Transcriptional Activity by Peroxisome Proliferator-activated Receptor γ Is Inhibited by Phosphorylation at a Consensus Mitogen-activated Protein Kinase Site*

(Received for publication, November 4, 1996, and in revised form, December 12, 1996)

The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) regulates transcription in response to prostanoid and thiazolidinedione ligands and promotes adipocyte differentiation. The amino-terminal A/B domain of this receptor contains a consensus mitogen-activated protein kinase site in a region common to PPARγ1 and γ2 isoforms. The A/B domain of human PPARγ1 was phosphorylated in vitro, and this was abolished either by mutation of serine 84 to alanine (S84A) or coexpression of a phosphoprotein phosphatase. In vitro, this domain was phosphorylated by ERK2 and JNK, and this was markedly reduced in the S84A mutant. A wild type Gal4-PPARγ(A/B) chimera exhibited weak constitutive transcriptional activity. Remarkably, this was significantly enhanced in the S84A mutant fusion. Ligand-dependent activation by full-length mouse PPARγ2 was also augmented by mutation of the homologous serine in the A/B domain to alanine. The nonphosphorylatable form of PPARγ was also more adipogenic. Thus, phosphorylation of a mitogen-activated protein kinase site in the A/B region of PPARγ inhibits both ligand-independent and ligand-dependent transcriptional functions. This observation provides a potential mechanism whereby transcriptional activation by PPARγ may be modulated by growth factor or cytokine-stimulated signal transduction pathways involved in adipogenesis.

Adipocyte differentiation is a complex process regulated by extracellular hormone and cytokine stimulation (1). Cultured preadipocyte cell lines differentiate into lipid-laden adipocytes following exposure to insulin, glucocorticoid, and inducers of intracellular cyclic AMP (2). Conversely, epidermal growth factor (EGF)3 and transforming growth factor α (TGFα) act via the EGF receptor to inhibit both primary and preadipocyte cell line differentiation (3, 4). Tumor necrosis factor α (TNFα) is also a potent inhibitor of differentiation. In addition, this cytokine promotes lipolysis and down-regulates adipocyte-specific gene expression in mature adipocytes (5, 6).

Adipocyte differentiation is driven by the coordinate expression of a range of transcription factors, including C/EBPγ, Per1, -2, and -4 (7) and ADD1 (8), which lead to the expression of adipocyte-specific genes. In addition, the peroxisome proliferator-activated receptor γ (PPARγ) has been shown to be selectively expressed in adipocytes (9, 10) and to modulate adipocyte-specific gene expression (10). Alternative splicing generates two receptor isoforms such that mouse PPARγ2 has a 28-residue extension at its amino terminus compared with human PPARγ1. PPARγ2 mRNA is highly expressed in murine adipocyte cell lines (10), whereas both receptor isoforms are abundant in freshly isolated mouse adipocytes (11). The early induction of PPARγ mRNA expression during adipogenesis, combined with the ability of retrovirally overexpressed PPARγ to induce lipid accumulation and the expression of adipocyte-specific genes (12), suggests that this receptor plays an important role in preadipocyte differentiation. This notion is strengthened by the observation that specific high affinity ligands for PPARγ including thiazolidinediones (which act as insulin sensitizers in vivo), as well as eicosanoids, promote the differentiation of murine preadipocyte cell lines (13–15).

PPARγ is an orphan member of the nuclear receptor family. These receptors share a conserved domain structure and mediate gene transcription through two transcription activation mechanisms: a hormone-dependent transcription activation function (AF-2) is located in the carboxyl-terminal hormone-binding domain, whereas the amino-terminal A/B domain contains a ligand-independent activation function (AF-1). Recently, the AF-1 activity of the estrogen receptor (ER), another member of this family, has been shown to be modulated following phosphorylation by mitogen-activated protein (MAP) kinase (16, 17). Three MAP kinase pathways have been identified in mammalian cells. Members of the extracellular signal-regulated kinases, ERK1 and ERK2, are activated predominantly by growth factor stimulation via a Ras-dependent signal transduction cascade (18). In contrast, activity of Jun NH₂-terminal kinase (JNK, also known as SAPK) and p38 kinase is increased by exposure of cells to environmental stress or to cytokines

*This work was supported by Grant DK49780 from the National Institutes of Health (to M. A. L.) and a grant from the Wellcome Trust (to V. K. C.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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including TNFα (19, 20). In turn, activated MAP kinases have been shown to regulate the activity of specific transcription factors including Elk-1, ATF-2, and c-Jun by phosphorylation of serine or threonine residues in the appropriate context (21).

Interestingly, it has been reported that PPARγ and PPARγ2 are similarly adipogenic but that a truncated receptor in which the amino-terminal domain of PPARγ2 is deleted is a more potent inducer of adipocyte differentiation (12). We noted that NH2-terminal domain of PPARγ contains a consensus MAP kinase site in a region conserved between PPARγ and PPARγ2 isoforms. Furthermore, we and others (11, 22) have observed that PPARγ proteins migrate on immunoblots as closely spaced doublets, a pattern suggestive of phosphorylation. In this report we show that this putative MAP kinase site is phosphorylated in vivo and also in vitro by ERK2 and JNK. Furthermore, we demonstrate that phosphorylation significantly inhibits both ligand-independent and ligand-dependent transcriptional activation by PPARγ. Finally, we show that mutation of this MAP kinase site in PPARγ increases its adipogenic activity. These findings provide a potential pathway by which extracellular hormones and cytokines might regulate adipocyte differentiation by phosphorylation-dependent modulation of PPARγ activity.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—The GAL4 UAS-TKLuc luciferase reporter contains two copies of the GAL4 17-mer binding site (23) and the Drosophila b-gal and CMV-b-gal reference plasmids have been described previously (23, 24). The GAL4-hPPARγ (A/B) wild type and S84A mutant expression vectors contain residues 1–108 of human PPARγ1 cloned into the EcoRI site of pSG424 (25). These domains were also cloned into pGEX4T for expression of glutathione S-transferase (GST) fusion proteins. pCMV-flag-p38, which contains the Flag epitope between codons 1 and 2 of p38, was provided free of charge from Dr. J. Han (Dept. of Immunology, Scripps Research Institute, San Diego, CA). RSV-PPI is an expression vector encoding protein phosphatase 1α (26). pSG-CL100 is an expression vector encoding a dual specificity MAP kinase phosphatase (27). pSPORT-mPPARγ2 encodes full-length mouse PPARγ2 (10), and the S112A point mutation was introduced into it by polymerase chain reaction-directed mutagenesis. Both PPARγ2 and PPARγ2-SA cDNAs were then subcloned into the SacI site of pCMX. All mutagenized and ligated junctures were confirmed by sequencing. The acyl-CoA-α 3-TK-LUC construct contains three copies of the acyl-CoA oxidase PPAR response element (5'-GATCTGGACCAGGAGACGCGGCAGG-3').

**Cell Culture and Transfection Studies**—For functional studies, JEG-3 cells were cultured in DMEM containing 10% fetal bovine serum and transfected to DMEM plus charcoal-stripped fetal calf serum immediately prior to transfection. Cells were transfected with luciferase reporter, receptor expression vector, b-gal expression vector, and phosphatase expression vector where indicated using the calcium phosphate precipitation method. 5 μCi BRL49653 (in MeSO) or vehicle control was added 16 h after transfection. Cells were lysed 24 h later, and luciferase and b-gal was measured as described (23, 28). Luciferase values were normalized to b-gal activity and fold activation was calculated.

**In Vivo Phospholabeling, Immunoprecipitation, and Western Blotting**—JEG-3 cells, plated on 10-cm plates, were transfected with 30 μg of GAL4-hPPARγ plus 10 μg of PP1 expression vector as indicated. 40 h later cells were incubated in phosphate-free DMEM for 30 min, followed by a 4-h incubation with 1 μCi/ml [32P]orthophosphate (DuPont NEN). Cells were lysed by a 30-min incubation at 4 °C in RIPA buffer (1% Nonidet P-40, 1% deoxycholate, 0.1% SDS, 0.15 mM NaCl, 0.1% NaPO₄, pH 7.2, 2 mM EDTA, 50 mM NaF, 0.2 mM sodium vanadate, 1 μg/ml leupeptin, and 1 μg/ml aprotinin). Extracts were cleared by centrifuging at 25,000 x g for 30 min at 4 °C. Samples were preclarified with 10 μl of whole rabbit serum (Cappel) in a 50% slurry of protein A-agarose and washed five times with RIPA buffer. The immunoprecipitate was analyzed by 12% SDS-PAGE and autoradiography.

For immunoprecipitation of p38 kinase, JEG-3 cells were maintained on 10-cm plates and transfected with pCMV-flag-p38 by a 6-h exposure to calcium phosphate. After 48 h cells were UV irradiated (40 J/m² for 30 s) and lysed by a 5-min incubation at 4 °C in buffer containing 20 mM Hepes, 2 mM EGTA, 50 mM b-glycerophosphate, pH 7.4, 1% Triton X-100, 10% glycerol, 1 mM diethiothreitol, 1 mM orthovanadate, 2 μM leupeptin, 1 mM phenylmethylsulfonyl fluoride, 5 μg/ml aprotinin, 0.1 μM okadaic acid, lysozymes were cleared by centrifugation at 10000 rpm for 10 min at 4 °C. Flag-p38 was immunoprecipitated by a 1-h incubation of cleared cell lysates with anti-FLAG M2 monoclonal antibody (Scientific Imaging Systems, New Haven, CT) followed by a 1-h incubation with protein G-Sepharose. Precipitates were washed three times with lysis buffer followed by three washes in lysis buffer containing 150 mM NaCl and a further three washes in kinase buffer (20 mM MOPS, pH 7.0, 1 mM EDTA, 5% glycerol, 0.1% b-mercaptoethanol, and 0.1 μg/ml okadaic acid).

Whole cell extracts were prepared from JEG-3 cells transfected with wild type or mutant mPPARγ2 as described above and analyzed by SDS-PAGE and Western blotting followed by chemiluminescent detection (Amersham Corp.) with anti-PPARγ IgG at a dilution of 1:1000.

**Expression of GST Fusion Proteins**—GST fusion proteins were expressed in Escherichia coli, induced with 1 mM isopropylthio-b-D-galactosidase, and purified using glutathione-Sepharose 4B affinity resin (Pharmacia Biotech Inc.) but not removed from the matrix. Following purification, proteins were resuspended in NETN buffer (20 mM Tris-HCl, 0.1 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, pH 8.0), and their concentrations were determined.

**Protein Kinase Assays**—Phosphorylation of 2 μg of GST fusion proteins by ERK2 (New England Biolabs, Beverly, MA, or JNK (Calbiochem-Novabiochem, Nottingham, UK) were carried out as recommended by the manufacturers. p38 kinase assays were performed with 2 μg of substrate at 30 °C for 30 min in kinase buffer containing 18 mM magnesium acetate, 90 μM ATP, and 2 μCi of [γ-32P]ATP. Myelin basic protein (Life Technologies, Inc.) was used as a control substrate for ERK2, and a GST fusion protein containing a truncated form of activating transcription factor-2 (GST-ATF2, residues 1–96, Santa Cruz Biotechnology, Wembley, UK) provided positive controls for JNK and p38. 32P incorporation was determined following SDS-PAGE fractioning. Gels were stained with Coomassie Blue to check equal loading of GST fusion proteins and autoradiographed.

**Retroviral Infection and Adipocyte Differentiation of 3T3-L1 Cells**—3T3-L1 cells were made to ectopically express PPARγ2 or PPARγ2-SI12A using retroviral gene transduction as described previously (22). Infected 3T3-L1 cells were selected in G418 and grown to confluence in DMEM containing 10% iron-enriched fetal bovine serum. 2 days after reaching confluency, cells were treated with BRL49653 dissolved in MeSO or MeSO alone. After 7 days, cells were washed three times with phosphate-buffered saline, fixed with 10% formalin in phosphate buffered saline for 1 h at room temperature, washed once again with phosphate-buffered saline, then stained with 60% filtered Oil Red O stock solution (0.5 g of Oil Red O (Sigma) in 100 ml isopropanol) for 15 min, washed four times with water, and photographed.

**RESULTS**

Sequence alignment of the amino-terminal A/B region that is common to PPARγ1 and PPARγ2 isoforms indicated that a consensual MAP kinase site is conserved between species (Fig. 1A). To determine whether the NH2-terminal region of PPARγ is phosphorylated in vivo, an expression vector encoding the A/B domain of human PPARγ1 (residues 1–108) fused to the heterologous DBD of GAL4 (residues 1–147) was transfected into JEG-3 cells. Orthophosphatate labeling followed by immunoprecipitation with an antibody directed against GAL4 revealed that the GAL4 PPARγ-A/B fusion protein was highly phosphorylated, whereas GAL4 DBD alone was not (Fig. 1B, lanes 2 and 3). In comparison, phosphorylation of transfected GAL4 PPARγ-A/B (S84A), in which a putative phosphoserine at position 84 has been mutated to alanine (S84A) was markedly reduced (Fig. 1B, lane 4), although this fusion is expressed and even more transcriptionally active than wild type GAL4-PPARγ (A/B) (see below). Furthermore, cotransfection of PP1, an activated form of a serine phosphatase, led to near complete loss of phosphorylation of the wild type PPARγ construct (Fig. 1B, compare lanes 3 and 6).

Several MAP kinase cascades have been defined, each with a slightly different substrate specificity and activation pathway (21). In view of this, we tested the ability of three kinases,
ERK2, JNK, and p38, to phosphorylate the isolated A/B domains of wild type and mutant hPPARγ expressed as GST fusion proteins in E. coli. Purified recombinant ERK2 was able to phosphorylate the wild type GST-PPARγ1 fusion protein (Fig. 2A). This phosphorylation was abolished by mutation of the serine at position 84 in hPPARγ1, which corresponds to the MAP kinase site to alanine (S84A) under conditions in which myelin basic protein was a good substrate for this kinase. Similarly, purified recombinant JNK was also able to phosphorylate wild type GST-PPARγ (A/B) in addition to a control GST-ATF2 fusion protein (Fig. 2B). Mutation of serine 84 to alanine markedly reduced but did not abolish phosphorylation by JNK, suggesting weak phosphorylation of a second kinase site in vitro, which is not phosphorylated in vivo. The predicted size of the GAL4 PPARγ (A/B) fusion protein is approximately 29 kDa.

**Fig. 1.** A, sequence alignment of PPARγ1 and PPARγ2 isoforms showing conservation of a putative MAP kinase site. Comparison of the amino-terminal regions of PPARγ with a consensus MAP kinase sequence (where X is one or two basic or neutral residues; Ref. 43). The putative phosphoserine is boxed. This residue is conserved between the A/B domains of human PPARγ1 (44), mouse PPARγ1 and PPARγ2 (45, 10), hamster PPARγ1 (46), and Xenopus PPARγ (29) isoforms. The codon nomenclature reflects two additional residues at the NH2 terminus of hPPARγ1 that are not represented in hamster or mouse PPARγ1. B, the amino-terminal A/B domain of hPPARγ1 is phosphorylated in vivo. JEG-3 cells were transfected with 30 μg of GAL4-DBD or wild type GAL4-PPARγ (A/B) or mutant (S84A) expression vectors. 10 μg of PPI protein phosphatase expression vector was also cotransfected where indicated. Following labeling with [32P]orthophosphate, cell lysates were immunoprecipitated using an antibody directed against the GAL4-DBD and 32P-labeled products were analyzed by SDS-PAGE and autoradiography. The predicted size of the GAL4-PPARγ (A/B) fusion protein is approximately 29 kDa. **Fig. 2.** Phosphorylation of the A/B domain of PPARγ1 by MAP kinases. In vitro phosphorylation of GST PPARγ (A/B) by ERK2 (A) and JNK (B). Bacterially expressed and purified GST, wild type PPARγ (A/B), of mutant PPARγ (A/B S84A) fusion proteins were incubated with recombinant ERK2 or JNK as detailed under “Experimental Procedures” (myelin basic protein (MBP) and ATF2 used as positive controls, respectively). Phosphorylation was analyzed by autoradiography following SDS-PAGE (upper panels). Lower panels indicate the same gels after staining with Coomassie Blue to show total protein. The predicted positions of full-length fusion proteins are indicated with arrowheads, and lower molecular weight species are presumed to be degradation products. Mutation of serine 84 to alanine, which abolished phosphorylation of the GAL4-PPARγ fusion protein (see above and Fig. 1B), markedly enhanced the AF1 transactivation function of the PPARγ A/B domain. Similar results were obtained following transfection of wild type and mutant GAL4-PPARγ fusions in COS-7 cells (data not shown). Furthermore, coexpression of CL100, the human homologue of murine MAP kinase phosphatase (MKP-1), also augmented the transcriptional activity of wild type Gal4-PPARγ A/B (Fig. 3) but had no effect on the
mutant GAL4-PPAR A/B S84A fusion. Thus, two independent paradigms that either prevent (S84A mutation) or reduce (CL100 coexpression) phosphorylation of the PPARγ A/B domain markedly enhance AF1 activity.

In addition to the constitutive AF1 function in the amino-terminal domain, PPARγ also contains a ligand-dependent transcription activation function (AF2). We therefore investigated whether phosphorylation of the MAP kinase site in the A/B domain could influence the ligand-dependent AF2 activity of full-length PPARγ. Because PPARγ is conserved between species and the residues encompassing the amino-terminal MAP kinase site are identical in PPARγ1 and PPARγ2 isoforms (Fig. 1A), murine PPARγ2, which is induced specifically during adipocyte differentiation, was used in these studies. Transfection of wild type mPPARγ2 expression vector together with a reporter construct containing three copies of the PPAR-response element from the acyl-CoA oxidase gene and the thiazolidinedione ligand BRL49653 was associated with significant transcriptional activation (Fig. 4). Mutation of the homologous serine within the putative MAP kinase site in mPPARγ2 to alanine (S112A) significantly increased ligand-dependent transactivation to approximately double that of the wild type receptor. These observations indicate that in addition to inhibiting the inherent AF1 activity of PPARγ, phosphorylation of the MAP kinase site within the A/B domain also attenuates ligand-induced transcription activation by this receptor.

We next tested whether the enhanced transactivation by PPARγ2-S112A resulted in increased adipogenic activity. Wild type PPARγ2 or PPARγ2-S112A were ectopically expressed in 3T3-L1 preadipocytes using a retroviral expression strategy that we have previously described (22). In the absence of adipogenic stimulation, confluent preadipocytes differentiate into adipocytes at a very low frequency (<1%). Ectopic expression of PPARγ2 resulted in ~10% adipocyte conversion, as shown by Oil Red O staining of accumulated intracellular lipid (Fig. 5). This effect is presumably mediated by low levels of an endogenous PPARγ2 ligand, although the possibility that this was a ligand-independent effect of PPARγ overexpression cannot be discounted. Remarkably, ectopic expression of PPARγ2-S112A at levels similar to those of the ectopically expressed wild type PPARγ2 (data not shown) induced adipocyte differentiation much more dramatically. In fact, Fig. 5 shows that the degree of adipocyte differentiation due to ectopic PPARγ2-S112A expression was comparable with that achieved by cells expressing wild type PPARγ and treated with the ligand BRL49653 (50 nM). At this concentration BRL49653 alone is a relatively weak adipogenic stimulus for control cells (13, 15, 30), resulting in ~10% adipocyte conversion. These results show clearly that mutation of the MAP kinase consensus site produces a more adipogenic form of PPARγ2, consistent with the increased transcriptional activity of this mutant receptor.

**DISCUSSION**

Our studies indicate that a consensus MAP kinase site located within the conserved amino-terminal A/B domains of PPARγ1 and PPARγ2 regulates the transcriptional activity of these adipogenic nuclear receptor isoforms. Phosphorylation of this site reduces the inherent transcriptional activity of the AF1 transactivation domain. In addition, ligand-dependent transactivation by the full-length receptor is also inhibited.

![Fig. 3. Phosphorylation of hPPARγ1 at S84 inhibits ligand-independent transcriptional activation.](Image)

![Fig. 4. Phosphorylation inhibits ligand-dependent transcriptional activation of full-length PPARγ2.](Image)

![Fig. 5. Phosphorylation inhibits the adipogenic activity of PPARγ2.](Image)
This observation contrasts with previous studies of the ER showing that MAP kinase-mediated phosphorylation of the amino-terminal A/B domain enhanced the transcriptional activity of this receptor (16, 17) and indicates that constitutive activity of the NH2-terminal domains of nuclear receptors can be modulated in response to other signal transduction pathways. Furthermore, the divergent transcriptional effects of phosphorylation in PPARγ versus ER suggest that the different sequences around the consensus MAP kinase site in each A/B domain may also influence the way AF1 activity is modulated.

Although the A/B domain sequence motif that we have identified represents a consensus MAP kinase site, we have demonstrated that this residue is amenable to phosphorylation by both ERK2 and JNK. Interestingly, both TNFα and EGF, which are potent inhibitors of adipocyte differentiation (3, 4, 5, 6) are known to activate these pathways. In addition to enhancing JNK activity, TNFα stimulation has also been reported to activate MAP kinase (31, 32), and EGF stimulation enhances MAP kinase activity in a variety of cell types (18). The present work suggests that activation of either pathway could phosphorylate and hence inhibit PPARγ activity, thus contributing to the anti-adipogenic effects of these agents.

It is also possible that other signal transduction pathways may be involved. For example, it is interesting to note that adipocyte differentiation is promoted by culture in conditions that raise intracellular cAMP (33, 34), and elevated cAMP may be involved. For example, it is interesting to note that altered PPARγ activity, thus contributing to the anti-adipogenic effects of these agents.

Phosphorylation has been shown to regulate the activity of transcription factors by a number of different mechanisms (21). Because equal levels of mutant and wild type PPARγ were detected in transfected cells (Fig. 4), phosphorylation does not appear to alter PPARγ protein stability, as has been shown for the transcription factor Fos (39). Furthermore, because the inhibitory effect of NH2-terminal PPARγ phosphorylation is transferable to the heterologous DNA binding domain of GAL4, we also consider it unlikely that phosphorylation alters the ability of PPARγ to bind to DNA. A third possibility is that phosphorylation inhibits the activity of PPARγ by altering receptor interaction with other transcription intermediary proteins. For example, an interaction between the A/B domain of thyroid hormone receptors TRβ2 and TRα1 and TFIIH, which might influence transduction by altering preinitiation complex formation or stability, has recently been described (40, 41). This raises the possibility of analogous interactions between basal transcription factors and the A/B region of PPARγ that are phosphorylation-sensitive. Alternatively, phosphorylation at this site might influence PPARγ interaction with specific coactivator or corepressor proteins, in the same way that phosphorylation of c-Jun enhances recruitment of the CREB-binding coactivator protein (42).

Our finding of increased adipogenicity of a PPARγ point mutant that is not phosphorylated by ERK and related MAP kinases in its A/B domain strongly suggests that NH2-terminal phosphorylation inhibits the adipogenic activity of the wild type receptor. This also provides a molecular basis for an earlier observation that retroviral expression of NH2-terminally truncated PPARγ induces murine preadipocyte differentiation more strongly than its wild type counterpart (12). The precise role of PPARγ phosphorylation in regulation of adipocyte differentiation remains to be elucidated. However, the ability to modulate the activity of this adipogenic transcription factor by altering its phosphorylation state might provide a novel strategy for development of anti-adipogenic therapeutic agents to treat obesity.

Acknowledgment—We thank Sam Krakow for technical assistance.

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