Ligand-independent Activation Domain in the N Terminus of Peroxisome Proliferator-activated Receptor γ (PPARγ)

DIFFERENTIAL ACTIVITY OF PPARγ1 AND -2 ISOFORMS AND INFLUENCE OF INSULIN*

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Peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear hormone receptor superfamily, and is an important regulator of adipogenesis and adipocyte gene expression. PPARγ exists as two isoforms, PPARγ1 and PPARγ2, that differ only in their N termini. Both isoforms are activated by ligands that include the antidiabetic thiazolinedione drugs and 15-deoxy-Δ12,14-prostaglandin J2, and potential differences in their function have yet to be described. We report that, in addition to a ligand-activated transcriptional activity, when studied under conditions of ligand depletion, intact PPARγ has a ligand-independent activation domain. To identify the basis for this ligand-independent activation, we used GAL4-PPARγ chimeric expression constructs and UAS-TK-LUC in CV1 cells and isolated rat adipocytes. In both cell systems, isolated PPARγ1 and PPARγ2 N termini have activation domains, and the activation function of PPARγ2 is 5–6-fold greater than that of PPARγ1. Insulin enhances the transcriptional effect mediated by both PPARγ1 and PPARγ2 N-terminal domains. These data demonstrate that 1) PPARγ has an N-terminal (ligand-independent) activation domain; 2) PPARγ1 and PPARγ2 N termini have distinct activation capacities; and 3) insulin can potentiate the activity of the N-terminal domain of PPARγ.

The peroxisome proliferator-activated receptor γ (PPARγ) is a member of the nuclear receptor superfamily that plays a pivotal role in the molecular determination of adipogenesis and the regulation of adipocyte gene expression (1–5). Under appropriate conditions, expression of PPARγ through retroviral infection of fibroblastic cell lines is sufficient to cause differentiation along an adipocyte lineage, as assessed by expression of adipocyte-specific genes, accumulation of lipid, and acquisition of adipocyte morphology (6). Recently, it has been shown that 15-deoxy-Δ12,14-prostaglandin J2 (PG J2) is a high affinity ligand for PPARγ (7, 8) and that PPARγ is also the receptor for the thiazolinedinedione class of insulin-sensitizing drugs (7, 8). PPARγ resembles other members of the nuclear receptor superfamily in that ligand-dependent receptor activation alters the rates of transcription of genes, specifically those that have peroxisome proliferator response elements (PPREs) within their promoters (e.g. aP2, phosphoenolpyruvate carboxykinase, and uncoupling protein) (9–11).

PPARγ exists as two isoforms, PPARγ1 and PPARγ2, that differ only in their N termini, with PPARγ2 having an additional 30 amino acids that are encoded by a single exon (9, 12). Expression of mRNA encoding the two isoforms is driven by alternative promoters within a single PPARγ gene (12), and their expression is differentially regulated in a tissue-specific manner. PPARγ2 is most abundantly expressed in adipocytes and is relatively specific for this tissue (9, 13). In contrast, while PPARγ1 is also expressed at a high level in adipocytes, it is also found at significant but lower levels in a number of other tissues, including muscle (13–15). Considering the relative abundance of PPARγ1 in many nondiapose tissues, it is likely that this isoform is capable of subserving roles apart from regulation of adipogenesis. In addition, although no functional differences between the γ1 and γ2 isoforms have been described to date, it is possible that these isoforms subserve different functions under some conditions. However, deletion of the N-terminal 129 amino acids of PPARγ did not diminish the adipogenic potency of PPARγ that was introduced into 3T3 fibroblasts by retroviral infection (6), and it has therefore been viewed as unlikely that the N terminus of PPARγ subserves a functionally important role.

Here, we provide evidence that PPARγ, in addition to being activated in a ligand-dependent manner, can also be activated in a ligand-independent manner, and we define a ligand-independent activation domain within the N terminus of PPARγ. We also demonstrate the first potential functional difference between the two PPARγ isoforms, wherein the N terminus of PPARγ2 more potently activates a heterologous promoter than does PPARγ1. We have mapped the overall activation domain to a region common to the two isoforms and demonstrate that the 30 amino acids unique to PPARγ2 can activate a heterologous promoter only in concert with the main N-terminal activation domain. Finally, we provide evidence that the ligand-independent activation function of the N terminus of PPARγ is augmented when cells are treated with insulin. We propose a model for the activation of PPARγ and discuss its possible implications.

MATERIALS AND METHODS

Plasmid Construction—The PPRE reporter construct consisted of two copies of the DR1 element upstream of the TK109 promoter in the vector pAγLuc (16). All GAL4 constructs were constructed by inserting
a PCR-generated fragment into the GAL4 vector (17). The γ1 N terminus, γ2 N terminus, each of the GAL4 constructs made to map the activation domain, and the PPARγ N terminus were constructed in a similar manner. Primers were designed to contain an EcoRI (GAATTC) site after a random pentamer (GGCGG) to ameliorate restriction enzyme cutting. Primers were designed to contain a stop codon before a PstI (CTATAAG) site and the same pentamer. The C-terminal GAL4 construct was designed in a similar manner except both primers contained a BamHI (GGATCC) site instead of EcoRI and PstI. All restriction sites were in frame with the template, which kept an open reading frame from GAL4 through the entire PCR fragment. PCR was performed under the following conditions: after heating to 94 °C for 30 s, each PCR cycle was performed, each as follows: 94 °C for 30 s, 54 °C for 1 min, and 72 °C for 1.5 min. Each PCR was purified from the buffer and enzyme using QiAquick gel electrophoresis (21), followed by transfer of the resolved polypeptides to nitrocellulose membranes using the system of Towbin et al. (22). The membranes were blocked with 10% nonfat dried milk in Towbin buffer (20 μl Tris-HCl, pH 7.4, 150 mm NaCl, 0.05% Tween 20) for 2 h at room temperature, then incubated with a polyclonal anti-yeast GAL4 DNA binding domain antibody (Upstate Biotechnology, Inc., Lake Placid, NY) (1:1000) in 5% milk overnight at 4 °C. After removal of unbound antibodies by three washes of 20 min in Towbin buffer at room temperature, membranes were incubated with horseradish peroxidase-conjugated anti-rabbit immunoglobulin (1:1000) in 2.5% milk for 1.5 h at room temperature and washed five times in Towbin buffer. The targeted proteins were detected using enhanced chemiluminescence (ECL) as described by the manufacturer (Amersham International, Buckinghamshire, UK).

RESULTS

We first assessed the capacity of PPARγ to mediate transcriptional activation under conditions of varying availability of endogenous ligand. To do this, we transfected CV-1 cells with an expression plasmid for PPARγ2 and assayed its ability to activate a reporter plasmid consisting of a PPRE upstream of a thymidine kinase (TK) promoter and the luciferase reporter gene. In an attempt to limit the availability of PPARγ ligand, we charcoal-stripped the serum twice before use (Fig. 1A). Despite this treatment of the serum, PPARγ transactivated the reporter plasmid in the absence of added ligand. Activity was further enhanced by the addition of the thiazolidinedione BRL49653, which is known to bind to and activate PPARγ (7, 8). Since this basal activation could have been due to residual ligand in the serum or to ligand produced by the cells during the course of the experiment, we constructed a plasmid containing the yeast GAL4 DBD upstream of the isolated PPARγ ligand binding domain (amino acids 193–505). This construct was co-transfected with a reporter gene containing the yeast GAL4 response element upstream of the TK promoter and luciferase as a reporter (UAS-TK-LUC), under serum and cell conditions identical to those of the TK experiment (Fig. 1B). Although BRL was able to activate this construct very efficiently, indicating that the intactness of the ligand binding domain, there was no activation in the absence of BRL, suggesting that the cellular level of endogenous ligand was extremely minimal under these conditions. The results additionally suggest that the high level of reporter activation mediated by the full-length PPARγ in the presence of the same stripped serum
and without any added ligand was indeed ligand-independent and did not map to the ligand binding domain. To further explore the possible existence of a ligand-independent activation domain in PPARγ, we constructed two separate plasmids containing sequence encoding the N termini of PPARγ1 or -2 (amino acids 1–98 and amino acids 1–128, respectively) downstream of GAL4's DNA binding domain. Upon cotransfecting each of these constructs with UAS-TK-LUC, reporter activation was seen with both N termini, with the activation by the PPARγ2 N terminus being about 5-fold greater than that of PPARγ1 (Fig. 2). In Western blotting experiments, using antibodies against the GAL4 DBD of the proteins, the expression levels of the two fusion proteins were the same (Fig. 3). PPARγ1 DBD and PPARγ2 DBD migrated with molecular masses of ~32 and ~36 kDa, respectively.

It is possible that the N-terminal 30 amino acids that are unique to PPARγ2 define a self-contained transactivation domain that might fully account for the difference between the activation potency of the two isoforms. A construct containing amino acids 1–30 of PPARγ2 downstream of the GAL4 DBD was therefore tested by co-transfection with UAS-TK-LUC. The PPARγ1-2 N-terminal activation domain either alone or upstream of amino acids 1–30, 1–66, 1–129, 31–66, 31–99, or 31–129 of PPARγ2, respectively, and cotransfected with UAS-TK-LUC. Cells were treated as in Fig. 1. Data are expressed as the mean of fold activation over the luciferase activity obtained in cells transfected with the GAL4 DNA binding domain. The results are the mean of two experiments, each performed in triplicate.

to repress the ability of these constructs to activate transcription. Together, these results suggest a complex basis for the structural determinants of the PPARγ ligand-independent activation domain.

To test whether N-terminal activation ability was present in a structurally homologous molecule, we constructed a plasmid containing the A/B domain of PPARβ (amino acids 1–72) downstream of GAL4's DBD, and this construct was unable to activate transcription of the reporter gene (Fig. 2B). Since the extent of transcriptional activation mediated by the PPARγ N termini might be dependent on the presence of tissue-specific co-activators, we transfected the PPARγ2 N-terminal constructs together with UAS-TK-LUC into freshly isolated rat adipocytes (Fig. 5A). The PPARγ2 N terminus showed a greater fold activation in adipocytes, the cell most relevant for PPARγ2 function, than in CV-1 cells. To determine whether the ligand-independent transcriptional activation mediated by PPARγ2 is regulated by insulin, we added 10 nM insulin to the transfected cells (Fig. 5B). Insulin enhanced the ligand-independent activation mediated by both PPARγ1 and -2 constructs, while having little or no effect on the vector containing the GAL4 DBD alone.
FIG. 5. PPARγ N terminus activates transcription in primary rat adipocytes and this is enhanced by insulin. A, isolated rat adipocytes were electroporated with a plasmid containing either the GAL4 DNA binding domain alone or GAL4 DBD upstream of the N-terminal amino acids of PPARγ1 or -2 (amino acids 1–99 and 1–129, respectively), and these were cotransfected with UAS-TK-LUC. B, 2 h after electroporation, some cells were treated with 10 nM insulin as indicated. Cells were harvested 20 h after transfection and assayed for luciferase activity. The data represent three separate experiments, each done in triplicate.

DISCUSSION

In this report we show that PPARγ resembles other members of the nuclear receptor superfamily in having the capacity to activate transcription in a ligand-independent manner. We have shown this in a number of ways. First, by co-transfecting intact PPARγ with a reporter plasmid under conditions designed to limit the availability of endogenous PPARγ ligand, we found that PPARγ activated transcription constitutively. To determine whether this activation was caused by residual endogenous ligand in the cells or media, and to identify a possible activation domain in the N terminus, we utilized the sensitive GAL4/UAS system. First, we created a construct that included PPARγ’s C terminus with both the LBD and its activation domain, but without the DNA binding and N-terminal domains. By cotransfecting this plasmid with the reporter UAS-TK-LUC, we created a sensitive assay for the presence of PPARγ ligand. Using this paradigm, we showed that, under identical cell and serum conditions to those of the experiment with intact PPARγ, this construct showed no transactivation activity. However, the addition to these cell transfectants of the PPARγ ligand BRL49653 produced more than 50-fold activation. Taken together, these results suggest that the activation seen with intact PPARγ in the presence of charcoal-stripped serum and without added ligand is due neither to residual ligand nor to a constitutively active ligand binding domain.

To pursue this possibility further, we created chimeras containing the GAL4 DBD with PPARγ1 and -2 A/B domains, in the absence of their DNA binding, ligand-binding, and C-terminal activation domains. Using these constructs, it is apparent that the N termini of both PPARγ1 and -2 do indeed contain activation domains, as assessed by the ability of these constructs to activate transcription of a GAL4-responsive reporter gene. Thus, we have shown that PPARγ can enhance transcription in both a ligand-dependent and ligand-independent manner.

The potential importance of this ligand-independent activation is worthy of discussion. Many members of the nuclear receptor superfamily, including receptors for estrogen, progesterone, thyroid hormone, glucocorticoids, vitamin D3, and retinoic acid contain two distinct domains responsible for activating transcription of specific genes (23). A critical activation domain is present in the receptor C terminus (the E domain), and this is activated upon binding of the cognate hormone/ligand to the adjacent ligand binding domain (reviewed in Ref. 24). In addition to this ligand-activated function, these receptors can also activate transcription independent of ligand binding, via an activation domain that resides within their N terminus (the A/B domain). This domain can be constitutively active and/or be regulated independently of ligand via phosphorylation (25, 26). Ligand-independent activation (AF-1) domains have been shown to have several attributes. In some cases they act synergistically with the ligand-dependent activation domain, as with the human estrogen receptor in HeLa cells (27), where neither activation domain in isolation was capable of activating transcription significantly but both domains together produced marked activation. The ligand-independent activation domain may also be necessary for the regulation of complex cellular events, as in the case of glucocorticoid-induced apoptosis in lymphocytes (28). Transfection of glucocorticoid receptors in which the N terminus was deleted ablated the apoptotic cause by the intact receptor, while the same receptor remained responsive to a number of other dexamethasone effects to induce gene expression, suggesting that regulation of different genes may be mediated by distinct activation domains of the same receptor (29). In the case of the vitamin D3 receptor, it has been shown that mutating serine 51 and thus preventing phosphorylation reduces activity (30), suggesting that phosphorylation/dephosphorylation may be involved in regulation of this activation domain.

These data also provide evidence that the two PPARγ isoforms may have differential abilities to activate target genes. To date, there have been no reports of functional differences between the PPARγ 1 and 2 isoforms, which have been described as having very similar capacities to be activated by known activators in co-transfection assays (14) and to be fully capable of bringing about the adipogenic program of differentiation in 3T3 cells under appropriate conditions. Here, we show that the N terminus of PPARγ2 is much more potent at activating a reporter independent of ligand than is the N terminus of PPARγ1. Interestingly, this difference between the two PPARγ isoforms is even more pronounced after transient transfection into isolated rat adipocytes, cells that normally express markedly higher levels of PPARγ2 than other tissues (1, 13). Whether this observation is due to higher expression in adipocytes of relevant but currently unknown co-activators is not known at this time. It is interesting to note that in adipocytes from both rodents (13) and humans (15), PPARγ2 is the isoform whose expression is more influenced by obesity and...
nutritional perturbations, while the expression of PPARγ1 is relatively stable under these conditions in vivo. The preferential regulation of PPARγ2 expression in physiologic states such as starvation is likely to serve a physiologic purpose that would not be evident if the two isoforms had identical profiles of biologic activity.

Tontonoz et al. (6) have shown that deletion of amino acids 1–127 in the N terminus of PPARγ2 does not reduce, but actually increases, the capacity of retrovirally expressed PPARγ2 to induce adipocyte differentiation of NIH-3T3 cells, as assessed by lipid accumulation and expression of adipose-specific genes. This could be viewed as conflicting with our results by suggesting that the N terminus does not play a physiological role in vivo. This apparent conflict can be resolved in several ways, however. First, Tontonoz et al. (6) performed their studies under conditions of excess ligand, including ETYA, which was at the time the best PPARγ activator, and they used nonstripped serum, another potential source of PPARγ ligand. Thus, while their results demonstrate that under conditions of abundant ligand, the N terminus of PPARγ is not necessary for fat cell differentiation, they do not conflict with the possibility that the N-terminal activation domain of PPARγ plays an important role under distinctly different conditions, i.e. when ligand is not abundant. Studies of differentiation induced by isolated PPARγ N termini using the retroviral approach will be necessary to further resolve the ability of the PPARγ N terminus to bring about the full program of adipogenesis or perhaps reveal an adipogenesis-unrelated role.

Finally, we observed in both CV-1 cells and isolated rat adipocytes that insulin is capable of regulating the ligand-independent transcriptional activity of PPARγ through its N-terminal activation domain. To understand the possible mechanism for this finding, it is necessary to review recent observations on the regulation of PPARγ activity by covalent modification. Shalev et al. (31) demonstrated that PPARγ is a phosphoprotein and that phosphorylation is capable of enhancing its transcriptional activation potency, although mapping of the responsible sites was not carried out. Zhang et al. (32) showed that insulin treatment of cells enhanced the ability of full-length PPARγ2 to stimulate cAMP gene expression and speculated that this might be mediated by the mitogen-activated protein kinase-dependent phosphorylation of PPARγ that they also demonstrated. In contrast, Hu et al. (33) clearly demonstrated that mitogen-activated protein kinase-dependent phosphorylation of PPARγ2 took place on serine 112 in the N-terminal activation domain in response to mitogens, but this phosphorylation inhibited, rather than stimulated, the ability of full-length PPARγ2 to promote specific gene expression and the process of adipogenesis. Very recently, Adams et al. (34) reported that mutation of the consensus mitogen-activated protein kinase site (serine 82) of PPARγ1 to alanine resulted in increased ligand-dependent transcriptional activity. In addition, these authors also described a weak constitutive transcriptional activity of the isolated PPARγ1 N terminus, and this was also increased when serine 82 was mutated. Whether or not the activity of insulin to enhance transactivation by intact PPARγ2 (32) or PPARγ2 N terminus as reported here is mediated by phosphorylation of PPARγ2, it is clear that signaling by PPARγ and insulin produce a number of common effects, including the ability to promote adipocyte differentiation (35, 36). Another indication of convergent pathways for these molecules is the fact that the insulin-sensitizing thiazolidinediones are now known to act by binding to and activating PPARγ (7, 8). The data presented here suggest a novel molecular basis for such a link, i.e. through convergence of their signaling pathways by an ability of insulin to enhance the function of the AF-1 activation domain of PPARγ, whether by direct phosphorylation or more likely through some other mechanism, such as an ability of insulin to modify a co-activator protein.

Interestingly, our mapping experiments of the N-terminal activation domain provide evidence for a complex mechanism by which PPARγ's N terminus can influence transactivation. Thus, the PPARγ2 GAL4 (1–128) construct was less active than the PPARγ2 GAL4 (1–99), and the PPARγ2 GAL4 (1–66) construct was less active than the PPARγ2 GAL4 (31–99). Although such results could be the consequence of a number of factors, they could be consistent with the existence of an N-terminal repression moiety under some conditions.

Prior to these studies and the report of Adams et al. (34), a ligand-independent N-terminal activation domain had not been described in the PPARγ gene family. Our results raise many questions about the functional roles of the ligand-independent and -dependent PPARγ activation domains under varying metabolic conditions that are likely to confront adipocytes and other cells that express these receptors. For example, it is possible that, under conditions of abundant PPARγ ligand, PPARγ would favor differentiation by causing growth arrest and transcription of adipocyte genes via the ligand-dependent AF-2 domain, whereas when ligand is limiting, as might be predicted to occur during starvation, PPARγ would act on promoters of genes needed for basal adipocyte homeostasis via the ligand-independent AF-1 domain. Such speculations on the possible functional domains of PPARγ will now need to be tested experimentally.

In summary, we have demonstrated that PPARγ, like many other members of the steroid receptor superfamily, contains a ligand-independent activation domain in its N terminus. We have further defined a functional difference between the two PPARγ isoforms; PPARγ2, which is expressed mainly in adipose tissue, activates ~5–10-fold more potently via its AF-1 activation domain than does PPARγ1. Lastly, we have shown that the N-terminal ligand-independent activation domain can be regulated by insulin, providing a new basis for the convergent signaling pathways of these molecules. Together, these results suggest a more complex regulation of PPARγ action than previously known and raise the possibility that differential regulation of PPARγ isoforms in vitro might account for different molecular activities and phenotypes.

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