Regulation of the Transcriptional Activity of the Peroxisome Proliferator-activated Receptor α by Phosphorylation of a Ligand-independent trans-Activating Domain

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Cristiana E. Juge-Aubry‡, Eva Hammar‡, Catherine Siegrist-Kaiser‡, Agnès Pernin‡, Akira Takeshita§, William W. Chin§, Albert G. Burger‡, and Christoph A. Meier‡‡

From the ‡Division of Endocrinology and Diabetes, University Hospital Geneva, CH-1211 Geneva 14, Switzerland, the §Division of Genetics, Brigham & Women’s Hospital, Boston, Massachusetts 02115, and the ‡‡Clinique de Médecine II, Department of Medicine, University Hospital Geneva, CH-1211 Geneva, Switzerland

The peroxisome proliferator-activated receptors (PPARs) are a subgroup of nuclear receptors activated by fatty acids and eicosanoids. In addition, they are subject to phosphorylation by insulin, resulting in the activation of PPARα, while inhibiting PPARγ under certain conditions. However, it was hitherto unclear whether the stimulatory effect of insulin on PPARα was direct and by which mechanism it occurs. We now demonstrate that amino acids 1–92 of hPPARα contain an activation function (AF)-1-like domain, which is further activated by insulin through a pathway involving the mitogen-activated protein kinases p42 and p44. Further analysis of the amino-terminal region of PPARα revealed that the insulin-induced trans-activation occurs through the phosphorylation of two mitogen-activated protein kinase sites at positions 12 and 21, both of which are conserved across evolution. The characterization of a strong AF-1 region in PPARα, stimulating transcription one-fourth as strongly as the viral protein VP16, is compatible with the marked basal transcriptional activity of this isoform in transfection experiments. However, it is intriguing that the activity of this AF-1 region is modulated by the phosphorylation of two serine residues, both of which must be phosphorylated in order to activate transcription. This is in contrast to PPARγ2, which was previously shown to be phosphorylated at a single site in a motif that is not homologous to the sites now described in PPARα. Although the molecular details involved in the phosphorylation-dependent enhancement of the transcriptional activity of PPARα remain to be elucidated, we demonstrate that the effect of insulin on the AF-1 region of PPARα can be mimicked by the addition of triiodothyronine receptor β1, a strong binder of corepressor proteins. In addition, a triiodothyronine receptor β1 mutant deficient in interacting with corepressors is unable to activate PPARα. These observations suggest that the AF-1 region of PPARα is partially silenced by corepressor proteins, which might interact in a phosphorylation-dependent manner.

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† To whom correspondence should be addressed. Tel.: 41-22-372-9039; Fax: 41-22-372-9329; E-mail: cameier@genet.ch.

1 The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; hPPAR, human PPAR; TR, triiodothyronine receptor; NCoR, nuclear receptor co-repressor; SMRT, silencing mediator for RAR and TR; MAP-K, mitogen-activated protein kinase; CAT, chloramphenicol acetyltransferase; AF, activation function.

2 A. Gorla-Bajsczczak and C. A. Meier, unpublished data.
viation of PPARα. Intriguingly, these MAP-K sites are distinct from the inhibitory MAP-K site described for PPARγ. Moreover, our results demonstrate that the transcriptional activation of PPARα by insulin may involve the dissociation from co-repressor proteins, which may be related to the nuclear receptor co-repressor (NCoR) and the silencing mediator for RAR and TR (SMRT) (21, 22). Hence, the insulin-induced phosphorylation of PPARα and γ is mediated through distinct phosphorylation motifs, resulting in transcriptional activation or repression, respectively.

**MATERIALS AND METHODS**

**Plasmid Constructs and Mutagenesis**—A fusion construct linking the GAL4-DNA-binding domain (amino acids 1–147) upstream of the amino-terminal region (amino acids 1–92) of hPPARα was constructed by cloning the cDNA corresponding to the first 92 amino acids of hPPARα amplified by polymerase chain reaction using the Pfu DNA polymerase (Life Technologies, Inc.) into the BamHI and PstI sites of the pGAL-4BD expression plasmid (CLONTECH, Stelhlin AG, Basel, Switzerland), resulting in the vector pGAL4BD-hPPARα. The expression plasmid pSG5-hPPARα was kindly provided by Dr. F. Gonzalez (NCI, National Institutes of Health, Bethesda, MD) (16). The final construct was amplified by automated sequencing (ABI 373, Perkin-Elmer).

Site-directed mutagenesis was performed by polymerase chain reaction amplification of the entire pGAL4BD-hPPARα1–92 or pSG5-hPPARα plasmids with Pfu DNA polymerase using sense and antisense primers containing the desired mutation: S77A mutation (GCC → GCG), S76A (TCC → GCC), S12A (TCC → GCC), S21A (AGC → GCC) according to the manufacturer’s protocol (QuickChange site-directed mutagenesis, Stratagene, Basel, Switzerland). The presence of the desired mutation and the absence of spurious mutations in the amplified cDNA was verified by automated sequencing.

The reporter plasmid pG5-chloramphenicol acetyltransferase (CAT) (CLONTECH, Stelhlin AG, Basel, Switzerland) contains five consensus GAL4 binding sites (UAS5). As a positive control, the plasmid pM3-VP16, encoding a fusion protein of the GAL4 BD and the VP16 activation domain, was used. The reporter plasmid pHL-I×PPRE-MEP-CAT8, containing the natural PPAR-response element from the malic enzyme promoter, was described previously (17).

The pSV2-TRβ1 expression vector encoding for the TR β1 was described previously (23). The triple mutant pcDNA/Amp-TRβ1-DNCor box (pA23C5/H224G/T227A) was generated by site-directed mutagenesis. This construct was shown previously to be deficient in its interaction with corepressor proteins (21).

**Transfection Experiments**—The human HepG2 hepatoma cell line was cotransfected using the calcium phosphate method as described previously with 4.2 μg of the expression plasmid for the parental vector pGAL4BD or the fusion protein pGAL4BD-hPPARα1–92, together with 0.42 μg of the reporter plasmid pG5-CAT (17, 24). Twenty-four hours prior to cell harvesting, cells were treated with 1 μM insulin and/or 30 μM of the MAP-K p42/p44 inhibitor PD98059 (Alexis Corp., Laüfelfingen, Switzerland) dissolved as described in the manufacturer instructions. CAT activity was determined as described by determining the incorporation of [3H](chloramphenicol (Hartmann Analytic, Braun-schweig, Germany) into acetyl-CoA as analyzed by thin layer chromatography (24). The results were quantitated by a PhosphorImager (Molecular Dynamics). CAT activity was normalized to the protein concentration as measured by the Bradford method (Bio-Rad).

**Phosphorylation Experiments**—HepG2 cells were transfected with 4.6 μg of pGAL4BD or either wild-type or mutant pGAL4BD-hPPARα constructs. Twenty-four hours before harvesting, cells were incubated in serum-free and phosphate-depleted Dulbecco’s modified Eagle’s medium (Life Technologies, Inc., Basel, Switzerland) followed by labeling with 32P orthophosphate for 2 h. Insulin (1 μM) was added for 20 min as described previously (10). Cells were lysed in radioimmuneprecipitation buffer (0.15 M NaCl, 10 mM sodium phosphate, pH 7.2, 2 mM EDTA, 1% Nonidet P-40, 1% deoxycholate, 50 mM NaF, Complete Complete protease inhibitors (Boehringer Mannheim) and 5 mM orthovanadate), and the expression proteins were immunoprecipitated with a polyclonal anti-GAL4BD antibody (1 μg) (Santa Cruz Biotechnology, Inc., Dr. Glaser AG, Basel, Switzerland). Immunoprecipitated products were analyzed by 10% SDS-polyacrylamide gel electrophoresis.

**Western Blotting**—As described above, HepG2 cells were transfected with 4.6 μg of pGAL4BD or the appropriate pGAL4BD-hPPARα constructs. After 48 h, cells were lysed in radioimmuneprecipitation buffer, and the fusion proteins were immunoprecipitated by using 1 μg of rabbit polyclonal anti-GAL4BD antibody. After electrophoresis, the proteins were electrotransferred to a 0.45-μm nitrocellulose membrane (Bio-Rad) for 45 min at 100 V (4 °C) in 25 mM Tris base, 190 mM glycine, 20% (v/v) methanol. Nonspecific binding was blocked by incubating the membrane for 2 h in 5% powdered milk in TBST (20 mM Tris, 150 mM NaCl, 0.1% Tween, pH 7.6). A monoclonal anti-GAL4BD antibody (CLONTECH, Stelhlin AG, Basel, Switzerland) was added to a solution of 1% powdered milk/TBST for 12 h. The membrane was washed twice with TBST, followed by five rinses of 15 min each in TBST. The second antibody (anti-mouse IgG horseradish peroxidase (Santa-Cruz Biotechnology, Inc., Dr. Glaser AG, Basel, Switzerland)) was diluted (1:1000 v/v) and was added for 2 h at 4 °C. After incubation, the membrane was washed as described above. Immunolabeled proteins were revealed by chemiluminescence as described by the manufacturer (Amersham Pharmacia Biotech, Zurich, Switzerland).

**RESULTS**

The **Amino Terminus of hPPARα (amino acids 1–92) Contains a Ligand-independent Activation Domain (AF-1), Which Is Regulated by Phosphorylation**—In order to explore the possibility that the previously described significant constitutive activity of the full-length hPPARα is due to an AF-1-like function in the amino terminus of this protein, we created a fusion construct linking the GAL4 DNA-binding domain to the first 92 amino acids of hPPARα (pM-GAL4BD-hPPARα). The transcriptional response of this construct was assessed in transient transfection experiments in HepG2 cells using a UAS-CAT reporter (pG5-CAT). Fig. 1 shows that the addition of the first 92 amino acids of hPPARα to the GAL4BD enhances the constitutive transcriptional activity over 20-fold, as compared with an 80-fold stimulation by the known strong transcriptional activator VP16.

To examine whether the previously described stimulation of the basal transcriptional activity of PPARα by insulin might involve this novel AF-1 domain, similar experiments were performed in the presence of 1 μM insulin for 24 h. As shown in Fig. 1, treatment with insulin enhances the activity of the amino terminus of PPARα over 4-fold, whereas no effect was seen on the control constructs GAL4BD and VP16.

**FIG. 1. Transcriptional activity of the fusion protein GAL4BD-hPPARα (1–92) on a GAL4 binding site UAS, 17 mer (→S) cloned upstream of a CAT reporter gene.** The effect of insulin and of the MAP-K p42/p44 inhibitor PD98059 were tested. HepG2 cells were transiently transfected with either pM-GAL4BD, pM-GAL4BD-hPPARα (amino acids 1–92), or the positive control plasmid pM3-VP16 together with the pM-CAT reporter construct. Twenty-four hours prior harvesting, cells were either left untreated (treated with vehicle (none)) or treated with insulin (1 μM), PD98059 (30 μM), or both. Transcriptional activity is expressed as relative CAT activity normalized to protein content (percentage of GAL4BD-hPPARα (1–92) activity with vehicle). Values (mean ± S.E. of four separate experiments) were performed in triplicate.
Because we have shown recently that insulin rapidly stimulates the phosphorylation of PPARα in adipose tissue and transfected cells, we also assessed the effect of insulin on PPARα in the presence of the specific inhibitor of the p42/44 MAP-K pathway PD98059 (25). Compatible with the hypothesis that the stimulation of the amino terminus of PPARα by insulin involves a MAP-K-mediated phosphorylation event, PD98059 completely abolished this effect (Fig. 1).

The Amino Terminus of hPPARα Contains Several Putative MAP-K Sites—Because the results described above suggest that insulin modulates the basal transcriptional activity of PPARα through the phosphorylation of a MAP-K site in its amino terminus, we examined the known sequences of PPARα from various species for conserved MAP-K motifs (PΦSP) (Fig. 2). Whereas several MAP-K motifs are present within the first 92 amino acids of hPPARα, three putative sites were highly conserved across evolution: serine 12, serine 21, and serine 77. Interestingly, the latter residue corresponds to a MAP-K motif described in mPPARγ2, which has been shown to be phosphorylated by MAP-K and results in an inhibition of transcription. Hence, we created the corresponding mutants of the pSG5-GAL4BD-hPPARα construct for the functional analysis of these sites (pM-GAL4BD-S12A, pM-GAL4BD-S21A, and pM-GAL4BD-S76A/S77A).

Serines 12 and 21, but Not 76/77, Mediate the Insulin Stimulation of the AF-1 Domain of PPARα—Using the various mutants of the putative MAP-K sites within the amino terminus of PPARα, we performed transient transfection experiments in HepG2 cells to assess their functional relevance. Fig. 3 shows that the serine to alanine mutations at positions 12 and 21 were both able to abolish the stimulatory effect of insulin, whereas similar mutations of another putative MAP-K site (Ser-76/77) did not alter the fold transcriptional stimulation by insulin. The lower transcriptional activity of the S76A/S77A mutant correlates with its lower expression as assessed in Western blot experiments (data not shown).

These results are thus compatible with a model in which insulin activates the AF-1 region of PPARα through the MAP-K-mediated phosphorylation of serines 12 and 21.

Serines 12 and 21 of hPPARα Are Phosphorylated in Response to Insulin—In order to test the hypothesis that insulin mediates the phosphorylation of serine residues 12 and 21, we performed phosphorylation experiments in HepG2 cells transfected with the appropriate wild-type and mutant pSG5-GAL4BD-PPARα constructs. Shown in Fig. 4 are the phosphorylated fusion proteins after precipitation with an anti-GAL4BD antibody, resulting in a main band at 39 kDa, corresponding to the apparent molecular mass of the GAL4BD-PPARα fusion protein. (CLONTECH document 5399–1), as well as a smaller band, which is likely to correspond to a proteolytic fragment. These experiments demonstrated a 2.8-fold increased phosphorylation of wild-type GAL4BD-PPARα in response to insulin, which is quantitatively comparable to the 4.5-fold enhancement in trans-activation observed above. However, whereas the single mutants S12A and S21A were still phosphorylated by insulin, the double mutant S12A/S21A did not show an increase in its phosphorylation status after treatment with insulin. These data demonstrate that serines 12 and 21 are both phosphorylated by insulin and that no other residues contribute to the insulin-enhanced phosphorylation of the amino terminus of PPARα.

Expression of GAL4BD-PPARα and Its Mutants at the Protein Level—In order to ascertain that the absent response of the GAL4BD-PPARα-S12A and S21A mutants was not due to the altered expression of these proteins, we performed Western blot analyses with an anti-GAL4BD antibody. As shown in Fig. 5, the S12A and S12A/S21A mutants were expressed at similar levels as the wild-type protein, whereas the S21A mutant was somewhat less abundant.

Serines 12 and 21 Are Required for the Insulin-mediated Increase in Transcriptional Activity of the Full-length PPARα—In order to examine the role of the Ser-12 and Ser-21 residues in the context of the intact hPPARα, both serines were mutated to alanine (pSG5-hPPARα-S12A/S21A). As shown in Fig. 6, the wild-type intact hPPARα was activated by insulin in a dose-dependent manner (p = 0.03, one-way analysis of variance), whereas the double-mutant S12A/S21A exhibited only a minimal and statistically not significant response to insulin (p > 0.2, one-way analysis of variance).

Phosphorylation of the AF-1 Region of PPARα May Result in the Dissociation of Co-repressor Proteins—Multiple scenarios can possibly explain how the phosphorylation of serines 12 and 21 activates transcription, such as the recruitment or dissociation of putative coactivator or corepressor proteins. Therefore, we hypothesized specifically that the phosphorylation event might result in the dissociation of a corepressor protein, such as NCoR or SMRT, thereby resulting in transcriptional derepression and hence activation. The testable prediction of this model is that the addition of a strong binder of such co-repressor proteins should exert an insulin-like stimulation on the AF-1 region of PPARα. In order to examine this hypothesis, we transfected HepG2 cells with GAL4BD-PPARα (amino acids 1–92) either alone or in the presence of increasing amounts of...
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**Fig. 4.** Effect of insulin on the level of phosphorylation of the GAL4BD-hPPARα (1–92) wild type (WT) and the mutated constructs S12A, S21A, and S12A/S21A. HepG2 cells were transfected with pmGal4BD, pmGal4BD-hPPARα (1–92) wild-type, or the appropriate mutants. Twenty-four hours before harvesting, cells were placed in serum-free and phosphate-free medium, followed by labeling with [32P]orthophosphate for 2 h, and stimulated with insulin (1 µM) for 20 min. After cell lysis, fusion proteins were immunoprecipitated with anti-GAL4BD antibodies and submitted to SDS-polyacrylamide gel electrophoresis. Dried gels were autoradiographed and exposed on a PhosphorImager.

**Fig. 5.** Level of expression of the wild-type and mutant GAL4BD-hPPARα constructs assessed by Western blotting. HepG2 cells were transfected with pmGal4BD, or the wild-type or mutant pmGal4BD-hPPARα (amino acids 1–92) constructs. After harvesting and lysis, the fusion proteins were immunoprecipitated with a polyclonal anti-GAL4BD antibody and submitted to SDS-polyacrylamide gel electrophoresis. Proteins were electrotransferred to a nitrocellulose membrane and blotted with a monoclonal anti-GAL4BD antibodies and revealed with an anti-mouse IgG-horseradish peroxidase antibody. Immunolabeled proteins were visualized by chemiluminescence.

**Fig. 6.** Effect of insulin on the transcriptional activity of intact wild-type PPARα and the mutated hPPARα-S12A/S21A. HepG2 cells were transfected with either pSG5-hPPARα or pSG5-hPPARα-S12A/S21A together with the reporter construct pBL-1xPPRE-MEpCAT8+. After transfection, the cells were exposed to vehicle or insulin (2 or 10 µM) for 24 h. Transcriptional activity is expressed as relative CAT activity, normalized to protein content. Values are the mean ± S.E. (n = 3). The insulin dose-response curves were analyzed by one-way analysis of variance. Whereas insulin had a significant effect on the transcriptional activity of wild-type PPARα (p = 0.03), the response of the S12A/S21A mutant was not statistically significant.

TRβ1, which is known to strongly interact with corepressor proteins (21, 22). Interestingly, the addition of TRβ1 was able to increase the transcriptional activity of the AF-1 domain of PPARα in a dose-dependent manner and to a level corresponding to that observed with insulin (Fig. 7A). However, to exclude the possibility that TRβ1 might squelch other proteins nonspecifically, a similar experiment was performed with a mutant TRβ1, known to be deficient in its interaction with corepressor proteins, such as NCoR and SMRT (TRβ1-ΔNCoR box). As shown in Fig. 7B, this mutant has lost its capacity to activate PPARα compatible with the model that TRβ1 competes for corepressor proteins, resulting in a derepression of the AF-1 domain of PPARα.

**DISCUSSION**

The human PPARα was previously shown to exhibit significant ligand-independent transcriptional activity, which can be modulated by insulin (10). The observation that insulin also enhances the phosphorylation of PPAR in adipocytes as well as in transfected cells raised the possibility that insulin might directly phosphorylate PPARα, thereby enhancing its transcriptional capacity. In the present paper, (i) we demonstrate that the amino-terminal 92 amino acids of PPARα contain an AF-1-like trans-activation domain, which is further activated by insulin, (ii) we demonstrate that the effect of insulin on the AF-1 region is dependent on MAP-K p42/44 and involves the phosphorylation of two serine residues at positions 12 and 21, and (iii) we present data indicating that the phosphorylation of the amino terminus of PPARα may result in the dissociation of corepressor proteins, which may then result in a further transcriptional enhancement of this domain.

The concept that nuclear hormone receptors contain a ligand-independent (AF-1) and a ligand-dependent (AF-2) trans-activation domain has been well demonstrated for the steroid and retinoid receptors (18, 20, 26). However, whereas the activity of the AF-2 domain is regulated by the binding of ligand resulting in the recruitment of coactivator proteins, the AF-1 region is conventionally thought to be constitutively active by molecular mechanisms that are poorly understood (27). The presence of a strong AF-1 region in the A/B domains of PPARα shown in the present paper explains, at least in part, the basal activity observed with this PPAR isoform in transient transfection experiments. Interestingly, the present data demonstrate that this activity is subject to regulation through phosphorylation of two MAP-K sites. Because we have previously shown that PPARα is a phosphoprotein in primary adipocyte cultures stimulated with insulin, it is quite likely that the...
cross-talk between these two signaling pathways is of functional relevance. This notion is supported by our observation that the full-length PPARα is also activated by insulin and that this ligand-independent trans-activation requires serines 12 and 21, compatible with the results obtained with the GAL4-PPARα 1–92 fusion construct. Although not statistically significant, the full-length S12A/S21A-PPARα retains a minimal residual response to insulin. However, this small effect is not mediated by the amino-terminal 92 amino acids, as supported by experiments with a PPARα deletion mutant lacking the amino terminus (data not shown). This residual stimulation by insulin may thus be mediated either through other regions of PPARα or through insulin effects on, for example, co-activators or the general transcriptional machinery. It is also of interest to note that treatment of primary hepatocytes with insulin for 3 days decreases the expression of the PPARα gene, suggesting that short-term exposure to insulin would increase trans-activation by PPARα (via the phosphorylation of PPARα), whereas a longer exposition to this hormone might result in the down-regulation of PPARα activity in liver (via the decreased expression of PPARα mRNA) (28).

In contrast to PPARα, the PPARγ2 isoform, which was recently shown to be phosphorylated in its amino terminus in response to activators of the MAP-K pathway, is inhibited after phosphorylation of a serine residue at position 112 as assessed by its adipogenic potential (11). Nevertheless, when examined in different experimental systems or in the context of GAL4-fusion constructs, the amino terminus of PPARγ can also be stimulated by phosphorylation. Hence, it appears that at least in adipose tissue, which coexpresses the PPARα and γ isoforms at high levels, the phosphorylation by insulin has opposite effects on PPARα and γ, the former being activated, whereas the latter is inhibited. This observation, together with their different ligand binding spectrum and differences in DNA binding, provides a novel mechanism for generating isoform-specific responses of PPAR (17).

The phosphorylation sites in PPARα are highly conserved across species, and they are distinct from the homologous MAP-K site shown to be phosphorylated in γ2 (see Fig. 2). Hence, the functionally opposite effects of insulin on PPARα and γ2 are reflected by the presence of distinct phosphorylation sites. The mechanisms mediating phosphorylation-dependent alterations in transcription rates are still poorly understood, as is the case for the PPARs. Because the AF-1 domain as well as the insulin-stimulated phosphorylation sites colocalize within the same domain of PPARα, it is tempting to speculate that the altered phosphorylation results in the differential recruitment of coactivator and/or corepressor proteins. However, the currently known coactivator proteins interact preferentially in a ligand-dependent manner with the AF-2 domain of nuclear receptors, although some cooperation with AF-1 may occur (29, 30). Moreover, the corepressor proteins, such as NCoR and SMRT, interact with different regions of the nuclear receptors in a ligand-independent manner. Therefore, our finding that TRβ1 can activate the amino terminus of PPARα is of interest, because the TRs are known to strongly interact with NCoR and SMRT, thereby resulting in a putative intranuclear sink for corepressor proteins. Because this effect is abolished by a mutation in the NCoR box of TRβ1, the presence of distinct phosphorylation sites colocalize within the same domain of PPARα, it is tempting to speculate that the altered phosphorylation results in the differential recruitment of coactivator and/or corepressor proteins. Because this effect is abolished by a mutation in the NCoR box of TRβ1, it is tempting to speculate that the amino terminus of PPARα interacts with a NCoR-like corepressor protein that is able to partially silence the activity of AF-1. However, whether the phosphorylation of serines 12 and 21 derepresses AF-1 by inducing the dissociation of corepressors is a matter of conjecture. Although PPARγ was shown to interact with NCoR in solution, this interaction involved the hinge region of PPARγ, rather than the amino terminus (31). Nevertheless, some recent data demonstrate that an anti-NCoR antibody blocks the transcriptional repression of PPARγ by insulin, suggesting at least indirectly a functional interaction between the phosphorylated amino terminus of PPARγ and NCoR (32). Preliminary experiments utilizing gel mobility shift and glutathione S-transferase pull-down assays examining the possibility of direct interactions of NCoR with the amino terminus of PPARα were inconclusive. These findings indicate that either the interactions between the AF-1 region of PPARα and NCoR are weak under the conditions used, or alternatively, novel corepressor proteins are involved, which have the potential to interact with the AF-1 region as well as the conventional NCoR box motif of nuclear receptors.

In summary, we have demonstrated that PPARα contains a strong ligand-independent AF-1 domain, which can be further

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3 C. Juge and C. A. Meier, unpublished data.
activated through the MAP-K-mediated phosphorylation of two serine residues, which are distinct from the inhibitory phosphorylation site present in PPARγ2. In addition, the AF-1 region of PPARα can be equally well activated by squelching corepressor proteins, suggesting that such proteins might silence the amino terminus of PPARα. It can thus be speculated that the phosphorylation of the AF-1 domain of PPARα might result in the dissociation of such corepressor proteins, thereby resulting in transcriptional activation.

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