**Block of Nuclear Receptor Ubiquitination**

**A MECHANISM OF LIGAND-DEPENDENT CONTROL OF PEROXISOME PROLIFERATOR-ACTIVATED RECEPTOR δ ACTIVITY**

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Peroxisome proliferator-activated receptor δ (PPARδ) is a ligand-activated transcription factor involved in many physiological and pathological processes. PPARδ is a promising therapeutic target for metabolic, chronic inflammatory, and neurodegenerative disorders. However, limited information is available about the mechanisms that control the activity of this nuclear receptor. Here, we examined the role of the ubiquitin-proteasome system in PPARδ turnover. The receptor was ubiquitinated and subject to rapid degradation by the 26 S proteasome. Unlike most nuclear receptors that are degraded upon ligand binding, PPARδ ligands inhibited the ubiquitination of the receptor, thereby preventing its degradation. Ligand binding was required for inhibition of the ubiquitination since disruption of the ligand binding domain abolished the effect. Site-directed mutagenesis showed that the DNA binding domain was also required, indicating that ligands preferentially stabilized the DNA-bound receptor. In contrast, the activation function-2 domain and co-repressor binding site were not involved in ligand-induced stabilization. Block of ubiquitination by ligands may be an essential step to avoid rapid degradation of a receptor, like PPARδ, with a very short half-life and sustain its transcriptional activity once it is engaged in transcriptional activation complexes.

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Activity and expression of PPARδ were inhibited by nonsteroidal anti-inflammatory drugs and cyclooxygenase-2 inhibitors in colon cancer cells, relating this nuclear receptor to the chemopreventive effects of this class of anti-inflammatory compounds (9, 14). Less is known about post-transcriptional mechanisms that control PPARδ protein level and activity. A better understanding of the multiple mechanisms that modulate ligand-dependent and -independent activity might provide hints into the involvement of PPARδ in human diseases and indicate ways to exploit its therapeutic potential.

In this study we examined the role of the ubiquitin-proteasome system (UPS) in basal and ligand-dependent turnover of PPARδ. The UPS is the major cellular machinery responsible for degradation of proteins and determines the level of critical regulatory factors, including nuclear receptors and transcription factors (28–31). We found that PPARδ was ubiquitinated and subject to rapid degradation by the 26 S proteasome. However, unlike most nuclear receptors that are degraded upon ligand binding, PPARδ was stabilized by its ligands. PPARδ agonists inhibited ubiquitination of the receptor with consequent block of its degradation. Thus, PPARδ ligands have a dual effect on the receptor. Ligands induce conformational changes, allowing co-activator binding and promoter transactivation, and at the same time prevent ubiquitination of the receptor engaged in transcriptional activation complexes. Block of ubiquitination may be an essential step to avoid rapid degradation of a receptor with very short half-life, like PPARδ, and sustain its transcriptional activity.

**EXPERIMENTAL PROCEDURES**

**Cell Lines, Plasmids, and Chemicals**—Human osteosarcoma U2OS cells and non-small cell lung cancer H358, H441, and A549 cells were purchased from American Type Culture Collection (LGC Promochem, Molsheim Cedex, F) and maintained in RPMI supplemented with 10% fetal bovine serum. In all experiments involving incubation with PPARδ ligands, cells were grown in phenol red-free RPMI supplemented with 5% charcoal-stripped serum (HyClone, Logan, UT). Full-length human wild type PPARδ (a gift of Bert Vogelstein, John Hopkins University, Baltimore, MD) was subcloned into pCMV (Stratagene, La Jolla, CA) and pcDNA3.1/His (Invitrogen) expression vectors. The C91A/C94A, F270A, and L432A/L925A mutants and the truncated form of PPARδ (1–299) were generated by site-directed mutagenesis of pcDNA3.1/His-PPARδ using the QuikChange site-directed mutagenesis kit (Stratagene). The HA-UB expression vector was kindly provided by Ronald M. Evans (Salk Institute, La Jolla, CA). Cells were transfected with expression and reporter vectors using Lipofectamine 2000 (Invitrogen). Transfections were separated by centrifugation at 4000 × g for 10 min at 4 °C. Protein concentration was determined using a BCA assay (Pierce).

Proteins were loaded on 10% polyacrylamide gels and analyzed by immunoblotting with antibodies against PPARδ (H-74, Santa Cruz Biotechnology, Santa Cruz, CA), tubulin (DM1B, Calbiochem), His tag (H1029, Sigma), FLAG tag (M2, Sigma) and HA tag (Roche Applied Science). Horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) were used for detection. Band intensity was assessed with the Alphalmer 3400 and AlphaEase Software (Alpha Innootech, San Leandro, CA).

**RNA Isolation and Analysis**—Cells (1 × 10⁶ cells/flask) were incubated with PPARδ ligands overnight and proteasome inhibitors for 4 h. For RNA interference studies, cells were transfected with 100 nM small interfering RNA (Ambion Ltd., Huntingtondon UK) specific for PPARδ (siPPARδ) or the firefly luciferase gene (siGL3) using Lipofectamine 2000, incubated for 48 h, and then treated with ligands and/or proteasome inhibitors. Total RNA was isolated using Trizol (Invitrogen) were dissolved in Me₃SO. Puromycin dihydrochloride (Sigma) was prepared in water.

**Reporter Assay**—Cells (2 × 10⁶ cells/well) were plated in 48-well plates and transfected with the PPREx3-tk-Luc, pRL-SV40 vectors and, when indicated, with PPARδ expression vectors. Dual-luciferase reporter assay was performed according to manufacturer’s instructions (Promega, Catalys AG, Wallisellen, CH) using a Turner luminometer (Turner Design, Sunnyvale, CA). Data were normalized for Renilla luciferase activity used as control for transfection efficiency.

**PPARδ Half-life**—U2OS cells were grown in 100-mm dishes to 90% confluence. Cells were transfected with 3 µg of His-PPARδ expression vector. The next day cells were plated into 6-well plates at a concentration of 3.5 × 10⁵ cells/well. After overnight incubation with or without a PPARδ ligand, cells were treated with 50 µM puromycin and harvested at the indicated times for Western blotting.

**PPARδ Ubiquitination**—U2OS cells were transfected with His-PPARδ and HA-UB and incubated with proteasome inhibitors and/or PPARδ ligands. After 24 h cells were lysed in a denaturing buffer consisting of 8 M urea, 0.1 M Na₂HPO₄/NaH₂PO₄, and 10 mM imidazole, pH 8.0. His-PPARδ was pulled down using His-select nickel affinity gel (Sigma) starting with ~100 µg of protein from cell lysates. After multiple washes with urea buffer, proteins were eluted from the beads with the same buffer supplemented with 250 mM imidazole, pH 6.0. Aliquots of whole lysate and flow-through samples corresponding to 15 µg of proteins and an equivalent fraction of the eluates were loaded on gels and analyzed by Western blotting.

**Western Blotting**—Cells were lysed in a buffer containing 25 mM Tris-HCl, pH 7.4, 150 mM KCl, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, and protease inhibitors mixture (Roche Applied Science). To isolate nuclear and cytoplasmic fractions, cells were lysed in 10 mM Tris-HCl, pH 8.0, 7.5 mM ammonium sulfate, 1 mM EDTA, 0.025% Nonidet P-40, and 1 mM dithiothreitol as previously described (32). After incubation on ice for 5 min, sucrose (0.3 M final concentration) was added to the cell homogenate, and the cellular fractions were separated by centrifugation at 4000 × g for 10 min at 4 °C. Protein concentration was determined using a BCA assay (Pierce). Proteins were loaded on 10% polyacrylamide gels and analyzed by immunoblotting with antibodies against PPARδ (H-74, Santa Cruz Biotechnology, Santa Cruz, CA), tubulin (DM1B, Calbiochem), His tag (H1029, Sigma), FLAG tag (M2, Sigma) and HA tag (Roche Applied Science). Horseradish peroxidase-conjugated secondary antibodies and the enhanced chemiluminescence (ECL) detection system (Amersham Biosciences) were used for detection. Band intensity was assessed with the Alphalmer 3400 and AlphaEase Software (Alpha Innootech, San Leandro, CA).
and further purified with RNeasy MiniKit (Qiagen). RNA concentration was determined using a NanoDrop spectrophotometer. RT-PCR was performed using the SuperScript One-Step RT-PCR system (Invitrogen) and gene-specific primers for the adipose differentiation-related protein (ADRP), glycerolaldehyde-3-phosphate dehydrogenase, and PPARδ. PCR products were run on 2% agarose gels, stained with ethidium bromide, and visualized using the AlphaImager 3400. Band intensity was determined using the AlphaEase software. For quantitative real time RT-PCR, 1 μg of total RNA was reverse-transcribed using the SuperScript First-Strand Synthesis system (Invitrogen). Real time PCR was performed on a 7900HT Fast Real Time PCR System (Applied Biosystems, Foster City, CA) using primer sets for ADRP and β-actin and Absolute SYBR Green ROX Mix (ABgene, Epsom, UK). Standard curves were generated for each primer set, and β-actin RNA was used as control to quantify ADRP RNA. Sequences of primers and small interfering RNAs are available as supplemental Table S1.

RESULTS

PPARδ Ligands Block Receptor Ubiquitination

PPARδ Ligands Stabilize the Receptor—Ligand-dependent transactivation of nuclear receptors is often associated with proteasome-mediated degradation of the receptor (33, 34). Ligand-induced proteolysis serves as a common mechanism to terminate transcriptional activity of the ligand-activated receptors (33, 34). To determine whether PPARδ was subject to a similar ligand-dependent control, human lung cancer cell lines expressing different levels of the receptor were incubated overnight with a PPARδ-selective ligand. PPARδ protein level increased after incubation with L165041 in cells expressing high (H441) and low (H358) levels of the receptor, whereas there was no effect in A549 cells in which PPARδ was undetectable in the immunoblots both before and after ligand treatment (Fig. 1A). The effect of the ligand on the receptor levels correlated well with the ligand ability to transactivate the PPARδ-responsive reporter in these cells (Fig. 1B). Ligand treatment increased reporter activity ~13- and 5-fold in H441 and H358 cells, respectively. The effect on A549 and U2OS cells was minimal (~2-fold), in agreement with the very low levels of PPARδ in these cells.

To better differentiate between transcriptional and post-translational effects, in subsequent experiments we used U2OS cells expressing recombinant PPARδ from a heterologous promoter. Increased levels of PPARδ were seen when PPARδ-expressing U2OS cells were treated with synthetic agonists (i.e. GW501516 and L165041) and a stable prostaglandin analogue (cPGI2 (carbobrostacyclin)) that binds to PPARδ (Fig. 1, C and D). On the other hand treatment of cells with prostaglandin E2, a prostaglandin that activates PPARδ indirectly and does not bind to the receptor (19), did not have any effect on the receptor protein level, suggesting that direct binding to the receptor was required to increase its stability. A time course experiment in PPARδ-expressing U2OS cells showed that the ligand acted rapidly (Fig. 1D). A substantial increase of PPARδ (~2-fold) was seen already after 4 h, and the levels continued to increase (up to ~4-fold) after 24 h. The effect on the protein level was reversible, and PPARδ returned to base-line levels within 4 h after the removal of the ligand (Fig. 1E). The effect seen in cells express-
His-PPARδ expression vector and monitored protein level by Western blotting after the addition of the protein synthesis inhibitor puromycin. His-tagged PPARδ level was greatly reduced within 1 h of puromycin treatment (Fig. 2A). The estimated PPARδ half-life under these conditions was ~30 min (Fig. 2C). Next, His-PPARδ expressing U2OS cells were incubated with puromycin in the presence of L165041 to determine the effects of the ligand on the receptor half-life. Incubation with L165041 increased PPARδ protein level and completely prevented the decline induced by puromycin (Fig. 2, B and C). Thus, the ligand increased the receptor protein level by extending its half-life considerably.

PPARδ Turnover Is Controlled by the Ubiquitin-Proteasome Pathway—The 26 S proteasome is the major cellular complex responsible for the degradation of proteins, including nuclear receptors and transcription factors (28–31). To determine whether proteasome-mediated degradation played a role in controlling the turnover of PPARδ, H441 and H358 cells expressing, respectively, high and low levels of the receptor were treated with the proteasome inhibitor PS341. In both cell lines PPARδ protein level increased, consistent with reduced degradation of the receptor in the presence of the proteasome inhibitor (Fig. 3A). Similar results were obtained by treating cells with another proteasome inhibitor, MG132 (data not shown). To further study the role of the 26 S proteasome in PPARδ turnover, U2OS cells expressing recombinant His-PPARδ were treated with PS341, and protein level was determined at different times by Western blotting. As shown in Fig. 3B, treatment with PS341 led to a significant accumulation of PPARδ (~3-fold) already after 4 h of incubation. Next, U2OS cells were treated either with puromycin, PS341, or both compounds together (Fig. 3C). PPARδ level decreased rapidly in the presence of puromycin, whereas treatment with puromycin and PS341 resulted in a higher level of the receptor, indicating that PPARδ turnover was under the control of the proteasome.

A necessary step for targeting proteins to the proteasome is the covalent attachment of ubiquitin (Ub) chains catalyzed by Ub ligases (28). To determine whether PPARδ was ubiquitinated, U2OS cells were transfected with His-PPARδ and HA-Ub expression vectors and then incubated with a proteasome inhibitor for 4 h. His-tagged PPARδ was pulled down with nickel affinity gel under denaturing conditions. PPARδ was detected in cell lysate, flow-through, and eluate fractions using an anti-His antibody, whereas ubiquitinated proteins were detected with an anti-HA antibody.
revealed limited specificity in preliminary immunoprecipitation experiments. In addition, it used highly stringent conditions to minimize non-covalent interactions of the receptor with potential ubiquitinated protein partners. Whole cell lysate and flow-through samples were examined along with the eluates to control for quantitative recovery of His-PPARα throughout the procedure (Fig. 3D, lanes 1–4). The specificity of the pulldown was demonstrated using cells transfected with His-tag empty vector (lanes 1, 3, and 5). In His-PPARα-expressing cells, high molecular weight protein species were detected with the anti-HA antibody indicating the presence of PPARα with covalently linked poly-Ub chains (lane 6). No such bands were detected in the eluate from empty vectortransfected cells (lane 5).

Proteasome activity is required for receptor turnover, and its inhibition generally leads to reduced transcriptional activity of nuclear receptors (34, 35). To determine whether a functional proteasome was required for PPARα transcriptional activity, U2OS cells stably expressing recombinant PPARα and H441 cells were transfected with PPARα-responsive reporter and incubated with PS341 in the presence or absence of a PPARα ligand (Fig. 4A). GW501516 increased reporter activity ~6 and 12-fold in U2OS and H441 cells, respectively. PS341 induced a ~2–3-fold increase in the absence of the ligand in both cell lines. The combination of PS341 and ligand increased reporter activity by ~13 and 26-fold in U2OS and H441, respectively. Thus, inhibition of proteasome activity did not negatively affect ligand-dependent activation of PPARα. Ligands and proteasome inhibitors had a positive and apparently synergistic effect on the activity of both recombinant and endogenous PPARα in the reporter assays. To determine whether ligands and proteasome inhibitors had similar effects on transcription of endogenous PPARα target genes, H358 and H441 cells were treated as described above, and the level of the ADRP RNA was monitored by real time RT-PCR or conventional RT-PCR. The ADRP gene contains a PPRE, and its transcription is activated by ligand-dependent activation of PPARα. Ligands and proteasome inhibitors led to the accumulation of ADRP RNA levels (by ~10 and 25-fold in H358 and H441 cells, respectively). This effect was enhanced in the presence of the proteasome inhibitor (up to ~25 and 100-fold in H358 and H441, respectively) (Fig. 4B). Neither the ligand nor proteasome inhibitor affected endogenous PPARα RNA levels in H358 and H441 cells, confirming that they acted post-transcriptionally, increasing the protein level of endogenous PPARα (Fig. 4C and data not shown). The specificity of these effects was further assessed by RNA interference. The increase induced by the PPARα ligand both alone and in combination with PS341 was attenuated in cells in which PPARα had been silenced (up to 75%) by a small interfering RNA against PPARα before the incubation with GW501516 and/or PS341 (Fig. 4C). Despite the evident reduction of PPARα RNA, ligand-dependent activation of ADRP transcription was reduced only partially by RNA interference because of the stabilizing effect of the ligand and proteasome inhibitor at the protein level. Taken together, these data indicate that inhibition of the proteasome did not reduce PPARα activity but, along with PPARα ligands, led to the accumulation of transcriptionally competent receptor. To determine whether the accumulated protein had also the proper subcellular localization, PPARα-expressing U2OS cells were treated with PS341 or a selective ligand before isolation of the nuclear and cytoplasmic fractions (Fig. 4D). Although a small amount was found in
the cytoplasm, PPARδ was detected predominantly in the cell nucleus of both PS341 and ligand-treated cells.

Ligands Prevent Proteasomal Degradation of PPARδ by Blocking Its Ubiquitination—Ligand-induced stabilization of PPARδ might be the consequence of inhibition of proteasome-mediated degradation. To exclude the possibility that PPARδ ligands might affect activity of the 26 S proteasome, U2OS cells expressing a Ub-enhanced green fluorescent protein fusion protein were treated with ligands or PS341 as a positive control and analyzed by flow cytometry. Although PS341 induced the expected accumulation of Ub-enhanced green fluorescent protein (EGFP), none of the PPARδ ligands changed Ub-EGFP levels (supplemental Fig. S2), suggesting that the ligands did not inhibit the 26 S proteasome and might affect PPARδ protein turnover by acting directly on the receptor.

To determine whether PPARδ ligands modulated the ubiquitination or proteasomal degradation of the receptor, we treated U2OS cells transfected with HA-Ub and His-PPARδ expression vectors with L165041 in the presence and absence of PS341. If the ligand blocked proteasomal degradation of the receptor, one would expect accumulation of ubiquitinated PPARδ. On the contrary, a block of ubiquitination would lead to reduced amounts of ubiquitinated PPARδ in the presence of ligand. As expected, L165041 and PS341 alone and in combination increased PPARδ in the cell lysates (Fig. 5A, top panel). However, although PS341 led to the accumulation of ubiquitinated PPARδ, treatment with the ligand both in the presence and absence of PS341 led to a reduction of the amount of ubiquitinated PPARδ (Fig. 5A, bottom panel, lanes 2 and 4) compared with control and PS341-treated cells (lanes 1 and 3). Thus, the ligand selectively affected the ubiquitination of the receptor rather than its proteasomal degradation. The effect of the ligand on PPARδ ubiquitination was reversible and depended on its continuous presence in the culture medium as seen before with the effect on PPARδ protein level (Fig. 1E). Removal of the ligand restored the ability of the cells to ubiquitinate the receptor within 4 h, concomitant with the decrease of the receptor level (Fig. 5B). To confirm that ligand binding was required to prevent PPARδ ubiquitination, a similar experiment was done with a truncated form of the receptor (PPARδ-(1–299)), which missed most of the ligand binding domain. Disruption of this domain abolished the effects of the ligand on PPARδ ubiquitination and stabilization (Fig. 5C). Thus, direct interaction of the ligand with the receptor was absolutely required for inhibition of PPARδ ubiquitination.

Ligand-induced Stabilization of PPARδ Involves the DNA Binding Domain of the Receptor—Like other nuclear receptors, PPARδ has well defined structural and functional domains (1). To understand the role played by distinct domains in ligand-induced stabilization of the receptor, we introduced inactivating mutations in the DNA binding domain (C91A/C94A), in the AF-2 transactivating domain (L432A/E435A), and within the region (F270A) known to be responsible for co-repressor binding (Fig. 6A). Mutations in the DNA binding domain (C91A/C94A) were similar to those known to prevent binding of murine PPARδ to DNA (37). The double mutant in the AF-2 domain (L432A/E435A) and the F270A mutation blocked the interaction of murine PPARδ with coactivators (38) and corepressors (37), respectively. The ability of the mutated receptors to activate a PPAR-responsive reporter was tested in luciferase assays along with wild type PPARδ (Fig. 6B). The DNA binding...
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A. PPARδ structural domains and positions of the mutations introduced in the wild type receptor. AF-1, activation function-1; DBD, DNA binding domain; Hinge, flexible hinge region; LBD, ligand binding domain; AF-2, activation function-2. B. U2OS cells were transfected with vectors encoding wild type PPARδ or mutated receptors along with PPREEx3-tk-Luc and pRL-SV40 reporter vectors. Cells were incubated overnight with or without L165041 (5 μM), and then luciferase activity was assessed as described in the legend for Fig. 1. Data represent fold increase (mean ± S.D. of triplicate experiments) relative to empty vector-transfected and untreated cells.* p ≤ 0.005 compared with control cells. ctrl, control. C. U2OS cells expressing wild type or mutated PPARδ were incubated with L165041 overnight and analyzed by immunoblotting using an anti-His antibody. Pull-down experiments were performed to detect ubiquitinated His-PPARδ as described in the legend for Fig. 3. D. PPARδ levels in cells transfected with wild type or mutated PPARδ and incubated with or without L165041 assessed by immunoblotting and densitometric analysis. Data (mean ± S.D. of triplicate experiments) represent the percentage of the band intensity relative to the corresponding untreated control.

**FIGURE 6. Structural requirements for ligand-induced stabilization of PPARδ.** A, PPARδ structural domains and positions of the mutations introduced in the wild type receptor. AF-1, activation function-1; DBD, DNA binding domain; Hinge, flexible hinge region; LBD, ligand binding domain; AF-2, activation function-2. B, U2OS cells were transfected with vectors encoding wild type PPARδ or mutated receptors along with PPREEx3-tk-Luc and pRL-SV40 reporter vectors. Cells were incubated overnight with or without L165041 (5 μM), and then luciferase activity was assessed as described in the legend for Fig. 1. Data represent fold increase (mean ± S.D. of triplicate experiments) relative to empty vector-transfected and untreated cells.* p ≤ 0.005 compared with control cells. ctrl, control. C, U2OS cells expressing wild type or mutated PPARδ were incubated with L165041 overnight and analyzed by immunoblotting using an anti-His antibody. Pull-down experiments were performed to detect ubiquitinated His-PPARδ as described in the legend for Fig. 3. D, PPARδ levels in cells transfected with wild type or mutated PPARδ and incubated with or without L165041 assessed by immunoblotting and densitometric analysis. Data (mean ± S.D. of triplicate experiments) represent the percentage of the band intensity relative to the corresponding untreated control.

The ability of ligands to stabilize wild type and mutated receptors was tested in transiently transfected U2OS cells. The domain mutant (C91A/C94A) was less active than the wild type receptor in the reporter assay. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutant in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability. The mutation in the putative co-repressor binding site (F270A) had a minimal effect on receptor stability.
Ub-processing enzymes (28, 31). Ubiquitinated proteins are generally marked for rapid degradation by the proteasome (28). However, they can be rescued by the action of deubiquitinating enzymes that remove Ub chains before proteolytic attack (40). Deubiquitinating enzymes receive increasing attention since they can participate, along with E3 Ub ligases, in ubiquitination-deubiquitination cycles capable of rapidly modulating the level of target proteins (40). Ligand-induced degradation of nuclear receptors is probably operated by Ub ligases and proteasomal subunits present as integral components of transcription regulatory complexes and acting within the cell nucleus (29, 30, 33, 34). Proteasome-dependent proteolysis serves to ensure both nuclear receptor turnover and timely termination of the transcriptional response (34, 35). Accordingly, inhibition of proteasome activity generally leads to reduced transactivation by nuclear receptors (34, 35).

In this study we observed that PPARδ was ubiquitinated and rapidly degraded by the 26 S proteasome. Brief incubation of cells expressing both endogenous and recombinant PPARδ with proteasome inhibitors led to a rapid increase of the receptor. Upon proteasome inhibition most of the receptor was in the cell nucleus similar to control and ligand-treated cells. Moreover, it was transcriptionally competent as shown by luciferase reporter assays and direct assessment of the level of an endogenous target gene. PPARδ behavior in this regard was quite different compared with other nuclear receptors, including the estrogen-, androgen-, thyroid hormone-, and retinoic acid receptors, whose transcriptional activity is reduced upon proteasome inhibition (34). Furthermore, although in the absence of ligand, PPARδ had a very short half-life of ~30 min, the addition of ligand considerably increased its half-life. The effect of the ligands was rapid and specific for compounds able to bind and activate the receptor directly. PPARδ protein level increased within 4 h after the addition of ligand to cells and remained high as long as the ligand was present in the culture medium. Removal of the ligand was followed by rapid reversal of the effect with return to the base-line level within 4 h. Also in this regard PPARδ behavior was almost unique among nuclear receptors, which generally are negatively regulated by their own ligands (34). Only vitamin D3 receptor has been previously shown to be stabilized by the ligand with similar kinetics (41). PPARγ was degraded rather rapidly upon exposure to ligands (42), whereas PPARα was stabilized in the presence of ligands but only transiently (43, 44). An increase of PPARα protein level was seen within 3 h and was followed by rapid proteolysis upon continuous exposure to ligands (43).

Thus, the system in place for PPARδ may be geared to prevent both accumulation of high levels and prolonged activation of the receptor. It is possible that overactivity of PPARδ may be detrimental to cells, owing perhaps to the anti-apoptotic function and oncogenic potential associated with this nuclear receptor. The level of PPARδ must be kept low and under constant control via UPS-dependent proteolysis. Only in the presence of high concentrations of the specific ligands PPARδ would be stabilized and activated. Furthermore, the transcriptional response mediated by the ligand-bound receptor would persist only as long as the ligand is present. Under physiological conditions, termination of the transcriptional response would be ensured by the short half-life and low abundance of natural PPARδ ligands, like prostaglandin I2. This hypothesis would be consistent with the observation that in processes such as wound healing, there is a sharp increase of PPARδ level concomitant with increased production of ligands (45). Also in cancer, up-regulation of PPARδ is apparently coordinated with up-regulation of cyclooxygenase-2 and consequent increased production of prostaglandin metabolites capable of stabilizing and activating PPARδ (10, 17). In the absence of this coordinated increase of ligand and receptor levels, PPARδ would not be able to exert its anti-apoptotic and growth-promoting functions.

Mechanism of Ligand-induced Stabilization of PPARδ—Ligand-induced stabilization of PPARδ was due to a selective block of receptor ubiquitination and not to interference with later steps of the proteolytic process, i.e. binding to and degradation by the proteasome. Like protein stabilization, reduced ubiquitination of PPARδ depended on the continuous presence of the ligand and was rapidly reversed after ligand removal. Disruption of the ligand binding domain in PPARδ (1–299) abolished the effect of the ligand on PPARδ ubiquitination and proteolysis, although the truncated form of the receptor was still ubiquitinated and degraded by the proteasome. This result supported the idea that ligand-induced stabilization of PPARδ was due to the direct interaction of the ligand with the receptor. Ligand binding may induce conformational changes that, in addition to allow co-activator binding and transactivation, block the interaction of PPARδ with Ub ligases or, alternatively, promote the activity of deubiquitinating enzymes. We are currently testing these hypotheses and attempting to identify the enzymes involved in ligand-dependent control of the PPARδ ubiquitination. Ub-processing enzymes involved in this process may represent critical elements in the pathways regulating the activity of this nuclear receptor.

Using site-directed mutagenesis we investigated the role of distinct receptor domains in the ligand-dependent regulation of PPARδ stability. This analysis also revealed additional differences between PPARδ and the other PPAR isotypes as far as the mechanisms involved. Mutations in the DNA binding domain reduced the effect of the ligand on receptor ubiquitination and protein level. This indicated that the ligand acted preferentially on DNA-bound receptor molecules preventing their ubiquitination. Interestingly, mutations in DNA binding domain of PPARγ had no effect, and DNA binding of this receptor was not a pre-requisite for ligand-induced degradation (42). Furthermore, we show that the AF-2 domain and the co-repressor binding site were not required for ligand-induced changes in PPARδ ubiquitination, indicating that the ligand effect was independent of co-activator and co-repressor binding. It is intriguing that the AF-2 domain of PPARδ did not have any role in this process since for most nuclear receptors the trans-activation function is tightly linked to proteolytic degradation and mutations in the trans-activating domain are expected to affect both ubiquitination and proteolysis (34, 35). This is also in contrast with the established role of the AF-2 domain in ligand-dependent degradation of PPARγ (42). The AF-2 domain of PPARγ mediated ligand-induced degradation of the receptor, suggesting that conformational changes induced by the ligand favored the interaction with both co-activators and the UPS.
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FIGURE 7. Model of ligand-dependent regulation of PPARα activity. In the absence of ligand, PPARα is bound to DNA as a heterodimer with retinoic X receptor forming a complex with co-repressor molecules and thereby functioning as a transcriptional repressor. PPARα can be ubiquitinated by ubiquitin ligases (Ub ligases) probably residing within the transcription regulatory complex, and its turnover is regulated by proteasome-dependent proteolysis. PPARα-selective ligands induce a conformational remodeling of the DNA-bound receptor that allows binding of co-activator molecules and displacement of co-repressors. Ligand-induced remodeling and block of ubiquitin ligases lead to increased stability of DNA-bound PPARα and promote transcriptional activation. Alternatively, deubiquitinating enzymes (DUB) may be activated upon ligand binding, resulting in reduced ubiquitination of the receptor. Deubiquitination of PPARα may also assist in switching between the distinct states of the receptor, favoring dissociation of co-repressor complexes or assembly of co-activator complexes.

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(42). Using a different approach, co-expression of the transcriptional co-activator CBP (cAMP-response element-binding protein (CREB)-binding protein) led to a decrease of PPARα level in the presence of ligand, showing that the interaction with co-activators via the AF-2 domain promoted proteolysis of the PPARα isotype (46). Thus, in the case of PPARα, the initial stabilization induced by ligands is probably followed by recruitment of co-activators along with other co-factors that trigger proteolysis of the receptor (43, 46). In contrast, our data show that transactivation and receptor ubiquitination are physically and functionally separated in the case of PPARα. It is probably the absence of a physical link between these processes that permits independent regulation of the transactivating function and ubiquitination of PPARα upon ligand binding.

Taken together, our data support a model of ligand-dependent regulation of PPARα activity that is in part different from what has been proposed for PPARγ and -α (Fig. 7). Under normal conditions, both unbound and DNA-bound PPARα may undergo constitutive ubiquitination and degradation to maintain low levels of the receptor in the absence of ligands. UPS-mediated proteolysis of the unliganded receptor may serve to control overall receptor level and, particularly, ligand-independent functions. In the absence of ligands, PPARα can regulate transcription by forming complexes with transcriptional repressors, like SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) (37) and Bcl-6 (36). We propose that upon ligand binding the DNA-bound receptor is protected from proteasome-dependent proteolysis by selective inhibition of receptor ubiquitination. This can be accomplished by blocking Ub ligases or, alternatively, activating deubiquitinating enzymes. The increased half-life of the DNA-bound receptor in the presence of ligand would allow time for transactivation of target genes to occur. Therefore, it is important that transactivation is functionally dissociated from ubiquitination of the receptor. In fact, this separation allows ligand binding, possibly via a conformational change independent of the AF2 domain and co-activator binding, to block ubiquitination of DNA-bound PPARα. This condition may be essential to prevent degradation of the DNA-bound receptor and elicit a proper transcriptional response. Receptor ubiquitination may also have effects independent of the proteolytic pathway and may assist in switching between the repressive and activated state of the receptor, perhaps favoring either dissociation or assembly of co-repressor and co-activator complexes.
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