Regulating the Balance between Peroxisome Proliferator-activated Receptor γ and β-Catenin Signaling during Adipogenesis

A GLYCOGEN SYNTHASE KINASE 3β PHOSPHORYLATION-DEFECTIVE MUTANT OF β-CATENIN INHIBITS EXPRESSION OF A SUBSET OF ADIPOGENIC GENES*

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The differentiation of preadipocytes into adipocytes requires the suppression of canonical Wnt signaling, which appears to involve a peroxisome proliferator-activated receptor γ (PPARγ)-associated targeting of β-catenin to the proteasome. In fact, sustained activation of β-catenin by expression of Wnt1 or Wnt 10b in preadipocytes blocks adipogenesis by inhibiting PPARγ-associated gene expression. In this report, we investigated the mechanisms regulating the balance between β-catenin and PPARγ signaling that determines whether mouse fibroblasts differentiate into adipocytes. Specifically, we show that activation of PPARγ by exposure of Swiss mouse fibroblasts to troglitazone stimulates the degradation of β-catenin, which depends on glycogen synthase kinase (GSK) 3β activity. Mutation of serine 37 (a target of GSK3β) to an alanine renders β-catenin resistant to the degradatory action of PPARγ. Ectopic expression of the GSK3β phosphorylation-defective S37A-β-catenin in Swiss mouse fibroblasts expressing PPARγ stimulates the canonical Wnt signaling pathway without blocking their troglitazone-dependent differentiation into lipid-laden cells. Analysis of protein expression in these cells, however, shows that S37A-β-catenin inhibits a select set of adipogenic genes because adiponectin expression is completely blocked, but FABP4/aP2 expression is unaffected. Furthermore, the mutant β-catenin appears to have no affect on the ability of PPARγ to bind to or transactivate a PPAR response element. The S37A-β-catenin-associated inhibition of adiponectin expression coincides with an extensive decrease in the abundance of C/EBPα in the nuclei of the differentiated mouse fibroblasts. Taken together, these data suggest that GSKβ is a key regulator of the balance between β-catenin and PPARγ activity and that activation of canonical Wnt signaling downstream of PPARγ blocks expression of a select subset of adipogenic genes.

β-Catenin is a component of cell-cell adherens junctions particularly in epithelial cells but is also involved in transducing the Wnt signal by coactivating Leβ/Tcf transcription factors (3, 4). Recent studies suggest that β-catenin might also act as a coactivator of other transcription factors including members of the nuclear hormone receptor family (5–9). The ability of cytoplasmic β-catenin to activate Wnt or other signaling pathways depends on its ability to escape proteosomal degradation and relocate to the nucleus. Degradation of β-catenin occurs in the absence of Wnt signaling in a cytoplasmic compartment containing the scaffold protein, Axin, the serine/threonine kinase GSK3β, and adenomatous polyposis coli protein (10). GSK3β phosphorolylates β-catenin on serine 33, serine 37, and threonine 41 following a priming phosphorylation by casein kinase I on serine 45, which targets β-catenin for degradation by the ubiquitin-proteosomal system (11–16). Inhibition of GSK3β activity by Wnt signaling or activation of Akt/PKB leads to accumulation of β-catenin in the cytoplasm and its subsequent translocation to the nucleus and activation of target genes (2, 17–20).

Recent studies have suggested that Wnt/β-catenin signaling plays a role in specifying the fate of mesenchymal progenitor cells (21, 22). For instance, it appears that constitutive activation of the canonical Wnt pathway favors myogenesis over adipogenesis (23). In fact, for preadipocytes to differentiate into adipocytes Wnt signaling needs to be actively suppressed because sustained overexpression of Wnt 1 or Wnt 10b blocks adipogenesis (23). In fact, studies have suggested that PPARγ may act as a suppressor of growth-related events to facilitate terminal differentiation into adipocytes (32, 33). In this regard, we recently demonstrated that the suppression of Wnt/β-catenin signaling

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† The abbreviations used are: GSK3, glycogen synthase kinase; PPARγ, peroxisome proliferator-activated receptor γ; C/EBP, CCAAT enhancer-binding protein; HA, hemagglutinin; EMSA, electrophoresis mobility shift assay; WT, wild type.
during adipogenesis involves a PPARγ-associated targeting of β-catenin for degradation in the proteasome (25). In contrast, forced overexpression of Wnt or a GSK3β phosphorylation-defective mutant of β-catenin in preadipocytes blocks adipogenesis in part by inhibiting PPARγ expression (23–25). It appears, therefore, that there is a balance between β-catenin and PPARγ signaling during adipogenesis.

In this study, our goal was to define the mechanisms regulating the balance between β-catenin and PPARγ signaling during the differentiation of mouse fibroblasts into adipocytes. We demonstrate that activation of PPARγ in mouse fibroblasts stimulates the degradation of phosphorylated β-catenin, whereas a GSK3β phosphorylation-defective β-catenin in which serine 37 has been modified to alanine (S37A) is resistant to the action of PPARγ. Interestingly, expression of the stable oncogenic S37A form of β-catenin in fibroblasts expressing PPARγ inhibits a select subset of adipogenic genes because it blocks adiponectin expression without a significant affect on aP2 expression. This selective effect of β-catenin appears to result from the inhibition of the PPARγ-associated induction of C/EBPα expression without blocking overall PPARγ activity.

**MATERIALS AND METHODS**

**Antibodies**—Anti-C/EBPα, anti-C/EBPβ, anti-PPARγ, anti-actin and anti-HA antibodies were purchased from Santa Cruz Biotechnology, Inc., (Santa Cruz, CA). Anti-Tcf4 was obtained from Upstate Biotechnology (Lake Placid, NY), whereas anti-β-catenin and anti-cyclin D1 were from BD Biosciences Transduction Laboratories (Lexington, KY). Anti-AcP30/adenopinectin was purchased from Affinity BioReagents (Golden, CO). Anti-aP2 antibody was kindly provided by Dr. David Bernlohr (University of Minnesota, MN), and anti-perilipin antibody was a gift of Dr. Andy Greenberg (New England Medical Center, Tufts University, Boston, MA).

**Plasmids and Stable Cell Lines**—The pRevTRE-HA-WT-β-catenin and pRevTRE-HA-S37A-β-catenin vectors were generated by subcloning a BamHI DNA coding fragment of pcDNA3-WT-β-catenin and pcDNA3-S37A-β-catenin (34), respectively, into the BamHI site of pRevTRE retroviral expression vector (Clontech Laboratories, Inc., Palo Alto, CA). The Swiss-Pγ mouse cell line was generated by infecting Swiss mouse fibroblasts constitutively expressing the tetracycline activator protein (Swiss TET-off cells; Clontech) with the pBabe-PPARγ retrovirus (puromycin-resistant). Swiss-Pγ WT-β-catenin and Swiss-Pγ S37A-β-catenin cell lines were obtained by infecting Swiss-Pγ cells with pRevTRE-HA-WT-β-catenin and pRevTRE-HA-S37A-β-catenin (hygromycin-resistant) retroviruses, respectively, following standard procedures as outlined previously (25). The cell lines were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 25 mm d-glucose and supplemented with 10% (v/v) fetal bovine serum, 1% penicillin/streptomycin, 100 μg/ml G418 (for Swiss Tet-Off cell line), plus 1 μg/ml puromycin (for Swiss-Pγ cell line), and 50 μg/ml hygromycin, with 2 μg/ml tetracycline (for Swiss-Pγ WT-β-catenin and Swiss-Pγ S37A-β-catenin cell lines). The differentiation of Swiss-Pγ cells was induced by growing cells in p100 dishes until confluent and then exposing them to 5 μM troglitazone. To induce expression of the ectopic β-catenin, Swiss-Pγ WT-β-catenin and/or Swiss-Pγ S37A-β-catenin cells were cultured without tetracycline for 3 days and then treated with or without 5 μM troglitazone to activate the adipogenic program.

**Western Blot Analysis of Proteins**—Equal amounts of protein extracted from either the total cell layer or from the nucleus were fractionated on 8% SDS-polyacrylamide gels and transferred to polyvinylidene difluoride membranes (PerkinElmer Life Sciences). Following transfer, the membranes were blocked with 4% nonfat dry milk in phosphate-buffered saline, 0.1% Tween 20 and probed with the antibodies specified above. Horseradish peroxidase-conjugated secondary antibodies (Sigma) and an ECL substrate kit (PerkinElmer Life Sciences) were used for detection of specific proteins. When the same membrane was used for sequential antibody detection, the membrane was stripped with 15% H2O2 at room temperature for 30 min.

**Analysis of RNA**—Total cellular RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer’s instructions. Equivalent amounts of total RNA were assayed for presence of ectopic and endogenous β-catenin mRNA by reverse transcriptase-PCR as described previously (25). Primer sequences used for amplification were synthesized (Integrated DNA Technologies Inc., Coralville, IA) to be specific for HA-tagged β-catenin mRNA (5′-GGC AGG ATG ATC CTA AGT CAT CTG AT-3′ and 5′-GTA GGC GAG GAC GTC GTA AG-3′) or for endogenous β-catenin mRNA (5′-ACA GAA AGG CTG TCA AGG GC-3′ and 5′-GTC TTA CTG CAT GAC AGT TCA-3′). The PCRs were performed in the linear range of cycle number for each set of primers, and the corresponding products were analyzed by 1.5% agarose gel electrophoresis.

**Electrophoresis Mobility Shift Assay—EMSA** was performed as described previously (35) with the following modifications. Double-stranded oligonucleotides corresponding to the transcription factor response elements (Tcf, 5′-GGC TTT GAT CTT TGC-3′; aP2, ARE7/DR-1 5′-GTC TTA CTG CAT GAG AGT TCA-3′; and C/EBP, 5′-TTT CA GTC TTG CAA TTG AG-3′) were labeled with [32P]dATP (PerkinElmer Life Sciences) using Klenow fragment of DNA polymerase. The binding reaction mixture contained 10,000 cpm radiolabeled oligonucleotide, 3 μg of nuclear protein (except for Tcf probe 5 μg of protein was used), 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 2 μg of poly(dI-dC) (Amersham Biosciences), and 10% glycerol in a final volume of 20 μl. The mixture was incubated for 30 min at room temperature, and the complexes were separated by PAGE (non-denaturing gel, 6% polyacrylamide in 1× Tris-glycine-EDTA, pH 8.3) at 4 °C for 1.5 h at 300 V. The gel was dried and visualized by autoradiography. For the competition analyses, a 100-fold molar excess of competitor oligonucleotide was incubated with the nuclear extracts (30 min at room temperature) before the addition of the labeled probe. For the supershift assays, 2 μg of the appropriate antibody were added to the reaction mixtures (30 min at room temperature) before addition of the labeled probe.

**Luciferase Reporter Gene Assay**—The cells were seeded in six-well plates in triplicate for 24 h before transfection. 200 ng of TOPFLASH (firefly reporter) or FOPFLASH (firefly reporter) or 3XARE7/DR1 (firefly reporter) plasmids plus 10 ng of Renilla luciferase plasmid, pRL_SV40, was transfected into each well using FuGENE 6 (DNA: FuGENE 6 = 1:6). Forty-eight hours later, the cells were washed twice with phosphate-buffered saline and lysed with 500 μl of passive lysis buffer. Luciferase/Renilla assays were then performed using the DLR11 kit (Promega, Madison, WI) and a Luminoskan Ascent luminometer (Thermo Labsystems, Franklin, MA). The average ratio (from three wells) of luciferase activity (relative light units) to Renilla activity was calculated. The same experiment was repeated at least three times. The final values/standard deviation was calculated based on all repeats.

**Oil Red O Staining**—The cells were seeded in a 6-well plate, and at the specified stage of differentiation they were rinsed with phosphate-buffered saline, fixed with 25.7% formaldehyde for 15 min, rinsed with distilled water, stained with 0.3% Oil Red O (Sigma) for 60–70 min, and then rinsed with distilled water prior to photography using a phase contrast microscope.

**RESULTS**

**PPARγ Induces a Decrease in β-Catenin Expression During Adipogenesis in Swiss Mouse Fibroblasts**—Previous studies have demonstrated that activation of PPARγ induces the down-regulation of β-catenin during the differentiation of preadipocytes into adipocytes (25). In an attempt to understand the mechanisms regulating this process, we constitutively expressed PPARγ in Swiss fibroblasts using a retroviral infection vector, pBabe-PPARγ, which gave rise to a population of cells (Swiss-Pγ cells) with adipogenic potential (see below). To show that the down-regulation of β-catenin responds to changes in PPARγ activity, Swiss-Pγ cells were exposed to troglitazone (a potent PPARγ ligand) for 5 days, and total cellular proteins were harvested at daily intervals for analysis of β-catenin abundance as well as PPARγ activity. The latter was assessed by measuring the expression of FABP4/aP2, a well characterized target of PPARγ. Fig. 1 demonstrates that activation of PPARγ within the Swiss fibroblasts leads to increased expression of aP2 and a dramatic down-regulation of total β-catenin expression as observed previously in 3T3-L1 preadipocytes (25). Treatment of the Swiss-Pγ cells with troglitazone also results in a decrease in the abundance of PPARγ, but the rate of decay of β-catenin is significantly faster than that of PPARγ. Exposure of Swiss fibroblasts, which do not express PPARγ, to troglitazone for 5 days (Fig. 1, lane 6) shows a negligible decrease in β-catenin, confirming that the down-regulation of
β-catenin is dependent on PPARγ and not some nonspecific effect of the PPARγ ligand.

The PPARγ-associated Decrease in β-Catenin Expression Requires GSK3 Activity—Our earlier studies suggested that the PPARγ-associated down-regulation of β-catenin requires a functional proteasome (25). Mechanisms responsible for this effect might involve phosphorylation of β-catenin by GSK3β and/or casein kinase I. Other investigators have suggested that the regulated degradation of β-catenin by the tumor suppressor p53 involves mechanisms that are both dependent and independent of GSK3β activity (36–39). To determine whether there is a role for GSK3β in the PPARγ-mediated down-regulation of β-catenin, we conditionally overexpressed an HA-tagged wild type β-catenin using the pRevTRE expression system in Swiss-PPARγ cells giving rise to a cell line referred to as Swiss-PPARγ WT-β-catenin cells. These cells were cultured for several days in the absence of tetracycline to induce expression of the HA-β-catenin, during which time the fibroblasts reached confluence. At this stage, the cells were exposed to troglitazone in the presence of LiCl or NaCl for 48 h, and the total cellular proteins were harvested for Western blot analysis of HA-β-catenin. Fig. 2A shows an extensive down-regulation of HA-tagged β-catenin in response to troglitazone (compare lanes 2 and 1). Exposure of the cells to LiCl, which inhibits GSK3 activity, prevents the decrease in β-catenin expression in response to activation of PPARγ (compare lanes 2 and 4). To confirm a role for GSK3β in regulating the PPARγ-mediated decrease in β-catenin expression, we also expressed a mutant form of the protein in which serine 37 has been modified to alanine (Swiss-PPARγ S37A-β-catenin cells). This mutation renders β-catenin resistant to the GSK3β-associated targeting to the proteasome (34). In the experiment shown in Fig. 2B, Swiss-PPARγ WT-β-catenin and Swiss-PPARγ S37A-β-catenin cells were cultured in the presence or absence of tetracycline for several days until confluent. The cells were then exposed to increasing concentrations of troglitazone for 2 days, and the total cellular extracts were subjected to Western blot analysis of HA-tagged β-catenin. The data show the expected down-regulation of wild type (WT) β-catenin in response to activation of PPARγ by increasing doses of troglitazone. In contrast, the mutant S37A protein appears to be completely resistant to the action of PPARγ because its abundance remains essentially constant at all concentrations of the ligand.

PPARγ Enhances the Degradation of β-Catenin—The down-regulation of the wild type β-catenin by PPARγ could be due to an increase in degradation or a decrease in synthesis. To investigate whether PPARγ enhances the turnover of β-catenin, Swiss-PPARγ WT-β-catenin and Swiss-PPARγ S37A-β-catenin cells were cultured in the absence of tetracycline to induce expression of the ectopic β-catenin, and the cells were treated with or without troglitazone for 16 h at which time cycloheximide (CHX) was added to the media to block protein synthesis. Total cellular proteins were collected at the indicated times and subjected to Western blot analysis of HA-tagged β-catenin, PPARγ, and actin.

![Figure 1](http://www.jbc.org/)

**Fig. 1.** Activation of PPARγ in Swiss fibroblasts induces the down-regulation of β-catenin. Swiss-PPARγ cells (Pγ) or control Swiss fibroblasts (S) were cultured in Dulbecco’s modified Eagle’s medium until confluent, at which time they were treated with 5 μM troglitazone for the indicated days. Total cellular proteins were collected and subjected to Western blot analysis of β-catenin, PPARγ, FABP4/aP2, and actin.

**Fig. 2.** The PPARγ-associated decrease in β-catenin expression requires GSK3 activity. A, Swiss-PPARγ WT-β-catenin cells were cultured without tetracycline for 3 days until confluent, at which time they were treated with 30 mM LiCl or 30 mM NaCl in the presence or absence of 5 μM troglitazone for 2 days. Total cellular proteins were then harvested and subjected to Western blot analysis of HA-tagged β-catenin, PPARγ, and actin. B, Swiss-PPARγ WT-β-catenin and Swiss-PPARγ S37A-β-catenin cells were cultured with or without tetracycline until confluent, at which stage they were treated with increasing doses of troglitazone (from 0 to 5 μM, as indicated in shaded triangle above the figure) for 2 days. Total cellular proteins were collected and subjected to Western blot analysis of HA-tagged β-catenin.

**Fig. 3.** PPARγ enhances the degradation of wild type β-catenin, whereas S37A β-catenin stabilizes PPARγ. Swiss-PPARγ WT-β-catenin and Swiss-PPARγ S37A-β-catenin cells were cultured without tetracycline for 3 days. The cells were then treated with or without 5 μM troglitazone (Trog) for 16 h, at which time cycloheximide (CHX) was added to the media to block protein synthesis. Total cellular proteins were collected at the indicated times and subjected to Western blot analysis of HA-tagged β-catenin, PPARγ, and actin.
both proteins (WT and S37A) are quite stable throughout the 8-h time period. It is interesting to note that the abundance and rate of turnover of PPARγ itself appears to be affected by both troglitazone and S37A β-catenin. Specifically, the level of expression of PPARγ following 16 h of exposure to troglitazone is significantly higher in the cells expressing S37A β-catenin than in those expressing WT protein. Furthermore, the rate of decay of PPARγ appears to be somewhat faster in the wild type cells, and this effect is particularly apparent when cells are cultured in the absence of troglitazone (Fig. 3, lanes 6–10).

Stimulation of β-Catenin Signaling during the Differentiation of Swiss Py Cells Inhibits Expression of Select Adipogenic Genes—Previous studies have shown that activation of the canonical Wnt signaling pathway through the ectopic expression of Wnt 1 or Wnt 10b blocks the differentiation in 3T3-L1 preadipocytes into adipocytes (23–25). To identify the molecular mechanisms responsible for this effect of Wnt signaling, we questioned whether ectopic expression of the S37A β-catenin inhibits PPARγ activity. In addressing this question, we considered it important to determine whether S37A β-catenin could stimulate the canonical Wnt signaling pathway in Swiss fibroblasts expressing abundant amounts of PPARγ. A reverse transcriptase-PCR analysis of total RNA from cells grown in the presence or absence of tetracycline for 3 days, at which time total RNA was isolated and subjected to reverse transcriptase-PCR analysis of total RNA from cells grown in the presence or absence of tetracycline demonstrates that activation of the ectopic S37A β-catenin gene induces expression of the corresponding mRNA to levels equivalent to those produced by the endogenous β-catenin gene (Fig. 4A). More importantly, expression of these moderate levels of S37A β-catenin mRNA resulted in expression of a transcriptionally active protein, which was capable of inducing expression of cyclin D1, a well known β-catenin target gene (Fig. 4B). The EMSA in Fig. 4C shows further that S37A β-catenin associates with Tcf4 to form a complex that binds to LeF/Tcf regulatory elements in the promoter of the cyclin D1 gene (compare lanes 5 and 10). Furthermore, ectopic expression of the mutant β-catenin trans-activates a Tcf-based reporter gene (TOPFLASH) following its transient transfection into Swiss-Py/WT-β-catenin and Swiss-Py/S37A-β-catenin cells (Fig. 4D). Taken together, these observations show that S37A-β-catenin is activating gene expression usually associated with the canonical Wnt signaling pathway in the presence of PPARγ.

To assess the effect of β-catenin on PPARγ activity, we chose to measure the expression of the adipogenic program in Swiss fibroblasts in response to activation of the ectopic PPARγ. Treatment of Swiss-Py/WT-β-catenin and Swiss-Py/S37A-β-catenin cells with troglitazone induces conversion of the fibroblast...
blasts into lipid-laden cells as revealed by the presence of many Oil Red O-positive lipid droplets (Fig. 5). Ectopic expression of either the WT or S37A forms of β-catenin appears to have no significant effect on the ability of PPARγ to induce this change in morphology (Fig. 5). Studies have previously shown, however, that analysis of lipid accumulation is not necessarily representative of expression of the mature adipogenic program (40-42). Consequently, we performed a more detailed analysis. Fig. 6A shows a Western blot of proteins isolated from Swiss-Pγ/WT-β-catenin and Swiss-Pγ/S37A-β-catenin cells in the presence or absence of tetracycline to control expression of either WT- or S37A-β-catenin with or without exposure to troglitazone. Culture of the cells in the absence of troglitazone fails to elicit the adipogenic program as revealed by the absence of adiponectin, perilipin, or aP2 expression. Exposure of WT-β-catenin cells to troglitazone stimulates adipogenic gene expression even under conditions in which the level of production of the wild type β-catenin is enhanced by culture in the absence of tetracycline (Fig. 6A, compare lanes 5 and 6). In contrast, expression of the mutant S37A form of β-catenin has a dramatic but selective effect on adipogenic gene expression. Specifically, expression of S37A-β-catenin completely blocks adiponectin and partially inhibits perilipin expression in response to stimulation of PPARγ activity (plus troglitazone) (Fig. 6A, lane 8). Interestingly, S37A-β-catenin has little, if any, effect on aP2 expression. This observation suggested that PPARγ might escape the inhibitory action of S37A-β-catenin for a subset of target genes, and this effect might also depend on the relative activity of PPARγ. To test this notion, we analyzed the inhibitory action of S37A-β-catenin in cells exposed to lower doses of troglitazone. Fig. 6B demonstrates a PPARγ ligand dose-dependent induction of adiponectin, perilipin, and aP2 expression when the Swiss-Pγ/WT-β-catenin cells are cultured in tetracycline to suppress S37A-β-catenin production (Fig. 6B, lanes 1, 3, 5, 7, 9, and 11). Ectopic expression of S37A-β-catenin, however, completely blocks adiponectin expression at all doses of troglitazone (Fig. 6B, lanes 2, 4, 6, 8, 10, and 12). It is interesting that at lower doses of the PPARγ ligand, the ectopic S37A-β-catenin appears to significantly attenuate expression of both perilipin and aP2 (Fig. 6B, compare lanes 7 and 8), but this inhibitory effect of aP2 is overcome in response to enhancing PPARγ activity by raising the concentration of troglitazone (Fig. 6B, compare lanes 7 and 8 with lanes 11 and 12). It is also interesting that ectopic expression of S37A-β-catenin results in more abundant levels of PPARγ as observed previously in Fig. 3.

In an attempt to identify the mechanism responsible for this selective inhibition of PPARγ activity, we determined whether β-catenin affected the ability of PPARγ to bind to a PPAR response element. Fig. 7A shows an EMSA in which nuclear proteins from both Swiss-Pγ/WT-β-catenin and Swiss-Pγ/S37A-β-catenin cells form a complex with an oligonucleotide corresponding to the DR-1 element present in the aP2/FABP4 enhancer (ARE7). It appears that formation of this complex is enhanced in the presence of the ectopic S37A-β-catenin (Fig. 7A, lane 4). The supershift EMSA presented in Fig. 7B demonstrates that this ARE7-protein complex contains PPARγ because incubation of nuclear extracts from Swiss-Pγ/S37A-β-catenin cells with anti-PPARγ prior to addition of the radiolabeled ARE7 probe forms a more slowly migrating com-
S37A-β-catenin enhanced the ability of PPARγ to bind to and transactivate the aP2/PPAR response element (ARE7/DR1). A, S37A-β-catenin enhances the DNA binding activity of PPARγ. Swiss-PPARγ WT-β-catenin (WT) and Swiss-PPARγ S37A-β-catenin (S37A) cells were cultured in presence or absence of tetracycline for 3 days, at which stage they were stimulated to differentiate by exposure to 5 μM troglitazone for 2 days. Nuclear extracts were prepared and subjected to EMSA using oligonucleotide probes corresponding to PPARγ DNA-binding sites of the aP2 enhancer (ARE7/DR1) as described under "Materials and Methods." B, supershift assay of PPARγ binding to ARE7/DR1. The same extracts used in A, lane 4 (S37A-β-catenin without tetracycline) was subjected to a supershift assay. Lane 1 is the regular EMSA as shown in A. Lane 2 is competitor EMSA (with 100-fold excess unlabeled oligonucleotide). Lanes 3–5 are supershifts with anti-PPARγ, anti-β-catenin, or both antibodies together, respectively. C, S37A-β-catenin enhances PPARγ transcriptional activity. Swiss-PPARγ WT-β-catenin and Swiss-PPARγ S37A-β-catenin cells were cultured in the absence of tetracycline for 3 days, at which stage they were seeded in 6-well plates in triplicate and stimulated with 5 μM troglitazone. 200 ng of ARE7-luciferase reporter plasmid along with 10 μg of Renilla luciferase plasmid was transfected into each population of cells using FuGENE 6 as described under "Materials and Methods."

It is interesting that incubation of the nuclear extract with an anti-β-catenin antibody either in the presence or absence of the anti-PPARγ antibody significantly reduces the association of proteins with the ARE7 probe. Because the radiolabeled probe is added to the nuclear extract following addition of the anti-β-catenin antibody, it is conceivable that the antibody binds to β-catenin within a PPARγ-associated complex and in so doing prevents the subsequent binding of ARE7 oligonucleotide to PPARγ. The most important conclusion from these EMSAs is that S37A-β-catenin did not prevent PPARγ from interacting with its target DNA-binding element. To address whether S37A-β-catenin is affecting the transcriptional activity of PPARγ, we performed a series of reporter gene assays using a construct in which luciferase expression is regulated by the ARE7 response element. The activity of this reporter gene was higher in Swiss-PPARγ cells expressing S37A-β-catenin compared with cells expressing WT-β-catenin (Fig. 7C), suggesting that the mutant β-catenin does not inhibit PPARγ activity at least on an ARE7 response element. These data are consistent with the observation in Fig. 6 showing that S37A-β-catenin does not block aP2 gene expression at least at the concentration of troglitazone (5 μM) used in these assays.

Previous studies have suggested that terminal differentiation of preadipocytes requires the expression of C/EBPα in addition to PPARγ (40, 41). It is conceivable, therefore, that S37A-β-catenin might be selectively blocking adiponectin without significantly affecting aP2 expression because it is acting at the level of C/EBPα rather than PPARγ. To address this possibility, we measured the DNA binding activity of C/EBPα in nuclear extracts isolated from Swiss-PPARγ WT-β-catenin and Swiss-PPARγ S37A-β-catenin cells. The supershift EMSA presented in Fig. 8A demonstrates that S37A-β-catenin results in a significant decrease in the binding of C/EBPα complexes to a C/EBP regulatory element. The Western blot in Fig. 8B suggests that this decrease in binding activity is primarily due to a significant decrease in the abundance of C/EBPα in the nuclei of S37A cells. In contrast, the abundance of PPARγ increases in response to expression of S37A β-catenin (Fig. 8B).

**DISCUSSION**

In this report, we demonstrate a balance between PPARγ and β-catenin signaling in fibroblasts undergoing differentiation into adipocytes. It appears that activation of PPARγ by exposure of mouse fibroblasts to a potent PPARγ ligand induces a programmed degradation of β-catenin, which depends on GSK3β activity. A mutant form of β-catenin (S37A), which escapes phosphorylation by GSK3β, is resistant to the action of PPARγ. In fact, expression of this stable form of β-catenin, which activates the Wnt signaling pathway, inhibits the ability of PPARγ to induce expression of a select set of adipogenic genes. Specifically, overexpression of S37A-β-catenin in Swiss fibroblasts expressing PPARγ inhibits the induction of adiponectin by troglitazone but has a lesser effect on other adipogenic genes such as aP2 and perilipin. This inhibition of adiponectin coincides with a corresponding decrease in C/EBPα expression in response to S37A β-catenin.

**What Are the Mechanisms by Which PPARγ Induces the Degradation of β-Catenin?**—Our earlier studies demonstrated that activation of PPARγ leads to the down-regulation of β-catenin by mechanisms involving the proteasome (25).
pathway operating to link p53 with β-catenin does not appear to be operating in this system.

It is conceivable, therefore, that activation of PPARγ by its ligand stimulates a signaling pathway leading to enhanced GSK3β activity. Phosphorylation of GSK3β by Akt is known to attenuate its activity; thus effectors that inhibit Akt are likely to enhance GSK3β activity. The tumor suppressor protein, PTEN, inhibits the phosphatidylinositol 3-kinase/Akt signaling pathway and is also induced by PPARγ under certain conditions (43, 44). Our preliminary studies, however, suggest that PPARγ is not activating PTEN expression in the Swiss-Pγ cells. Similarly, Ben-Ze’ev and co-workers (37) demonstrated that PTEN is not responsible for the p53-mediated degradation of β-catenin. In fact, these authors suggest that p53 is affecting the kinetics of Axin transition between a Triton X-100-soluble and -insoluble compartment within the cell. Specifically, they propose that p53 enhances the rate at which Axin recruits β-catenin from a soluble pool into an insoluble pool where phosphorylation and the subsequent degradation of the phosphorylated β-catenin in the proteasome occur. It is conceivable that PPARγ also induces a similar change in the kinetics of recycling of Axin between these two cellular compartments.

How Does β-Catenin Inhibit Expression of Select PPARγ Target Genes?—Activation of Wnt signaling at the onset of adipogenesis in 3T3-L1 preadipocytes blocks expression of both PPARγ and C/EBPα expression in part by facilitating the translocation of a stable β-catenin into the nucleus, which induces expression of Wnt target genes (23–25). Recent studies by MacDougald and co-workers (45) demonstrate that targeted expression of Wnt 10b to adipose tissue through the use of the adipose-specific aP2/FABP4 promoter/enhancer blocks adipogenesis in vivo. Because aP2/FABP4 promoter/enhancer is activated by PPARγ, these observations suggest that Wnt-10b is inhibiting adipogenesis at a step downstream of PPARγ. In the present study, we questioned whether stable nuclear β-catenin is capable of inhibiting the adipogenic activity of PPARγ. We demonstrate that expression of S37A-β-catenin in Swiss fibroblasts induced canonical Wnt signaling in the presence of abundant quantities of PPARγ. In fact, this oncogenic form of β-catenin is capable of suppressing PPARγ activity by blocking expression of the terminal adipogenic program. The major effect of β-catenin is to inhibit adiponectin and C/EBPα expression with a significantly reduced effect on the other genes analyzed (i.e. FABP4/aP2). We have recently shown that expression of adiponectin during the differentiation of mouse fibroblasts requires C/EBPα as well as PPARγ, whereas expression of aP2 occurs in the absence of C/EBPα (46). Consequently, it is possible that the selective inhibitory action of β-catenin on adipogenic gene expression results from its ability to block the induction of C/EBPα by PPARγ. The fact that adipogenesis in these fibroblasts is driven by an ectopic PPARγ prevented β-catenin from completely blocking adipogenic gene expression. Without the ectopic PPARγ it is likely that β-catenin would also block the cross-regulation between C/EBPα and PPARγ, resulting in a dramatic decrease in expression of the endogenous PPARγ gene. In fact, the block in adipogenesis in animals expressing Wnt 10b in adipose tissue (45) was conceivably caused by a similar set of inhibitory events activated downstream of PPARγ but resulting eventually in an inhibition of PPARγ expression.

The mechanisms responsible for the selective inhibition of PPARγ activity by β-catenin might involve expression of specific Wnt target genes or an interaction of β-catenin with transcriptional complexes containing PPARγ. A notable product of Wnt signaling that has the potential to inhibit PPARγ activity is cyclin D1 (47, 48). Recent studies by Pestell and co-workers...
demonstrated that cyclin D1 inhibits ligand-induced PPARγ function by mechanisms that appear to involve a direct interaction between cyclin D1 and PPARγ. Myc, another product of Wnt signaling (50), is also a candidate for the inhibitory action of β-catenin but at the level of C/EBPα gene expression rather than a direct effect on PPARγ (51). Specifically, studies have shown that overexpression of Myc during the early phase of adipogenesis blocks adipogenic gene expression by inhibiting C/EBPα transcription. Mechanisms involving interaction of β-catenin with PPARγ-associated transcriptional complexes might include sequestration of critical coactivators of PPARγ through an association with β-catenin. For instance, it has recently been demonstrated that β-catenin binds to the androgen receptor and its associated coactivator, TIF2/GRIP-1, and in so doing enhances the transcriptional activity of the androgen receptor (5–9, 52). In the case of PPARγ, an interaction of β-catenin and GRIP-1 might interfere with the ligand-dependent association of GRIP-1 with PPARγ and consequently block transcriptional activity. The data presented in Fig. 7B suggest that β-catenin might exist in PPARγ-associated complexes because incubation of nuclear extracts with an anti-β-catenin antibody prevents the binding of PPARγ to its cognate ARE7 response element. Further investigation into the nature of various PPARγ-coactivator complexes and the identity of their downstream targets will provide greater insight into the versatility of PPARγ in regulating terminal adipogenesis.

It is possible that the cross-talk between β-catenin and PPARγ signaling contributes to the response of a variety of cells to extracellular effectors. For instance, β-catenin and PPARγ are abundantly expressed in the lining epithelial cells of the colon. In fact, mutations in the Wnt signaling pathway including β-catenin contribute significantly to the progression of these cells into tumors (53). In addition, some investigators have suggested that PPARγ is a tumor suppressor that operates in the colon to maintain normal epithelial growth and that thiazolidinediones (PPARγ ligands) may be effective chemotherapeutic agents (54, 55). Consequently, knowledge of the interplay between β-catenin and PPARγ should provide a greater understanding of the events leading to the development of colon cancer. In the case of adipocytes, which express abundant quantities of PPARγ, activation of β-catenin by effectors that attenuate GSK3β activity could selectively block adipogenic gene expression in a manner like that outlined above. In fact, the physiological consequences of stimulating the Wnt signaling pathway in adipose tissue by the targeted expression of Wnt10b to adipocytes appear to be quite dramatic because the resulting mice have a significantly reduced fat mass. In this regard, targeting β-catenin signaling may be an effective therapy for obesity and its related disorders.

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Regulating the Balance between Peroxisome Proliferator-activated Receptor γ and β-Catenin Signaling during Adipogenesis: A GLYCOGEN SYNTHASE KINASE 3β PHOSPHORYLATION-DEFECTIVE MUTANT OF β-CATENIN INHIBITS EXPRESSION OF A SUBSET OF ADIPOGENIC GENES

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