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Z. Elizabeth Floyd
Louisiana State University

Jacqueline M. Stephens
Louisiana State University

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Interferon-γ-mediated Activation and Ubiquitin-Proteasome-dependent Degradation of PPARγ in Adipocytes*

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Z. Elizabeth Floyd and Jacqueline M. Stephens‡

From the Department of Biological Sciences, Louisiana State University, Baton Rouge, Louisiana 70803

Interferon-γ (IFNγ) treatment of adipocytes results in a down-regulation of the peroxisome proliferator-activated receptor γ (PPARγ). The decrease in PPARγ expression is mediated by inhibition of PPARγ synthesis and increased degradation of PPARγ. In this study, we demonstrate that both PPARγ1 and PPARγ2 are targeted to the proteasome under basal conditions and that PPARγ1 is more labile than PPARγ2. The IFNγ-induced increase in PPARγ turnover is blocked by proteasome inhibition and is accompanied by an increase in PPARγ-polyubiquitin conjugates. In addition, IFNγ treatment results in the transcriptional activation of PPARγ. Similar to ligand-dependent activation of PPARγ, IFNγ-induced activation was greater in the phosphorylation-deficient S112A form of PPARγ when compared with wild-type PPARγ. Moreover, the inhibition of ERKs 1 and 2 with a MEK inhibitor, U1026, lead to an inhibition in the decay of PPARγ proteins, indicating that serine phosphorylation influences the degradation of PPARγ in fat cells. Our results also demonstrate that the proteasome-dependent degradation of PPARγ does not require nuclear export. Taken together, these results indicate that PPARγ is targeted to the ubiquitin-proteasome pathway for degradation under basal conditions and that IFNγ leads to an increased targeting of PPARγ to the ubiquitin-proteasome system in a process that is affected by ERK-regulated serine phosphorylation of PPARγ proteins.

PPARγ[1] is a member of the nuclear hormone receptor family, a group of transcription factors that are activated by small lipophilic ligands (1). PPARγ exists as two isoforms, PPARγ1 and PPARγ2, which are produced by a combination of different promoters and alternative splicing (2). There is also a *PPARγ3* gene that codes for a protein that is identical to PPARγ1 (3). PPARγ1 is predominantly expressed in fat cells but occurs in low levels in multiple tissues. PPARγ2 has an N-terminal extension of 30 amino acids and is very highly expressed in adipocytes (4, 5). Deletion of the *PPARγ* gene in mice results in placental dysfunction and embryonic lethality (6, 7).

PPARγ has been implicated in the regulation of systemic insulin sensitivity. This was first demonstrated when PPARγ was shown to be a functional receptor for the synthetic antidiabetic thiazolidinediones (TZDs) (8). Thiazolidinediones are specific high affinity ligands for PPARγ and the order of their receptor binding affinities *in vitro* mirrors their antihyperglycemic activity *in vivo* (9). Direct evidence for the association between PPARγ and insulin sensitivity comes from genetic studies showing that mutations in the ligand-binding domain of PPARγ are associated with severe insulin resistance. Although not obese, these patients developed type 2 diabetes as well as early onset hypertension (10). Also, insulin has been shown to acutely regulate the expression of PPARγ in human adipocytes (11), and mice that only express one copy of the *PPARγ* gene have been shown to be more sensitive to insulin (12). We have recently demonstrated that IFNγ results in a substantial loss of PPARγ expression by regulating two cellular events: 1) targeting PPARγ to the proteasome for degradation, and 2) inhibiting the synthesis of PPARγ (13). Moreover, prolonged IFNγ treatment of 3T3-L1 adipocytes also results in the development of insulin resistance (13) and supports the hypothesis that PPARγ is involved in conferring insulin sensitivity.

Interferon-γ (IFNγ) is a cytokine that is primarily known for its roles in immunological responses but has also been shown to affect fat metabolism and adipocyte gene expression. In adipocytes, IFNγ treatment results in a decrease of lipoprotein lipase (LPL) activity and increased lipolysis (14). In 3T3-F442 adipocytes, exposure to IFNγ results in a decreased expression of lipoprotein lipase and fatty acid synthase. Also, in various rodent preadipocyte cell lines, IFNγ inhibits the differentiation of preadipocytes (15–17). Acute IFNγ treatment of cultured and native rat adipocytes results in a dose- and time-dependent activation of STATs 1 and 3 (18). Moreover, there are studies (19–21) linking IFNγ and insulin resistance in humans. IFNγ has been implicated in the development of insulin resistance during viral infections (20), and IFNγ therapy of cancer patients has been associated with the development of hyperglycemia (21).

The ubiquitin-proteasome pathway is essential for the degradation of short lived proteins, the levels of which are regulated constitutively or in response to changes in the cellular environment (22, 23). Transcription factors and tumor suppressors are among the proteins regulated by the ubiquitin-proteasome pathway, and included in this group are members of the nuclear hormone receptor superfamily (24, 25). Ligand-dependent down-regulation by the ubiquitin-proteasome system has been demonstrated for several members of the nuclear hormone receptor family, including the estrogen (26, 27), progesterone (28), thyroid hormone (29), and aryl hydrocarbon receptors (30).

Substrates of the ubiquitin-proteasome system are targeted to the proteasome after covalent attachment of multiple ubiquitin molecules. Ubiquitin, a 76 amino acid protein, is initially

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‡ To whom correspondence should be addressed: Louisiana State University, Dept. of Biological Sciences, 508 Life Sciences Bldg., Baton Rouge, LA 70803. Tel.: 225-578-1749; Fax: 225-578-2597; E-mail: jsteph1@lsu.edu

[1] The abbreviations used are: PPARγ, peroxisome proliferator-activated receptor γ; TZD, thiazolidinedione; IFNγ, interferon-γ; STAT, signal transducer and activator of transcription; HA, hemagglutinin; DMEM, Dulbecco's modified Eagle's medium; PBS, phosphate-buffered saline; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; MEK, MAPK/ERK kinase; LMB, leptomycin B.

activated by E1, the ubiquitin-activating enzyme. Activated ubiquitin is then transferred to a ubiquitin-conjugating enzyme (E2), which generally shuttles ubiquitin to ubiquitin ligase (E3). E3 is bound to the targeted substrate and catalyzes the covalent attachment of ubiquitin to the substrate. Once the first ubiquitin is transferred to the substrate, a polyubiquitination chain is generated via a series of isopeptide linkages. The multiubiquitinated substrate protein is then degraded by the 26 S proteasome in an ATP-dependent manner (31).

Our recent studies (13) have shown that acute IFNγ treatment of 3T3-L1 adipocytes results in a repression of PPARγ transcription that is independent of new protein synthesis. Yet, we also demonstrated that the half-life of PPARγ proteins was shorter following IFNγ treatment. In the current investigation, we observed that proteasomal inhibitors attenuate the TZD- and IFNγ-induced decrease in PPARγ expression. Moreover, we demonstrate that IFNγ treatment is associated with an increase in the formation of polyubiquitin-PPARγ conjugates in 3T3-L1 adipocytes. Together, these data indicate that IFNγ signaling results in the increased targeting of PPARγ to the ubiquitin-proteasome system in adipocytes. In addition, we have shown that like TZDs, IFNγ increases the transcriptional activity of PPARγ. Also, the IFNγ-induced activation of a phosphorylation-deficient mutant of PPARγ2 (S112A) is substantially greater than the IFNγ activation of wild-type PPARγ2. Our results suggest that phosphorylation of PPARγ2 at Ser^{112} contributes to the targeting of PPARγ to the ubiquitin-proteasome pathway. Finally, these studies indicate that the IFNγ-mediated ubiquitin-proteasome-dependent degradation of PPARγ occurs in the nucleus.

EXPERIMENTAL PROCEDURES

Materials—Dulbecco's modified Eagle's medium (DMEM), Opti-MEM, and fetal bovine serum were purchased from Invitrogen. Calf serum was purchased from Sigma. Murine IFNγ was purchased from Roche Molecular Biochemicals. PPARγ monoclonal (E-8, no. sc-7273) and polyclonal (H-100, no. sc-7196) antibodies, Mdm2 monoclonal (SMP14, no. sc-965) antibody, and a STAT 5A polyclonal (L-20, no. sc-1081) antibody were purchased from Santa Cruz Biotechnology. Monoclonal anti-ubiquitin (no. 13–1600) was purchased from Zymed Laboratories Inc. The proteasome inhibitors epoxomicin, lactacystin, and MG132 (N-carbobenzoxyl-Leu-Leu-Leucinal) were purchased from Boston Biochemicals. A luciferase assay system, pSV-β-galactosidase control vector, and a β-galactosidase enzyme assay kit were purchased from Promega. FuGENE 6 was purchased from Roche Molecular Biochemicals. Darglitazone was kindly provided by Pfizer.

Constructs—The pSVSport plasmids encoding wild-type PPARγ and the S112A PPARγ mutant as well as DR-1 luciferase were the generous gift of Dr. Bruce Spiegelman (Dana Farber Cancer Institute). The HA-ubiquitin plasmid and leptomycin B (LMB) were kindly provided by Dr. Dirk Bohmann (European Molecular Biology Laboratories) and Dr. Minoru Yoshida (The University of Tokyo), respectively.

Cell Culture—Murine 3T3-L1 preadipocytes were plated and grown to 2-days postconfluence in DMEM with 10% calf serum. The medium was changed every 48 h. Cells were induced to differentiate by changing the medium to DMEM containing 10% fetal bovine serum and 0.5 mM 3-isobutyl-methylxanthine, 1 μM dexamethasone, and 1.7 μM insulin (MDI). After 48 h, this medium was replaced with DMEM supplemented with 10% fetal bovine serum, and the cells were maintained in this medium until used for experimentation. NIH 3T3 cells were grown in DMEM with 10% calf serum.

Preparation of Whole Cell Extracts—Cell monolayers were rinsed with phosphate-buffered saline (PBS) and harvested in a lysis buffer containing 10 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% Nonidet P-40, 1 μM phenylmethylsulfonyl fluoride, 1 μM pepstatin, 50 trypsin inhibitory milliunits of aprotinin, 10 μM leupeptin, and 2 mM sodium vanadate. Samples were extracted on ice for 30 min prior to centrifugation at 10,000 × *g* for 15 min. The resulting supernatants were analyzed for protein content by BCA analysis (Pierce) according to the manufacturer's instructions and stored at −80 °C.

Preparation of Nuclear/Cytosolic Extracts—Cell monolayers were rinsed with PBS and harvested in a nuclear homogenization buffer (NHB) containing 20 mM Tris-Cl, pH 7.4, 10 mM NaCl and 3 mM $MgCl_2$. Nonidet P-40 was added to a final concentration of 0.15%, and the cells were homogenized with 16 strokes in a Dounce homogenizer. The resulting homogenate was centrifuged at 1500 rpm for 5 min, and the supernatant was saved as cytosolic extract. The nuclear pellet was twice resuspended in 0.5 volume of a nuclear homogenization buffer and centrifuged as before. The nuclear pellet was then resuspended in an extraction buffer containing 20 mM HEPES pH 7.9, 420 mM NaCl, 0.2 mM EDTA and 25% glycerol. Nuclei were extracted for 30 min on ice followed by incubation with 200 units of DNase I at room temperature for 15 min. Finally, the sample was centrifuged at 15,000 rpm for 10 min at 4 °C. The resulting nuclear extract and the previously obtained cytosolic extract were analyzed for protein content by BCA analysis (Pierce) according to the manufacturer's instructions and stored at −80 °C.

Gel Electrophoresis and Immunoblotting—Proteins were separated in 12% polyacrylamide (National Diagnostics) gels containing SDS according to Laemmli (32) and transferred to nitrocellulose (Bio-Rad) in 25 mM Tris, 192 mM glycine, and 20% methanol. Following transfer, the membrane was blocked in 4% milk overnight at 4 °C. The immunoblots were visualized with horseradish peroxidase-conjugated secondary antibodies (Sigma) and enhanced chemiluminescence (Pierce).

Transient Transfection and Luciferase Assay—NIH 3T3 cells were grown to 60–70% confluence and transiently transfected with either wild-type PPARγ2 or PPARγ2 S112A. To measure PPARγ activity, the cells were cotransfected with DR-1 luciferase and pSV-β-galactosidase to normalize for transfection efficiency. FuGENE 6 was used according to the manufacturer's instructions and a FuGENE 6 to DNA ratio of 3:2 was used in the transfections. Transient transfections were carried out in OptiMEM for 8 h. After 8 h, the media were replaced with DMEM supplemented with 10% calf serum, and the cells were incubated overnight. Twenty-four hours after transfection, the cells were treated with IFNγ (100 units/ml) or darglitazone (TZD) (2.5 μM), and the cells were harvested 6 h later. Cell lysates were prepared and analyzed for luciferase activity and β-galactosidase activity according to the manufacturer's instructions (Promega). PPARγ transcriptional activity was reported as the ratio of luciferase activity (relative light units) to β-galactosidase activity.

Ubiquitin Conjugation Assay—NIH 3T3 cells were transfected with 2 μg of PPARγ alone or in combination with 4 μg of HA-ubiquitin per 100 mm plate using FuGENE 6 as described above. After 24 h, the cells were treated with 10 μM MG132 for 2 h prior to the addition of IFNγ (100 units/ml). The cells were harvested after 15- and 30-min incubations and lysed on ice in PBS, pH 7.0, containing 1% Triton X-100, 10 mM N-ethylmaleimide, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin and 10 μM leupeptin. Immunoprecipitations were performed by incubation with a polyclonal anti-PPARγ followed by incubation with protein A-Sepharose (RepliGen). PPARγ-ubiquitin complexes were detected by Western blotting with an anti-HA antibody.

3T3-L1 adipocytes were serum-deprived overnight in OptiMEM, followed by incubation with 10 μM MG132 for 2 h. At the end of 2 h, IFNγ (100 units/ml) was added, and the cells were harvested after 15- and 30-min incubations and lysed on ice in PBS containing 1% Triton X-100, 10 mM *N*-ethylmaleimide, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 μM pepstatin, and 10 μM leupeptin. Immunoprecipitations were performed with a polyclonal anti-PPARγ, and PPARγ-ubiquitin complexes were detected by Western blotting using both anti-PPARγ (monoclonal) and anti-ubiquitin antibodies.

PPARγ Stability in Vivo—Experiments using 3T3-L1 adipocytes were carried out in the presence or absence of cycloheximide (5 μM) to examine the effect of IFNγ on the half-life of PPARγ proteins. The half-lives of PPARγ1 and PPARγ2 were calculated based on first order decay after quantitation of Western blot data using Un-Scan-It software (Silk Scientific, Inc). IFNγ was added at 100 units/ml and darglitazone was added at 2.5 μM, where indicated. The adipocytes were incubated with one of three proteasome inhibitors (5 μM lactacystin, 100 nM epoxomicin, or 10 μM MG132) in experiments designed to assay proteasome targeting of PPARγ. In these experiments, the cells were preincubated with the proteasome inhibitor for 15–30 min prior to adding the ligand or cycloheximide. A MAPK/ERK kinase (MEK) inhibitor, U0126 (5 μM), was used to assay involvement of ERK1/2 in the turnover of PPARγ, and the cells were preincubated with U0126 for 30–45 min. Leptomycin B (10 nM) was added as an inhibitor of CRM-1-dependent nuclear export (33). Cells were pretreated with leptomycin B for 0.5–1 h prior to the addition of ligand or cycloheximide. Vehicle control additions were performed with either Me_2SO (for proteasome inhibitors, TZDs, and U0126) or ethanol (for leptomycin B).

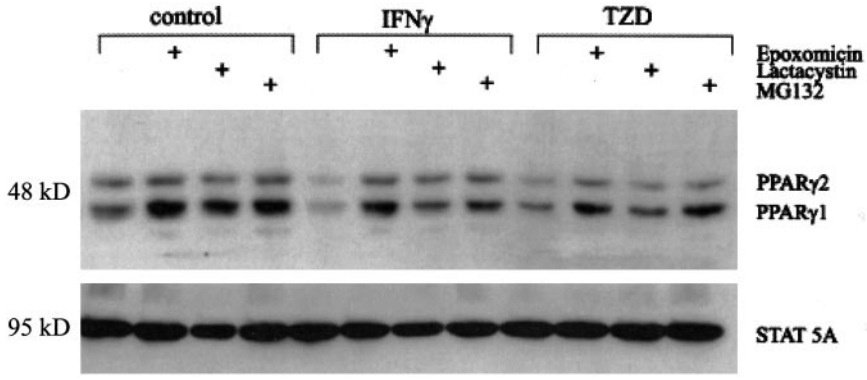

FIG. 1. **PPARγ is targeted to the proteasome under basal conditions and after IFNγ or TZD treatment.** Whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes that were untreated or treated with 100 units/ml IFNγ or 2.5 μM TZD. Proteasome activity was inhibited with epoxomicin (100 nM), lactacystin (5 μM), or MG132 (10 μM). Steady-state levels of PPARγ were measured after 6 h. One hundred micrograms of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. The molecular mass of each protein is indicated to the left of the blot in kDa. The detection system was horseradish peroxidase-conjugated secondary antibodies (Sigma) and ECL (Pierce). This was a representative experiment independently performed three times.

RESULTS

Basal and IFNγ-mediated Targeting of PPARγ to the Proteasome—We have previously shown that treatment of 3T3-L1 adipocytes with IFNγ leads to a decrease in the half-life of both PPARγ proteins (13). Recent studies by Spiegelman and coworkers (34) have shown that TZDs target PPARγ for proteasome-mediated degradation. These results suggest that targeting to the proteasome is an important regulatory event in the control of PPARγ expression. Therefore, we examined PPARγ expression in the presence of three distinct proteasome inhibitors. As shown in Fig. 1, treatment of 3T3-L1 adipocytes with either epoxomicin, lactacystin, or MG132 resulted in an increase in the levels of PPARγ proteins under basal conditions or in the presence of IFNγ or TZD. Lactacystin and epoxomicin are highly specific proteasome inhibitors and confirm that the observed effects on degradation are due to proteasomal targeting (35, 36). As shown in Fig. 1, under steady-state conditions, IFNγ treatment of 3T3-L1 adipocytes leads to a substantial loss of PPARγ when compared with control levels. The decrease in PPARγ after IFNγ treatment is slightly greater than the decrease associated with the presence of synthetic ligand (TZD). Inhibition of the proteasome substantially reduces the IFNγ-induced decrease in PPARγ expression. These results indicate that the loss of PPARγ following IFNγ treatment is mediated by the targeting of PPARγ to the 26 S proteasome. Interestingly, PPARγ levels in both IFNγ- and TZD-treated adipocytes in the presence of proteasome inhibitors are less than the control levels under the same conditions. This result is consistent with studies that demonstrate that both IFNγ and TZDs can also down-regulate PPARγ at the mRNA level (13, 37).

IFNγ-mediated Ubiquitin-PPARγ Conjugation—Ubiquitin-proteasome-dependent degradation of a substrate requires two separate steps. First, the substrate is targeted to the proteasome via covalent tagging of the substrate with a polyubiquitin chain. The polyubiquitin-conjugated substrate is then recognized by the 26 S proteasome (22). These polyubiquitin-substrate conjugates are short lived, high molecular mass intermediates of the ubiquitin-proteasome pathway. Because IFNγ affects PPARγ decay and this effect can be modulated by proteasome inhibitors, we hypothesized that there would be an increase in polyubiquitin-PPARγ conjugates after IFNγ treatment. To test this theory, we examined the formation of endogenous PPARγ-ubiquitin adducts in 3T3-L1 adipocytes. PPARγ proteins were immunoprecipitated from whole cell extracts that had been incubated in the presence or absence of IFNγ for the times indicated in Fig. 2. The immunoprecipitations were analyzed by immunoblotting using either an anti-PPARγ antibody (Fig. 2*A*) or an anti-ubiquitin (Fig. 2*B*) antibody. As shown in Fig. 2, PPARγ was detected in high molecular mass forms that are present under basal conditions and with increased intensity after IFNγ treatment. We also ectopically expressed octameric HA-tagged ubiquitin and PPARγ2 in NIH 3T3 cells and observed ubiquitin conjugation of PPARγ under basal conditions and a significant increase in PPARγ ubiquitin conjugation following IFNγ treatment (data not shown).

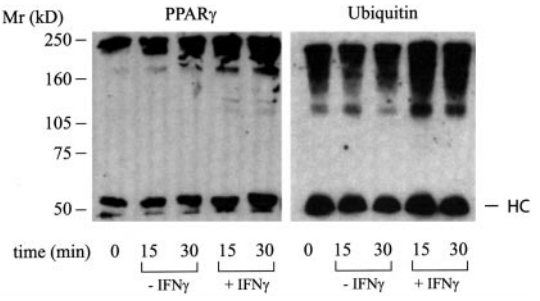

FIG. 2. **IFNγ treatment is associated with an increase in PPARγ-ubiquitin conjugates in 3T3-L1 adipocytes.** Fully differentiated 3T3-L1 adipocytes were treated with IFNγ (100 units/ml) for 15 and 30 min after preincubation with MG132 (20 μM) for 2 h. Control samples were incubated for the same time period without the addition of IFNγ. Whole cell extracts were harvested and immunoprecipitations were performed as described under "Experimental Procedures" using anti-PPARγ. Western analysis was performed using either anti-PPARγ (*left*) or anti-ubiquitin (*right*). HC represents IgG heavy chain.

IFNγ-mediated Activation of PPARγ—Based on our previous studies showing that IFNγ treatment of cultured adipocytes has the dual effect of suppressing PPARγ transcription and increasing PPARγ turnover (13), we hypothesized that IFNγ treatment may also decrease the transcriptional activity of PPARγ. To test this prediction, we assayed the transcriptional activity of PPARγ in NIH 3T3 cells using a luciferase reporter (DR1 luciferase) construct containing three PPARγ response elements. This construct has previously been used to measure PPARγ activity (34, 38). In this experiment, we also examined the effect of IFNγ on the transcriptional activity of the phosphorylation-deficient PPARγ2 S112A mutant. Numerous studies have shown that this mutant is more transcriptionally active and that phosphorylation at this site is associated with reduced PPARγ activity (39–41). To measure PPARγ activity, NIH 3T3 cells were transiently cotransfected with DR1 luciferase and PPARγ2 or PPARγ2 S112A in pSVSport vectors in the presence and absence of IFNγ or TZD. As shown in Fig. 3, IFNγ treatment activates PPARγ2 to the same extent as the ligand-dependent activation associated with TZD treatment. In

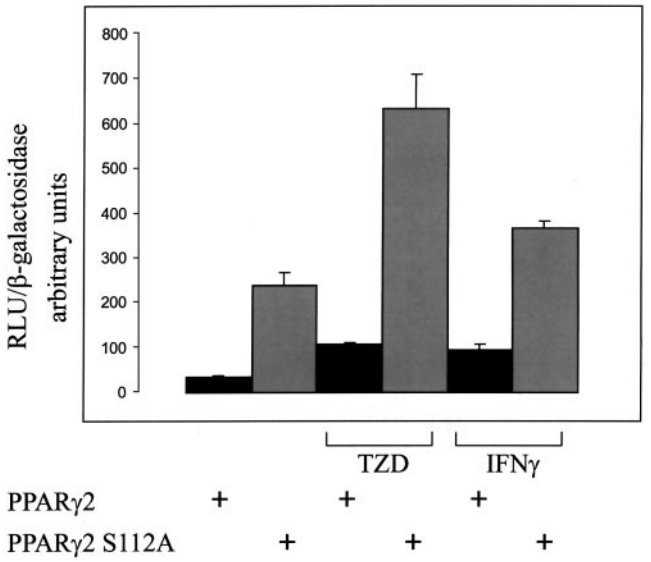

FIG. 3. **IFNγ mediates transcriptional activation of both wild-type PPARγ and PPARγ S112A.** NIH 3T3 cells were cotransfected with pDR1-luciferase and either wild-type PPARγ or PPARγ S112A. The cells were also transfected with pSV-β-galactosidase to correct for variability in transfection efficiency. After 24 h, the cells were incubated with IFNγ (100 units/ml) or darglitazone (*TZD*, 2.5 μM) and harvested 6 h later. PPARγ transcriptional activity was determined by calculating the ratio of luciferase activity (relative light units, *RLU*) to β-galactosidase activity. The experiment was independently performed in duplicate.

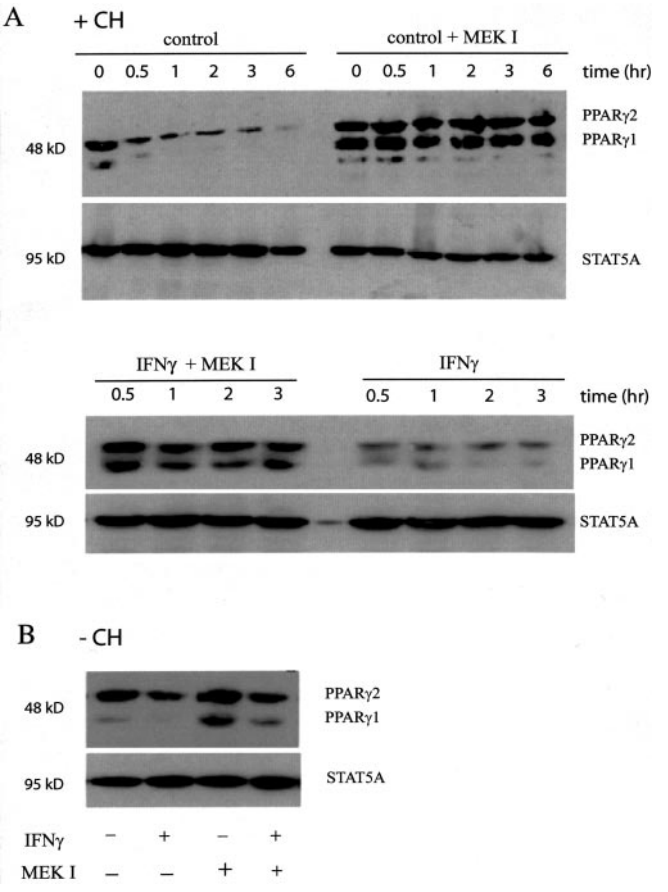

FIG. 4. **Inhibition of ERK1/2 prolongs the half-life of PPARγ proteins in adipocytes.** PPARγ expression was measured in the presence of 5 μM cycloheximide (*CH*) (*A*) or under steady-state conditions (*B*) under control or IFNγ (100 units/ml)-treated conditions. Where indicated, the 3T3-L1 adipocytes were pretreated for 45 min with the MEK inhibitor (MEK I), U0126 (5 μM). *B*, the cells were harvested after a 2-h incubation in the presence or absence of IFNγ. One hundred micrograms of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the Fig. 1 legend. The molecular mass of each protein is indicated to the left of the blots in kilodaltons. This was a representative experiment independently performed three times.

addition, activity of the PPARγ2 S112A was greater than wild-type PPARγ2, and the transcriptional activity of the mutant was also significantly induced by IFNγ treatment. However, the mutant was more potently activated by TZD treatment.

The Role of Ser^{112} Phosphorylation in the Decay of PPARγ—Because IFNγ and TZDs both activate PPARγ and target it for degradation, we hypothesized that regulators of PPARγ activation could also contribute to PPARγ degradation. Therefore, we examined the contribution of PPARγ Ser^{112} phosphorylation on PPARγ degradation because phosphorylation at this site has profound effects on PPARγ activation. Fully differentiated 3T3-L1 adipocytes were pretreated with the MEK inhibitor, U0126, prior to the addition of IFNγ or a vehicle control. Turnover of PPARγ was then measured in the presence or absence of cycloheximide. As shown in Fig. 4*A*, the turnover of both PPARγ1 and γ2 was prolonged in the presence of the MEK inhibitor (*control + MEK I*). We also observed that inhibition of ERK1/2 activity abrogates the IFNγ-mediated decrease in the half-life of PPARγ (Fig. 4*A*, *IFNγ + MEK I*). The results in Fig. 4*A* clearly demonstrate that the presence of the MEK inhibitor suppresses the decay of PPARγ proteins in adipocytes under control and IFNγ-treated conditions. We also examined the effect of IFNγ and/or MEK I on PPARγ levels in the absence of cycloheximide. The results in Fig. 4*B* confirm that ERKs 1 and 2 and play a role in degradation of PPARγ proteins under basal as well as IFNγ-mediated conditions. In Fig. 4, *A* and *B*, the expression of STAT 5A is shown as a loading control. The results in Fig. 4*A* also indicate that the decay of PPARγ is much quicker than the decay of PPARγ2. Therefore, we performed an additional decay experiment to compare the decay of γ1 and γ2. Fully differentiated 3T3-L1 adipocytes were treated with cycloheximide, and whole cell extracts were isolated at various times over a 6 h period. Fig. 5*A* shows the decay of PPARγ proteins under basal conditions. The γ1 and γ2 half-lives were calculated to be 58 min and 1.45 h, respectively. The *bottom panel* of Fig. 5*A* represents an enlarged display of four of the time points from the *top panel*. As shown in this panel, we were also able to resolve the two bands of PPARγ1, which represent the Ser^{112}-phosphorylated (*upper band*) and unphosphorylated forms of the protein. The decay experiment in Fig. 5*A* clearly demonstrates that PPARγ1 is more labile than γ2. In addition, the unphosphorylated γ1 disappears quicker than the phosphorylated form of γ1. This pattern was also observed in the presence of IFNγ. (Fig. 5*B*).

Cellular Location of PPARγ Degradation—The majority of PPARγ proteins are found in the nucleus, and this raises the possibility that the nuclear, rather than cytosolic, ubiquitin-proteasome components may mediate the degradation of PPARγ. To address this question, we treated 3T3-L1 adipocytes with IFNγ alone or in the presence of either MG132 or leptomycin B (Fig. 6). LMB acts as an irreversible inhibitor of the CRM-1-dependent nuclear export pathway via the modification of Cys^{529} of CRM-1 (33) and has been used to determine whether nuclear export is required for the degradation of nuclear proteins (42–44). We examined the decay of PPARγ proteins following IFNγ treatment in the presence of either MG132 or LMB. The results in Fig. 6 indicate that MG132 prolongs the half-life of PPARγ proteins, and the presence of LMB has no effect on PPARγ decay. To confirm LMB activity, we assayed the cellular location of Mdm2 in 3T3-L1 adipocytes

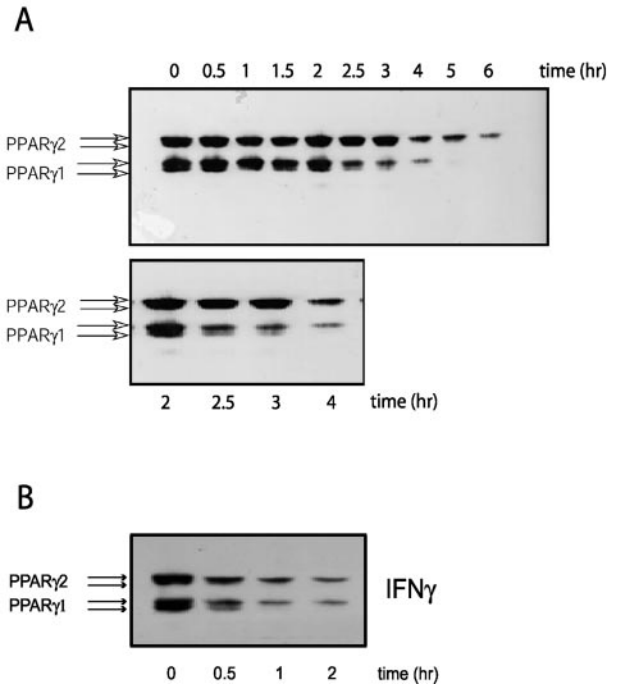

FIG. 5. **PPARγ1 is more labile than PPARγ2, and the higher mobility form of PPARγ1 decays after the lower mobility form.** *A*, whole cell extracts were prepared from fully differentiated 3T3-L1 adipocytes after incubation in the presence of 5 μM cycloheximide for the indicated time points. Incubations were carried out in the absence (*A*) or presence (*B*) of IFNγ (100 units/ml). The *lower panel* (*A*) is an enlargement of the indicated time points from the *upper panel*. One hundred micrograms of each extract was separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the Fig. 1 legend. This was a representative experiment independently performed three times.

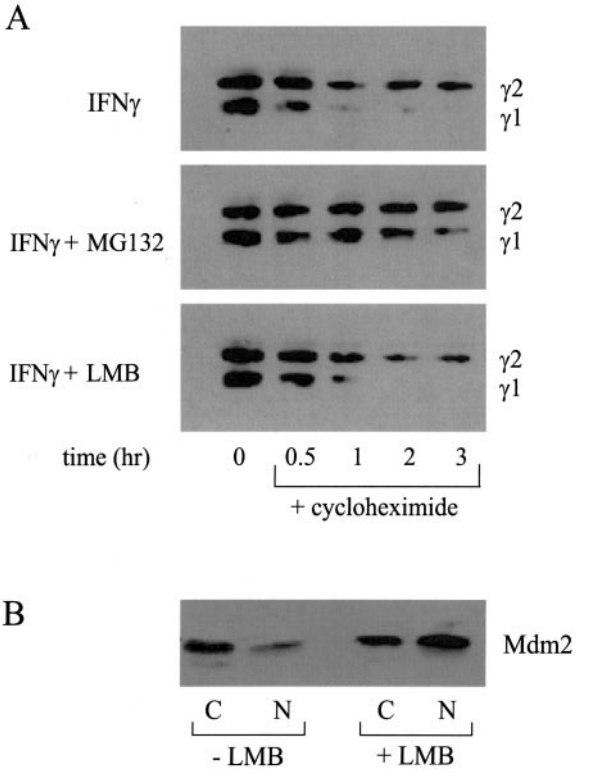

FIG. 6. **PPARγ degradation in adipocytes does not depend on nuclear export.** *A*, fully differentiated 3T3-L1 adipocytes were incubated in the presence of cycloheximide (5 μM) and harvested at the indicated time points. The adipocytes were treated with IFNγ alone or in the presence of MG132 (20 μM) or leptomycin B (10 nM) as indicated. *B*, fully differentiated 3T3-L1 adipocytes were harvested after a 4-h incubation in the presence of ethanol (−*LMB*) or leptomycin B (+*LMB*, 10 nM). Cytosolic and nuclear extracts were obtained as described under "Experimental Procedures." One hundred micrograms of each extract were separated by SDS-PAGE, transferred to nitrocellulose, and subjected to Western blot analysis. Samples were processed, and results were visualized as described in the Fig. 1 legend. This was a representative experiment independently performed two times.

in the absence or presence of LMB. Mdm2 has been characterized as an ubiquitin ligase (E3) that shuttles between the nucleus and cytoplasm and is required for the degradation of p53 (22). Although p53 expression is down-regulated during differentiation of 3T3-L1 adipocytes, Mdm2 expression is maintained in fully differentiated 3T3-L1 adipocytes (45). Fig. 6*B* demonstrates that Mdm2 accumulates in the nucleus in the presence of LMB, indicating the effectiveness of LMB in these experiments. These results demonstrate that CRM-1-dependent nuclear export is not required for the degradation of PPARγ following IFNγ treatment and strongly suggests that PPARγ is degraded in the nucleus.

DISCUSSION

The novel observations in this study include the increased ubiquitin conjugation of PPARγ following IFNγ treatment, the activation of PPARγ transcriptional activity by IFNγ, evidence that PPARγ1 is substantially more labile than PPARγ2, evidence that serine phosphorylation of PPARγ contributes to the turnover of PPARγ proteins in adipocytes, and evidence that PPARγ proteins are degraded by the nuclear ubiquitin-proteasome system. These results and recent findings by Spiegelman and co-workers (34) indicate that ubiquitin-proteasome-mediated degradation of PPARγ is an important contributor to the cellular levels of PPARγ proteins. Moreover, the cellular levels of PPARγ appear to be important because transgenic mice that express half the normal amount of PPARγ have been shown to be more insulin sensitive (12).

In light of our current findings and the studies cited above (13, 34, 39–41), we have formulated a model for the degradation of PPARγ proteins in adipocytes. This model, illustrated in Fig. 7, suggests that activation of PPARγ by IFNγ, TZDs, or endogenous ligands is followed by ubiquitin-proteasome-mediated degradation. This model also suggests that serine phosphorylation contributes to PPARγ degradation. The validity of this model is addressed in the following paragraphs.

Our results demonstrate that both PPARγ1 and PPARγ2 are targeted to proteasome under basal conditions and following IFNγ treatment of adipocytes. We have also observed ubiquitin conjugation of PPARγ under basal conditions and demonstrated a substantial increase in ubiquitin conjugation of PPARγ after IFNγ exposure. The increase in PPARγ-ubiquitin conjugates occurred within 15 min of IFNγ treatment and precedes the decrease in PPARγ observed in experiments measuring PPARγ degradation. Our results demonstrating that proteasome inhibitors reduce the effect of IFNγ on PPARγ expression and the results demonstrating the appearance of PPARγ-polyubiquitin conjugates indicate that IFNγ treatment in adipocytes results in the rapid degradation of PPARγ via the ubiquitin-proteasome pathway.

The rapid reduction in PPARγ mRNA and protein levels following IFNγ treatment (13) led us to predict that IFNγ treatment would suppress PPARγ activity in adipocytes. Surprisingly, IFNγ treatment of 3T3-L1 adipocytes was associated with the transcriptional activation of PPARγ2. Although unexpected, this result is consistent with the idea that nuclear hormone receptor turnover occurs concomitantly with transcriptional activation of these transcription factors (24). Ligand-

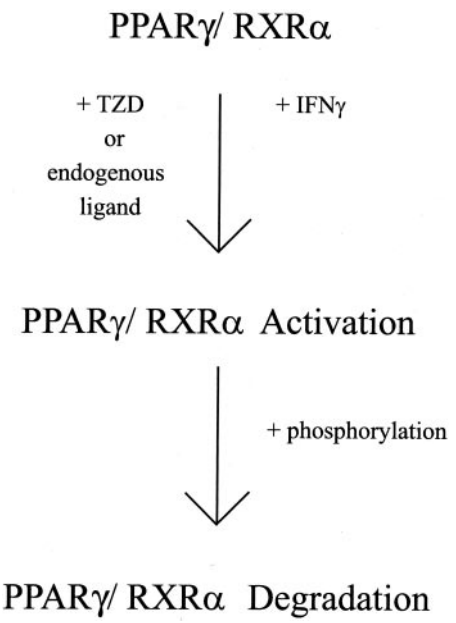

FIG. 7. **Proposed model for degradation of PPARγ.** PPARγ activation is mediated by ligand binding or exposure to IFNγ. Phosphorylation of PPARγ influences the IFNγ and ligand-dependent degradation of PPARγ.

dependent activation and subsequent degradation has been demonstrated for several other nuclear hormone receptors (26–30), and the paradigm of activation followed by ubiquitin-proteasome-dependent degradation has been extended to proteins such as protein kinase C (46). Although IFNγ has not been shown to be a ligand for PPARγ, the activation of PPARγ is a ligand-dependent process (47), and a recent study has demonstrated that PPARγ2 degradation is associated with the TZD-induced activation of PPARγ2 (34). Our data demonstrating that IFNγ treatment results in both the activation of PPARγ2 and the ubiquitin-proteasome-mediated degradation of PPARγ suggest that IFNγ-mediated signaling in adipocytes may be associated with the binding of an endogenous ligand and the activation and subsequent degradation of PPARγ. Moreover, IFNγ-induced PPARγ2 transcriptional activation is enhanced in the phosphorylation-deficient S112A mutant of PPARγ2. This result is consistent with previous findings showing that the mutation of Ser^{112} to alanine in PPARγ (Ser^{82} in PPARγ1) is associated with increased transcriptional activity (39, 40, 48).

The phosphorylation of PPARγ by MAPKs has been described in various studies (39–41, 48, 49). Although neither IFNγ nor TZDs directly activate ERKs 1 and 2 in adipocytes, we found that inhibition of these MAPKs resulted in an inhibition of PPARγ decay. Therefore, the mechanism(s) by which MAPKs influence PPARγ degradation is not clear. However, phosphorylation plays an important role in targeting many substrates for ubiquitination and can either inhibit or increase the targeting of substrates to the ubiquitin-proteasome system (22, 23). In our experiments, we observed that both PPARγ1 and PPARγ2 migrate as a doublet on gels that have been run for 24–30 h (refer to Fig. 5). This doublet is easily distinguishable for PPARγ1. We confirmed that the slower migrating form corresponds to serine-phosphorylated PPARγ1, and the faster migrating form represents the unphosphorylated PPARγ1 proteins (data not shown), as has been previously described (34). The results in Fig. 5 demonstrate that the faster migrating form of PPARγ1 disappears prior to the phosphorylated form of the protein. The observed difference in the decay of these two forms of PPARγ1 suggest that phosphorylation of PPARγ proteins may serve as a ubiquitin-proteasome targeting signal in which PPARγ is converted to the phosphorylated form prior to degradation by the ubiquitin-proteasome pathway. This hypothesis is also consistent with the increased activation the S112A mutant, and we predict that the ubiquitin-conjugating machinery may not recognize the phosphorylation-deficient PPARγ as well as the wild-type protein. We hypothesize that this may contribute to the increased activation observed with the S112A mutant. This model is also supported by our data demonstrating that inhibition of PPARγ serine phosphorylation with the MEK inhibitor prolongs the half-life of PPARγ proteins. All of these results support the hypothesis that serine phosphorylation of PPARγ may influence its targeting to the ubiquitin-proteasome system. However, recent work from the Spiegelman laboratory (34) has shown that both the wild-type and the S112A form of PPARγ2 are degraded after ligand activation, but they did not determine whether the half-lives of these forms of the protein were different. Nonetheless, because the phosphorylation-deficient mutant can be degraded, it seems unlikely that serine phosphorylation is the only means by which PPARγ proteins are targeted to the ubiquitin-proteasome system. Interestingly, the MAPK-regulated serine phosphorylation of the progesterone receptor has been shown act as a targeting signal for the degradation of this protein (28, 50).

We also investigated the cellular location of the IFNγ-mediated ubiquitin-proteasome-dependent degradation of PPARγ. PPARγ proteins are predominantly localized in the nucleus, and recent studies have demonstrated that the nuclear ubiquitin-proteasome is active in the degradation of selected substrates (42, 51, 52). Our results demonstrate that the IFNγ-mediated degradation of PPARγ does not require CRM1-dependent nuclear export, indicating that IFNγ-induced PPARγ degradation likely occurs in the nucleus. In the absence of serum deprivation, we observe active ERKs 1 and 2 in the nucleus of 3T3-L1 adipocytes (data not shown) and hypothesize that the presence of these kinases influences the nuclear decay of PPARγ proteins. Finally, the observation that PPARγ1 is substantially more labile than PPARγ2 suggests that recognition of PPARγ proteins by the ubiquitin-proteasome system in adipocytes is influenced by the 30-amino acid N-terminal extension found in PPARγ2. However, examination of the N-terminal residues of both forms of PPARγ reveals that neither region contains the characteristic residues involved in the N-end rule targeting to the ubiquitin-proteasome system (53). Moreover, neither form contains a lysine residue necessary for ubiquitin conjugation (22). However, this study does not address the mechanisms underlying the differences in the half-lives of PPARγ1 and PPARγ2.

Recent studies (12, 54) have shown that reduced PPARγ expression in mice ($PPAR\gamma^{+/-}$) is associated with resistance to weight gain along with protection from the insulin resistance that typically accompanies weight gain. In addition, genetic evidence indicates that decreased PPARγ activity may protect against insulin resistance in humans (55). Conversely, PPARγ is required for the formation of fat cells, and a lack of adipose cells is associated with insulin resistance and hyperglycemia (56). These studies suggest that a careful balance between PPARγ expression and activity levels must be maintained to avoid development of diseases such as type II diabetes and obesity. The current study, along with a previous study showing that ligand activation of PPARγ leads to ubiquitin-proteasome-dependent degradation of PPARγ (34), suggests that the ubiquitin-proteasome pathway plays an important role in the regulation of PPARγ expression in adipocytes.

REFERENCES

1. Egea, P. F., Klaholz, B. P., and Moras, D. (2000) *FEBS Lett.* **476,** 62–67
2. Zhu, Y., Qi, C., Korenberg, J. R., Chen, X. N., Noya, D., Rao, M. S., and Reddy, J. K. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92,** 7921–7925

3. Fajas, L., Fruchart, J. C., and Auwerx, J. (1998) *FEBS Lett.* **438,** 55–60
4. Tontonoz, P., Hu, E., Graves, R. A., Budavari, A. I., and Spiegelman, B. M. (1994) *Genes Dev.* **8,** 1224–1234
5. Chawla, A., Schwarz, E. J., Dimaculangan, D. D., and Lazar, M. A. (1994) *Endocrinology* **135,** 798–800
6. Barak, Y., Nelson, M. C., Ong, E. S., Jones, Y. Z., Ruiz-Lozano, P., Chien, K. R., Koder, A., and Evans, R. M. (1999) *Mol. Cell* **4,** 585–595
7. Rosen, E. D., Sarraf, P., Troy, A. E., Bradwin, G., Moore, K., Milstone, D. S., Spiegelman, B. M., and Mortensen, R. M. (1999) *Mol. Cell* **4,** 611–617
8. Lehmann, J. M., Moore, L. B., Smith-Oliver, T. A., Wilkison, W. O., Willson, T. M., and Kliewer, S. A. (1995) *J. Biol. Chem.* **270,** 12953–12956
9. Willson, T. M., Cobb, J. E., Cowan, D. J., Wiethe, R. W., Correa, I. D., Prakash, S. R., Beck, K. D., Moore, L. B., Kliewer, S. A., and Lehmann, J. M. (1996) *J. Med. Chem.* **39,** 665–668
10. Barroso, I., Gurnell, M., Crowley, V. E., Agostini, M., Schwabe, J. W., Soos, M. A., Maslen, G. L., Williams, T. D., Lewis, H., Schafer, A. J., Chatterjee, V. K., and O'Rahilly, S. (1999) *Nature* **402,** 880–883
11. Rieusset, J., Andreelli, F., Auboeuf, D., Roques, M., Vallier, P., Riou, J. P., Auwerx, J., Laville, M., and Vidal, H. (1999) *Diabetes* **48,** 699–705
12. Miles, P. D., Barak, Y., He, W., Evans, R. M., and Olefsky, J. M. (2000) *J. Clin. Invest.* **105,** 287–292
13. Waite, K. J., Floyd, Z. E., Arbour-Reily, P., and Stephens, J. M. (2001) *J. Biol. Chem.* **276,** 7062–7068
14. Doerrler, W., Feingold, K. R., and Grunfeld, C. (1994) *Cytokine* **6,** 478–484
15. Gregoire, F., De Broux, N., Hauser, N., Heremans, H., Van Damme, J., and Remacle, C. (1992) *J. Cell. Physiol.* **151,** 300–309
16. Keay, S., and Grossberg, S. E. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77,** 4099–4103
17. Grossberg, S. E., and Keay, S. (1980) *Ann. N. Y. Acad. Sci.* **350,** 294–300
18. Stephens, J. M., Lumpkin, S. J., and Fishman, J. B. (1998) *J. Biol. Chem.* **273,** 31408–31416
19. Khanna, S., Roy, S., Packer, L., and Sen, C. K. (1999) *Am. J. Physiol.* **276,** R1327-R1333
20. Koivisto, V. A., Pelkonen, R., and Cantell, K. (1989) *Diabetes* **38,** 641–647
21. Shiba, T., Higashi, N., and Nishimura, Y. (1998) *Diabetes Med.* **15,** 435–436
22. Hershko, A., and Ciechanover, A. (1998) *Annu. Rev. Biochem.* **67,** 425–479
23. Weissman, A. M. (2001) *Nat. Rev. Mol. Cell. Biol.* **2,** 169–178
24. Dennis, A. P., Haq, R. U., and Nawaz, Z. (2001) *Front. Biosci.* **6,** D954-D959
25. McKenna, N. J., Xu, J., Nawaz, Z., Tsai, S. Y., Tsai, M. J., and O'Malley, B. W. (1999) *J. Steroid Biochem. Mol. Biol.* **69,** 3–12
26. Lonard, D. M., Nawaz, Z., Smith, C. L., and O'Malley, B. W. (2000) *Mol. Cell* **5,** 939–948
27. Nawaz, Z., Lonard, D. M., Dennis, A. P., Smith, C. L., and O'Malley, B. W. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 1858–1862
28. Lange, C. A., Shen, T., and Horwitz, K. B. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 1032–1037
29. Dace, A., Zhao, L., Park, K. S., Furuno, T., Takamura, N., Nakanishi, M., West, B. L., Hanover, J. A., and Cheng, S. (2000) *Proc. Natl. Acad. Sci. U. S. A.* **97,** 8985–8990
30. Roberts, B. J., and Whitelaw, M. L. (1999) *J. Biol. Chem.* **274,** 36351–36356
31. Hershko, A., Ciechanover, A., and Varshavsky, A. (2000) *Nat. Med.* **6,** 1073–1081
32. Laemmli, U. K. (1970) *Nature* **227,** 680–685
33. Kudo, N., Matsumori, N., Taoka, H., Fujiwara, D., Schreiner, E. P., Wolff, B., Yoshida, and Horinouchi, S. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 9112–9117
34. Hauser, S., Adelmant, G., Sarraf, P., Wright, H. M., Mueller, E., and Spiegelman, B. M. (2000) *J. Biol. Chem.* **275,** 18527–18533
35. Lee, D. H., and Goldberg, A. L. (1998) *Trends Cell Biol.* **8,** 397–403
36. Meng, L., Mohan, R., Kwok, B. H., Elofsson, M., Sin, N., and Crews, C. M. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 10403–10408
37. Rosenbaum, S. E., and Greenberg, A. S. (1998) *Mol. Endocrinol.* **12,** 1150–1160
38. Forman, B. M., Tontonoz, P., Chen, J., Brun, R. P., Spiegelman, B. M., and Evans, R. M. (1995) *Cell* **83,** 803–812
39. Hu, E., Kim, J. B., Sarraf, P., and Spiegelman, B. M. (1996) *Science* **274,** 2100–2103
40. Camp, H. S., and Tafuri, S. R. (1997) *J. Biol. Chem.* **272,** 10811–10816
41. Camp, H. S., Tafuri, S. R., and Leff, T. (1999) *Endocrinology* **140,** 392–397
42. Floyd, Z. E., Trausch-Azar, J. S., Reinstein, E., Ciechanover, A., and Schwartz, A. L. (2001) *J. Biol. Chem.* **276,** 22468–22475
43. Alt, J. R., Cleveland, J. L., Hannink, M., and Diehl, J. A. (2000) *Genes Dev.* **14,** 3102–3114
44. Henderson, B. R. (2000) *Nat. Cell. Biol.* **2,** 653–660
45. Berberich, S. J., Vaughn, L., Mayo, L. D., Tabesh, D., and Morris, D. (1999) *Differentiation* **64,** 205–212
46. Lu, Z., Liu, D., Hornia, A., Devonish, W., Pagano, M., and Foster, D. A. (1998) *Mol. Cell. Biol.* **18,** 839–845
47. Rosen, E. D., Walkey, C. J., Puigserver, P., and Spiegelman, B. M. (2000) *Genes Dev.* **14,** 1293–1307
48. Adams, M., Reginato, M. J., Shao, D., Lazar, M. A., and Chatterjee, V. K. (1997) *J. Biol. Chem.* **272,** 5128–5132
49. Zhang, B., Berger, J., Zhou, G., Elbrecht, A., Biswas, S., White-Carrington, S., Szalkowski, D., and Moller, D. E. (1996) *J. Biol. Chem.* **271,** 31771–31774
50. Shen, T., Horwitz, K. B., and Lange, C. A. (2001) *Mol. Cell. Biol.* **21,** 6122–6131
51. Blondel, M., Galan, J. M., Chi, Y., Lafourcade, C., Longaretti, C., Deshaies, R. J., and Peter, M. (2000) *EMBO J.* **19,** 6085–6097
52. Lenk, U., and Sommer, T. (2000) *J. Biol. Chem.* **275,** 39403–39410
53. Varshavsky, A., Turner, G., Du, F., and Xie, Y. (2000) *Biol. Chem. Hoppe-Seyler* **381,** 779–789
54. Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., Eto, K., Tsubamoto, Y., Okuno, A., Murakami, K., Sekihara, H., Hasegawa, G., Naito, M., Toyoshima, Y., Tanaka, S., Shiota, K., Kitamura, T., Fujita, T., Ezaki, O., Aizawa, S., and Kadowaki, T. (1999) *Mol. Cell* **4,** 597–609
55. Deeb, S. S., Fajas, L., Nemoto, M., Pihlajamaki, J., Mykkanen, L., Kuusisto, J., Laakso, M., Fujimoto, W., and Auwerx, J. (1998) *Nat. Genet.* **20,** 284–287
56. Willson, T. M., Lambert, M. H., and Kliewer, S. A. (2001) *Annu. Rev. Biochem.* **70,** 341–367