Peroxisome Proliferator-activated Receptor Coactivator-1α (PGC-1α) Coactivates the Cardiac-enriched Nuclear Receptors Estrogen-related Receptor-α and -γ

IDENTIFICATION OF NOVEL LEUCINE-RICH INTERACTION MOTIF WITHIN PGC-1α*

Received for publication, June 25, 2002, and in revised form, August 8, 2002 Published, JBC Papers in Press, August 13, 2002, DOI 10.1074/jbc.M206324200

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The transcriptional coactivator PPARγ coactivator-1α (PGC-1α) has been characterized as a broad regulator of cellular energy metabolism. Although PGC-1α functions through many transcription factors, the PGC-1α partners identified to date are unlikely to account for all of its biologic actions. The orphan nuclear receptor estrogen-related receptor α (ERRα) was identified in a yeast two-hybrid screen of a cardiac cDNA library as a novel PGC-1α-binding protein. ERRα was implicated previously in regulating the gene encoding medium-chain acyl-CoA dehydrogenase (MCAD), which catalyzes the initial step in mitochondrial fatty acid oxidation. The cardiac perinatal expression pattern of ERRα paralleled that of PGC-1α and MCAD. Adenoviral-mediated ERRα overexpression in primary neonatal cardiac myocytes induced endogenous MCAD expression. Furthermore, PGC-1α enhanced the transactivation of reporter plasmids containing an estrogen response element or the MCAD gene promoter by ERRα and the related isoform ERRγ. In vitro binding experiments demonstrated that ERRα interacts with PGC-1α via its activation function-2 homology region. Mutagenesis studies revealed that the LXXLL motif at amino acid position 142–146 of PGC-1α (L2), necessary for PGC-1α interactions with other nuclear receptors, is not required for the PGC-1α-ERRα interaction. Rather, ERRα binds PGC-1α primarily through a Leu-rich motif at amino acids 209–213 (Leu-3) and utilizes additional LXXL-containing domains as accessory binding sites. Thus, the PGC-1α-ERRα interaction is distinct from that of other nuclear receptor PGC-1α partners, including PPARα, hepatocyte nuclear factor-4α, and estrogen receptor α. These results identify ERRα and ERRγ as novel PGC-1α interacting proteins, implicate ERR isoforms in the regulation of mitochondrial energy metabolism, and suggest a potential mechanism whereby PGC-1α selectively binds transcription factor partners.

Cellular energy production is tightly linked to metabolic demand, which is, in turn, dictated by diverse developmental, physiologic, and environmental conditions. The capacity for cellular ATP production is controlled, in part, by the expression levels of nuclear genes involved in mitochondrial oxidative metabolism. Thus, tight regulation of cellular energy metabolism necessitates transduction of diverse signals related to cellular energy demands to the nucleus. Although numerous factors involved in the transcriptional regulation of metabolic gene expression have been identified, the precise pathways involved in the physiologic control of cellular energy metabolism have not been delineated. The recent discovery of PPARγ coactivator-1α (PGC-1α), PGC-1β, and the PGC-1-related protein, a family of inducible transcriptional coactivators responsive to selective physiological stimuli, have provided new insights into the link between extracellular events and the regulation of genes involved in energy metabolism. PGC-1α, the first member of this novel coactivator family to be identified, was initially characterized as a key regulator of thermogenesis in brown adipose tissue (BAT) and skeletal muscle via its coactivation of the adipose-enriched nuclear receptor, PPARγ (1, 2). Subsequent studies have revealed a broader role for PGC-1α in a variety of cellular energy metabolic processes including mitochondrial biogenesis, mitochondrial fatty acid oxidation (FAO), and gluconeogenesis (2–6). The function of PGC-1β and PGC-1-related protein remain to be defined.

PGC-1α is unique from the p160 and p300/cAMP response element-binding protein-binding protein classes of transcriptional coactivators in its tissue-restricted expression pattern, its developmental regulation, and its inducibility by specific physiological stimuli. PGC-1α is enriched in tissues reliant on oxidative metabolism for ATP generation (heart, skeletal muscle) or heat (BAT) but is also expressed in liver, brain, and kidney (1). Immediately after birth, PGC-1α expression increases in heart coincident with a shift from reliance on glycolysis to mitochondrial FAO as the chief energy source in the adult myocardium (4). PGC-1α expression is induced in adult skeletal muscle, BAT, and heart in response to stimuli that increase energy demands. For example, cold exposure leads to

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* This work was supported by National Institutes of Health Grants R01 DK54416, RO1 HL58493, P30 DK56341, and P30 DK52574. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Supported by National Institutes of Health National Research Service Award F32 HL10410.

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This paper is available on line at http://www.jbc.org
a rapid induction of PGC-1α gene expression in BAT (1, 7). In addition, fasting and short-term exercise induces PGC-1α gene expression in heart and skeletal muscle, respectively (4, 8, 9).

Recent studies have shown that PGC-1α protein is phosphorylated in response to cytokine stimulation of the p38 mitogen-activated protein kinase pathway resulting in stabilization of the protein (10). Furthermore, we have shown that activation of the p38 pathway enhances ligand-dependent PGC-1α activation of PPARs (11). Finally, Knauf et al. (12) demonstrated p38-mediated activation of PGC-1α via release of a repressor.

The results of recent gain-of-function studies have demonstrated that PGC-1α serves as a global regulator of mitochondrial metabolic capacity. Spiegelman and co-workers (2) have shown that overexpression of PGC-1α in myogenic cells activates the mitochondrial biogenic program leading to an increase in mitochondrial number and respiration rates. Our laboratory has shown that forced expression of PGC-1α in primary cardiac myocytes and in hearts of transgenic mice leads to the transcriptional activation of genes encoding mitochondrial FAO enzymes, such as medium-chain acyl-CoA dehydrogenase (MCAD), and triggers a robust increase in mitochondrial cellular volume density (4). Collectively, these recent studies suggest that PGC-1α serves to transduce stimuli linked to physiologic demands to the transcriptional control of mitochondrial functional capacity.

The regulatory effects of PGC-1α are thought to be mediated primarily by its ability to interact with and coactivate numerous nuclear receptors, as well as non-nuclear receptor transcription factors, based on in vitro and cell culture studies (1–3, 6, 12–14). The effects of PGC-1α on mitochondrial FAO enzyme gene expression occur, at least in part, through its activation of the nuclear receptor PPARα (3, 4). The mitochondrial biogenesis response involves the transcription factors nuclear respiratory factor-1 and nuclear respiratory factor-2 (2). However, not all of the downstream effects of PGC-1α on cellular energy metabolism have been ascribed to particular PGC-1α transcription factor partners. The role of PGC-1α as a “master” regulator of cellular energy metabolism is likely mediated by multiple transcription factors, some of which could be novel. In addition, the mechanisms involved in partner selection by PGC-1α in the context of coexpressed transcription factors are also unknown.

In an attempt to identify PGC-1α interacting proteins relevant to the postnatal heart, we performed a two-hybrid screen of an adult human heart cDNA library in yeast using PGC-1α as bait. We focused on the adult mammalian heart because of its extraordinary capacity to match mitochondrial energy production with high demands. The orphan nuclear receptor, estrogen-related receptor α (ERRα; NR3B1), was identified as a novel PGC-1α interacting protein. PGC-1α enhanced the transcriptional activities of ERRα and the related isoform ERRγ. The PGC-1α/ERRα complex was shown to directly activate the MCAD promoter, a gene implicated previously as an ERRα target. Functional assays and in vitro binding studies demonstrated that ERRα binds PGC-1α via a novel set of leucine-rich domains compared with other characterized nuclear receptor PGC-1α partners. These results identify a novel PGC-1α target in heart. In addition, our results suggest that the utilization of distinct binding interfaces within PGC-1α provides one mechanism whereby this versatile coactivator may differentially activate multiple downstream effectors in diverse cellular and physiologic contexts.

**Experimental Procedures**

**Mammalian Cell Culture and Transient Transfections**

CV1, 293, and HepG2 cells were maintained at 37 °C and 5% CO2 in Dulbecco’s modified Eagle’s medium/10% fetal calf serum. For experiments requiring estradiol, HepG2 cells were plated the day before transfection in Earle’s minimum essential medium without phenol red/10% stripped fetal calf serum. Transient transfections were performed by the calcium phosphate coprecipitation method as described (15). Reporter plasmids (4 μg/ml) were cotransfected with Rous sarcoma virus-β-galactosidase (0.5 μg/ml), expressing the β-galactosidase gene driven by the Rous sarcoma virus promoter, to control for transfection efficiency. For cotransfection experiments, mammalian expression vectors (see below) for nuclear receptors, PGC-1α constructs, or the corresponding empty vectors were used. Ligands were added immediately following transfection protocol at the concentrations indicated in the figure legends, and cells were collected and assayed 48 h later. Luciferase and β-galactosidase activities were measured as described (16).

Ventricular cardiac myocytes were prepared from 1-day-old Harlan Sprague-Dawley rats as described (15). After 24 h cells were infected with adenovirus expressing GFP (Ad-GFP) or ERRα (Ad-ERRα) driven by a cytomegalovirus promoter. The latter construct also expresses GFP from an independent promoter. Infection rate of 90–95% was achieved by 18 h as assessed by quantitation of GFP-expressing cells using fluorescence microscopy. Whole cell protein extracts were prepared from cells 72 h post-infection. The adenosiviral construct, Ad-ERRα, was constructed by subcloning a Saull/Not fragment from the pBk-ERRα (generously provided by C. Teng) construct containing the full-length human ERRα cDNA encoding amino acids 1–422 into the pAd-Track-cytomegalovirus vector. Recombination and propagation of adenovirus expressing ERRα was performed as described (17).

**Plasmid Constructs**

**Reporter Plasmids**—The MCAD promoter-lucerase plasmids have been described (15, 18). The VitP36.Luc, containing two copies of the vitellogenin estrogen responsive element upstream of the prolactin minimal promoter, was generously provided by S. Adler (Southern Illinois University). The (UAS)3.TK.Luc was a gift from D. Moore (Baylor College of Medicine). The (AP0CIII)2.TK.Luc was constructed by ligation of annealed oligonucleotides (sense strand 5’-GATCCCTGATC-TCCACTGGTCAGCAGGTGACCTTTGCCCAGCGCCCTGGGA-3’) into the BamHI/Bg/III site upstream of the thymidine kinase promoter of the pGL2-TK.Luc reporter plasmid. The sequence is based on the HNF-4 response element contained in the human apolipoprotein CIII gene promoter (19).

**Mammalian Expression Plasmids**—The mammalian expression vector pCDNA3.1-Myc/His.PGC-1α has been described elsewhere (3). Site-directed mutations in PGC-1α were introduced by a PCR-based strategy (Quickchange Mutagenesis kit; Stratagene) using the Myc/His.PGC-1α as template for single-site mutants. The double- and triple-site mutations were made using the single- and double-mutant templates, respectively. The PGC-1α deletion series, PGC-330, PGC-260, and PGC-200, have been described (3). Additional FLAG-tagged PGC-1α deletion constructs were generated by PCR introducing an in-frame Bg/III at the start codon and a stop codon at 273, 213, 208, or 191. PCR fragments were then subcloned (Bg/III) into cytomegalovirus promoter-Tag1 (Stratagene) to fuse a cassette encoding a FLAG epitope to 5’ end of the PGC-1α sequence. The PGC-1α constructs were subsequently cloned into the NotI site of the pCDNA3.1 mammalian vector for cotransfection studies. The pCDNA3.1-FLAG-ERRα full-length and deletion constructs were generated by the same procedure described above for PGC-1α using PCR to introduce a stop codon at 403, 359, or 209. The Gal4-ERRα and Gal4-ERRα were generated by subcloning the BamHI fragment from the corresponding pCDNA3.1 construct into the pCMX-Gal4 plasmid (a kind gift from D. Moore). The Gal4-PPARα has been described (3). The pS55-hemagglutinin-ERRγ, the pBk-Rous sarcoma virus-ERRα, and the pCMX-HNF-4 expression vectors were kind gifts from M. Stallcup (University of Southern California), S. Adler, and J. Ladas (Harvard Medical School), respectively.

**Mammalian Cell Culture and Transient Transfections**

**Bacterial Expression Plasmids**—Construction of the pGex4T-3-PGC338 has been described elsewhere (3). The pGex4T-3-ERRα was generated by subcloning the BamHI fragment from the pCDNA3.1-ERRα into the pGex4T-3 vector (Amersham Biosciences).

**Northern Blot Analysis**

Total cellular RNA isolation and blotting was performed as described (16). Blots were hybridized with radiolabeled probes derived from the following cDNA mouse clones: MCAD, ERRγ, PPARα, and PGC-1α. In addition, human ERRα, rat M-CPT-I, and universal actin probes were used.

**Immunoblotting**

Protein extracts were resolved by SDS-PAGE (7.5%). Transfer and detection were performed as described (20). Immunodetection of ERRα...
FIG. 1. A yeast two-hybrid screen identifies ERRα as a PGC-1α interacting protein. A, top, schematic of PGC-1α protein showing its functional domains: transactivation domain (TAD), transcription factor binding domain, and the Arg/Ser-rich/RNA recognition motif (RS/ERM). L1, L2, and L3 indicate conserved LXXLL motifs. The transcription factor binding domain (aa 122–403) was used as “bait” in a yeast two-hybrid screen of a human adult cardiac cDNA library. Bottom, schematic of the hERRα protein, highlighting the conserved DBD and LBD. Two distinct populations of ERRα clones, both containing the LBD, were isolated in the screen. The numbers in parentheses indicated the number of clones isolated within each group. B, bacterially expressed GST-PGC338 (top) or GST-ERRα (middle) was used in GST pull down assays with 35S-labeled full-length ERRα or PGC-1α, respectively. Bottom, binding of the ERRα-isoform with PGC-1α was assessed using GST-PGC338 with 35S-labeled ERRγ. GST alone was used to control for nonspecific binding of 35S-labeled proteins. 20% of the radio-labeled protein used in the binding reactions (20% Input) was run in parallel lanes for comparison.

and COUP-TF were performed using polyclonal anti-ERRα or anti-COUP-TF antibodies generously provided by V. Giguere (McGill University) and M.-J. Tsai (Baylor College of Medicine), respectively. MCAD was detected using the anti-MCAD antibody described previously.

GST Pull Down Assays

In vitro protein-protein interaction assays have been described previously (3). 35S-Labeled proteins were synthesized in the TNT T7 quick-coupled in vitro transcription/translation system (Promega). In pull down reactions, 50 μl of a 50% slurry of GST fusion protein bound to glutathione-Sepharose was incubated with 10 μl of 35S-labeled protein in 500 μl of binding buffer (20 mM Tris, 7.5, 100 mM KCl, 0.1 mM EDTA, 0.05% Nonidet P-40, 10% glycerol, 1 mg/ml bovine serum albumin, 0.5 mM phenylmethylsulfonyl fluoride, and 1× Complete (Roche Molecular Biochemicals)) for 1 h at 4°C. The beads were pelleted and washed five times with cold binding buffer. SDS-PAGE reducing buffer was added to the beads, samples were boiled for 5 min, and the eluted proteins were analyzed by SDS-PAGE. The gels were fixed and dried, and band intensities were quantified by phosphorimage analysis using the Bio-Rad GS 525 molecular imaging system.

RESULTS

Identification of ERRα as a PGC-1α Interacting Partner—Using a yeast two-hybrid approach, a human adult cardiac cDNA library was screened to identify proteins that interacted with the region of PGC-1α encompassing amino acids (aa) 122–403. This region of the PGC-1α protein contains all of the domains known to mediate interactions between PGC-1α and transcription factors partners identified to date (21). In a screen of 4 × 107 transformants, ~40% of the clones encoded the orphan nuclear receptor, human ERRα. Importantly, two groups of unique clones encoding human ERRα were identified (Fig. 1A). Group 1 encodes aa 206–422 of ERRα, which includes the ligand binding domain (LBD). Group 2 encodes aa 121–422, which contains a portion of the DNA binding domain (DBD) and the hinge region, in addition to the LBD.

To demonstrate that PGC-1α interacts directly with ERRα, in vitro GST pull down assays were performed using a GST-ERRα fusion protein produced in bacteria and full-length 35S-PGC-1α generated by in vitro translation. Conversely, a PGC-1α-GST fusion construct, containing aa 1–338 (GST-PGC338), was incubated with 35S-ERRα. An interaction was observed between PGC-1α and ERRα in both configurations, whereas no significant binding occurred between the 35S-labeled proteins and GST alone, confirming the specificity of the interaction (Fig. 1B). Given the structural homology between ERRα and the related isoform, ERRγ, and the observation that both are cardiac-enriched (22–25), we also examined whether PGC-1α could bind ERRγ. Using GST-PGC338, a specific interaction between PGC-1α and ERRγ was demonstrated, thereby extending our findings to encompass multiple ERR isoforms (Fig. 1B).

These results demonstrate that PGC-1α physically interacts with ERRα and ERRγ and that the interacting region is contained within amino acids 1–338 of the PGC-1α molecule.

The Expression of ERRα Parallels That of PGC-1α and Several of Its Target Genes during Cardiac Development—The identification of ERRα as a PGC-1α interacting protein was intriguing for several reasons. First, we and others (22, 23) have identified a likely role for ERRα in the transcriptional regulation of the gene encoding MCAD, which catalyzes the initial step in the mitochondrial FAO spiral. PGC-1α coactivates genes involved in mitochondrial FAO and biogenesis (2–4). Second, the expression of ERRα is enriched in heart and BAT, a pattern similar to that of PGC-1α (23). As an initial step to determine whether the ERRα interaction with PGC-1α was of potential biological relevance, the expression patterns of each were compared in hearts from different developmental stages with varying degrees of mitochondrial oxidative capacity. Consistent with its role in the transcriptional regulation of mitochondrial oxidative metabolism, cardiac PGC-1α expression is induced following birth, during a period of intense mitochondrial biogenesis, coincident with the shift toward reliance on FAO as the chief source of energy (4). Therefore, the cardiac perinatal-to-adult expression profile of the ERRα gene was compared with that of PGC-1α. Northern analysis revealed that the ERRα transcript was in relatively low abundance before birth but displayed a postnatal day 1 spike followed by a gradual increase to its maximum expression levels in the adult mouse heart (Fig. 2A). This pattern paralleled that of PGC-1α, the putative ERRα target gene MCAD, and PPARα, a known regulator of postnatal cardiac energy metabolism (16, 18). Levels of ERRα protein correlated with mRNA abundance during the cardiac metabolic transition (Fig. 2B). In contrast, expression of the COUP-TF, a known antagonist of nuclear receptor signaling and MCAD expression (26, 27), was expressed in a reciprocal pattern, falling to low levels in the adult heart. These data demonstrate that the expression of ERRα parallels that of PGC-1α and downstream targets involved in mitochondrial energy production during perinatal cardiac development.

Forced Overexpression of ERRα Induces MCAD Gene Expression—Because ERRα and FAO enzyme genes appeared to be coordinately expressed in heart, we next wanted to determine whether ERRα could directly regulate the expression of the endogenous MCAD gene. For these studies, ERRα was overexpressed in cardiac myocytes using adenoviral constructs expressing either GFP alone (to monitor infection efficiency) or both GFP and ERRα from independent promoters. Analysis of whole cell extracts by immunoblotting revealed that MCAD expression was significantly induced in the ERRα-infected cells
PGC-1α Coactivates ERR Isoforms

Fig. 2. PGC-1α and ERRα are coordinately expressed with FAO enzyme genes, and ERRα induces endogenous MCAD expression. A, the results of Northern analyses performed with total RNA (15 μg) from hearts of mice at different stages of development. B, immunoblot analyses using α-ERRα and α-COUP-TF antibodies with nuclear protein extracts prepared from hearts of mice at the indicated developmental stages. C, autoradiograph representing immunoblot analyses of whole cell extracts (WCE) from primary neonatal cardiac myocytes that were either uninfected (C) or infected with adenovirus expression GFP alone (GFP) or GFP and ERRα (ERRα). The blot was hybridized sequentially with antibodies to ERRα or MCAD as indicated.

compared with GFP controls (Fig. 2C). No further increase in MCAD expression was observed upon coexpression of ERRα and PGC-1α (data not shown) likely because of the presence of endogenous ERRα coactivators in these cells. These results strongly suggest that the parallel expression pattern of ERRα with FAO enzyme genes reflects a biologically relevant role for ERRα in the regulation of mitochondrial fatty acid metabolism.

The PGC-1α-ERRα Complex Directly Activates Transcription through a Consensus ERE and through the NRRE-1 of the MCAD Gene Promoter—To determine whether PGC-1α can function as a coactivator of ERRα and ERRγ, transient transfections were first performed using the ERRα-responsive VitP36.Luc reporter construct, containing two copies of the estrogen receptor response element derived from the vitellogenin promoter. No effect on reporter activity was observed upon cotransfection with either PGC-1α or ERRα alone (Fig. 3A). However, a marked activation of VitP36.Luc activity (≥22-fold) was observed when ERRα and PGC-1α were coexpressed. PGC-1α also coactivated ERRγ albeit to a lesser magnitude (6-fold) (Fig. 3A).

Based on the results of previous studies and the data shown above, ERRα is predicted to regulate MCAD gene transcription in BAT and heart (22, 23). However, despite the observation that ERRα binds a complex NRRE-1 within the MCAD gene promoter, transient transfection studies in a variety of mammalian cell lines have failed to reveal a direct regulatory effect of ERRα on MCAD gene transcription. Indeed, studies of ERRα-mediated activation of most target genes identified to date have demonstrated relatively modest transactivation potency suggesting that an ERRα coactivator is absent or is expressed at low levels in the cell lines used to evaluate ERRα transactivating properties. Given that most mammalian cell lines are devoid of PGC-1α, we hypothesized that PGC-1α might be a relevant coactivator for the ERRα-mediated regulation of MCAD gene transcription. Accordingly, the regulatory effects of ERRα on the MCAD promoter were evaluated in the absence or presence of overexpressed PGC-1α (Fig. 3B). For these experiments, a luciferase reporter construct driven by a segment of the human MCAD gene promoter containing the ERRα binding site, NRRE-1, was used (376.MCAD.Luc). As expected, 376.MCAD.Luc activity was not affected by either factor alone. However, overexpression of both ERRα and PGC-1α induced a synergistic activation (≥8-fold) of −376.MCAD.Luc (Fig. 3B). When the same experiment was performed with an MCAD gene promoter-reporter construct containing an inactivated NRRE-1 (NRRENmut.MCAD.Luc) (15), the PGC-1α-ERRα-mediated activation was abolished (data not shown). Collectively, these results demonstrate that PGC-1α coactivates ERRα and ERRγ and that the PGC-1α-ERRα complex transcriptionally regulates at least one gene target involved in cardiac mitochondrial energy metabolism.

The PGC-1α-ERRα Interaction Maps to the AF-2 Region of ERRα—Generally, nuclear receptor-coactivator interactions involve domains within the receptor LBD. Indeed, the smallest ERRα clone isolated in our yeast two-hybrid screen contained only the LBD (Fig. 1A, Group 1). Therefore, to evaluate the role of the LBD in the PGC-1α-ERRα interaction, a series of ERRα C-terminal deletion mutants were generated and evaluated using GST pull downs and transient transfection assays. As shown above, full-length ERRα bound strongly with GST-PGC-388. In contrast, deletion of the C-terminal 19 aa, containing the consensus AF-2 domain, resulted in an ~80% reduction in binding activity (Fig. 4A). No further decrease was observed when subsequent deletions were made to residue 209, removing all but the N-terminal 12 aa of the LBD. Considering the clones isolated in the yeast two-hybrid library screen (Fig. 1A), the 209–422 region of the LBD is likely sufficient to interact with ERRα (Group 1 clone). The small degree of residual binding observed in the ERR209 mutant suggests that a low affinity binding region exists within residues 1–209 of the ERRα protein, possibly in the hinge region, which was contained in half of the clones isolated in the library screen.

Given that deletion of the AF-2 region in ERR209 had the most profound influence on binding, the effect of an AF-2 deletion on PGC-1α-mediated ERRα coactivation was assessed using a Gal4 system in which the entire ERRα protein was fused to the yeast Gal4 DNA binding domain (Gal4-DBD) (Fig. 4B). Using this system, PGC-1α-ERRα interactions are assessed in mammalian cells without potential background from endogenous PGC-1α interacting partners. Compared with the Gal4-DBD control, Gal4-ERRα repressed activity of the (UAS)₅TK.Luc reporter by 65%. Addition of PGC-1α coactivated Gal4-ERRα modestly (3-fold), essentially raising activity back to control levels. Interestingly, deletion of the AF-2 region
PGC-1α Coactivates ERR Isoforms

Fig. 3. ERRα and ERRγ are coactivated by PGC-1α; the PGC-1αERRα complex activates the MCAD gene promoter. A, transient transfections using the heterologous promoter-reporter VitP36.Luc were performed in 293 cells. The reporter construct (4 µg) was cotransfected with 0.5 µg of empty pcDNA3.1 expression vector (−), ERRα (left), or ERRγ (right) in the absence or presence of pcDNA3.1-PGC-1α expression vector (0.5 µg), as indicated. B, transient transfections were performed in NIH 3T3 cells using the homologous MCAD promoter construct, 376.MCAD.Luc, containing the ERRα binding site (NRRE-1). Transfections were performed as described in A. The β-galactosidase expression vector, Rous sarcoma virus promoter-β-galactosidase, was cotransfected (0.5 µg) in all conditions to control for transfection efficiency. Bars represent mean (± S.E.) β-galactosidase-corrected RLU, normalized (= 1.0) to the activity of the reporter cotransfected with pcDNA3.1 (−). Data represent three independent trials performed in triplicate. The asterisks indicate the mean values are significantly different (p < 0.05) from the vector control values.

resulted in a significant increase in baseline activity 2.5-fold above the control (Gal4-DBD) levels. No further increase in activity was observed upon cotransfection of PGC-1α. These data support the GST pull down results localizing the major PGC-1α interaction domain to the consensus AF-2 region of ERRα. The results also suggest that the coactivating effects of PGC-1α on ERRα in this assay system involves, at least in part, displacement of a repressor bound to the AF-2 region of the ERRα molecule.

The ERRα Interacting Domain Maps to a Region of the PGC-1α Protein Containing an Inverted LXXLL Motif—Next, in vitro mapping experiments were performed to identify the PGC-1α domain essential for its interaction with ERRα. A number of C-terminal truncated PGC-1α mutants were analyzed for their ability to interact with GST-ERRα (Fig. 5A). No decrease in binding affinity was observed with either PGC338 or PGC285 compared with the full-length protein. However, a significant drop was observed when the region of PGC-1α encompassing aa 191–285 was removed. This region contains L3, one of three consensus LXXLL motifs (L1, L2, L3; see Fig. 1A and Fig. 5A) within the PGC-1α protein (12). The LXXLL motifs of coactivator proteins are predicted to be embedded within short α-helical stretches. Typically one or more LXXLL α-helical domains within a coactivator protein are involved in AF-2-dependent interactions with nuclear receptors. The LXXLL motifs of PGC-1α are contained within the region encompassing aa 86–213 of the protein (Fig. 1A). To date, only L2 has been shown to be necessary for PGC-1α/nuclear receptor interactions (3, 6, 12–14, 28). Surprisingly, deletion of the region encompassing aa 191–285 of PGC-1α, which contains L3 (aa 209–213), resulted in a dramatic loss of ERRα binding (Fig. 5A). Deletion of PGC-1α amino acids 191–120, which contains L2, abolished the residual low affinity interaction.

The mutant PGC-1α proteins were evaluated in functional assays using Gal4-ERRα as a target in transient transfections. As shown in Fig. 5B, the relative activity of each PGC-1α truncation mutant paralleled its binding efficiency. Specifically, full-length PGC-1α displayed 4-fold activation, whereas the PGC238 and PGC285 mutants activated Gal4-ERRα to a greater degree than wild-type PGC-1α. This enhanced activity of truncated PGC-1α proteins has been described previously (1, 3) and is thought to be because of removal of an autoinhibitory domain located in the C-terminus of PGC-1α. As predicted by the binding studies, subsequent removal of the 285 to 191
PGC-1α Coactivates ERR Isoforms

**Fig. 4.** PGC-1α interacts with ERRα through the consensus AF-2 domain. **A**, top, schematic of ERRα C-terminal truncation mutants to used to localize the PGC-1α interacting domain within the LBD. All constructs begin with aa 1; mutant designations indicate their C-terminal ends. Bottom, GST pull downs were performed as described in Fig. 1B using GST alone or GST-PGC338 with the radiolabeled mutant proteins. wt, wild-type. **B**, transient cotransfections using the (UAS)₃.TK.Luc heterologous reporter (4 μg) were performed in CV1 cells with 0.5 μg of either the Gal4-ERRα or Gal4-ERRα₄₀₃ fusion construct. Empty vector (−) or PGC-1α expression vector (+) were cotransfected with Gal4 constructs, as indicated. Bars represent mean (± S.E.) β-galactosidase-corrected RLU, normalized (wt = 1.0) to the activity of the reporter cotransfected with Gal4-DBD. The Gal4-DBD control was unaffected by PGC-1α cotransfection (data not shown). Data represent three trials performed in triplicate. The asterisk indicates the mean values are significantly different (p < 0.05) from the Gal4-ERRα value. n.s., not significant.

The functional mapping and binding studies suggested that the region of PGC-1α containing L3 was necessary for its interaction with ERRα. These findings were surprising, because the interaction between PGC-1α and other nuclear receptors characterized to date have mapped to the L2 motif at aa 142–146 (Fig. 5C). A second point of interest is that two p38 mitogen-activated protein kinase (p38-MAPK) phosphorylation sites are located between residues 285 and 191 at Thr-262 and Ser-265 of PGC-1α (see Fig. 5C and Refs. 10 and 12). Therefore, the activities of additional PGC-1α truncation mutants were analyzed to determine whether the p38-MAPK sites or L3 influenced coactivation ERRα by PGC-1α. As observed with PGC285, the PGC273 and PGC213 mutants displayed strong activation of Gal4-ERRα (Fig. 5C). This suggests that the phosphorylation sites at 262/265 are not essential for the functional interaction of PGC-1α with ERRα. Further removal of the five residues corresponding to the L3 motif, an inverted LXXLL, resulted in nearly a complete loss of PGC-1α activity with ERRα (Fig. 5C). Parallel analyses of these mutant PGC-1α constructs performed with the nuclear receptors, PPARα and ERα, which have been shown to interact with PGC-1α via L2, demonstrated that all of the mutants were fully active with these receptors (data not shown). The binding activity of the PGC-1α mutants with GST-ERRα paralleled their activity in the functional assays, displaying a significant reduction in binding affinity for ERRα upon deletion of the L3 motif (Fig. 5D). Collectively, these results indicate that PGC-1α-mediated coactivation of ERRα involves L3 but not the p38-MAPK phosphorylation sites. The results identify a unique nuclear receptor interaction site within the PGC-1α molecule.

The PGC-1α-ERRα Interaction Interface Is Distinct from That of Other Nuclear Receptor PGC-1α Partners—The results shown above suggest that the PGC-1α-ERRα interaction involves the L3 site, a novel PGC-1α/nuclear receptor interface. However, a cooperative role for the other LXXLL motifs was not excluded in the above analysis. Furthermore, it is important to evaluate the role of the L3 site in the context of the full-length PGC-1α protein. Accordingly, the leucine residues in L1, L2, and L3 were substituted with phenylalanines in the full-length PGC-1α protein (Fig. 6A). The binding and functional activities of these PGC-1α mutants were assessed with ERRα (Fig. 6). As predicted, the single L1 (mL1) and L2 (mL2) PGC-1α mutants bound GST-ERRα with the same affinity as wild-type PGC-1α and were fully active with ERRα on the Vit2P36.Luc reporter in 293 cells (Fig. 6). Unexpectedly, ERRα coactivation by the PGC-1α mutant harboring substitutions in L3 remained intact (Fig. 6B). However, disruption of L3, in combination with either of the other two sites, markedly re-
duced the ability of PGC-1α to bind and coactivate ERRα. These data suggest that although the L3 motif is sufficient to mediate the functional interaction of PGC-1α with ERRα (PGCmL1/2 mutant displays full activity) and is likely the primary binding site for ERRα, L3 is not absolutely required when multiple alternate LXXLL motifs are available on PGC-1α. Hence, the alternate sites (L1 and L2) may serve a low affinity accessory function in the PGC-1α interaction with ERRα.

The results shown above suggest that the binding of certain nuclear receptors with PGC-1α involves distinct combinations of Leu-rich (LXXLL) interacting motifs. To explore this possibility further, the LXXLL motif mutants were also tested for their ability to coactivate ERRγ, ERRα, and HNF-4α. Interestingly, the pattern of ERRγ coactivation by the PGC-1α mutants was somewhat different from that of ERRα (Fig. 6B). Whereas ERRα appeared to primarily interact with the L3 motif of PGC-1α, ERRγ displayed a dependence on both L2 and L3. Only when both sites were simultaneously eliminated was PGC-1α activation of ERRγ abolished indicating that ERRγ binds either L2 or L3 interchangeably. ERRα and HNF-4α are PGC-1α targets reported previously to interact with PGC-1α via the L2 motif. Consistent with their L2 dependence, disruption of the L2 motif alone or in any combination with the other sites completely abrogated PGC-1α enhancement of receptor-mediated activation (Fig. 6B). Importantly, the mL1/3 mutant, which was inactive with ERRα, displayed full activity with the L2 binding class of receptors. Collectively, these data confirm that ERRα and ERRγ define distinct classes among nuclear receptor PGC-1α partners with regard to binding site specificity.

**DISCUSSION**

The data presented here provide several lines of evidence that the PGC-1α-ERRα interaction represents a functional transcriptional complex involved in the regulation of cardiac and skeletal muscle metabolism. First, multiple independent ERRα clones were isolated as PGC-1α interacting proteins using a two-hybrid screen. The interaction of PGC-1α with ERRα and -γ isoforms was verified using in vitro binding assays. Specific binding domains were mapped within the PGC-1α-ERRα binding interface on both ERRα and PGC-1α, thereby indicating the specificity of the interaction. Interestingly, the ERRα binding domain within the PGC-1α molecule is unique compared with other transcription factor binding sites. Second, PGC-1α coactivated both ERRα and ERRγ isoforms in heterologous promoter-reporter assays. Third, involvement of PGC-1α-ERRα in regulating cellular metabolism is suggested by parallel tissue-specific and developmental expression profiles of ERR isoforms, PGC-1α, and mitochondrial FAO enzyme.

**Fig. 5.** ERRα interaction with PGC-1α involves the Leu-rich L3 motif. A, GST pull down assays were performed to localize the ERRα interacting domain within the PGC-1α protein. All PGC-1α truncation mutants begin with aa 1, and mutant designations indicate their C-terminal ends. Binding assays were performed as described in the legend for Fig. 1B using GST-ERRα with the various 35S-labeled PGC-1α proteins. B, transient cotransfections were performed with the PGC-1α mutants and Gal4-ERRα in CV1 cells as described in the legend for Fig. 4B. Activities of Gal4-ERRα cotransfected with the various PGC-1α expression vectors were normalized (= 1.0) to the activity of Gal4-ERRα alone (= 1.0) to the activity of Gal4-ERRα alone (= 1.0) to the activity of Gal4-ERRα alone (= 1.0) to the activity of Gal4-ERRα alone (= 1.0) to the activity of Gal4-ERRα alone (= 1.0) to the activity of Gal4-ERRα alone (= 1.0). C, top, schematic of PGC-1α protein showing the location and sequence of the Leu-rich motifs. The MAPK phosphorylation sites at Thr-262 and Ser-265 are indicated. Bottom, additional PGC-1α truncation mutants were constructed to analyze the ERRα functional interaction within the aa 191-285 region; mutant designations indicate the C terminus. Transient cotransfections to measure activities of the 285 to 191 PGC-1α mutants were performed as described in B. Bars represent mean (± S.E.) β-galactosidase-corrected RLU normalized (= 1.0) to the activity of the reporter cotransfected with Gal4-ERRα. Data represent three trials performed in triplicate. Asterisks indicate the mean values are significantly different (p < 0.05) from the Gal4-ERRα control value. D, GST pull downs were performed with the PGC-1α aa 285 to 191 C-terminal truncation mutants as in A.
Mapping studies of the PGC-1α-ERRα binding interface revealed a novel LXXLL-type nuclear receptor binding motif on PGC-1α. Conserved LXXLL motifs within coactivator proteins have been shown to mediate AF-2 domain-dependent interactions with nuclear receptors. The Leu-rich motif adopts an α-helical conformation that fits into a hydrophobic binding pocket formed by several helices of the receptor LBD. PGC-1α contains three potential LXXLL motifs (L1–L3) although only one (L2) has been shown previously to play a major role in binding nuclear receptors. Our mutagenesis studies demonstrated that the interaction of ERRα with PGC-1α involves the L3 motif. Identification of an LXXLL motif as the ERRα binding site is consistent with the observation that the ERRα AF-2 domain is required for the interaction with PGC-1α. However, this is the first example in which the L3 motif of PGC-1α was found to mediate the interaction of PGC-1α with a nuclear receptor beyond a role as an accessory site for GR interaction with PGC-1α.

Although LXXLL defines the consensus signature motif through which coactivator proteins interact with nuclear receptors, numerous studies have established the importance of residues flanking the Leu-rich sequence in determining receptor selectivity and binding affinity (29, 30). Chang et al. (29) recently defined three distinct classes of LXXLL domains based upon conserved flanking residues. According to this scheme, the PGC-1α L2 site is a class III binding site determined in part by the presence of Leu and Ser residues positioned immediately upstream of the first Leu of the LXXLL motif. The L3 motif, through which ERRα primarily interacts, appears to be an inverted LXXLL and, therefore, does not readily conform to this classification scheme. However, recent crystal structure studies performed with ERRα demonstrated that the coactivator binding pocket of ERRα could recognize an LXX̅ωL motif within an NR box derived from the transcription intermediary factor 2 coactivator (31). Interestingly, despite the presence of a consensus LXXLL motif in the transcription intermediary factor 2 NR box, constraints placed by basic residues N-terminal to the α-helical motif shifted the binding site by one residue changing the recognition motif to LXXYL. The L3 motif of PGC-1α does, in fact, conform to an LXXYL consensus. Furthermore, a series of basic residues lie upstream of the L3 LXXYL motif that may contribute to its recognition by ERRα and ERRγ. Finally, consistent with these findings, the L1 motif (L[LavV]) does not match either of these consensus sequences and has not been identified as a high affinity binding site in any nuclear receptor AF-2-dependent interaction with PGC-1α (12) (present study).

The basis for the unique interaction of ERRα with PGC-1α compared with other L2-dependent PGC-1α nuclear receptor partners is unknown but is presumably related to structural differences within the nuclear receptor LBDs. The nuclear receptor interface with PGC-1α has not been precisely defined. However, p160 coactivator binding sites have been mapped for a number of receptors, including ERRα, PPARγ, and retinoid X receptor α (31–33). ERRα interacts with NR boxes within transcription intermediary factor 2 via direct contacts between the LXXLL α-helix and ERRα residues Gln-542, which resides within LBD helix 12, and Lys-362, which resides within helix 3.
Both of these residues are conserved in ERR isoforms, suggesting that other structural differences within the LBD of ERRα and ERRγ account for their differential binding with PGC-1α. Modeling of the ERRα LBD based on homology with ERα reveals that 16 of 19 residues involved in ligand binding to ERα are either identical or conservative mismatches in ERRα (34). Of the three distinct residues, Phe-329 was shown to be essential for the constitutively active conformation adopted by the LBD of ERRα (34). Interestingly, the same residues are also distinct between ERRα and ERRγ. These findings suggest that, although these residues are not directly involved with coactivator binding, the amino acid differences may contribute to slight differences in LBD conformation among these receptors to influence the binding interface with PGC-1α.

Modeling of the ERRα LBD of ERRα and ERRγ share some common targets, and ERRα have focused on the potential for ERRα-mediated regulation of MCAD gene transcription. The ability of PGC-1α to utilize distinct binding sites with different partners provides an additional basis for receptor selection. According to this model, a post-translational event, such as phosphorylation, could influence the accessibility of specific nuclear receptor binding sites within the PGC-1α molecule. Hence, when the L2 site is inaccessible, the branch of the PGC-1α regulatory network mediated by L2-dependent receptors would be inactive whereas PGC-1α is recruited to alternate partners, such as ERRα. Such a mechanism would allow dynamic control of PGC-1α-mediated activation of related metabolic gene targets downstream of its numerous partners.

The ERR isoforms (α, β, and γ) comprise a subfamily of nuclear receptor transcription factors involved in transcriptional activation or repression of target genes (40, 41). Although ERRα was the first orphan nuclear receptor described within the mammalian nuclear receptor superfamily, little is known about its biological function. Some proposed models have focused on the potential for ERRα modulation of estrogen signaling. ERRα and ERRγ share some common targets, and ERRα has been shown to modulate ERRα-mediated transcriptional responses (42–44). ERRα has also been implicated in the regulation of cellular differentiation and metabolism (22, 23).

Regarding the latter, several lines of evidence suggest that ERRα serves to regulate PGC-1α target genes involved in mitochondrial metabolism. First, ERRα and ERRγ expression is enriched in tissues that utilize mitochondrial FAO as a primary source for energy or heat generation, such as heart and BAT. We also found that ERRα and γ expression parallels FAO capacity among skeletal muscle types.2 ERR isoforms and PGC-1α isoforms are highly expressed in rodent skeletal muscles comprised of slow-twitch oxidative fibers compared with fast-twitch fiber types in which these factors are nearly undetectable. Second, the coordinated developmental pattern of ERRα and PGC-1α expression suggests a role in metabolic matura-

Acknowledgments—Special thanks to Mary Wingate for assistance with preparation of the manuscript.

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PGC-1α Coactivates ERR Isoforms

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