1-1-2004

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Recommended Citation

Floyd, Z., & Stephens, J. (2004). Control of peroxisome proliferator-activated receptor γ2 stability and activity by SUMOylation. *Obesity Research, 12* (6), 921-928. https://doi.org/10.1038/oby.2004.112

Control of Peroxisome Proliferator-Activated Receptor γ2 Stability and Activity by SUMOylation

Z. Elizabeth Floyd and Jacqueline M. Stephens

Abstract

FLOYD, Z. ELIZABETH AND JACQUELINE M. STEPHENS. Control of peroxisome proliferator-activated receptor γ2 stability and activity by SUMOylation. *Obes Res.* 2004;12:921–928.

Objective: To determine whether small ubiquitin-related modifier (SUMO)ylation of lysine 107 plays a role in regulating the activity of peroxisome proliferator-activated receptor γ (PPARγ).

Research Methods and Procedures: Transient expression of wild-type and K107R-PPARγ2 in the NIH 3T3 fibroblast cell line was carried out in conjunction with half-life studies, luciferase activity assays, and indirect immunofluorescence localization studies. Additional in vitro analysis was carried out using recombinant SUMOylation pathway proteins along with in vitro transcribed and translated wild-type or K107R-PPARγ2 to examine the SUMO-1 modification state of wild-type and SUMO-deficient K107R-PPARγ2.

Results: While examining PPARγ2 for potential ubiquitylation sites, we identified a strong consensus site for SUMO modification that contains lysine 107. In vitro, SUMOylation studies showed that lysine 107 of PPARγ2 is a major SUMOylation site and that at least one other SUMOylation site is present in PPARγ. In addition, our results demonstrated that SUMO-1 affects PPARγ stability and transcriptional activity but not the nuclear localization of PPARγ.

Discussion: These results indicated that SUMOylation plays a role in regulating PPARγ, both indirectly and directly by modification of lysine 107. Because PPARγ is regulated in numerous animal models of obesity, understanding the covalent modifications of PPARγ may enhance our understanding of the metabolic syndrome.

Key words: fat cells, adipocytes, peroxisome proliferator-activated receptor γ, small ubiquitin-related modifier-1, ubiquitin

Introduction

The peroxisomal proliferator-activated receptor γ (PPARγ)[1] is a member of the nuclear hormone receptor superfamily that is essential for the development of adipocytes (1). PPARγ protein is expressed in adipocytes in two forms, PPARγ2 and PPARγ1, that are produced by a combination of alternative promoter usage and alternative splicing (2,3). PPARγ1 is expressed at low levels in multiple tissues, whereas PPARγ2 is expressed predominantly in fat cells and differs from PPARγ1 by an N-terminal extension of 30 amino acids (4). As a member of the nuclear hormone receptor family, PPARγ is composed of an N-terminal region or activation function (AF)-1 domain followed by the DNA binding and hinge domains. The carboxy-terminal or AF-2 domain is composed of a dimerization and ligand-binding domain and is responsible for ligand-dependent activation (5).

Recent studies have focused on understanding the underlying mechanisms that regulate the activity of the nuclear hormone receptors. In particular, the ubiquitin-proteasome system has emerged as an important regulator of nuclear receptors, including PPARγ (6–8). Moreover, a growing number of studies have also shown a role for the ubiquitin-like protein, small ubiquitin-like modifier (SUMO), in regulating nuclear receptors (9–13). SUMO conjugation (SUMOylation) to a substrate occurs by a pathway that is

Received for review February 6, 2004.
Accepted in final form March 25, 2004.
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[1] Nonstandard abbreviations: PPARγ, peroxisomal proliferator-activated receptor γ; AF, activation function; SUMO, small ubiquitin-related modifier; RXR, retinoid × receptor; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; GR, glucocorticoid receptor.

distinct from ubiquitin conjugation and functions to alter the substrate's cellular location, stability, or activity (14,15). SUMOylation of the progesterone receptor (9,10), glucocorticoid receptor (12,16), and androgen receptor (11) at the N-terminal AF-1 domain has been shown to alter transcriptional activity, indicating a general role for SUMOylation in controlling the transcriptional activity of nuclear hormone receptors.

We found that PPARγ contains a strong consensus site for SUMOylation in the AF-1 domain that contains lysine 107. We now report that SUMOylation plays a role in PPARγ stability and transcriptional activity and that lysine 107, a major SUMOylation site, is a determinant of PPARγ activity and stability.

Research Methods and Procedures

Materials

Dulbeccos' modified Eagle's medium and OptiMEM were purchased from Invitrogen (Carlsbad, CA). Calf serum was purchased from Atlanta Biological (Atlanta, GA). PPARγ monoclonal (E-8, sc-7273), PPARγ polyclonal (H-100, sc-7196), retinoid × receptor (RXR)α polyclonal (D-20, sc-553), and ubc9 polyclonal (H-81, sc-10,759) antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MG132 (*N*-carbobenzoxyl-Leu-Leu-Leucinal) was purchased from Boston Biochemicals (Cambridge, MA). A luciferase assay system, pSV-β-galactosidase control vector, and β-galactosidase enzyme assay kit, as well as the TnT T7 coupled reticulocyte lysate system, were obtained from Promega (Madison, WI). Polyfect was purchased from Qiagen (Valencia, CA), and the Quick-Change Mutagenesis kit was obtained from Stratagene (La Jolla, CA). The in vitro SUMOylation kit was obtained from LAE Biotechnology (Rockville, MD).

Cell Culture and Mutagenesis

NIH 3T3 cells were cultured in Dulbeccos' modified Eagle's medium supplemented with 10% calf serum and antibiotics (100 units/mL penicillin G and 100 μg/mL streptomycin). The cells were maintained at 37 °C. The pSVSportPPARγ2-K107R and pSVSportPPARγ2-K329R were generated from wild-type PPARγ2 by site-directed mutagenesis according to the manufacturer's directions (Stratagene), and the mutations were confirmed by dideoxy-sequencing.

Transient Transfections and Immunoblotting

NIH 3T3 cells were grown to 30% confluence, and transfections were carried out using a total of 1.5 μg DNA/well and Polyfect according to the manufacturer's directions (Qiagen). Cells were assayed 48 hours after transfection. Whole cell lysates were rinsed once with phosphate-buffered saline (pH 7.4) and harvested in a lysis buffer containing 10 mM Tris-Cl (pH 7.4), 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Igepal, 1 μM phenylmethylsulfonylfluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Lysates were analyzed by separation on 12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to Laemmli (17), followed by immunoblotting as previously described (8).

SUMOylation Assay

Wild-type PPARγ2, K107R-PPARγ2, and RXRα were transcribed and translated in vitro according to the manufacturer's suggestions (Promega). Both forms of PPARγ2 were labeled with [^{35}S] Met (Amersham, Piscataway, NJ) during translation, and RXRα was translated in the presence of unlabeled methionine. Translation of both forms of PPARγ2 was confirmed by autoradiography, and translation of RXRα was confirmed by Western blot analysis. The in vitro SUMOylation assays were carried out according to the manufacturer's instructions. In addition, ATP was added fresh for a final concentration of 2 mM in each assay. The reactions were analyzed by 10% SDS-PAGE followed by autoradiography.

Immunolocalization

HeLa cells were transfected with pSVSport-PPARγ2 wild-type or pSVSport-K107R-PPARγ2 and cotransfected with pSVSport-PPARγ2 wild-type and pCMVubc9C93S (ubc9DN). Twenty-four hours later, localization of both forms of PPARγ2 was determined by indirect immunofluorescence with monoclonal anti-PPARγ (Santa Cruz), and localization of ubc9DN was assayed using a polyclonal antiubc9 (Santa Cruz). Secondary antibodies were AlexaProbe 568 (goat anti-mouse) and 488 (goat anti-rabbit) (Molecular Probes, Eugene, OR). Images were photographed using a Nikon Microphot-FXA microscope.

Stability of PPARγ2 in Vivo

Experiments using transiently transfected NIH 3T3 cells were carried out in the presence or absence of cycloheximide (5 μM) to examine the half-life of the wild-type, K107R, and K329R forms of PPARγ2. When examining the role of SUMOylation in PPARγ2 stability, wild-type and K107R-PPARγ2 were cotransfected with a dominant negative ubc9, pCMVubc9C93S (ubc9DN) (18), in the presence or absence of MG132 (10μM) as a proteasome inhibitor.

Pulse-chase analysis of wild-type and the K107R form of PPARγ2 was carried out in NIH 3T3 cells at 24 hours after transfection. Cells were transfected with either pSVSport-PPARγ2 or pSVSportK107R-PPARγ2 and were metabolically labeled with [^{35}S] Met/Cys (Perkin-Elmer, Boston, MA) for 30 minutes in media devoid of exogenous methionine and cysteine. The cells were washed and chased in complete media containing unlabeled methionine and cys-

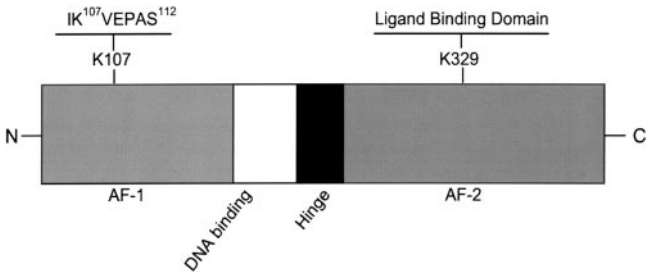

Figure 1: Schematic of PPARγ2. Lysine 107 of the AF-1 domain is contained in the consensus sequence for SUMO modification. Lysine 107 is also in close proximity to serine 112, which plays a role in PPARγ activation. Lysine 329 is located in the ligand-binding domain of PPARγ and has been implicated in PPARγ transcriptional activity (20).

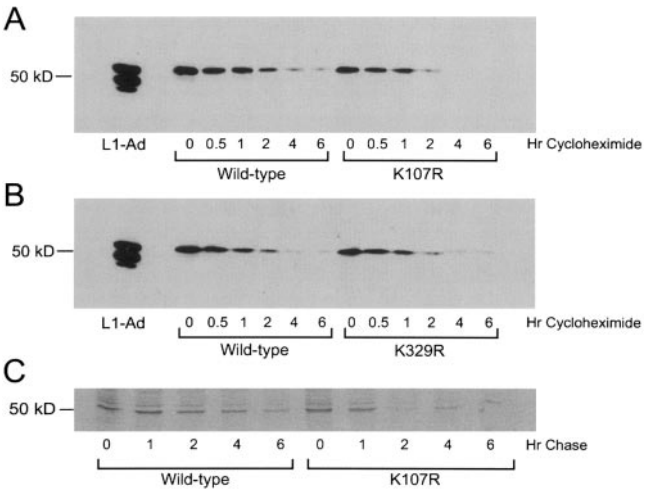

Figure 2: The K107R form of PPARγ2 is less stable than wild-type. The K107R, K329R, and wild-type forms of PPARγ2 were transiently transfected into NIH 3T3 cells. (A and B) Forty-eight hours after transfection, the cells were incubated with 5 μM cycloheximide (CHX) and harvested at the indicated time-points. Whole cell extracts were examined for PPARγ2 expression by Western blot analysis. A lane containing whole cell extract from 3T3-L1 adipocytes (L1-Ad) is included as a marker for PPARγ1 and PPARγ2. (C) Transfected NIH 3T3 cells were metabolically labeled with [^{35}S] Met/Cys, washed extensively, and chased in media containing unlabeled Met/Cys. At the indicated time-points, the cells were harvested and subjected to immunoprecipitation.

teine and harvested at the indicated time-points. Cells were resuspended in lysis buffer as described above and sonicated. After centrifugation, the resulting supernatant was precleared by incubation with protein A-Sepharose (RepliGen, Waltham, MA) at 4 °C for 1 hour. Immunoprecipitations were performed by incubation with polyclonal anti-PPARγ antibody (2 μg, H-100; Santa Cruz,) followed by incubation with protein A-Sepharose. The beads were washed extensively, and the immunoprecipitates were analyzed by 10% SDS-PAGE followed by autoradiography.

Transcriptional Activity Assay

Wild-type or K107R-PPARγ2 was transfected into NIH 3T3 cells along with pPPREx3TK-luciferase (19) and pSV-β-galactosidase and, where indicated, ubc9DN. An equal amount of each construct was transfected into the cells, with empty vector used to balance the total amount of DNA in each case. Forty-eight hours later, PPARγ2 was activated by the addition of 5 μM darglitazone in the presence or absence of MG132 (10μM). Whole cell extracts were harvested after 6 hours and analyzed for luciferase and β-galactosidase activity according to the manufacturer's instructions (Promega). PPARγ2 transcriptional activity is reported as the ratio of luciferase activity (relative light units) to β-galactosidase activity.

Results

Lysine 107 of the N-terminal AF-1 Domain Was a Determinant of PPARγ2 Stability

Recent reports have shown that PPARγ is targeted to the ubiquitin-proteasome system for degradation under basal and ligand-activated conditions (7,8). In an effort to understand how PPARγ degradation is regulated, we began a systematic search for regions of PPARγ2 that are required for targeting to the ubiquitin-proteasome pathway. In particular, we were interested in determining which lysine(s) are targeted for ubiquitylation. As shown in Figure 1, 2 lysines from a total of 36 were initially chosen for site-directed mutagenesis. Lysine 329 of PPARγ2 (numbered based on GenBank accession BCO21798), located in helix 3 of the AF-2 ligand-binding domain (5), is highly conserved in nuclear receptors and may play a role in coactivator binding and transcriptional activity (20). Lysine 107 of PPARγ2 is located in the N-terminal AF-1 domain in close proximity to serine 112 and is contained in a strong consensus sequence for SUMO recognition, ψKXE (where ψ is a hydrophobic residue) (21,22). SUMOylation has been shown to directly affect ubiquitylation in the case of IκB-α and Mdm2 (15). As shown in Figure 2, half-life experiments carried out in the presence of cycloheximide indicated that substitution of arginine for lysine 329 had no effect on the turnover of PPARγ2, whereas the K107R mutated form of PPARγ2 was more labile than wild-type. Using pulse-chase experiments, we confirmed that the K107R form of PPARγ2 is less stable than wild-type PPARγ2. The increased decay rate indicated that lysine 107 of PPARγ2 was not conjugated to ubiquitin but did have a modest effect on the stability of PPARγ2.

PPARγ Was SUMOylated at Lysine 107 in Vitro

Based on the results shown in Figure 2, we hypothesized that SUMOylation of PPARγ at lysine 107 is a determinant

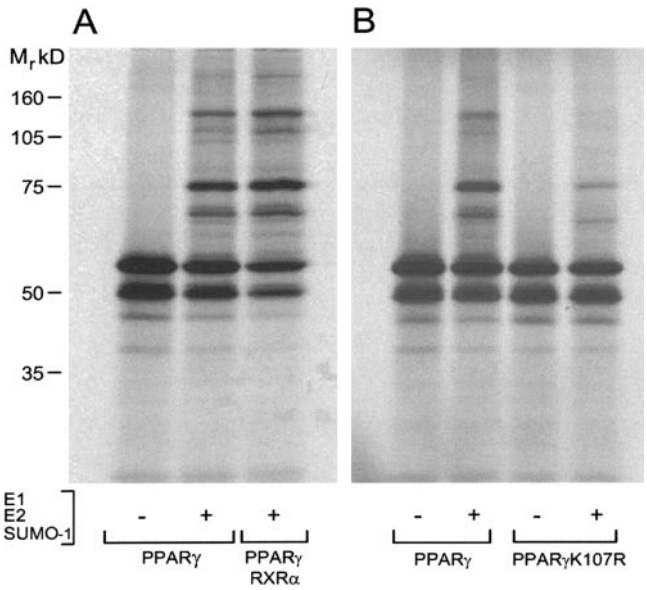

Figure 3: PPARγ is SUMOylated at lysine 107. Wild-type PPARγ2 and K107R-PPARγ2 were ^{35}S-labeled through in vitro transcription/translation (Promega) reactions. RXRα was generated by in vitro transcription/translation without ^{35}S-labeling. The presence of RXRα was confirmed by Western blotting. (A) Wild-type PPARγ2 was incubated in buffer only (−) or in the presence (+) of SAEI/II (E1), ubc9 (E2), and SUMO-1 at 37 °C for 1 hour. RXRα was included in the reaction as indicated. (B) Wild-type PPARγ2 or K107R-PPARγ2 was incubated at 37 °C for 1 hour in buffer only (−) or in the presence (+) of SAEI/II, ubc9, and SUMO-1. In each reaction, ATP was added fresh to yield a final concentration of 2 mM ATP.

of PPARγ stability. To confirm that PPARγ is SUMOylated at lysine 107, we carried out an in vitro SUMOylation experiment using ^{35}S-labeled PPARγ2 wild-type in the presence of SUMO-1, along with ubc9, the essential E2, and SAEI/SAEII, the SUMO-activating enzyme (E1) (14). As shown in Figure 3A, several higher molecular weight bands were apparent when wild-type PPARγ was incubated in the presence of the SUMOylation enzymes, indicating that PPARγ was modified by SUMO-1 in vitro. The most prominent bands were consistent with the addition of one or two SUMO-1 proteins. The presence of RXRα may increase the efficiency of PPARγ SUMOylation, but RXRα is not required for PPARγ SUMOylation in vitro. Figure 3B shows that replacement of lysine 107 with arginine resulted in the loss of the higher molecular weight bands along with the greatly decreased intensity of the band corresponding to the addition of a single SUMO-1. This is consistent with lysine 107 being a direct target of the SUMOylation machinery and the primary SUMO-1 site in PPARγ2. However, at least one other nonconsensus site in PPARγ was modified by SUMO-1, albeit at reduced levels in the in vitro experiment.

SUMOylation Did Not Alter Localization of PPARγ2 to the Nucleus

To determine whether SUMOylation affects the cellular localization of PPARγ, we carried out indirect immunolocalization experiments with wild-type PPARγ2 in the presence or absence of the dominant negative form of ubc9 (ubc9DN). In addition, we compared the nuclear localization of wild-type PPARγ2 with the K107R form of PPARγ2. The experiments shown in Figure 4 indicated that both wild-type PPARγ2 and K107R-PPARγ2 were located

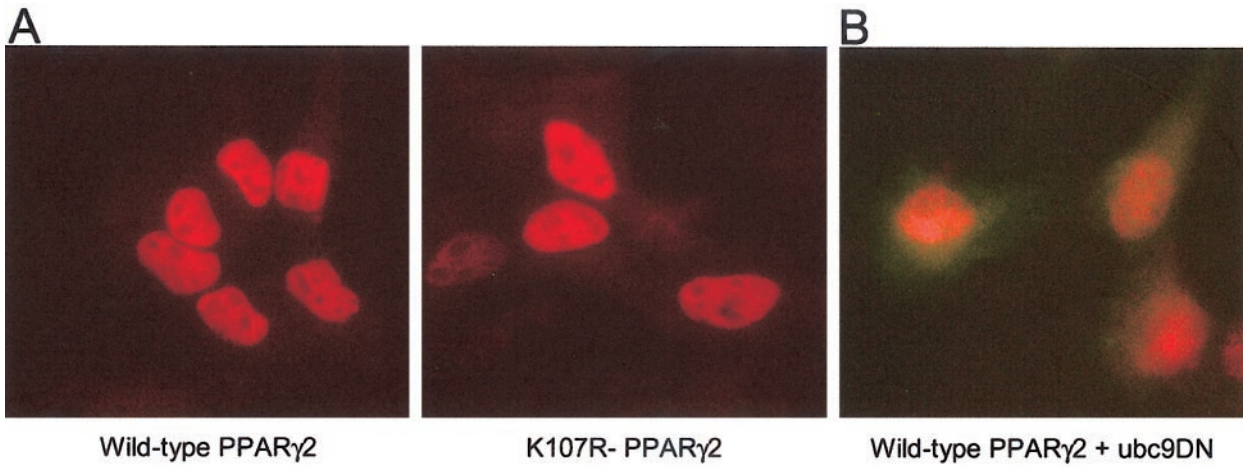

Figure 4: SUMOylation did not alter localization of PPARγ2 to the nucleus. HeLa cells were transfected with pSVSport-PPARγ2 wild-type or pSVSport-K107R-PPARγ2 (A) or cotransfected with pSVSport-PPARγ2 wild-type and pCMVubc9DN (B). Twenty-four hours later, localization of both forms of PPARγ2 was determined by indirect immunofluorescence. AlexaProbe 568 (rhodamine) secondary antibody was used to detect both forms of PPARγ2, and AlexaProbe 488 (fluorescein) was used to detect ubc9DN. The overlap of wild-type PPARγ2 and ubc9DN in the nucleus appears orange in B. Images were photographed using a Nikon Microphot-FXA microscope.

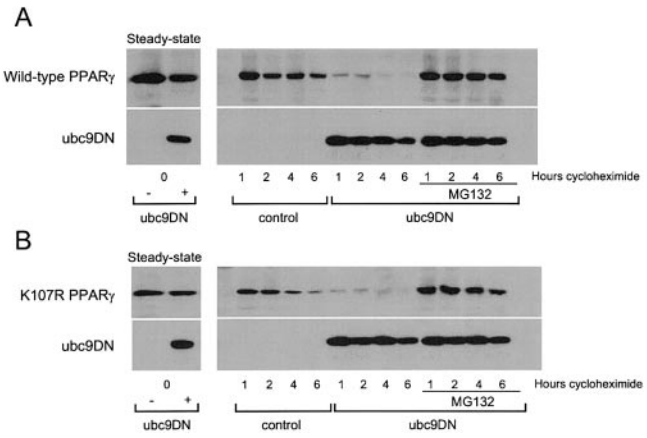

Figure 5: Wild-type-PPARγ2 and K107R-PPARγ2 decay rates were higher in the presence of ubc9DN. Transient transfections of wild-type and K107R-PPARγ2 alone or in the presence of ubc9DN were carried out in NIH-3T3 cells. After forty-eight hours, the cells were incubated with 5 μM cycloheximide (CHX) and 10 μM MG132 where indicated, harvested at the indicated time-points, and subjected to Western blot analysis.

in the nucleus. In addition, cotransfection of wild-type PPARγ2 with ubc9DN did not alter the nuclear localization of PPARγ2, indicating that SUMOylation was not required for localizing PPARγ to the nucleus. However, this result cannot rule out the possibility that SUMOylation can affect the subnuclear localization of PPARγ.

SUMOylation Affected PPARγ2 Stability

To examine the role of SUMOylation in PPARγ2 turnover, we examined the decay rate of both wild-type PPARγ2 and K107R-PPARγ2 in the presence or absence of ubc9DN, a dominant negative form of the essential conjugating enzyme (E2) for SUMO modification (18). Figure 5 shows that the steady-state levels of wild-type PPARγ2 were decreased in the presence of ubc9DN, whereas the levels of K107R-PPARγ2 were unchanged. However, the turnover rate of both forms of PPARγ2 was significantly increased in the presence of ubc9DN, suggesting that lysine 107 was not the only possible SUMOylation site in PPARγ or that the effect was due to disruption of SUMOylation of an interacting factor that influences PPARγ turnover. In addition, the increased turnover rate introduced by inhibiting SUMOylation was abrogated in the presence of MG132. This indicates that SUMOylation may function, in part, to regulate targeting of PPARγ to the 26S proteasome.

SUMOylation Affected the Transcriptional Activity of PPARγ2

SUMOylation has been shown to regulate protein activity, as well as protein stability and cellular localization (15). Recent studies have revealed that SUMOylation is involved in regulating the transcriptional activity of several members of the nuclear hormone receptor superfamily (10,12,13). We examined the transcriptional activity of wild-type PPARγ2 and K107R-PPARγ2 in the absence or presence of ubc9DN to block SUMOylation (Figure 6). For each condition, the cells were treated with darglitazone, a synthetic PPARγ ligand. As shown in Figure 6A, the transcriptional activity of wild-type PPARγ2 increased in the presence of ubc9DN, indicating that SUMOylation acts to attenuate PPARγ2 activity. Moreover, K107R-PPARγ2 was more active than wild-type PPARγ2. The 2.6-fold increase in activity of K107R-PPARγ2 over wild-type PPARγ2 (Figure 6B) under control conditions was largely eliminated in the presence of ubc9DN, suggesting that SUMO modification at lysine 107 is involved in determining PPARγ2 activity. However, we also observed that the increased activity obtained for wild-type PPARγ2 in the presence of ubc9DN was greater that the activity found in K107R-PPARγ2 under control conditions. These results support the notion that additional nonconsensus SUMOylation sites are present in PPARγ2 or that SUMOylation of an interacting protein also influences the transcriptional activity of PPARγ2.

We also carried out these experiments in the presence or absence of MG132 to examine the effect of proteasome inhibition on PPARγ2 activity. For both wild-type PPARγ and K107R-PPARγ, proteasome inhibition was associated with increased transcriptional activity (Figure 6), suggesting a link between transcriptional activation and degradation, as has been shown for a variety of transcription factors (23).

Discussion

Nuclear hormone receptors undergo an intricate set of posttranslational modifications that modulate their function, including phosphorylation, acetylation, and ubiquitylation. Recent studies have shown that these receptors can also be conjugated to SUMO-1, a small ubiquitin-like modifier protein. To date, it has been shown that SUMOylation plays a role in the transcriptional activity of the aryl hydrocarbon, progesterone, glucocorticoid, and androgen receptors (16). Our work indicated that SUMOylation also modulates the stability and transcriptional activity of PPARγ, a nuclear receptor essential for the development of fat cells.

Like other nuclear receptors, PPARγ contains a strong SUMOylation consensus site in the AF-1 domain at the N-terminal region. We established that PPARγ2 is SUMOylated at lysine 107 and at least one other (nonconsensus) site. RXRα, the heterodimeric PPARγ partner required for DNA binding, was not required for PPARγ SUMOylation in vitro, indicating that DNA binding may not be essential for PPARγ2 SUMOylation.

Substitution of lysine 107 (K107R) in the consensus site resulted in increased PPARγ turnover, suggesting that, although lysine 107 is not an ubiquitylation site, it does play a role in PPARγ stability, possibly through SUMOylation.

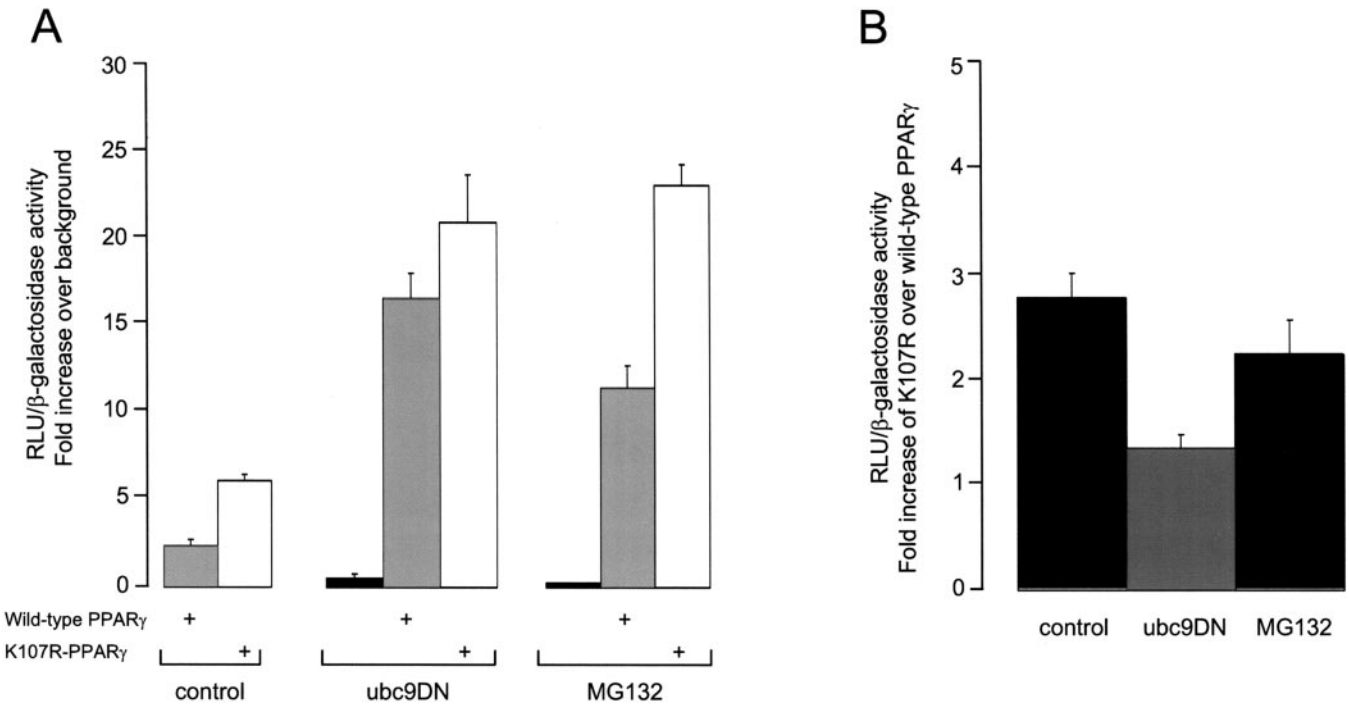

Figure 6: SUMOylation and degradation affected PPARγ2 transcriptional activity. Wild-type PPARγ2or K107R-PPARγ2 were transfected into NIH-3T3 cells along with PPREx3-TK-luciferase, β-galactosidase, and, where indicated, ubc9DN. An equal amount of each construct was transfected into the cells with empty vector (pBluescriptSK) used to balance the total amount of DNA in each case. Forty-eight hours later, 5 μM darglitazone (TZD) was added as a synthetic PPARγ ligand. Where indicated, the cells were preincubated for 1 hour with 10 μM MG132 before the addition of darglitazone. Whole cell extracts were harvested after 6 hours. The experiment was independently performed three times, and activity is reported in relative light units (RLU)/β-galactosidase as (A) fold-increase of wild-type and K107R-PPARγ2 over background (wild-type PPARγ2 without ligand) and (B) fold-increase of K107R over wild-type PPARγ2. Activity levels (black bars) were also measured to determine the contribution of ubc9DN or MG132 in the absence of either form of PPARγ2.

Indeed, generalized inhibition of SUMOylation using a dominant negative form of ubc9 destabilized both wild-type and K107R-PPARγ2. However, proteasome inhibition abrogated the increased turnover of PPARγ observed in the presence of the dominant negative form of ubc9, indicating that SUMOylation either directly or indirectly affects targeting of PPARγ to the proteasome.

Our studies also showed that generalized inhibition of SUMOylation resulted in increased PPARγ transcriptional activity. Moreover, mutation of lysine 107 (K107R) enhanced PPARγ activity, indicating that lysine 107 acts as a determinant of PPARγ activity. This supports the notion that SUMOylation of lysine 107 plays a role in regulating PPARγ transcriptional activity. Interestingly, we also observed that inhibition of the proteasome resulted in increased PPARγ transcriptional activity. This observation suggests that SUMOylation and targeting to the proteasome function to down-regulate PPARγ activity, although these processes have opposite effects on the stability of PPARγ. This situation is similar to findings reported for the glucocorticoid receptor (GR) (24). In that case, it was shown that GR-mediated transcriptional activity is down-regulated by the proteasome, whereas a separate study has found that generalized SUMOylation both destabilizes GR and increases GR transcriptional activity (16).

The suggestion that SUMOylation of PPARγ attenuates PPARγ transcriptional activity is consistent with other results showing that SUMOylation of the androgen receptor (11) and GR (12) at N-terminal lysines results in decreased transcriptional activity, as does SUMOylation of the progesterone receptor in the AF-1 domain (9,10). In these studies, the influence of SUMOylation on the transcriptional activity of the receptors occurs in a promoter context-dependent manner. In each case, attenuation of activity is associated with the presence of more than one response element. In this study, the observed effects of SUMOylation on PPARγ transcriptional activity were also measured in the presence of more than one response element, and further studies are planned to examine the importance of promoter context in regulating PPARγ transcriptional activity. In this context, it is important to note that an earlier study using two copies of the PPARγ response element has shown that amino acids 99–129 of PPARγ2 repress PPARγ2 transcriptional activity (25).

Moreover, recent studies have defined a motif in the N-terminal region of the GR (26) and the CCAAT/enhancer-binding proteins α, β, δ, and ε (27,28) that inhibits transcriptional activity. This motif also occurs in the otherwise divergent AF-1 domain of the nuclear receptors and has been named the synergy control motif by Iniguez-Lluhi and Pearce (26) because of its role in regulating higher order interactions of the identified transcription factors. These studies have shown that the synergy control motif overlaps the consensus SUMOylation motif, and SUMOylation of the conserved lysine is associated with transcriptional attenuation (26,28). This synergy control motif is also found in the AF-1 domain of PPARγ and overlaps the SUMOylation motif, which is near serine 112, a residue well known to participate in the regulation of PPARγ transcriptional activity (29). Our findings suggested that this region of the AF-1 domain of PPARγ, as found in other nuclear hormone receptors, modulates both transcriptional activity and PPARγ stability and that SUMOylation plays a role in this process.

Acknowledgments

This work was supported by NIH Grant R01DK52968-02 and a Research Award from the American Diabetes Association to J.M.S. and a Postdoctoral Fellowship from the American Heart Association to Z.E.F. We thank Bruce Spiegelman (Dana Farber Cancer Institute) for the gift of the pSVSportPPARγ2 and pPPREx3-TK-luciferase plasmids and Zhiyuan Shen (University of New Mexico) for the dominant negative ubc9 (pCMV-Myc-ubc9C93S-54) plasmid. We also thank Mitchell Lazar (University of Pennsylvania School of Medicine) for the plasmids containing PPARγ2 and RXRα under control of the T7 promoter.

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