Modulation of Estrogen Receptor-α Transcriptional Activity by the Coactivator PGC-1*

Received for publication, February 18, 2000
Published, JBC Papers in Press, March 9, 2000, DOI 10.1074/jbc.M001364200

Irina Tcherepanova†, Pere Puigserver‡, John D. Norris‡, Bruce M. Spiegelman§, and Donald P. McDonnell†‡

From the †Department of Pharmacology and Cancer Biology, Duke University Medical Center, Durham, North Carolina 27710 and ‡Dana-Farber Cancer Institute, Harvard Medical School, Boston, Massachusetts 02115

A transcriptional coactivator of the peroxisome proliferator-activated receptor-γ (PPARγ), PPARγ-coactivator-1 (PGC-1) interacts in a constitutive manner with the hinge domain of PPARγ and enhances its transcriptional activity. In this study we demonstrate that PGC-1 is a coactivator of estrogen receptor-α (ERα)-dependent transcriptional activity. However the mechanism by which PGC-1 interacts with ERα is different from that of PPARγ. Specifically, it was determined that the carboxyl terminus of PGC-1 interacts in a ligand-independent manner with the ERα hinge domain. In addition, an LXXLL motif within the amino terminus of PGC-1 was shown to interact in an agonist-dependent manner with the AF2 domain within the carboxyl terminus of ERα. The ability of PGC-1 to associate with and potentiate the transcriptional activity of an ERα-AF2 mutant that is unable to interact with the p160 class of coactivators suggests that this coactivator may have a unique role in estrogen signaling. It is concluded from these studies that PGC-1 is a bona fide ERα coactivator, which may serve as a convergence point between PPARγ and ERα signaling.

The steroid hormone estrogen is a key regulator of cellular processes involved in the development and maintenance of reproductive function. In addition, estrogen exhibits biological actions in bone, the cardiovascular system, and the central nervous system (1, 2). In these target organs the biological action(s) of estrogen are manifest through either one of two action(s) of estrogen are manifest through either one of two specific, high affinity, estrogen receptors (ERα or ERβ)1 located within target cells (3, 4). Given the similarity in the structure of these two ER subtypes, it is likely that they share a similar mechanism of action; however, most studies thus far have focused on defining the ERα signal transduction pathway. These studies have revealed that the apo-receptor resides in the nucleus of target cells in an inactive form associated with a large inhibitory heat-shock protein complex (5). Upon binding ligand, the receptor undergoes a conformational change, an event that leads to the displacement of heat-shock proteins, receptor dimerization, and subsequent interaction of ERα with specific estrogen response elements (EREs) located within the regulatory regions of target genes (6–8). Depending on the cell and promoter context, the DNA-bound receptor can exert either a positive or negative effect on target gene transcription (9).

Although the precise mechanism by which ERα modulates target gene transcription remains to be determined, significant insights in this regard have come from studies that probed the molecular pharmacology of different ERα-ligands (10). Notable was the finding that the biological activity of the same compound can differ between cell types, indicating that ERα does not function in an identical manner in all contexts (10–12). For example, the drug tamoxifen has been shown to function as an antiestrogen in most ERα-positive breast cancer cells where it opposes the mitogenic activity of estradiol (13). In other tissues, such as the uterus and the cardiovascular system, this compound exhibits estrogenic activity (1, 14, 15). The molecular basis underlying the distinct activities of estrogen and tamoxifen became apparent from crystallography studies, which demonstrated that ERα bound to estradiol and tamoxifen assumes different conformations (16). Specifically, it was determined that the structural changes, which occur in the ligand binding domain of ERα when it is bound to estradiol, but not tamoxifen, facilitated the interaction of this receptor with cellular adaptors or coactivators, i.e. proteins that link ERα to the general transcriptional machinery (17, 18). The best studied of these factors are SRC-1, GRIP-1, and AIB-1, which are members of the p160 superfamily of coactivators (19–24). The ability of these coactivators, when overexpressed in cells, to potentiate the transcriptional activity of estradiol-activated ERα confirmed the functional significance of these interactions (20, 22, 25). The p160 coactivators exhibit a relatively ubiquitous tissue distribution pattern, making it unlikely that they play a major role in determining the tissue-selective actions of different ERα-ligand complexes. However, the recent identification of factors, other than those of the p160 class, that interact with ERα and that are expressed in a tissue-selective manner adds to the complexity of estrogen action. One of these factors, PGC-1, was originally identified as a transcriptional coactivator of the peroxisome proliferator-activated receptor-γ (PPARγ) (26), and it was determined that PGC-1 also interacts with ERα in vitro, although its role as a coactivator of ERα has not yet been established.

It has been shown previously that PGC-1 plays a role in adaptive thermogenesis where, among many activities, it enhances the ability of PPARγ and nuclear respiratory factors (NRF-1 and NRF-2) to induce the synthesis of the enzymes required for oxidative metabolism (27). Commensurately, PGC-1 has been shown to be expressed and highly regulated in...
brown adipose tissue and skeletal muscle in rodents (26). In addition, PGC-1 is expressed in the heart, kidney, and brain, suggesting that it is involved in processes other than thermogenesis (26). Given that ERα target cells also exist in the heart, kidney, and brain, we were intrigued by the possibility that PGC-1 may function as an ERα coactivator. If PGC-1 could serve as such a coactivator, it might provide a point of convergence between PPARγ and ERα-regulated signaling pathways (28).

**EXPERIMENTAL PROCEDURES**

**Enzymes and Chemicals**—Restriction and modification enzymes were obtained from Roche Molecular Biochemicals. Glutathione-Septarose 4B was purchased from Amersham Pharmacia Biotech. The TNT translation system was obtained from Promega. QuickChange® kits were purchased from Stratagene (La Jolla, CA). 17β-Estradiol and 4-hydroxytamoxifen were obtained from Sigma.

**Plasmids**—A vector expressing the ER-LL mutant was generated using oligo-directed mutagenesis as described previously (29). The ER-1F and ER 282-stop mutants were generated as described (12, 30), respectively. The ER 253-stop mutant was generated by introducing a stop codon (indicated by underscoring) into the ER open reading frame using oligo-directed mutagenesis as described previously (29). The ER-282-stop mutants were generated as described (12, 30), respectively. The ER-253-stop mutant was generated by introducing a stop codon (indicated by underscoring) into the ER open reading frame using oligo-directed mutagenesis as described previously (29).

**Definition of the ER-interacting Sites within PGC-1**—Multiple sites in PGC-1 govern its interaction with ERα in vitro. A, schematic diagram of PGC-1 and the PGC-1 deletion mutants used for the in vitro GST pulldown assay. B, PGC-1 or a PGC-1 mutant fused to GST were immobilized on glutathione-Sepharose beads and incubated with in vitro-translated [35S]methionine-labeled ERα without hormone (NH), with 10−6 M 17β-estradiol (E2) or 10−6 M 4-hydroxytamoxifen (T) as indicated. A control experiment evaluated the nonspecific binding of ERα to the GST alone (C).

**RESULTS**

**Definition of the ER-interacting Sites within PGC-1**—We examined the ability of PGC-1, and a series of PGC-1 mutants, to interact with ERα using in vitro pulldown assays. The results of these analyses are shown in Fig. 1. Full length PGC-1 binds to ERα in the absence of ligand (Fig. 1B). This interaction is enhanced significantly by addition of the agonist 17β-estradiol (E2), whereas addition of the antagonist tamoxifen has no impact on binding (Fig. 1B). By assessing the interaction of ERα with fragments of PGC-1 we were able to define one domain whose interaction with the receptor was ligand-dependent and a second that bound in a ligand-independent manner. Specifically, we observed that polypeptides encoding...
either the amino-terminal 400 or 170 amino acids bound efficiently to E2-activated ERα, but not to apo-ERα or that complexed with tamoxifen. The most carboxyl-terminal peptide studied (residues 604–797) interacted with ERα in a ligand-independent manner. Fragments of PGC-1 encoding amino acids 1–588 or 1–670 bound ERα in the absence of ligand; however, their interaction was enhanced by the addition of E2 (Fig. 1B). Cumulatively, these data suggest that there are two major contact sites for ERα within PGC-1: a hormone-dependent binding site located between residues 1 and 170 and a hormone-independent binding site located within the carboxy-terminal half of PGC-1.

An LXXLL Motif within PGC-1 Is Required for the Ligand-dependent Binding to ERα—The PGC-1 fragment 1–170 interacts with ERα in an agonist-dependent manner (Fig. 1B). This fragment contains an LXXLL sequence (residues 142–146), a motif that has been shown to mediate ligand-dependent recruitment of the p160-type of coactivators to nuclear receptors (32). Mutation of the conserved leucines within the p160 LXXLL motifs has been shown to disrupt these interactions (23, 32, 33). We tested whether the LXXLL motif present within PGC-1 is required for the hormone-dependent binding of the amino-terminal fragment of PGC-1 to ERα. Using an in vitro GST pulldown assay we were able to demonstrate that a PGC-1 fragment (PGCLA), harboring an L142A mutation, was unable to bind to ERα in the presence or absence of E2 (Fig. 2). These data suggest that the LXXLL motif is required for the ligand-dependent component of the ERα/PGC-1 interaction.

Definition of the Regions within ERα That Interact with PGC-1—To define the region(s) within ERα that bind PGC-1, we performed a series of in vitro binding assays using either full length ERα, a mutant lacking the AF2 domain (ER-351), or mutants lacking either part or all of the hinge region of ERα (ER-282 and ER-253) (Fig. 3A). These studies revealed that the ER-351 mutant, which contains an intact hinge domain, but lacks the AF2 domain, binds PGC-1 (Fig. 3B). The ER-282 fragment also binds to PGC-1 in a ligand-independent manner; however, it does not bind as well as ER-351 or ERα-wild type.

The ERα fragment 1–253, lacking the entire hinge region, does not interact with PGC-1. These data indicate that the hinge region of ERα between amino acids 253 and 283 mediates the ligand-independent component of the ERα/PGC-1 interaction. We confirmed the importance of this domain by deleting residues 253–282 in the full-length receptor (ER-Δ30) and showing that this mutant protein did not exhibit constitutive ERα binding. However, as expected, ER-Δ30 was able to bind PGC-1 when tested in the presence of E2 (Fig. 3C).

Many nuclear receptor coactivators have been shown to use LXXLL to mediate the interaction with the AF2 domain of ERα. The demonstration that a fragment of PGC-1, which contains an LXXLL motif, was also able to interact with ERα in a ligand-dependent manner suggests that the AF2 domain of ERα may be responsible for this protein-protein interaction. This hypothesis was tested by assessing the ability of PGC-1 to interact with an ERα mutant in which the AF2 domain was disrupted (Fig. 4A). Mutation of the hydrophobic residues in the ERα AF2 domain (ER-LL) abolishes the ligand-induced activation function and prevents the interaction of the LXXLL-containing coactivators with ERα (34, 35). We evaluated the ability of PGC-1 to interact with ER-LL and observed that the ligand-dependent, but not ligand-independent, component of the ERα/PGC-1 interaction was abrogated (Fig. 4B).

Mutation of the three charged amino acid residues (D538, E542, D545 → N538, Q542, N545) in helix 12 of the AF2 domain (ER-3X) leads to the loss of ERα transcriptional activity in some, but not all, cell contexts (12, 36). This mutation abrogates recruitment of p160-type coactivators such as GRIP-1 and SRC-1 but not RIP-140 (19, 25, 37). Although these coactivators all interact with ERα through their LXXLL motifs, it is clear that not all LXXLL motifs interact with ERα in the same manner (35). Because the LXXLL motif and the surrounding amino acids in PGC-1 resemble those in RIP-140, we determined whether or not PGC-1 is capable of interacting with the ERα mutant (ER-3X). The results of these experiments are...
shown in Fig. 4B and demonstrate that full length PGC-1 and the ER-3X mutant interact and that their association is enhanced by the addition of E\(_2\). Thus, PGC-1, through the LXXLL motif, interacts with the AF2 domain of ER\(a\) in a manner that is different from that of the p160-type of coactivators. A double mutant in which the hinge region and AF2 domains have been disrupted (ER\(30/\mathrm{LL}\)) is unable to bind PGC-1 in either the presence or absence of E\(_2\) (Fig. 4B). This result confirms the importance of both the AF2 domain and the hinge region in mediating the interaction of ER\(a\) with PGC-1 and indicates that, if other binding sites on ER\(a\) are involved in this interaction, they do not contribute in a significant manner. We conclude that the ER\(a\)/PGC-1 interaction differs from that of PPAR\(\gamma$/PGC-1 and, unlike the latter, is enhanced by the addition of the hormone.

PGC-1 as Coactivator of ER\(a\)-dependent Transcriptional Activity—The functional significance of the ER\(a$/PGC-1 interactions were next evaluated using estrogen-responsive transcription systems reconstituted in heterologous cells. Initially, the ability of PGC-1 to potentiate the transcriptional activity of wtER\(a\) was assessed in HeLa cells (Fig. 5). In this cell line, ER\(a\) functioned as an estradiol-dependent transactivator when assayed on a TATA promoter containing either one (1XERE) or three (3XERE) copies of a canonical ERE. However, when PGC-1 was coexpressed in these cells, ER-dependent transcriptional activity was significantly elevated. Thus, for the first time we demonstrate that PGC-1 is capable of activating ER\(a\)-dependent transcriptional activity and therefore is a bona fide coactivator of ER\(a\). Interestingly, the partial agonist activity of tamoxifen was not affected by PGC-1 overexpression. To determine the functional significance of ER\(a$/PGC-1 contact sites we have identified, we next evaluated the impact of PGC-1 on the transcriptional activity of ER\(a\) mutants. When compared with wtER\(a\), the transcriptional activity of ER-LL, ER\(30\), and ER-3X, and the double mutant ER\(30/\mathrm{LL}\) were greatly reduced, re-affirming the importance of both the AF2 and the hinge regions in ER\(a\) action. The transcriptional activity of the mutant ER-LL, which contains an intact hinge domain and which interacts with PGC-1 in vitro, is not potentiated by PGC-1 overexpression. Thus, although PGC-1 interacts well with the hinge domain of ER\(a\) in vitro, this interaction alone is not sufficient for coactivation. In contrast, deletion of the PGC-1 binding site within the hinge region of ER\(a\) (ER\(30/\mathrm{LL}\)) reduces, but does not eliminate, the ability of this coactivator to potentiate ER\(a/-mediated transcriptional activity. Indeed, in PGC-1-expressing cells, wtER\(a\) and ER\(30\) displayed similar transcriptional activity when assayed using the 3XERE-reporter vector. Thus, although PGC-1 can bind to both the hinge and AF2 domains, it appears that, with respect to function, the contact mediated by the AF2 domain is the most important for the transcriptional activation. This conclusion is consistent with the observation that an intact AF2 domain is important for ER\(a$/coactivator interactions. The recent solution of the co-crystal structure of the ER\(a/-ligand binding domain with a fragment of the coactivator GRIP-1 provides a universal model with which to explain ER\(a$/coactivator interactions (16). The interaction...
between GRIP-1 and ERα could be abrogated by mutating the charged amino acid residues in the AF2 domain (ER-3X). Therefore, it was surprising that PGC-1 can function as a very charged amino acid residues in the AF2 domain (ER-3X).

The recent discovery of coactivators, proteins that can interact with and enhance the transcriptional activity of agonist-activated ERα, and the demonstration that their overexpression in target cells influences ERα pharmacology indicate that differential co-factor expression is a primary determinant of a cell’s ability to respond to different agonists and antagonists (18, 37, 40, 41). The first bona fide steroid hormone receptor coactivator, SRC-1, was identified by virtue of its ability to interact with the hormone binding domain of agonist-activated progesterone receptor (20). Subsequently, it was demonstrated for ERα-mediated transcriptional activity in this context. When the transcriptional activity of these ERα proteins was assayed in cells overexpressing PGC-1, it was observed that the activity of wtERα and ER-3X were greatly enhanced. A minor enhancement of ERΔ30 activity was also observed, but the transcriptional activity of the AF1-containing ER-LL mutant was unaffected. To exclude the possibility that the activation of ERα-dependent transcription is a result of increased ERα protein expression in cells expressing PGC-1, we performed a Western immunoblot analysis using anti-ERα-specific antibodies. This analysis revealed that ERα, and mutants thereof, were expressed at the same level in the presence and absence of PGC-1 (data not shown). Thus, the mutations considered previously to disrupt AF2 activity (ER-3X) were sufficient to block the interaction of ERα with most, but not all, coactivators. The ability of ER-3X to function in a cell-selective manner in some contexts, therefore, may reflect the cell-specific expression and regulation of cofactors like PGC-1, which can interact with and potentiate the transcriptional activity of ER-3X.

**FIG. 6.** PGC-1 potentiates estrogen receptor transcriptional activity in HepG2 cells. HepG2 cells were transfected with vectors expressing either wtERα or its mutants (ER-LL, ER-Δ30, ER-Δ30/LL, or ER-3X) along with C3 Luc reporter. To assess the ability of PGC-1 to enhance ERE-dependent transcription, we also introduced either empty vector or vector expressing PGC-1. Luciferase activity was normalized to the activity of the cotransfected, pcMV-bgal plasmid. Cells were grown in the absence of hormone (gray bars) or were treated either with 10⁻³ M of 17β-estradiol (black bars), or with 10⁻³ M 4-hydroxytamoxifen (white bars). Transfections were performed in triplicate (n = 3). The error is presented as standard error of the mean.

DISCUSSION

The recent discovery of coactivators, proteins that can interact with and enhance the transcriptional activity of agonist-activated ERα, and the demonstration that their overexpression in target cells influences ERα pharmacology indicate that differential co-factor expression is a primary determinant of a cell’s ability to respond to different agonists and antagonists (18, 37, 40, 41). The first bona fide steroid hormone receptor coactivator, SRC-1, was identified by virtue of its ability to interact with the hormone binding domain of agonist-activated progesterone receptor (20). Subsequently, it was demonstrated...
that SRC-1 was able to interact efficiently with most of the nuclear receptors. The physiological relevance of this interaction was confirmed by demonstrating that mice bearing a genetic disruption of the SRC-1 gene display only a mild form of resistance to estrogens and progestins (42). The subtle phenotypes exhibited by the SRC-1 knockout mice are probably due to the fact that other coactivators can functionally substitute for SRC-1. Indeed, two proteins closely related in structure to SRC-1, GRIP-1 and ACTR, have been identified, and each has been shown in cell transfection studies to exhibit the properties of a coactivator (21, 43). Because of the similarity in their structure and function, the latter proteins have collectively been described as the p160 coactivators. Specifically, we and others had determined that PGC-1 may interact with the AF2 domain differently from the p160-type coactivators. Specifically, we and others had determined that mutation of the charged residues (D538, E542, H5, and H12) within the ligand binding domain and the subsequent formation of a hydrophobic cleft (16). This hydrophobic cleft constitutes the functional AF2 domain and forms a pocket for the LXXLL motif contained within the p160 coactivators. The functional importance of the hydrophobic cleft in binding PGC-1 was confirmed by showing that mutations, which alter the hydrophobicity (ER-LL) of the AF2 domain, prevent the interaction of ERα with the LXXLL domain and significantly reduce the ability of PGC-1 to coactivate ERα. However, we also demonstrated that an intact hinge region in ERα was required for maximal coactivation by PGC-1. Our unexpected results have led us to conclude that PGC-1 is a versatile coactivator, which interacts with ERα and PPARγ by different mechanisms.

Further dissection of the interaction between the LXXLL motif of PGC-1 and ERα mutated at the AF2 domain revealed that PGC-1 may interact with the AF2 domain differently from p160-type coactivators. Specifically, we and others had determined that mutation of the charged residues (D538, E542, D545 → N538, Q542, N545) within the AF2 domain (ER-3X) prevented ERα from interacting with the p160 class of coactivators (35). This loss of interaction was thought to occur as a consequence of a perturbation in the formation of a “charge clamp,” which was required to position the LXXLL motif in the coactivator groove. In contrast, these charged residues in ERα were not required for PGC-1 binding. This indicates that the mechanism by which p160 proteins and PGC-1 interacted with the AF2 domain of ERα is different and that, although the LXXLL motif within PGC-1 or the p160 coactivators is required for this interaction, these motifs are not equivalent. Our recent studies using phage display revealed that, based on their primary amino acid sequence, there are at least three different types of LXXLL motifs (35). Although all three classes of LXXLL motifs interacted with the AF2 domain of ERα, we demonstrated that one class alone (class III) was able to interact with the ER-3X mutant. Interestingly, the LXXLL motif within PGC-1 is a class III member, whereas LXXLL motifs present in p160-type coactivators are mostly class I or II members. Thus, the differences in binding characteristics of PGC-1 and p160-type coactivators to ERα can be attributed to the differences in flanking amino acid sequences in LXXLL motifs.

The discovery of the class III LXXLL motif and the demonstration that this motif is found in PGC-1 could explain transcriptional activity of the ER-3X mutant in some cell environments. Taken together the results of our studies and those of others indicate that the ER-3X mutation may not totally dis-
rupt AF2 function but rather block the interaction of ERα with coactivators that contain specific types of LXXLL motifs (class I or II) (35). Thus coactivators, like PGC-1, which contain a class III LXXLL motif and which display a tissue-restricted expression pattern, are likely to be important for the ERα action in some cell and promoter contexts.

We demonstrated in these studies that PGC-1 is an ERα coactivator, which is functionally and mechanistically distinct from the p160 family of coactivators. Although the physiological relevance of this interaction requires further investigation, we believe that PGC-1 may serve as a point of convergence for mitochondrial function and in an indirect manner enhances the transcription of genes encoded by the mitochondrial genome (27). Indeed, we have recently shown in MCF-7 breast cancer cells that estradiol induces the transcription of genes required for mitochondrial function and in an indirect manner enhances the transcription of genes encoded by the mitochondrial genome. Our findings also demonstrate that coactivators can interact with ERα in different ways. This result, combined with the finding that the structure of ERα is influenced by the nature of the bound ligand, provides a molecular model with which to explain how different ligands acting through the same receptor can manifest different biological activities in different cells (34).

Acknowledgments—We thank Drs. C.-Y. Chang and P. Giangrande for comments and suggestions.

REFERENCES
1. Love, R. R., Mazess, R. B., Barden, H. S., Epstein, S., Newcomb, P. A., Jordan, V. C., Carpenter, P. P., and Demets, D. L. (1992) N. Engl. J. Med. 326, 852–856
2. Migliaccio, S., Davis, V. L., Gibson, M. K., Gray, T. K., and Korach, K. S. (1992) Endocrinology 130, 2617–2624
3. Kipper, G. G. J. M., Esumark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, J.-A. (1992) Proc. Natl. Acad. Sci. U. S. A. 93, 5925–5930
4. Mol. Cell. Biol. 13, 869–876
5. Smith, C. L., Nawaz, Z., and O’Malley, B. W. (1999) Mol. Cell. Biol. 19, 4350–4355
6. Klein-Heipp, L., Schorr, M., Wagner, U., and Ryffel, G. U. (1986) Cell 46, 1053–1061
7. Beato, M., and Sánchez-Pacheco, A. (1996) Endocrinol. Rev. 17, 587–609
8. Wijayaratne, A. L., Nagel, S. C., Paige, L. A., Christensen, D. J., Norris, J. D., Fowlkes, D. M., and McDowell, D. P. (1999) Endocrinology 140, 5828–5840.
9. McDonald, D. P., Clemm, D. L., Hermann, T., Goldman, M. E., and Pike, J. W. (1995) Mol. Endocrinol. 9, 659–668
10. Trzaskan, M. T., Esty, A., Santiso-Mere, D., Danielian, P., Parker, M. G., Stein, R. B., Pike, J. W., and McDowell, D. P. (1994) Mol. Cell. Biol. 14, 387.
11. Parker, M. G. (1993) Breast Cancer Res. Treat. 26, 131–137

*J. Hall and D. P. McDonnell, unpublished data.