Transcriptional Activity of Peroxisome Proliferator-Activated Receptor-γ is Modulated by SUMO-1 Modification.

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Running title: Regulation of PPARγ activity by sumoylation

The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; GST, glutathione S-transferase; HA, hemagglutinin; RT, reverse transcriptase; PCR, polymerase chain reaction; E1, ubiquitin-activating enzyme; E2, ubiquitin carrier protein; E3, ubiquitin-protein isopeptide ligase; SUMO, small ubiquitin-like modifier; PIAS, protein inhibitors of activated STAT.
Summary

Covalent modification of many transcription factors with SUMO-1 is emerging as a key role of trans-activational regulation. Here, we demonstrate that peroxisome proliferator-activated receptor (PPAR)γ, which is a ligand-activated nuclear receptor, is modified by SUMO-1. Sumoylation of PPARγ mainly occurs at a lysine residue within the activation function 1 (AF1) domain. Furthermore, we show that the PIAS family proteins, PIAS1 and PIASxβ, function as E3 ligases for PPARγ. PPARγ interacts directly with PIASxβ in a ligand-independent manner. Analysis using a PPARγ mutant with a disrupted sumoylation site shows that modification of PPARγ by SUMO-1 represses its transcriptional activity. Interestingly, PIASxβ and Ubc9 enhance the transcriptional activity of PPARγ independent of PPARγ sumoylation. Furthermore, PPARγ ligand-induced apoptosis in a human hepatoblastoma cell line, HepG2, is significantly enhanced by ectopic production of the sumoylation-mutant PPARγ. These results suggest that the PPARγ-dependent transactivation pathway seems to be modulated by SUMO-1 modification and may serve as a novel target for apoptosis-induction therapy in cancer cells.
Introduction

PPARγ is a member of the nuclear hormone receptor superfamily of ligand-activated transcription factors (1), which regulates diverse biological functions including cell differentiation, growth inhibition, lipid metabolism, and apoptosis (2-5). Two isoforms of PPARγ, PPARγ1 and PPARγ2, are generated by alternative promoter usage. PPARγ2, which contains an additional 30 amino acid residues at the amino terminus compared to PPARγ1, is predominantly expressed in adipose tissue, whereas PPARγ1 is widely expressed (6).

The role of PPARγ in adipogenesis has been extensively studied. Many adipocyte-specific genes, such as adipocytokines, contain PPARγ-responsive elements in their promoter and/or upstream enhancer regions (7-10). PPARγ plays a role as a central transcription factor in cellular differentiation and lipid accumulation during adipogenesis. Recent investigations have demonstrated that treatment of a variety of human cancer cell lines with PPARγ ligands leads to growth inhibition and apoptosis (2, 11-13). The use of PPARγ ligands in the treatment of cancer is a potentially promising nontoxic and selective chemotherapeutic approach and, consequently, increased understanding of the mechanisms of PPARγ in tumor suppression are needed.

Post-translational modifications regulate the function of many proteins. In the case of PPARγ, transcriptional activity is reduced by mitogen-activated protein kinase (MAPK) induced phosphorylation of serine residue 112 (14-16). Knock-in mice expressing PPARγ
with a Ser→Ala mutation at this residue exhibit preserved insulin sensitivity in the setting of diet-induced obesity by changing fat cell size, generation of adiponectin, and increasing the amount of free fatty acid levels in serum (17).

Recently, a number of ubiquitin-like proteins (Ubl) have been identified that are covalently linked to lysine residues in target proteins (18, 19). One Ubl, SUMO-1, also known as PIC1, UBL1, sentrin, GMP1, and SMT3, is an 11-kDa protein that is structurally homologous to ubiquitin (20-22). SUMO-1 modification plays an important role in altering the function of modified proteins, including transcriptional activation, nuclear localization, and increased turnover (23-25). SUMO-1 is conjugated to proteins through a series of enzymatic steps (26). Initially, the ATP-dependent formation of a thioester bond between SUMO-1 and the E1 enzyme complex (SAE1/Uba2) is formed, and SUMO-1 is then transferred to the E2-conjugating enzyme Ubc9. Finally, SUMO-1 is conjugated from Ubc9 directly to a lysine residue of target proteins in vitro. The E3 ligase that conjugates SUMO-1 to target molecules in vitro and in vivo has only recently been identified (27-30). One group of such E3 ligases, protein inhibitor of activated STAT (PIAS) family proteins homologous to the yeast Siz family protein, has a conserved RING-finger domain, which regulates transactivation of many transcription factors including STAT1 (31, 32), LEF-1 (33) and nuclear receptors (34, 35) by conjugating SUMO-1.

In order to understand the molecular mechanisms of PPARγ transcriptional function through post-translational modifications, we explored the possible modification of PPARγ by SUMO-1. In this paper we demonstrate that PPARγ is a target for SUMO-1
modification, and PIAS proteins function as E3 ligases for SUMO-1 modification. The main sumoylation site of PPARγ was mapped to a lysine residue at position 107, located in close proximity to the regulatory Ser-112. Sumoylation at this lysine residue reduced PPARγ-dependent transcriptional activation significantly. However, reporter gene assays suggested that PIAS proteins enhanced the transcriptional activity of PPARγ by a mechanism independent of PPARγ sumoylation. We also observed that a PPARγ sumoylation-mutant displayed enhanced ligand-induced apoptosis in a human hepatoblastoma cell line, HepG2, which suggests a possible new target for cancer therapy.
Materials and Methods

Cell culture, transfection, and luciferase reporter assay Cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Invitrogen) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin, and 100 µg/ml streptomycin. Transfection was performed using FuGENE6 (Roche Diagnostics Corp.), according to the manufacturer’s instructions. Luciferase activity was normalized to *Renilla* luciferase activity derived from co-transfected pRL-CMV-Luc (Promega). All reporter assays were performed in triplicate, and standard errors (S.E.) are denoted by bars in figures.

Antibodies and reagents Rat anti-HA (3F10; Roche), mouse and rabbit anti-FLAG (Sigma), and mouse anti-GFP (Clontech) antibodies were purchased commercially. Horseradish peroxidase (HRP)-linked goat antibodies to rat IgG were from Jackson ImmunoResearch Lab. HRP-linked goat antibodies to mouse or rabbit IgG were from Amersham Biosciences. Rosiglitazone was purchased from Alexis Biochemicals, Germany.

Plasmid construction Plasmids producing GST fusion proteins and pcDNA3 (Invitrogen) based plasmids expressing epitope-tagged human SUMO-1, human UBC9, and mouse PIAS families have been described previously (36). A luciferase reporter plasmid, p4xPPRE-Luc, was constructed by inserting four copies of the PPAR response element (PPRE) (5'- TTGACCTTTGACCTTTGACCTTTGACCTTTGACCTTAGATC-3') into the luciferase reporter plasmid pGL2 (Promega). The mouse PPARγ2 gene containing the entire coding region was isolated by RT-PCR from 3T3-L1 cells and subcloned into
epitope-tagged pcDNA3 and pM (Clontech) to generate pcDNA3-FLAG-PPARγ2, -HA-PPARγ2, and pM-PPARγ2. The cDNAs for mutant PPARγ2 with substitution of Lys-107 to Arg, PPARγ2(K/R1), and Lys-159 to Arg, PPARγ2(K/R2), were created using site-directed mutagenesis and subcloned into expression vectors to obtain pcDNA3-FLAG-PPARγ2(K/R1) and -PPARγ2(K/R2). The expression vector for PPARγ1 was obtained using PCR from PPARγ2 as a template. pSG5-mRXRα, encoding mouse RAR, was kindly provided by P. Chambon of the Institut de Genetique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP/College de France, and S. Kato at the Institute of Molecular and Cellular Biosciences, The University of Tokyo.

**GST pull-down analysis** GST and GST fusion proteins were expressed in the *Escherichia coli* strain BL21 (DE3) and affinity purified with glutathione-S-Sepharose beads according to the manufacturer’s instructions (Amersham Biosciences). PPARγ2 protein was metabolically labeled in the TNT-coupled reticulocyte lysate system (Promega) with T7 RNA polymerase and 35S-methionine. GST pull-down analysis was carried out as described previously (37).

**Immunoprecipitations** HEK-293T cells (1 x 10⁵ per 6 cm-diameter dish) were transfected using FuGENE6 (Roche Applied Science) according to the manufacturer’s instructions. After incubation, cells were lysed in 1 ml lysis buffer (25 mM Tris-HCl [pH 8.0], 150 mM NaCl, 1% NP-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM DTT, 5 mM EDTA, 10 mM N-ethylmaleimide [NEM], 200 µM indole-3-acetic acid, and a complete protease inhibitor cocktail tablet [Roche]) for sumoylation analysis or RIPA buffer (25 mM Tris-HCl [pH 8.0], 125 mM NaCl, 0.1% NP-40, 1 mM DTT, 1 mM...
EDTA, and a complete protease inhibitor cocktail tablet) for co-immunoprecipitation analysis. Cell debris was removed by centrifugation for 15 min. Lysates were first cleared with protein G beads for 30 min, followed by incubation with antibodies for 1 h at 4°C. Finally, the antibody complexes were captured with protein G beads for 1 h. Beads were washed four times with the same buffer and immunoprecipitates were eluted and analyzed by Western blot.

**Detection and measurement of apoptosis and indirect immunofluorescence observation.** HepG2 cells were grown to subconfluency on 8-well Lab-Tec Chamber (NUNC) in DMEM with 10% FCS. Cells were transfected with 500 ng pcDNA3-FLAG-PPARγ1 or -PPARγ1(K/R1) expression vectors. Forty-eight hours after transfection, rosiglitazone was added to culture medium to a final concentration at 1 µM for 24 h. Cells were fixed at room temperature with 3.7% formaldehyde for 3 min and then permeabilized with 0.5% Triton X-100 in PBS for 5 min. After blocking with 3% BSA and 0.1% Triton X-100 in PBS, the cells were incubated with anti-HA antibody for 1 h at 37°C and stained with Alexa Fluor 568 anti-rat secondary antibody (Molecular Probes) for 1 h at room temperature. Apoptotic cells were detected by using the In Situ Cell Death Detection Kit, Fluorescein following the manufacturer's instructions (Roche Applied Science). The ratio of apoptotic cells was quantitated by analysis of DNA fragmentation using the Cell Death Detection ELISA according to the manufacturer's instructions (Roche Applied Science).
Results

**PPARγ is a substrate for SUMO-1 modification.** SUMO-1 modification of certain transcription factors including nuclear hormone receptors is known to affect transcriptional activity, and, consequently, we wished to address whether PPARγ was a substrate for SUMO-1 modification. We first examined whether PPARγ is modified by SUMO-1 in cells transiently expressing FLAG-PPARγ2 and HA-SUMO-1. Western blot analysis using anti-FLAG antibody revealed the presence of FLAG-tagged PPARγ2 in all cells transfected with the plasmid expressing FLAG-PPARγ2. When HA-SUMO-1 was co-expressed, additional slower migrating bands were detected by the FLAG antibody (Fig. 1A, left panel, lane 3). Moreover, to determine whether these slower migrating bands represent PPARγ2 conjugated to SUMO-1, the membrane was re-probed with anti-HA antibody, which detects proteins conjugated to HA-SUMO-1. The result showed that the slower migrating forms of PPARγ2, about 90 and 130 kDa, were indeed sumoylated (Fig. 1A, right panel, lane 6). These data suggest that PPARγ is modified by SUMO-1 at least two sites. We next examined whether a specific PPARγ ligand, rosiglitazone, affected PPARγ sumoylation. As shown Fig.1 B, SUMO-1 conjugated PPARγ2 was detected in cells co-producing SUMO-1. In lysates prepared from cells treated with rosiglitazone, the amount of SUMO-1 conjugated PPARγ2 was lower than in mock-treated cells (lane 2 and 4), suggesting that PPARγ ligand negatively regulates SUMO-1 conjugation to PPARγ. Two lysine residues, K107 and K347 in the
AF1 and AF2 domains, respectively, of PPARγ2 conform to the proposed consensus motif, ψKxD/E (where ψ is a hydrophobic amino acid residue, x represents any residue and D or E is an acidic residue), for SUMO-1 conjugating sites (18, 20) (Fig. 1C). To determine whether these lysine residues are targets for sumoylation, mutants with lysine to arginine substitutions, K107R (K/R1) and K347R (K/R2) as shown in Fig. 1C, were generated and analyzed for sumoylation. Two bands migrating slower than the original band were detected with almost same intensity as cells producing both wild type PPARγ1, PPARγ2 and the mutant PPARγ2(K/R2) (Fig. 1D). In contrast, the slowest band disappeared in cells producing the mutant PPARγ2(K/R1) and, moreover, the amount of the slower migrating band was also reduced in the cells expressing the mutant PPARγ2(K/R1) (Fig. 1D, lane 2). These results imply that Lys-107 is the major site for sumoylation, and this site may function as the master switch of sumoylation, because mutation of this lysine residue greatly impaired sumoylation for PPARγ. The fact that mutation of lysine residue at 347 did not affect the efficiency of sumoylation suggests the presence of lysine residues other than those not in the consensus motif for SUMO-1 modification in PPARγ.

**PIAS family proteins act as E3-ligases for PPARγ sumoylation.** Recent studies indicated that members of the PIAS family enhanced sumoylation of many proteins including nuclear receptors (25, 34, 35). Therefore, we investigated whether PIAS family proteins function as E3-ligases for PPARγ. We generated a mutant of PIASxβ, PIASxβ(C/S), in which the conserved cysteine residue at position 353 within the RING-finger domain was changed to serine. This mutant was not able to interact with Ubc9
SUMO-1 conjugation to PPARγ2 was analyzed in cells producing either wild-type PIASxβ, PIASxβ(C/S) or PIAS1. Small amounts of SUMO-1-conjugated PPARγ2 were detected in cells expressing only HA-SUMO-1 ectopically (Fig. 2, lane 2). PPARγ2 sumoylation was enhanced by exogenous expression of PIAS1 and PIASxβ but not PIASxβ(C/S) (lanes 3 to 5). These findings indicate that PIAS family proteins function as E3-ligases for PPARγ2.

Next, to investigate the association of PPARγ2 with PIASxβ, we employed a GST pull-down analysis. Full-length PIASxβ expressed in bacteria as a GST fusion protein was coupled to glutathione-S-Sepharose beads, and this complex was incubated with in vitro translated 35S-labeled PPARγ2 in buffer with or without rosiglitazone, a specific ligand for PPARγ. As shown Fig. 3A, PPARγ2 interacted both in the presence and absence of rosiglitazone with GST-PIASxβ but not with GST alone. To analyze the physical interaction of PPARγ2 with PIASxβ in cells, a co-immunoprecipitation experiment was conducted using extracts from HEK-293T cells co-expressing HA-PPARγ2 and FLAG-PIASxβ, treated with or without rosiglitazone. Proteins precipitated with anti-HA antibody were resolved by SDS-PAGE, and Western blot using anti-FLAG was conducted. A FLAG reactive species was detectable in the complex precipitated with the anti-HA antibody. The PIASxβ in the immunocomplex was increased in cells treated with rosiglitazone, indicating that the binding efficiency of PPARγ2 and PIASxβ was significantly enhanced by treatment with this ligand (Fig. 3B, lanes 2 and 3).

SUMO-1 conjugation to PPARγ represses the transcriptional activity of
To evaluate the effect of PPARγ sumoylation on its transcriptional function, we analyzed the effects of PPARγ sumoylation on expression of the p4xPPRE-Luc reporter gene in which the luciferase gene is driven by a PPAR responsive promoter. NIH3T3 cells were transfected with various combinations of plasmids expressing wild-type PPARγ1, wild-type PPARγ2, PPARγ1(K/R1), PPARγ2(K/R2), and mRXRα, a component of a heterodimeric complex with PPARγ, together with p4xPPRE-Luc. Cells were then treated with or without rosiglitazone. Additional production of mRXRα in cells enhanced reporter activity by PPARγ. Reporter activity was enhanced by treatment with rosiglitazone and this was highest in cell lysates containing PPARγ2(K/R1) (Fig. 4A). The fact that transcriptional activity of PPARγ2 was higher than that of PPARγ1 was in good agreement with previous report (38). Next, to analyze the direct effect of the SUMO-1 conjugation-dependent transcriptional activity of PPARγ, we utilized PPARγ fused with GAL4 to analyze gene expression from pGL2-Luc containing five GAL4 binding sites in the promoter region. GAL4-PPARγ1(K/R1) and -PPARγ2(K/R1) showed about five-fold higher luciferase activities than the activity observed by GAL4-wild-type-PPARγ1 and GAL4-wild-type-PPARγ2, respectively, in a ligand-dependent manner (Fig. 4B). Taken together, these data suggest that sumoylation of PPARγ represses the transcriptional activity of PPARγ itself.

Both Ubc9 and PIASxβ enhance PPARγ-dependent transactivation. To further investigate the transcriptional role of sumoylated PPARγ, we examined the effects of Ubc9, an essential factor for sumoylation, on PPARγ-dependent transcription. GAL4-fused wild-type PPARγ2 and -PPARγ2(K/R1) were expressed in HEK-293T cells with
increasing amounts of Ubc9 (Fig. 5A). Co-production of Ubc9 enhanced transcription by PPARγ2 and PPARγ2(K/R1) in a dose-dependent fashion. We next examined the effects of PIASxβ and PIASxβ(C/S) on the transcriptional activation of PPARγ2. Luciferase activities were significantly enhanced by the co-production of PIASxβ. However, co-production of PIASxβ(C/S) only slightly enhanced the activity (Fig. 5B). Similar results were also observed for PPARγ1 (data not shown). These data suggest that the SUMO-1 conjugation activity of Ubc9 and PIAS positively regulate PPARγ-mediated transactivation. The observation that the transcriptional activity of PPARγ2(K/R1) was not only significantly enhanced by co-production of Ubc9 but also by PIASxβ, suggests that Ubc9 and PIAS proteins function as positive regulators for PPARγ-dependent transcription possibly through SUMO-1 conjugation of factor(s) other than PPARγ involved in transcriptional regulation.

Ligand-induced apoptosis by PPARγ is enhanced in cells producing PPARγ(K/R1). Recent studies have demonstrated that specific ligands for PPARγ inhibit cell growth and induce apoptosis in several human cancer cells (2, 11-13). PPARγ activation seems to be important for inducing apoptosis in some cells. However, the molecular mechanisms of PPARγ-dependent apoptosis, particularly the relationship between the transcriptional activity of PPARγ and apoptosis, remain unclear. To investigate the effect of sumoylation on PPARγ-dependent apoptosis, we compared the apoptotic potential of wild-type PPARγ1 to that of PPARγ1(K/R1) in HepG2 cells. Plasmids expressing FLAG-PPARγ1 or -PPARγ1(K/R1) were transfected into HepG2 cells, and forty-eight hours after transfection, cells were treated with 1 μM rosiglitazone.
for 24 h. PPARγ expression in cells and apoptotic cells were detected by immunostaining and TUNEL assay, respectively. Approximately 5% of PPARγ1-transduced cells became TUNEL positive, which stained strongly by the anti-FLAG antibody. In contrast, approximately 40% of PPARγ1(K/R1)-transduced cells became TUNEL positive and almost all cells expressed high levels of PPARγ1(K/R1) (Fig. 6A). The numbers of apoptotic cells producing PPARγ1 or PPARγ1(K/R1) were verified by measurement of the accumulation of fragmented nucleosomes. Ligand-induced apoptosis is significantly enhanced when PPARγ1(K/R1) was produced in cells (Fig. 6B). These results suggest that transcriptional activation of PPARγ is involved in enhancing ligand-mediated apoptosis.
Discussion

In this study, we showed that sumoylation of PPARγ significantly affected its transcriptional activity. PPARγ was predominantly modified by SUMO-1 at Lys-107 within the AF1 domain. Our result suggest that there is a lysine residue(s) in addition to Lys-107 targeted for sumoylation which is likely to lie in a non-consensus SUMO-1 conjugation motif, because mutational analysis of the lysine residues lying in other consensus SUMO-1 conjugation motif in this protein did not affect SUMO-1 conjugation (Fig. 1D). Since mutation of Lys-107 reduced SUMO-1 conjugation of PPARγ severely, Lys-107 is the primary site for modification. Similar observations of the presence of hierarchic lysine residues for SUMO-1 conjugation were reported in other proteins such as PML (39), androgen receptor (40), aryl hydrocarbon receptor (ARNT) (41), and DNA topoisomerase I (42). PIAS1 and PIASxβ acted as E3-ligase factors for SUMO-1 conjugation to PPARγ (Fig. 2). We also showed that PIASxβ associated with PPARγ2 in vitro and in vivo in a ligand-independent manner, but the association was enhanced by the presence of the ligand in vivo. (Fig. 3). Interestingly, ligand treatment led to a reduction in the amount of SUMO-1 conjugated to PPARγ2 (Fig. 1B). Because of conformational alteration of nuclear receptors, association of co-activator complexes with nuclear receptor seems to be regulated by specific ligands. Thus, it is likely that PPARγ sumoylation is suppressed by the association of the co-activator complex with the ligated PPARγ in which the sumoylation sites of PPARγ may be masked, and/or the E3-ligase activity of PIAS
proteins may be blocked.

Using a reporter gene assay, we demonstrated that the transcriptional activity of PPARγ was negatively regulated by sumoylation. It has been reported that phosphorylation of Ser-112, adjacent to the sumoylation site as revealed by this work, on PPARγ by MAP kinase significantly inhibited both ligand-independent and ligand-dependent transcriptional activation by PPARγ (15). Mutation analysis of the phosphorylation site revealed that this phosphorylation-mediated transcriptional repression was not due to a reduced capacity to make PPARγ/RXRα complexes or the impairment of recognition of its DNA binding site (14). An AF-1 domain of PPARγ may be negatively regulated by phosphorylation and sumoylation. Alternatively, SUMO-1 conjugated PPARγ may recruit the transcriptional repressor complex by providing a novel interaction site. Recently it has been shown that sumoylation of the ETS domain transcription factor, Elk-1, results in the recruitment of histone deacetylase activity to promoters (43). Similarly, SUMO-1-conjugated PPARγ may recruit additional cellular factors that repress PPARγ-dependent transcription.

Our data clearly showed that Ubc9 enhanced the transcriptional activities of both PPARγ and PPARγ(K/R1), possibly by a mechanism independent of SUMO-1 conjugation to PPARγ. PIASxβ also enhanced PPARγ activity through a RING-finger domain dependent mechanism. Thus, it seems that ectopically produced PIASxβ regulates PPARγ-mediated transactivation through not only sumoylation of PPARγ itself, but also in the conjugation of SUMO-1 to other cellular factor(s) involved in transcriptional regulation. In agreement with our observations, key-
molecules in the SUMO-1 conjugation system, including SUMO-1, Ubc9 and PIAS, have been shown to modulate the transcriptional activities of p53 (44), androgen receptor (40), ARNT (41) and LEF-1 (33), even when these target molecules lacked major sumoylation site(s) by mutation. Moreover, it has been shown that Ubc9 modulates the transcriptional activity of ETS-1 and TEL independent of its E2 enzymatic activity (45, 46). In view of these reports, mechanism(s) other than the direct SUMO-1 conjugation to PPARγ by Ubc9 and PIASxβ seem to be important for the regulation of transactivation of PPARγ. Further studies to clarify the molecular basis of the transcriptional activation of PPARγ-dependent transcription by Ubc9 and PIAS should provide significant insight.

A role for PPARγ in adipogenesis is well characterized. In addition, a novel function of PPARγ in tumor pathogenesis has been reported recently, which includes PPARγ-ligand dependent growth inhibition and/or apoptosis in a variety of human cancer cells (2, 11-13). Several studies have demonstrated that induction of apoptosis was accompanied by the up-regulation of several pro-apoptotic genes, Bax, caspase-3 and –9, and down-regulation of the anti-apoptotic gene Bcl-2 (47, 48), suggesting that transactivation of PPARγ is likely to regulate expression of apoptosis modulators at the transcriptional level and contribute as an important modulator of tumor suppression. In this study, we demonstrated that the trans-activation function of PPARγ was up-regulated by mutation of Lys-107, the major target for sumoylation in PPARγ. Since HepG2 cells expressing this mutant form of PPARγ displayed enhanced PPARγ-ligand dependent apoptosis, the increased transactivation function of PPARγ seems to play an
important role in inducing apoptosis. Sumoylation regulates the transacting function of PPARγ that could play a role in the regulation of apoptosis. We suggest here that the sumoylation of PPARγ may be a good target for a novel therapeutic agent in cancer cells.
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Figure legends

Fig. 1. PPARγ is covalently modified by SUMO-1. A, HEK-293T cells were transfected with 2 µg of each plasmid expressing FLAG-PPARγ2 together with (+) or without (-) 2 µg of plasmid expressing HA-SUMO-1. Thirty-six hours after transfection, cell lysates were prepared and immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitates were subjected to 7.5% SDS-PAGE and analyzed by immunoblot (IB) with anti-FLAG antibody (left panel). After ECL development, the membrane shown in the left panel was stripped and re-probed with anti-HA antibody (right panel). The asterisk indicates the immunoglobulin heavy chain. B, Sumoylation of PPARγ was repressed by ligand treatment. HEK-293T cells were transfected with (+) or without (-) 2 µg of each plasmid expressing FLAG-PPARγ2 or HA-SUMO-1. Twenty-four hours after transfection, cells were treated with (+) or without (-) 5 µM rosiglitazone for 24 h. Cell extracts were prepared and immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitants were subjected to SDS-PAGE and analyzed by immunoblot (IB) with anti-FLAG antibody. C, Schematic representation of murine PPARγ2. The activation function 1 (AF1) domain, the DNA-binding domain (DBD), and ligand-gated activation function 2 (AF2) domain are shown. Two lysine residues, at positions 107 and 347, in the putative SUMO-1 acceptor sites are indicated below with the neighboring amino acid residues. D, PPARγ was predominantly modified by SUMO-1 at Lys-107 within the AF1 domain. HEK-293T cells were cotransfected with 2 µg of
each plasmid expressing FLAG-wild-type PPARγ2 (γ2), -mutant PPARγ2 with substitution of Lys-107 to Arg (K/R1), -mutant PPARγ2 with substitution of Lys-347 to Arg (K/R2), and FLAG-wild-type PPARγ1 (γ1) together with plasmid expressing HA-SUMO-1. Thirty-six hours after transfection, cell extracts were immunoprecipitated (IP) with anti-FLAG antibody. The immunoprecipitants were subjected to SDS-PAGE and analyzed by immunoblot (IB) with anti-FLAG antibody. Unmodified (arrowheads) and sumoylated (arrows with a and b) forms of PPARγ1 (white arrows and arrowhead) and PPARγ2 (black arrows and arrowhead) are indicated.

**Fig. 2. PIAS family proteins function as E3-ligases for PPARγ.** HEK-293T cells were cotransfected with 2 µg plasmid expressing FLAG-PPARγ2 together with (+) or without (-) 2 µg plasmid expressing HA-SUMO-1, GFP-PIAS1 (1), -PIASxβ (xβ), or -PIASxβ(C/S) (C/S). Thirty-six hours after transfection, cell extracts were prepared and subjected to immunoprecipitation using anti-FLAG antibody (IP) followed by anti-FLAG immunoblot (IB). Levels of PIAS protein in whole cell lysates (WCL) are analyzed by immunoblot using anti-GFP antibody.

**Fig. 3. Association of PIASxβ with PPARγ2 in vitro and in vivo.** A, GST and GST-PIASxβ fusion proteins were immobilized on glutathione-Sepharose beads and incubated with 35S-labeled PPARγ2 translated in vitro in pull-down buffer containing 5 µM rosiglitazone as indicated. The pull-down complexes were analyzed by SDS-PAGE followed by image analyzer. The input lane represents 20% of total volume of whole cell lysates used for pull-down assay. B, HEK-293T cells were transfected with 2 µg plasmid expressing FLAG-PIASXβ together with (+) or without (-) 2 µg plasmid
expressing HA-PPARγ2. Twenty-four hours after transfection, cells were treated with (+) or without (-) 5 µM rosiglitazone for 12 h. Cell extracts were then prepared and subjected to immunoprecipitation using anti-HA antibody (IP). The immunoprecipitants were subjected to SDS-PAGE and analyzed by immunoblot (IB) with anti-FLAG antibody (top panel) or anti-HA antibody (second panel). The protein levels of FLAG-PIASxβ in each cell lysate are indicated (third panel).

Fig. 4. Mutant PPARγ has higher transcriptional activity than wild-type PPARγ. A, NIH3T3 cells were cotransfected with 25 ng p4xPPRE-Luc and 100 ng of each plasmid expressing HA-wild-type PPARγ2 (γ2(WT)), -mutant PPARγ2 with substitution of Lys-107 to Arg (γ2(K/R1)), -wild-type PPARγ1 (γ1(WT)), -mutant PPARγ1 with substitution of Lys-77 to Arg (γ1(K/R1)), mRXRα or an empty plasmid (-). Twenty-four hours after transfection, cells were treated with or without 5 µM rosiglitazone. Luciferase activities were then measured 18 h after treatment. Open bars denote no treatment, and closed bars indicate rosiglitazone treatment. The activity in control cells was arbitrarily given a value of 1, and the activities in the other cells were relative to the value of control cells. B, HEK-293T cells were cotransfected with 25 ng GAL4-luciferase reporter plasmid (pGAL4-Luc) and 100 ng of plasmids expressing fusion proteins of the GAL4 DNA binding domain to PPARγ1 (γ1(WT)), PPARγ1(K/R1) (γ1(K/R1)), PPARγ2 (γ2(WT)), PPARγ2(K/R1) (γ2(K/R1)), or an empty plasmid (-). Twenty-four hours after transfection, cells were treated with or without 5 µM rosiglitazone. Luciferase activities were then measured 18 h after treatment. Open bars denote no treatment, and closed bars indicate rosiglitazone treatment. The activity
in control cells was arbitrarily given a value of 1, and the activities in the other cells were relative to the value of control cells.

**Fig. 5.** PPARγ-dependent transactivation is enhanced by Ubc9 and PIASxβ independently of PPARγ sumoylation. A and B, HEK-293T cells were cotransfected with 25 ng pGAL4-Luc and 100 ng of plasmids expressing fusion proteins of the GAL4 DNA binding domain to PPARγ2 (WT), PPARγ2(K/R1) (K/R1), or an empty plasmid (-) together with 50 or 100 ng plasmids expressing Myc-Ubc9, FLAG-PIASxβ, or –PIASxβ(C/S), respectively. Twenty-four hours after transfection, cells were treated with 5 µM rosiglitazone. Luciferase activities were then measured 18 h after treatment. The activity of the reporter plasmid alone in control cells was arbitrarily given a value of 1, and the activities of the other transfections were adjusted relative to this assay.

**Fig. 6.** Comparison of ligand-induced apoptosis between wild-type and sumoylation-mutant PPARγ1 in HepG2 cells. A, HepG2 cells were transfected with plasmids expressing FLAG-PPARγ1 (WT), FLAG-PPARγ1(K/R1) (K/R1). Forty-eight hours after transfection, cells were treated with (+Rosi) or without (-Rosi) 1 µM of rosiglitazone for 24 h. TUNEL assay and immunostaining were performed as described in Materials and Methods. B, HepG2 cells were transfected with plasmids expressing FLAG-PPARγ1 (WT), FLAG-PPARγ1(K/R1) (K/R1), or an empty vector (-). Forty-eight hours after transfection, cells were treated with 1 µM rosiglitazone. Cells were then collected for 0, 12, and 24 h after treatment and the ratio of apoptotic cells were quantitated by analysis of accumulation of fragmented nucleosomes. Open bars denote 0 h, shaded bars 12 h, and closed bars 24 h after treatment.
FIG. 1. T. Ohshima et al.

A

B

Rosiglitazone

|               | IP:αFLAG | IB:αFLAG |
|---------------|----------|-----------|
| FLAG-PPARγ2   |          |           |
| HA-SUMO-1     |          |           |

(kDa)

50 75 100 150

IP:αFLAG IB:αFLAG
FIG. 1. T. Ohshima et al.

C

mPPARγ2

AF1 DBD AF2

IK10VE (K/R1)
LK34YG (K/R2)

D

FLAG-PPARγ

γ2 K/R1 K/R2 γ1

50 75 100 150 (kDa)

IP:αFLAG
IB:αFLAG

PPARγ2
PPARγ1

a b
FIG. 2. T. Ohshima et al.

| GFP-PIAS | HA-SUMO-1 |
|---------|-----------|
| -       | -         |
| -       | +         |
| 1       | +         |
| xβ      | +         |
| C/S     | +         |

**IP:** αFLAG
**IB:** αFLAG

**WCL**
**IB:** αGFP

**FLAG-PPARγ2**

(kDa)

100 75

PPARγ2

- SUMO

PPARγ2
FIG. 3. T. Ohshima et al.

A

Rosiglitazone

|   | INPUT | GST  | GST-α | GST-β |
|---|-------|------|-------|-------|
| - | -     | +    | +     | +     |

35S-PPARγ2

B

Rosiglitazone

| HA-PPARγ2 | FLAG-PIASxβ |
|-----------|-------------|
| - | - | + | + | + |

IP:αHA  
IB:αFLAG

(kDa)  
75  
100

WCL  
IB:αFLAG

PIASxβ
FIG. 4. T. Ohshima et al.

A

Luciferase activity (fold)

p4xPPRE-Luc

- control
- + ligand

HA-PPARγ

- γ2(WT) γ2(K/R1) γ2(WT) γ2(K/R1) γ1(WT) γ1(K/R1)

RXRα

- - - + + + +

B

Luciferase activity (fold)

pGAL4-Luc

- control
- + ligand

GAL4-PPARγ

- γ1(WT) γ1(K/R1) γ2(WT) γ2(K/R1)
FIG. 5. T. Ohshima et al.

A

Luciferase activity (fold)

GAL4-PPARγ2
Myc-Ubc9

WT WT K/R1 K/R1

B

Luciferase activity (fold)

GAL4-PPARγ2
FLAG-PIASxβ
FLAG-PIASxβ(C/S)

WT WT K/R1 K/R1 K/R1 K/R1
FIG. 6. T. Ohshima et al.

A

\( \alpha \text{FLAG} \)  |  TUNEL  |  merge

| WT + Rosi |  |  |
| K/R1 + Rosi |  |  |
| K/R1 − Rosi |  |  |

B

| 0 h | 12 h | 24 h |
|---|---|---|
| (−) | WT | K/R1 |

absorbance (A405nm - A490nm)
Transcriptional activity of peroxisome proliferator-activated receptor-gamma is modulated by SUMO-1 modification
Takayuki Ohshima, Hiroshi Koga and Kunitada Shimotohno

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