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An adipogenic cofactor bound by the differentiation domain of PPARγ

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Ligand activation of the nuclear receptor PPARγ induces adipogenesis and increases insulin sensitivity, while activation of other PPAR isoforms (-α and -δ) induces little or no fat cell differentiation. Expression and activation of chimeras formed between PPARγ and PPARδ in fibroblasts has allowed us to localize a major domain of PPARγ responsible for adipogenesis to the N-terminal 138 amino acids, a region with AF-1 transcriptional activity. Using this region of PPARγ as bait, we have used a yeast two-hybrid screen to clone a novel protein, termed PGC-2, containing a partial SCAN domain. PGC-2 binds to and increases the transcriptional activity of PPARγ but does not interact with other PPARs or most other nuclear receptors. Ectopic expression of PGC-2 in preadipocytes containing endogenous PPARγ causes a dramatic increase in fat cell differentiation at both the morphological and molecular levels. These results suggest that interactions between PGC-2, a receptor isoform-selective cofactor and PPARγ contribute to the adipogenic action of this receptor.

Keywords: adipogenesis/coactivator/differentiation/PGC-2/PPARγ

Introduction

The nuclear receptors represent a very large family of >120 proteins. These factors enable cells to respond to changes in the extracellular environment with an altered pattern of gene expression. In addition to the classical receptors for steroid hormones, this family also includes receptors for retinoids, thyroid hormone and vitamin D. Much interest has focused in more recent years on the so-called orphan receptors, those members of this family whose endogenous ligands are not yet known. Examples of orphan nuclear receptors are Nurr-1 (Law *et al*., 1992; Saucedo-Cardenas *et al*., 1997), SRF-1 (Politz *et al*., 1987) and the PPARs. Although several low-affinity natural ligands for the PPARs have been described (Forman *et al*., 1995; Kliewer *et al*., 1995, 1997), there is not yet a consensus about the identity of endogenous ligands that activate these receptors in cell nuclei.

The PPARs have attracted a great deal of attention because they play several important and relatively well-defined roles in lipid homeostasis and energy metabolism. PPARα is crucial in the expression of certain enzymes of β-oxidation and in the process of peroxisome proliferation, as illustrated by deficiency of these processes in the PPARα knockout mouse (Lee *et al*., 1995). PPARγ is expressed at the highest level in fat and at lower levels in many other tissues (Dreyer *et al*., 1992; Tontonoz *et al*., 1994a; Flier, 1995). This receptor exists as two isoforms, γ2 expressed only in fat and γ1, which is expressed in non-adipose tissues; the functional significance of these splice variants is not clear. PPARγ plays a dominant role in lipogenesis, adipocyte differentiation and systemic insulin sensitivity. Ligand activation of PPARγ in fibroblasts or preadipocytes causes full adipogenic differentiation, including lipid accumulation, increased hormone sensitivity and the cessation of cell growth (Tontonoz *et al*., 1994b; Altiok *et al*., 1997). Ligand administration *in vivo* causes an increase in fat cell number (Okuno *et al*., 1998) and an increase in systemic insulin sensitivity that involves fat, muscle and liver. The first ligands described for PPARγ were the thiazolidinedione (TZD) drugs, such as troglitazone, pioglitazone and BRL 49653 (Forman *et al*., 1995; Lehmann *et al*., 1995). These compounds have affinities for PPARγ in the 50–700 nM range and were developed without knowledge of their molecular targets. They increase insulin sensitivity *in vivo*, and one of them, troglitazone (Rezulin™), is currently used in the treatment of Type II diabetes (reviewed in Spiegelman, 1998). They are also under clinical investigation for the treatment of other conditions involving insulin resistance, such as polycystic ovary syndrome (Dunaif *et al*., 1996). In addition, they have promising differentiation-inducing, anti-proliferative activities in liposarcoma and other malignancies (Tontonoz *et al*., 1997; Elstner *et al*., 1998; Mueller *et al*., 1998; Sarraf *et al*., 1998; Demetri *et al*., 1999).

It is now appreciated that nuclear receptors serve as platforms for the docking of multiprotein complexes that carry much of the transcriptional activation function initiated by the receptors. These factors are generally termed 'coactivators' (Glass *et al*., 1997; Torchia *et al*., 1998). Proteins such as SRC-1/NCoA-1 (Zhu *et al*., 1996), CBP/p300, pCAF, TRAP220 (Yuan *et al*., 1998) and PGC-1 (Puigserver *et al*., 1998) modulate the activity of PPARγ in both ligand-dependent and independent fashions. The docking of many of these factors occurs at the extreme C-terminus of the receptors, on the AF-2 or helix 12 region of the ligand-binding domain, which shows sequence

conservation among most nuclear receptors. On the coactivator side, ligand-dependent binding is mediated primarily through one or more short LXXLL sequence motifs that are both necessary and sufficient to achieve coactivator–receptor interaction. Many of the coactivator complexes are associated with histone acetyl transferase (HAT) activity, which is thought to modify histones and place chromatin in a permissive state for gene transcription (Ogryzko *et al*., 1996; Yang *et al*., 1996; Korzus *et al*., 1998). In addition to the coactivators mentioned above, the laboratories of Roeder (Fondell *et al*., 1996; Yuan *et al*., 1998) and Freedman (Rachez *et al*., 1998) have identified a multiprotein complex that interacts with the thyroid receptor and vitamin D receptor (so-called TRAPs and DRIPs) but contains a completely different set of proteins.

One key question that has not been explored thoroughly is how the specificity of function of the different nuclear receptors is generated. One contributing factor could be through the developmental and/or physiological regulation of particular coactivators linked to particular biological actions. While most coactivators have not been found to be highly regulated *in vivo*, we have recently described a coactivator (PGC-1) that is expressed in a limited set of tissues and is induced by cold exposure in those tissues (muscle and brown fat) that play a primary role in adaptive thermogenesis (Puigserver *et al*., 1998). When expressed ectopically in cultured cells, it specifically activates several aspects of mitochondrial function that are part of the thermogenic program.

The sharp distinction in biological actions shown by the variety of nuclear receptors suggests that certain key interactions with coactivators or other proteins may be highly selective. In this report, we have attempted to study in detail the molecular mechanisms giving rise to biological specificity by comparing two receptors, PPARγ and PPARδ, that bind to very similar target sequences but have completely different biological actions. While ligand activation of PPARγ readily promotes fat cell formation, activation of PPARδ does not lead to adipogenesis (Brun *et al*., 1996). Functional analysis of chimeras between these two receptors reveals an N-terminal region that contains much of the adipogenic action of PPARγ. We have also identified a novel sub-type selective cofactor that binds to this region and promotes the transcriptional and adipogenic action of PPARγ.

Results

The N-terminus of PPARγ has strong adipogenic action

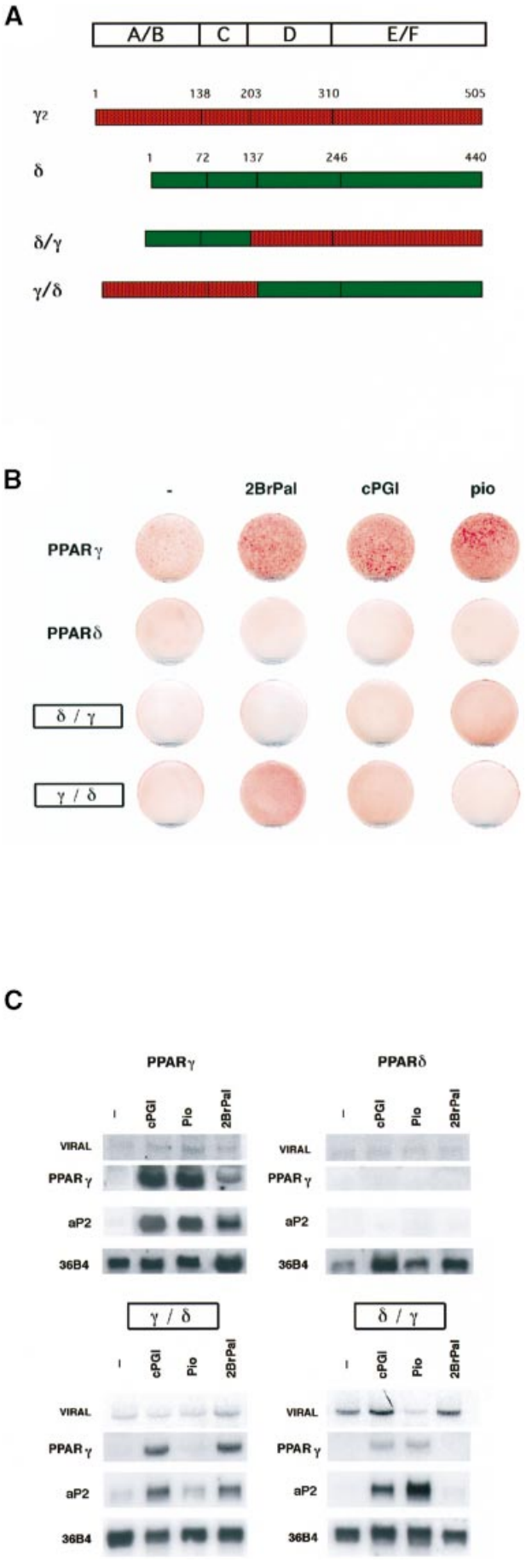
PPARγ is highly adipogenic while PPARδ has no adipogenic action (Brun *et al*., 1996). This difference provides an opportunity to map the region(s) of PPARγ responsible for its adipogenic actions through the use of chimeric molecules. Both of these factors have the same overall domain structure typical of nuclear receptors: an A/B domain that, in some receptors, including PPARγ, contains a ligand-independent transcriptional activation function (AF-1); a C domain responsible for DNA binding; a D hinge region and a large E/F region that is responsible for ligand binding, dimerization and the major transcriptional activation function (AF-2).

To perform these studies, we have utilized pioglitazone, a highly selective agonist for PPARγ of the TZD class (Forman *et al*., 1995), 2-bromopalmitate (Amri *et al*., 1995), an activator of both PPARγ and PPARδ and carbaprostacyclin (cPGI), an activator of both receptors, with some preference for PPARδ (Brun *et al*., 1996). As illustrated in Figure 1A, we first used reciprocal swaps of the A/B and C domains into the D and E/F region of the opposite receptor. cDNAs encoding wild-type and chimeric receptors were cloned into retroviral vectors and packaged, and the resulting virions were used to infect NIH 3T3 cells. mRNA for all of these receptors was expressed at similar levels (Figure 1C). As shown in Figure 1B and C, and as in Brun *et al*. (1996), cells expressing wild-type PPARγ2 are highly adipogenic when treated with any of these ligands/activators. In contrast (again, as shown in Brun *et al*., 1996), no adipogenesis was seen in cells expressing wild-type PPARδ. Cells that expressed the N-terminal half of PPARδ fused to the C-terminus of PPARγ (δ/γ) were very weakly adipogenic, showing some differentiation only in response to the strongest and most selective agonist for PPARγ, pioglitazone. The weak agonist, 2-bromopalmitate, was sufficient to induce adipogenesis through wild-type PPARγ, but failed to induce differentiation through the δ/γ chimera. The reverse chimera, γ/δ, showed a more robust differentiative response in response to 2-bromopalmitate and carbaprostacyclin, but no response to pioglitazone, consistent with activation through the ligand-binding domain. At the mRNA level (Figure 1C), it is also clear that the differentiative response of the δ/γ chimera was rather minimal, despite the fact that this molecule contains the major transcriptional activation and ligand-binding domains of PPARγ. In contrast, the γ/δ chimera expresses two markers of differentiation, endogenous PPARγ and aP2, at robust levels. These results suggest that a large portion of the adipogenic activity detectable in these chimeras lies in the N-terminal portion of PPARγ, though there is also some residual activity in the C-terminal half.

The A/B and C domains of PPARγ were further analyzed individually by exchanging these regions with the equivalent segments of PPARδ. After infection into NIH 3T3 cells and treatment with various ligands/activators, only the chimera containing the A/B domain of PPARγ (NH_2-γ2/δ) induced the differentiation program, while the chimera containing only the C (DNA-binding) domain (DBD-γ/δ) did not (Figure 2A, B and C). Experiments performed with the translation product of both chimeras showed that they bound to DNA in a manner comparable with that of the wild-type proteins (data not shown). Figure 2B and C also illustrates that only ligands that activate PPARδ could induce differentiation of the NH_2-γ2/δ chimera. The overall level of differentiation stimulated by this chimera, as assayed by lipid accumulation and induction of fat-specific mRNAs, was >50% of that seen with the wild-type PPARγ. Taken together, these results indicate that a major portion of the adipogenic action of PPARγ, as assayed on a PPARδ backbone, resides in the A/B domain.

Cloning of a novel cofactor that binds to the N-terminus of PPARγ

We next sought to determine whether the differentiation-inducing A/B domain of PPARγ might function as a



binding site for a cofactor that modulates or contributes to this function. Previous work done by others (Adams *et al*., 1997; Werman *et al*., 1997) has shown that the N-terminal A/B domain of PPARγ contains an AF-1, ligand-independent transcriptional activity when fused to the Gal4 DNA-binding domain. Consistent with this, we found that this region is a very powerful activator of transcription in yeast, complicating our efforts to use this region as bait in yeast two-hybrid screens. However, by using high concentrations of the drug 3-amino-1,2,4-triazole, we could suppress the levels of the *His3* gene driven by the A/B domain of PPARγ alone. We were then able to detect interactions with other proteins in the yeast two-hybrid screen (see Materials and methods for detail).

A small protein of 142 amino acids, termed PGC-2 (PPAR gamma coactivator-2), emerged from the screen and showed strong and selective interactions with the A/B domain of PPARγ (see below). As shown in Figure 3, PGC-2 is not identical to any known proteins in available databases, but displays homology to several proteins containing a 'SCAN' box such as the zinc-finger proteins 174 (ZNF174), 165 (ZNF165) and 192 (ZNF192) (Williams *et al*., 1995; Lee *et al*., 1997). In fact, PGC-2 contains half of the domain identified as a SCAN motif. This motif has no known function, but has been seen in several zinc-finger-containing transcriptional repressors of the Kruppel type. PGC-2 itself has neither obvious zinc fingers nor Kruppel A or B motifs.

Like PPARγ, PGC-2 mRNA is expressed in most tissues; unlike PPARγ, however, its expression is not elevated in fat tissues (Figure 4A) nor is it induced during adipogenesis (Figure 4B). Transfection of an allele of PGC-2 fused to green fluorescent protein (GFP) illustrates that while wild-type GFP protein accumulates in both the nucleus and cytoplasm, GFP–PGC-2 resides exclusively in the cell nucleus.

Glutathione *S*-transferase (GST) fusion proteins were used in combination with reticulocyte lysate-translated material to analyze PPARγ–PGC-2 interactions *in vitro*. *In vitro*-translated PGC-2 appears as two major bands of 28 and 25 kDa when produced in a reticulocyte lysate (Figure 5A); the biochemical basis of this doublet is not clear. About 20% of the input PGC-2 is retained on beads containing the 138 amino acids of the N-terminal A/B domain of PPARγ2 linked to GST, while there is no detectable binding to beads containing similar amounts (data not shown) of only the GST protein or the A/B domain of PPARδ. A key question is whether PGC-2 can interact with the full-length PPARγ2. As shown in Figure 5B, PGC-2 is bound as well by full-length PPARγ as it is by the isolated N-terminal A/B domain.

The selectivity of the interaction between PGC-2 and

Fig. 1. Adipogenic potential of PPAR γ/δ chimeras (half swaps): PPARs were ectopically expressed in NIH 3T3 cells and assayed for their ability to induce the adipocyte differentiation program. (**A**) Schematic diagram of recombinant PPARs. (**B**) Differentiation assayed by cellular lipid acumulation: the cells were fixed and stained with Oil Red O, which stains the neutral lipid accumulated in differentiated adipocytes. (**C**) Differentiation assayed by gene expression: Northern blot analysis of total RNA of the cells expressing the chimeric PPARs shown in (A) probed for the adipocyte-specific genes PPARγ and aP2. 36B4 was used as a control for loading.

a wide variety of nuclear receptors was investigated utilizing full-length PGC-2 fused to GST (GST–PGC-2, Figure 5C and D). Consistent with the data shown above, the *in vitro*-translated PPARγ interacts with GST–PGC-2 but not with GST alone. As might be expected for an interaction through the N-terminal domain, it is not affected by the presence of a PPARγ ligand. No interaction is detectable between PGC-2 and PPARα or PPARδ (Figure 5C). There is also no detectable interaction with RARα, RORγ or RXRα (Figure 5D; data not shown). A very small amount of the thyroid hormone receptor β (TRβ) (<3% of input) was retained on these beads, while ~10% of the input of the estrogen receptor (ER)-α was bound by these PGC-2 beads (Figure 5D). These data suggest that the physical interaction between PPARγ and PGC-2 appears completely specific among the PPARs and is selective, but not entirely specific, within the nuclear receptor gene family.

We also asked whether PGC-2 could associate with PPARγ in a cellular context. Cells were transfected with vectors expressing His-tagged PGC-2, PPARγ or both. Extracts from these cells were subjected to precipitation with nickel-linked beads to capture the His-tagged protein and then immunoblotted for PPARγ. As shown in Figure 5E, PPARγ and PGC-2 form a complex when these two factors are cotransfected into COS-7 cells.

PGC-2 augments the transcriptional and adipogenic activities of PPARγ

The ability of PGC-2 to affect the transcriptional activity of the three PPAR isoforms was investigated using full-length PPARs. These were combined with the heterodimeric partner of PPARs, RXRα and an appropriate PPAR ligand: pioglitazone (selective for PPARγ), Wyeth14,653 (selective for PPARα) and carbaprostacyclin (somewhat selective for PPARδ). Transcriptional activity was determined using a luciferase reporter gene linked to multiple PPAR response elements. Co-expression of PGC-2 stimulates no increase in the transcriptional activity of PPARα or PPARδ, with or without added ligand (Figure 6A). PGC-2 does not significantly increase the transcriptional activity of PPARγ without added ligand. However, when pioglitazone is added, PGC-2 stimulates an increase of at least 3-fold in activity relative to the activity obtained from cells that received ligand but no PGC-2. These results demonstrate that, among the PPARs, PGC-2 specifically increases the transcriptional activity of PPARγ.

Because the protein-binding data in Figure 5 suggest that PGC-2 physically interacts with ERα, we investigated whether PGC-2 can increase the transcriptional activity of this receptor. To do this, a PGC-2 expression vector was cotransfected into MCF7, a cell line expressing endogenous ERα and β, along with a plasmid containing several copies of an ER response element linked to a luciferase reporter gene. As shown in Figure 6B, PGC-2 increases the transcriptional activity through the ERs

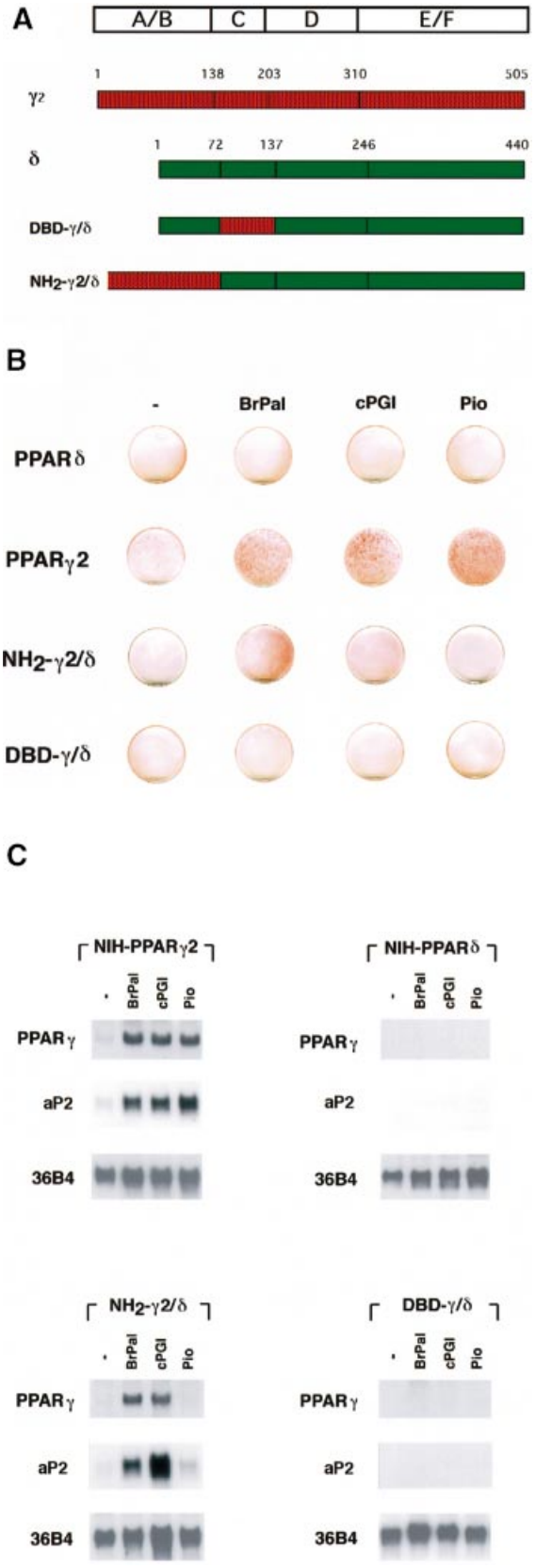


Fig. 2. Adipogenic potential of PPAR γ/δ N-terminal chimeras. PPARs were ectopically expressed in NIH 3T3 cells and assayed for their ability to induce the adipocyte differentiation program. (**A**) Schematic diagram of recombinant PPARs. (**B**) Differentiation assayed by cellular lipid accumulation: the cells were fixed and stained with Oil Red O, which stains the neutral lipid accumulated in differentiated adipocytes. (**C**) Differentiation assayed by gene expression: Northern blot analysis of total RNA of the cells expressing the chimeric PPARs shown in (A) probed for the adipocyte-specific genes PPARγ and aP2. 36B4 was used as a control for loading.

2-fold. These data indicate that PGC-2 activates both PPARγ and ER.

We next investigated whether PGC-2 could augment the ligand-independent transcriptional activity associated with the AF-1 region of PPARγ. This was done by fusing the GAL4 DBD to the first 138 amino acids of PPARγ2 and assaying the transcriptional activity using a luciferase reporter containing GAL4 target sequences. As shown in Figure 6C, PGC-2 does not increase the transcriptional activity of the isolated AF-1 domain, suggesting that other domains of PPARγ are also required for PGC-2 to function.

Finally, to determine whether PGC-2 displays intrinsic transcriptional activity, as do many coactivators, we made fusion proteins between PGC-2 and the DNA-binding domain of GAL4. Activity was then assayed through a luciferase reporter containing GAL4 target sequences. As shown in Figure 6D, GAL4 fusion proteins of PGC-2 do not activate transcription strongly. This was true whether GAL4-DBD was fused to the N-terminus or the C-terminus of PGC-2. In contrast, a GAL4 fusion with a known coactivator, PGC-1, greatly increased the GAL4 reporter activation. Thus, PGC-2 appears not to bear an intrinsic transcriptional activation domain.

PPARγ has powerful adipogenic action when it is expressed in fibroblasts and activated by ligands. While the data above show that PGC-2 binds to PPARγ and augments its transcriptional activity, these experiments have all been in the context of reporter genes and transient transfections. Thus, it is of great interest to determine whether this protein can alter the biological activity of PPARγ. To investigate this, full-length PGC-2 was cloned into a retroviral vector, and the resulting virus was used to infect a cell type that expresses endogenous PPARγ, 3T3-L1 preadipocytes. Instead of using the standard differentiation-inducing cocktail with dexamethasone and isobutylmethylxanthine, which gives nearly complete differentiation of 3T3-L1 cells, we provided cells with either no PPARγ ligand or a limited amount of pioglitazone. When cells received no ligand, PGC-2 expression did not stimulate significant morphological differentiation (Figure 7A and B). However, examination of mRNA levels indicates that there is a low but significant level of expression of fat differentiation-linked genes in the PGC-2-expressing cells. mRNA of PPARγ, aP2 and adipsin are all elevated (Figure 7C). Interestingly, endogenous PGC-2 mRNA is also elevated in the cells expressing the viral PGC-2. When suboptimal levels of pioglitazone are applied (1 μM), a major difference in morphological differentiation and lipid accumulation is observed between control cells and those expressing PGC-2 (Figure 7A and C). At the

A

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   1 gaattcggcacgagagcttcgcgggtcgagacgcaggcgaggcgccggactgcgaaacaa
  61 agggggacgccaacagccgtagtcATGGCGGCGCCGCAGCCCAGCCAGGACCCCCAAAGC
   1                          M  A  A  P  Q  P  S  Q  D  P  Q  S

 121 CCCGCGGCGCCTCCCGAGCAGCAGGAAGGAGCCGGCGACTGTGCGGCCCCGTCGCCCGAT
  13 P  A  A  P  P  E  Q  Q  E  G  A  G  D  C  A  A  P  S  P  D

 181 TCCGGGAGCTCCCCGGCCCCGGAGCTCCCCGGCGCGCCCGCGGCTTTGAAcACGGCGCCC
  33 S  G  S  S  P  A  P  E  L  P  G  A  P  A  A  L  N  T  A  P

 241 TACGCTGACgCCGTCCTGCGGCCCGGAGCCTCTCGGCCCGGCCCCGAGACCTTTCGCCAG
  53 Y  A  D  A  V  L  R  P  G  A  S  R  P  G  P  E  T  F  R  Q

 301 CGCTTCCGGCAGTTTCGCTACCAGGACGCGGCCGGGCCCCGGGAAGCCTTCCGGCAGCTG
  73 R  F  R  Q  F  R  Y  Q  D  A  A  G  P  R  E  A  F  R  Q  L

 361 CGGGAGCTGTCCCGCCAGTGGCTGCGGCCGGACATCCGCACCAAGGAGCAGATCGTGGAG
  93 R  E  L  S  R  Q  W  L  R  P  D  I  R  T  K  E  Q  I  V  E

 421 ATGCTGGTGCAGGAGCAGCTGCAGGCCATTCTACCGGAGGCGGCCCGGGCTCGGCGGCTG
 113 M  L  V  Q  E  Q  L  Q  A  I  L  P  E  A  A  R  A  R  R  L

 481 CGGCGCCGCGCGGACGTGCGCATCACCGGCTGAGCGGCGGAGCCTGGGGCTCCTTGGACT
 133 R  R  R  A  D  V  R  I  T  G
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B

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 541 gacaccctacccctgagtt**aataaa**agttgattttgacagct
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E T F R Q R F R Q F R Y Q D A A G P R E A F R Q L R E L S R Q W L R P D I R T K E Q I V E M L V Q E Q L Q A I L P E  PGC2
E T S H L H F R Q L R Y H E T S G P Q E A L S R L R E L C R R W L R P E A R T K A Q I L E L L V L E Q F L S I L P G  AF011573
E A C R Q R F R Q F C Y G D V H G P H E A F S Q L W E L C C R W L R P E L R T K E Q I L E L L V L E Q F L T V L P    AF017433
- - - - - - F R Q L R Y Q E V A G P E E A L S R L R E L C R R W L R P E L L S K E Q I L E L L V L E Q F L T I L P E  bbs|153833
E I S R Q R F R Q F G Y H D T P G P R E A L S Q L R V L C C E W L Q P E I H T K E Q I L E L L V L E Q F L T I L P    CTFIN51
E A S H L R F R R F R F Q E A A G P R E A L S R L Q E L C H G W L R P E M R T K E Q I L E L L V L E Q F L T I L P Q  D88827
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E V F R L R F R Q L R Y Q E T L G P R E A L I Q L R A L C H Q W L R P D L N T K E Q I L E L L V L E Q F L T I L P E  U57796
E I F R R H F R Q L C Y Q E T P G P R E A L T R L Q E L C Y Q W L R P H V S T K E Q I L D L L V L E Q F L S I L P K  U62392
E I F R Q R F R Q F G Y Q D S P G P R E A V S Q L R E L C R L W L R P E T H T K E Q I L E L V V L E Q F V A I L P -  U68536
E I S R Q R F R Q F G Y H D T P G P R E A L S Q L R V L C C E W L Q P E I H T K E Q I L E L L V L E Q F L T I L P    X63747
E L C R Q L F R Q F C Y Q D S P G P R E A L S R L R E L C C Q W L K P E I H T K E Q I L E L L V L E Q F L T I L P    X84801
E I F R Q R F R Q F G Y Q D S P G P R E A V S Q L R E L C R L W L R P E T H T K E Q I L E L V V L E Q F V A I L P -  ZNF191
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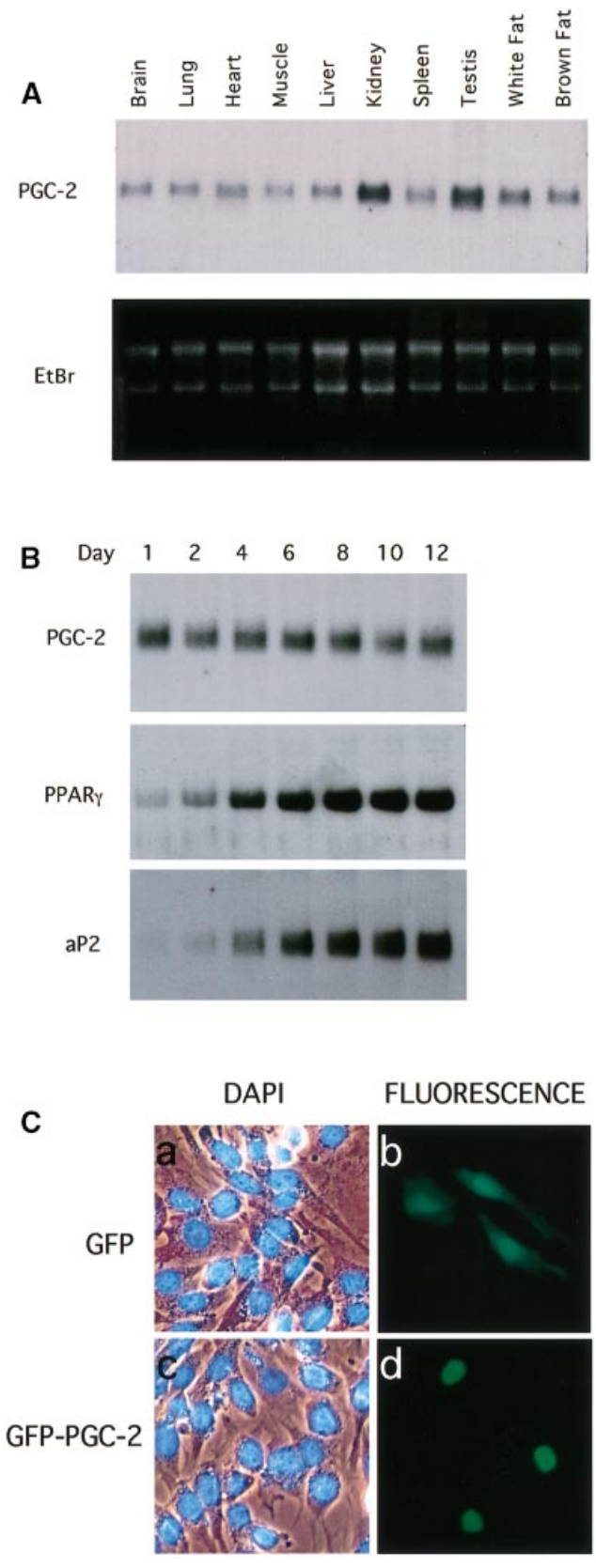
Fig. 3. cDNA sequence of PGC-2. (**A**) Nucleotide sequence of PGC-2. The single polyadenylation sequence is shown in bold. The putative SCAN domain is boxed. (**B**) Sequence alignment of the PGC-2 SCAN domain and the known SCAN domain-containing genes as shown by their DDBJ/EMBL/GenBank accession numbers.

mRNA level, this difference is very large, at least a 10-fold increase in expression of PPARγ, aP2 and adipsin. These results clearly demonstrate that PGC-2 dramatically augments the adipogenic activity of PPARγ in a ligand-dependent way.



Discussion

The molecular basis for the great specificity of action of the nuclear receptors is still largely obscure. Certainly, part of the difference in biological functions must derive from differences in the DNA binding specificity of various receptors. While most or all nuclear receptors bind to core 'hormone response elements', these can be arranged as direct repeats or inverted repeats, with a variable but critical number of bases between repeats. On the other hand, many receptors bind to the same or closely related sequences and still carry out very different biological actions upon binding of cognate ligands. For example, numerous receptors bind to direct repeats separated by one base, so-called DR-1 elements. These include all three PPARs, COUP-TF and HNF4. None of these other receptors have the well-known adipogenic or lipogenic actions of PPARγ. This suggests that there are likely to be specific or selective protein–protein interactions that allow each receptor to carry out its distinct biological role.

The known coactivators for nuclear hormone receptors are typically quite large proteins which dock on receptors through the C-terminal AF-2 domain in a manner that is usually ligand dependent. They promote ligand-dependent transcriptional activity that is often linked to histone acetyltransferase (HAT) activity (Ogryzko *et al*., 1996; Yang *et al*., 1996; Korzus *et al*., 1998). The coactivators which interact with PPARγ, such as SRC-1, pCAF and CBP/p300, form very large complexes that generate powerful transcriptional activity (Zhu *et al*., 1996; Yuan *et al*., 1998). A biological role for most coactivators is not known. However, data from mice bearing null mutations in SRC-1 support a critical role for this factor in the development of the breast epithelium, at least partly through its interaction with the estrogen receptor (Xu *et al*., 1998).

The coactivators of nuclear receptors represent something of a paradox. Despite the documented and powerful roles for several of the known coactivators, they show little or no selectivity among the nuclear receptors. Most show the ability to interact with all or nearly all members of the nuclear receptor family and mediate transcription upon ligand binding. We have recently identified one coactivator, PGC-1, that is linked to a particular biological response, in that it is induced in brown fat and skeletal muscle upon cold exposure and activates a genetic program of thermogenesis (Puigserver *et al*., 1998). However, it appears that execution of this biological program by PGC-1 is regulated primarily via regulated expression in tissues and physiological states and not by selectivity among nuclear receptor targets.

Fig. 4. Expression and subcellular localization of PGC-2. (**A**) Northern blot analysis of mouse tissues. Ethidium bromide (EtBr) staining is shown as a loading control. (**B**) Expression of PGC-2 during differentiation of 3T3-F442A cells. The induction of PPARγ and aP2 is shown as a control. The numbers show days post-induction with insulin. (**C**) NIH 3T3 cells were transfected with GFP or GFP fused to PGC-2. Panels a and c show phase contrast/DAPI staining and panels b and d show green fluorescence of cells transfected with GFP (a and b) or GFP–PGC-2 (c and d).

The detailed studies described here attempt to understand how a particular cellular function, adipogenesis, is controlled by PPARγ. This is a useful biological program through which the mechanistic basis of receptor specificity may be approached, in that the other PPARs either have little (PPARα) or no (PPARδ) adipogenic action, despite binding to similar or identical DR-1 sites. As shown in Figures 1 and 2, domain swaps between PPARδ and PPARγ clearly indicate that a major portion of the differentiation-inducing activity of PPARγ resides in the N-terminal 138 amino acids. When this region substitutes the equivalent region of PPARδ, this chimeric receptor has strong adipogenic action, despite the fact that the activators of the PPARδ ligand-binding domain used (2-bromopalmitate or carbaprostacyclin) are not as potent or effective as the thiazolidinedione ligand (pioglitazone) that is used to

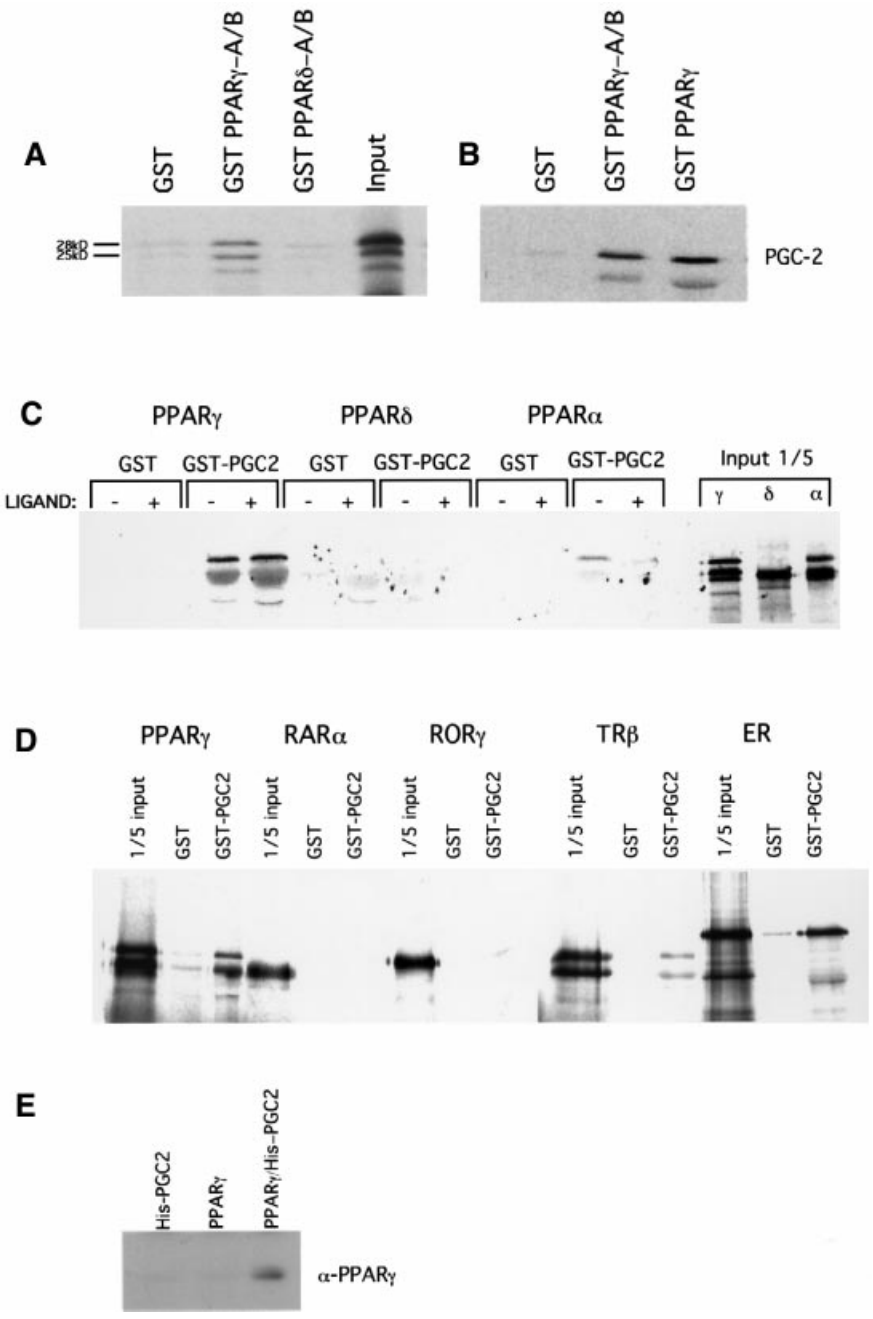


Fig. 5. Interaction of PGC-2 and nuclear hormone receptors *in vitro*. (**A**) The N-terminal regions of PPARγ2 (amino acids 1–18) or PPARδ (amino acids 1–72) were fused to GST, immobilized on glutathione beads and incubated with ^{35}S-labeled *in vitro*-translated PGC-2. The beads were washed, and the bound proteins were eluted, separated by SDS–PAGE and visualized by autoradiography. (**B**) Same as (A), but using full-length PPARγ or the N-terminal region of PPARγ. (**C**) Interaction of PGC-2 with the three PPARs. PGC-2 fused to GST and immobilized on glutathione beads was incubated with *in vitro*-translated PPARα, PPARγ or PPARδ in the absence or presence of pioglitazone, carbaprostacyclin or Wyeth14,643, respectively. Bound proteins were analyzed as described above. (**D**) Interaction of PGC-2 with various nuclear hormone receptors. PGC-2 fused to GST and immobilized on glutathione beads was incubated with *in vitro*-translated PPARγ, RARα, RORγ, TRβ or ERα. The bound proteins were analyzed as described above. (**E**) Interaction of PGC-2 with PPARγ in cells. COS-7 cells were transfected with PPARγ and PGC-2 containing an N-terminal His tag. The cells were harvested and extracts were incubated with beads containing Nickel (Ni^{2+}) ions. After extensive washings, the bound proteins were eluted and separated by SDS–PAGE, followed by Western blotting using an anti-PPARγ antibody.

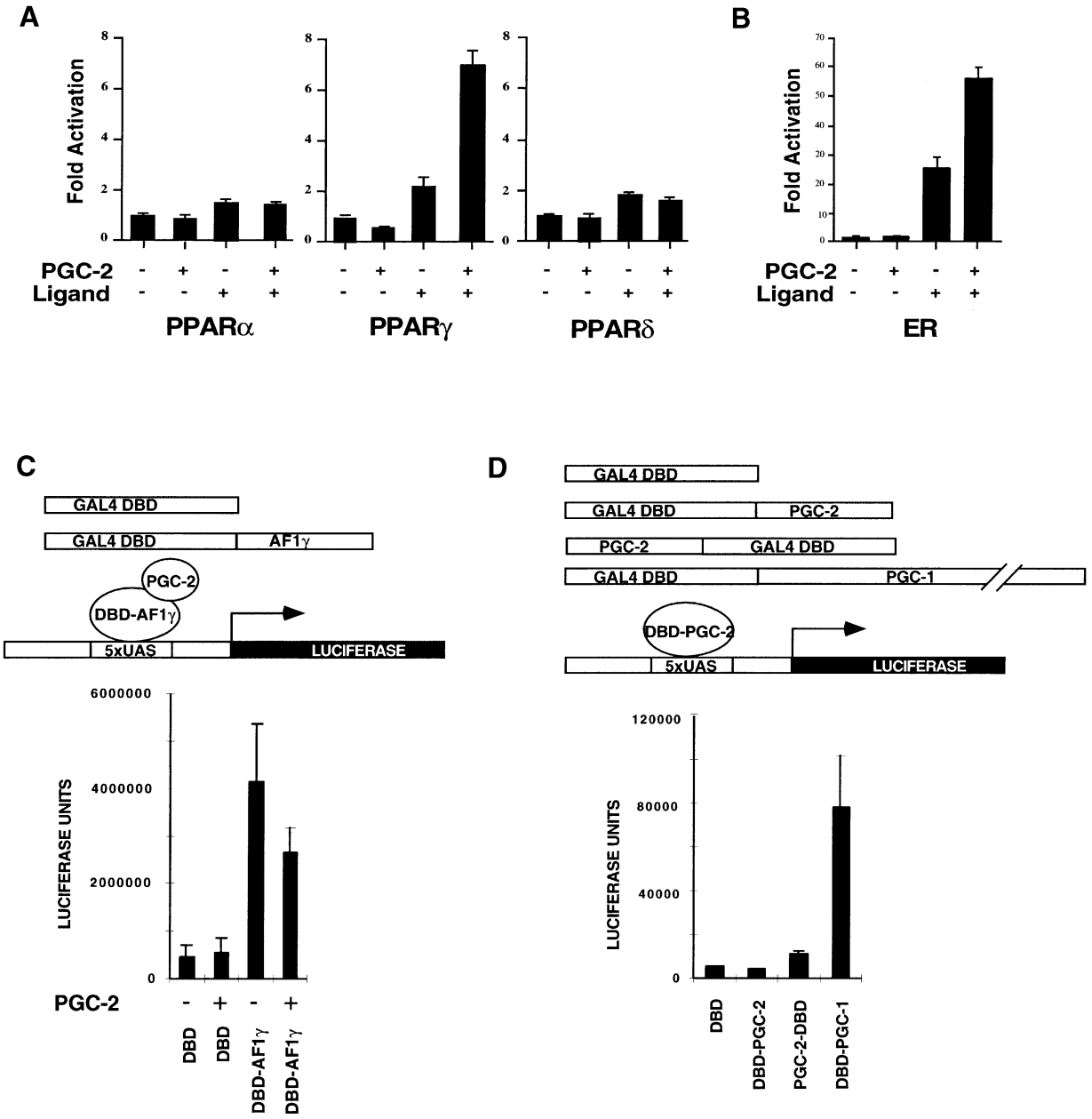


Fig. 6. Coactivation of the PPARγ and ER transcriptional activity by PGC-2. (**A**) PGC-2 coactivates PPARγ, but not PPARα or PPARδ. COS-7 cells were cotransfected with PGC-2, RXRα and either PPARγ, α or δ in the presence or absence of 10 μM pioglitazone, 50 μM Wyeth14,643 or 10 μM carbaprostacyclin, respectively. The transcriptional activity was analyzed using a reporter construct containing three copies of a DR-1 sequence upstream of the luciferase gene. (**B**) PGC-2 coactivates ER. MCF7 cells were cotransfected with PGC-2 and a plasmid containing an ER response element upstream of a luciferase gene. Cells were then treated with or without β-estradiol. (**C**) PGC-2 does not modulate PPARγ AF-1 activity. The AF-1 region of PPARγ was fused to the GAL4 DBD (amino acids 1–147) and cotransfected with PGC-2. Transcription was assayed with a reporter plasmid containing five copies of the UAS linked to luciferase. (**D**) PGC-2 does not display autonomous transcriptional activation. PGC-2 was fused to either the C- or the N-terminus of the GAL4 DBD and expressed in cells. DBD–PGC-1 is shown as a positive control. Transcription was assayed with a reporter plasmid containing five copies of the UAS linked to luciferase. The values represent the mean ± SD of triplicates.

activate the PPARγ ligand-binding domain. While quantitative comparisons are approximations at best, this NH2-γ2/δ chimera induces levels of adipogenic genes that are at least half of that seen with ectopic expression of wild-type PPARγ. On the other hand, it is also clear that the reciprocal swap containing the N-terminal 72 amino acids of PPARδ in a PPARγ backbone also has some adipogenic activity, though the recipient cells show much less lipid accumulation and considerably lower levels of adipogenic gene expression (data not shown). That there is adipogenic action outside of the N-terminal region of PPARγ was also clearly shown in our earlier work. Deletion of the N-terminal 138 amino acids of PPARγ results in a hyperactive allele of PPARγ, relative to the wild-type gene (Tontonoz *et al*., 1994b). This was later shown to be due largely to a phosphorylation site at the N-terminus (serine 112 of PPARγ) that has a very powerful negative effect on PPARγ transcriptional activity (Hu *et al*., 1996; Adams *et al*., 1997). Thus, a simple deletion of the N-terminus causes a deletion of both a major adipogenic domain and a dominant-negative site, resulting in an allele with increased activity. However, a single point mutation

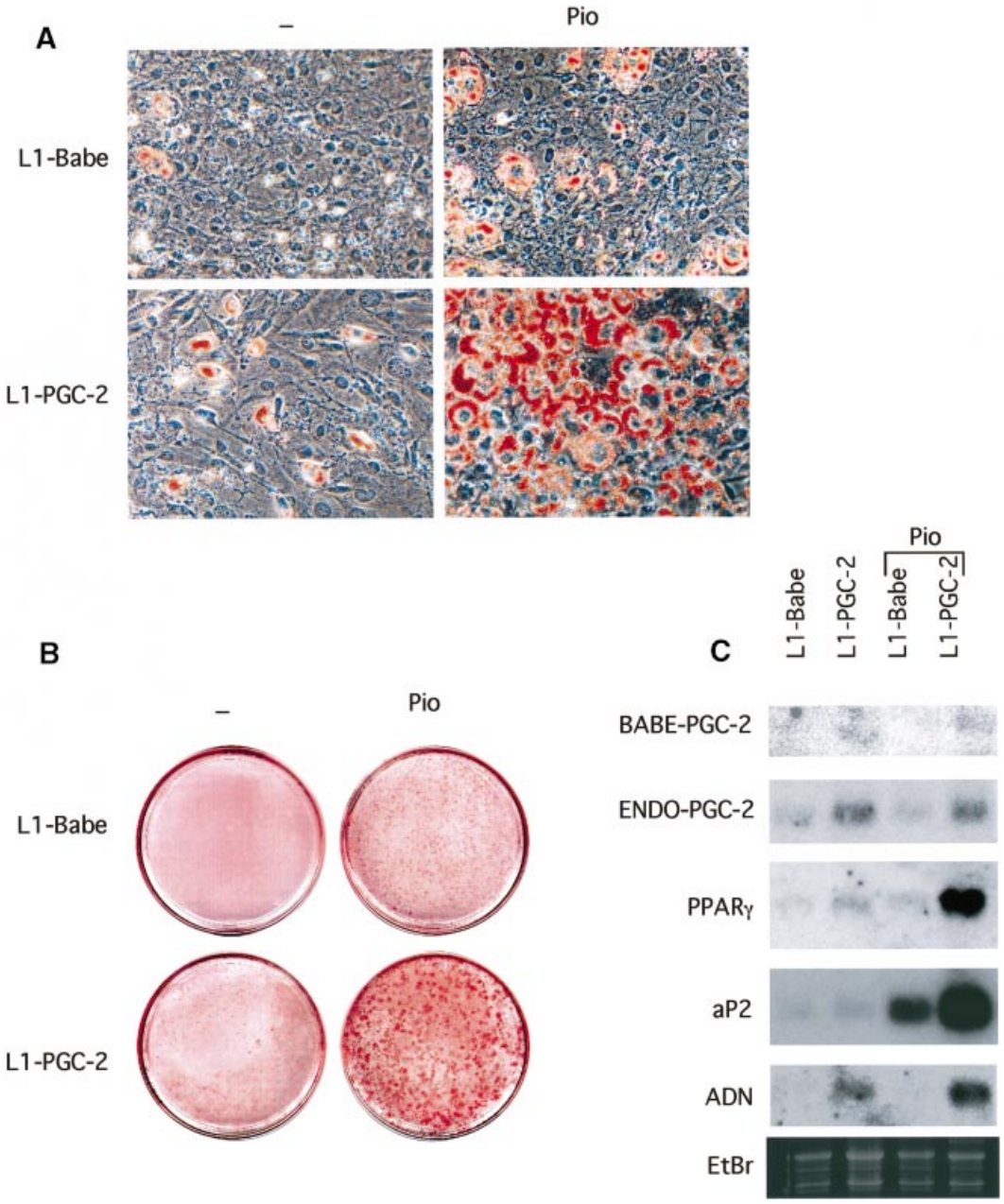


Fig. 7. Ectopic expression of PGC-2 stimulates adipogenesis of 3T3-L1 cells. 3T3-L1 cells were infected with a retrovirus containing either pBABE vector or PGC-2-pBABE. The resulting cells were induced to differentiate with a suboptimal level (1 μM) of pioglitazone for 7 days. The cells were then either fixed and stained with Oil Red O, or RNA was extracted and analyzed by Northern blotting. (**A**) Microscopic views of the Oil Red O-stained cells at 40× magnification. (**B**) Macroscopic views of the Oil Red O-stained dishes. (**C**) Northern blot analysis of the cells showing the ectopic and endogenous expression of PGC-2, as well as the expression of the adipocyte-specific markers PPARγ, aP2 and adipsin (ADN). Ethidium bromide staining is shown as a loading control.

of serine 112 results in an allele that is much more active than the wild-type or N-terminal deletion (Hu *et al*., 1996; Adams *et al*., 1997; data not shown).

Definition of a relatively short region of PPARγ with adipogenic activity allowed a search for a factor that might mediate or contribute to this function of the receptor. PGC-2 has no motifs that have been associated with particular biological actions; however, the C-terminal half of PGC-2 (amino acids 73–132) displays high homology to a region of a so-called SCAN domain (Williams *et al*., 1995; Lee *et al*., 1997) that has been found in several transcriptional regulators and is believed to interact with other nuclear proteins. A more precise function has not been defined.

PGC-2 can increase several important activities of PPARγ. When co-expressed with holoreceptors, it binds to and increases transcription of PPARγ, but not PPARα or PPARδ. These activities appear quite selective for PPARγ but are not entirely specific in that the transcriptional activity of the ER is also increased by PGC-2. Despite the fact that binding of PGC-2 to PPARγ does not require ligand binding, substantial coactivation by

this factor occurs mainly in a ligand-dependent fashion. Presumably, this reflects the participation of other ligand-dependent coactivators for a full transcriptional response. The ligand dependence of PGC-2 was also observed in adipogenic assays. While a very small level of gene activation was observed when 3T3-L1 preadipose cells were not provided with any exogenous ligand or adipogenic stimuli, a very large effect of PGC-2 was seen when cells were provided with a suboptimal concentration of pioglitazone, a PPARγ ligand. Hence, these data argue strongly that PGC-2 interacts in some way with the ligand-gated coactivator machinery that is known to dock at the C-terminal AF-2 helix.

That PGC-2 collaborates with other factors that bind distally on PPARγ is also strongly suggested by other data. First, PGC-2 can not alter the transcriptional activity of the isolated AF-1 domain fused to the GAL4 DBD, despite the fact that this is the major binding site for PGC-2 on PPARγ. Transcriptional coactivation by PGC-2 absolutely requires other parts of this receptor. Secondly, we have found that the N-terminal domain of PPARγ docks several other proteins in addition to PGC-2 when this region is used as an affinity matrix in biochemical studies using whole nuclear extracts of preadipose cells (unpublished data). Finally, PGC-2 itself has no significant transcriptional activation function when fused to the GAL4 DBD. Most, but not all, transcriptional coactivators have intrinsic transcriptional activity in this context. This again suggests that PGC-2 is a cofactor that interacts with certain other coactivators in a manner that increases their transcriptional activity. Of course, since PGC-2 binds selectively to PPARγ, it would presumably cause a selective increase in transcription utilizing components that may not themselves have much binding selectivity. Since functional collaboration between AF-1 and AF-2 regions of the nuclear receptors has long been noted (Tasset *et al*., 1990) it is tempting to suggest that PGC-2 might activate such collaboration and contribute to the specific functions of PPARγ. The components and regions of PPARγ that interact structurally and functionally with PGC-2 remain to be determined.

It has been noted that phosphorylation of PPARγ at the N-terminus (serine 112 of murine PPARγ2) causes a dramatic decrease in transcriptional activity (Hu *et al*., 1996; Adams *et al*., 1997). Most recently, Lazar and colleagues (Shao *et al*., 1998) have observed that modification of this site, by phosphorylation or replacement with an acidic amino acid, lowers the affinity of the holoreceptor for ligand. Interaction of PGC-2 with PPARγ does not seem to be affected by phosphorylation of PPARγ, nor is phosphorylation of this site affected by PGC-2 (data not shown). However, it remains to be determined whether interaction of PGC-2 with the N-terminus of PPARγ can induce conformational changes in PPARγ that could result in increased ligand affinity.

Materials and methods

Plasmids

The retroviral constructs containing cDNAs encoding murine PPARγ2 and PPARδ have been described previously (Tontonoz *et al*., 1994b; Brun *et al*., 1996). cDNAs encoding chimeric receptors were constructed using PCR and cloned as *Sal*I fragments into the *Sal*I site of pBabe-puro (Pear *et al*., 1993). The δ/γ receptor was constructed by fusing amino acids 1–137 of PPARδ to amino acids 204–505 of PPARγ2. The γ/δ receptor contains amino acids 1–203 of PPARγ2 fused to amino acids 137–440 of PPARδ. NH2-γ2/δ contains amino acids 1–138 of PPARγ2 (the A/B domain) fused to amino acids 73–440 of PPARδ. In the DBD-γ/δ receptor, the C domain of PPARδ (amino acids 73–137) has been replaced with the C domain of PPARγ2 (amino acids 139–203). His–PGC-2 was constructed by inserting the full-length cDNA of PGC-2 in-frame into the pCDNA3.1/His expression vector (Invitrogen). GFP–PGC-2 was constructed by inserting the full-length cDNA of PGC-2 in-frame into the pEGFP vector (Clontech).

Yeast two hybrid system

Amino acids 1–138 of PPARγ2 were fused to the GAL4-DBD in the PAS2 yeast vector. A 3T3-F442A adipocyte library was constructed in the PACII vector containing the activation domain of GAL4. Two-hybrid screening was carried out as described (Clontech Matchmaker Two Hybrid System Protocols). PAS2-PPARγ (A/B region) was transfected into the Hf7c yeast strain by the lithium acetate method. PACII-F442A cDNA library was transfected into the yeast containing PAS2-PPARγ and selected for growth in minimal synthetic drop-out media lacking leucine, tryptophan and histidine, in the presence of 50 mM 3-amino-1,2,4-triazole. The clone expressing PGC-2 contained a full-length PGC-2 cDNA.

Viral infection of cell lines

Stable cell lines expressing wild-type and chimeric PPARs were derived as described previously (Tontonoz *et al*., 1994b). BOSC23 cells were cultured in 100 mm dishes and transfected by calcium phosphate coprecipitation with 15 μg of pBabe-puro expression vector as described (Pear *et al*., 1993). Seventy-two hours after transfection, viral supernatants were collected and used to infect NIH 3T3 cells at 50% confluence in 100 mm dishes. Polybrene (2 μg/ml) was added to the viral supernatants before their application. After 24 h incubation with viral supernatant, NIH 3T3 cells were split 1:4 and plated in Dulbecco's modified Eagle's medium (DMEM) containing 10% bovine calf serum (Hyclone) and 2 μg/ml puromycin to select infected cells.

PGC-2-BABE viral expression vector was constructed by inserting the *Eco*RI–*Xho*I fragment from PACII-PGC-2 into pBABE digested with *Eco*RI–*Sal*I. BING cells were cultured in 100 mm dishes and transfected at 70% confluence with 10 μg of pBABE or pBABE-PGC-2. Viral supernatants were collected after 72 h and used to infect 3T3-L1 preadipocytes.

Cell culture and differentiation

Following drug selection, virally infected cell lines were cultured to confluence in DMEM containing 10% cosmic calf serum (Hyclone). Cells were treated at confluence with 1 μM dexamethasone for 24 h. PPAR ligands were added to the media at confluence in a minimal volume (<0.1%). Pioglitazone (5-{4-[2-(5-ethyl-2-pyridyl)-ethoxy]benzyl}-2,4-thiazolidinedione) (Pharmacia–UpJohn) was dissolved in dimethylsulfoxide. Carbaprostacyclin (BioMol) and 2-bromopalmitate (Sigma) were dissolved in ethanol. Cells were refed every 24 h. At 7 days post-confluence, total RNA was isolated or cells were fixed and stained with Oil Red O (Green and Kehinde, 1976).

3T3-F442A cells were differentiated as described previously (Green and Kehinde, 1974). 3T3-L1 cells infected with PGC-2 or the vector alone were grown to confluence in DMEM containing 10% bovine calf serum (Hyclone). At confluence the media was changed to DMEM containing 10% cosmic calf serum, 5 μg/ml insulin and 1 μM pioglitazone for 7 days. After treatment the cells were stained with Oil Red O or total RNA was extracted as described below.

Transient transfection assays

COS-7 cells were transiently transfected at 60% confluence in six-well plates by mixing plasmid DNA (see figures) with Superfect (Qiagen) for 3 h according to the manufacturer's instructions. Cells were maintained in DMEM containing 10% cosmic calf serum (Hyclone). Ligands were added 3 h after transfection as indicated in the figure legends. The cells were analyzed after 18–24 h for luciferase activity. Relative transfection efficiency was determined by cotransfection with a β-galactosidase-expressing vector.

MCF7 cells were maintained in DMEM with 10% fetal bovine serum (FBS) (Hyclone). On the day of transfection, the medium was switched to phenol red-free DMEM (Gibco-BRL) with 10% charcoal-stripped FBS. The cells were transfected with Superfect at 80% confluence. Twenty-four hours after transfection, 100 nM β-estradiol (Sigma) was

added. The cells were analyzed 48 h after transfection for luciferase activity. Relative transfection efficiency was determined by cotransfection with a β-galactosidase-expressing vector.

RNA analysis

Total RNA was extracted from cells using Tri-Reagent™ (Molecular Research Center) according to the manufacturer's instructions. RNA was separated on formaldehyde-containing agarose gels as described (Maniatis *et al*., 1989) and transferred to Genescrene membrane (DuPont). All blots were hybridized with a cDNA for human acid ribosomal phosphoprotein PO (36B4, Laborda, 1991) to control for equivalency of RNA loading and transfer. Using the random-priming method (Feinberg and Vogelstein, 1984), cDNA probes were labeled with [α-^{32}P]dCTP (6000 Ci/mmol) to a specific activity of at least 10^9 c.p.m./mg. The membranes were UV-crosslinked, hybridized and washed in 2× SSC as described (Castillo *et al*., 1995), and visualized by autoradiography.

Tissues were obtained from three adult BALB/c By mice, pooled and homogenized in a Polytron homogenizer. RNA was extracted using Tri-Reagent™ according to the manufacturer's instructions.

Protein analysis

PGC-2 fused to GST was generated by inserting the full-length PGC-2 cDNA into the pGEX-6P2 vector (Pharmacia). Amino acids 1–138 of PPARγ and 1–72 of PPARδ were inserted in-frame into pGEX-2TK to generate GST–AB-PPARγ and GST–AB-PPARδ, respectively. The fusion proteins were expressed in BL-21 *Escherichia coli* (Novagen) and purified with a glutathione resin (Pharmacia). *In vitro* translation was performed using the TNT coupled transcription/translation kit (Promega) in the presence of [^{35}S]methionine and 0.5 μg of the indicated plasmids (see figures). Ten microlitres of the GST–PGC-2 beads were incubated in binding buffer (20 mM HEPES pH 7.7, 75 mM KCl, 0.1 mM EDTA, 2.5 mM $MgCl_2$, 0.05% NP-40, 2 mM dithiothreitol, 10% glycerol, 600 mM NaCl) with 5 μl of the *in vitro*-translated nuclear hormone receptors indicated for 1 h at room temperature. The beads were washed four times with binding buffer containing the ligands indicated, and the bound material was eluted by boiling in SDS–PAGE sample buffer. The eluted material was separated by SDS–PAGE and visualized by autoradiography.

For the experiment in Figure 5E, 100 mm dishes of COS-7 cells were transiently transfected with 10 μg of PGC-2, PPARγ and/or pSV-Sport. Forty-eight hours after transfection, the cells were washed once in phosphate-buffered saline (PBS) and then lysed in RIPA buffer [1× PBS, 1% NP-40, 0.5% deoxycholate, 0.1% SDS, Complete™ protease inhibitors (Boehringer Mannheim)]. Lysates (100 μg of protein) were diluted 1:100 with binding buffer (see above) and incubated with Ni^{2+} agarose beads which were equilibrated in binding buffer. After extensive washing, the beads were boiled for 2 min in SDS–PAGE sample buffer. The eluted material was separated by SDS–PAGE and analyzed by Western blot with a rabbit polyclonal anti-PPARγ antibody (Hu *et al*., 1996).

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