Differential activation of adipogenesis by multiple PPAR isoforms

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Peroxisome proliferator-activated receptor γ (PPARγ) is a nuclear hormone receptor expressed predominantly in adipose tissue, where it plays a central role in the control of adipocyte gene expression and differentiation. Because there are two additional PPAR isoforms, PPARα and PPARδ, and these are also expressed at some level in certain adipose depots, we have compared directly the adipogenic potential of all three receptors. Ectopically expressed PPARγ powerfully induces adipogenesis at a morphological and molecular level in response to a number of PPARγ activators. PPARα is less adipogenic but is able to induce significant differentiation in response to strong PPARα activators. Expression and activation of PPARδ did not stimulate adipogenesis. Of the three PPARs, only PPARγ can cooperate with C/EBPα in the promotion of adipogenesis. To begin to investigate the functional basis for the differential adipogenic activity of the PPAR isoforms, we have examined their ability to bind to several PPAR DNA response sequences. Compared with PPARα and PPARδ, PPARγ shows preferential binding to two well-characterized regulatory sequences derived from a fat-specific gene, ARE6 and ARE7. These data strongly suggest that PPARγ is the predominant receptor regulating adipogenesis; however, they also suggest that PPARα may play a role in differentiation of certain adipose depots in response to a different set of physiologic activators or in certain disease states.

[*Key Words*: Adipogenic activity; PPAR isoforms; nuclear hormone receptor; adipose tissue; gene expression; differentiation]

As a central component of the energy balance system, adipocytes store triglycerides in periods of nutritional abundance and release free fatty acids in times of caloric deficiency. Obesity, an excessive development of adipose tissue, affects ~30% of all adults in the United States. This disorder is a major health problem, representing a significant risk factor for non-insulin-dependent diabetes mellitus, coronary artery disease, and hypertension. Thus, new approaches to the prevention and treatment of these disorders may result from a thorough understanding of the molecular aspects of adipocyte differentiation and lipogenesis.

Several recent advances have led to new insights in the molecular control of adipogenesis and adipocyte-specific gene expression. One important advance has come from the analysis of the tissue-specific enhancer from the adipocyte P2 (aP2) gene (Ross et al. 1990; Graves et al. 1991). The key transcriptional regulator of this enhancer was identified as a novel activity termed ARF6 (Graves et al. 1992). This same factor was also identified as a regulator of the PEPCK gene (Tontonoz et al. 1994b). The

observation that the ARF6 DNA-binding sequence was similar to the DR-1-type hormone response element led to the identification of a member of the nuclear hormone receptor family, peroxisome proliferator-activated receptor γ2 (PPARγ2), which is expressed at high levels specifically in adipose tissue. PPARγ binds to and activates transcription through DR-1 sites in a heterodimeric complex with retinoid X receptor α (RXRα) (Kliewer et al. 1994; Tontonoz et al. 1994a, b). The PPARγ gene contains two promoters that produce two isoforms, PPARγ1 and PPARγ2, via differential splicing (Zhu et al. 1995). Expression and activation of PPARγ1 or PPARγ2 in fibroblasts is sufficient to trigger the adipocyte differentiation cascade (Tontonoz et al. 1994c).

The adipogenic potential of members of the CCAAT/enhancer-binding protein (C/EBP) family has also been studied intensively. Although expression of the C/EBPs is not restricted to fat, they are expressed at high levels in this tissue and are induced during adipogenesis (Cao et al. 1991). C/EBPα has been demonstrated to bind and *trans*-activate the promoters of a number of adipocyte genes (Ntambi et al. 1988; Christy et al. 1989, 1991; Herrera et al. 1989; Park et al. 1990; Christy et al. 1991; Cornelius et al. 1994). In addition, C/EBPα was shown to

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play an important role in terminal differentiation of preadipocytes (Samuelsson et al. 1991; Umek et al. 1991; Lin and Lane 1994). Although C/EBPα is not expressed until relatively late in the time course of adipogenesis (Cao et al. 1991), ectopic expression of C/EBPα can convert fibroblastic cells to adipocytes (Freytag et al. 1994). Recently, C/EBPβ, which is expressed early in the differentiation process (Cao et al. 1991), has also been shown to induce adipocyte differentiation (Yeh et al. 1995), possibly through the induction of PPARγ (Wu et al. 1995). Because the promoters of several fat-specific genes contain both C/EBP-and PPAR-binding sites, it is likely that members from both families cooperate in the transcription of many fat cell-specific genes (Christy et al. 1989; Herrera et al. 1989; Park et al. 1990; Tontonoz et al. 1994a, 1995). Recent experiments have shown that PPARγ and C/EBPα promote differentiation synergistically, allowing adipogenesis in the absence of exogenously added PPARγ activator (Tontonoz et al. 1994c). This combination of PPARγ and C/EBPα is so powerfully adipogenic it causes the transdifferentiation of determined myoblasts into fat cells (Hu et al. 1995).

Three PPAR isoforms have been identified that define a subfamily of nuclear hormone receptors. The PPARs contain highly conserved DNA-binding domains but more divergent ligand-binding domains (Kliewer et al. 1994). Thus, although they bind to the same consensus DR-1 DNA sequences in vitro, the activation profiles of these three receptors are pharmacologically distinct. PPARα, expressed mainly in heart, liver, and kidney, is thought to regulate transcription of many genes involved in lipid metabolism, especially the enzymes involved in the β-oxidation of fatty acids (Gottlicher et al. 1992; Gulick et al. 1994; Krey et al. 1995; Lee et al. 1995). Although PPARα is not expressed at significant levels in white fat, it is expressed in brown fat (Isseman and Green 1990; Beck et al. 1992). PPARδ, also referred to as NUC-1 (Schmidt et al. 1992) and FAAR (Amri et al. 1995), is expressed in nearly all tissues (Kliewer et al. 1994; Tontonoz et al. 1994c; Amri et al. 1995) and has been re-

ported to regulate expression of some fat-specific genes (Amri et al. 1995). As its expression appears to be induced with the cessation of cell growth at confluence, it has been suggested that PPARδ may play a significant role in the adipogenic process (Amri et al. 1995). Although the other PPAR isoforms remain orphan receptors, ligands for PPARγ have been identified recently that are potent inducers of adipogenesis in vitro. These include thiazolidinediones, a class of antidiabetic drugs, and the arachidonic acid derivative 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-J2) (Forman et al. 1995; Kliewer et al. 1995; Lehmann et al. 1995).

As all of the PPAR isoforms are expressed to some degree in certain fat depots, we have examined directly the adipogenic potential of the three PPAR isoforms when expressed in fibroblasts. Although PPAR is the most adipogenic member of this family, we find that PPARα is also capable of promoting adipocyte differentiation in response to strong PPARα activators. PPARδ did not stimulate adipogenesis even in response to the strongest PPARδ activators. Only PPARγ showed marked synergy with C/EBPα to activate the adipogenic program. These results may be at least partially explained by a preferential binding of PPARγ to non-consensus PPAR response elements (PPRE) found in certain fat cell-specific genes.

Results

PPAR isoforms can be distinguished pharmacologically

To assess the adipogenic potentials of the three PPAR isoforms, we first characterized the activation profile of each of the receptors (Fig. 1). Each PPAR was transfected into CV-1 cells and assayed for its ability to activate multiple copies of a PPRE placed upstream of a luciferase reporter gene. As reported previously, the peroxisome proliferator Wy14,643 efficiently activates PPARα, but not PPARγ or PPARδ (Kliewer et al. 1994). In contrast,
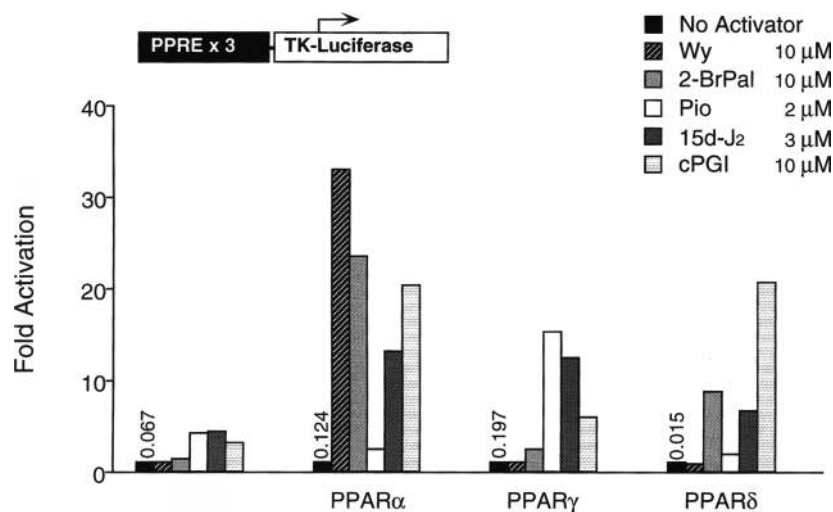

Figure 1. Transcriptional activation of the PPAR isoforms by PPAR activators. The reporter construct contains three copies of the acyl-CoA oxidase PPRE cloned upstream of the TK–LUC reporter. CV-1 cells were transfected with the PPRE₃TK–LUC reporter (300 ng/10⁵ cells), CMX–PPARα, CMX–PPARγ, or CMX–PPARδ (100 ng/10⁵ cells), and the internal control, CMX-β-gal (500 ng/10⁵ cells). Cells were treated for 40 hr with media containing the indicated compounds. Normalized luciferase activity was determined and plotted as fold activation relative to untreated cells. The absolute value of luciferase units for the untreated controls is indicated. (Wy) Wy14,643; (2BrPal) 2-bromopalmitate; (pio) pioglitazone; (15d-J2) 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; (cPGI) carbacyclin.

pioglitazone, a thiazolidinedione that has been shown to be a ligand for PPARγ (Forman et al. 1995; Lehmann et al. 1995), efficiently activates only PPARγ. The other activators examined did not demonstrate such a marked isoform specificity. 15d-J2, which has also been shown to bind directly to PPARγ (Forman et al. 1995; Kliewer et al. 1995), also activates the α and δ isoforms. 2-Bromopalmitate, a stable form of a dietary fatty acid, has been reported to be an efficient activator of PPARδ (Amri et al. 1995). As shown in Figure 1, this compound also activates the other two receptors.

Prostacylin is known to possess many biological activities, including the promotion of adipogenesis (Catalioto et al. 1991; Vassaux et al. 1992). These effects are thought to be mediated by binding to a cell surface receptor (Namba et al. 1994) and the activation of cAMP and Ca^{2+} second messenger pathways (Vassaux et al. 1995). The recent identification of 15-dJ2 as a ligand for PPARγ raises the possibility that other arachidonate metabolites might also function as activators of PPARs. We have found that carbacyclin, a stable analog of prostacyclin, is an effective activator of all three PPAR isoforms and may be the most efficacious activator of PPARδ identified to date.

Adipogenic action of the three PPARs

We used retroviruses to express the three PPARs in fibroblastic cells. It is beneficial to use a retroviral system in differentiation studies, as it allows similar levels of ectopic gene expression of a construct in hundreds of thousands of cells and avoids the isolation of individual clones. This is extremely important because individually selected fibroblastic clones exhibit tremendous variability in their ability to undergo adipogenesis (Green and Kehinde 1976). This retroviral system has been used previously to investigate the biological function of PPARγ (Tontonoz et al. 1994c) and C/EBPβ (Yeh et al. 1995). The pBabe–Puro expression vectors containing the cDNAs encoding PPARα, PPARδ, and PPARγ were packaged into virus, and the resulting viral stocks were used to infect NIH-3T3 cells (Tontonoz et al. 1994c). In this manner, stable cell lines were isolated that contained the parental expression vector (NIH–vector) or that expressed PPARα (NIH–PPARα), PPARγ (NIH–PPARγ), or PPARδ (NIH–PPARδ).

As shown in Figure 2A, each retroviral construct is expressed at significant levels in NIH-3T3 cells. In NIH–PPARγ cells, the viral transcript encoding PPARγ (lane 2) is produced at approximately one-third the level of endogenous PPARγ observed in fully differentiated adipocytes (lane 1). NIH-3T3 cells do not normally express significant levels of PPARγ (lane 5). NIH–PPARα cells (lane 3) express much more PPARα mRNA than is expressed in 3T3–L1 adipocytes (lane 1) or NIH–vector cells (lane 5). PPARδ mRNA is expressed in fully differentiated adipocytes at approximately the same level as in NIH–vector cells (lanes 1,5). The retroviral expression vector increases this level of PPARδ expression by three-to four-fold (lane 4). Western blot analysis reveals that
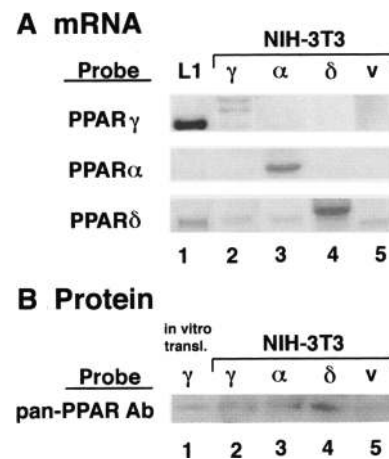

Figure 2. Endogenous and ectopic expression of PPAR isoforms in NIH-3T3 cells and 3T3–L1 adipocytes. NIH-3T3 cells were infected with retroviruses carrying pBabe-derived expression vectors as described in Materials and methods. (*A*) Total RNA (15 μg/lane) was isolated from virally transduced NIH-3T3 cells and differentiated 3T3–L1 adipocytes, electrophoresed, transferred to nylon membranes, and hybridized with ^{32}P-labeled PPARγ, PPARα, and PPARδ cDNA. An equivalent amount of intact RNA was run in each lane, as indicated by hybridization to a 36B4 cDNA probe (data not shown). (*B*) Western blot analysis of nuclear extracts (50 μg/lane) from virally transduced NIH-3T3 cells (lanes *2–4*) was subjected to SDS-PAGE, transferred to membrane, and probed with an anti-PPAR antibody that recognizes all three isoforms (Affinity Bioreagents). (Lane *1*) In vitro-translated PPARγ.

the protein level of each of the PPAR isoforms is similar (Fig. 2B). In addition, expression of all three PPAR isoforms results in a similar increase in PPRE-binding activity in extracts from these cell lines (data not shown).

These cell lines were then cultured under conditions permissive for adipogenesis. At confluence, they were treated with various PPAR activators, and after 7 days, they were fixed and stained for accumulated neutral lipid with Oil Red O. Macroscopic examination of the stained dishes demonstrated that NIH–PPARγ cells readily accumulated lipid when treated with pioglitazone, 2-bromopalmitate, 15d-J2 or carbacyclin (Fig. 3). Pioglitazone, a direct ligand of PPARγ, stimulated the most abundant lipid accumulation. PPARα-expressing cells also accumulated considerable stainable lipid when treated with the highly efficacious PPARα activators 2-bromopalmitate or carbacyclin. Wy14,643 also clearly induced lipid accumulation, although this was somewhat less than seen with 2-bromopalmitate and carbacyclin. However, chronic treatment of cells with this agent was accompanied by signs of toxicity, including the detachment of many cells. Little or no lipid accumulation in the PPARα cells was observed in response to either of the known PPARγ ligands pioglitazone or 15d-J2. In contrast to the other two receptors, lipid accumulation was not observed in NIH–PPARδ cell cultures regardless of the activator used. Even carbacyclin, which was the most effective inducer of the transcriptional activity of PPARδ
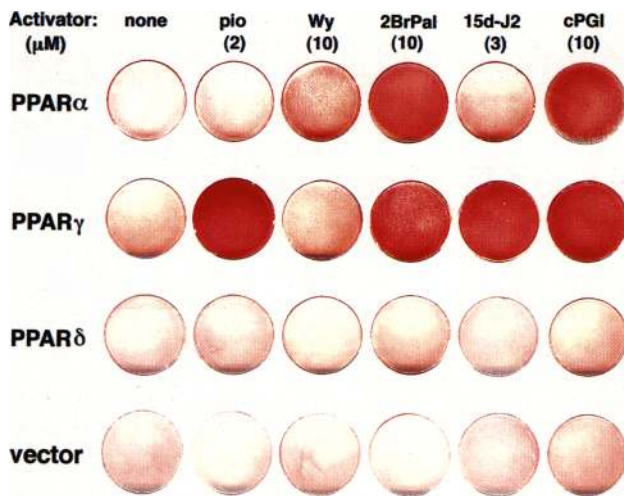
Figure 3. PPARγ and PPARα stimulate adipogenesis in NIH-3T3 cells. Stable NIH–PPARα, NIH–PPARγ, NIH–PPARδ ,and NIH–vector cell lines were cultured in differentiation media (see Materials and methods) with the indicated concentration of PPAR activator. Macroscopic views of cells are shown that were fixed and stained with Oil Red O after 7 days of confluent culture. (Wy) Wy14,643; (2BrPal) 2-bromopalmitate; (pio) pioglitazone; (15d-J2) 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; (cPGI) carbacyclin.

(Figure 1) and which promoted differentiation in the PPARγ- and PPARα-expressing cells, failed to promote differentiation through PPARδ. NIH–vector cells failed to undergo adipogenesis in response to any activator.

Microscopic examination of differentiated NIH–PPARγ (Fig. 4C) and NIH–PPARα (Fig. 4B) cells revealed a morphological appearance characteristic of cultured adipocytes, with many small lipid droplets seen in the cytoplasm. However, the NIH–PPARγ cells generally exhibited more extensive differentiation, reflected both in the amount of lipid accumulated in each cell and the percentage of cells that accumulated lipid (80%–95% for PPARγ in eight experiments versus 30%–60% for PPARα in eight experiments). The time course of differentiation of the PPARα cells also appeared slower than that seen with cells expressing PPARγ (see below). NIH–PPARδ cells and NIH–vector cells retained a fibroblastic morphology under all conditions examined (Fig. 4A). These experiments were performed with at least five different cell lines generated by retroviral infection for each receptor.

To characterize the differentiated state of the cells at a molecular level, Northern analysis was performed on RNA isolated at two time points in the differentiation process (Fig. 5). In the experiment shown, ~90% of the PPARγ cells treated with pioglitazone had differentiated morphologically, as compared with 60% of the PPARα cells treated with 2-bromopalmitate or carbacyclin. When examined after 5 days of treatment with pioglitazone, 2-bromopalmitate, or 15d-J2, NIH–PPARγ cells had induced expression of many adipocyte-specific genes, including endogenous PPARγ, adipsin, and aP2

(Fig. 5A). However, no or very little induction of these genes was seen in the PPARα cells at this time point (Fig. 5A). After 7 days of treatment with various PPAR activators, both NIH–PPARγ and NIH–PPARα cells expressed high levels of mRNA for endogenous PPARγ, adipsin, and aP2 (Fig. 5B). At this time point, NIH–PPARγ and NIH–PPARα differentiated cells also express similar levels of mRNA for lipoprotein lipase, ADD1, and acyl coenzyme A (acyl-CoA) synthase (data not shown). Thus, the pattern of gene expression of both NIH–PPARγ and NIH–PPARα-differentiated cells indicate a bona fide adipogenic process. These data are also consistent with the morphological evidence suggesting that differentiation directed by PPARα may occur at a slower rate than differentiation directed by PPARγ.

Significant levels of adipsin or PPARγ were not detected in NIH–vector or NIH–PPARδ cells under any condition, even after a week of treatment with PPAR activators (Fig. 5B). A longer exposure of the films shown in Figure 5 revealed low levels of aP2 mRNA in NIH–PPARδ cells that were treated for 7 days with 2-bromopalmitate and carbacyclin, the compounds that activated PPARδ most effectively (Fig. 5C). aP2 expression was also stimulated in NIH–vector cells treated with carbacyclin, albeit to a lesser extent. This may be attributable to the normal, low level of PPARδ or PPARγ expression in NIH-3T3 cells or to another biological effect of carbacyclin. These data demonstrate that PPARδ can stimulate some expression of aP2 mRNA but does not appear to be capable of fully activating the adipocyte differentiation program.

C/EBPα cooperates with PPARγ, but not PPARα or PPARδ, to induce adipogenesis

Expression of both C/EBPα and PPARγ in fibroblasts allows powerful differentiation in the absence of added PPAR activators (Tontonoz et al. 1994c). This implies that forced expression of C/EBPα in fibroblasts overrides the need to provide PPARγ activators exogenously. The cooperation between PPARγ and C/EBPα may be very important in the adipogenic process, as both factors are present in most adipocytes examined. We have investigated whether PPARα and PPARδ can also cooperate with C/EBPα. Using a retroviral construct which contained the C/EBPα cDNA and a neomycin-selectable marker (Tontonoz et al. 1994c), NIH-3T3 cells were sequentially infected with retroviruses to generate cell lines that expressed C/EBPα and one of the PPAR isoforms. These cells were cultured for 7 days after confluence under conditions permissive for adipogenesis but without exogenously added PPAR activators. Macroscopic views of the Oil Red O-stained dishes are shown in Figure 6A. As demonstrated by lipid accumulation, NIH–C/EBPα cells differentiated into adipocytes at a low but detectable level. Coexpression of PPARγ with C/EBPα strikingly increased the degree of differentiation. Coexpression of PPARα or PPARδ with C/EBPα did not appear to increase the level of differentiation induced
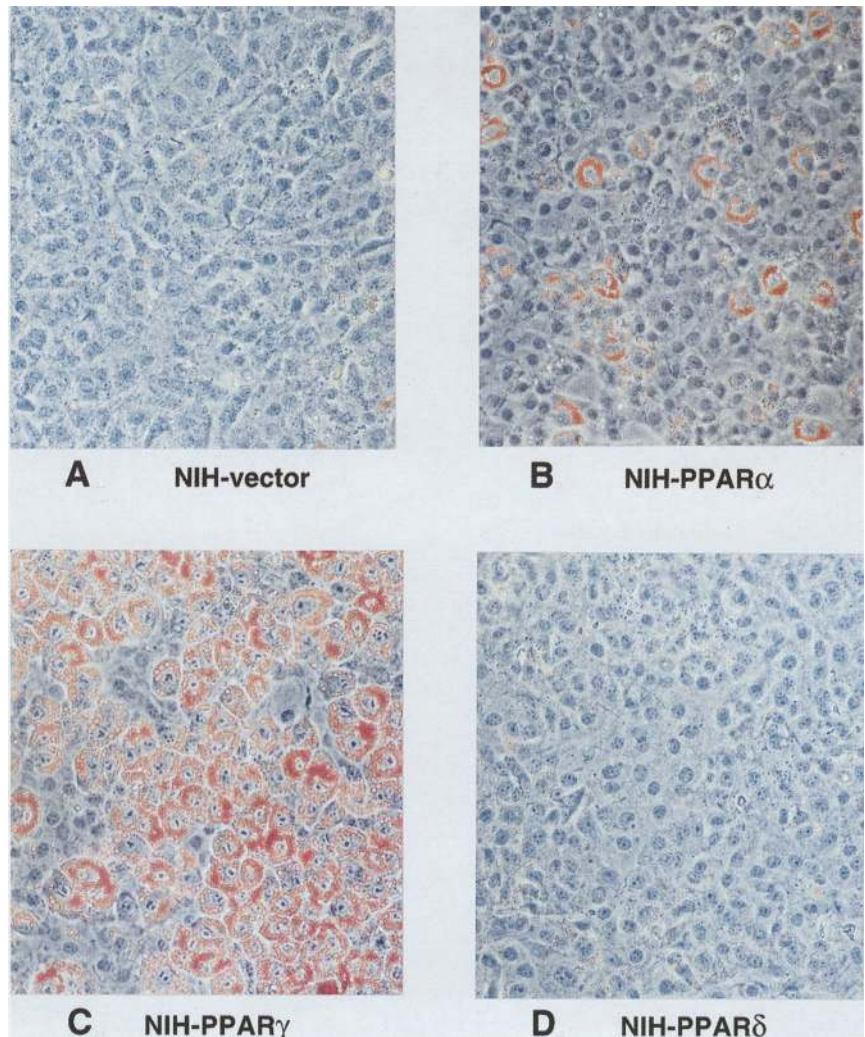
Figure 4. Morphological differentiation through PPAR expression. Stable NIH–PPARα, NIH–PPARγ, and NIH–PPARδ cell lines were cultured in differentiation media (see Materials and methods) with their most efficacious PPAR activator for 7 days postconfluence. NIH–PPARα cells were treated with 10 μM 2-bromopalmitate (B), NIH–PPARγ cells were treated with 2 μM pioglitazone (C), and NIH–vector (A) and NIH–PPARδ (D) cells were treated with 100 μM 2-bromopalmitate. Cells were then fixed and stained with Oil Red O. Microscopic views of cells at 40× magnification are shown.

by C/EBPα alone. PPARα and PPARδ actually appeared to have an inhibitory effect.

The expression of adipocyte-specific genes in these cell lines was consistent with the morphologic data (Fig. 6B). NIH–C/EBPα cells expressed elevated levels of adipocyte-specific genes (lane 2) when compared to cells transfected with only the parental expression vectors (lane 1). Coexpression of PPARγ markedly increased the expression of all adipocyte marker genes examined (lane 4). However, neither PPARα nor PPARδ had this effect. In fact, coexpression of PPARδ actually inhibited expression of endogenous PPARγ, aP2, and especially adipsin, when compared to cells transfected with only the C/EBPα expression vector (lane 5). PPARα likewise inhibited the induction of these marker genes, albeit to a lesser extent (lane 3). Thus, only PPARγ demonstrates the ability to cooperate with C/EBPα in the induction of adipogenesis, at least under these experimental conditions. These data indicate that while PPARα possesses some adipogenic potential, PPARγ is a more effective inducer of adipocyte differentiation in combination with C/EBPα.

PPARγ has higher affinity for fat-specific PPREs than PPARα or PPARδ

All three PPAR isoforms have highly conserved DNA-binding domains and bind to the same consensus DR-1 sequences. However, the possiblity that the three receptors possess differences in DNA-binding characteristics has not been thoroughly explored. To determine whether differences in DNA binding might contribute to the differential adipogenic activity shown by the PPAR isoforms, we tested by electorphoretic mobility shift assay the ability of each isoform to bind to two regulatory DR-1-type elements present in the fat-specific enhancer of the aP2 gene (Graves et al. 1992; Tontonoz et al. 1994a), a functional DR-1 sequence present in an enhancer of the liver-specific acyl-CoA oxidase gene (Tugwood et al. 1992) and an ideal consensus DR-1 sequence.

Of these DNA sequences, each PPAR isoform demonstrated the strongest binding to the optimal, consensus DR-1 element (PPRE-op). The binding of each PPAR isoform to the AOX sequence was similar to its binding of the PPRE-op (Fig. 7B). The binding of all three isoforms
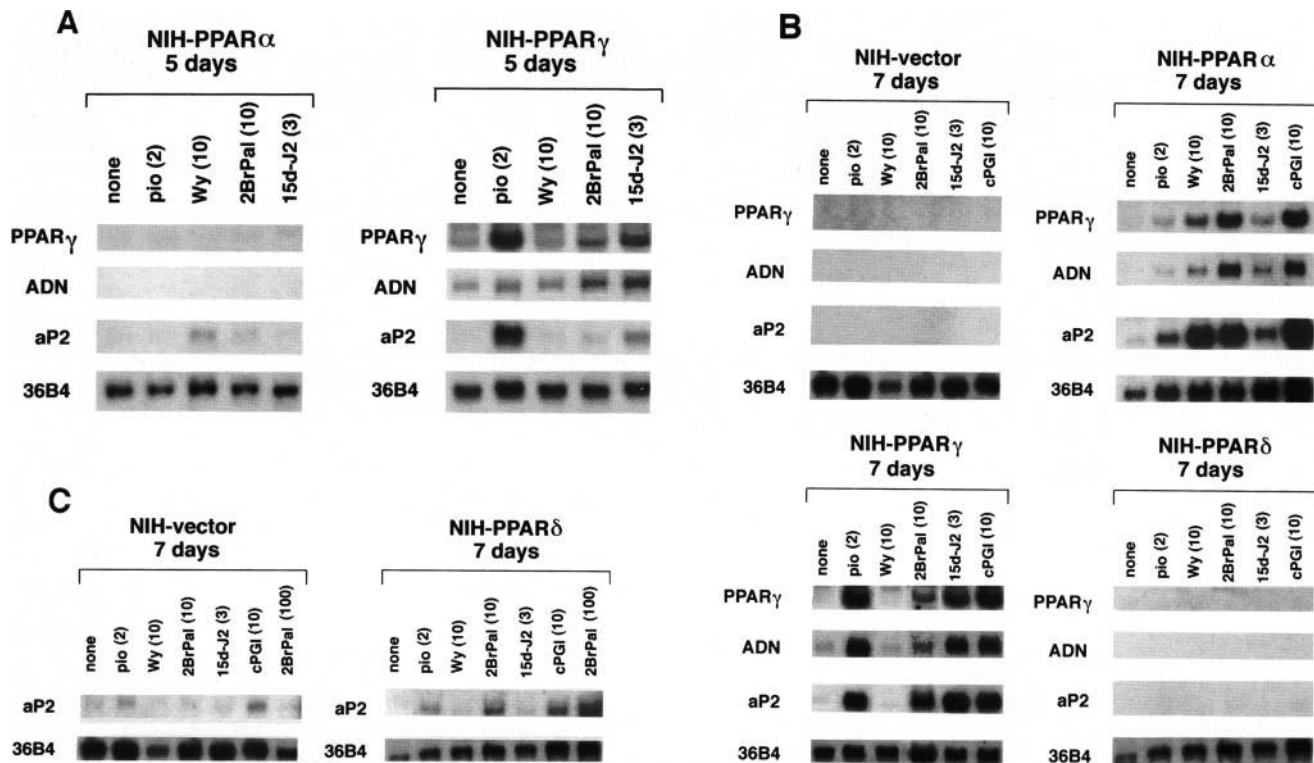
Figure 5. Induction of adipocyte-specific gene expression by PPAR isoforms. (*A*) NIH–PPARα and NIH–PPARγ cells were treated as in Fig. 3 with the indicated concentration of PPAR activator for 5 days after confluence. Total RNA (15 μg/lane) was isolated, electrophoresed, blotted onto nylon membranes, and hybridized with the indicated ^{32}P-labeled cDNA probes. An equivalent amount of intact RNA was run in each lane as indicated by hybridization to a 36B4 probe. (*B*) NIH–vector, NIH–PPARα, NIH–PPARγ, and NIH–PPARδ cells were treated as in Fig. 3 with the indicated concentration of PPAR activator for 7 days after confluence. Total RNA (15 mg/lane) was isolated, electorphoresed, blotted onto nylon membranes, and hybridized with the indicated ^{32}P-labeled cDNA probes. (*C*) The filters hybridized with aP2 from *B* were exposed for 15 hr, vs. 3 hr in *B*, to show induction of low levels of aP2 expression. (Wy) Wy14,643; (2BrPal) 2-bromopalmitate; (pio) pioglitazone; (15d-J2) 15-deoxy-$\Delta^{12,14}$-prostaglandin J2; (cPGI) carbacyclin; (ADN) adipsin; (aP2) adipocyte fatty acid-binding protein; (36B4) human acid ribosomal phosphoprotein PO.

to the ARE6 and ARE7 oligonucleotides was decreased compared with the other two sequences (Fig. 7C,D). Although the nonconsensus, fat-specific elements are less optimal binding sites for all of the PPARs, PPARγ clearly shows a higher affinity for ARE6 and ARE7 relative to the other two isoforms. When normalized to binding on the consensus site, PPARγ shows a two-fold higher binding to the ARE7 site compared with PPARα or PPARδ (Fig. 7C). The binding of PPARγ to ARE6, which is even more divergent from the consensus DR-1 sequence, was 2.5-fold higher than PPARδ and 4-fold higher than PPARα (Fig. 7D). This suggests that the differences in adipogenic potential between PPARα and PPARγ may be at least partially attributable to differences in the ability to bind to DR-1 sequences of some fat regulatory genes.

Discussion

Roles in adipogenesis have been suggested for all three mammalian PPAR isoforms. To date, the only datum addressing this issue directly is the demonstration that PPARγ, when expressed and activated in fibroblasts, ef-ficiently induces the adipocyte differentiation program (Tontonoz et al. 1994c). A role for PPARα in adipogenesis has been suggested by the observation that a PPARα-selective activator stimulates terminal differentiation of 3T3-L1 preadipocytes (Yu et al. 1995). Studies using thi-azolidinediones to stimulate adipogenesis have sug-gested that PPARδ plays an important role in this pro-cess (Teboul et al. 1995). Given these data, it is impor-tant to assess directly the ability of all three isoforms to induce adipocyte differentiation by expressing and acti-vating each of the receptors in fibroblastic cells. We ob-served previously that PPARα could induce aP2 expres-sion but could not activate the differentiation cascade in response to the weak PPARα activator ETYA (Tontonoz et al. 1994c). Using the highly efficacious PPARα activa-tors Wy14,653, 2-bromopalmitate, or carbacyclin, we now show that PPARα is capable of initiating the adipo-cyte program. Fibroblasts expressing PPARα undergo morphologic changes, accumulate lipid, and induce ex-pression of an array of adipocyte-specific genes when treated with very efficient activators of this receptor. In contrast, expression and activation of PPARδ in these experiments did not cause adipogenesis. The expression
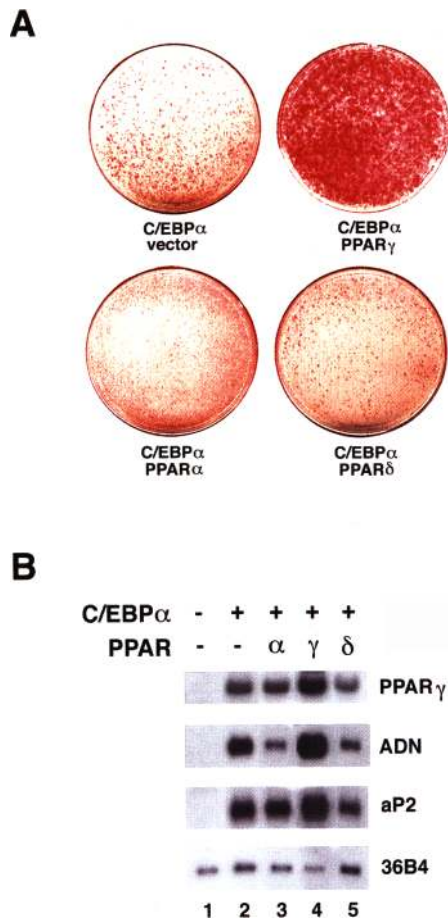
A

B

Figure 6. Cooperation of PPAR isoforms with C/EBPα. (A) NIH–C/EBPα/vector, NIH–C/EBPα/PPARγ, NIH–C/EBPα/ PPARα, and NIH–C/EBPα/PPARδ stable cell lines were cultured in differentiation media containing 1 μM dexamethasone for 7 days postconfluence. Macroscopic views of cells which were fixed and stained with Oil Red O are shown. (B) Total RNA from stable cell lines cultured as described in A was isolated, electrophoresed, blotted to nylon membrane, and hybridized with the indicated ^{32}P-labeled cDNA probes. An equivalent amount of intact RNA was run in each lane as indicated by hybridization to a 36B4 probe. (ADN) Adipsin; (aP2) adipocyte fatty acid-binding protein; (36B4) human acid ribosomal phosphoprotein PO.

of PPARδ actually reduces the level of adipogenesis promoted by C/EBPα expression.

It has been shown recently that overexpression of C/EBPβ activates adipogenesis in a manner dependent on PPARγ activity (Wu et al. 1995). Because adipocytes derived from PPARα-expressing cells express PPARγ, it is possible that PPARα stimulates differentiation through the expression of PPARγ. Three lines of evidence argue against this possibility. First, PPARα-induced differentiation does not require the addition of an exogenous PPARγ activator. As seen in Figures 3–5, treatment with Wy14,643, which does not activate PPARγ and had no effect on NIH–PPARγ cells, stimu-

lates adipogenesis in NIH–PPARα cells. Second, induction of endogenous PPARγ expression in NIH–PPARα cells does not occur particularly early in the course of differentiation, as has been demonstrated with overexpression of C/EBPβ (Wu et al. 1995). Endogenous PPARγ expression seems to be induced by PPARα with the other markers of the adipocyte differentiation program (Fig. 5). Finally, addition of pioglitazone, a PPARγ-specific activator, to NIH–PPARα cells treated with Wy14,643 did not further stimulate lipid accumulation or adipocyte specific gene expression early in the differentiation process (data not shown). Some effect of pioglitazone was observed only relatively late in the differentiation process, after induction of endogenous PPARγ.

Although PPARα activates the differentiation program, it appears to do so less efficiently than PPARγ. PPARγ induces differentiation in response to diverse PPARγ activators, even those that are not particularly strong activators of its transcriptional activity, such as carbacyclin and 2-bromopalmitate. PPARα-directed differentiation, on the other hand, only occurs in the presence of the strongest PPARα activators. In addition, the differentiation induced by PPARα is less extensive in terms of the percentage of cells undergoing differentiation, the amount of lipid accumulated, and also occurs at a slower rate compared to that driven by PPARγ. As shown in Figure 7, the PPARs do differ in their relative binding to divergent sequences that still may be considered DR-1-type elements. Hence, this could underlie some of the differences in adipogenic activity among these receptors. This is supported by the fact that PPARγ possesses higher affinity for two nonconsensus, DR-1-type sequences present in a fat specific enhancer (from the aP2 gene) compared to the other receptors. The notion that the DNA-binding characteristics of the PPARs may contribute to differences in adipogenic potential may be definitively determined by studying chimeric receptors that switch DNA-binding domains.

Cooperation between the C/EBP and PPAR families is likely to play an important role in the process of adipocyte differentiation. Several fat-specific genes have been shown to contain binding sites for both PPARs and C/EBPs (Christy et al. 1989; Herrera et al. 1989; Park et al. 1990; Tontonoz et al. 1994a, 1995). We have shown here that only PPARγ is able to cooperate with C/EBPα to induce adipogenesis in the absence of exogenously added PPAR activator. This suggests a special relationship between these two factors that does not appear to exist between C/EBPα and the other two receptors. There are several possible explanations, which are not mutually exclusive, for this cooperative interaction. One is that C/EBPα and PPARγ synergistically activate a factor in the basal transcriptional machinery. Another possibility is that there is a direct protein–protein interaction between PPARγ and C/EBPα that increases the activity of either or both factors. The observation that the PPAR isoforms have subtle DNA-binding site preferences raises the possibility that PPARα does not synergize with C/EBPα because it is unable to interact with C/EBPα on the promoters of some important, fat-specific
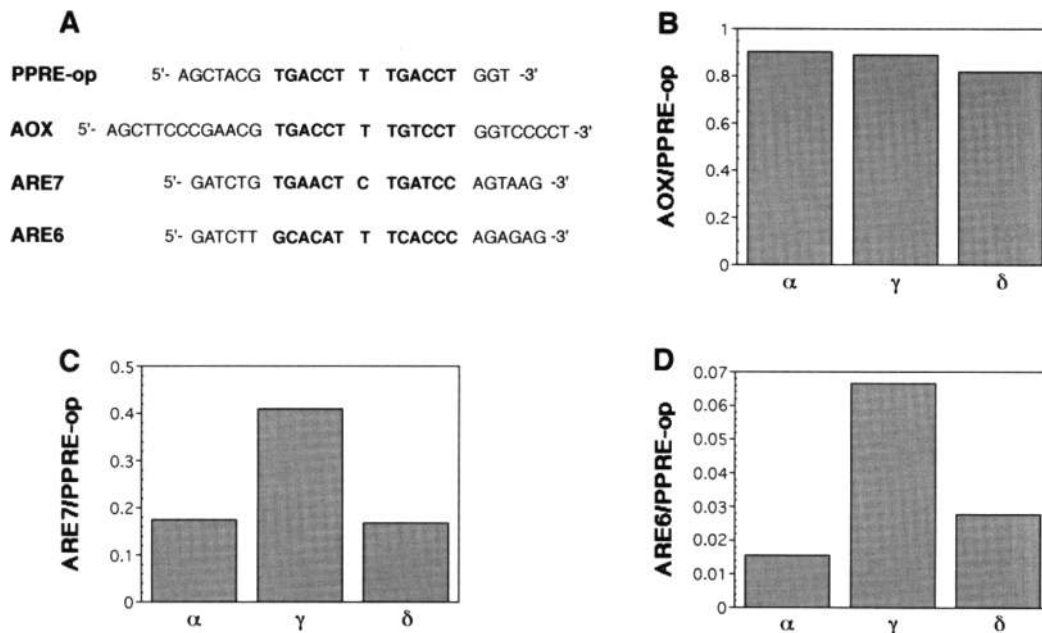
Figure 7. Binding of PPAR isoforms to different DR-1 sequences. (*A*) Sequences (top strand only) of double-stranded oligonucleotides that were radiolabeled and used as probes in EMSA with in vitro-translated RXRα and equal amounts of in vitro-translated PPARα, PPARγ, or PPARδ. Nucleotides of the DR-1-type sequence are in boldface type. Amounts of specifically bound acyl CoA oxidase PPRE (*B*) and the aP2 enhancer elements ARE7 (*C*) and ARE6 (*D*) were quantitated and normalized to bound PPRE-op for each isoform. (PPRE-op) Consensus PPRE; (AOX) acyl CoA oxidase PPRE.

genes. Alternately, the lack of cooperativity between C/EBPα and PPARα might suggest that the region of PPARγ that interacts with C/EBPα is not conserved in PPARα. Finally, as these receptors are pharmacologically distinct, C/EBPα may regulate genes involved in the production of a ligand that activates only PPARγ. Thus, coexpression of PPARγ and C/EBPα could lead to differentiation in the absence of added PPARγ activator, but C/EBPα expression would not necessarily result in the efficient activation of PPARα. Although the exact nature of the cooperativity between C/EBPα and PPARγ remains to be elucidated, it appears to be specific to the γ isoform.

It has also been postulated that PPARδ plays a prominent role in the activation of adipogenesis (Amri et al. 1995; Teboul et al. 1995). These reports suggest that PPARδ mediates induction of adipocyte-specific gene expression in fibroblasts and adipogenesis in myocytes in response to treatment with 2-bromopalmitate and thiazolidinediones, respectively. In this study we have directly addressed the potential role of this receptor in adipogenesis by overexpressing and activating PPARδ in fibroblasts. No evidence of adipocyte differentiation was observed in NIH–PPARδ cells under any condition, including treatment with thiazolidinediones and 2-bromopalmitate. Both of these compounds efficiently induced differentiation in fibroblasts expressing PPARγ. Furthermore, we and others have reported that PPARγ, and not PPARδ, directly binds and is activated by thiazolidinediones (Forman et al. 1995; Lehmann et al. 1995). Taken together, these data suggest that the reported ef-

fects of 2-bromopalmitate and thiazolidinediones are not attributable to the activation of PPARδ, but, rather, are probably the results of activation of the low levels of PPARγ present in these cell lines. However, it still remains possible that much more potent and efficacious activators of PPARδ might reveal some adipogenic action of this receptor that cannot be revealed with the compounds currently available.

Previous studies have shown that the stable prostacyclin analog carbacyclin can promote the differentiation of cultured adipocytes (Catalioto et al. 1991; Vassaux et al. 1992). We demonstrate here that carbacyclin is an activator of all three known mammalian PPARs and is the best activator of PPARδ described to date. Although some biological effects of prostacyclin are known to be mediated by its cell-surface receptor (Namba et al. 1994), our results suggest that the ability of this compound to promote differentiation may be mediated by nuclear PPARs. As the prostanoid 15d-J2 recently has been demonstrated to promote adipogenesis by binding directly to PPARγ (Forman et al. 1995; Kliewer et al. 1995), our results raise the possibility that carbacyclin may also bind directly to one or more PPARs.

Although the physiological meaning of the finding that PPARα can be adipogenic is not clear, it may lie in the heterogeneity of adipose cells. Mammalian organisms possess at least two distinct types of adipose tissue, white and brown. Whereas the main function of white fat is to store energy and mobilize it in times of nutritional deficiency, brown fat plays an important role in theromogenesis. Brown fat expresses the mitochondrial

uncoupling protein (UCP), which allows the cell to dissipate energy in the form of heat rather than generate ATP. The molecular basis underlying the decision to differentiate into brown or white fat is not understood. Interestingly, brown fat is known to expresses both PPARα (Isseman and Green 1990; Beck et al. 1992), and PPARγ (P. Tontonoz and B.M. Spiegelman, unpubl.). This is one of the few known differences in gene expression between white and brown fat. Thus, although PPARγ is likely to be the predominant receptor regulating the differentiation of white fat, PPARγ and PPARα may both contribute to the development of brown fat.

Given that PPARγ and PPARα are pharmacologically distinct, our results suggest that there may be several different lipid signaling pathways involved in the regulation of adipocyte gene expression and differentiation. A novel pathway involving prostaglandin J2 metabolites recently has been implicated in the activation of PPARγ (Forman et al. 1995; Kliewer et al. 1995). Although true ligands for PPARα have not been described, this receptor is activated by high concentrations of dietary fatty acids (Keller et al. 1993). β-Adrenergic stimulation of brown adipose cells leads to the generation of free fatty acids and the induction of UCP. It is possible that PPARα has a role in the expression of UCP and other genes linked to brown fat thermogenesis.

Finally, it must be remembered that hormones play a great role in the promotion of adipogenesis. Insulin, insulin-like growth factor (IGF-1), growth hormone, glucocorticoids, and catecholamines all affect fat differentiation in vitro and in vivo. The activity of all three isoforms of PPAR may be modified by one or more of these factors and make them more or less adipogenic. A full appreciation of the role of the PPARs in fat differentiation and lipogenesis must include a greater understanding of hormonal influences and the identity and quantity of their respective endogenous ligands.

Materials and methods

Transient transfection assay

Luciferase assays were performed as described previously (Forman et al. 1995, and references therein). CV-1 cells were grown in Dulbecco's modified Eagle medium (DMEM) containing 10% resin–charcoal-stripped bovine calf serum. One day prior to transfection, cells were plated to 50%–80% confluence. Cells were transfected in phenol-red free DMEM–FBS (DMEM containing 10% resin-charcoal stripped fetal bovine serum) by lipofection using N-[1-(2,3-dioleoyloxy)propyl]-N,N,N-ammonium methylsulfate according to the manufacturer's instructions (DOTAP, Boehringer Mannheim). After 2 hr, the liposomes were removed and cells were treated for ~40 hr with phenol-red free DMEM–FBS alone or with the indicated compounds. After exposure to PPAR activators, cells were harvested and assayed for luciferase and β-galactosidase activity.

Plasmids

Construction of the mammalian expression vectors used in the transfection assays, pCMX–PPARα, pCMX–PPARγ, and pCMX–PPARδ were described previously (Kliewer et al. 1994). The lu-

ciferase reporter gene PPRE₃/thymidine kinase/luciferase (PPRE$_3$–TK-LUC) has been described (Forman et al. 1995). The retroviral constructs containing cDNAs encoding murine PPARγ (pBabe–PPARγM1) and C/EBPα (pLJ–C/EBPα) were described previously (Tontonoz et al. 1994c). cDNAs encoding amino acid residues 1–440 of murine PPARδ and amino acid residues 88–469 of murine PPARα were derived from pCMX–PPARα and pCMX–PPARδ (Kliewer et al. 1994) by PCR and cloned as SalI fragments into the SalI site of pBabe–Puro (Pear et al. 1993). The PPARγ cDNA sequences from pBabe–PPARγM1 and pBabe–PPARα were derived by PCR and inserted into pCMX and pCRII (Invitrogen) for in vitro transcription and translation.

Stable cell lines

Stable cell lines expressing adipogenic transcription factors were derived as described previously (Tontonoz et al. 1994c). BOSC23 cells were cultured in 90-mm dishes and transfected at 80% confluence by calcium phosphate coprecipitation with 15 μg of pBabe- or pLJ-derived expression vector as described (Pear et al. 1993). Viral supernatants were collected 48 hr after transfection. NIH-3T3 cells at 50% confluence in 90-mm dishes were infected with equal titers of recombinant virus. The supernatants were applied in DMEM containing 10% cosmic calf serum (Hyclone) and 4 μg/ml of polybrene. Cells were split 1:4, 24 hr after infection and plated in DMEM containing 10% calf serum and either 2 μg/ml of puromycin or 400 μg/ml of neomycin to select infected cells. Cell lines expressing both C/EBPα and a PPAR isoform were established by infecting NIH–C/EBPα–pLJ cells with virus containing a pBabe–PPAR expression vector.

Cell culture and induction of differentiation

Following drug selection, virally infected stable cell lines were cultured to confluence in differentiation medium [DMEM containing 10% cosmic calf serum (Hyclone), 5 μg/ml of insulin, and 1 nM triiodothyronine]. At confluence, cells were treated with 1 μM dexamethasone for 24 hr. PPAR activators were added to the media at confluence in a minimal volume (<5 μl), where indicated. Pioglitazone (5-[4-[2-(5-ethyl-2-pyridyl)ethoxy]benzyl]-2,4-thiazolidinedione) (UpJohn), and Wy14,643 [4-chloro-(6-(2,3-xylindino)-2-pyrimidinylthio)acetic acid] (Chemsyn) were dissolved in DMSO, 15d-J2 (Cayman Chemical) in methyl acetate, and carbacyclin (Biomol), and 2-bromopalmitate (Sigma) in ethanol. Cells were refed every 24 hr. At 5 or 7 days postconfluence as indicated, either total RNA was isolated or cells were fixed and stained with Oil Red O (Green and Kehinde 1974).

RNA and protein analysis

Total RNA was isolated from cultured cells by guanidine isothiocyanate extraction (Chirgwin et al. 1979). RNA was denatured in formamide and formaldehyde and electrophoresed through formaldehyde-containing agarose gels as described (Maniatis et al. 1989). RNA was blotted to BioTrans nylon (ICN Pharmaceuticals), and membranes were cross-linked, hybridized, and washed as directed by the manufacturer. To control for equivalency of RNA loading and transfer, all blots were hybridized with the cDNA for human acid ribosomal phosphoprotein PO [36B4 (Laborda 1991)]. cDNA probes were labeled with [α-^{32}P]dCTP (6000 Ci/mmole) by the random-priming method (Fineberg and Vogelstein 1984) to a specific activity of at least 10^9 cpm/μg.

Nuclear extracts were obtained from virally transduced NIH-

3T3 cells as described previously (Tontonoz et al. 1994b) and subjected to Western blot analysis with a polyclonal anti-PPAR antibody raised against a peptide of a sequence in the DNA-binding domain that is conserved in all three isoforms (Affinity Bioreagents, Inc.). The proteins were separated on a 10% poly-acrylamide–SDS gel, and transferred to Immobilon P membrane (Millipore). Immunodetection was performed with ECL reagents (Amersham, Inc.) according to the manufacturer's directions.

Electorphoretic mobility shift assays

In vitro-translated RXRα and PPARs were obtained by transcribing and translating the pCMX–RXRα, pCMX–PPARγM1, pCRII–PPARα, and pCMX–PPARδ expression plasmids using the TNT T7-coupled reticulocyte lysate system (Promega). Proteins were quantitated using parallel [^{35}S]methionine-labeled reactions. DNA mobility retardation assays were performed as described (Graves et al. 1991) with the reactions containing equal amounts of in vitro translated products in 3 μl of reticulocyte lysate, 20 mM HEPES at pH 7.9, 150 mM NaCl, 5% glycerol, and 0.5 μg of poly [d(I-C)] (Pharmacia). DNA–protein complexes were resolved from free probe on a 5% polyacrylamide gel, which was dried and exposed to film The autoradiograph was digitally scanned, and specifically shifted probe was quantitated using NIH-Image v1.44.

Acknowledgments

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