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A Cold-Inducible Coactivator of Nuclear Receptors Linked to Adaptive Thermogenesis

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Summary

Adaptive thermogenesis is an important component of energy homeostasis and a metabolic defense against obesity. We have cloned a novel transcriptional coactivator of nuclear receptors, termed PGC-1, from a brown fat cDNA library. *PGC-1* mRNA expression is dramatically elevated upon cold exposure of mice in both brown fat and skeletal muscle, key thermogenic tissues. PGC-1 greatly increases the transcriptional activity of PPARγ and the thyroid hormone receptor on the uncoupling protein (*UCP-1*) promoter. Ectopic expression of PGC-1 in white adipose cells activates expression of *UCP-1* and key mitochondrial enzymes of the respiratory chain, and increases the cellular content of mitochondrial DNA. These results indicate that PGC-1 plays a key role in linking nuclear receptors to the transcriptional program of adaptive thermogenesis.

Introduction

Obesity, an excessive accumulation of adipose tissue, affects nearly 30% of adults in the US. This high prevalence has led to increases in the medical conditions that often accompany obesity, especially diabetes (NIDDM), cardiovascular disorders, and certain cancers. Obesity results from a chronic imbalance between energy intake (feeding) and energy expenditure. Energy expenditure has several major components including basal metabolism, physical activity, and adaptive (nonshivering) thermogenesis. This latter process refers to energy that is dissipated in response to changing environmental conditions, most notably exposure to cold or excessive caloric intake (so-called diet-induced thermogenesis).

Adaptive thermogenesis is regulated by at least two major hormonal effectors: β-adrenergic agents and thyroid hormone (Himms-Hagen, 1989). Both of these hormone systems have an impact on energy dissipation through actions on the number and function of mitochondria, especially in brown fat and skeletal muscle. Thyroid hormone is known to act through its specific nuclear receptors while the β-adrenergic agents function through the cell surface β-adrenergic receptors, the widely expressed β-1 and β-2 and the fat-selective β-3 receptor. At a cellular level, the key processes of adaptive thermogenesis are the biogenesis of mitochondria and the expression of genes that control the rate of fuel oxidation and ATP synthesis in those mitochondria (Nicholls et al., 1986). Of particular interest here are the mitochondrial uncoupling proteins (UCPs; Flier and Lowell, 1997). These small proteins reside in the inner membrane of the mitochondria where they facilitate proton transport, dissipating the proton gradient linked to the oxidation of metabolic fuels, thus generating heat. Three UCPs have been reported. UCP-1 is specific for brown adipose cells (Jacobsson et al., 1985; Bouillaud et al., 1986). UCP-2 is expressed widely (Fleury et al., 1997; Gimeno et al., 1997), while UCP-3 is expressed primarily in skeletal muscle and brown fat (Boss et al., 1997; Gong et al., 1997; Vidal-Puig et al., 1997). The relative contributions of these three molecules to both adaptive thermogenesis and basal metabolic rate are under active study.

While many of the genes that function directly in adaptive thermogenesis are being uncovered, very little is known about the transcriptional regulation of this process. One promising approach may be through studying differentiation and gene expression in the cell type specialized to carry out adaptive thermogenesis: brown fat cells. These cells function specifically to dissipate energy in the form of heat in response to cold or overfeeding. The role of brown adipose tissue (BAT) as a defense against obesity has been clearly illustrated through targeted ablation of this tissue; BAT-less mice develop a severe obesity with insulin resistance and other metabolic abnormalities typical of obesity (Lowell et al., 1993). Analysis of the role of BAT in humans and other large mammals has proven more complex. Adult humans do not have well-defined BAT depots, but UCP-1 expression can be detected in white adipose tissue (WAT) depots, suggesting that brown fat cells may be dispersed among white fat cells (Garruti and Ricquier, 1992). Chronic exposure to β-3-adrenergic agonists causes BAT hypertrophy in both rodents and larger mammals, including humans, and has thermogenic, antiobesity effects (Champigny et al., 1991).

Recent data has pointed to an important role for several nuclear hormone receptors in the differentiation of brown fat cells and *UCP-1* gene expression, particularly the thyroid hormone receptor (TR) (Silva, 1995) and PPARγ (Sears et al., 1996). Thyroid hormone is required for brown fat cell differentiation, and the *UCP-1* promoter contains a binding site for TR (Cassard-Doulcier et al., 1994). The potential involvement of PPARγ in these processes is particularly interesting because the PPARγ/RXR heterodimer has been shown to be a major regulator of fat cell–specific gene regulation and differentiation in white adipose cells (Tontonoz et al., 1994a; Spiegelman and Flier, 1996). Recently, activation of PPARγ by synthetic ligands, the antidiabetic thiazolidinediones (TZDs), has been shown to promote the differentiation of brown fat precursor cells. These agents also cause a hypertrophy of BAT when given in vivo to obese rats

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MAWDMCSQDSVWSDIECAALVGEDQPLCPDLPELDLSELDVNDLDTDSFL   50
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B)

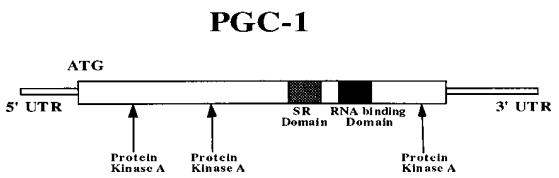

Figure 1. Analysis of PGC-1 Sequence

(A) Amino acid sequence of PGC-1. Shown by underlining are the SR domains (aa 565–598 and 617–631), RNA-binding (aa 677–709), three consensus sites for phosphorylating protein kinase A (aa 238–241, 373–376, and 655–658), and an LXXLL motif (aa 142–146).

(B) Schematic representation of the structure of PGC-1. Arrows indicate possible protein kinase A phosphorylation sites [(R,K)2x(ST)]. Gray box indicates the SR rich region domain and black box indicates the RNA-binding domain.

(Foellmi-Adams et al., 1996; Tai et al., 1996). While these data have indicated a role for PPARγ in brown fat differentiation, it is also clear that this transcription factor cannot per se determine whether fat cells proceed along the energy storage (white) or energy dissipation (brown) pathway. One clue to this issue may have come from a recent series of studies that identified a functional binding site for PPARγ/RXR in the brown fat selective enhancer of the *Ucp-1* gene (Sears et al., 1996). Transactivation studies showed that PPARγ could activate this element, but only in brown fat cells, not in fibroblasts. This suggested that PPARγ might have a regulated cofactor that allows it to function specifically in the context of brown fat cell lineage and the thermogenic genes that characterize this cell type. In this report, we describe the cloning and characterization of a novel coactivator of PPARγ and other nuclear receptors from brown adipose cells. This factor, termed PGC-1, is dramatically induced in both brown fat and skeletal muscle by exposure of animals to cold and can regulate several key mitochondrial genes that contribute to the program of adaptive thermogenesis.

Results

Isolation of PGC-1, a Novel PPARγ Binding Protein

We used a yeast two-hybrid system to identify PPARγ-interacting proteins from murine brown fat cells (see Experimental Procedures). A screen of 1×10^6 primary transformants made using cDNAs prepared from HIB 1B brown fat cells yielded about 130 clones. These were then analyzed for preferential expression in brown versus white fat with RNA blots. One partial cDNA whose open reading frame encodes a novel protein received more detailed study. We termed the protein encoded by this cDNA **P**PAR **G**amma **C**oactivator (PGC)-1.

A 3 kb *PGC-1* cDNA containing the entire open reading frame was obtained by screening an oligo dT λZAP cDNA library made from HIB-1B cells. This encodes a protein of 795 amino acids with a predicted molecular mass of 92 kDa (Figure 1A). Databank searches indicate that PGC-1 represents a novel protein with no close homologs in any databases except expressed sequence tag (EST) databases. It does, however, contain recognizable peptide motifs. As shown in Figures 1A and 1B, PGC-1 contains a putative RNA-binding motif (aa 677–709) and two so-called SR domains, regions that are rich in serine and arginine residues (aa 565–598 and 617–631). Interestingly, proteins containing paired RNA-binding motifs and SR domains have been shown to interact with the C-terminal domain (CTD) of RNA polymerase II (Yuryev et al., 1996). Except for these two regions, however, PGC-1 shares no other sequence similarity with other proteins that contain these domains. In addition to these domains, PGC-1 also contains three consensus sites for phosphorylation by protein kinase A. However, no significant homology was discovered between PGC-1 and any known coactivator of nuclear receptors. PGC-1 does, however, contain one LXXLL motif (aa 142–146), recently identified as an element that can mediate nuclear receptor–coactivator interactions (Heery et al., 1997; Torchia et al., 1997).

PGC-1 Interacts Specifically with PPARγ and Other Members of the Nuclear Hormone Receptor Superfamily

To investigate the interaction between PGC-1 and PPARγ first observed in yeast, GST-purified fusion proteins were immobilized on glutathione agarose beads

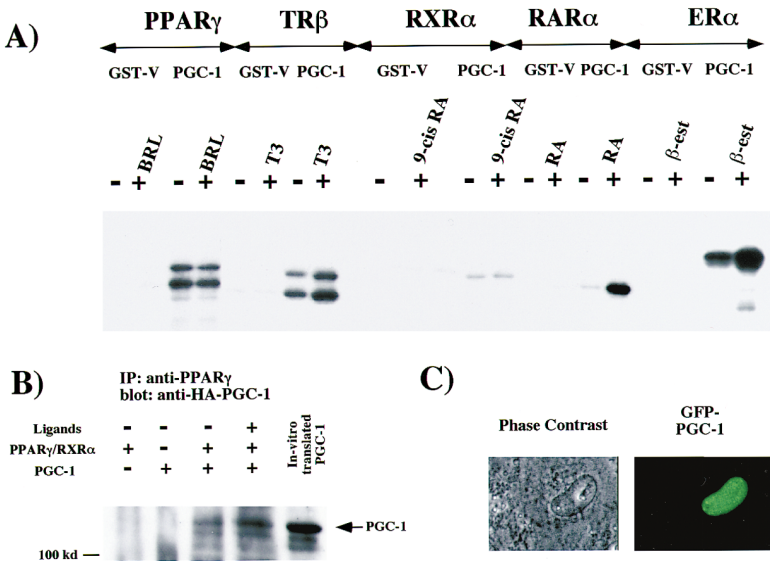

Figure 2. Interaction of PGC-1 with Several Nuclear Receptors

(A) Interaction between proteins in vitro. Control GST protein alone or PGC-1 (aa 36–797) fused to GST were immobilized on glutathione agarose beads and incubated with different in vitro–translated [^{35}S]methionine-labeled nuclear receptors and appropriate ligands or vehicle (see Experimental Procedures for details). For PPARγ, BRL 49653 was used at 10 μM; for TRβ, T_3 was used at 1 μM; for RXRα, 9-*cis* RA was used at 1 μM; for RARα, RA was used at 1 μM; and for ERα, estradiol was used at 1 μM. After the beads were washed, bound proteins were eluted and resolved by SDS-PAGE and visualized by autoradiography.

(B) Interaction between PGC-1 and PPARγ in cells. Vectors expressing HA-tagged *PGC-1* and *PPAR*γ were transfected into COS cells. Ligands pioglitazone (5 μM), 9-*cis* RA (1 μM), and 8-Br-cAMP (1 mM) were added 3 hr before cells were harvested. Cells extracts and Western blots were performed as described in the Experimental Procedures.

(C) PGC-1 is localized to the nucleus. The full-length PGC-1 was fused to a green fluorescent protein (GFP), and this was expressed in COS cells with a pEGFP expression vector. Fluorescent GFP-PGC-1 was visualized with a Nikon Diaphot 200 microscope.

and incubated with in vitro–translated, [^{35}S]methionine-labeled PPARγ and other nuclear receptors. As shown in Figure 2A, PPARγ associates with GST–PGC-1 but fails to bind to the control GST protein. This interaction is not ligand-dependent, in that addition of BRL49653 (a thiazolidinedione ligand for PPARγ) at 10 μM does not significantly alter this binding. A similar lack of ligand dependence for this interaction was seen when bacterially expressed PPARγ was immobilized on beads and used with reticulocyte-translated PGC-1 (data not shown). The TRβ also binds specifically to PGC-1, though in this case ligand (T_3) addition causes a 2- to 3-fold increase in binding. A strong ligand-dependent binding is seen between PGC-1 and the retinoic acid (RA) receptor, and between PGC-1 and the estrogen receptor (ERα). In contrast, little or no binding is seen between PGC-1 and the retinoid X receptor (RXRα), with or without ligand addition. These data indicate that PGC-1 interacts specifically with PPARγ and several other nuclear receptors in vitro. There is a broad range of dependence on ligand for these interactions, from no ligand dependence (PPARγ) to a strong dependence on ligand addition (RARα).

The interaction between PPARγ and PGC-1 can also be seen in mammalian cells. Even in the absence of added ligand, an association is observed between these two proteins in immunoprecipitation assays (Figure 2B). When cells are treated with pioglitazone (a PPARγ ligand), a very modest increase in association is observed. To ask whether PGC-1 does indeed reside in the cell nucleus, we constructed a fusion protein between PGC-1 and green fluorescent protein (GFP). When GFP–PGC-1 is expressed in COS cells, it is observed entirely in the cell nucleus (Figure 2C).

Identification of the Domains that Mediate the PGC-1–PPARγ Interaction

The interaction between nuclear receptors and certain coactivators such as SRC-1 or CBP is ligand dependent (Kamei et al., 1996) and involves an LXXLL motif in the coactivators and the C-terminal AF-2 domain in the receptors (Heery et al., 1997; Torchia et al., 1997). To identify the domains responsible for PGC-1–PPARγ interactions, we first generated different C-terminal deletions of PGC-1 as reticulocyte translation products and mixed these with a GST-PPARγ fusion protein. As shown in Figures 3A–3C, 35% of the input of both the full-length PGC-1(1–797) and the 1–675 deletion is bound to the immobilized PPARγ. The binding of PGC-1 1–503, which lacks the SR and RNA-binding domains, is modestly decreased to 18%. A similar level of binding can be seen for PGC-1 1–403 and 1–338. However, PGC-1 1–292, which still contains the LXXLL motif, completely loses the ability to interact with PPARγ. As shown in Figure 1, residues 292–338 contain no distinct domains known to mediate protein–protein interaction, though it is very rich in proline residues.

Most of the nuclear hormone receptor coactivators identified to date interact with the C-terminal AF-2 domain, which is responsible for ligand-dependent transcriptional activation. To determine if PGC-1 also interacts with this part of PPARγ, we used several deletions of PPARγ prepared as GST fusion proteins and combined them with in vitro–translated PGC-1. Figure 4 shows that amino acids 181–505 of PPARγ (the original fragment used in the yeast two-hybrid screen) interact strongly with PGC-1, pulling down 23% of the input. On the other hand, a further deletion of 45 amino acids (228–505) is not able to bind to full-length PGC-1. Both of these PPARγ deletions were able to bind SRC-1 (data not shown), indicating that they have not lost their general ability to interact with other proteins. These data demonstrate that PPARγ utilizes part of its DNA-binding and hinge domains to bind PGC-1. It apparently does not interact through the C-terminal AF-2 domain that docks other coactivators such as SRC-1 and CBP.

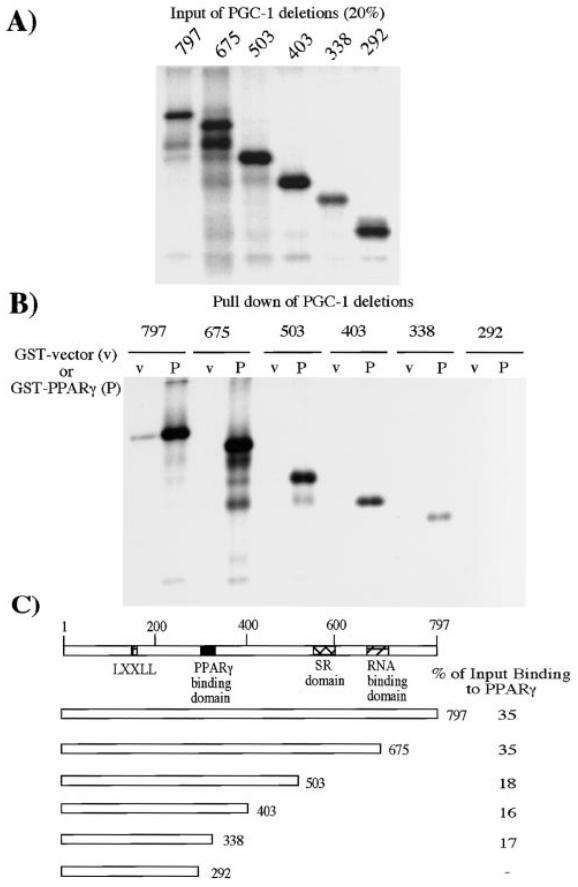

Figure 3. Mapping the Domain of PGC-1 Interacting with PPARγ

(A) Input of deleted PGC-1 proteins. Deletions of PGC-1 were made using specific rectriction sites in the *PGC-1* cDNA cloned in pBluescript. The following restriction enzymes were used for these deletions: full-length XhoI (aa 1–797), HaeII (aa 1–675), NcoI (aa 1–503), XbaI (aa 1–403), KpnI (aa 1–338), and StuI (aa 1–292). These were then translated in vitro with an [^{35}S]methionine-label. One microliter of each in vitro translation reaction was resolved by SDS-PAGE and autoradigoraphed.

(B) PPARγ interactions with PGC-1 deletions. A control GST fusion protein (V) or a GST fusion with PPARγ (P) was incubated with the different PGC-1 deletion shown in (A). Incubations, washing, and analysis were done as in Figure 2, without addition of ligand. The percentage of binding was quantified by phosphorimage analysis.

(C) Diagram of different PGC-1 deletions with the corresponding percentage of input material that bound to PPARγ. The LXXLL motif is located at amino acid residues 142–146. The black box corresponds to the PPARγ-binding domain of PGC-1 (aa 292–338).

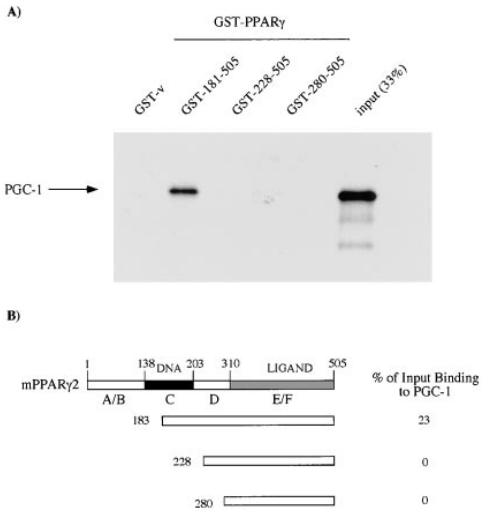

Figure 4. Mapping the Domain of PPARγ Interacting with PGC-1

(A) Full-length, [^{35}S]methionine-labeled PGC-1 was prepared in reticulocyte lysates and was incubated with various deletions of PPARγ prepared as GST fusion proteins. The pull-down assays were done exactly as in Figure 3A.

(B) Diagram of different PPARγ deletions with the corresponding percentage binding to PGC-1. Quantitative analysis was done as in Figure 3.

Expression of *PGC-1* mRNA Is Induced by Exposure to Cold or a β-Adrenergic Agonist

The pattern of *PGC-1* expression was determined by Northern blot analysis of mRNA prepared from a variety of mouse tissues. As shown in Figure 5A, using mice kept at ambient temperature (24°C), a major species of approximately 5 kb is observed in brown fat, heart, kidney, and brain. In addition, a minor species of approximately 8 kb is also observed in all of these tissues. In contrast, no *PGC-1* mRNA expression is observed from white fat, lung, skeletal muscle, liver, testes, or spleen.

Exposure to cold is a classical inducer of adaptive thermogenesis, especially in brown fat and skeletal muscle (Himms-Hagen, 1989). As shown in Figure 5B, *PGC-1* is highly induced (about 30- to 50-fold) in brown fat after 3 hr or 12 hr at 4°C, in parallel with *UCP-1* mRNA. Figure 3 also shows that *PGC-1* mRNA expression in white fat and liver remains undetectable after cold exposure. Although *PGC-1* mRNA expression is not detectable in skeletal muscle from mice kept at ambient temperature, exposure of mice to cold for 12 hr induces expression of the *Pgc-1* gene in this tissue. Heart and kidney, which express *PGC-1* mRNA at room temperature, do not elevate this expression upon cold exposure.

Figure 5C illustrates that the cold-induced expression of *PGC-1* in the brown fat of these mice also correlates other key mitochondrial proteins including ATP-synthetase (β subunit) and cytochrome c-oxidase subunits (*COX II* and *COX IV*). Although chronic cold exposure has been reported to lead to elevated activities for these mitochondrial proteins in skeletal muscle (Bourhim et al., 1990), no induction of mRNA for ATP-synthetase, *COX II*, or *COX IV* can be seen in muscle with this relatively brief exposure to cold.

Cold is sensed in the central nervous system and results in increased sympathetic output to peripheral tissues, including muscle and brown fat (see Himms-Hagen, 1989, and references therein). Cold exposure can be mimicked, in terms of brown adipocyte precursor cell growth and the induction of UCP-1, by exposure of cultured brown fat cells to β-adrenergic agonists (Rehnmark et al., 1990). To determine if *Pgc-1* gene expression is also sensitive to β-adrenergic agonists, HIB1B brown fat cells were treated with isoproterenol, a nonsubtype selective β agonist, for 10 hr. As shown in Figure 6, this results in a sharp increase in both *PGC-1* mRNA and *UCP-1* mRNA. Exposure of brown fat cells to 9-*cis* retinoic acid has previously been shown to potentiate the

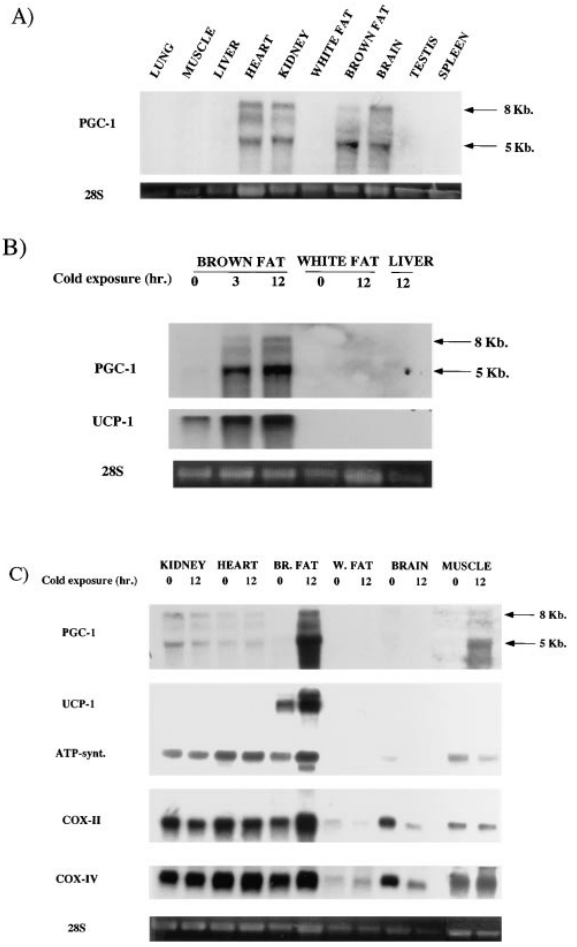

Figure 5. Expression of *PGC-1* mRNA in Mouse Tissues and Induction by Exposure to Cold

(A) Northern blot analysis of total RNA isolated from the indicated mouse tissues using radiolabeled *PGC-1* cDNA as a probe. Ethidium bromide–stained 28S RNA is shown as a control for quantity of RNA. Arrows indicate the size of the *PGC-1* bands.

(B) *PGC-1* and *UCP-1* mRNAs are induced in brown fat during cold exposure. Mice were maintained at 4°C for 3 or 12 hr, sacrificed, and tissues were dissected and used for preparation of RNA. Ten mice were pooled for each sample.

(C) mRNA analysis of *PGC-1* and genes of mitochondrial function in different mouse tissues after cold exposure. Animal treatment was as in (B). Probes used for hybridization were *PGC-1*, *UCP-1*, ATP synthetase (β subunit), cytochrome c-oxidase II (*COX-II*), and cytochrome c-oxidase IV (*COX-IV*).

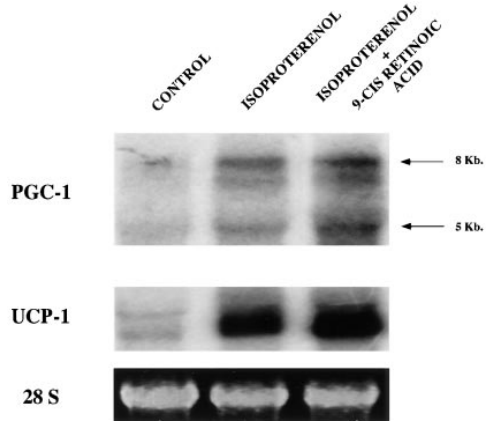

Figure 6. Expression of *PGC-1* mRNA Is Induced by β-Adrenergic Agonists

HIB 1B brown fat preadipocytes were differentiated as described in the Experimental Procedures. After 6 days, cells were approximately 80% differentiated. They were then treated with isoproterenol (1 μM) for 6 hr and 9-*cis* retinoic acid (1 μM) for 24 hr. Total cellular RNA was isolated and analyzed using *PGC-1* and *UCP-1* cDNA probes.

effects of β agonists to induce UCP-1 expression (Puigserver et al., 1996). Addition of this retinoid (which activates both RXR and RAR) and isoproterenol to the HIB1B cells results in a small, further increase in both *PGC-1* and *UCP-1* expression. These results suggest that β-adrenergic agonists may play an important role in mediating the effects of cold on the induction of both *UCP-1* and *PGC-1*.

PGC-1 Is a Powerful Transcriptional Coactivator for PPARγ and the TR

To test for transcriptional coactivation by PGC-1, we utilized the *UCP-1* promoter in combination with PPARγ or the TR. The *UCP-1* promoter has been shown to have binding sites for both PPARγ and the TR (Cassard-Doulcier et al., 1994; Sears et al., 1996). Figure 7A shows that the PPARγ/RXRα combination transactivates the *UCP-1* promoter very poorly in fibroblasts, as shown by Sears et al. (1996). The addition of a cocktail of 8-bromocyclic AMP, 9-*cis* retinoic acid, and troglitazone, a PPARγ ligand, has been used previously to give an optimal induction of *UCP-1* in brown fat cells (Silva and Rabelo, 1997). These agents cause only a small increase in the transcriptional activity of the PPARγ/RXR combination above that seen with the control vector. The expression of PGC-1 alone does not induce transcriptional activity above the control vector. However, the addition of PGC-1 to the PPARγ/RXRα combination causes a very dramatic increase in transcriptional activity that is dependent on the ligand cocktail (see below). Similarly, TRβ/RXRα combination alone induced very little transcriptional activity, even when stimulated with a ligand cocktail including T_3 (Figure 7B). However, the combination of PGC-1 with the TRβ/RXRα pair induced powerful transactivation, again in a ligand-dependent manner. These results clearly indicate that PGC-1 can function as a potent transcriptional coactivator for PPARγ and the TR. It is interesting that the optimal transcriptional response is seen when PPARγ ligand is added, despite the fact that the binding of PGC-1 and PPARγ is not ligand dependent. It is likely that this results from simultaneous, ligand-dependent docking of another coactivator, such as SRC-1, CBP, or others (see Discussion).

The role of different hormones and ligands used to achieve maximal transcriptional activation with PPARγ and PGC-1 is dissected in Figure 7C. The individual components—troglitazone (trog.), 9 *cis*-retinoic acid (9cRA), and 8-bromo cyclic AMP (cAMP)—each stimulate a 2- to 4-fold increase in transcriptional activity. However, the most robust responses are seen when they are used in combination. The synergistic effect of

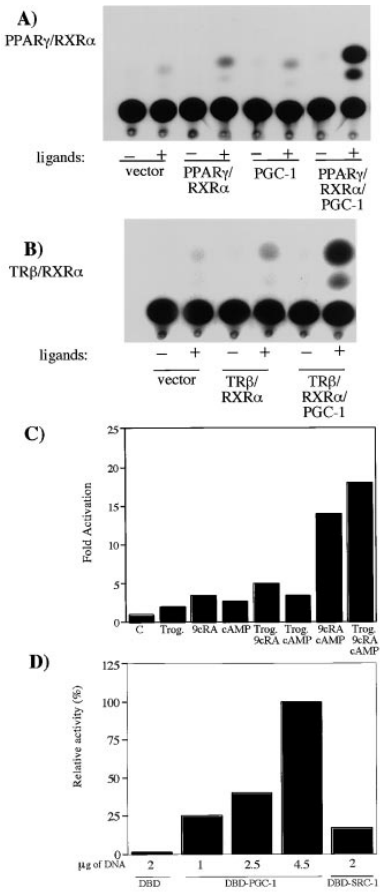

Figure 7. PGC-1 Powerfully Stimulates the Transactivation of the *UCP-1* Promoter by PPARγ and the TR

(A) PGC-1 coactivates the transcriptional activity of PPARγ/RXRα. A CAT reporter gene linked to the *UCP-1* promoter (−3740/+110 kb) was transfected into Rat1 IR cells along with expression vectors for *PPAR*γ, *RXR*α, and *PGC-1* (see Experimental Procedures). Cells were changed to medium containing 0.5% BSA 12 hr after transfection and the following ligands were added: 10 μM troglitazone and 1 μM 9-*cis*-retinoic acid for 24 hr treatment, while 1 mM 8-bromo cAMP was added for the last 12 hr. Cells were harvested 36 hr after transfection. Results from CAT assays were controlled for transfection efficiency by cotransfection of a β-galactosidase reporter gene under the control of the *CMV* promoter.

(B) PGC-1 activates the transcriptional activity of TRβ/RXRα. CAT reporter assays were done as in (A), except that 1 μM T_3 was used instead of troglitazone.

(C) Ligand/hormone dependence of PGC-1-stimulated activity from the *UCP-1* promoter. CAT reporter gene assays were performed as described in (A), using *PPAR*γ, *RXR*α, and *PGC-1* vectors. The fold activation was compared to the value observed in cells transfected with the same vectors but not treated with ligand.

(D) Transcriptional activity function of PGC-1 linked to GAL4 DBD. The full-length PGC-1 was fused in-frame to the DNA-binding domain of GAL4. This was expressed in cells with pCMX vector, along with a control fusion between GAL4 DBD and full-length murine *SRC1*. Transcription was assayed with a reporter plasmid containing five copies of the UAS linked to CAT. The activity stimulated by 4.5 μg of the DBD-PGC-1 was set as 100%.

9-*cis* retinoic acid and 8-bromo cyclic AMP is particularly striking (14-fold), while all three agents together cause an 18-fold increase above the untreated control.

The data shown above do not indicate whether PGC-1 has its own transcriptional activation domain or contains some activity that might unmask or augment the transcriptional activator properties of the nuclear receptors. To approach this, we made a fusion protein between PGC-1 and the DNA-binding domain (DBD) of GAL4 and assayed transcription through a GAL4 DNA binding target sequence, the UAS. As shown in Figure 7D, PGC-1 can activate transcription readily when tethered to DNA by the GAL4 DBD. For comparison, the results obtained by fusion of the GAL4 DBD with another coactivator of nuclear receptors, SRC-1, is shown. Thus, PGC-1 does not absolutely require docking to a nuclear receptor to demonstrate transcriptional activation function; it is likely that its interaction with these receptors serves primarily to bring PGC-1 to appropriate DNA sites.

Ectopic Expression of PGC-1 Induces Molecular Components of Adaptive Thermogenesis

To examine directly the ability of PGC-1 to regulate the genes of adaptive thermogenesis, we have utilized retroviral vectors to express this protein in white fat precursor cells, and 3T3-F442A preadipocytes were then stimulated to differentiate. The cells were subsequently maintained for 4 days in medium containing 10 or 100 nM T_3. To induce *UCP-1* expression, 1 μM 8-bromo-cAMP and 1 mM 9-*cis*-retinoic acid were added to the medium, and total RNA was extracted from the cells 6 hr later. As shown in Figure 8A, *PGC-1* mRNA was barely detectable in these white fat cells infected with empty vectors but was obvious in cells infected with the viruses containing the *PGC-1* cDNA. The expression of this mRNA in the cultured cells was approximately 6% of that seen in the brown fat of cold exposed mice (data not shown). mRNA for *UCP-1*, the classic marker of brown fat cells that is encoded in the cell nucleus, is barely detectable in the control 3T3-F442A cells but is significantly induced in the cells expressing PGC-1. mRNA for ATP synthetase, a key mitochondrial protein involved in oxidative phosphorylation that is also encoded in the nucleus, is likewise increased in the cells expressing PGC-1. The mitochondrial respiratory enzyme cytochrome c-oxidase subunits COX II and IV are encoded in the mitochondrial and nuclear genome, respectively. Both of these mRNAs increase 2- to 3-fold in the cells ectopically expressing PGC-1. For comparison, we show expression of *aP2*, a white and brown fat cell gene not linked to thermogenesis, and 36B4, a ribosomal protein used as a loading control. These data clearly demonstrate that PGC-1 can stimulate the expression of several key genes of mitochondrial function and adaptive thermogenesis, even when expressed at levels far below those seen in cold-exposed animals.

Finally, the ability of PGC-1 to affect the expression of mRNA for a protein (COX-II) encoded in the mitochondrial genome suggests that PGC-1 could affect the biogenesis of mitochondria per se. Changes in the cellular content of mitochondrial DNA have been used as a simple biochemical assay for mitochondrial proliferation (Martin et al., 1995; Klingenspor et al., 1996). As shown by Southern blot in Figure 8B, cells expressing PGC-1 have twice the mitochondrial DNA content compared to control cells. The same blots were also probed with

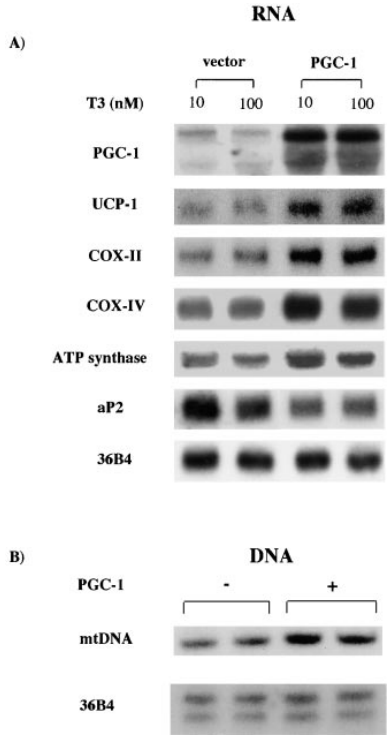

Figure 8. Ectopic Expression of PGC-1 Induces Mitochondrial Gene Expression and Increased Mitochondrial DNA

3T3F442A preadipose cells were infected with a control pBabe retroviral vector or one expressing PGC-1. Cells were differentiated under standard conditions for 6 days when 80%–90% of cells showed an adipocyte phenotype. Cells were then treated for 4 days in medium containg 10% cosmic calf serum, 17 nM insulin, and 10 or 100 nM T_3. To induce *Ucp-1* gene expression, 9-*cis*-retinoic acid (1 μM) and 8-Br-cAMP (1 mM) were added for 24 and 6 hr, respectively. After these treatments, total RNA was isolated and analyzed. (A) RNA blot analysis for *UCP-1*, *COX-II*, *COX-IV*, and ATP synthetase. Probes and hybridization were as in Figure 5. Control hybridization with cDNAs for *aP2* and the ribosomal protein 36B4 are shown in the bottom panel.

(B) Southern blot analysis of mitochondrial genome DNA. 3T3-F442A cells were differentiated as in (A). Total cellular DNA was isolated (Maniatis et al., 1989) and was digested with Nco I. Ten micrograms of DNA was electrophoresed, and the Southern blot was hybridized using *COX-II* cDNA as a probe for mitochondrial DNA. The blot was then stripped and hybridized with the nuclear gene *36B4*. Duplicate samples of DNA from cells containing empty vector (−) or PGC-1 (+) are shown.

cDNA for 36B4, a ribosomal protein encoded in the nucleus. These results show that ectopic PGC-1 expression can stimulate an increase in mitochondrial DNA, indicating an increased biogenesis of mitochondria.

Discussion

The nuclear receptor superfamily, now known to be comprised of more than 100 members, controls an amazing array of physiological processes in metazoan organisms. Recent research has demonstrated that the nuclear receptors function by serving as platforms for the assembly of other important transcriptional components into macromolecular complexes at specific DNA sequences. These accessory proteins, termed coactivators and corepressors, appear to mediate gene activation or gene repression at promoters even quite distant from the binding sites for the nuclear receptors (Chakravarti et al., 1996; Glass et al., 1997; Shibata et al., 1997). Some examples of ligand-dependent coactivators are SRC-1/TIFα (Onate et al., 1995), TIF-2 (Voegel et al., 1996), CBP/p300 (Chakravarti et al., 1996; Kamei et al., 1996), RIP140 (Cavailles et al., 1995), ACTR (Chen et al., 1997), and PBP (Zhu et al., 1997).

Despite the high degree of specificity in the biological processes influenced or controlled by particular nuclear receptors, most of the coactivators or corepressors described to date have very little specificity in terms of where and when they are expressed, or the nuclear receptors with which they interact. There may also be many new coactivators still to be discovered whose expression is regulated spatially or temporally, or that have selectivity with respect to the receptors with which they interact. In the category of nuclear receptor coactivators, one coactivator (termed AIB or SRC-3) has recently been described that is overexpressed in many human breast cancers and increases the transcriptional activity of the estrogen receptor (Anzick et al., 1997). A small number of coactivators have been described for transcription factors outside the nuclear receptor family that are regulated in a tissue-selective fashion, including Bob1/OCA-B/OBF1 in B limphocytes (Luo, et al., 1992) and TRF in the *Drosophila* nervous system and gonads (Hansen et al., 1997).

The data presented here show that PGC-1 is unusual among known nuclear receptor coactivators in that its expression is dramatically regulated with respect to both tissue selectivity and the physiological state of the animal. The expression of *PGC-1* in BAT but not WAT distinguishes it from most known transcriptional components in these tissues and its induction by cold is even more dramatic than that observed for *UCP-1*. PGC-1 is also distinct from the known coactivators in that it appears to use different sequence motifs for protein–protein docking, on both sides of the receptor–coactivator pair. Nearly all of the known coactivators and corepressors utilize LXXLL sequences to bind at the ligand-regulated helix 12 in the carboxy terminal AF-2 domain (Heery et al., 1997; Torchia et al., 1997). In contrast, PGC-1 utilizes a domain rich in proline residues to bind to a region that overlaps the DNA binding and hinge region of PPARγ. For PPARγ, this opens the possibility that PGC-1 is not an alternative coactivator to one or more of the ligand-controlled coactivators but, rather, may bind in concert with these proteins to give a larger macromolecular complex. On the other hand, ligand-dependent docking is seen with some other receptors such as the retinoic acid receptor, the estrogen receptor, and to a certain degree, the thyroid receptor. Since PGC-1 has one LXXLL sequence, a motif shown in several contexts to be both necessary and sufficient for ligand-dependent receptor docking, it is entirely possible that the binding of PGC-1 to those receptors will depend on this sequence and the receptor AF-2 domains.

It is now appreciated that most of the coactivators or corepressors that bind to receptors at AF-2 domains

carry either histone acetyltransferase or histone deacetylase activities (Pazin and Kadonaga, 1997). These activities may be intrinsic to certain coactivators such CBP and SRC-1 (Bannister and Kouzarides, 1996; Spencer et al., 1997) or reside in proteins that form complexes with corepressors, as illustrated by the complex between SMRT and mammalian histone deacetylase (Nagy et al., 1997; Torchia et al., 1997). Based on primary sequence data, PGC-1 does not contain any motifs that would be suggestive of histone acetylase or deacetylase activity. It also has no significant sequence homologies with any of the known nuclear receptor coactivators or corepressors. It may be noteworthy that PGC-1 has paired SR and RNA-binding domains that have been identified in a number of proteins, including several that bind to the regulatory carboxy terminal domain (CTD) of RNA polymerase II (Yuryev et al., 1996). Whether PGC-1 can recruit RNA pol II into nuclear receptor complexes or has some other positive action on the gene transcription machinery remains to be determined. Of course, the data presented here could also be explained by PGC-1 relieving a gene repression mechanism. The hinge region of at least one nuclear receptor (TR) has been shown to be involved in binding a corepressor (N-CoR; Horlein et al., 1995). Hence, it is possible that an important part of PGC-1's action may be to derepress transcription by interfering with corepressor binding. Regardless of its biochemical activity, it seems highly likely that PGC-1 will be a coactivator that falls outside of the current paradigm of ligand-dependent AF-2–docking proteins.

Adaptive thermogenesis refers to a component of energy expenditure, which is separate from physical activity and which can be elevated in response to changing environmental conditions, most notably cold exposure and overfeeding (Himms-Hagen, 1989). Although there has been little progress in understanding the molecular control of adaptive thermogenesis, there is considerable interest in this subject because of its potential roles in both the pathogenesis and therapy of human obesity.

A role for PGC-1 in adaptive thermogenesis is indicated first by its connection to the key tissues and hormones implicated in this process. Our current understanding suggests an especially important role for skeletal muscle and brown fat. PGC-1 is induced by cold exposure in both muscle and brown fat but not in other tissues. The thermogenic and antiobesity properties of brown fat are conclusively established in rodents (Himms-Hagen, 1995), but the role of BAT is less clear in humans due to the fact that adult humans and other large mammals do not have well-defined brown fat depots. The expression of UCP-1 in the white fat depots of adults suggests that brown adipocytes may be incorporated into depots that appear white and can be recruited upon adrenergic stimulation (Garruti and Ricquier, 1992).

With regard to hormones, thyroid hormone and β-adrenergic agonists appear to play the most important roles in both cold and diet-induced thermogenesis in muscle and brown fat (Himms-Hagen, 1989; Cannon and Nedergaard, 1996). β-adrenergic agonists appear to affect PGC-1 function in at least two distinct ways. First, they can induce *PGC-1* expression, as shown in Figure 5. Second, cyclic AMP (the intracellular mediator of β-adrenergic receptor activity) increases the transcriptional activity mediated by PGC-1 when expression is driven ectopically, as shown in Figure 7C. While the molecular basis of this is not known, the presence of three consensus phosphorylation sites for protein kinase A suggests that the protein may be posttranslationally activated by this pathway. The thermogenic effects of thyroid hormone and its receptors are well known. One of the clearest effects of increasing thyroid hormone levels is the stimulation of mitochondrial respiration rates in skeletal muscle, brown fat, heart, and kidney. Abnormally low respiration rates, characteristic of a hypothyroid state, can be increased by raising thyroid hormone levels (Pillar and Seitz, 1997). Based on the tissues where it is expressed and its ability to coactivate the TR, PGC-1 appears to be a very good candidate to mediate some of these effects.

Recent evidence has also suggested interesting effects of the TZDs in thermogenesis. These PPARγ ligands can increase energy expenditure when given systemically to rodents, perhaps due to increased formation of brown fat and an increase in *Ucp-1* gene expression. These effects have also been seen in cultured cells (Foellmi-Adams et al., 1996; Tai et al., 1996; P. P. and B. M. S., unpublished data). The ability of PGC-1 to coactivate the function of PPARγ on the *UCP-1* promoter, and presumably other promoters in thermogenic pathways, may provide some explanation for these effects.

In addition to these associations described above, ectopic expression experiments presented here show more directly that PGC-1 can regulate components of thermogenesis. At a cellular and molecular level, adaptive thermogenesis consists of at least three separable processes: the biogenesis of mitochondria, the expression of the mitochondrial enzymes of the respiratory chain, and the expression of specific uncoupling proteins. There are now three known members of the *Ucp* gene family: *Ucp-1*, expressed exclusively in brown fat; *Ucp-2*, expressed widely, and *Ucp-3*, expressed primarily in skeletal muscle and brown fat. Depending on the length of time and severity of a given physiological challenge, one or more of these aspects of thermogenesis may be affected in muscle, BAT, or other tissues.

In our initial experiments, we have used retroviral expression of PGC-1 in white fat cells. This cell type was chosen because it has little endogenous PGC-1 expression and is known to have relatively low numbers of mitochondria and little expression of UCP-1 or UCP-3. Although we were only able to get a relatively low level of *PGC-1* mRNA expression (6% of that seen in cold-induced BAT), it is clear that several molecular components of the adaptive thermogenesis system are altered. First, expression of the *Ucp-1* gene is turned on from the almost undetectable level that is characteristic of these white cells. Second, several mitochondrial genes of the respiratory chain that are ordinarily expressed in these cells, such as ATP synthetase, *Cox-II* and *Cox-IV*, are significantly increased. Finally, mitochondrial content is doubled, as evidenced by the increase in mitochondrial DNA per unit of total cellular DNA. Although

not shown here, we have been unable to detect any increases in the expression of *UCP-2* or *UCP-3* in these cells. UCP-3 is of special interest because its tissue major sites of synthesis (muscle, BAT; Vidal-Puig et al., 1997), and hormones that are reported to induce it (β-adrenergic agonists and thyroid hormone; Gong et al., 1997) fit very well with the expression patterns and activities of PGC-1. The inability to elevate *UCP-3* in the experiments may represent a strict limitation on the cell types that are permissive for *UCP-3* expression, the fact that we have so far attained an insufficient quantity of *PGC-1* expression, or the lack of a role for this coactivator in the regulation of UCP-3.

How might PGC-1 regulate mitochondrial processes linked to adaptive thermogenesis? For genes such as *UCP-1* that are encoded in the nucleus and are responsive to PPAR, TR, or other nuclear receptors, PGC-1 could act directly as a coactivator to increase transcription rates. For genes that are encoded in the mitochondrial genome (such as *Cox-II*), PGC-1 could be acting directly or indirectly. Certain genes within the mitochondria have been shown to have functional thyroid response elements (TREs; Pillar and Seitz, 1997). While we show here that PGC-1 is observed mainly in the nucleus (Figure 2C), a small percentage of the TR and PGC-1 are transported into the mitochondria and function directly at these sites. Similarly, with regard to mitochondrial DNA replication, the D loop of the mitochondrial genome is a site of heavy strand replication and contains a TRE-DR2 sequence (Wrutniak et al., 1995), suggesting that the TR and PGC-1 could act here directly. On the other hand, PGC-1 and nuclear receptors could regulate the expression of other nuclear factors, such as NRF or mitochondrial factor A, that have been shown to function in the mitochondria to stimulate gene transcription and/or DNA replication (Pillar and Seitz, 1997).

A full accounting of the role of PGC-1 in adaptive thermogenesis and perhaps other physiological processes will require both genetic gain-of-function and loss-of-function experiments in mice. A more detailed knowledge about the function and regulation of PGC-1 could provide new opportunities to manipulate energy expenditure in vivo and treat disorders of energy homeostasis such as obesity and obesity-linked diabetes.

Experimental Procedures

Yeast Two-Hybrid System and cDNA Cloning

Amino acids 183–505 of the murine PPARγ were cloned in-frame into the GAL4 DNA-binding domain plasmid pAS2. A HIB 1B cDNA expression library was constructed in the GAL4 activation domain plasmid pACT II. Yeast two-hybrid screening was performed as described in the CLONTECH Matchmaker two-hybrid system protocol. pAS-PPARγ was transformed into Y190 yeast cells by the lithium acetate method and maintained by selection in leucine$^-$ plates. A pACT-HIB 1B cDNA library was constructed using isoproterenol-treated differentiated HIB 1B cells. The pACT II-HIB 1B cDNA library was transformed into Y190-PPARγ yeast cells, and positive clones were assayed for β-galactosidase activity in a filter assay as described in the CLONTECH protocol. pAS1 lamin cDNA was used to test the specificity of interaction between PGC-1 and PPARγ. To obtain full-length *PGC-1*, the positive yeast cDNA clone was used as a probe to screen a oligo dT λZAP cDNA library from HIB 1B cells. After three rounds of screening, the cDNA inserts of positive phage clones were excised into pBluescript and both strands were sequenced by standard methods.

Protein Binding and Cellular Localization Studies

PGC-1 (EcoRI-XhoI fragment from pBluescript) was cloned into the SmaI site of pGEX 5X3. The PPARγ deletions were generated by performing PCR using specific oligonucleotides and there were cloned in-frame in pGEX 5X2. These fusion proteins were expressed, and purified from *E. coli* on beads containing glutathione. Of 50% slurry beads containing approximately 1 μg of protein (either GST, or alone, or fused to PGC-1), 30 μl was resuspended in the binding buffer (20 mM HEPES [pH 7.7], 75 mM KCl, 0.1 mM EDTA, 2.5 mM MgCl2, 0.05% NP40, 2 mM DTT, 10% glycerol). This was mixed with 5 μl of different nuclear receptors made in an in vitro reticulocyte translation reaction using [^{35}S] methionine (Promega TNT reticulocyte lysate system kit). Specific nuclear receptor ligands or vehicle (5 μl) was added as described in the figure legends. Binding was performed for 60 min at room temperature. The beads were then washed four times with the binding buffer with or without ligands and resuspended in SDS-PAGE sample buffer. After electrophoresis, fixation, and enhancement, the radiolabeled proteins were visualized by autoradiography.

Full-length PGC-1 with an HA-tagged N terminus was generated by PCR and cloned into SmaI of pSV-SPORT. Cell extracts and immunoprecipitation from transfected cells were done as in Lassar et al. (1991). Rabbit anti-murine PPARγ (Hu et al., 1996) was used as a 1:500 dilution for immunoprecipitation. An anti-HA mouse monoclonal antibody (Boehringer Mannheim) was used at 1:200 dilution for Western blot that was developed using ECL (Amersham).

GFP fused to the full-length PGC-1 was generated by cloning this coactivator in-frame into the SalI and BamHI sites of pEGFP-C1 (Clontech). Cellular localization was visualized 24 hr after transfection using a Nikon Diaphora 200 microscope.

Transcriptional Activation Assays

An expression plasmid for full-length PGC-1 was constructed by first ligating the entire 3 kb cDNA as a SmaI-XhoI fragment into SmaI-SalI sites of pSV-SPORT (GIBCO-BRL). The −3740/+110 bp *UCP* promoter was described previously (Kozak et al., 1994). Rat1 IR fibroblasts were cultured in DMEM containing 10% cosmic calf serum and transfected at 80%–90% confluence by the calcium phosphate method. Ligands were dissolved in a vehicle containing 0.1% DMSO (9-*cis* retinoic acid and troglitazone) or water (8-bromo-cAMP). Transfections were performed in duplicate and repeated at least three times. CAT activity was assayed as described (Kim and Spiegelman, 1996).

For GAL4 fusion constructs, full-length PGC-1 generated by PCR was cloned in-frame into the SalI-EcoRV sites of pCMX-GAL4 plasmid. Murine full-length SRC-1 was cloned into the SmaI site of RSV.GAL4. COS cells were transfected in the same way as Rat1 IR fibroblasts and the reporter was the 5xUASg-CAT.

Northern and Southern Blot Analysis

Total RNA was isolated from cultured cells and tissues of mouse by guanidine isothiocyanate extraction. RNA samples were processed as previously described (Tontonoz et al., 1994b). For Southern blot, genomic DNA was isolated and processed from cells as described (Maniatis et al., 1989).

Cell Culture, Retroviral Infection, and Differentiation

The *PGC-1* viral expression vector (pBabe-PGC-1) was constructed by ligating the BamHI-XhoI fragment from pBluescript-PGC-1 plasmid into BamHI/SalI sites of pBabe-puro (Pear et al., 1993). Following drug selection, virally infected 3T3F442A-PGC-1 and 3T3F442A-vector cell lines were grown to confluence in DMEM with 10% BCS. Differentiation of these cells was initiated by culturing them in DMEM containing 10% cosmic calf serum (CCS, Hyclone) and 850 nM insulin. They were refed every 2 days with this medium. Specific treatments of the cells are indicated in the figure legends. HIB-1B cells were grown in DMEM with 10% CCS to confluence. They were then treated with 1 μM dexamethasone, 0.5 mM of methyl-isobutyl-xanthine, 125 μM indomethacin, 17 nM insulin, and 1 nM T_3 for 48 hr to induce differentiation. Cells were subsequently maintained

in DMEM containing 10% CCS, 17 nM insulin, and 1 nM T_3 and replenished every 2 days.

Animal Protocols

Four-week-old male C57BL/6J mice were used in animal experiments. These were fed ad libitum and 10 animals were grouped per cage. A control group was kept at 24°C, while experimental groups were kept at 4°C for 3 or 12 hr. Animals were sacrificed and tissues were dissected and collected immediately.

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