Regulation of Peroxisome Proliferator–Activated Receptor-α by MDM2

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Peroxisome proliferator–activated receptor-alpha (PPARα) belongs to the nuclear receptor (NR) family of transcription factors and regulates lipid and glucose metabolism. Like other NRs, the regulation of gene expression by PPARα depends on cofactor recruitment to the transcription complex and multiple protein-protein interactions. In this study, Murine Double Minute 2 (MDM2), an E3 ubiquitin ligase, is identified as a PPARα–interacting protein that regulates PPARα transcriptional activity. MDM2 modulated the transcriptional activity of PPARα and PPARβ/δ, but not PPARγ in reporter assays. Knockdown of MDM2 by small interfering RNA in rat hepatoma cells inhibited ligand-induced mRNA levels of several PPARα target genes involved in lipid metabolism. MDM2 associated with PPARα on target gene promoters, and this association increased in response to Wy14,643 treatment. MDM2 interacted with PPARα and this interaction occurred with the A/B domain of PPARα. Coexpression of MDM2 increased PPARα ubiquitination and the E3 ubiquitin ligase activity of MDM2 affected PPARα protein expression and transcriptional activity. MDM2 expression was decreased in response to clofibrate in wild-type (WT), but not in PPARα null mice, indicating a PPARα-dependent regulation. These studies identify a role for MDM2 in regulating PPARα-mediated pathways of lipid metabolism.

Key Words: peroxisome proliferator-activated receptor alpha; MDM2; nuclear receptor.

Peroxisome proliferator–activated receptors (PPARs) regulate lipid and glucose metabolism, and are critical to the maintenance of cellular energy homeostasis (Desvergne et al., 2004). In addition, they regulate several biological processes such as inflammation, differentiation, apoptosis, and wound healing (Desvergne et al., 2004). The PPARs perform these diverse functions by acting as ligand-activated transcription factors that modulate the expression of numerous genes involved in these processes. Three different subtypes of PPARs mediate these responses; PPARα, PPARβ, and PPARγ. PPARα is highly expressed in tissues with high rates of fatty acid catabolism including the liver, kidney, heart, and skeletal muscle. It is activated by naturally occurring and metabolized fatty acids and by peroxisome proliferators (PPs), a diverse group of xeno-biotics that include fibrate hypolidemic drugs, phthalate esters, and herbicides (Isemann and Green, 1990). Upon long-term exposure to PPs, rodents develop hepatocellular carcinoma, and studies with PPARα null mice have shown that this subtype is responsible for the tumor-promoting activity (Hays et al., 2005; Peters et al., 1997). Humans, however, appear to be resistant to the carcinogenic effects of fibrate drugs, and the underlying molecular mechanisms are under investigation (Cheung et al., 2004; Morimura et al., 2005; Yang et al., 2008).

Regulation of gene expression by PPARα follows the classical ligand-dependent transcription factor mechanism. Upon ligand binding, conformational changes occur in the receptor complex that facilitates dissociation of corepressor molecules and recruitment of coactivators and coactivator–associated proteins. Following activation, PPARα binds to PPAR-response elements (PPREs) in the promoter of target genes as a heterodimer with retinoid X receptor (RXR). The multiple protein-PPARα interactions that occur in the transcription complex are important for proper target gene regulation. Besides its heterodimeric partner RXRα, PPARα associates with heat shock proteins hsp70 and hsp90, coregulators such as PPAR binding protein, SRC-1, CBP (Tien et al., 2006), and cAMP response element binding-binding protein/p300-interacting transactivator with ED-rich tail 2 (CITED2, also called p35srj/mrg1/msg1) (Tien et al., 2004).

Studies in our laboratory identified ribosomal protein L11 as a PPARα-associated protein that inhibited PPARα transcriptional activity (Gray et al., 2006). Because ribosomal biogenesis is a crucial part of cell growth, ribosomal proteins such as L11 have recently come into focus as potential components of cell cycle control. This particular ribosomal protein interacts with MDM2, an E3 ubiquitin ligase that is well known for its degradation and negative regulation of p53 (Bhat et al., 2004; Lohrum et al., 2003; Zhang et al., 2003). MDM2 also has p53-independent roles and interacts with several other proteins. Of importance, are MDM2-interacting proteins, Rb and E2F that play prominent roles in cell cycle regulation (Loughran and La Thangue, 2000; Martin et al., 1995; Uchida et al., 2005).
MDM2 also ubiquitinates and regulates nuclear receptors (NRs) such as estrogen receptor (ER), glucocorticoid receptor (GR), and androgen receptor (AR) (Duong et al., 2007; Gaughan et al., 2005; Kinyamu and Archer, 2003; Lin et al., 2002; Reid et al., 2003; Sengupta and Waslylk, 2004). This study demonstrates that MDM2 interacts with and regulates the transcriptional activity of PPAR-α.

MATERIALS AND METHODS

Plasmids. MDM2 plasmids were purchased from Addgene (Cambridge, MA). The plasmid pVP16-MDM2 was generated by subcloning the coding sequence of MDM2 from pSG5-MDM2 (a kind gift from Dr Mariikki Laiho at the Haartman Institute and Molecular and Cancer Biology Program, University of Helsinki, Finland). The plasmid pcDNA3.1-MDM2 was kindly provided by Dr Christine Blattner (Institut für Genetik, Forschungszentrum Karlsruhe, Germany). The construction of the two-hybrid PM-PPARα constructs has been described previously (Tien et al., 2004). The plasmid pFR-luciferase (US luciferase) was purchased from BD Biosciences Clontech (Palo Alto, CA), whereas pRL/TK and pRL-CMV were from Promega (Madison, WI). The PPRE reporter pACO (−581–471) G·L·u·c was supplied by Dr Jonathan Tugwood (AstraZeneca Macclesfield, UK) and has been described previously (Tien et al., 2004). The plasmid pcDNA3.1/V5-His-PPARα has been described previously (Gray et al., 2006). Plasmids pDNA3.1/FLAG-PPARβ and pDNA3.1-PPARγ were a kind gift from Dr Curtis Omiecinski (Department of Veterinary and Biomedical Sciences, Pennsylvania State University). MT-123-HA-ubiquitin was kindly provided by Dr Dirk Bohmann (European Molecular Biology Laboratory, Heidelberg, Germany).

In vitro interaction studies. In vitro translations were performed by using the TNT-reticulocyte lysate system (Promega). The plasmid pcDNA3.1-PPARα was in vitro translated in the presence of 35S-labeled methionine, and mixed with in vitro translated pSG5-MDM2 for 2 h at 4°C in MEGN buffer (25mM morpholinepropanesulfonic acid [MOPS], 2mM ethylenediaminetetraacetic acid [EDTA], 0.02% Na3S, 10% glycerol [pH 7.5]) with 2 mg/ml bovine serum albumin, followed by immunoprecipitation with anti-MDM2 antibody (SMP14, Santa Cruz Biotechnology, Santa Cruz, CA) and protein G beads (Sigma, St Louis, MO) overnight. Following washes in MEGN + 150mM NaCl, protein complexes were eluted from the beads with 2× Tris-Glycine sample buffer, and resolved on sodium dodecyl sulfate (SDS-PAGE). PPARα was visualized by autoradiography on the dried gels.

In the Maltose binding protein (MBP)-pull down assays, PPARα (full length [FL] and domains A/B, C, D, E/F) was expressed as a MBP fusion protein and extracted from DH5α as previously described (Gray et al., 2006). The plasmid pcDNA3.1-MDM2 was in vitro translated in the presence of 35S-labeled methionine, and incubated with equimolar amounts of the FL or different domains of PPARα-MBP in MEGN containing 2 mg/ml BSA, 50mM Wy-14,643 and amylose resin for 2 h at 4°C. Following washes in MEGN + 150mM NaCl, protein complexes were eluted from the beads with 2× Tris-Glycine sample buffer, and resolved on SDS-PAGE. MDM2 was visualized by autoradiography on the dried gels.

Transfections and reporter assays. Lipofectamine (Invitrogen, Carlsbad, CA) was used to transfect COS-1 and 293T cells (maintained in high glucose-Dulbecco’s modified Eagle’s medium (HG-DMEM) with 8% serum and 100 units each of penicillin and streptomycin) according to the manufacturer’s instructions. For reporter assays examining transient PPRE activity, all transfections included pRL/TK (Promega) to control for transfection efficiency and ACO (Acyl-CoA oxidase)-luciferase. For reporter assays examining transient Gal4 response element activity, all transfections included pRL/CMV (Invitrogen) to control for transfection efficiency and pFR-Luciferase. Following treatment for 6 h with 0.1% dimethyl sulfoxide (DMSO) or 50mM Wy-14,643, cells were lysed and reovirus and firefly luciferase activities were examined using the Dual Luciferase Assay kit (Promega). Luciferase activity was corrected for transfection efficiency (pRLTK/pRLCMV) and extraction yield (via total protein assay).

Immunoprecipitations and Western analysis. COS-1 cells (maintained in HG-DMEM with 8% serum and 100 units each of penicillin and streptomycin) were transfected with plasmids expressing V5-PPARα, MDM2, or MDM2-C464A using Lipofectamine (Invitrogen), according to manufacturer’s instructions. Following an overnight recovery, cells were treated with 0.1% DMSO or 50mM Wy-14,643 for 4 h. COS-1 cells were then lysed in RIPA buffer and cell lysates were precleared 30 min with protein G-sepharose beads (Invitrogen) at 4°C and then subjected to immunoprecipitation with anti-V5 antibody (Invitrogen) or anti-MDM2 antibody (SMP14, Santa Cruz Biotechnology) and protein G-sepharose beads overnight at 4°C. Following four washes in RIPA buffer, the bound protein complexes were eluted in 2× Tris-Glycine sample buffer, and subjected to SDS/PAGE. Proteins were transferred to Immobilon-PVDF membrane (Millipore, Billerica, MA), followed by western using anti-V5 (Invitrogen) or anti-MDM2 (SMP14, Santa Cruz Biotechnology) antibodies.

Ubiquitination experiment. COS-1 cells were transfected with plasmids expressing HA-ubiquitin, V5-PPARα, MDM2, or MDM2-C464A. Following an overnight recovery, cells were treated with 5μM MG-132. Cells were lysed in RIPA buffer with protease inhibitors (Sigma) and 10mM N-iodoacetamide. The lysate was precleared for 30 min with protein G-sepharose beads at 4°C and then subjected to immunoprecipitation with anti-HA antibody (Santa Cruz) and protein G-sepharose beads overnight at 4°C. Following four washes in RIPA buffer, the bound protein complexes were eluted in 2× Tricine sample buffer, and subjected to SDS/PAGE. Proteins were transferred to Hybond-ECL-Ni-NiCl2 membrane, followed by western using anti-V5 (Invitrogen) antibody.

siRNA and real-time PCR. FaO cells (maintained in DMEM/Nutrient F-12/F16 Ham with 8% serum and 100 units each of penicillin and streptomycin) were transfected with MDM2 ON-TARGETplus small interfering RNA (siRNA) (catalog # D-001810-02-05) purchased from Dharmacon (Lafayette, CO), or control ON-TARGETplus Nontargeting siRNA (Dharmacon, catalog # D-001810-05-0010) purchased from Dharmacon (Lafayette, CO), or control ON-TARGETplus Nontargeting siRNA (Dharmacon, catalog # D-001810-02-05). Transfections were performed using Lipofectamine RNAiMax reagent (Invitrogen), according to the manufacturer’s instructions. Following an overnight recovery, cells were treated with 50mM Wy-14,643 or 0.1% DMSO for 6 h. Total RNA was isolated using Tri Reagent (Sigma) according to the manufacturer’s protocol. The total RNA was reverse transcribed using the ABI High Capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA). Standard curves were made using serial dilutions from pooled cDNA samples. Real-time PCR was performed using the SYBR Green PCR Mater Mix (Applied Biosystems) according to the manufacturer’s protocol and amplified on the ABI Prism 7300 Sequence Detection system. mRNA levels of genes measured were normalized to β-actin mRNA. Sequences for primers used are listed in Table 1. For protein analysis, following siRNA transfection, FaO cells were lysed in RIPA buffer with protease inhibitors (Sigma). Cell lysates were subjected to SDS/PAGE and proteins were transferred to Immobilon-PVDF membrane (Millipore), followed by western blotting using anti-PPARα (Cayman Chemicals, Ann Arbor, MI) or anti-MDM2 (SMP14, Santa Cruz Biotechnology) antibodies. Visualization was performed by phophoimaging and band intensity was quantitated using Optiquant Acquisition and Analysis Software (Packard Biosciences, Meriden, CT).

Chromatin immunoprecipitations and real-time PCR. Chromatin immunoprecipitation (ChIP) was performed by modifications of the procedure by Upstate Biotechnology (Lake Placid, New York), using FaO cells grown to 95% confluence, and then treated with 0.1% DMSO or 50mM Wy-14,643 for 2 h. Briefly, chromatin was cross-linked using 1% formaldehyde for 10 min at room temperature, the cells were collected after two washings with PBS in 1 ml of lysis buffer (1% SDS, 10mM EDTA, 50mM Tris-HCl, pH 8.1), and incubated on ice for 10 min. They were then sonicated 10 times for 10 s at an output control set at 6 and duty cycle at 90% (Sonifier 250, Branson Ultrasounds, Danbury, CT), followed by centrifugation. For MDM2 ChIP or PPARα ChIP, 20 μl of supernatant was saved as input, and the rest diluted 10 times in dilution buffer (2mM EDTA, 100mM NaCl, 20mM Tris-HCl, 0.5% Triton X-100).
MDM2 was examined in the presence of MDM2, by the beads through a 30-min incubation with 1% SDS, 0.1M NaHCO₃. This step was repeated using 15-min incubation time. For re-ChIP, 10% of the eluate from the PPARα was saved for input, and the rest diluted 40 times.

**Note.** Suitable real-time PCR primers were designed using PrimerExpress (Applied Biosystems). All primers are listed 5′–3′.

Triton-X, followed by preclearing with 40 µl of Salmon sperm DNA/Protein A agarose (Upstate) for 1 h at 4°C. Immunoprecipitation was performed overnight at 4°C with 2 µg anti-MDM2 (Santa Cruz, SMP14), anti-PPARα (Cayman, 10710, Michigan) or control antibodies. After immunoprecipitation, 60 µl of Salmon sperm DNA/Protein A agarose (Upstate) was added and the incubation continued for 3 h. Precipitates were washed sequentially for 5 min each in 1 ml of low salt wash buffer (0.1% SDS, 1% Triton-X 100, 2mM EDTA, 20mM Tris-HCl [pH 8.1], 500mM NaCl), LiCl wash buffer (0.1% SDS, 1% Triton-X 100, 2mM EDTA, 20mM Tris-HCl [pH 8.1], 150mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton-X 100, 2mM EDTA, 20mM Tris-HCl [pH 8.1], 500mM NaCl, LiCl), and then twice with 1 ml of Tris-EDTA buffer (1mM Triton-X 100, 2mM EDTA, 20mM Tris-HCl [pH 8.1]). Precipitated complexes were extracted from the beads through a 30 min incubation with 1% SDS, 0.1M NaHCO₃. This step was repeated using with 15-min incubation time. For re-ChIP, 10% of the eluate from the PPARα ChIP was saved for input, and the rest diluted 40 times in dilution buffer, followed by immunoprecipitation with anti-MDM2 antibody as described above. Eluates were pooled and heated along with inputs at 65°C overnight to reverse the formaldehyde cross-linking. All buffers contained 1:100 dilution of protease-inhibitor cocktail (Sigma). DNA was purified using phenol-chloroform precipitation. Real-time PCR was performed using the SyBr Green master mix (Applied Biosystems) according to the manufacturer’s protocol on the ABI Prism 7000 sequence detection system. Standard curves were made using serial dilutions from the inputs. Sequences for primers used are listed in Table 1.

**Mice.** Eight to ten week old male WT, PPARα-null mice (Lee et al., 1995) on a SV129 genetic background were housed in a light (12 h light/12 h dark) and temperature (25°C) controlled environment in microisolator cages. Mice were gavaged daily with either vehicle control (corn oil) or 500 mg clofibrate/kg body weight for 14 days. Mice were euthanized, livers weighed and frozen until lysed. Luciferase activity was measured and values were corrected for transfection efficiency and protein. Values are represented after normalization to DMSO. Asterisks indicate a significant difference in ligand induction when compared with the 0 ratio group (*p < 0.05 with statistical analysis using ANOVA). The graph is representative of four independent experiments.

### RESULTS

**MDM2 Modulates the Ligand-Dependent Transcriptional Activity of PPARα and PPARβ**

The transcriptional activity of PPARα, PPARβ and PPARγ isotypes was examined in the presence of MDM2, by measuring the activity of a reporter gene under the control of a natural PPRE. As indicated in Fig. 1, transfecting increasing amounts of MDM2 modulated Wy-14,643-induced PPARα activity in a dose-dependent manner. Interestingly, lower amounts of MDM2 increased reporter activity, whereas higher amounts resulted in inhibition. Transfecting increasing amounts of MDM2 inhibited GW501516-dependent PPARβ reporter activity in a dose-dependent manner. No changes were seen in the absence of ligand (data not shown). PPARγ activity, in the presence (Fig. 1) or absence of rosiglitazone activity, was determined by real-time PCR as described above.

![FIG. 1.](image-url) MDM2 modulates ligand-induced transcriptional activity of PPARα and PPARβ. HEK 293T cells were transfected with plasmids expressing 4X-ACO-Luciferase, pRL/TK, MDM2, and PPARα, PPARβ, or PPARγ. Cells were treated with 0.1% DMSO or 50µM Wy-14,643 (PPARα), 50µM GW501516 (PPARβ), or 2µM Rosiglitazone (PPARγ) for 6 h, and then lysed. Luciferase activity was measured and values were corrected for transfection efficiency and protein. Values are represented after normalization to DMSO. Asterisks indicate a significant difference in ligand induction when compared with the 0 ratio group (*p < 0.05 with statistical analysis using ANOVA). The graph is representative of four independent experiments.
MDM2 Regulates mRNA Levels of PPARα Target Genes

To examine the effect of MDM2 on the transcriptional activity of PPARα, siRNA was used to abrogate MDM2 expression in FaO hepatoma cells. These cells were transfected with MDM2 siRNA or control siRNA, and subsequently treated with PPARα ligand Wy-14,643. Protein levels MDM2 was reduced by 54% in the siRNA harboring cells (Fig. 2A) and mRNA levels by 75% (Fig. 2B). A decrease in MDM2 protein was accompanied by decreased expression of PPARα protein (Fig. 2B). The effects of reduced MDM2 expression on endogenous PPARα target genes was measured. The genes examined were chosen based on their role in PPARα-mediated lipid metabolism, or were previously identified in gene expression microarrays in FaO cells (Tien et al., 2003).

MDM2 Associates with the A/B Domain of PPARα

To examine if the effects of MDM2 on the transcriptional activity of PPARα were due to an interaction between the two proteins, FL PPARα was in vitro translated in the presence of [35S] methionine and incubated with FL in vitro translated MDM2. As shown in Figure 4A, PPARα co-immunoprecipitated with MDM2. The association occurred in the absence or presence (data not shown) of Wy-14,643. In order to identify the domain in PPARα that MDM2 interacted with, PPARα (FL and domains A/B, C, D, E/F) was expressed in bacteria as a MBP fusion. MDM2 was in vitro translated in the presence of [35S] methionine, incubated with PPARα-MBP, and the resultant complex was affinity purified in the presence of amyllose resin. MDM2 copurified with FL and the A/B domain of PPARα. A weak interaction was also observed with the C domain of PPARα (Fig. 4B).

To verify the interaction of MDM2 with the A/B domain of PPARα, mammalian-two-hybrid assays were performed using plasmids expressing MDM2 fused to the pVP16 activation domain, and the different domains of PPARα (A/B, C, D, E/F) and full-length (FL) PPARα in the pM vector. The Gal4 response element reporter (pFR-luciferase) was used to assess the interaction between MDM2 and PPARα. As seen in Fig. 4C, transfecting pVP16-MDM2 in HEK 293T cells resulted in increased ligand-induced reporter activity with full-length PPARα in (top panel). The pM construct expressing different domains of PPARα was cotransfected with pVP16-MDM2 to identify the PPARα domain that was required for interaction with MDM2. An increase in reporter activity was observed with FL and pM-PPARα constructs expressing the A/B and A–D (A/B, C, and D) domains (Fig. 4C, bottom panel). No changes in reporter activity were seen with domains C, D, E/F, or C–F (C, D, and E/F), indicating that the A/B domain of PPARα is required for interaction with MDM2.

To further characterize the interaction, COS-1 cells were transiently transfected with plasmids expressing V5-PPARα and MDM2. Cell lysates were immunoprecipitated with anti-V5 antibody and protein complexes were detected by western blotting with anti-V5 and anti-MDM2 antibodies. As seen in Figure 5B, MDM2 co-immunoprecipitated with PPARα following overexpression.

Finally, to detect if the two proteins interacted at physiological levels of expression, FaO cell lysates were immunoprecipitated with control rat IgG, anti-PPARα or anti-MDM2 antibodies, and protein complexes detected by western blotting. As indicated in Figure 4D, PPARα co-immunoprecipitated with MDM2. No significant difference was found in the interaction between PPARα and MDM2 in the presence of Wy-14,643 (data not shown).
The E3 Ubiquitin Ligase Activity of MDM2 Regulates PPARα Protein Expression and Transcriptional Activity

Because knockdown of MDM2 expression by siRNA resulted in decreased protein levels of PPARα (Fig. 2A) and no changes in PPARα mRNA were seen with MDM2 siRNA (data not shown) in FaO cells, MDM2/p53 null MEFs were used to determine if MDM2 modulated the transcriptional activity of PPARα by regulating its protein expression.

FIG. 2. MDM2 regulates mRNA levels of PPARα target genes. FaO cells were transfected with MDM2 siRNA or control siRNA. Cells were treated with 0.1% DMSO or 50 μM Wy-14,643 for 6 h. (A) Total RNA was isolated from the cells and real-time PCR was performed on reverse transcribed RNA. Letters (a, b, c) indicate a significant difference (p < 0.05 with statistical analysis using ANOVA). The graphs represent mean values obtained from three independent experiments. (B) FaO cell lysates were resolved by SDS-PAGE and proteins were detected by western blotting with anti-MDM2 and anti-PPARα antibodies. The western is representative of three independent experiments. The graph depicts the mean band intensity (relative to glyceraldehyde 3-phosphate dehydrogenase) from the three independent experiments. Quantitation of bands was performed using Optiquant Image Acquisition and Analysis software. Asterisks indicate a significant difference when compared with the corresponding control siRNA group (*p < 0.05 with statistical analysis using ANOVA).
Because MDM2 null MEFs are not available, MDM2/p53 null MEFs increased PPARα protein levels and restored the response to ligand (Fig. 5A).

In order to determine if the above effects of MDM2 on PPARα protein expression was mediated by the E3 ubiquitin ligase function of MDM2, COS-1 cells were transiently transfected with plasmids expressing PPARα, MDM2 WT, or MDM2-C464A that carries a point mutation in the RING finger, disrupting its E3 ubiquitin ligase function. Following transfections, cells were treated with 0.1% DMSO or 50 μM Wy-14,643 and immunoprecipitated with anti-V5 antibody, and protein complexes detected by western blotting with anti-V5 and anti-MDM2 antibodies. As shown in Figure 5B, both WT and RING finger mutant MDM2 co-immunoprecipitated with PPARα. Interestingly, in contrast to increased PPARα expression that was observed when MDM2 was expressed in MEFs (Fig. 5A), association of PPARα with WT MDM2 in COS-1 cells resulted in decreased protein levels of PPARα. However, association with MDM2-C464A did not change protein levels of PPARα, suggesting that the E3 ubiquitin ligase function of MDM2 is required for its regulation of PPARα protein levels. When protein bands were quantitated from multiple (n = 4) experiments, no significant difference was found in the interaction between PPARα and MDM2 in the presence of Wy-14,643. The MDM2-C464A mutant expressed slightly higher than MDM2 WT under these experimental conditions, and the increased levels of MDM2-C464A that co-immunoprecipitated with PPARα in comparison with MDM2 WT may be a result of the differences in expression levels. However, in spite of its higher expression levels, the MDM2-C464A mutant had no effect on PPARα protein expression (Fig. 5B).

To determine if the ubiquitin ligase function is required for MDM2-mediated modulation of PPARα-dependent transactivation, the activity of a reporter gene under the control of a natural PPRE was measured in HEK 293T cells. As indicated in Figure 6, transfecting increasing amounts of MDM2 WT modulated ligand-mediated induction of reporter activity in a dose-dependent manner (left panel). Interestingly, lower amounts of MDM2 WT resulted in an increase, whereas higher amounts resulted in a decrease in reporter activity. However, transfecting MDM2-C464A did not result in any changes in reporter activity (right panel), suggesting that a functional E3 ubiquitin ligase domain is required for modulating PPARα transactivation. These differences were not due to different transfection efficiencies because both
MDM2 WT and MDM2-C464A expressed at similar levels in HEK 293T cells (data not shown).

MDM2 is Regulated In Vivo in a PPARα-Dependent Manner

Finally, the regulation of MDM2 in response to PPARα ligand was examined in vivo. Wild-type and PPARα null mice (Lee et al., 1995) were maintained on a clofibrate or control diet for 2 weeks, following which their livers were analyzed for protein levels of MDM2. A decrease in MDM2 protein expression was detected in response to clofibrate in WT, but not in PPARα null (Fig. 7), indicating a PPARα-dependent regulation.

DISCUSSION

The ubiquitin-proteasome system has emerged as an important regulator of NR function. Both NRs and their coregulators are targeted to the ubiquitin-proteasome system for degradation. Several ubiquitin-proteasome pathway enzymes have been characterized as coactivators for NRs and ubiquitin-proteasome components are recruited to the promoters of NR regulated genes (Kinyamu et al., 2005; Rochette-Egly, 2005). This study identifies an E3 ubiquitin ligase, MDM2 that associates with and regulates the transcriptional activity of PPARα. Ligand-induced mRNA levels of PPARα target genes involved in lipid metabolism were decreased with MDM2 siRNA, indicating that MDM2 affects PPARα transcriptional activity.

To understand the mechanism behind MDM2-mediated regulation of PPARα, PPARα protein expression was examined in FaO cells transfected with MDM2 siRNA and in MDM2/p53 null MEFs. Both cells showed decreased protein levels of PPARα and PPARα null MEFs were less responsive to ligand, when compared with WT MEFs which showed a significant increase in PPARα protein in the presence of ligand. The response to ligand was restored in the null MEFs when MDM2 was transfected, suggesting that MDM2 stabilizes PPARα protein levels in response to ligand. This was in contrast to studies in COS-1 cells where MDM2 coexpression decreased PPARα protein levels. This effect with PPARα-MBP (FL and domains A/B, C, D, E/F), and the resultant complex was affinity purified in the presence of amylase resin. (C) HEK 293T cells were transfected with plasmids expressing pFR-luciferase, pRLCMV, pVP16-MDM2, pM-PPARα (full length/FL and domains A/B, C, D, E/F). Cells were treated with 0.1% DMSO or 50 μM Wy-14,643 for 6 h, and then lysed. Luciferase activity was measured and values were corrected for transfection efficiency and protein. Letters (top panel) indicate a significant difference. Asterisks (bottom panel) indicate a significant increase when compared with the corresponding pM-PPARα group (*p < 0.05 with statistical analysis using ANOVA). The data are representative of three independent experiments. (D) FaO cell lysates were immunoprecipitated with control anti-IgG, anti-PPARα or anti-MDM2 antibodies, protein complexes resolved by SDS-PAGE and detected by western blotting as indicated. The Western is representative of three independent experiments.
required the ubiquitin ligase function of MDM2. Because the COS-1 studies used overexpressed PPARα, whereas the MEF studies examined endogenous PPARα, it is possible that MDM2 responds differently to different expression levels of PPARα. Lower expression of PPARα may provoke MDM2-mediated nonproteolytic stabilization events such as monoubiquitination and/or recruitment of coactivators, contributing to increased PPARα activity. This has been observed for MDM2-mediated regulation of the HIV-1 transactivator, Tat. MDM2 positively regulates Tat transactivation by nonproteolytic ubiquitination (Bres et al., 2003). Higher expression of PPARα may provoke MDM2-mediated polyubiquitination and degradation as a means of controlling the transcriptional response. This could explain the decreased transcriptional activity of PPARα that was observed in PPRE-dependent reporter assays. MDM2-mediated ubiquitination of the AR destabilizes AR and attenuates AR activity (Gaughan et al., 2005), indicating that MDM2 can act as a negative regulator of NR function.

Because increased transcriptional activity of PPARα was observed in PPRE-dependent reporter assays at lower amounts of coexpressed MDM2, another possibility is that polyubiquitination and increased turnover contribute to increased transcriptional activity. Degradation of NRs such as retinoic acid receptor (RARγ2), progesterone receptor (PR) and GR is linked to transcriptional activation (Gianni et al., 2002; Shen et al., 2001; Wallace and Cidlowski, 2001). It has been proposed that degradation by the ubiquitin-proteasome pathway might provide an efficient mechanism for regulating the cyclic interaction of NRs with the promoter (Rochette-Egly, 2005). This is in contrast to previous reports which show that inhibition of PPARα degradation increases its transcriptional activity (Blanquart et al., 2002). These differences may be attributed to analysis of PPARα activity at a single time point. It is likely that PPARα degradation is regulated in a timely manner to allow for accurate sensing of ligand concentrations and tighter control over transcriptional events. This has been

FIG. 5. E3 ubiquitin ligase function of MDM2 regulates PPARα protein expression. (A) Wild-type MEFs were transfected with an empty vector or plasmid expressing MDM2, following which they were treated with 0.1% DMSO or 50μM Wy-14,643 for 4 h. Cell lysates were resolved by SDS-PAGE and proteins were detected by western blotting with anti-PPARα antibody. The western is representative of three independent experiments. The graph depicts the mean band intensity from the three independent experiments. Quantitation of bands was performed using Optiquant Image Acquisition and Analysis software. Asterisks indicate a significant difference when compared with the corresponding control (no Wy-14,643 treatment) group. (*p < 0.05 with statistical analysis using ANOVA). (B) COS-1 cells were transfected with plasmids expressing V5-PPARα, MDM2, or MDM2-C464A and treated with 0.1% DMSO or 50μM Wy-14,643 for 4 h. Cell lysates were immunoprecipitated with anti-V5 antibody, protein complexes resolved by SDS-PAGE and detected by western blotting with anti-V5 or anti-MDM2 antibodies. The Western is representative of three independent experiments. (C) COS-1 cells were transfected with plasmids expressing V5-PPARα, MDM2, and HA-Ubiquitin, and treated with 5μM MG-132. Cell lysates were immunoprecipitated with anti-HA antibody, protein complexes resolved by SDS-PAGE and detected by Western blotting with anti-V5 antibody. The Western is representative of three independent experiments.
observed with the ER, whose transcriptional activity is enhanced under the influence of MDM2, possibly by affecting the periodicity of receptor proteasomal degradation and the association of ER to target gene promoters in response to ligand concentrations (Reid et al., 2003).

MDM2 siRNA studies indicate that MDM2 enhances Wy-14,643-induced transcriptional activity of PPARα, and ChIP experiments revealed increased occupancy of MDM2 on PPREs in the presence of Wy-14,643. In contrast to MDM2-mediated increase in PPARα activity seen in FaO cells, the protein expression of MDM2 was decreased in mice treated with Wy-14,643. This decrease was observed in WT but not in PPARα null mice, indicating a PPARα-dependent regulation of MDM2. Although these results were from experiments in different model systems (hepatoma cells and mice), it is possible that PPARα-mediated inhibition of MDM2 expression represents a feedback regulation to control activation of PPARα in the presence of ligand. The expression of MDM2 is also negatively regulated by the orphan receptor TR3 (Zhao et al., 2006). Other NRs that regulate MDM2 expression are the ER (Hori et al., 2002), thyroid hormone receptor (Qi et al., 1999), and constitutive androstane receptor (CAR) (Huang et al., 2005), indicating a broad role for MDM2 as a regulator of NR function. Although the current studies indicate an involvement for PPARα in regulation of MDM2 expression, it will be of interest to determine if PPARα is involved directly or if other cofactors are involved.

MDM2 interacted with the A/B domain of PPARα as revealed by in vitro assays and mammalian-two-hybrid assays. This domain contains the ligand-independent AF-1 transactivation function, and our laboratory has demonstrated that phosphorylation in the A/B domain by GSK3β regulates PPARα activity (unpublished results; Burns and Vanden Heuvel, 2007). Preliminary data indicate that a PPARα mutant defective in GSK3β-mediated phosphorylation is regulated differentially by MDM2. GSK3β is important in insulin signaling and understanding the interplay between MDM2 and GSK3β in regulating PPARα activity may be important in understanding the regulation of glucose metabolism by PPARα. A role for GSK3β in regulating MDM2 has already been demonstrated with regards to p53 stabilization (Kulikov et al., 2005).

MDM2 plays a central role in regulating p53 stability and this pathway is critical for cellular response to aberrant oncocgenic signaling. Hepatocarcinogenesis induced by PPs in rodents has been attributed to increased cell proliferation and suppression of apoptosis leading to imbalanced hepatocyte growth control (Klaunig et al., 2003). One compelling case for the role of p53 in PP-induced cell proliferation is the increased levels of p53 in Wy-14,643-treated humanized PPARα mice, in contrast to the comparatively lower p53 expression in Wy-14,643-treated WT mice (Morimura et al., 2006). The lack of significant increase of p53 levels in WT mice may allow cells to escape p53-mediated cell cycle surveillance, leading to hepatocellular proliferation and carcinogenesis, effects that are absent in humanized PPARα mice (Morimura et al., 2006). Interestingly, p53 and p21 protein are increased in mice administered Wy-14,643 (Ma et al., 1997). Other studies with p53 and its target genes such as p21 following PP administration have shown varied results (Hoivik et al., 2004; Knight et al., 2005; Ma et al., 1997; Peters et al., 1997; Simbula et al., 2004). Cell cycle regulators such as cyclins and cyclin-dependent kinases (CDKs) are increased in mice administered PPs (Hays et al., 2005; Ma et al., 1997; Rininger et al., 1996, 1997). This study demonstrated a decrease in MDM2 protein expression in mice administered clofibrate. In addition to regulating p53, MDM2 also promotes

FIG. 6. E3 ubiquitin ligase function of MDM2 regulates transcriptional activity. HEK 293T cells were transfected with plasmids expressing 4X-ACO-Luciferase, pRL/TK, V5-PPARα, MDM2 WT, or MDM2-C464A. Cells were treated with 0.1% DMSO or 50μM Wy-14,643 for 6 h, and then lysed. Luciferase activity was measured and values were corrected for transfection efficiency and protein. Asterisks indicate a significant difference in Wy-14,643 induction when compared with the 0 ratio group (*p < 0.05 with statistical analysis using ANOVA). The graph is representative of three independent experiments.
cell cycle progression by regulating the Rb/E2F complex (Levav-Cohen et al., 2005). The relevance of this role in PP-induced cell proliferation is worthy of investigation. Because studies with PPARα null mice demonstrated a PPARα-dependent regulation of MDM2, it will be of interest to identify the mechanism of this regulation. Characterizing the role of PPARα as direct or involving other coregulators in regulating MDM2 may contribute to understanding the effects of PPs on cell cycle regulation.

The studies presented here provide a basis for examining the role of PPARα in regulating the MDM2-p53 pathway during cancer development. Additionally, these studies identify for the first time, a role for MDM2 in regulating PPARα-mediated pathways of lipid and glucose metabolism. The various biological processes regulated by PPARs are crucial in control of disorders such as diabetes, inflammation, and cardiovascular ailments. Further work on the role of MDM2 and the ubiquitin-proteasome system in regulating PPARs holds promise towards the understanding and treatment of these diseases.

**REFERENCES**


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