Coactivators PGC-1β and SRC-1 Interact Functionally to Promote the Agonist Activity of the Selective Estrogen Receptor Modulator Tamoxifen*§

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Dieter Kressler†1, M. Benjamin Hock‡2, and Anastasia Kralli‡§1

From the †Division of Biochemistry, Biozentrum of the University of Basel, Klingelbergstrasse 70, CH-4056 Basel, Switzerland and the ‡Department of Cell Biology, The Scripps Research Institute, La Jolla, California 92037

PGC-1β is a transcriptional coactivator that enhances strongly and in a hormone-dependent manner the activity of the estrogen receptor α (ERα) while having only weak effects on similar steroid hormone receptors, such as ERβ or the glucocorticoid receptor. Notably, PGC-1β enhances ERα transcriptionsal activity not only in response to agonist ligands, such as estradiol, but also to selective ER modulators, such as tamoxifen. Here, we dissect the molecular mechanisms underlying the ability of PGC-1β to act selectively on ERα and to promote the agonist activity of tamoxifen. We show that receptor selectivity is achieved by PGC-1β interactions with not just the ligand binding domain (LBD), which is highly conserved among nuclear receptors, but also the N-terminal domain and the hinge/AF-2a region of ERα, which are less well conserved. PGC-1β interacts directly with the hinge/AF-2a and LBD regions but indirectly and via the coactivator SRC-1 with the N-terminal domain. The three ERα surfaces and SRC-1 collectively enable efficient coactivation by PGC-1β. Similar ERα surfaces and interactions enable PGC-1β to coactivate transcription by tamoxifen-bound ERα. Surprisingly, PGC-1β coactivation of tamoxifen-bound ERα depends partially on one of the LXXLL motifs of PGC-1β and on Lys362 of the ERα LBD (i.e. surfaces implicated in agonist-dependent interactions). Our findings suggest that tamoxifen-induced changes in the ERα LBD promote interactions with the coactivator PGC-1β, which then cooperates with SRC-1 to enable tamoxifen agonism.

Estrogens play important roles in the female reproductive system and impact on the physiology of a broad range of non-reproductive systems, including bone, the cardiovascular and central nervous systems, and glucose and lipid metabolism (1–3). Estrogens also exert proliferative effects on hormone-responsive breast cancers (i.e. ~50% of breast cancer cases). The action of estrogens is mediated by two estrogen-regulated transcription factors that belong to the nuclear receptor family, and are referred to as estrogen receptors α and β (ERα and -β)3 (2, 4). Because ligand-activated ERs promote beneficial effects in some contexts, such as bone density maintenance, and harmful effects in others, such as the proliferation of breast cancer cells, there is a clinical interest in synthetic ER ligands that act as agonists in some tissues and antagonists in others. Such selective estrogen receptor modulators (SERMs) (e.g. tamoxifen) are used to treat estrogen-responsive breast cancers, where they antagonize endogenous estrogen action while maintaining beneficial agonist effects in bone and the cardiovascular system (1, 5). The cellular factors that determine the agonistic versus antagonistic properties of tamoxifen and other SERMs are only partially understood (5, 6). These factors are likely to be important not only for determining the tissue selectivity of SERM action, but also for understanding how breast cancers eventually develop resistance to treatment and start recognizing tamoxifen as an agonist that promotes proliferation (5).

ERs has the typical modular structure of nuclear receptors (4). A central DNA binding domain (DBD) mediates recruitment to specific DNA sequences, termed estrogen response elements (EREs). A C-terminal ligand-binding domain (LBD), separated from the DBD by a short hinge region, serves as the molecular switch that conveys information on the presence and type of ligand. Ligand-activated, DNA-bound ERα regulates the expression of genes via two transcriptional activation functions, denoted AF-1 and AF-2. AF-1 is located in the N-terminal domain of ERα, a region that is not conserved among different nuclear receptors and can activate transcription in a ligand-independent manner. AF-2 is encoded by highly conserved parts of the LBD and is tightly regulated by ligand. The ability of different ERs ligands to act as agonists or antagonists relies largely on their ability to induce distinct conformational changes in the LBD and accordingly modulate the recruitment of coregulators. Agonists direct a small amphipathic helix (helix 12 of the LBD and part of AF-2) to complete the fold of a hydrophobic pocket, which enables the docking of diverse transcriptional coactivators (7, 8). Antagonists and SERMs, like tamox-

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‡ Present address: Biochemie-Zentrum der Universität Heidelberg, Im Neuenheimer Feld 328, D-69120 Heidelberg, Germany.

§ To whom correspondence should be addressed: Dept. of Cell Biology, The Scripps Research Institute, 10550 N. Torrey Pines Rd., La Jolla, CA 92037. Tel.: 858-784-7287; Fax: 858-784-9132; E-mail: kralli@scripps.edu.

The abbreviations used are: ER, estrogen receptor; E2, 17β-estradiol; NR, nuclear receptor; NR box, nuclear receptor-interacting motif; DBD, DNA binding domain; aa, amino acid; ERE, estrogen response element; LBD, ligand binding domain; AF-1 and -2, activation function 1 and 2, respectively; SERM, selective estrogen receptor modulator; SMRT, silencing mediator of retinoid and thyroid hormone receptor; SRC, steroid receptor coactivator; GR, glucocorticoid receptor; PBS, phosphate-buffered saline; HA, hemagglutinin.
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ifen and raloxifene, block the agonist-dictated positioning of helix 12 and instead allow this helix to dock itself in part of the hydrophobic pocket (7, 8). This conformation occludes interactions with coactivators and instead is thought to promote the recruitment of corepressors (9, 10). Since SERMs appear to block AF-2, their agonistic properties depend largely on AF-1. Consequently, SERMs act as agonists in contexts where the AF-1 activity is strong (11, 12). However, SERMs that differ in their agonistic properties induce distinct conformational changes in the ERα LBD (7, 8, 13), suggesting that SERM-specific LBD conformations are communicated to AF-1. The mechanism of such communication is not clear. As one possibility, ligand binding may affect intramolecular interactions of AF-1 with the LBD (14). Alternatively, the different SERMs may affect differentially interactions with corepressors or enable the recruitment of yet unidentified factors that synergize with AF-1 (13, 15, 16).

A large number of coactivators that interact with estradiol-activated ERα and enable its transcriptional function have been identified (17, 18). Coactivators of the p160 steroid receptor coactivator (SRC) family, (SRC-1/NcoA1, GRIP1/TIF2/NcoA2, and pCIP/ACTR/AIB1/RAC3/SRC-3/NcoA3), interact with the AF-2 pocket of the LBD via short amphipathic α-helical motifs with the consensus sequence LXXLL, also called NR boxes (19). In addition, SRC coactivators interact with the AF-1 of ERα in a ligand-independent manner (20). Thus, in the presence of ERα agonists, when both AFs are active, SRC coactivators promote a transcriptional synergy between AF-1 and AF-2 (21, 22). In the presence of SERMs, SRC coactivator interactions with AF-1 are thought to promote SERM-induced transcription (6, 20, 23). Among the SRC family members, SRC-1 in particular has been shown to be important for the agonist activity of tamoxifen in uterine cells, where SRC-1 is expressed at high levels (6). The ability of SRC coactivators to enhance tamoxifen-induced transcription can be inhibited by corepressors (23). These findings have led to the notion that the relative levels or activity of coactivators and/or corepressors may be responsible for the distinct activities of SERMs in different tissues, as well as for the resistance of breast cancers to tamoxifen treatment (6, 23, 24).

PGC-1β (also known as PERC) is a tissue-specifically expressed coactivator of selective nuclear receptors (25, 26). It is a member of the PGC-1 family of coactivators, which includes also PGC-1α and PRC (27). PGC-1β shares functional similarities with PGC-1α, such as the ability to interact with NRF-1 and the orphan estrogen-related receptors, and to promote mitochondrial biogenesis and oxidative metabolism (25, 28, 29). However, PGC-1β is an inefficient coactivator of many nuclear receptors that are activated by PGC-1α, including the glucocorticoid receptor (GR) and HNF4 (26, 30). Notably, PGC-1β activates potently ERα- but not ERβ-mediated transcription, whereas PGC-1α shows no preference between the two ERs (26). Moreover, PGC-1β enhances the agonist activity of tamoxifen, whereas PGC-1α does not (26). The mechanism by which PGC-1β coactivates ERα seems similar to that of other AF-2 coactivators of ERα. Two LXXLL motifs of PGC-1β (termed NR1 and NR2), mediate an interaction with the LBD of ERα in an AF-2- and agonist-dependent manner, whereas a potent N-terminal bipartite activation domain enhances transcription (26).

The molecular mechanisms that enable PGC-1β to coactivate preferentially ERα over other nuclear receptors and to enhance the agonist activity of tamoxifen are not clear. In this study, we elucidate these mechanisms by dissecting the physical and functional interactions of PGC-1β with ERα and with SRC-1. We identify multiple interaction domains that enable PGC-1β to act synergistically with SRC-1 to enhance agonist- as well as SERM-dependent ERα-mediated transcription. Importantly, our findings reveal a role for the ERα LBD, and specifically for an AF-2-like surface, in promoting interactions with PGC-1β in the presence of tamoxifen. These results suggest that specific AF-2 coactivators, such as PGC-1β, can recognize the tamoxifen-induced conformation of the ERα LBD and contribute to the agonistic properties of tamoxifen.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—All recombinant DNA techniques were done according to established procedures using Escherichia coli DH5α for cloning and plasmid propagation. All cloned DNA fragments generated by PCR amplification were verified by sequencing. More information on the plasmids is available on request. Expression plasmids pcDNA3/HA-PGC-1β, pcDNA3/HA-PCG-1β.nrl, pcDNA3/HA-PCG-1β.nr2, pcDNA3/HA-PCG-1β.nr1/2, and pcDNA3/HA-PCG-1α as well as the luciferase reporter plasmids pA(VERE)x1-Luc, pTAT3-Luc, and pGK1 have been described (26, 31). The following expression and luciferase reporter plasmids were generously provided: pSG5/ SRC-1e, pSG5/SRC-1e-M123 (M. Parker), pSG5/GRIP1 (M. Stallcup and P. Webb), pSG5/myc-SmRMT (M. Privalsky), pCMV/HA-CBP (R. Eckner), pC3-Luc (D. McDonnell).

Cell Culture, Transfections, and Ligands—COS7 and U2OS cells were cultured in Dulbecco’s modified Eagle’s medium supplemented with 9% fetal bovine serum. Medium lacking phenol red and supplemented with charcoal-stripped or charcoal/dextran-treated (HyClone) fetal bovine serum was used when assaying ligand responses. Cells used for luciferase assays were seeded into 6-well plates 24 h prior to transfection by the calcium phosphate precipitation method. All transfections included 0.2 μg of plasmid and 0.8 μg of p6RlacZ for normalization of transfection efficiency. Amounts of expression and reporter plasmids per transfection are indicated in the figure legends. After overnight exposure to the DNA-calcium phosphate precipitate, cells were washed and incubated for an additional 24 h in fresh medium containing the indicated ligands. Assays for luciferase and β-galactosidase activities were performed as described (31). Luciferase values normalized to β-galactosidase activity are referred to as luciferase units. Data shown represent the mean ± S.D., unless otherwise indicated, of at least four values from two independent experiments performed in duplicates. 17β-estradiol (E2), tamoxifen, and 4-OH-tamoxifen were from Sigma (E8875, T9262, and H7904) and dissolved in 100 or 70% ethanol. The raloxifene analogue LY117018 (dissolved in 100% ethanol) was kindly provided by Lilly.

GST Pull-down Interaction Assay—Expression of GST fusion proteins in the E. coli strain BL21 was induced by the addition of 250 μl of 100 mM isopropyl 1-thio-β-d-galacto-
The activity in the presence of hormone was assessed by coactivator in the presence of hormone; the activity in the presence of hormone and the absence of coactivator was set equal to 1 for all receptor variants.

RESULTS

Efficient Coactivation by PGC-1β Requires the AF-1 Domain of ERα—The agonist activity of tamoxifen has been attributed to the ability of this ligand to promote the transcriptional function of the AF-1 domain of ERα (11, 12). Because PGC-1β enhances the agonist activity of tamoxifen (26), we reasoned that PGC-1β may interact functionally with the AF-1 domain. To test this hypothesis, we compared the activity of PGC-1β to coactivate full-length ERα versus a variant of ERα that lacks AF-1 (ERαΔAF-1). As previously reported, PGC-1β expression in COS7 cells enhanced potent the E2-dependent activation of an ERE-driven luciferase reporter by full-length ERα (Fig. 1A). This enhancement was significantly lower for the ERαΔAF-1 variant, suggesting that AF-1 is required for an efficient response to PGC-1β. A similar requirement for AF-1 was seen in HeLa cells (data not shown). Full-length ERα and ERαΔAF-1 were expressed at comparable levels, suggesting that differences in expression could not account for the different responses to PGC-1β (Fig. 1B). Moreover, a requirement for AF-1 was not evident when ERα was coexpressed with a different coactivator, PGC-1α (Fig. 1A).

Next, we asked whether the AF-1 of ERα was sufficient to convert a nuclear receptor that is poorly coactivated by PGC-1β, like GR (26), to one that responds efficiently to PGC-1β. To this end, we replaced the AF-1 of GR with the AF-1 of ERα and compared the responses of the chimeric ERα(AF-1)-GR and of wild-type GR to PGC-1β. As seen in Fig. 1C, ERα(AF-1)-GR was coactivated robustly, around 20-fold, by PGC-1β. In contrast, PGC-1β had at most a 2-fold effect on the activity of wild-type GR, as shown previously (26). In conclusion, the AF-1 of ERα is not only required for efficient coactivation by PGC-1β but can also confer responsiveness to PGC-1β when fused to a heterologous receptor DBD and LBD.
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PGC-1β Is Not a Direct AF-1 Coactivator—The AF-1 requirement for efficient coactivation by PGC-1β may reflect a direct physical interaction between AF-1 and PGC-1β, analogous to interactions of AF-1 with other coactivators, such as SRC-1 (20). To address this possibility, we first asked whether PGC-1β enhances the transcriptional activity of the N-terminal domain of ERα fused to the DBD of Gal4 (AF-1/G4). Established AF-1 coactivators, such as SRC-1e, GRIP1, and CBP, stimulated the activity of AF-1/G4, as expected (Fig. 2A). In contrast, PGC-1β decreased the activity of the ERα AF-1 (Fig. 2A). Similarly, whereas a VP16-SRC-1e fusion protein was even more potent than SRC-1e in activating AF-1/G4, VP16-PGC-1β reduced ERα AF-1 activity (data not shown). Next, we asked if PGC-1β and the AF-1 of ERα interact physically in vitro. Using bacterially expressed AF-1 of ERα and in vitro translated PGC-1β, we did not detect an interaction (Fig. 2B).

Under the same conditions, SRC-1e and GRIP1 interacted efficiently with GST-ERα AF-1, as expected for AF-1 coactivators (Fig. 2B). Taken together, these findings suggest that PGC-1β is not a direct AF-1 coactivator.

The Hinge/AF-2a Region of ERα Contributes to the Coactivation of ERα by PGC-1β—Because PGC-1β does not interact directly with the AF-1 of ERα and yet enhances the activity of tamoxifen-bound ERα (26), a context where coactivator interactions with AF-2 are thought to be blocked, we next asked whether PGC-1β interacts with other domains of ERα. Indeed, PGC-1β enhanced the activity of the variant ERαaN341, which includes AF-1, the DBD, the hinge region, and most of AF-2a, a domain (aa 282–351) previously shown to carry an independent activation function (32) (Fig. 3, A and B). A partial deletion (ERαaN307) or the complete removal of the AF-2a domain (ERαaN280) abolished responsiveness to PGC-1β (Fig. 3B), indicating a role for this region in the coactivation by PGC-1β. To gain an insight into the mechanism by which this region contributes, we tested the ability of the hinge/AF-2a domain to interact physically with PGC-1β. As seen in Fig. 3C, in vitro translated PGC-1β bound, albeit weakly, to bacterially expressed GST-hinge/AF-2a fusion protein, suggesting a direct physical interaction.

Next, we tested the ability of the hinge/AF-2a domain to confer responsiveness to PGC-1β by itself or in combination with other domains of ERα. To ensure that any activation seen in the presence of PGC-1β was mediated by the ERα variants we wished to analyze, we performed these assays in the context of ERα fusions to the Gal4 DBD, measuring luciferase activity of a Gal4-responsive reporter. PGC-1β did not coactivate a Gal4 DBD fusion to just the hinge/AF-2a region (data not shown), suggesting that the PGC-1β/AF-2a physical interaction is not sufficient by itself. PGC-1β did, however, coactivate modestly a Gal4 DBD fusion protein that harbors AF-1 and the hinge/AF-2a region (AF-1/G4/341; Fig. 3, A and D), suggesting that these two domains together enable responsiveness to PGC-1β. Moreover, the presence of the hinge/AF-2a region enhanced significantly the ability of PGC-1β to coactivate the ERα LBD. The -fold activation by which PGC-1β enhanced the E2-dependent activity of the ERα LBD was higher when the complete hinge/AF-2a region was present (G4/251C) than when its N-terminal part was missing (G4/308C) (Fig. 3, A and E). Finally, we tested the impact of the hinge/AF-2a region in the context of both AF-1 and LBD/AF-2, using Gal4 DBD chimeras that have AF-1 and the LBD either with the hinge/AF-2a region (AF-1/G4/251C) or without it (AF-1/G4/308C) (Fig. 3A). The presence of the hinge/AF-2a region allowed for a much stronger response to PGC-1β (Fig. 3F). Notably, the combined presence of AF-1, hinge/AF-2a region, and the LBD/AF-2 led to a protein whose activity was potently enhanced by PGC-1β (Fig. 3F). In conclusion, although the hinge/AF-2a domain is not sufficient by itself to respond to PGC-1β, it enables coactivation when together with AF-1 (Fig. 3D) and/or the ERα LBD (Fig. 3E).

PGC-1β Interacts Physically with the AF-1 Coactivator SRC-1e—Because the AF-1 domain of ERα clearly contributes to the response to PGC-1β, we next considered the possibility that although PGC-1β does not bind directly to AF-1, it may interact with an AF-1 coactivator. An appealing candidate is SRC-1, an ERα AF-1 coactivator that enables tamoxifen to act as an agonist (6, 23) and that interacts physically with the activation domain of the PGC-1β-related coactivator PGC-1α (33). In a mammalian two-hybrid assay, PGC-1β, like PGC-1α, interacted with SRC-1e (Fig. 4B). Furthermore, the N-terminal 128 aa of PGC-1β, expressed as a GST fusion protein in bacteria, interacted in vitro with full-length SRC-1e (Fig. 4C). As control, the activation domain of VP16 that has an acidic nature, like aa 1–128 of PGC-1β, showed no interactions with SRC-1e (Fig. 4C). To map the regions of SRC-1e that mediate the interaction with PGC-1β, we tested the ability of different domains of SRC-1e (Fig. 4A) to bind PGC-1β in vitro and in a mammalian two-hybrid assay. These studies identified two regions of SRC-1e, aa 254–422 and 1048–1140, that interact with PGC-1β (Fig. 4, C and D). Next, we asked whether the existence of two binding surfaces for PGC-1β is a common feature of p160 family coactivators. Indeed, the corresponding regions of GRIP1 interacted efficiently with PGC-1α and PGC-1α (Fig. 4E). In conclusion, PGC-1β interacts with two distinct regions of the
AF-1 coactivator SRC-1e, suggesting that SRC-1 could mediate the functional interaction between PGC-1β and the AF-1 domain of ERα.

PGC-1β and SRC-1e Interact Functionally and Coactivate Tamoxifen-bound ERα—The physical interaction between PGC-1β and SRC-1e suggests that the two coactivators may cooperate in enhancing the transcriptional activity of ERα. To test this notion, we evaluated the effect of PGC-1β and SRC-1e coexpression on ERα activity in different contexts (Fig. 5). First, we tested the ability of PGC-1β to synergize with a variant of SRC-1 that has its three NR boxes mutated and can therefore no longer interact with the AF-2 of ERα (SRC-1e.M123) (34). This SRC-1e NR box mutant has similar activity to that of wild-type SRC-1e in the coactivation of ERα(AF-1) (data not shown), suggesting that it retains interactions with the AF-1 of ERα. As seen in Fig. 5A, SRC-1e.M123 synergized with PGC-1β in the enhancement of E2-activated ERα at an ERE-driven luciferase reporter. This effect of SRC-1e.M123 was not seen with an ERα variant that lacks the AF-1 (ERα.DAF-1; Fig. 5A), indicating that the PGC-1β/SRC-1e synergy requires the AF-1 domain.

tamoxifen, creates a cellular context where tamoxifen activates ERα-mediated transcription.

A Role for the ERα AF-2 in Tamoxifen Agonist Activity—The fact that the responsiveness of SERM-activated ERα to PGC-1β is sensitive to subtle differences in the LBD conformation, as seen in tamoxifen- versus raloxifene-bound ERα, prompted us to examine a potential role for the AF-2 domain of tamoxifen-bound ERα. To investigate this possibility, we examined the transcriptional activity of tamoxifen-bound ERα in the presence of PGC-1β and SRC-1e cotransfection. We observed again a synergistic enhancement of tamoxifen-dependent ERα activation when PGC-1β and SRC-1e were coexpressed (Fig. 5B), consistent with a functional interaction between PGC-1β and SRC-1e.

Finally, we asked whether the PGC-1β/SRC-1 interaction could influence the ability of selective modulators, such as tamoxifen and raloxifene, to act as ERα agonists. Indeed, we observed a ~10-fold activation of tamoxifen-bound AF-1/G4/251C when PGC-1β and SRC-1e were coexpressed, whereas PGC-1β and SRC-1e alone had a ~3- and ~1.5-fold effect, respectively (Fig. 5C). Interestingly, PGC-1β and SRC-1e did not enhance the activity of ERα bound to the raloxifene analogue LY117018, a different SERM, suggesting that subtle structural differences in the LBD of ERα affect interactions with PGC-1β and/or SRC-1e. In conclusion, PGC-1β and SRC-1e synergize to enhance the transcriptional activity of tamoxifen-bound ERα. Notably, coexpression of the two coactivators in COS7 cells, a cell line with inherently low agonist activity of ERα, further corroborates the synergism between PGC-1β and SRC-1, we next tested the effect of these coactivators on the activity of a Gal4DBD fusion protein that bears the AF-1 and hinge/AF-2a domains of ERα (AF-1/G4/341; see Fig. 3A). We observed again a synergistic activation when PGC-1β and SRC-1e were coexpressed (Fig. 5B), consistent with a functional interaction between PGC-1β and SRC-1e.
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FIGURE 4. Two distinct regions of the AF-1 coactivators SRC-1e and GRIP1 interact physically with PGC-1β. A, schematic representation of SRC-1e (full-length, 1399 aa) and the constructs used in B–D. BH1, basic helix-loop-helix region; PAS, period/aryl hydrocarbon receptor/single-minded motif; CID, CBP/p300-interacting domain; Q-rich, glutamine-rich region. aa 1048–1399 interact with the AF-1 of ERα. B, COS7 cells were cotransfected with 0.1 μg of pSG5/GRIP1 or pSG5/GRIP1-SRC-1e, 0.5 μg of the Gal4-responsive luciferase reporter pGK1, and 1 μg of either pcDNA3 control vector, pcDNA3/HA-PGC-1β, or pcDNA3/HA-PGC-1α. Cells were assayed for luciferase activity 40 h after transfection. Data are expressed as -fold activation of the transcriptional activity of the Gal4 fusion proteins by PGC-1β or PGC-1α. Data are expressed as -fold activation of the transcriptional activity of the Gal4-responsive luciferase reporter pGK1, and 1 μg of either pcDNA3 control vector, pcDNA3/HA-PGC-1β, or pcDNA3/HA-PGC-1α. Cells were assayed for luciferase activity 40 h after transfection. D and E, COS7 cells were cotransfected with 0.1 μg of expression vectors for the indicated Gal4 fusion proteins, 0.5 μg of the Gal4-responsive luciferase reporter pGK1, and 1 μg of either pcDNA3 control vector, pcDNA3/HA-PGC-1β, or pcDNA3/HA-PGC-1α. Cells were assayed for luciferase activity 40 h after transfection. Data are expressed as -fold activation of the transcriptional activity of the Gal4 fusion proteins by PGC-1β or PGC-1α.

FIGURE 5. PGC-1β and SRC-1e interact functionally and coactivate tamoxifen-bound ERα. A, COS7 cells were cotransfected with 0.1 μg of pSG5/ERα or pSG5/ERα.AAF-1, 0.5 μg of the luciferase reporter pΔ(3EExE)x1-Luc, 1 μg of pSG5 control vector or pSG5/SRC-1e.M123, and 0.05 μg of either pcDNA3 control vector or pcDNA3/HA-PGC-1β. Cells were treated with 50 nm 17β-estradiol for 24 h and assayed for luciferase activity. Data are expressed as -fold activation of ERα or ERα.AAF-1 activity in the presence of hormone. B, COS7 cells were cotransfected with 0.1 μg of pSG5/AF-1/G4/341, 0.5 μg of the Gal4-responsive luciferase reporter pGK1, 0.5 μg of pSG5 control vector or pSG5/SRC-1e, and 0.5 μg of pcDNA3 control vector or pcDNA3/HA-PGC-1β. Cells were assayed for luciferase activity 40 h after transfection. Data are expressed as -fold activation of AF-1/G4/341 activity by coactivator(s). C, COS7 cells were cotransfected with 0.5 μg of pSG5/AF-1/G4/251C, 0.5 μg of the Gal4-responsive luciferase reporter pGK1, 1 μg of pSG5 control vector or pSG5/SRC-1e, and 0.1 μg of pcDNA3 control vector or pcDNA3/HA-PGC-1β. Cells were treated with 5 μM tamoxifen or 5 μM LY117018 for 24 h and assayed for luciferase activity. Data are expressed as -fold activation of AF-1/G4/251C activity in the presence of ligand.

tamoxifen-bound AF-1/G4/251C by ~50%, whereas mutations in NR box 2 alone (nr2 mut) had no effect (Fig. 6C). The effects of the single NR box mutations on the response to tamoxifen were comparable with the effects of the same mutations on the specific mutations in the AF-2 domain of the LBD. The K362A mutation (helix 3 of the ERα LBD) reduces interactions with selective coactivators and corepressors; the L372R mutation (helix 5) selectively decreases binding of corepressor NR box response to estradiol (Fig. 6C). The double nr1/nr2 mutant activated tamoxifen-bound ERα to a similar extent as the nr1 single mutant (~50% of that of wild-type PGC-1β), suggesting that NR box 2 does not contribute to the tamoxifen response. In contrast, the nr1/nr2 double mutant was severely deficient in the coactivation of E2-bound receptor, consistent with our previous report that both NR1 and NR2 can mediate the interaction of PGC-1β with E2-activated AF-2 (26) (Fig. 6C). Taken together, these findings indicate that PGC-1β contains an unusual nuclear receptor interaction surface, NR1, that contributes to the coactivation of tamoxifen-bound ERα. In contrast, NR2 is similar to other coactivator LXXL motifs that interact only with bona fide agonist-bound and not with SERM-bound ERα. Clearly, mutations in NR1 decrease but do not abolish the ability of PGC-1β to enhance the agonist activity of tamoxifen. In a search for other important PGC-1β domains in this context, we observed that the region C-terminal to aa 426, which does not include known nuclear receptor interaction motifs, is essential for the activation of tamoxifen-bound receptor (Fig. 6C). Notably, deletion of this PGC-1β region had only a weak effect on E2-activated receptor, suggesting that it affects selectively the tamoxifen-activated receptor. In conclusion, these experiments show that one of the NR boxes of PGC-1β, NR1, contributes to tamoxifen-induced as well as E2-induced transcription. Additional PGC-1β surfaces, such as the C-terminal part (aa 427–1023) or NR2, interact selectively with the tamoxifen- or E2-bound ERα, respectively.

To further investigate the contribution of an interaction of PGC-1β with a tamoxifen-induced structure of the ERα LBD, we next tested the responsiveness of ERα variants with
peptides; and the L539A/L540A mutation in helix 12 diminishes the interaction with coactivator LXXLL motifs and may lead to increased corepressor binding (13, 15, 35–37). First, we assessed the ligand responses of the different AF-2 mutants in the context of the AF-1/G4/251C chimeric receptor in U2OS cells in the absence of PGC-1β (Fig. 7A). The L539A/L540A mutation reduced the already weak response to tamoxifen (by 50%) and almost completely abolished the response to E2. The K362A and L372R mutants showed similar responses to that of wild-type receptor to tamoxifen but differed in their responses to estradiol. K362A showed a strongly reduced although still detectable response to E2 (30-fold lower than that of wild-type receptor), whereas L372R had a slightly increased E2 response. These results indicate that the endogenous U2OS coactivators interact weakly with the E2-bound K362A mutant. Next, we assessed the effect of PGC-1β on the ligand-dependent activity of the different ERα variants (Fig. 7B). Notably, the ability of PGC-1β to coactivate the tamoxifen-bound receptor was clearly decreased for the K362A mutant (2-fold activation, as opposed to 5- and 9.6-fold activation for the wild-type and L539A/L540A variants, respectively). This finding is consistent with a role for an AF-2-like surface, and in particular Lys362 in promoting an interaction with PGC-1β in the presence of tamoxifen. Interestingly, PGC-1β coactivated the E2-bound K362A variant by more than 1000-fold, thereby recovering the transcriptional activity of K362A in the presence of estradiol to just 3-fold lower than that of wild-type receptor (Fig. 7B, right). The ability of PGC-1β to interact with the E2-bound K362A variant depended largely on NR1 (Fig. 7C). Altogether, our findings support a role for NR1 of PGC-1β and for residue Lys362 of the AF-2 of ERα in the functional interaction of PGC-1β with tamoxifen-bound ERα. Notably, residue Lys362, although important for interaction of tamoxifen-bound ERα with PGC-1β, seems dispensable for the interaction of E2-bound ERα with PGC-1β.

**The Corepressor Silencing Mediator of Retinoid and Thyroid Hormone Receptor (SMRT) Antagonizes PGC-1β-mediated Activation of Tamoxifen-bound ERα**—The corepressor/corepressor ratio has been proposed to determine whether tamoxifen acts as a partial agonist or an antagonist (6, 23, 24). Moreover, the interaction of ERα with corepressors requires a hydrophobic cleft of the ERα LBD as well as the hinge region (13, 38, 39) (i.e. surfaces similar to the ones contributing to the interaction with PGC-1β). To determine the extent to which PGC-1β and corepressors act antagonistically to each other, we determined the effect of the corepressor SMRT on the PGC-1β-mediated enhancement of tamoxifen-dependent transcription (Fig. 8A). SMRT had no effect on the weak basal activity of the luciferase reporter, either in the absence of the ERα-Gal4 fusion protein or in the presence of the fusion protein but absence of ligand (data not shown). Tamoxifen by itself was a weak agonist of AF-1/G4/251C (~3.5-fold), and SMRT reduced this transcriptional activity by 50%, consistent with SMRT interacting with the tamoxifen-bound AF-1/G4/251C. Importantly, coexpression of SMRT reduced dramatically and in a dose-dependent manner the PGC-1β-mediated enhancement of the activity of tamoxifen-bound receptor (Fig. 8, A and B). The negative effect of SMRT on the transcriptional activity of E2-bound receptor was less pro...
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In this study, we show that the ability of PGC-1α via these surfaces facilitate response not only to full agonists, like estradiol, but also to specific SERMs, such as tamoxifen. Surprisingly, one of the LXXLL motifs of PGC-1β and part of the AF-2 of the ERα LBD contribute to the enhancement of tamoxifen agonist activity, suggesting that PGC-1β recognition of the tamoxifen-bound LBD enables part of the agonist activity of tamoxifen.

We have previously shown that the ability of PGC-1β to coactivate estradiol-bound ERα depends on an interaction between LXXLL motifs (NR1 and NR2 boxes) of PGC-1β and the agonist-induced AF-2 domain of ERα (26). Nevertheless, PGC-1β is a potent coactivator of ERα but not of other nuclear receptors that bear similar AF-2 regions, such as ERβ and GR (26). Our findings in this study explain this apparent paradox by showing that

FIGURE 7. A role for the ERα AF-2 in PGC-1β-dependent agonist activity of tamoxifen. A, U2OS cells were cotransfected with 0.5 μg of pSG5/AF-1/G4/251C, wild type (wt) or bearing the indicated amino acid substitutions, and 0.5 μg of the Gal4-responsive luciferase reporter pGK1. Cells were treated with no ligand (−), 5 μM tamoxifen, or 50 nM E2 for 24 h and assayed for luciferase activity. Data are expressed as -fold activation by ligand, with activity for wild-type AF-1/G4/251C in the absence of ligand set equal to 1. B, U2OS cells were cotransfected with 0.5 μg of pSG5/AF-1/G4/251C, wild type (wt) or bearing the indicated amino acid substitutions, 0.5 μg of the Gal4-responsive luciferase reporter pGK1, and 1 μg of pcDNA3 control vector or pcDNA3/HA-PGC-1β. Cells were treated with 5 μM tamoxifen (left) or 50 nM 17β-estradiol (E2; right) for 24 h and assayed for luciferase activity. Data are expressed as -fold activation by AF-1/G4/251C-K362A by coactivator in the presence of estradiol.

pronounced and was similar in the absence and presence of PGC-1β. These findings suggest that SMRT antagonizes selectively the PGC-1β-mediated coactivation of tamoxifen-bound receptor, consistent with SMRT and PGC-1β acting antagonistic to each other in the context of tamoxifen.

DISCUSSION

The transcriptional activity of nuclear receptors is tuned by ligands and coregulators, which collectively determine the context in which the receptors function (18, 40). Despite the wealth of information on the conformational changes that agonists, antagonists, and SERMs confer to the ERα LBD, it is still not clear how a specific SERM acts as an agonist in one context and an antagonist in another. One hypothesis is that the distinct ERα conformations are recognized selectively by specific coregulators and that the repertoire of the different coregulators in different cells determines the agonist or antagonist capacity of a SERM (5, 40). In support of this hypothesis, SERMs can promote interactions of ERα with corepressors (13, 24, 38, 41, 42). However, we do not know yet of any coactivators that recognize a SERM-bound LBD and contribute to SERM agonist activity. In this study, we show that the ability of PGC-1β to promote ligand-activated ERα transcription relies on multiple interaction surfaces, among them three surfaces of ERα: AF-1, the hinge/AF-2 region, and the LBD AF-2. Interaction of PGC-1β with AF-1 is indirect and mediated by SRC-1, which synergizes with PGC-1β in the coactivation of ERα. In contrast, PGC-1β binds directly the hinge/AF-2a region and AF-2. The interactions of PGC-1β with ERα via these surfaces facilitate response not only to full agonists, like estradiol, but also to specific SERMs, such as tamoxifen. Surprisingly, one of the LXXLL motifs of PGC-1β and part of the AF-2 of the ERα LBD contribute to the enhancement of tamoxifen agonist activity, suggesting that PGC-1β recognition of the tamoxifen-bound LBD enables part of the agonist activity of tamoxifen.

PGC-1β coactivates weakly the ERα LBD alone, reminiscent of the effect of PGC-1β on other receptors (26) and suggesting that AF-2 is important but not sufficient for efficient coactivation. The weak effect of PGC-1β via AF-2 is strengthened by two other domains of ERα: AF-1 and the hinge/AF-2a region. The AF-1 of ERα enables coactivation by PGC-1β when transferred to a receptor that is not responsive to PGC-1β, as shown here for GR, consistent with an interaction of AF-1 with PGC-1β. However, AF-1 by itself does not interact functionally or physically with PGC-1β, suggesting that other factors (e.g. SRC-1) and ERα domains bridge an AF-1 communication with PGC-1β. In the case of the hinge/AF-2a, the region following the DBD and extending up to aa 341 (i.e. including the AF-2a activation domain) (32) interacts physically with PGC-1β, albeit weakly, suggesting a role for direct binding and stabilization of a PGC-1β-ERα complex. All together, the combined presence of the three ERα domains and the bridging factor SRC-1 enables PGC-1β to enhance robustly ERα transcription. The lack of sequence conservation in AF-1 and hinge regions across nuclear receptors may therefore explain how PGC-1β acts selectively on a subset of receptors. The use of multiple weak interaction surfaces that function cooperatively may enable a large degree of flexibility as to when
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and where PGC-1β coactivates ERα. For example, modifications in AF-1, DNA response element-driven constraints on the presentation of the hinge/AF-2a region, and the levels and availability of bridging factors such as SRC-1 would be predicted to impact on the ability of PGC-1β to enhance ERα-mediated responses. Consistent with this notion, the ability of PGC-1β to coactivate ERα is highly dependent on promoter context (26).

Many coactivators serve as platforms that recruit other coactivators. For example, PGC-1α interacts with SRC-1 and the histone acetyltransferases CBP and p300 via its N-terminal activation domain and with the Mediator complex and the arginine methyltransferase PRMT1 via its C-terminal region (33, 43, 44). It is thus not surprising that PGC-1β interacts physically and functionally with SRC-1 and that the two proteins synergize in coactivating ERα. However, our findings suggest that PGC-1β does not act simply as a platform for recruiting SRC-1. The synergism of PGC-1β and SRC-1 in the coactivation of ERα is strongest when both AF-1, which binds SRC-1 but not PGC-1β, and the hinge/AF-2a and LBD domains, which bind PGC-1β, are present (Fig. 5) (data not shown). These results suggest that the two coactivators stabilize each other’s interaction with ERα and, more specifically, that SRC-1 mediates the functional interaction of PGC-1β with the ERα AF-1. It is possible that other proteins serve a similar mediating role and stabilize a PGC-1β complex with ERα. As shown here, both PGC-1β interaction surfaces of SRC-1 are conserved in other p160 coactivators, such as GRIP1. Notably, these two interaction surfaces are distinct from SRC-1 regions that bind ERα (aa 1048–1140 of SRC-1 do not interact with the ERα AF-1) (data not shown), suggesting that SRC-1 could interact simultaneously with PGC-1β and ERα. The PGC-1β-interacting domains of SRC-1 are, however, subdomains of larger regions shown to interact with other coactivators (CoCoA, CARM1, and PRMT1) and to contribute to the coactivation of nuclear receptors (45–47). Future studies will be needed to address whether PGC-1β competes with these other proteins for binding to SRC-1 or is incorporated together with them into large multicoactivator complexes.

In previous studies, PGC-1β but not PGC-1α enhanced tamoxifen-induced, ERα-mediated transcription from the complement C3 promoter in U2OS cells (26). We now demonstrate that PGC-1β acts as a coactivator of tamoxifen-bound ERα, using a chimeric ERα, where the cognate DBD is replaced by the Gal4 DBD and a minimal synthetic promoter under the control of Gal4 binding sites. The use of this defined system whose activation depends entirely on the chimeric ERα is important, because it minimizes any contributions first by transcription factors that may respond to PGC-1β and act via other binding sites (e.g. the 1.8-kb C3 promoter fragment contains sites for transcription factors other than ERα) and, second, by factors other than ERα that can act via EREs (e.g. estrogen-related receptors α and γ are coactivated by PGC-1α and PGC-1β and stimulate transcription via simple EREs (29, 48–50)). In the defined Gal4-based system, PGC-1β acts synergistically with SRC-1 in promoting the agonist activity of tamoxifen. Notably, coexpression of PGC-1β and SRC-1 is sufficient to convert COS7 cells, a cell line with inherently low agonist activity of tamoxifen, into a cell line where tamoxifen-bound ERα activates transcription efficiently. Using the same system, we have also defined the roles of specific PGC-1β and ERα domains in the response to tamoxifen. In PGC-1β, the C-terminal region, which seems dispensable for the activation of estradiol-bound ERα, is essential for the activation of tamoxifen-bound ERα. In ERα, the hinge/AF-2a region is important for efficient coactivation by PGC-1β (supplemental material). Surprisingly, point mutations in the first PGC-1β LXXLL motif and around the AF-2 of ERα (i.e. surfaces usually implicated in agonist-dependent interactions) also reduce the ability of PGC-1β to activate tamoxifen-bound ERα, suggesting that PGC-1β interacts with the tamoxifen-induced conformation of the ERα LBD.

Nuclear receptor ligands with partial agonist activity are thought to induce specific LBD conformations that promote selective interactions with a subset of coregulators (51, 52). The ability of tamoxifen to enable the presentation of a protein interaction surface is supported by studies showing that tamox-
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ifen promotes the interaction of ERα with corepressors, which recognize a surface that is similar to the agonist-induced AF-2 pocket (13, 14, 39, 42). Moreover, phage display screens have identified synthetic peptides that interact with the LBD of ERα in a tamoxifen-dependent manner (15, 16, 53). Overexpression of some of these peptides inhibits tamoxifen-induced transcription, presumably by competing with endogenous coactivators that contribute to the agonist activity of tamoxifen (16). However, no coactivators that interact with the tamoxifen-bound ERα LBD have been identified to date. Two findings in our study suggest that PGC-1β is such a coactivator. First, mutations in the PGC-1β NR1 box, which mediates interactions with the AF-2 of the ERα LBD (26), reduce the ability of PGC-1β to coactivate tamoxifen-bound ERα. Notably, the NR1 box of PGC-1β and the corresponding LXXLL motif of PGC-1α (L2), which does not enhance tamoxifen agonist activity, share a common core but differ in the flanking sequences. Whereas the L2 motif of PGC-1α has prolines in positions −3 and +8 (numbers relative to the first leucine of L56). Second, mutation of Lys362 of the ERα LBD may promote the interaction of ERα with coactivator complexes. SRC-1 interactions with AF-1 are likely to be strengthened by PGC-1β interactions with the hinge/AF-2a and an AF-2-like pocket of the LBD as well as interactions of SRC-1 with PGC-1β. Notably, SRC-1 has been implicated as a key player in determining the agonist activity of tamoxifen in uterine cells (6). Future studies will be required to address potential roles of PGC-1β in contributing to tamoxifen agonist activity in physiological systems, such as bone, liver, cardiovascular system, or uterus, or in pathophysiological situations, such as in tamoxifen resistance (i.e. development of tamoxifen agonism) in breast cancer.

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