The transcription factor peroxisome proliferator-activated receptor γ (PPARγ) belongs to the family of nuclear hormone receptors and consists of two isoforms, PPARγ1 and PPARγ2. Our earlier studies have shown that troglitazone (TZD)-mediated activation of PPARγ2 in hepatocytes inhibits growth and attenuates cyclin D1 transcription via modulating CREB levels. Because this process of growth inhibition was also associated with an inhibition of β-catenin expression at a post-translational level, our aim was to elucidate the mechanism involved. β-Catenin is a multifunctional protein, which can regulate cell-cell adhesion by interacting with E-cadherin and other cellular processes via regulating target gene transcription in association with TCF/LEF transcription factors. Two adenomatous polyposis coli (APC)-dependent proteasomal degradation pathways, one involving glycogen synthase kinase 3β (GSK3β) and the other involving p53-Siah-1, degrade excess β-catenin in normal cells. Our immunofluorescence and Western blot studies indicated a TZD-dependent decrease in cytoplasmic and membrane-bound β-catenin, indicating no increase in its membrane translocation. This was associated with a reduction in E-cadherin expression. PPARγ2 activation inhibited GSK3β kinase activity, and pharmacological inhibition of GSK3β activity was unable to restore β-catenin expression following PPARγ2 activation. Additionally, this β-catenin degradation pathway was operative in cells, with inactivating mutations of both APC and p53. Inhibition of the proteasomal pathway inhibited PPARγ2-mediated degradation of β-catenin, and incubation with TZD increased ubiquitination of β-catenin. We conclude that PPARγ2-mediated suppression of β-catenin levels involves a novel APC/GSK3β/p53-independent ubiquitination-mediated proteasomal degradation pathway.

The peroxisome proliferator-activated receptor (PPAR) family of transcription factors belongs to the nuclear hormone superfamily, which consists of at least three members PPARα, γ, and δ and members of which are involved in regulating growth, differentiation, and metabolism in a variety of cell types (1). The PPARγ subfamily consists of two isoforms, PPARγ1 and PPARγ2, of which PPARγ1 is expressed in many tissues, whereas PPARγ2 is preferentially expressed in adipose tissue (2). Expression of functional PPARY in the liver is elevated during obesity (3–5) as well as in hepatocellular carcinoma (6, 7). The PPAR isoatypes can regulate transcription of target genes in response to a corresponding ligand (8, 9). The ligands for PPARγ include nicotinic acid, eicosanoid derivatives (example 15-deoxy-Δ12,14-prostaglandin J₂ or 15d-PGJ₂), and thiazolidinedione (for example TZD) family of insulin sensitizers (which were developed as drugs to treat type II diabetes) (8). PPAR-mediated transactivation of target genes involves their dimerization with the retinoid X receptor (RXR) following ligand activation and binding of the resulting heterodimer to specific PPAR response elements (PPREs) located within the promoters/enhancers of target genes (10). PPARγ also utilize different coactivators to specify selective target gene transcription, which include p300 (or CBP) (11, 12), the SRC-1 class of coactivators (13, 14), PGC-1 and PGC-2 (15, 16), ARA70 (17), and DRIP205 (or TRAP220) (18, 19).

In the adipose tissue, PPARγ plays a key role in regulating differentiation of pre-adipocytes into mature adipocytes (20, 21), which involves cross-talk with the Wnt-signaling pathway. This is evident from the fact that PPARγ-mediated adipocyte differentiation is associated with an extensive down-regulation of β-catenin expression coincident with terminal differentiation (22). Additionally, activation of the Wnt-signaling pathway in the adipocytes antagonizes adipogenesis, inhibition of which results in spontaneous adipogenesis (23, 24). These results indicated that PPARγ pathway functions via antagonism of the β-catenin pathway.

β-Catenin is involved in mediating two major functions in a normal cell: (a) in the cell membrane, as part of the E-cadherin/catenin adherens complex and regulating cell-cell adhesion and (b) in the cytoplasm and nucleus as a mediator of the proliferating signal through the Wingless/Wnt pathway (25–27). Signaling via β-catenin involves post-translational stabilization and translocation into the nucleus, where it interacts with transcription factors of the T cell factor/lymphoid enhancer factor (TCF/LEF) family to activate target gene transcription involved in cell growth control and apoptosis (25, 26). Free pools of β-catenin are tightly regulated via two major pathways: MT APC; mutant APC; PBS, phosphate-buffered saline; PPRE, PPAR response element; RXR, retinoic X receptor; TCF, T cell factor; TZD, troglitazone; DMEM, Dulbecco’s modified Eagle’s medium; TBTB, Triton-X-buffered saline with Tween; MOPS, 4-morpholinepropane-sulfonic acid. This paper is available online at http://www.jbc.org

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‡ The abbreviations used are: PPAR, peroxisome proliferator-activated receptor; APC, adenomatous polyposis coli; CHX, cycloheximide; CREB, CRE-binding protein; FBS, fetal bovine serum; FL, APC, full-length APC; GSK3β, glycogen synthase kinase 3β; LEF, lymphoid enhancer factor; MT APC, mutant APC; PBS, phosphate-buffered saline; PPRE, PPAR response element; RXR, retinoic X receptor; TCF, T cell factor; TZD, troglitazone; DMEM, Dulbecco’s modified Eagle’s medium; TBTB, Triton-X-buffered saline with Tween; MOPS, 4-morpholinepropane-sulfonic acid.
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involving the tumor suppressor protein adenomatous polyposis coli (APC). In one pathway, in the presence of axin and functionally active APC, the serine-threonine kinase glycogen synthase kinase 3β (GSK3β) phosphorylates β-catenin at critical serine and threonine residues in its N terminus. N-terminal phosphorylated β-catenin is recognized by the F box protein βTrCP and targeted toward ubiquitination-mediated proteasomal degradation (28). Activation of phosphatidylinositol 3-kinase (PI3K) and Wnt-signaling pathways as well as signals originating from adhesion of cells to extracellular matrices can affect β-catenin stabilization and signaling via regulating GSK3β activity. In a second pathway, p53 inducible Siah-1, in the presence of APC targets β-catenin toward the ubiquitin-mediated proteasomal degradation pathway (29, 30). Mutations of either APC, p53, or β-catenin itself can lead to inhibition of β-catenin degradation, resulting in increased cytoplasmic pools (31–36) and activation of TCF/LEF-mediated transcription. Cancer-chemotherapeutic drugs targeted toward suppression of β-catenin expression or activation thus might be a successful means of combating tumor growth.

Activation of PPARγ in different systems have resulted in attenuation of growth (6, 7, 37–42). In our recent studies with mouse hepatocytes, PPARγ2 activation attenuated growth and cyclin D1 transcription (43), which involved modulation of CREB. In those studies PPARγ2-mediated attenuation of hepatocyte growth was also associated with a corresponding inhibition of β-catenin expression as reported in other systems (22, 44). Because the mechanism involved in this process of β-catenin degradation is still unclear, we designed studies to elucidate the pathway by which activation of PPARγ2 inhibited β-catenin expression. Our results indicated that PPARγ2 inhibited β-catenin expression via an APC-GSK3β- and APC-p53-independent pathway. This novel mechanism of β-catenin regulation, however, involved a ubiquitination-mediated proteasomal degradation pathway.

EXPERIMENTAL PROCEDURES

Reagents and Plasmids—DMEM-F12 and DMEM medium, fetal bovine serum (FBS), penicillin-streptomycin, trypsin, LipofectAMINE, and β-galactosidase assay kits were obtained from Invitrogen; insulin-transferrin-sodium selenite media supplement and dexamethasone were obtained from Sigma; cycloheximide, GSK3 inhibitor, TZD, and protein A/G-agarose were obtained from BIOMOL Research Laboratory (Plymouth Meeting, PA), Tau protein from Santa Cruz Biotechnology (Santa Cruz, CA), E-cadherin and total GSK3β from BD Transduction Laboratories (Franklin Lakes, NJ), Phospho-β-catenin (Ser15/18/19) and Phospho-GSK3β (Ser9) from Cell Signaling Technology (Beverly, MA), HA.11 from BabCO (Richmond, CA), β-catenin from Zymed Laboratories (San Francisco, CA), APC from Oncogene (San Diego, CA), and 9E10 as described earlier (52). HA-tagged wild-type β-catenin or S37A-β-catenin constructs were obtained from Dr. Stephen Byers (46), the His/Myc-tagged wild-type ubiquitin construct (H,M-Ub) (47) was obtained from Dr. Ron Kopito, the HT29-APC and HT29-βS48A cells, and pGLOT, pGL3OP reporter constructs were obtained from Dr. Bert Vogelstein (48), the tk-PPREx3-luc reporter construct was obtained from Dr. Ron Evans (49) and the SW-480, HT-29, HCT-15, and RKO-31(131–781 amino acids) and 5′-CCGGATCCGCGCATGCTAAGCTTATGG-3′ and 5′-ATAGTTTATGCGGCGCATGCTAAGCTTATGG-3′ for N-terminal β-catenin (1–131 amino acids), 5′-CCGGATCCGCGCATGCTAAGCTTATGG-3′ and 5′-ATAGTTTATGCGGCGCATGCTAAGCTTATGG-3′ for C-terminal β-catenin (131–781 amino acids) and 5′-CCGGATCCGCGCATGCTAAGCTTATGG-3′ and 5′-ATAGTTTATGCGGCGCATGCTAAGCTTATGG-3′ for full-length β-catenin (1–781 amino acids). Following subcloning of the β-catenin fragments in the pcDNA 3.1 Myc/His vector, plasmid DNA was purified and sequences were verified by DNA sequencing.

Luciferase Reporter Assays—These assays were performed as described previously (43). Briefly, cells plated at a density 0.15 × 10⁶ cells/well of a 6-well plate were allowed to adhere overnight followed by transient transfection with the respective luciferase vectors along with a β-galactosidase vector using LipofectAMINE. The cells were recovered in serum-containing medium and then treated with either Me2SO or TZD. Luciferase assays were performed using a Microplate Luminometer LB 96V (EG&G Berthold, Bad Wildbad, Germany), and β-galactosidase assays were performed using a platereadere. Each assay was performed in duplicate, and each transfection was repeated at least six to nine times. The results obtained were calculated as the ratio of relative light units to the β-galactosidase values (relative light units/β-galactosidase) and expressed as the percent inhibition of luciferase activity considering those obtained from the TZD untreated controls as 100%.

Western Blot Analyses—For Western analysis, total cellular or nuclear protein was extracted from the cells at different time intervals according to the procedures described previously (50). Equal amounts of total cellular or nuclear protein were fractionated by SDS-PAGE, transferred to PVDF membranes, and blotted with the respective antibodies. In the experiments with inhibitors, cells were pretreated with the respective inhibitors prior to the addition of TZD. In the cycloheximide experiments, TZD treatment was performed for 48 h before incubation of the cells with cycloheximide. The Triton-soluble and Triton-insoluble cell extracts were prepared according to the protocol described previ- ously (51). Briefly, Triton-soluble fraction was isolated by extracting cells in lysis Buffer A containing 1% Triton X-100, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 5 mM DTA, 140 mM NaCl, 10% glycerol, 1 mM MgCl2, and protease inhibitors. The supernatant obtained following centrifugation of these extracts was termed Triton-soluble extracts. The pellet obtained following extraction with Buffer A was further extracted in lysis Buffer B containing 1% SDS, 50 mM Tris-HCl, pH 7.5, 1 mM EGTA, 5 mM EDTA, and protease inhibitors. The supernatant obtained following extraction with Buffer B was termed “Triton-insoluble extracts.”

Immunoprecipitation Studies—For immunoprecipitation studies cell extracts were prepared in lysis buffer (20 mM HEPES, 2 mM EGTA, 50 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM dithiothreitol, 1 mM vanadate, and 0.04 mM phenylmethylsulfonyl fluoride) containing a mixture of protease inhibitors. Equal amounts of cell extracts were incubated with specific antibodies, which were pre-adsorbed previously on protein A-Sepharose beads for 1 h at 4 °C. Following a 2-h incubation at 4 °C the antibody-protein complex was purified by centrifugation of the beads and washing twice with ice-cold lysis buffer and once with 1× PBS. Finally SDS sample buffer was added to the pellets, and the denatured protein was resolved on SDS-PAGE followed by Western blotting. To determine any interaction between PPARγ2 and β-catenin, an in vivo cross-linking of the proteins using dithiobis(succinimidylpropionate) (Pierce, IL) was performed prior to immunoprecipitation as described earlier (52).

Pulse-chase Analysis—Subconfluent populations of P7 hepatocytes grown in growth medium were first treated in the absence or presence of TZD for ~48 h. At the end of this incubation the cells were washed in methionine-, cysteine-, and glutamine-free DMEM (Invitrogen), starved for 1 h in the same medium and pulse-labeled using 500 μCi of Tran35S- L-leucine for 1 h. The cells were then washed with acid-free medium and then chased in complete growth medium for different lengths of time in the presence or absence of TZD. At the end of the chase, total protein was harvested and 35S-labeled β-catenin was immunoprecipitated. The immunoprecipitates were resolved on 10% SDS-PAGE, transferred on PVDF membranes, and subjected to β-catenin immunoblotting.

Northern Blot Analysis—Total RNA extracted from cells grown in the presence or absence of TZD for different lengths of time were subjected to Northern blot analysis, as described (53), using a β-catenin cDNA as probe or an 18 S ribosomal RNA DECAprobe (Ambion, TX) as control.
Immunofluorescence Analysis—Immunofluorescence assays were carried out according to a modification of the protocol described (54). Briefly, cells were plated on double well chamber slides (0.125 × 10⁶ cells per well) and treated with TZD (10 μM) for the indicated periods of time. They were fixed in 4% methanol-free formaldehyde in PBS, pH 7.4, at 4°C for 25 min and permeabilized in PBS containing 0.2% Triton X-100. This was followed by two washes in PBS and incubation with a monoclonal antibody against β-catenin (Zymed Laboratories Inc., CA) in 1% bovine serum albumin-TBST for 2 h at room temperature. Following five washes with TBST, cells were incubated with rhodamine-conjugated anti-murine secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature, washed, and mounted in Antifade (Molecular Probes, OR). Images were acquired using an OlymPix 2000 RGB camera and OlymPix 2000 software (1280 × 1024 pixels/image).

In Vitro Kinase Assay—The in vitro kinase assays were performed according to the protocol described elsewhere (55). Equal amounts of total protein lysate, extracted from cells treated as indicated, were immunoprecipitated by an antibody against GSK3β pre-adsorbed to protein A-Sepharose beads. This was followed by washing of the protein A-Sepharose beads twice with lysis buffer and twice with assay buffer (20 mM MOPS, pH 7.2, 2 mM EGTA, 10 mM MgCl₂, 1 mM dithiothreitol, and 0.1% Triton X-100). Kinase assays were carried out by incubating the sample beads in 40 μl of kinase buffer (containing 2 mM cold ATP and 10 mM MgCl₂) in presence of Tau recombinant protein (as GSK3β substrate) and 10 μCi of [γ⁻³²P]ATP, for 30 min at 30°C. Samples were fractionated by SDS-PAGE and transferred to PVDF membranes. [³²P] incorporation into Tau was quantified using a PhosphoImager (Storm Scanner model 860 and ImageQuant 5.2 software, Amersham Biosciences).

RESULTS

PPARγ2 Inhibited Expression of Endogenous and Ectopic β-Catenin in AML-12 Hepatocytes—To determine the effect of PPARγ2 on β-catenin expression, we utilized the AML-12 hepatocytes (56). Luciferase assays designed with a PPARγ-responsive luciferase reporter (tk-PPAREx₃-luc) indicated a significant activation of the reporter activity following transient overexpression of PPARγ2 (Fig. 1A, lane 3). This activity was further enhanced following activation of PPARγ2 with an exogenous ligand TZD (lane 4). These results indicated that PPARγ-mediates the pathway was functional in these hepatocytes.

To study the effect of PPARγ2 on β-catenin expression in hepatocytes, β-catenin levels were estimated following transient expression and activation of PPARγ2 in the AML-12 hepatocytes. Results from these studies indicated a dramatic decrease in the expression of β-catenin in AML-12 cells (Fig. 1B, lanes 3 and 4), coincident with PPARγ expression and activation. To confirm the effect of PPARγ2 in attenuating β-catenin expression, we also utilized the control, Py, and Py-wt-β-catenin hepatocytes, stably overexpressing either an empty vector or a PPARγ2-containing vector or a combination of PPARγ2 and wt-β-catenin vectors, respectively (43). Activation of PPARγ2 following addition of TZD was associated with a significant reduction of endogenous β-catenin protein levels in the Py cells and not in the control cells (Fig. 1C, compare Py and control lanes). In addition, PPARγ2 activation was capable of reducing levels of ectopically expressed β-catenin as evident from a TZD-mediated reduction in HA expression in Py-wt-β-catenin hepatocytes (Fig. 1D, compare +/− lanes in the HA panel). Estimation of β-catenin levels in the nuclear protein showed a TZD-dependent reduction of β-catenin levels in the nucleus of Py hepatocytes (Fig. 1E). Western analysis of Py hepatocytes with increasing concentrations of TZD indicated that 10 μM TZD was sufficient to reduce β-catenin expression optimally compared with the TZD-un-treated cells (Fig. 1F). These results indicated that PPARγ2 activation was capable of attenuating both ectopic and endogenous β-catenin expression in AML-12 hepatocytes.

PPARγ Activation Inhibited Expression of β-Catenin in Different Cell Types—To determine whether PPARγ-mediated suppression of β-catenin expression was a generalized event, MCP-7 breast cancer cells and NIH-3T3 fibroblasts were utilized. Addition of TZD to the MCF-7 cells, which showed growth inhibition following activation of endogenous PPARγ in other studies (57), also showed inhibition of β-catenin expression with 25 and 50 μM TZD (Fig. 2A). In addition, β-catenin expression was significantly attenuated in the NIH-3T3 fibroblasts following ectopic expression and activation of PPARγ2 (Fig. 2B, lanes 3 and 4). These results indicated that PPARγ activation-mediated suppression of β-catenin expression was a generalized event.

PPARγ2 Activation Was Unable to Increase Membrane Translocation of β-Catenin—In a recent report it was shown that ligand activation of the PPARγ-modulated E-cadherin/β-catenin system by inducing E-cadherin expression and increasing β-catenin membrane translocation (58) in pancreatic cancer cells. To determine whether a similar mechanism was also operating in the hepatocytes to regulate the cytosolic levels of β-catenin following PPARγ2 activation, immunofluorescence analysis was carried out in the Py hepatocytes using an antibody against β-catenin. The results from these studies showed that in the absence of TZD β-catenin was distributed evenly in the membrane as well as in the cytoplasm of these hepatocytes (Fig. 3A, vehicle panel). Incubation of these hepatocytes with TZD resulted in a dramatic reduction in the levels of both membrane-bound as well as cytoplasmic β-catenin (TZD panel), indicating no increase in the membrane translocation of β-catenin following activation of PPARγ2. To confirm these results further, Triton-soluble (cytoplasmic) and Triton-insoluble (membrane-bound) cell extracts were prepared from Py cells following treatment in the absence or presence of TZD. Western analysis of these extracts indicated a TZD-dependent decrease in β-catenin as well as E-cadherin expression in both cytoplasmic and membrane-bound fractions (Figs. 3, B and C). This suggested that PPARγ2-mediated attenuation of β-catenin expression in the hepatocytes did not involve an increase in membrane translocation of the protein and recruitment by E-cadherin.

Activation of PPARγ2 Inhibited β-Catenin Expression via a Post-translational Mechanism—Because PPARγ can modulate β-catenin expression via regulating transcriptional (44) as well as post-translational events (22), we determined whether this process in the hepatocytes involved a post-translational modulation of the protein. To address this, we estimated changes in β-catenin mRNA levels in the Py hepatocytes following TZD-mediated activation of PPARγ2. The results from the Northern blot studies indicated no significant variation in β-catenin mRNA levels following exposure of the Py hepatocytes to TZD (Fig. 4A), despite significant down-regulation of β-catenin protein expression during this time period (43). To determine whether PPARγ2 affected β-catenin expression via a post-translational modification, we studied the effect of TZD on β-catenin expression in the presence of the protein synthesis inhibitor cycloheximide. In these studies, addition of TZD to the Py hepatocytes was capable of attenuating β-catenin levels even in the presence of cycloheximide as shown in Fig. 4B (compare +CHX/+TZD samples in lanes 6–8 with +CHX/−TZD samples in lanes 3–5), indicating that attenuation of β-catenin levels involve a post-translational mechanism. To further establish that PPARγ2 effects β-catenin protein stability, pulse-chase analysis was performed with [³⁵S]labeled β-catenin. Addition of TZD to the Py hepatocytes resulted in a significantly faster decay of [³⁵S]labeled β-catenin compared with the TZD-un-treated control (Fig. 4, C and D). Combined together, these results confirmed that PPARγ2 activation in the hepatocytes attenuated β-catenin expression via a post-translational event. These observations are supported by a recent study in 3T3 L1-adipocytes, which indicated that PPARγ...
activation inhibited expression of $\beta$-catenin via a proteasome-mediated pathway (22).

**PPARγ-mediated Inhibition of $\beta$-Catenin Expression Involved a Ubiquitination-mediated Proteasomal Degradation Pathway**—In the conventional APC-dependent pathways, $\beta$-catenin is degraded via a ubiquitination-mediated pathway involving the proteasomes. To determine whether PPARγ-mediated degradation of $\beta$-catenin involved the proteasomal pathway in hepatocytes, Pγ hepatocytes were treated separately with two proteasome inhibitors MG-132 and lactacystin prior to the treatment with TZD. MG-132 or lactacystin-mediated inhibition of the proteasomal degradation pathway in the Pγ hepatocytes antagonized PPARγ-mediated degradation of endogenous $\beta$-catenin as shown in Figs. 5 (A and B). In addition, pretreatment of these hepatocytes with MG-132 antagonized PPARγ-mediated degradation of ectopic $\beta$-catenin (Fig.
we determined the effect of TZD on the expression of the S37A-mutant-β-catenin. Due to a mutation of serine to alanine at amino acid residue 37, GSK3β is unable to phosphorylate S37A-β-catenin, and S37A-β-catenin serves as a stabilized β-catenin mutant for the GSK3β pathway. To determine the effect of PPARγ2 on S37A-β-catenin expression, we utilized the stable Py-S37A-β-catenin cell lines, created by stable overexpression of HA-tagged-S37A-β-catenin in the Py hepatocytes as described elsewhere (43). Western analysis with an HA antibody indicated a time-dependent decrease in HA expression following treatment of Py-S37A-β-catenin cells with TZD (Fig. 6B, HA panel). Luciferase assays carried out with a β-catenin-responsive reporter construct (pGL3OT) also showed a TZD-mediated reduction of luciferase activity in the Py-S37A-β-catenin cells in a similar fashion as in the Py-wt-β-catenin cells (Fig. 6C, compare lanes 1 and 2 with lanes 5 and 6). This degradation (Fig. 6B) and inhibition (Fig. 6C) of the S37A mutant form of β-catenin by PPARγ2 indicated that this pathway of β-catenin degradation might not involve GSK3β. Similarly, pretreatment of the Py hepatocytes with LiCl (inhibitor of GSK3β) or a commercial GSK3β inhibitor was unable to antagonize TZD-mediated attenuation of β-catenin expression (Fig. 6D), thus confirming that PPARγ2-mediated degradation of β-catenin involved GSK3β-independent pathways.

**Activation of PPARγ2 Inhibited GSK3β Activity**—Because the results described in Fig. 6 indicated that PPARγ2-mediated pathway of β-catenin degradation was independent of GSK3β, we determined the status of GSK3β activity following activation of PPARγ2 in the hepatocytes. The activity of GSK3β is regulated via a phosphorylation at its Ser9 site via multiple pathways (61), and an increase in Ser9 phosphorylation results in inhibition of the enzyme activity. Western analysis of TZD-treated Py cell extracts with a phospho-antibody specific to GSK3βSer9 phosphorylation showed an increase in GSK3βSer9 phosphorylation indicating inhibition of its activity following incubation with TZD (Fig. 7A, phospho-GSK3β panel, +/+ TZD lanes), whereas the total GSK3β levels remained unchanged (total GSK3β panel). Additionally, GSK3β kinase assays performed with TZD-treated Py cell extracts and the GSK3β-specific substrate Tau indicated a PPARγ2 activation-mediated inhibition of Tau phosphorylation (Fig. 7B). These results suggested that PPARγ2 activation was associated with a reduction in GSK3β kinase activity and established further that β-catenin degradation through this pathway was not mediated through GSK3β.

**PPARγ2-mediated Degradation of β-Catenin Was Independent of APC and p53**—Although GSK3β is the major mediator of β-catenin degradation in the APC pathway, β-catenin can also be degraded by APC in a GSK3β-independent and p53/Siah-1-dependent pathway (29, 30). Because a recent report suggested that intact/full-length APC was required for the suppression of β-catenin levels and colon carcinogenesis following PPARγ activation (62), we determined whether PPARγ-mediated suppression of β-catenin involved APC and p53. To address this, HT-29 and SW480 colon cancer cell lines were utilized, both of which harbor inactivating mutations of APC as well as p53 (29, 30, 32, 63–65). We have previously reported that addition of TZD to the HT-29 cells was unable to down-regulate β-catenin expression (43). To determine whether expression of full-length APC in these cells restored β-catenin degradation following TZD stimulation, we utilized the stable HT-29-APC and HT-29-p53-β-galactosidase cells (48), which can express either full-length APC or the control β-galactosidase vector, respectively, in a zinc-dependent manner. Incubation of HT-29-APC cells with 100 μM zinc induced expression of full-length APC (Fig. 8B, FL APC), whereas the HT-29-β-galactosidase cells showed

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**Fig. 2. Activation of PPARγ inhibited β-catenin expression in other cell types.** A, exponentially growing MCF-7 cells were treated with different concentrations of TZD and harvested at 24 and 72 h. Western analysis was carried out as in Fig. 1 with β-catenin and actin antibodies. B, NIH3T3 cells were transiently transfected with either an empty vector (EV lanes) or a PPARγ2 vector (Py lanes) followed by treatment in the absence (−) or presence (+) of TZD. Western analyses with β-catenin and actin antibodies were carried out as in A.

5C), suggesting that this pathway of β-catenin degradation in hepatocytes also involved the participation of the proteasomes. To understand any role of ubiquitination in mediating this process, Py hepatocytes were transiently cotransfected with HA-tagged-WT-type-β-catenin along with either empty vector or a His-Myc epitope-tagged ubiquitin vector (H1M-Ub) (47) followed by treatment in the absence (−) or presence (+) of TZD. The cells were harvested, and equal amounts of cell extracts were immunoprecipitated with HA antibody (to immunoprecipitate ectopic β-catenin) followed by immunoblotting with a Myc antibody. The results from Fig. 5D indicated an increase in β-catenin ubiquitination following activation of PPARγ2 by TZD in the presence of the proteosomal inhibitor MG-132 (compare lanes 7 and 8). These data indicated that PPARγ2-mediated attenuation of β-catenin levels involved a ubiquitination-mediated proteasomal degradation pathway.

**PPARγ2-mediated Degradation of β-Catenin Involved a GSK3β-independent Pathway**—In the conventional pathway, GSK3β-mediated phosphorylation of N-terminal β-catenin is a key step toward β-catenin degradation involving the ubiquitination-mediated proteasomal degradation pathway. To gain further insight into the mechanism by which PPARγ2 activation degraded β-catenin, we determined whether it involved GSK3β-mediated phosphorylation of β-catenin. In case β-catenin degradation in this pathway involved GSK3β, we would expect an increase in β-catenin phosphorylation at its GSK3β phosphorylation sites, Ser37/Thr41 (59, 60). However, Western analysis of Py cell extracts with a phospho-β-catenin antibody (directed against phospho-Ser37/Thr41 of β-catenin) indicated a specific decrease in the levels of phospho-β-catenin in presence of TZD (Fig. 6A). Because this decrease in phospho-β-catenin levels could be due to a corresponding decrease in the steady-state levels of β-catenin following incubation with TZD,
the expression of only the mutant forms (MT APC). Expression of full-length APC in the HT-29-APC cells resulted in an overall decrease in β-catenin levels irrespective of TZD when compared with the HT29-β-galactosidase cells, indicating that full-length APC was functional. However, treatment of HT-29APC cells with TZD following a pretreatment with zinc showed no further decrease in β-catenin expression and was similar to the control HT-29-β-galactosidase cells (HT29-βGal panel), indicating that overexpression of full-length APC in these cells was not enough to mediate TZD-dependent β-catenin degradation. Because HT-29 cells also have a mutation of p53, we determined whether p53 participated in mediating TZD-induced β-catenin degradation. To achieve this, we utilized the SW-480 cells with inactivating mutations of both APC and p53, which showed a TZD-mediated reduction of β-catenin expression (Fig. 8C). Similar studies carried out in HCT-116 cells, which express full-length (wild-type APC) (63), as well as functional PPARγ (66) showed that addition of TZD was unable to degrade β-catenin even in the presence of wild-type APC (Fig. 8D). Taken together, these results confirmed that PPARγ-mediated degradation of β-catenin involved APC- and p53-independent pathways.

**The N-terminal Amino Acids of β-Catenin Mediated Its Degradation following Activation of PPARγ2**—To further elucidate the mechanism by which PPARγ2 activation degraded β-catenin, we mapped the region of β-catenin that was responsible for mediating the effects of TZD. To achieve this, we utilized the SW-480 cells with inactivating mutations of both APC and p53, which showed a TZD-mediated reduction of β-catenin expression (Fig. 8C). Similar studies carried out in HCT-116 cells, which express full-length (wild-type APC) (63), as well as functional PPARγ (66) showed that addition of TZD was unable to degrade β-catenin even in the presence of wild-type APC (Fig. 8D). Taken together, these results confirmed that PPARγ-mediated degradation of β-catenin involved APC- and p53-independent pathways.
PPARγ Regulates β-Catenin Levels

Several recent studies have indicated a PPARγ-mediated suppression of β-catenin expression, although the mechanism involved is still largely unknown. In one study, PPARγ activation in 3T3L1 adipocytes resulted in down-regulation of both mRNA and protein levels of β-catenin (44). In a separate study with pancreatic cancer cell line, however, PPARγ activation increased membrane translocation of β-catenin due to an increase in E-cadherin expression (58). In the studies described here our aim was to elucidate the pathway by which PPARγ attenuated β-catenin expression. Our results demonstrated that activation of PPARγ in the Pγ hepatocytes following incubation with TZD resulted in a dramatic down-regulation of β-catenin protein expression via a post-translational mechanism. Results from our Western blot and immunofluorescence studies indicated a TZD-mediated reduction in the levels of both cytoplasmic and membrane-bound fractions of β-catenin. This was associated with a corresponding decrease in E-cadherin expression, suggesting that PPARγ activation in these hepatocytes was unable to increase the membrane translocation of β-catenin.

Because there are multiple pathways that can regulate β-catenin expression post-translationally, we focused our studies to determine the contribution of the conventional β-catenin degradation pathways in mediating PPARγ2-induced β-catenin degradation. In the conventional pathway, in the presence of the tumor suppressor protein APC and axin, casein kinase I, and GSK3β phosphorylates the N-terminal amino acids of β-catenin. This N-terminal phospho-β-catenin is then recognized by the F-box protein β-TrCP and targeted toward a ubiquitin-proteasomal degradation pathway (28, 67). In a second APC-dependent pathway the F-box protein Ebi mediates β-catenin degradation, which also involves the p53-inducible Siah-1 (29, 30). This pathway does not involve any phosphorylation of β-catenin. In a recent study it has been suggested that PPARγ-mediated suppression of colon carcinogenesis and

Fig. 4. PPARγ2 activation inhibited β-catenin expression via a post-translational mechanism. A, equal amounts of total RNA extracted from subconfluent Pγ hepatocytes cultured in the absence (−) or presence (+) of TZD for the indicated periods of time were subjected to Northern blot analysis using a β-catenin cDNA probe (top panel). The same blot was probed with an 18 S ribosomal RNA probe to indicate equal RNA loading (bottom panel). B, exponentially growing Pγ hepatocytes were cultured in the presence (+) or absence (−) of 10 μM TZD for 48 h, followed by incubation in the presence of 20 μg/ml cycloheximide (CHX + lanes) for the indicated periods of time. The first two lanes were cultured in the absence of CHX and in the presence (+) or absence (−) of TZD for 48 h. Western analysis was performed with the antibodies indicated. C, pulse-chase analysis to determine the effect of PPARγ2 activation on the stability of β-catenin protein. Pγ hepatocytes treated in the absence (−) or presence (+) of TZD were labeled with Tran35S-labeled [35S]S-labeled β-catenin and immunoprecipitated by an antibody against β-catenin followed by fractionation on 10% SDS-PAGE and autoradiography. D, the autoradiographic bands obtained from the pulse-chase experiments of C were analyzed by densitometric scanning and expressed graphically as a percentage of the values obtained from the corresponding 0-h time points.

The C-terminal 131–781 aa fragment (lanes 3 and 4). Addition of TZD to these Pγ hepatocytes, however, resulted in down-regulation of the endogenous β-catenin levels similarly under all conditions (Fig. 9A, endogenous β-catenin panel). These results indicated that the N-terminal 131 amino acids of β-catenin were involved in mediating its degradation following activation of PPARγ2.

PPARγ2 Interacted with β-Catenin in Vivo, Which Was Enhanced in the Presence of TZD—Recent studies by Xiao et al. (52) have shown that degradation of β-catenin by the retinoic acid receptor RXR was also mediated by an APC-independent proteasomal degradation pathway involving an in vivo interaction of RXR with β-catenin. To determine whether PPARγ2 and β-catenin also interacted with each other in vivo, we performed studies utilizing the Pγ and Pγ-S37A-β-catenin cells and subjected them to cross-linking prior to immunoprecipitation following treatment with TZD (see “Experimental Procedures”). Equal amounts of cell extracts were immunoprecipitated with PPARγ-specific antibody followed by immunoblotting with either β-catenin antibody (for endogenous β-catenin in Pγ cells) or HA antibody (for ectopic β-catenin in Pγ-S37A-β-catenin cells). These results indicated an interaction of PPARγ with endogenous as well as ectopic β-catenin (Fig. 9B, lanes 1 and 3), which was enhanced following addition of TZD (Fig. 9B, compare lanes 1 and 2 and lanes 3 and 4). Reciprocal immunoprecipitation of the Pγ cell extracts with an antibody against β-catenin followed by immunoblotting with PPARγ antibody indicated the presence of PPARγ in these immunoprecipitates, which was increased following addition of TZD (Fig. 9B, lanes 5 and 6).

DISCUSSION

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different concentrations of MG-132. Western blot analysis was performed with these cell extracts with antibodies against PPARγ-pathway (22). To delineate the detailed mechanism by which the APC/GSK3β pathway in the 3T3L1 cells have been shown to involve a proteasome-activated degradation. In A: top panel, Pγ hepatocytes were treated with TZD (for 24 h) following a 1-h pretreatment in the absence (− lanes) or presence (+) of different concentrations of MG-132. Western blot analysis was performed with these cell extracts with antibodies against β-catenin or actin. Bottom panel, the autoradiographic bands obtained following blotting with β-catenin and actin in A were individually scanned and the ratios of β-catenin and actin were plotted as a percentage of controls, considering those obtained from TZD-untreated controls as 100%. In B: top panel, similar Western analysis as in A was carried out with Pγ hepatocytes following a 1-h pretreatment in the absence (−) or presence (+) of 10 μM lactacystin. Bottom panel, the ratio of the β-catenin and actin bands obtained from the results of B were expressed as percentage of control, considering those obtained from TZD-untreated controls as 100%, and plotted as bar graphs. C, Pγ cells were transiently transfected with HA-tagged, wild-type β-catenin vector and treated with TZD (for 24 h) in the absence (−) or presence (+) of a 1-h pretreatment with 15 μM MG132. Western analysis was performed with antibodies against HA (for ectopic β-catenin) or actin (as control). D, subconfluent Pγ hepatocytes were transfected with HA-tagged wild-type β-catenin along with either an empty vector (− lane) or the Myc-tagged ubiquitin vector H6M-Ub (+ lane). They were then treated with TZD as in C in the presence (+) or absence (−) of a 1-h pretreatment with MG-132. Equal amounts of cell extracts were immunoprecipitated with HA antibody followed by immunoblotting with 9E10 antibody (against the Myc tag) as shown by the ubiquitinated HA-β-catenin in the top panel. The two bottom panels represent Western analysis of the same cell extracts with HA or actin antibodies.

β-catenin expression is dependent upon a functional APC (62). Additionally, β-catenin degradation following PPARγ activation in the 3T3L1 cells have been shown to involve a proteasome-pathway (22). To delineate the detailed mechanism by which PPARγ activation degraded β-catenin expression, we determined whether it involved the APC/GSK3β pathway. Data generated from our studies indicated that this process was independent of GSK3β due to the following reasons. Pre-treatment of these hepatocytes with two independent inhibitors of GSK3β (LiCl and a commercially available inhibitor) showed no effect on PPARγ-mediated suppression of β-catenin expression. Furthermore, PPARγ was capable of degrading the mutant S37A-β-catenin to a similar extent as wild-type-β-catenin, despite its mutation at the GSK3β phosphorylation site (serine 37). Additionally, PPARγ activation in these hepatocytes resulted in an inhibition of GSK3β kinase activity. All these results indicated that this pathway of β-catenin degradation was independent of GSK3β. Because APC can degrade β-catenin in a GSK3β-independent and p53/Siah-1-dependent pathway, we addressed whether PPARγ-mediated degradation of β-catenin involved APC. To address this, the HT-29APC and HT-29-Gal cells were utilized, which can express either full-length APC or the control vector, respectively, in a Zinc-dependent manner (48). In a previous study (43) we reported that, although HT-29 cells showed TZD-mediated inhibition of cyclin D1 expression, they were unable to down-regulate β-catenin expression possibly due to the inactivating mutation of APC in these cells. Addition of TZD, however, was unable to induce β-catenin degradation even following incubation of the HT-29APC cells with zinc, which can induce expression of full-length APC in these cells. Additionally, in the HT-116 colon cancer cells, which express full-length (wild-type) APC (63) as well as functional PPARγ (66), TZD was unable to down-regulate β-catenin expression. Interestingly, TZD-mediated activation of PPARγ was capable of attenuating β-catenin expression in SW-480 cells, which contain inactivating mutations of both APC and p53 (29, 30, 32, 63–65). Taken together these studies indicated that PPARγ-mediated suppression of β-catenin expression was independent of APC and involved a novel degradation pathway.
To gain more insight into the mechanism by which PPAR\(^\gamma\)/H9253 degraded \(/\)/H9252-catenin, we also determined whether it involved ubiquitination and was mediated through proteasome-dependent pathways. Our studies with H6M-Ub ubiquitin vector indicated a TZD-dependent increase in ubiquitination of \(/\)/H9252-catenin in P\(/\)/H9253 hepatocytes. Additionally, preincubation of these cells with the proteasomal inhibitors MG-132 or lactacystin abolished the effects of TZD, indicating the involvement of proteasomes in the process. In our studies we also detected an interaction between PPAR\(^\gamma\)/H9253 and \(/\)/H9252-catenin in vivo, which was enhanced following addition of TZD. Interaction with other hormone receptors has also been reported previously (68). Because PPAR\(^\gamma\)/H9253 can also be degraded in a ligand-dependent manner through the ubiquitin-proteasome pathway (69), it is likely that these two proteins are degraded via the same mechanism. The PPAR\(^\gamma\)/H9253-activated pathway has also been reported to degrade cyclin D1 via the ubiquitination-proteasome pathway (70). It is thus possible that PPAR\(^\gamma\) stimulation leads to the activation of a novel proteasomal pathway, which is capable of degrading proteins involved in regulating important cellular events (example: growth and differentiation). In a very recent study a similar novel mechanism of \(/\)/H9252-catenin degradation has been reported to be activated by the retinoid X receptor (RXR) (52). Additionally, studies have shown that suppression of \(/\)/H9252-catenin-mediated signaling via nuclear receptors was performed as in Fig. 1A following treatment in the presence (+) or absence (−) of TZD and expressed as percentage of control. Each transfection was performed in triplicate and each experiment was repeated at least two times. The data represent the mean ± S.D. of two independent experiments. D: exponentially growing P\(/\)/H9253 hepatocytes were treated with TZD for 48 h following a 3-h pretreatment with 20 mM LiCl, or 10 \(/\)/H9252 of a commercial GSK3\(\beta\) inhibitor, or none. Western analysis was performed utilizing antibodies against \(/\)/H9252-catenin and actin.

**Fig. 6.** PPAR\(^\gamma\)/H9253-mediated inhibition of \(/\)/H9252-catenin expression was independent of GSK3\(\beta\). A. Western analysis of TZD-treated P\(/\)/H9253 cell extracts with phospho-\(/\)/H9252-catenin (Ser\(^{33/37}/\)/Thr\(^{41}\)), total \(/\)/H9252-catenin or actin antibodies. B, equal amounts of cell extracts obtained from exponentially growing P\(/\)/H9253 cell lines (PTA3\(\gamma\)/H9253, P\(/\)/H9253 wt, P\(/\)/H9253 37A) were transiently transfected with the TCF-responsive promoters pGL3OT, containing wild-type TCF sites (OT lanes) or pGL3OF, containing mutated TCF sites (OF lanes). Luciferase and \(/\)/H9252-galactosidase assays were performed as in Fig. 1A following treatment in the presence (+) or absence (−) of TZD and expressed as percentage of control. Each transfection was performed in triplicate and each experiment was repeated at least two times. The data represent the mean ± S.D. of two independent experiments. B, exponentially growing P\(/\)/H9253 hepatocytes were treated with TZD for 48 h following a 3-h pretreatment with 20 mM LiCl, or 10 \(/\)/H9252 of a commercial GSK3\(\beta\) inhibitor, or none. Western analysis was performed utilizing antibodies against \(/\)/H9252-catenin and actin.

**Fig. 7.** PPAR\(^\gamma\)/H9253\(\beta\) inhibition of GSK3\(\beta\) kinase activity. A. Western analysis of TZD-treated P\(/\)/H9253 cell extracts with phospho-GSK3\(\beta\) (Ser\(^{9}\)) or total GSK3\(\beta\) antibodies. B, total GSK3\(\beta\) immunoprecipitated from P\(/\)/H9253 hepatocytes at the indicated time points treated in the absence (−) or presence (+) of TZD was subjected to in vitro kinase assay using recombinant Tau as a substrate and [\(\gamma\)/H9253P]ATP. The samples were fractionated via SDS-PAGE and transferred to membranes. The radiolabeled bands were detected by exposure of the membranes to phosphorimaging screens. The first lane indicates \(\gamma\)/H9253P incorporation in the substrate alone. The Total GSK3\(\beta\) panel represents a Western blot analysis of the same samples with a total GSK3\(\beta\) antibody to indicate equal input.
tors pathways (including vitamins A and D and androgen receptors) required the N-terminal 1–151 amino acids of β-catenin and involved the participation of the coactivator p300 (71). A similar mechanism might be activated following stimulation of PPARγ, because the PPARγ2-mediated pathway of β-catenin degradation also involved the N-terminal (1–131 amino acids) fragment of β-catenin. Proteasome-mediated degradation of PPARγ was shown to be independent of the transcriptional activity of the receptor and was dependent upon the activation function 2 (AF2) domain (69) as was also reported in the case of RXR-mediated β-catenin degradation (52). It is still unclear whether the same domain of PPARγ2 was involved in mediating β-catenin degradation.

Activation of PPARγ has been shown to attenuate growth in a multiple variety of cell lines (1). In some cases this growth suppression leads to induction of differentiation. The oncoprotein β-catenin is capable of promoting growth via TCF/LEF-mediated transactivation of downstream target genes (e.g. cyclin D1 and c-myc) and is a key player in regulating the tumorigenic pathways. Based on this we hypothesized that PPARγ activation attenuated growth via regulating expression...
of β-catenin. Surprisingly, our earlier studies (43) indicated that overexpression of β-catenin in these hepatocytes was unable to antagonize the inhibitory effects of PPAR-γ on cyclin D1 transcription as well as PCNA expression and growth. It thus seems unlikely that β-catenin is a major player in mediating the growth-suppressive effects of PPAR-γ and might be involved in mediating other processes. It is important to mention that overexpression of a stable mutant form of β-catenin, induced polyp formation in the small intestine, but was unable to induce tumorigenesis in hepatocytes (72), indicating tissue-specific functions of the protein. Recent reports have indicated that down-regulation of β-catenin expression might be an important event toward the induction of PPAR-γ-mediated differentiation. Because PPAR-γ activation in hepatocytes was also associated with increased differentiation (73), it is conceivable that β-catenin is involved in mediating PPAR-γ-induced differentiation in hepatocytes. Most importantly, because the N terminus of β-catenin (particularly the GSK3β phosphorylation sites) is mutated frequently in cancers (63), and because PPAR-γ attenuated β-catenin expression in a GSK3β-APC-independent manner, it is possible that tumors harboring inactivating mutations of APC or certain forms of β-catenin (GSK3β-resistant) will still be susceptible to PPAR-γ-mediated degradation. An understanding of this pathway of β-catenin degradation is thus an important step toward utilizing PPAR-γ ligands for cancer control and increasing the potency of this response through PPAR-γ activation.

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