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## Degradation of the Peroxisome Proliferator-activated Receptor $\gamma$ Is Linked to Ligand-dependent Activation\*

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**The nuclear hormone receptor peroxisome proliferator-activated receptor (PPAR)  $\gamma$  is a ligand-activated transcription factor that regulates several crucial biological processes such as adipogenesis, glucose homeostasis, and cell growth. It is also the functional receptor for a new class of insulin-sensitizing drugs, the thiazolidinediones, now widely used in the treatment of type 2 diabetes mellitus. Here we report that PPAR $\gamma$  protein levels are significantly reduced in adipose cells and fibroblasts in response to specific ligands such as thiazolidinediones. Studies with several doses of different ligands illustrate that degradation of PPAR $\gamma$  correlates well with the ability of ligands to activate this receptor. However, analyses of PPAR $\gamma$  mutants show that, although degradation does not strictly depend on the transcriptional activity of the receptor, it is dependent upon the ligand-gated activation function 2 (AF2) domain. Proteasome inhibitors inhibited the down-regulation of PPAR $\gamma$  and ligand activation enhanced the ubiquitination of this receptor. These data indicate that, although ligand binding and activation of the AF2 domain increase the transcriptional function of PPAR $\gamma$ , these same processes also induce ubiquitination and subsequent degradation of this receptor by the proteasome.**

The peroxisome proliferator-activated receptor (PPAR)  $\gamma$ ,<sup>1</sup> a member of the nuclear hormone receptor family of transcription factors, has recently been implicated in the regulation of a variety of biological processes. Two forms of PPAR $\gamma$ , PPAR $\gamma$ 1 and PPAR $\gamma$ 2, are generated by alternative splicing. PPAR $\gamma$ 2 bears an additional 30 amino acids at the amino terminus and is predominantly expressed in adipose tissue. PPAR $\gamma$  plays a central regulatory role in adipogenesis, where it acts in concert with members of the CAAT/enhancer-binding protein (C/EBP)

family of transcription factors (reviewed in Ref. 1). The expression of PPAR $\gamma$  mRNA and protein are induced early during adipogenesis. Many of the adipocyte-specific genes harbor PPAR $\gamma$  binding sites in their promoters or upstream enhancer regions. Gain-of-function experiments have shown that PPAR $\gamma$  is sufficient to induce adipogenesis, specifically, ectopic expression and activation of PPAR $\gamma$  in fibroblasts efficiently induces an adipocytic phenotype (2). Recent genetic studies conclusively demonstrate that PPAR $\gamma$  is also absolutely required for fat cell formation. Cells lacking both alleles for PPAR $\gamma$  do not differentiate into adipocytes *in vitro* or *in vivo*; a PPAR $\gamma$ -/- mouse lived only briefly after birth and lacked visible fat pads (3–5). The gene dosage of PPAR $\gamma$  is apparently important, because differentiation of cells that contain only a single PPAR $\gamma$  allele shows a phenotype intermediate to wild-type and null cells (3, 5).

PPAR $\gamma$  has also been implicated in the regulation of systemic insulin sensitivity. This was first suggested when PPAR $\gamma$  was found to be the functional receptor for a group of synthetic insulin-sensitizing agents, the thiazolidinediones (TZDs), which are currently used for the treatment of type 2 diabetes mellitus (6, 7). This role is now supported by the finding that certain mutations within PPAR $\gamma$  are associated with severe insulin resistance and diabetes mellitus, even though these patients are not obese (8).

Although expressed at lower levels than in adipose cells, PPAR $\gamma$  has also been implicated in the growth and/or differentiation of several cell types such as monocytes, breast, and colonic epithelium. The ability of PPAR $\gamma$  to arrest growth in many of these cell types has suggested a possible connection to tumor biology (reviewed in Refs. 9, 10). Indeed, loss-of-function mutations of PPAR $\gamma$  have been found in human colon cancers, suggesting a possible role as a tumor suppressor in this cell lineage (11). Recently, clinical data show that treatment of liposarcoma patients with TZDs induced tumor differentiation and a reduction in tumor cell growth (12).

PPAR $\gamma$  is a ligand-activated transcription factor that binds to DR-1 sites as a heterodimeric complex with the retinoic X receptor (RXR). Synthetic ligands for PPAR $\gamma$  are the aforementioned TZDs (*e.g.* troglitazone, pioglitazone, and rosiglitazone) and certain nonsteroidal anti-inflammatory drugs (6, 13). Natural ligands include 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub> (15-dPGJ<sub>2</sub>), certain polyunsaturated fatty acids such as linoleic acid, and endogenous constituents of oxidized low density lipoprotein particles such as 9- and 13-hydroxyoctadecadienoic acid and 15-hydroxyicosatetraenoic acid (14–17). All of these natural compounds are low affinity ligands. So far, there is no consensus on the existence or the nature of a high affinity endogenous ligand.

To date, the major regulatory events identified in PPAR $\gamma$

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<sup>1</sup> The abbreviations used are: PPAR $\gamma$ , peroxisome proliferator-activated receptor  $\gamma$ ; TZD, thiazolidinedione; RXR, retinoic X receptor; 15d-PGJ<sub>2</sub>, 15-deoxy- $\Delta^{12,14}$ -prostaglandin J<sub>2</sub>; ER, estrogen receptor; RAR, retinoic acid receptor; PR, progesterone receptor; DMEM, Dulbecco's modified Eagle's medium; MAP kinase, mitogen-activated protein kinase; PBS, phosphate-buffered saline; ALLN, N-acetyl leucine leucine norleucinal; C/EBP, CAAT/enhancer-binding protein; CREB, cAMP response element binding protein; pio, pioglitazone; rosi, rosiglitazone.

function are ligand binding, coactivator docking, and phosphorylation at an inhibitory serine (serine 112 of murine PPAR $\gamma$ ) by mitogen-activated protein (MAP) kinase (18–21). As for most nuclear receptors, binding of agonist ligands induces a conformational change in a conserved helix in the carboxyl terminus of the ligand binding domain (22). This helix, also known as the AF2 helix, is a docking site for a number of coactivators that stimulate transcription via acetylation of core histones or interaction with the basal transcription machinery (reviewed in Ref. 23). PPAR $\gamma$  also is susceptible to negative regulation via covalent modification. Phosphorylation of serine 112 in murine PPAR $\gamma$ 2 by MAP kinases (Erk 1, Erk 2, and stress-activated protein kinase/c-Jun amino-terminal kinase) results in a strong suppression of PPAR $\gamma$  activity (18–21), at least in part by influencing ligand binding (24).

We describe here a novel regulatory mechanism for PPAR $\gamma$  via ligand binding: the induction of ubiquitin-dependent degradation of this receptor by the proteasome. Because ligands induce both the transcriptional activation and the destruction of the receptor, these results illustrate a feedback system for balancing the transcriptional activity of PPAR $\gamma$ .

#### EXPERIMENTAL PROCEDURES

**Chemicals**—Pioglitazone was purchased from Upjohn, Wy14,643 from Chemsyn, and 15d-PGJ2 from Cayman Chemical. Troglitazone, M2, and rosiglitazone were gifts from A. Saltiel (Parke-Davis Pharmaceuticals). LG268 was a gift from R. Heyman (Ligand Pharmaceuticals). The proteasome inhibitors MG132 and *N*-acetyl leucine leucine norleucinal (ALLN) were obtained from Calbiochem, Calpain inhibitor II from Roche Molecular Biochemicals. Serum for tissue culture was purchased from Hyclone, and dexamethasone and insulin were obtained from Sigma.

**Adipocyte Differentiation and Ligand Treatment**—3T3-F442A and 3T3-L1 cells were differentiated for 6–9 days as described previously (25). Ligands and proteasome inhibitors were added at the concentration and for the times indicated in the figure legends. Degradation occurred both in the presence (Dulbecco's modified Eagle's medium (DMEM) + 10% fetal bovine serum) or the absence (DMEM + 2% bovine serum albumin) of growth factors.

**Transient Transfection Assays**—Expression of all PPAR $\gamma$  alleles is under the control of an SV-40 promoter. The plasmids for wild-type PPAR $\gamma$  (2), PPAR $\gamma$ 2-S112A (19), PPAR $\gamma$ 2- $\Delta$ DNA (originally named PPAR $\gamma$ 2-M2) (2) have been described. PPAR $\gamma$ 2-E499Q was cloned by polymerase chain reaction, thereby changing codon 499 from GAG to CAG.

To analyze PPAR $\gamma$  degradation in NIH-3T3 cells, 60-mm dishes (Figs. 2B, 3B–D, and 4A) or 100-mm dishes (Fig. 5) at 60–70% confluence were transiently transfected by mixing plasmid DNA (see figures) with Superfect transfection (Qiagen) reagent for 3 h according to the manufacturer's instructions. Ligands were added 3 h after transfection (Fig. 3, B–D), and whole cell lysates were prepared 15 h after treatment. For Figs. 2B, 4A, and 5, ligands and inhibitors were added 24 h after transfection for the time indicated in the figure legends.

To test for transcriptional activity of PPAR $\gamma$ , NIH-3T3 cells, grown in 24-well cell culture plates in DMEM + 10% bovine calf serum, were transfected with pSV-Sport plasmids (500 ng each) encoding PPAR $\gamma$ , and DR-1 Luciferase, and 100 ng of  $\beta$ -galactosidase plasmid utilizing Superfect transfection reagent 3 h after transfection, cells were exposed to ligands for 15 h, lysed, and assayed for luciferase and  $\beta$ -galactosidase activity using a 96-well luminometer and spectrophotometer. Transfections were performed in triplicate.

**Whole Cell Lysates, Immunoprecipitations, and Western Blot Analysis**—Cells were washed once in phosphate-buffered saline (PBS) and then lysed in PBS supplemented with 1% Triton X-100, 0.5% deoxycholate, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 100 mM sodium fluoride, 10  $\mu$ g/ml aprotinin, 10  $\mu$ g/ml leupeptin, 100  $\mu$ g/ml phenylmethylsulfonyl fluoride (and 25  $\mu$ g/ml ALLN for Figs. 4 and 5). Concentration of the soluble proteins was determined by Bradford assays (Bio-Rad), and for each sample the same amount of protein (50–100  $\mu$ g) was analyzed by SDS gel electrophoresis after trichloroacetic acid precipitation (19). Western blots were performed as described previously (19). The antibody against PPAR $\gamma$  is described in a previous study (19), antibodies against RXR $\alpha$ , C/EBP $\alpha$ , and cAMP response element binding protein (CREB) were obtained from Santa

Cruz. Supernatant of 9E10 hybridoma cultures was a gift from S. Gaubatz.

Immunoprecipitations were performed with an anti-PPAR $\gamma$  antibody (diluted 1:100) in lysis buffer. After 4 h of incubation, 50  $\mu$ l of a 50:50 slurry of protein A-Sepharose (Amersham Pharmacia Biotech) was added for another 2 h. Immunocomplexes were then washed three times with PBS + 1% Nonidet P-40. Immunocomplexes were analyzed by SDS-polyacrylamide gel electrophoresis and Western blot as described above.

**Northern Blot Analysis**—Total RNA was isolated using the Trizol reagent (Life Technologies, Inc.) according to the manufacturer's instructions. 10  $\mu$ g of total RNA was analyzed for PPAR $\gamma$  mRNA with an *Eco*RI fragment of the PPAR $\gamma$  cDNA as described previously (2).

#### RESULTS

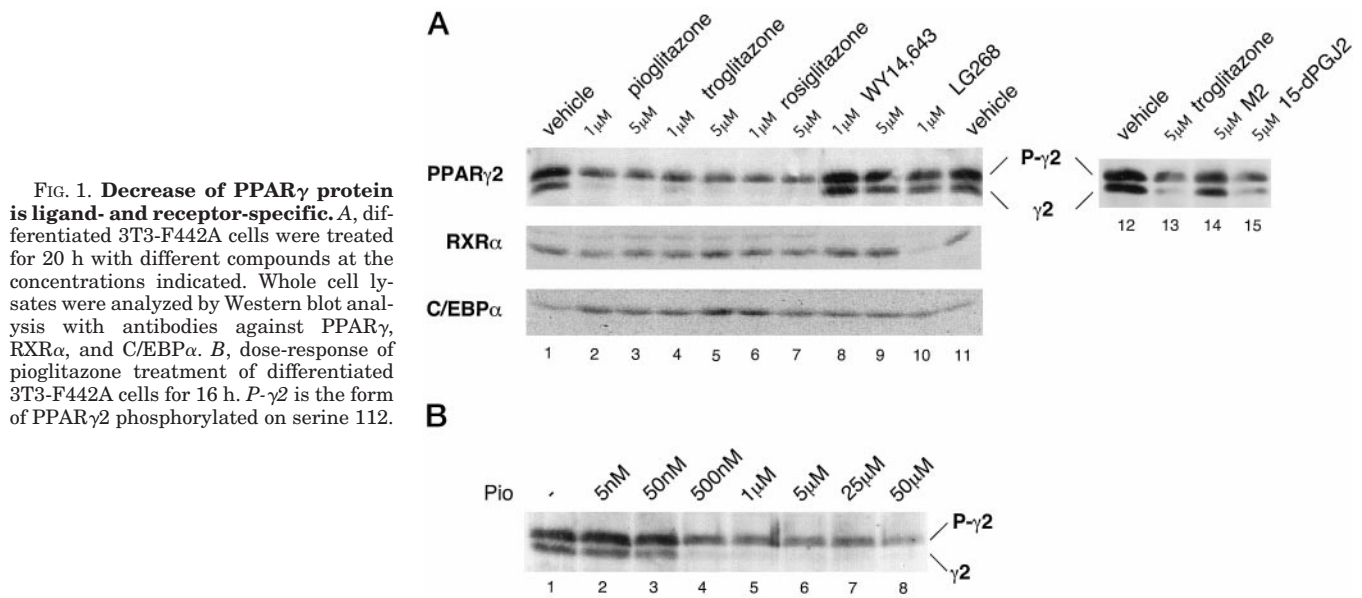
**PPAR $\gamma$  Protein Amount Is Decreased by Its Specific Ligands**—To investigate the effect of PPAR $\gamma$  ligands on the level of this receptor, differentiated adipocytes were treated with either vehicle or pioglitazone, a ligand of the TZD class. Whole cell lysates were analyzed by Western blot analysis with an anti-PPAR $\gamma$  antibody. PPAR $\gamma$ 2 appears as a doublet in solvent-treated cells; as shown earlier, the upper band is an inactive form of PPAR $\gamma$ , phosphorylated by MAP kinases on serine 112 (19). The lower band represents the nonphosphorylated, active form of this protein (Fig. 1A, lanes 1, 11, and 12). Interestingly, treatment with pioglitazone resulted in a dramatic loss of PPAR $\gamma$  protein, with the active, nonphosphorylated form of this receptor lost preferentially (Fig. 1A, lanes 2 and 3). The levels of two other proteins, RXR $\alpha$ , the heterodimerization partner of PPAR $\gamma$ , and C/EBP $\alpha$  did not change when the cells were exposed to pioglitazone. This indicates that the decrease in protein is selective or specific for PPAR $\gamma$ .

To analyze more critically whether the decrease of PPAR $\gamma$  protein is related to specific ligand binding, differentiated adipocytes were treated with several different TZDs (pioglitazone, troglitazone, and rosiglitazone) and the natural ligand 15-dPGJ2. As controls we also used M2, a urinary metabolite of troglitazone that no longer binds to PPAR $\gamma$ , the PPAR $\alpha$ -selective ligand Wy14,643, and the RXR-selective ligand LG268. Treatment with the different TZDs and with 15-dPGJ2 again resulted in a loss of the nonphosphorylated, active form of PPAR $\gamma$  (Fig. 1A, compare lanes 2–7, 13, and 15 with lanes 1, 11, and 12). In contrast, treatment with the inactive troglitazone metabolite M2 or Wy14,643 and LG268 did not down-regulate PPAR $\gamma$  (Fig. 1A, lanes 8–10 and 14). Interestingly, LG268 did decrease the amount of its own receptor, RXR $\alpha$  (Fig. 1A, compare lane 10 with lanes 1–9 and 11). Thus, the decrease of the PPAR $\gamma$  protein appears to be specific for PPAR $\gamma$  ligands.

The experiments described were performed with concentrations of ligand (1 and 5  $\mu$ M) that are above the  $K_d$  of most of these ligands for PPAR $\gamma$  (40 nM for rosiglitazone to 3  $\mu$ M for 15-dPGJ2). Therefore, a dose response was performed on differentiated adipocytes to determine the relationship between this reduced amount of protein and the affinity for a particular ligand. As shown in Fig. 1B, an effect of pioglitazone on PPAR $\gamma$  levels could be detected at 50 nM, whereas strong effects could be seen at 500 nM (Fig. 1B, compare lanes 1 and 4), which is close to its  $EC_{50}$  of 690 nM (26). Recently, Shao *et al.* (24) proposed that the phosphorylated form of PPAR $\gamma$  binds to ligand with lower affinity than the nonphosphorylated form. In our experiments, ligand leads to the preferential loss of the nonphosphorylated PPAR $\gamma$ . Thus, the loss of the receptor generally correlates well with ligand binding.

**Decrease of PPAR $\gamma$  Is Regulated at the Protein Level**—Rosenbaum and Greenberg (27) and Camp *et al.* (28) recently reported that PPAR $\gamma$  mRNA levels are decreased upon exposure of cells to specific ligands; hence the loss of protein shown here could be a consequence of mRNA metabolism. To address this, we first performed time course studies of the amount of PPAR $\gamma$





**FIG. 1. Decrease of PPAR $\gamma$  protein is ligand- and receptor-specific.** *A*, differentiated 3T3-F442A cells were treated for 20 h with different compounds at the concentrations indicated. Whole cell lysates were analyzed by Western blot analysis with antibodies against PPAR $\gamma$ , RXR $\alpha$ , and C/EBP $\alpha$ . *B*, dose-response of pioglitazone treatment of differentiated 3T3-F442A cells for 16 h. P- $\gamma$ 2 is the form of PPAR $\gamma$ 2 phosphorylated on serine 112.

protein and PPAR $\gamma$  mRNA in response to a PPAR $\gamma$  ligand. Total protein and RNA of differentiated adipocytes were prepared after 10 or 24 h of treatment with either pioglitazone or vehicle. Fig. 2*A* shows that, in agreement with the published reports, a 24-h treatment of adipocytes with a TZD down-regulated PPAR $\gamma$  mRNA and both bands of PPAR $\gamma$  protein. However, 10 h of pioglitazone treatment left PPAR $\gamma$  mRNA largely intact. In contrast, most of the unphosphorylated form of the PPAR $\gamma$  protein has disappeared by this time, whereas the phosphorylated form was barely affected. These data strongly suggest that PPAR $\gamma$  ligands have an effect on PPAR $\gamma$  protein that is separate from effects at the mRNA levels.

To separate the effects of PPAR $\gamma$  ligands at transcriptional and post-translational levels more definitively, we expressed PPAR $\gamma$  ectopically with the SV-40 promoter, which is not subject to regulation by PPAR $\gamma$  ligands. Fibroblasts transiently transfected with the empty vector or with a PPAR $\gamma$  expression plasmid were treated for 24 h with pioglitazone and analyzed by Western and Northern blot. As can be seen in Fig. 2*B* (compare lanes 1 and 3), substantial PPAR $\gamma$  mRNA and protein could only be detected in cells receiving the expression vector for PPAR $\gamma$ . Northern blot analysis showed that the PPAR $\gamma$  mRNA is expressed at similar levels in the absence or presence of ligand (Fig. 2*B*, lanes 3 and 4). However, following the treatment with ligand, the amount of nonphosphorylated PPAR $\gamma$  protein was reduced, whereas the amount of phosphorylated PPAR $\gamma$  was largely unaffected (Fig. 2*B*, lanes 3 and 4), similar to what is described alone for endogenous PPAR $\gamma$  in adipocytes (see Fig. 1). Importantly, the protein levels of two other transcription factors, RXR $\alpha$  and CREB, were not affected by the treatment (Fig. 2*B*). These data clearly demonstrate an effect of PPAR $\gamma$  ligands on PPAR $\gamma$  protein that is separable from effects at the mRNA level; in addition these results show that the decrease of PPAR $\gamma$  protein is not unique to adipocytes.

**The AF2 Domain of PPAR $\gamma$  Is Important for Ligand-induced Down-regulation**—To study the structural requirements for PPAR $\gamma$  down-regulation, we transfected wild-type PPAR $\gamma$  and various mutant alleles into NIH-3T3 cells (Fig. 3*A*). Although the experiments above indicate that the nonphosphorylated form of PPAR $\gamma$  is preferentially degraded, any conversion between phosphorylated and nonphosphorylated forms upon ligand binding would complicate these data. To clarify and simplify this issue, we used an allele of PPAR $\gamma$  with the serine 112 phosphorylation site converted to alanine (PPAR $\gamma$ 2-S112A)

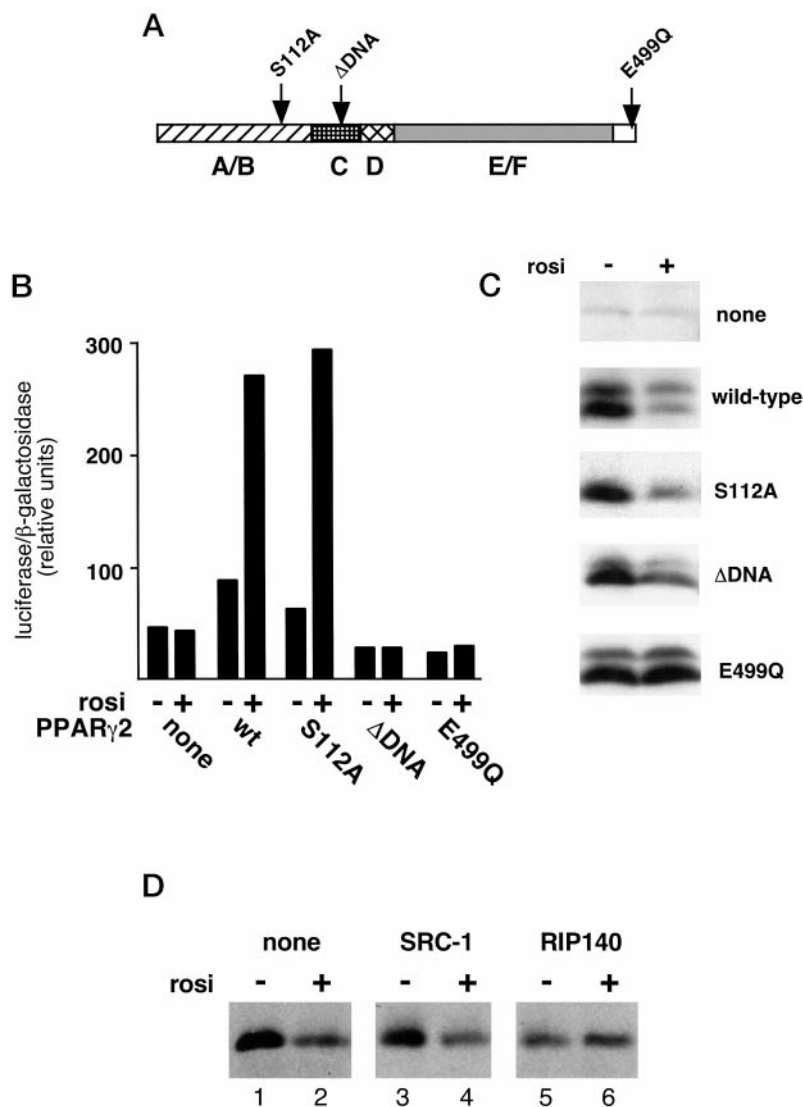
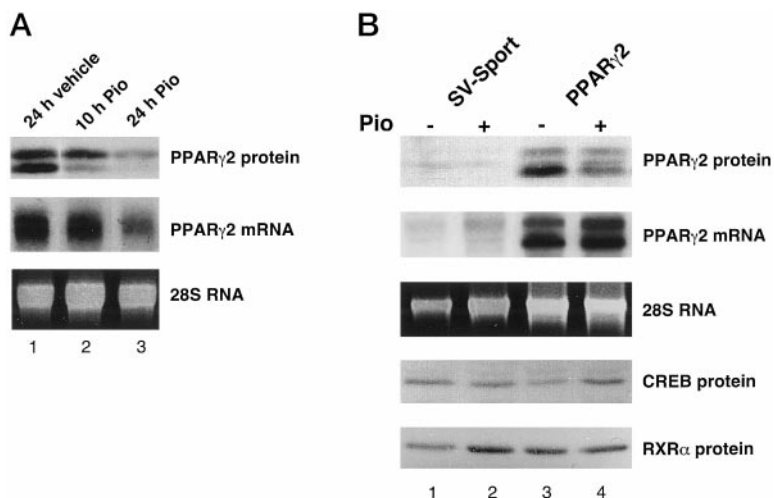
(19). Transient transfections and subsequent treatment with pioglitazone revealed that both the wild-type PPAR $\gamma$ 2 as well as PPAR $\gamma$ 2-S112A were transcriptionally active under the conditions used (Fig. 3*B*). Both were also subject to down-regulation (Fig. 3*C*). Phosphorylation of PPAR $\gamma$ 2 on serine 112 is therefore not required for its down-regulation.

Because ligand binding results in transcriptional activation, we next analyzed whether transcriptional activation of PPAR $\gamma$  is required for the ligand-dependent down-regulation. Two different point mutants were used to address this question: PPAR $\gamma$ 2- $\Delta$ DNA, a double point mutant in the DNA binding region that can bind ligand but is unable to bind to DNA (2), and PPAR $\gamma$ 2-E499Q, a mutant in the AF2 domain of the receptor. The latter also binds to PPAR $\gamma$  ligands with comparable affinity as the wild-type receptor (data not shown). Both of these mutants showed no ligand-dependent transcriptional activation (Fig. 3*B*). Upon ligand exposure, PPAR $\gamma$ - $\Delta$ DNA was subject to normal ligand-dependent degradation, whereas the levels of PPAR $\gamma$ -E499Q did not change (Fig. 3*C*). These data indicate that transcriptional activation of the receptor *per se* is not a prerequisite for ligand-induced down-regulation. However, an intact AF2 domain is required, suggesting that a ligand-dependent conformational change of the AF2 region and/or docking of cofactors to the AF2 region is important for the down-regulation.

Proteins known to associate with the AF2 region of nuclear hormone receptors are coactivators and some corepressors. We therefore investigated whether coexpression of a ligand-dependent coactivator (SRC-1) or corepressor (RIP140) would influence the degradation of PPAR $\gamma$ . Although most corepressors bind to nuclear hormone receptors in a nonligand-dependent way, RIP140 is unusual in that it is a corepressor whose association through the AF2-domain is stimulated by ligand binding. As shown in Fig. 3*D*, down-regulation in the presence of SRC-1 occurs rather normally. In contrast, RIP140 completely blocked degradation of PPAR $\gamma$ . These results illustrate that protein down-regulation is not linked simply to ligand binding but is closely associated with a transcriptionally active conformation of the AF2 domain of the receptor. Two different ways of interfering with the activity of the AF2 region but not ligand binding, mutation in the AF2-domain or binding of RIP140, prevent protein down-regulation.

**PPAR $\gamma$  Is Degraded by the Proteasome**—We next addressed the mechanisms by which PPAR $\gamma$  is degraded. Because the

**FIG. 2. Ligands stimulate a reduction of PPAR $\gamma$  protein that is distinct from mRNA regulation.** *A*, differentiated 3T3-F442A cells were treated for the times indicated with 5  $\mu$ M pioglitazone or vehicle. Whole cell lysates were analyzed by Western blot with an antibody against PPAR $\gamma$ . Total RNA was prepared and analyzed in Northern blots with a probe against PPAR $\gamma$ . Ethidium bromide staining of the 28 S rRNA is shown as a loading control. *B*, NIH-3T3 cells were transiently transfected with either the empty vector or an expression vector for PPAR $\gamma$ 2. On the following day cells were treated overnight with or without 5  $\mu$ M pioglitazone. Whole cell lysates were analyzed in Western blots with antibodies against PPAR $\gamma$ , CREB, and RXR $\alpha$ . Total RNA was analyzed in Northern blots with a probe against PPAR $\gamma$ . Ethidium bromide staining of the 28 S rRNA is shown as a loading control.

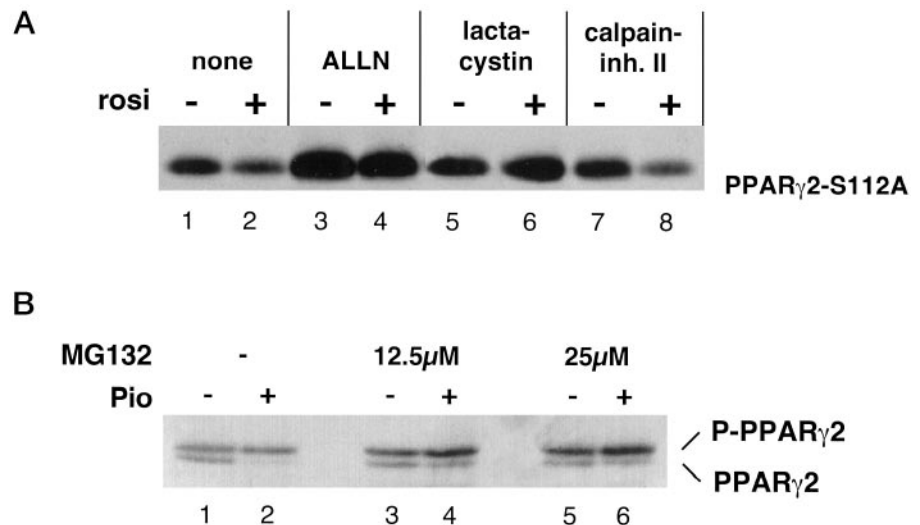


**FIG. 3. The AF2 region of PPAR $\gamma$  is important for the ligand-dependent decrease of PPAR $\gamma$  protein.** *A*, schematic representation of PPAR $\gamma$  with indications of the sites of the point mutations. (*A* and *B*, AF1 domain; *C*, DNA binding domain; *D*, hinge domain; *E* and *F*, ligand binding, heterodimerization, and AF2 domain). *B*, NIH-3T3 cells were transfected with expression plasmids for PPAR $\gamma$  wild-type or PPAR $\gamma$  mutants in the presence of a luciferase reporter plasmid with three PPAR $\gamma$  response elements (DR1-sites) and an expression plasmid for  $\beta$ -galactosidase. 3 h after transfection, the cells were treated for 15 h with or without 1  $\mu$ M rosiglitazone (*rosi*). Cell lysates were then analyzed for luciferase and  $\beta$ -galactosidase activity. *C*, NIH-3T3 cells were transiently transfected with expression vectors for PPAR $\gamma$  wild type or mutants and treated for 15 h with or without 2.5  $\mu$ M rosiglitazone. Whole cell lysates were analyzed in Western blots with an antibody against PPAR $\gamma$ . *D*, NIH-3T3 cells were cotransfected with expression plasmids for PPAR $\gamma$ 2-S112A and SRC-1 or RIP140. Cells were treated for 15 h with or without 2.5  $\mu$ M rosiglitazone. Whole cell lysates were analyzed in Western blots with an antibody against PPAR $\gamma$ .

ubiquitin-proteasome pathway regulates the stability of many proteins, including transcription factors (29), we analyzed whether proteasome inhibitors, ALLN and lactacystin, can block the down-regulation. NIH-3T3 cells expressing PPAR $\gamma$ 2-S112A were treated for 15 h in the absence or presence of ligand with or without proteasome inhibitors. Because ALLN is

known to also inhibit calpains, we also studied the effects of calpain inhibitor II, which does not affect proteasome-dependent degradation (30). As shown in Fig. 4A, ligand-induced down-regulation was largely inhibited by all three proteasome inhibitors but was not affected by calpain inhibitor II. In addition to the inhibition of the ligand-induced degradation, basal

**FIG. 4. Inhibition of the proteasome blocks ligand-dependent decrease of PPAR $\gamma$  protein.** *A*, NIH-3T3 cells were transiently transfected with an expression vector for PPAR $\gamma$ 2-S112A. On the following day cells were treated for 15 h with the indicated compounds (10  $\mu$ g/ml ALLN, 5  $\mu$ M lactacystin, or 25  $\mu$ g/ml calpain inhibitor II) and 1  $\mu$ M rosiglitazone. Whole cell lysates were analyzed in Western blots for the presence of PPAR $\gamma$ . *B*, differentiated 3T3-L1 adipocytes were pretreated for 30 min with the indicated concentrations of MG132 and then treated for 30 min with or without 25  $\mu$ M pioglitazone. Whole cell lysates were analyzed in Western blot with antibodies against PPAR $\gamma$ .



levels of PPAR $\gamma$  are increased by the presence of the proteasome inhibitors for the long-term treatment. This suggests that the normal turnover of the receptor is also mediated by the proteasome.

To test the importance of the proteasome in ligand-induced degradation of PPAR $\gamma$  in adipocytes, differentiated 3T3-L1 cells were incubated with pioglitazone or vehicle following treatment with or without the proteasome inhibitor MG132. To minimize cell stress caused by the proteasome inhibitors in fat cells, we used a shorter treatment with proteasome inhibitors and PPAR $\gamma$  ligands at higher concentrations. As shown in Fig. 4*B*, PPAR $\gamma$  is effectively degraded under these conditions (Fig. 4*B*, lanes 1 and 2). Treatment with the proteasome inhibitor completely inhibited the down-regulation (Fig. 4*B*, lanes 3–6). These experiments suggest that PPAR $\gamma$  is degraded by the proteasome upon ligand-mediated activation in both fibroblasts and fat cells.

**Ligand Enhances Ubiquitination of PPAR $\gamma$** —Most proteasome substrates are ubiquitinated prior to their degradation and can be detected by the formation of high molecular weight complexes (for review see Ref. 31). To investigate whether PPAR $\gamma$  is ubiquitinated in response to ligand, we expressed either PPAR $\gamma$ , myc-tagged ubiquitin, or both in NIH-3T3 cells. The cells were treated with or without ligand in the absence or presence of ALLN, a proteasome inhibitor that has proven useful in preserving short-lived ubiquitin conjugates. Immunoprecipitations were performed with an antibody against PPAR $\gamma$ , followed by a Western blot analysis with the myc-tag specific antibody 9E10. In the absence of myc-tagged ubiquitin, no higher weight molecular complexes were observed (Fig. 5, lanes 1–4). Similarly, in the presence of both PPAR $\gamma$  and myc-tagged ubiquitin, but without proteasome inhibitor, almost no high molecular weight complex can be detected (Fig. 5, lanes 5 and 6). When the cells were treated with ALLN, a small amount of a high molecular weight ubiquitin complex could be observed without ligand treatment (Fig. 5, lane 7). Following ligand addition, the amount of this complex was greatly increased (Fig. 5, lane 8). As expected, the overall levels of PPAR $\gamma$  detected by Western blots were unchanged in the presence of ALLN (data not shown). The formation of the higher molecular weight complex in response to ligand depends upon PPAR $\gamma$ , because it cannot be observed when no PPAR $\gamma$  was expressed (Fig. 5, lanes 9–12). We conclude that PPAR $\gamma$  is ubiquitinated prior to degradation by the proteasome, and that ubiquitination is increased by a PPAR $\gamma$  ligand.

## DISCUSSION

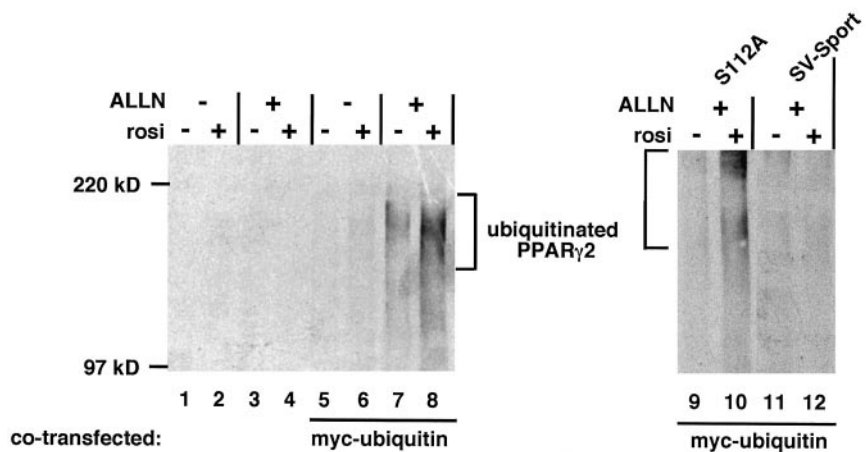
Homeostasis in many physiological systems is maintained by the action of hormones, including those that bind to either cell surface or nuclear receptors. The precise balance of hormone actions requires both positive and negative controls. Negative modulation of signaling can occur through several mechanisms. In one case, receptor signaling is reduced through a covalent modification such as phosphorylation, as for example the phosphorylation of PPAR $\gamma$  on serine 112 (18–21). Downstream signaling can also be prevented by receptor destruction, which may occur after ligand binding via direct proteolytic degradation of a receptor; for cell surface receptors this often requires the internalization of the receptors bound to their ligands (32).

Nuclear hormone receptors are transcription factors whose activity can be induced by ligand binding. Many of these receptors have ligands with long half-lives. Several mechanisms have been proposed to attenuate or terminate the actions of hormones through this class of receptors. Chen *et al.* (33) recently proposed a possible mechanism for attenuation of the estrogen receptor (ER) function, involving signaling through the acetylation of the coactivator ACTR, which causes the disruption of this ER transcriptional complex. Another mechanism that has been reported recently is the reduction of the amount of nuclear hormone receptors through their ligand-dependent degradation by the proteasome. This has been shown for the ER, the retinoic acid receptor (RAR), the RXR, and the progesterone receptor (PR) (34–38). Generally, the structural requirements for the turnover of nuclear hormone receptors have not been well established. For RAR, however, it was shown that its degradation is dependent on the DNA binding function and certain amino acids in the AF2 region (35), suggesting that transcriptional activation of this receptor is required for its degradation.

We show here that PPAR $\gamma$  ligands induce the degradation of this receptor by the proteasome. This process is induced only by ligands that bind specifically to this receptor and in a concentration range that correlates well with ligand binding. Our experiments using proteasome inhibitors demonstrate that PPAR $\gamma$  is degraded by the proteasome upon ligand activation. Proteins targeted for proteasomal degradation are often modified by polymers of ubiquitin, which confer specificity for the degradation process (31). Indeed, PPAR $\gamma$  is ubiquitinated upon ligand binding prior to its degradation by the proteasome. In the current model of the ubiquitination process, attachment of



**FIG. 5. Ligand enhances ubiquitination of PPAR $\gamma$ .** NIH-3T3 cells were transiently transfected with expression plasmids for either PPAR $\gamma$ 2-S112A, myc-tagged ubiquitin, or both. In the absence of PPAR $\gamma$ 2-S112A, SV-Sport was used as a vector control. Cells were then treated for 15 h with or without 25  $\mu$ g/ml ALLN and 2.5  $\mu$ M rosiglitazone. Western blot analysis was performed with an anti-myc antibody after immunoprecipitation of the whole cell lysates with a PPAR $\gamma$  antibody.



ubiquitin to lysine residues in the substrate is mediated by the serial action of three enzymes, the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2), and the ubiquitin-protein ligase (E3). There are many E2 and E3 enzymes, and a complex of E2 and E3 mediates substrate-specificity (31). PPAR $\gamma$  degradation might very well require a specific E2-E3 complex, because different E2 enzymes have already been shown to mediate the basal turnover of ER, PR, and thyroid hormone receptors (38).

A crucial question here is which features of PPAR $\gamma$  are recognized by the proteolytic machinery upon ligand binding. One possibility is that the signal for receptor degradation is the recruitment of PPAR $\gamma$  into transcriptionally activated complexes on target promoters, a process known to be stimulated by ligand binding. However, we can essentially rule out this possibility, because a PPAR $\gamma$  mutant defective in DNA binding is degraded as well as the wild-type receptor. This is in direct contrast to the proposed mechanisms for the degradation of RAR, which requires intact DNA binding capacity (35). We conclude, based on the data shown here, that transcriptional activity of PPAR $\gamma$  is not required for its degradation.

However, our results make it likely that a key requirement for PPAR $\gamma$  degradation is the ligand-induced conformational change associated with transcriptional activation. Crystal structures of ligand-bound nuclear receptors, including that of PPAR $\gamma$ , suggest that an important result of ligand binding is the translocation of the AF2 helix (22). It is this feature that is recognized by many coactivator and corepressor proteins, particularly those with LXXLL motifs. We show here that a point mutant of PPAR $\gamma$  in the AF2 helix that is transcriptionally inactive is not degraded upon ligand exposure. Furthermore, a corepressor protein, RIP140, which docks via the AF2 motif, blocks PPAR $\gamma$  degradation. However, a coactivator protein such as SRC-1 does not interfere with receptor destruction. Hence, it is tempting to speculate that a protein associated with the degradation machinery might also bind to the AF2 domain and compete with a corepressor like RIP140 for receptor docking. A coactivator might bind differently to the AF2 region and still allow the degradation machinery to make contact with the AF2 domain. Consistent with this is recent data illustrating that the contact of coactivators and corepressors in the AF2 domain are not identical (39). In an alternative model, the degradation apparatus might recognize a complex of a coactivator and ligand-activated PPAR $\gamma$ . There is precedence for the involvement of a coactivator in the degradation of a transcription factor; the turnover of p53 requires the formation of a ternary complex between p53, the coactivator p300/CBP, and the ubiquitin ligase mdm2 (40). Clearly, more study is required to understand the role of the AF2 in the docking of a

very large array of proteins that can potentially bind there *in vivo*.

The complex biology of PPAR $\gamma$  is only beginning to be understood. However, it is notable that ligands that activate this receptor are already clinically useful. Many patients with type 2 diabetes mellitus can achieve improved glucose homeostasis through the use of synthetic PPAR $\gamma$  ligands, and increasing clinical data suggest dramatic improvement in patients with polycystic ovarian syndrome, another disease involving insulin resistance (7). Most recently, differentiation of human tumors in patients with liposarcoma was achieved (12), raising the possibility that PPAR $\gamma$  ligands may also have utility in the cancer clinic. PPAR $\gamma$  ligands also modulate several important functions in monocytes, including cytokine generation, differentiation to macrophages, and lipid accumulation (9, 10). However, even in many clinical situations where benefits have been established, such as in type 2 diabetes, the effects of PPAR $\gamma$  ligands are still far from ideal. In particular, many patients do not achieve a large enough response to eliminate the use of insulin or other insulinotropic drugs (7). Hence, an understanding of how to achieve a more robust response through the PPAR $\gamma$  response system holds particular interest and promise. A further understanding of the receptor destruction process induced by PPAR $\gamma$  ligands could eventually offer the possibility of therapeutic modulation of receptor number and improved responses through this system.

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## **Degradation of the Peroxisome Proliferator-activated Receptor $\gamma$ Is Linked to Ligand-dependent Activation**

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