Identification of a Specific Molecular Repressor of the Peroxisome Proliferator-activated Receptor γ Coactivator-1 α (PGC-1α)*

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The nuclear co-activator PGC-1α is a pivotal regulator of numerous pathways controlling both metabolism and overall energy homeostasis. Inappropriate increases in PGC-1α activity have been linked to a number of pathological conditions including heart failure and diabetes mellitus. Previous studies (Puigserver, P., Adelman, G., Wu, Z., Fan, M., Xu, J., O’Malley, B., and Spiegelman, B. M. (1999) Science 286, 1368–1371) have demonstrated an inhibitory domain within PGC-1α that limits transcriptional activity. Using this inhibitory domain in a yeast two-hybrid screen, we demonstrate that PGC-1α directly associates with the orphan nuclear receptor estrogen-related receptor-α (ERR-α). The binding of ERR-α to PGC-1α requires the C-terminal AF2 domain of ERR-α. PGC-1α and ERR-α have a similar pattern of expression in human tissues with both being present predominantly in organs with high metabolic needs such as skeletal muscle and kidney. Similarly, we show that in mice physiological stimuli such as fasting coordinately induces PGC-1α and ERR-α transcription. We also demonstrate that under normal conditions PGC-1α is located within discrete nuclear speckles, whereas the expression of ERR-α results in PGC-1α redistributing uniformly throughout the nucleoplasm. Finally, we show that the expression of ERR-α can dramatically and specifically repress PGC-1α transcriptional activity. These results suggest a novel mechanism of transcriptional control wherein ERR-α can function as a specific molecular repressor of PGC-1α activity. In conclusion, our results suggest that other co-activators might also have specific repressors, thereby identifying another layer of combinatorial complexity in transcriptional regulation.

Co-activators represent an important class of molecules that can regulate transcription although they are unable to directly bind to DNA. In general, co-activators are thought to modulate gene expression through specific protein-protein interactions with classic transcription factors that possess DNA-binding domains. Similarly, another class of molecules called co-repressors can interact with transcription factors and subsequently inhibit downstream activation of gene expression. Although regulation of co-activator or co-repressor activity is incompletely understood, a number of recent reports have suggested that co-activators can be post-translationally modulated by targeted ubiquination as well as by various intracellular signaling pathways (1, 2). Given that a single co-activator can potentially interact with a number of different transcription factors, such regulation will undoubtedly be important in ultimately specifying the specificity of transcriptional regulation.

The nuclear co-activator PGC-1α was initially identified as a PPAR-γ co-activator (3) but has since been recognized to function in cooperation with a number of other members of the nuclear receptor family including the glucocorticoid receptor, the thyroid hormone receptor, the mineralocorticoid receptor, and the estrogen receptor (4–7). Binding of PGC-1α to these nuclear receptors requires a leucine-rich protein interaction domain, termed an LXXLL motif, which is found adjacent to the N-terminal activation domain of the protein (5, 6, 8, 9). In the presence of DNA and transcriptional binding partners, a recent study (10) has demonstrated that PGC-1α appears capable of further recruiting other transcriptional regulators such as CBP, SRC-1, and the RNA II polymerase machinery. This study also demonstrated an inhibitory domain within PGC-1α that under normal circumstances appears to repress transcriptional activity of the protein. This inhibitory domain was revealed in part by the observation that an N-terminal 400-amino acid fragment of PGC-1α fused to a heterologous DNA-binding domain was significantly weaker at stimulating transcription than a construct containing only the N-terminal 200 amino acid activation domain (10). Interaction with a nuclear receptor partner and DNA was demonstrated to activate PGC-1α transcriptional activity presumably due to a conformational change in the inhibitory domain of the molecule.

Activation of PGC-1α appears to be increasingly important in a number of critical biological processes including cellular respiration and adaptive thermogenesis (11). Experiments in white fat, skeletal muscle, fibroblasts, and heart have demonstrated that overexpression of PGC-1α is sufficient to induce mitochondrial biogenesis (9, 12–15). Consistent with these observations, PGC-1α activation can dramatically alter cellular energy homeostasis and has been noted to regulate a host of intracellular enzymes involved in oxidative-phosphorylation, glucose metabolism, and mitochondrial energetics (11, 16). Consistent with a central role in metabolism, physiological stimuli such as cold, fasting, or exercise have been demonstrated to regulate the intracellular levels of PGC-1α (9, 10, 13, 17). Given the pleotropic effects of PGC-1α, it is not surprising that there are significant pathological effects when the co-activator is not properly regulated. For instance, continuous
expression of PGC-1α in the myocardium results in a dilated cardiomyopathy (13). Similarly, activation of PGC-1α in the liver has been linked to hepatic gluconeogenesis (9, 18), a metabolic response to starvation that is inappropriately stimulated in patients with diabetes.

Fully understanding the regulation of PGC-1α activity will undoubtedly provide significant insight into cellular energy homeostatic control. In addition, PGC-1α would appear to be an attractive therapeutic target for a number of conditions including heart failure and diabetes that have an underlying metabolic component. Recent evidence suggests that in skeletal muscle, cytotoxic activation of the p38/MAPK pathway results in phosphorylation of PGC-1α with a concomitant increase in the half-life and hence activity of the protein (19). Similarly, in cardiac myocytes p38/MAPK activation has been demonstrated to potentiate PGC-1α-mediated co-activation (20). Interestingly, another report based on genetic evidence postulated that a specific repressor of PGC-1α might exist (8). In general, specific molecular repressors of co-activators have not been described. Nonetheless, this postulated repressor appeared to bind to a potential leucine-rich motif contained within the inhibitory domain of PGC-1α, which is distinct from the LXXL motif that a variety of nuclear receptors interact with. The interaction of PGC-1α with this unidentified repressor has been postulated to be regulated in part by the p38/MAPK pathway (8, 21). Consistent with these studies, we report here the identification of a specific PGC-1α repressor and demonstrate that this molecule is the orphan nuclear receptor ERR-α.

EXPERIMENTAL PROCEDURES

Protein-Protein Interaction— Yeast two-hybrid analysis was performed using the Matchmaker Gal4-Two-Hybrid System 3 along with a human heart pre-transformed Matchmaker library (Clontech). Screening was performed according to manufacturer’s recommendations. The region of human PGC-1α used as bait corresponded to amino acids 199–406. To confirm interaction of PGC-1α and ERR-α in mammalian cells, HeLa cells were harvested 24 h after transfection in lysis buffer (20 mM HEPES-KOH, pH 7.9, 125 mM NaCl, 0.1% Nonidet P-40, 1 mM EDTA, 10 mM Na-pyrophosphate, 5 mM NaF, 5 mM β-glycerolphosphate, 0.2 mM sodium pyrophosphate, and protease inhibitor mixture) followed by vigorous vortexing, one freeze-thaw cycle, and then centrifugation at 14,000 rpm for 10 min. For co-immunoprecipitation experiments, 1 mg of protein lysate was used and immunoprecipitated for 3 h with 5.0 μl of Anti-FLAG M2 monoclonal antibody (Sigma). Immunoprecipitated samples were washed three times in ice cold phosphate-buffered saline, re-suspended in SDS-sample loading buffer, and analyzed by Western blot analysis using enhanced chemiluminescence. Plasmids and Cells—Epitope-tagged full-length human PGC-1α and ERR-α were respectively prepared by PCR amplification from a human heart and skeletal muscle cDNA library (Clontech) followed by in-frame subcloning into a Myc- (Invitrogen) or FLAG-tagged (Stratagene) expression vector. Similarly, GFP-PGC-1α was created by an in-frame fusion of full-length PGC-1α (amino acids 1–794) with green fluorescent protein. Transcription mutants of ERR-α were created by PCR amplification using full-length ERR-α as a template. All constructs were confirmed by direct nucleotide sequencing. GAL4-PGC-1α containing full-length PGC-1α or GAL4-PGC-120 containing only the first 120 amino acid N-terminal activation domain have been previously described (5) and were a kind gift of Daniel Kelly (Washington University, St. Louis, MO). Similarly, the constitutively active Mek3b and the wild type Mek6, both upstream activators of the p38/MAPK pathway, were gifts of Silvio Gutkind (National Institutes of Health). The phophoenolpyruvate carboxykinase promoter luciferase construct (p1PEPCK) consisting of the upstream elements (−467) of the phosphoenolpyruvate carboxykinase promoter has been previously described (9) and was a kind gift of D. Granger (Vanderbilt University, Nashville, TN).

HeLa cells and human hepatocellular carcinoma cells (HepG2) were obtained from ATCC (Rockville, MD) and maintained in Dulbecco’s Modified Eagle Medium supplemented with antibiotics and 10% fetal calf serum. In general, cells were plated 24 h prior to transient transfection using LipofectAMINE 2000 (Invitrogen) according to the manufacturer’s recommendations.

RESULTS

To gain further insight into the molecular regulation of PGC-1α we performed a yeast two-hybrid screen using a region of PGC-1α (amino acids 199–406) that overlaps with the previously described inhibitory domain of the molecule. This region of PGC-1α lacks a classic LXXL motif (amino acids 143–148) previously implicated in binding to a number of nuclear receptors (5–9) but does contain a leucine-rich motif (amino acids 209–213) that is required to bind the putative PGC-1α repressor (8, 21). We identified a number of potential interacting partners including the cytoskeletal proteins filamin C, tropomyosin, and titin. The most abundant partner was, however, the nuclear orphan receptor ERR-α. Interestingly, ERR-α has been previously implicated in transcriptional regulation of genes involved in energy metabolism (22, 23). Very recently, using a similar strategy as we have described, another report has also demonstrated an interaction between ERR-α and PGC-1α (24). This study demonstrated that this interaction required the leucine-rich motif (amino acids 209–213) of PGC-1α and the AF2 domain of ERR-α. We have come to similar conclusions (data not shown), and as noted in Fig. 1, in vivo, full-length ERR-α co-immunoprecipitated with PGC-1α.
There was a very weak interaction noted between PGC-1\textalpha\ and the AF2-deleted construct of ERR\textalpha\ that lacked the terminal 15 amino acids, although this binding was substantially less than with the full-length protein. Thus, as recently described (24), high affinity binding to PGC-1\textalpha\ requires the AF2 domain of ERR\textalpha. If ERR\textalpha\ is a physiological regulator of PGC-1\textalpha\ we reasoned that the tissue distribution of the two molecules should be similar. As noted in Fig. 2A, in human tissues, PGC-1\textalpha\ was most abundant in skeletal muscle and kidney, two tissue with high metabolic needs. Interestingly, ERR\textalpha\ was similarly highly expressed in these two tissues. We next sought to understand if the physiological stimuli that regulate PGC-1\textalpha\ also affect ERR\textalpha. It has been recently described that hepatic PGC-1\textalpha\ is strongly induced by starvation (9). We therefore took either \textit{ad libitum} fed mice or subjected mice to 24 h of starvation and compared the levels of hepatic PGC-1\textalpha\ and ERR\textalpha. As noted in Fig. 2B, \textit{inset}, similar to what has been previously reported for PGC-1\textalpha, levels of ERR\textalpha\ rose significantly in fasting animals. In a group of six animals, levels of PGC-1\textalpha\ and ERR\textalpha\ both rose \textasciitilde10-fold with starvation (ERR\textalpha/\beta-actin, control = 0.39 \pm 0.16 to fasting = 3.35 \pm 0.68; PGC-1\textalpha/\beta-actin control = 0.63 \pm 0.08 to fasting = 9.97 \pm 1.69; arbitrary fluorescent units, \(n = 6\) mean \pm S.E.).

Given that ERR\textalpha\ and PGC-1\textalpha\ interact and appear to be coordinately expressed and regulated, we next sought to further understand the biological effects of this interaction. Expression of full-length PGC-1\textalpha\ fused to green fluorescent protein (GFP-PGC-1\textalpha) demonstrated that PGC-1\textalpha\ was predominantly expressed in nuclear speckles (Fig. 3A). This result is consistent with previous studies (25) suggesting that PGC-1\textalpha\ also participates in mRNA processing, because a number of splicing factors have been demonstrated to co-localize with PGC-1\textalpha within these nuclear speckles. Interestingly, expression of ERR\textalpha\ results in a significant alteration in PGC-1\textalpha\ distribution so that the protein was now seen to be widely and evenly distributed within the nucleoplasm (Fig. 3B). As seen in Fig. 3C, consistent with the observation that the p38/MAPK pathway regulates the half-life of PGC-1\textalpha\ (19), expression of a constitutively active form of MEK3b resulted in a slight overall increase in GFP-PGC-1\textalpha\ intensity. Nonetheless, activation of
the p38/MAPK pathway did not reverse the effects that ERR-α expression had on PGC-1α sub-cellular distribution.

We next sought to understand the effects of ERR-α on PGC-1α-mediated co-activation. As noted in Fig. 4A, full-length PGC-1α fused to a heterologous DNA-binding domain (GAL4-PGC-1α) was able to activate transcription of a reporter construct. Expression of a truncated form of ERR-α lacking the AF2 domain and therefore unable to interact with PGC-1α had a slight stimulatory effect on GAL4-PGC-1α activity. The basis for this stimulatory effect is unknown. In contrast, as seen in Fig. 4A, expression of full-length ERR-α significantly repressed PGC-1α activity in this one-hybrid assay. Consistent with previous studies (5, 10), a truncated form of PGC-1α containing only the N-terminal activation was a more potent activator of transcription than the full-length PGC-1α construct. In contrast to the dramatic effects of ERR-α on full-length PGC-1α, ERR-α expression had only a small effect on this truncated PGC-1α construct that lacked the inhibitory domain of the molecule (Fig. 4B). A similar 10–20% decrease in activity was also seen when ERR-α was co-expressed with a GAL4-VP16 construct containing the potent viral activation domain (Fig. 4C).

We next assessed the effects of activating the p38/MAPK pathway on ERR-α repression of PGC-1α. As noted in Fig. 4D, transfection of a constitutively active MEK3b or overexpression of wild type MEK6 did not substantially alter or relieve the ERR-α-mediated repression of PGC-1α. Finally, we analyzed the effects of ERR-α on an authentic target promoter. A previous report (9) has demonstrated that in hepatoma cells, the promoter of PEPCK is regulated by PGC-1α. This effect is consistent with the known effects of PGC-1α as a regulator of hepatic gluconeogenesis (9, 18). As demonstrated in Fig. 4D, PGC-1α was able to function as a transcriptional co-activator of the PEPCK promoter. Although expression of ERR-α alone had no effect on the promoter, co-expression of ERR-α and PGC-1α resulted in a near complete repression of PGC-1α activity.

**DISCUSSION**

Regulation of co-activator activity represents an area of emerging interest. Previous studies (1, 2) have demonstrated that co-activators are subject to a host of post-translational modifications that regulate their activity. Specific repressors of co-activators, however, have not, to our knowledge, been widely described. We have demonstrated in this report that the inhibitory domain of PGC-1α binds to the nuclear orphan receptor ERR-α and that this binding alters the nuclear distribution of PGC-1α and represses its ability to act as a co-activator. These results complement a recent study (24) that has also demonstrated an interaction between PGC-1α and ERR-α. The implications of these two studies fundamentally differ, however,
because whereas the previous report assessed the ability of PGC-1α to co-activate ERR-α, we have concentrated on the ability of ERR-α to repress PGC-1α. This unique property of ERR-α is consistent with its unique binding site that lies within the inhibitory domain of PGC-1α rather than the classic LXXLL motif (amino acids 143–148), the site of interaction for all previous nuclear receptors.

The inhibitory domain within the PGC-1α molecule has previously been demonstrated to be relieved when PGC-1α binds to other transcription factors and DNA (10). Our results are consistent with these previous studies but suggest that repression of PGC-1α-mediated co-activation may also involve binding of additional proteins. Our studies are also consistent with recent speculation based predominantly on genetic arguments suggesting the existence of a specific molecular repressor of PGC-1α (5, 21). Given the importance of PGC-1α in a number of physiological and pathophysiological conditions, the interaction of ERR-α and PGC-1α would appear to represent a promising therapeutic target. We hypothesized that one potential regulator of the ERR-α/PGC-1α interaction might be the widely used anti-diabetic drug metformin, an agent that inhibits gluconeogenesis by an incompletely understood mechanism. Nonetheless, to date, we have been unable to observe any effect of metformin on either ERR-α transcript levels or repressor activity.2

Two previous reports have demonstrated that upstream activation of p38/MAPK stimulates PGC-1α activity presumably by relieving the effects of the repressor (8, 21). It is important to note, however, that in these studies the postulated repressor was not directly identified. It should be noted that still other reports have noted a direct effect of p38/MAPK on PGC-1α protein levels (19). These latter studies raise the possibility that the effects of p38/MAPK to augment PGC-1α function may not be mediated by altering the interaction with a repressor but rather via a direct on PGC-1α levels or activity. Our results do not support an effect of p38/MAPK on ERR-α-mediated repression, nor did we observe any effects on the overall degree of protein-protein interaction (data not shown). We cannot exclude the possibility that other repressors of PGC-1α could be identified that are regulated by the p38/MAPK pathway.

The tissue expression pattern of ERR-α and PGC-1α are similar, suggesting that ERR-α may function in vivo as an important regulator of PGC-1α activity. Release of PGC-1α from ERR-α would presumably be stimulated by conditions that required an alteration in cellular energetics. It is tempting to speculate that the endogenous ligand of ERR-α might therefore somehow be linked to the energetic state of the cell. Previous reports (26) have demonstrated that ERR-α is also involved in regulating gene products linked to metabolism. Thus, whether PGC-1α and ERR-α appear bound to each other or free might provide a mechanism to coordinate and integrate a wide range of gene products involved in both glucose and fatty acid metabolism. Further studies aimed at understanding the regulation of this interaction should therefore provide important insight into cellular energetics.

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