibility of the gene promoter to transcriptional factors and the basal transcription machinery complex. Many of these coactivators possess a signature motif, LXXLL, which is necessary and sufficient to permit their interactions with nuclear hormone receptors. The best-characterized coactivators are p300, CBP,1 the p300/CBP-associated factor and the members of the p160 families, including, SRC-1, GRIP1/TIF2/SRC-2, and ACTR/AIB1/RAC3/SRC-3 (see Ref. 3 for review). These proteins are generally ubiquitously expressed, although several of them, such as AIB1 (4), FHL2 (5), and NIX-1 (6), are limited to specific tissues.

Using a yeast two-hybrid approach to screen a brown adipocyte cell line-derived cDNA library, Puigserver et al. (7) cloned a tissue-selective coactivator for the peroxisome proliferator-activator receptor (PPARγ), named PGC-1. It has been reported that PGC-1 is highly expressed in the brown adipose tissue, heart, kidney, and brain (7, 8), and its expression is dramatically induced in brown fat by the exposure of cold and a stimulation of β3-adrenergic receptor (7, 9). In addition, PGC-1 gene expression is induced in the mouse heart in response to short-term fasting (10). Interestingly, PGC-1 interacts with PPARγ in a ligand-independent manner (7), whereas the interaction of PGC-1 with the thyroid-hormone receptor β3 (7), retinoic-acid receptor α (7), estrogen receptor α (7, 11), and glucocorticoid and mineralocorticoid receptors (8) occurs in a ligand-dependent manner. Wu and co-workers (12) demonstrated that PGC-1 stimulates mitochondrial biogenesis and respiration in skeletal muscle cells by regulating the transcriptional activity of the nuclear respiratory factor and by regulating uncoupling protein gene transcription. Moreover, cardiac-specific overexpression of PGC-1 in mice results in mitochondrial hyperproliferation, which leads to dilated cardiomyopathy (10). In addition, Michael and co-workers (13) reported that PGC-1 regulates GLUT4 gene expression and thereby increases glucose uptake through, at least in part, an interaction with the muscle-specific transcription factor MEF2C. Recently, it has been shown that the expression of PGC-1 in liver is dramatically induced in fasting mice. In addition, PGC-1 functions as a key modulator in regulating hepatic gluconeogenesis by increasing phosphoenolpyruvate carboxykinase and glucose-6-phosphatase gene transcription via a coactivation of the glucocorticoid receptor and the liver-specific transcription factor HNF-4α (14).

It has been reported that PGC-1 and retinoid X receptor (RXR) possess an overlapping tissue distribution (7, 15), and several of the RXR dimerization partners, such as PPARs and TRα, have been reported to interact with PGC-1 (7). We, therefore, hypothesized that PGC-1 could function as a coactivator and augment RXR-mediated transcription. In this study, we...

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1 The abbreviations used are: CBP, CREB-binding protein; PPAR, peroxisome proliferator-activator receptor; RXR, retinoid X receptor; GST, glutathione S-transferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; TK, thymidine kinase; HA, hemagglutinin; CAT, chloramphenicol acetyltransferase; Eu, europium; NR, nuclear receptor; LBD, ligand-binding domain.
show that PGC-1 acts as a bona fide coactivator for the RXR family members in transient transfection assays. GST pull-down and immunoprecipitation assays demonstrate that RXRα and PGC-1 indeed physically interact in a ligand-dependent manner both in vitro and in vivo, respectively. Moreover, these interactions were confirmed using a mammalian two-hybrid analysis. Finally, we show that PGC-1 and TIF2 synergistically activate RXRα-mediated transcription.

**MATERIALS AND METHODS**

**Cell Culture and Reagents**—HeLa cells (ATCC, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 2 mM glutamine and 10% (vol/vol) fetal calf serum (FCS) in an atmosphere of 5% CO2 at 37 °C. 9-cis-Retinoic acid was obtained from Sigma Chemical Co.

**Plasmids**—The plasmids (DR-1)-TK-pGL3 and pSG5-RXRα have been previously described (16). The G5-Luc plasmid, containing five copies of the DNA-binding site of the yeast GAL4 transcription factor cloned in front of the minimal thymidine kinase (TK) promoter, was purchased from Promega. The pCDNA3-HA-PGC-1 vector was a kind gift from Dr. Kralli (University of Basel, Basel, Switzerland) and has already been described (8). The GAL4-Luc expression plasmid was obtained by cloning a full-length PGC-1 cDNA into the pM vector (CLONTECH). The VP16-PGC-1 and GST-PGC-1 plasmids were constructed with a PCR-amplified PGC-1 fragment (amino acid residues 100–411) using pCDNA3/HA-PGC-1 as a template. The resulting PCR products were cloned in VP16 (CLONTECH) or pGEX-4T2 (Amersham Biosciences, Inc.) yielding VP16/PGC-1 and GST/PGC-1, respectively. The mutations of LKKLL to AKKAI in the NR box of the PGC-1 were obtained using site-directed mutagenesis (Stratagene). All constructs were verified by DNA sequence analysis. Finally, the mCRBPII-CAT, GAL4/RXRα, GAL4/RXRβ, GAL4/RXRγ, VP16/RXRα, and VP16/RXRα-AP2 were provided by Dr. Sunil Nagpal (Eli Lilly Research Laboratories).

**Transient Transfection Assays**—HeLa cells, plated in 24-well plates at 50–60% confluence in DMEM supplemented with 10% FCS, were transfected with reporter and receptor expression plasmids using LipofectAMINE Plus reagent (Invitrogen) as indicated in the figure legends. The phosphohydrate kinase β-galactosidase expression plasmid was cotransfected to assess transfection efficiency. After an overnight incubation, cells were collected and were assayed for luciferase and β-galactosidase activities. Chloramphenicol acetyltransferase expression was measured by enzyme-linked immunosorbent assay (Roche Molecular Biochemicals) following manufacturer’s protocol. All experiments were repeated at least three times.

**Immunoprecipitation and Western Blot Analysis**—5 × 105 HeLa cells were transiently transfected with pSG5-RXRα (4 μg) and pCDNA3/HA-PGC-1 (4 μg) or empty vector, pCDNA3. After 24 h, cells were treated with 9-cis-retinoic acid (1 μM) or Me2SO (0.1%) for an additional 24 h. At the end of the treatment period, cells were washed with ice-cold phosphate-buffered saline and the pellet was re-suspended in an immunoprecipitation lysis buffer and incubated for 10 min on ice. Cell debris was spun down for 10 min at 14,000 × g, and supernatants were collected. Lysates were diluted in 1 ml of incubation buffer (20 mM Hepes, pH 8.0, 150 mM KCl, 2.5 mM MgCl2, 1 mM dithiothreitol, and protease inhibitors (Roche Molecular Biochemicals)) containing RXRα antibody (5 μg/ml) (Santa Cruz Biotechnology, v-20). Samples were incubated overnight at 4 °C. Protein G-agarose beads (Invitrogen) were then added, and samples were incubated for an additional hour. After centrifugation, beads were washed three times with incubation buffer and subsequently re-suspended with 2× Laemmli loading buffer for a SDS-PAGE and Western blotting. Membranes were probed with a monoclonal HA antibody (Santa Cruz Biotechnology, F-7) or an RXRα antibody. After incubation with a secondary peroxidase-conjugated antibody, signals were visualized by chemiluminescence (Amersham Biosciences, Inc.).

**GST Pull-down Assays**—An in vitro protein-protein interaction assay was performed as previously described (17). Briefly, glutathione-Sepharose 4B beads containing a GST fusion protein (0.5 μg) were incubated with an in vitro translated [35S]methionine-labeled protein, in the presence or absence of 9-cis-retinoic acid (1 μM), in a total volume of 500 μl of incubation buffer (20 mM Tris, pH 7.5, 75 mM KC1, 50 mM NaCl, 1 mM EDTA, 0.1% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and protease inhibitors (Roche Molecular Biochemicals)), for 2 h at room temperature. After centrifugation, the beads were washed five times with incubation buffer and re-suspended in 2× Laemmli buffer, boiled for 5 min, and centrifuged. The supernatant was subjected to electrophoretic analysis on a 4–20% SDS-polyacrylamide gel (Invitrogen). After drying, gels were exposed for autoradiography.

**Time-resolved Fluorescence Assay**—The time-resolved fluorescence-based coactivator interaction assay was performed as previously described (18). Briefly, while 96-well plates were coated overnight with full-length GST-RXRα. Protein-coated plates were washed five times and then blocked for at least 1 h followed by extensive washing. NR box peptide europium (Eu) conjugate was prepared by incubating the biotin-labeled NR box peptide with Eu-labeled streptavidin on ice. The 96-well plate coated with RXRα protein was then incubated with increasing amounts of NR box peptide-Eu conjugate in the presence of 9-cis-retinoic acid (1 μM) incubated for at least 1.5 h. Plates were washed three times followed by incubation with gentle shaking in the presence of enhancement solution for 5 min allowing for release of the bound europium label. Plates were read in a Wallac Victor II plate reader using a protocol specific for the detection of europium (PerkinElmer Wallac, Inc.). Assays were repeated for three times and representative experiments are shown. Kd values were determined using GraphPad Prism.

**RESULTS AND DISCUSSION**

To determine whether PGC-1 serves as a coactivator and augments RXRα-stimulated transcriptional activity, a transient transfection assay was performed in HeLa cells using a luciferase reporter gene, which contains three copies of a DR-1 response element cloned in front of the minimal thymidine kinase (TK) gene promoter. Cotransfection of RXRα, in the presence of 9-cis-retinoic acid, results in a strong activation (10-fold) of the reporter gene (Fig. 1A), whereas no significant effect was observed in the absence of RXRα, a result in agreement with previous reports (19). A 2-fold induction of a reporter gene activity was observed after transient transfection of a plasmid containing PGC-1 alone, which it is likely due to the presence of endogenous RXRs in HeLa cells. Cotransfection of PGC-1 significantly enhanced RXRα-mediated transcription further (an additional 4-fold enhancement) in a ligand-dependent manner (Fig. 1A), which suggests that PGC-1 functions as an RXRα coactivator. Similar results were obtained using
The mouse cellular retinol-binding protein II promoter (19) (Fig. 1B).

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Therefore, a mammalian two-hybrid experiment in which the PGC-1 and RXRα constructs were prepared in a reciprocal manner that is consistent with the results of our protein-protein interaction assays (Fig. 3B). Altogether, these data demonstrate that there is a direct interaction of PGC-1 and RXRα.

Using a time-resolved fluorescence assay (18), we next evaluated the ability of RXRα to recruit the NR box of PGC-1 in the presence of its cognate ligand, 9-cis-retinoic acid. In this assay, an increased recruitment of a NR box peptide to RXRα was observed with an apparent Kd around 40 nM. (Fig. 5A). In addition, similar experiments were conducted with TIF2 NR boxes, and the Kd values of NR1 and NR2 were 34 and 50 nM, respectively. Because RXRα does not have a particular preference to the NR boxes of PGC-1 and TIF2, a coactivator function of either PGC-1 or TIF2 alone and/or in combination was studied in a transient transfection assay. RXRα-stimulated reporter gene activity was enhanced by either TIF2 or PGC-1, and this transcriptional activation was augmented further as these two cDNAs were cotransfected (Fig. 5B). These results are in agreement with those from Puigserver et al. (22) in which PPARγ docking induces an active conformational change in PGC-1, which then leads to the recruitment of SRC-1 and CBP simultaneously to activate transcription synergistically. Based on our results, it seems that such a mechanism, transcription synergy mediated by PGC-1 and TIF2, also applies to RXRα.

Using PPARγ as bait, PGC-1 was originally cloned in a yeast two-hybrid assay by screening a cDNA library that was derived from a brown adipocyte cells (7). Unlike PPARγ, which binds to PGC-1 via its hinge region (7), we found that RXRα interacts with PGC-1 both in vivo and in vitro via its LBD, in a strict ligand-dependent manner (Fig. 3). It has been reported that the interactions of PGC-1 with TRβ and ERs are enhanced by their cognate ligands (7, 11). Tcherepanova et al. (11) demonstrated that both AF2 and the hinge regions of these receptors are required for maximal interaction with PGC-1. Our results suggest that RXRα interacts with different nuclear receptors by various mechanisms. It would be interesting to
determine how PGC-1/RXR interaction may modulate class II nuclear receptor transcriptional activity.

Transcriptional coactivators have been shown to interact with nuclear hormone receptors through their LXXLL motifs in the NR boxes (2). These peptides bind to a hydrophobic groove created by helices 3, 4, 5, and 12 within the LBD of the nuclear hormone receptor (24). In addition, helix 12 plays a crucial role in a ligand-induced conformational change that generates a surface sufficient for interaction with a coactivator (25). Consistent with this model, we found that a partial deletion of the helix 12 of RXRα resulted in a complete loss of its interaction with PGC-1 in a mammalian two-hybrid assay (Fig. 4A). In addition, mutations of the LXXLL motif in the NR box completely abolished its binding to RXRα (Figs. 3B and 4B); a similar finding with the glucocorticoid receptor has also recently been reported (21). Using a library, enriched in LXXLL peptides, in a phage-display assay, Chang and co-workers (23) isolated three classes of peptides with different LXXLL motifs that interact with a ligand-bound ER. They found that the NR boxes of the p160 family of coactivators contain both class I and II peptides. In contrast, the NR box of the PGC-1 contains a class III peptide (23). In addition, they found that RXRα pref-
erentially interacts with a class III peptide in a mammalian two-hybrid assay, a finding that is further supported by our data.

Although the physiological relevance of an interaction between PGC-1 and RXR remains unclear, the ability of these two proteins to interact in vitro and in vivo provides a biochemical basis for likely cellular function. In conclusion, we demonstrate that PGC-1 is a bona fide coactivator for RXRs and its interaction requires the presence of its cognate ligand. Because RXR is the obligate partner for the class II nuclear receptors, our data suggest a potential involvement of PGC-1 in a wide range of physiological and pathophysiological regulatory pathways.

Acknowledgments—We thank Drs. A. Kralli and S. Nagpal for providing valuable reagents.

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J. Biol. Chem. 2002, 277:3913-3917.
doi: 10.1074/jbc.M109409200 originally published online November 19, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109409200

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