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Stabilization of Peroxisome Proliferator-Activated Receptor α by the Ligand

Masaki Hirotani, Toshiro Tsukamoto, Jessie Bourdeaux, Hiroyuki Sadano, and Takashi Osumi[1]
Department of Life Science, Faculty of Science, Himeji Institute of Technology, 3-2-1 Koto, Kamigori, Hyogo 678-1297, Japan

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Peroxisome proliferator-activated receptor (PPAR) constitutes a subfamily among a large group of ligand-activated transcription factors, the nuclear receptor superfamily. We studied the effects of ligand on the intracellular behaviors of PPARα. Although nuclear localization of PPARα was not affected by a selective ligand, Wy14643, we observed that exogenously expressed PPARα was rapidly degraded in HeLa cells, and the ligand significantly stabilized the protein. The stability of PPARα was also improved by coexpression of the heterodimer partner retinoid X receptor (RXR) α, and further stabilization was not observed with the ligand. These results indicate that PPARα is stabilized through heterodimerization with RXR, and the excess protein unpaired with RXR is rapidly turned over, if not bound by an appropriate ligand. These observations on PPARα are in sharp contrast to the ligand-stimulated degradation reported on PPARγ. The ligand-dependent stabilization would have physiological significance when the synthesis of PPARα is elevated exceeding the available level of RXR.

Key Words: **peroxisome proliferator-activated receptor; retinoid X receptor; PPAR; RXR; ligand; nuclear hormone receptor.**

PPAR constitutes a subfamily of nuclear hormone receptor superfamily. PPARα is mainly involved in the regulation of lipid metabolism (1), whereas PPARγ has versatile functions including stimulation of adipocyte differentiation (2) and regulation of macrophage functions (3). No clear regulatory target is known for PPARδ.

PPAR family accepts a wide variety of ligands of highly diverged structures. The ligands bind to the carboxy-terminal ligand-binding domain that is functionally separable from the DNA-binding domain. Upon binding of the ligands, conformation of the former domain changes, causing rearrangement of the orientation of a carboxy-terminal helix (4–7). This allows the association of coactivators, hereby leading to the activation of target genes.

Ligands also cause important biological changes in the behaviors of nuclear receptors. Nuclear translocation of glucocorticoid receptor is strictly dependent on the ligand (8), whereas that of many other nuclear receptors seems ligand-independent [see (9)]. For PPARα, conflicting results were reported on the ligand-dependence of nuclear translocation (10, 11). Ligands also affect the protease sensitivity of the nuclear receptors, through the change in protein conformation. PPARγ is degraded by proteasome in adipose cells when activated by a ligand, and this has been postulated to have an important regulatory role (12). Proteasome-dependent degradation in the presence of ligands is also known for other nuclear receptors (13–17). On the other hand, ligand-mediated change in the stability *in vivo* is not known for PPARα, though alteration in the proteolytic digestion pattern *in vitro* was reported (18).

In this study, we expressed green fluorescent protein (GFP)- or hemagglutinin (HA)-tagged version of PPARα in HeLa cells, and followed their behavior in either the presence or absence of ligand. We observed that the ectopically expressed PPARα is rapidly degraded, but significantly stabilized by the ligand.

MATERIALS AND METHODS

Plasmids. For constructing the GFP fusion protein of mouse PPARα, we used GFP105, a mutant version of GFP having an improved fluorescence intensity at 37°C (19). An expression vector of HA-tagged protein, pHA-C1, was created from pGFP–C1 (Clontech), by replacing the GFP-coding portion with an oligonucleotide encoding the HA peptide. Both PPARα-fusion proteins contained a linker peptide of nine amino acid residues, derived from the 5′ noncoding region of PPARα cDNA, between the amino-terminal tags and carboxy-terminal PPARα sequence.

Abbreviations used: PPAR, peroxisome proliferator-activated receptor; RXR, retinoid X receptor; GFP, green fluorescent protein; HA, hemagglutinin; FBS, fetal bovine serum; RT, reverse transcription.

[1] To whom correspondence and reprint requests should be addressed. Fax: +81-791-58-0193. E-mail: osumi@sci.himeji-tech.ac.jp.

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Cell culture and transfection. HeLa cells were used throughout the study. The cells were cultured in Ham's F12 medium containing 10% fetal bovine serum (FBS). For electroporation, trypsinized cells were suspended in phosphate-buffered saline at the density of 5×10^5 cells/ml. To each electroporation cuvette, 400 μl of cell suspension was added, together with 10 μg of the plasmid to be tested. After applied with an electric pulse at the settings of 250 V and 400 μF, the cells were divided into two equal portions, and each cultured in a 35-mm dish, where a glass cover slip was placed at the bottom. Cultures were performed with or without 100 μM of a peroxisome proliferator, WY14643, which is a selective PPAR ligand (20). For some experiments to show clearer ligand-dependence, FBS was extracted with *n*-heptane and further treated with charcoal and an ion-exchange resin, AG1-X8 (Bio-Rad). The resulting ultrastripped serum was substituted for the usual FBS after transfection.

Transfection by calcium phosphate method was performed as described (21). For usual experiments, 1 μg of the expression vectors of PPARα fusion proteins was used per dish, while the total amount of DNA was kept at 6 μg per dish, using an empty vector, pCMX. After removal of the calcium phosphate precipitates, cells were cultured in the presence or absence of 100 μM WY14643.

Measurement of the expression of PPARα fusion proteins. Fluorescence microscopy was done as described (22). HA-PPARα was detected by immunofluorescence staining, using a rat monoclonal anti-HA IgG as the primary antibody.

Western blotting was performed, using rabbit polyclonal anti-GFP serum or mouse monoclonal anti-HA IgG as the primary antibody. Bands were detected using an ECL kit (Amersham), according to the protocol recommended by the supplier.

Reverse transcription (RT)-PCR. Total RNA was prepared from the transfected HeLa cells by the guanidine thiocyanate method. For the detection of GFP–PPARα, an upstream primer 5′-CCAGAATTCATGGTGGACACAGAGAGCCCC-3′ and a downstream primer 5′-CGTTGATCACTCGATGTTCAGGGCACTGCC-3′ were used. This primer pair amplifies a 322-bp cDNA fragment corresponding to the A/B domain of PPARα (21). For the correction of transfection efficiency, a β-galactosidase expression vector, pCMVβ, was cotransfected. For amplifying β-galactosidase cDNA, an upstream primer 5′-CCAGAATTCCTTAATCGCCTTGCAGCACAT-3′ and a downstream primer 5′-CGTTGATCAGACGTCACGGAAAATGCCGCT-3′ were used. RT was performed using a mixture of the downstream primers, and PCR was done using a mixture of all primers to amplify both cDNAs simultaneously. The reaction conditions were as described, and from the 50-μl reaction mixtures, 5-μl aliquots were withdrawn after 21st, 24th, and 27th cycles and analyzed by agarose gel electrophoresis.

Pulse-chase experiment. HeLa cells seeded in 35-mm dishes were transfected with 5 μg of the GFP–PPARα expression vector by calcium phosphate method, and cultured for 12 h. Medium was changed to Dulbecco's modified Eagle medium without methionine and cysteine, while containing 10% dialyzed FBS. The cells were labeled with 50 μCi of [^{35}S]methionine/cysteine (Perkin–Elmer Life Sciences) for 1 h. Medium was then changed to Ham's F12 containing the normal FBS, and cultured for 1, 3, or 6 h. Cells were lysed, GFP–PPARα immunoprecipitated and analyzed by SDS–PAGE, as described (23), except that an anti-GFP antibody was used. Cells were cultured with or without 100 μM WY14643, throughout the period after transfection.

RESULTS

Ligand-Independent Nuclear Localization and Ligand-Dependent Accumulation of PPARα

HeLa cells were transfected by electroporation with the expression vector of GFP–PPARα, and observed 6 h later by fluorescence microscopy. It was essential to perform the observation at such an early point, because the accumulation of the protein was poorer at later points (see below). The expressed fusion protein virtually all localized in the nuclei, both in the presence and absence of the ligand, Wy14643 (Figs. 1A–1D). HA-PPARα was also distributed solely in the nuclei, even without a ligand (data not shown). Similar results were obtained using the ultrastripped FBS, which was highly depleted of hydrophobic or acidic substances (data not shown). Thus, we did not obtain evidence for the ligand dependence of nuclear translocation of PPARα. At higher magnification, heavy aggregates containing GFP were often seen in the nuclei, in the absence, but not presence of ligand (Figs. 1E and 1F), probably reflecting the ligand-dependent conformational change. Such an aggregate was not observed for HA-PPARα (data not shown).

We were aware that the percentage of the cells expressing GFP–PPARα was abnormally low. Indeed, it was difficult to find an expressing cell in every microscopic field at lower magnification, 24 h after transfection. In our experience, electroporation under similar conditions usually gave transfection efficiency of at least 50%. We found that more cells were positive at earlier points, and addition of the ligand significantly improved the ratio of GFP-positive cells (compare Figs. 1A and 1C). Fluorescence intensity of individual expressing cells was also increased with the ligand. Ligand-dependent increase in the proportion of positive cells was evident upon counting the cells in microscopic fields (Fig. 1G). Similar results were obtained by calcium phosphate transfection, with other peroxisome proliferators, clofibrate and ciprofibrate, and also for HA-PPARα (data not shown). Thus, the ligand-dependent accumulation is an intrinsic character of PPARα.

Estimation of the Expression of PPARα Fusion Proteins

We determined the amounts of PPARα fusion proteins by Western blotting. Accumulation of GFP–PPARα was threefold higher in the presence than in the absence of ligand, 6 h after electroporation (Figs. 2A and 2B), being consistent with the microscopic observation. The accumulation increased up to 12 h, whereas it severely decreased 24 h after electroporation. Calcium phosphate transfection gave a similar result, though the accumulation of the protein was delayed, compared with the case of electroporation (data not shown). To show the effect of ligand more clearly, we used the ultrastripped FBS. As expected, the accumulation of GFP–PPARα was almost completely diminished in the absence of ligand (Fig. 2C). Ligand-dependent accumulation was also evident with HA-PPARα, though doublet bands were detected at

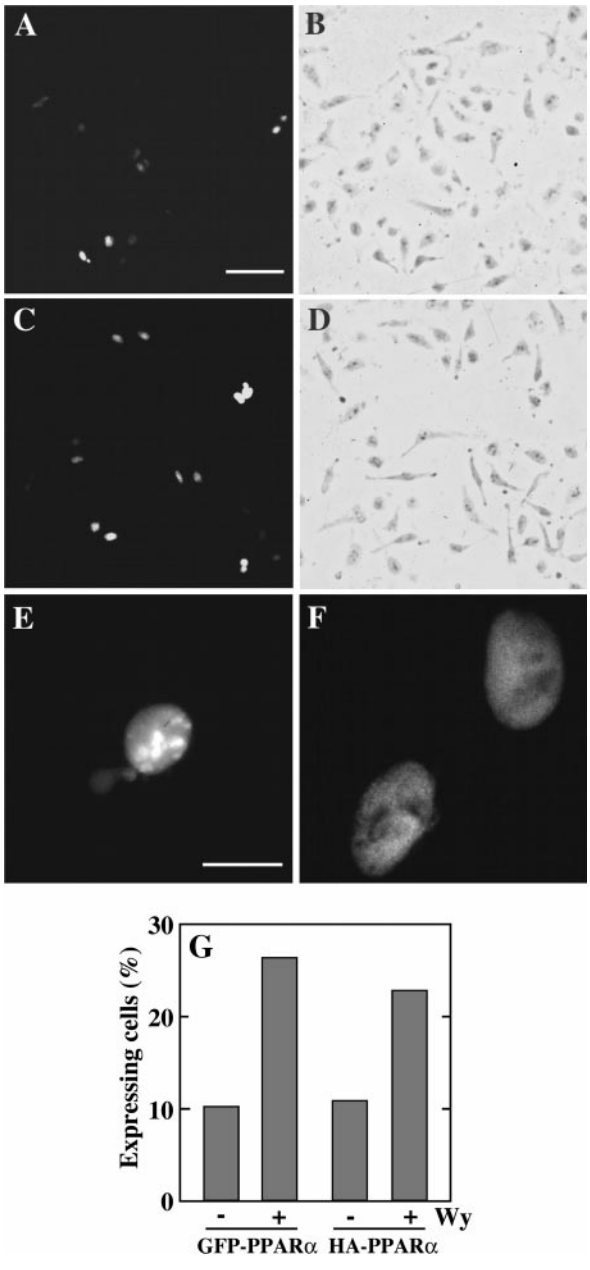

FIG. 1. Expression of GFP- and HA-tagged PPARα in HeLa cells in the presence and absence of ligand. Plasmids were introduced by electroporation, and cells were observed by a fluorescence microscope 6 h later. (A, C, E and F) Fluorescence micrographs; (B and D) phase-contrast micrographs of the fields identical with those of A and C, respectively. (A, B, and E) Cells cultured without the ligand; (C, D, and F) those with the ligand. Bar, 100 μm for A–D, and 20 μm for E and F. (G) Percentage of the cells expressing the PPARα fusion proteins 6 h after electroporation. Fluorescence-positive cells were counted among 1200 to 1600 cells for each sample. − and + indicate the cells cultured without and with Wy14643, respectively.

12 h, possibly reflecting partial clipping at the carboxy-terminus (Fig. 2D).

PPARα forms a heterodimer with RXR. Hence, we examined the effect of coexpression of RXRα on the accumulation of PPARα. Remarkably, a higher amount

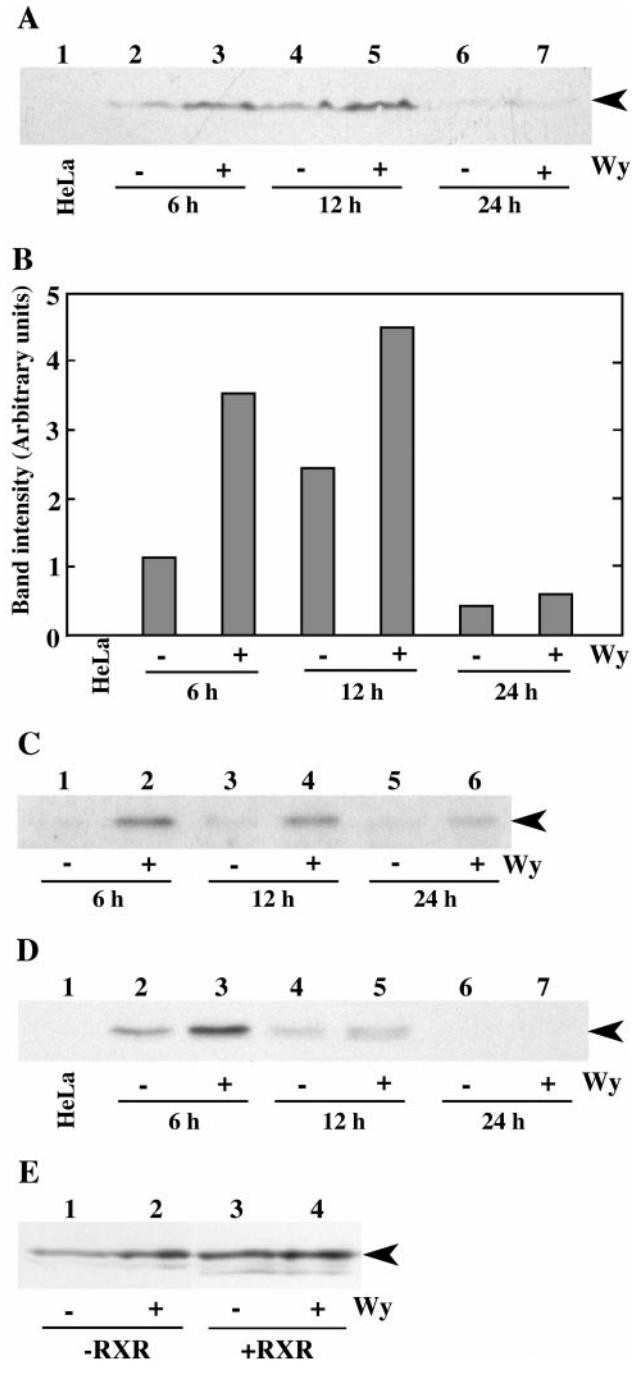

FIG. 2. Estimation of the PPARα fusion proteins by Western blotting. Each lane was applied with cell lysates corresponding to one-third of cells obtained from a dish. Cells were cultured for 6, 12, or 24 h after electroporation, in the absence (marked −) or presence (+) of Wy14643. Lane "HeLa" contained a lysate from untransfected HeLa cells. (A) GFP–PPARα expressed in the culture with normal FBS. (B) Band intensities of A, determined by analyzing the images with a NIH Image software. (C) GFP–PPARα and (D) HA-PPARα, both expressed in the culture with the ultrastripped FBS. (E) GFP–PPARα, coexpressed or not with RXRα, 12 h after transfection by calcium phosphate method. The amounts of two expression vectors were both 2 μg. Arrowhead indicates the PPARα fusion protein.

of GFP–PPARα accumulated in the presence of RXRα, and the ligand did not enhance the accumulation any more (Fig. 2E).

Ligand-Dependent Stabilization of PPARα

The GFP– and HA–PPARα fusion genes used in the present study were under the control of human cytomegalovirus promoter, of which function is unlikely to be affected by peroxisome proliferators. To confirm this, we estimated the mRNA of GFP–PPARα by RT-PCR (Fig. 3). As expected, the RNAs from the cells treated or not treated with the ligand exhibited indistinguishable intensities of bands at all PCR cycles, most importantly at cycle 21 when the reaction was not saturating (lanes 2 and 3).

We next examined the rates of synthesis and degradation of GFP–PPARα by a pulse-chase experiment (Fig. 4). After 1 h pulse, the fusion protein exhibited a slightly more intense band in the presence than in the absence of ligand (lanes 2 and 3). After 1, 3, and 6 h chase, the band intensity rapidly decreased, but the ligand retarded the decrease significantly (lanes 4–9). Thus, the PPARα ligand exerted a protective effect on GFP–PPARα against degradation.

DISCUSSION

The present results indicate that ectopically expressed PPARα is highly unstable, but the ligand significantly improves the stability. This effect seems to be due to conformational change caused by the ligand binding. Ligand-induced structural transition is the base of gene-activating functions of nuclear receptors (24), which also affects the susceptibility to proteases *in vitro*, as reported (18). Release of corepressors and/or binding of coactivators resulted by ligand binding may also be the cause of different protease sensitivity.

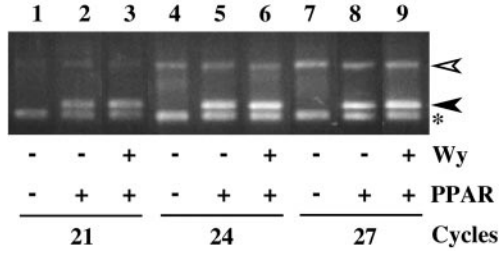

FIG. 3. Indistinguishable accumulation of the mRNA of GFP–PPARα either in the presence or absence of ligand. RNA was prepared from the cells cultured for 6 h after electroporation, in the absence (−) or presence (+) of Wy14643. Lanes 1, 4, and 7, cells transfected with an empty vector, pCMX, containing the cytomegalovirus promoter. All other lanes, cells transfected with the expression vector of GFP–PPARα. Filled arrowhead, GFP–PPARα; open arrowhead, β-galactosidase; *, nonspecific PCR product. Note that comparative intensities of β-galactosidase bands were observed for all samples, confirming the comparative efficiencies of transfection.

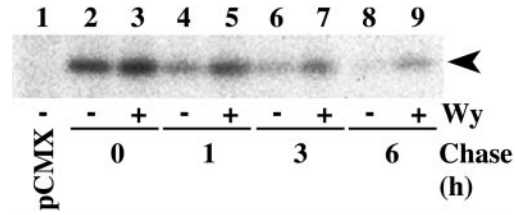

FIG. 4. Pulse-labeling and chase of GFP–PPARα in the presence or absence of ligand. Transfected cells were radiolabeled for 1 h (lanes 2 and 3), and chased for 1, 3, and 6 h, in the absence (marked −) or presence (+) of Wy14643. Lane 1, cells transfected with an empty vector, pCMX, and labeled for 1 h. Arrowhead indicates GFP–PPARα.

Stabilization of PPARα by the ligand is in sharp contrast with the case of PPARγ. PPARγ is turned over more rapidly in the presence of the ligand, thiazolidinedione, and this seems to have a regulatory significance in adipocyte functions (12). Ligands may cause stability or instability depending on the nuclear receptor species, though destabilization seems more general (13–17). We examined whether the ligand affected the amount of endogenous PPARα in rat hepatoma H4IIEC3. We failed to show an increase in the amount of PPARα, under the conditions (25) where acyl-CoA oxidase was induced (data not shown). This was possibly because the hepatoma cells contained enough RXR to associate with PPARα. The result of Fig. 2E suggests that PPARα complexed with RXR is stable, and the ligand has a protective effect only on the excess PPARα. The expression of PPARα is affected by glucocorticoid (26), and susceptible to diurnal rhythm (27). Ligand-dependent stabilization would have a regulatory importance when the expression of PPARα peaks and exceeds the available level of RXR.

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