Molecular and Cellular Biochemistry **286:** 33–42, 2006.
DOI: 10.1007/s11010-005-9052-z

Aspects of the regulatory mechanisms of PPAR functions: Analysis of a bidirectional response element and regulation by sumoylation

Makoto Shimizu,* Daisuke Yamashita,* Tomohiro Yamaguchi, Fumiko Hirose and Takashi Osumi

Graduate School of Life Science, Himeji Institute of Technology, University of Hyogo, Kamigori, Hyogo 678-1297, Japan

Received 1 November 2004; accepted 1 December 2004

Abstract

Peroxisome proliferator-activated receptors (PPARs) constitute a subfamily of nuclear receptor superfamily. A wide variety of compounds including hypolipidemic agents, antidiabetic drugs, and long-chain fatty acids are the potential ligands of PPARs. To approach the regulatory mechanisms of PPARs, we studied on two subjects in this work. First, we identified a functional PPAR-binding site in the spacer region between the PEX11α and perilipin genes, which are arranged in tandem on the mouse genome. By gene reporter assays and in vivo as well as in vitro binding assays, we show that these genes are regulated tissue-selectively through this common binding site: The PEX11α gene is activated by PPARα in the liver, whereas the perilipin gene by PPARγ in the adipose tissue. As the second subject, we found that PPARγ2 is conjugated with small ubiquitin-related modifier (SUMO) at a specific lysine residue in the amino-terminal region. By site-directed mutagenesis combined with gene reporter assays and sumoylation analyses, we show that sumoylation represses the ligand-independent transactivating function carried by this region, and hence negatively regulates the whole transactivating competence of PPARγ2. In addition, phosphorylation at a specific site in the amino-terminal region represses the transactivation by PPARγ2 possibly through enhancing sumoylation. (Mol Cell Biochem **286:** 33–42, 2006)

Key words: peroxisome proliferator-activated receptor, peroxisome, PPAR, PEX, perilipin, SUMO, transcriptional regulation

Introduction

The peroxisome proliferator-activated receptor (PPAR) subfamily is a branch of nuclear receptor superfamily [1]. PPAR, now named the α subtype, was first cloned as a nuclear receptor activated by hypolipidemic agents called peroxisome proliferators [2]. These compounds cause proliferation of peroxisomes in the liver when administered to rodents [3]. It is supposed that the activated PPARα stimulates the expression of certain genes that lead to the proliferation of peroxisomes as well as the enhancement of lipid-metabolizing activities.

PPAR binds to DNA as a heterodimer with another nuclear receptor, RXR [4]. The most preferable binding sequence is a direct repeat of AGGTCA (or its complement) half-sites spaced by one nucleotide [5] and carrying four extra conserved nucleotides on the 5′ side [6–8]. PPARα is particularly enriched in the liver, and stimulates the expression of genes for lipid metabolism such as mitochondrial and peroxisomal β-oxidation enzymes [1]. PPARγ, especially the γ2 isoform, is predominantly expressed in the adipose tissue, playing a key role in adipocyte differentiation [9]. PPARδ is also suggested to play important roles in the regulation of lipid

Address for offprints: T. Osumi, Graduate School of Life Science, Himeji Institute of Technology, University of Hyogo, Kamigori, Hyogo 678-1297, Japan (E-mail: osumi@sci.u-hyogo.ac.jp.)
*MS and DY equally contributed to this work.

metabolism [10, 11]. As a whole, the PPAR subfamily controls body lipid metabolism and mobilization [12]. A wide variety of hydrophobic compounds are known as potential PPAR ligands [13]. Peroxisome proliferators are selective ligands of PPARα, while the thiazolidinedione class of antidiabetic compounds are virtually specific for PPARγ [14–16]. Long-chain fatty acids are agonists of all PPAR subtypes, and possibly the important endogenous ligands of PPARs [16].

As described above, PPARα was first identified as a mediator of peroxisome proliferation in rat liver caused by hypolipidemic compounds. Although many PPARα target genes were found, none of the genes implicated in peroxisome proliferation was reported as a PPARα target. As a candidate for such a gene, we paid attention to PEX11α. PEX11 is one of the peroxisome biogenesis genes called PEXs [17], and three subtypes are known [18]. Among them, PEX11α is enriched in the liver, and induced by peroxisome proliferators [19]. Most importantly, peroxisomes are proliferated by overexpression of PEX11 in cultured cells [20, 21]. Based on these observations, we asked whether PEX11α is a target gene of PPARα, as the first subject of present study. We identified a PPAR-binding site (peroxisome proliferator-response element; PPRE) downstream of the PEX11α gene. Moreover, we found that this PPRE also served for the transactivation of an adjacent gene, perilipin. We show that this is a bidirectional response element, specifically acting with PPARα on the PEX11α gene, whereas with PPARγ on the perilipin gene.

Like many other nuclear receptors, PPARγ consists of four discrete domains, and contains a ligand-independent transactivating function, AF-1, in the amino-terminal region [22, 23]. AF-1 modulates the whole function of nuclear receptors by cooperating with the ligand-dependent transactivating function, AF-2, which is located in the carboxyl-terminal region. A previous study suggested that the amino-terminal region of PPARγ2 also contains a repression domain that negatively regulates AF-1 [23]. A mitogen-activated protein (MAP) kinase phosphorylation site is located in this domain, and phosphorylation at this site was suggested to be important for the negative regulation of PPARγ2 activity [22–24]. On the other hand, we were aware that there is a consensus motif for conjugation with small ubiquitin-related modifier (SUMO) in the repression domain, adjacent to the phosphorylation site. Accordingly, as the second subject of this work, we asked whether the function of PPARγ2 is regulated by sumoylation.

Materials and methods

Plasmids

Genomic DNA fragments of the PEX11α gene [25] were inserted into the luciferase reporter plasmid pGVP, containing a SV40 promoter, or pGVB basic vector (Toyo Ink). When the genomic fragments were placed on the downstream side, they were inserted downstream of the poly(A) addition site of the luciferase gene. Site-directed mutagenesis was carried out using a QuickChange mutagenesis kit (Stratagene), according to the manufacturer's protocol, and mutations were verified by nucleotide sequencing. A PPARγ reporter plasmid, pPEX11α/perilipin-PPREx3-luc, was constructed by inserting three copies of the PPRE derived from mouse PEX11α/perilipin gene pair (see below) in pGVP.

pAOXPPREluc, a mouse PPARα expression vector pNCMVPPARα, and an empty plasmid pCMVNot were as described previously [7]. The cDNAs of mouse PPARγ1, PPARγ2 and PPARδ were subcloned into a mammalian expression vector pCMX. Mammalian expression plasmids of myc-tagged PPARγ2 (amino acid residues 2–505), CFP-tagged SUMO-1 and CFP-tagged Ubc9 were as described [26].

For the assay of AF-1 activity of PPARγ2, wild-type and mutant sequences coding for residues 2–138 and 2–99 of the A/B region of mouse PPARγ2 were amplified by PCR from appropriate plasmids, and inserted in-frame into pCMX-GAL4-N, a vector containing a coding sequence of the yeast GAL4 DNA-binding domain (BD) (residues 1–147), driven by the human cytomegalovirus promoter. The resulting plasmids were pCMX-GAL4-PPARγ2<2–138> and <2–99>, and their derivatives. The activities of GAL4-fusion constructs were assayed as described [27], using a reporter plasmid, tk-GALpx3-luc, containing three copies of the GAL4-binding site and the herpes simplex virus thymidine kinase (*tk*) promoter.

Cell culture and DNA transfection

HeLa cells were cultured in 96-well plates with F-12 medium containing 10% fetal bovine serum (FBS), and transfection was carried out by the calcium phosphate method [28]. NIH3T3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Transfection was performed using Lipofectamine Plus (Invitrogen), according to the manufacturer's instructions. Wy14,643, BRL49,653, and carbaprostacyclin were used as the ligands for PPARα, γ, and δ, at the concentrations of 100 μM, 1 μM, and 10 μM, respectively. Same volume, 0.1% the culture medium, of vehicle (dimethyl sulfoxide, DMSO) was added to control samples.

Luciferase assays

In 96-well plates, cells were solubilized with 20 μl of cell lysis buffer [7], and measured for luciferase activity using

a PicaGene reagent kit (Toyo Ink) in a Lucy2 microplate luminometer (Anthos). The assays were performed in triplicate, and the averages are given, together with the standard deviations. When transfection was carried out in 24-well plates, cells were lysed with 62.5 μl of the cell lysis buffer, and 10 μl of the lysate was used for luciferase assay. A β-galactosidase expression plasmid, pCMVβ, was included in transfection, and the enzyme activity was used to normalize luciferase activity for transfection efficiency.

Protein analysis

Transfected cells were washed once with PBS containing 20 mM N-ethylmaleimide and immediately dissolved in heated SDS-PAGE sample buffer. Immunoblotting was performed using the following antibodies: mouse anti-myc monoclonal antibody (9E10) (Santa Cruz), rabbit anti-GFP polyclonal antibody (BD Biosciences Clontech), and anti-mouse and anti-rabbit IgG species-specific antibodies linked to horseradish peroxidase (Amersham). Immunocomplex was visualized by the ECL method according to the manufacturer's instructions (Amersham).

Electrophoretic gel-mobility shift assay (EMSA)

A double-stranded oligonucleotide composed of 5′-TCGACTTCCCTTGTCACCTTTCACCCACATCCTAGA ATCC-3′ and 5′-TCGAGGATTCTAGGATGTGGGTGAA AGGTGACAAGGGAAG-3′ encompassing the PEX11α/perilipin PPRE (underlined) was used as a probe. A mutant version of this sequence, in which the PPRE sequence was changed to TCACCTTTCgggC, was also used. The probes were ^{32}P-labeled at the 3' end by filling-in reaction. The assay was performed using maltose-binding protein-PPARα and glutathione *S*-transferase-RXRα fusion proteins, as described previously [7], or with PPARγ2 and RXRα synthesized *in vitro*, in a rabbit reticulocyte lysate system (Amersham).

Chromatin immunoprecipitation (ChIP) assay

For the detection of *in vivo* binding of PPARα and PPARγ to the PEX11α/perilipin PPRE, H4IIEC3 rat hepatoma cells and 3T3-L1 adipocytes were used, respectively. H4IIEC3 cells were grown in DMEM/10% FBS to 80% confluency, followed by the treatment with 100 μM Wy14,643 for 2 days. 3T3-L1 cells were differentiated as described [29], and cultured for 5 days after initiation of the cocktail treatment. Approximately 1×10^7 cells were processed for ChIP assay using a reagent kit (Upstate). Immunoprecipitation was performed with polyclonal antibodies against PPARα, PPARγ, pan-RXR, and CBP (Santa Cruz), and a preimmune rabbit IgG. PCR was carried out using primer pairs amplifying a region containing the PEX11α/perilipin-PPRE and a region distal to the PPRE. One-tenth the DNA recovered from each immunoprecipitate was used for PCR, and the products were analyzed on an agarose gel after 35 cycles of amplification.

Results

Identification of the PEX11α/perilipin PPRE

We found that the expression of PEX11α was increased with a peroxisome proliferator, Wy14,643, in the livers of wild-type mice but not PPARα knockout mice [25], confirming that PEX11α is a target gene of PPARα. To seek a PPRE of the PEX11α gene, we connected various portions of the mouse PEX11α gene and its flanking regions to the SV40 basal promoter and luciferase reporter gene. The reporter plasmids were introduced into HeLa cells with or without a PPARα expression vector, and the cells were cultured in the presence or absence of a ligand. We found activation of luciferase expression dependent on PPARα or γ, but not δ, with a downstream sequence of the PEX11α gene, in the presence of respective ligands (Fig. 1C). On the other hand, we did not observe significant activation of the reporter expression with the upstream region or the structural gene region (Fig. 1A and B). No PPAR-dependent gene activation was observed with the upstream region, up to 12.5 kb upstream of the cap site [25]. These results suggest that a functional PPRE of PEX11α gene is located downstream. By inspecting the nucleotide sequence of the downstream region, we found a PPRE-like sequence motif, TCACCTTTCACCC. This motif was located 8.4 kb downstream of the cap site of PEX11α gene, being the most nearby PPRE-like sequence to the PEX11α promoter.

We next asked if this PPRE-like sequence is the genuine PPRE of the PEX11α gene. We constructed a reporter plasmid that contained the basal PEX11α promoter region and the downstream region. In this construct, we tried to keep the relative position of the candidate PPRE as close as possible to the natural gene arrangement. We observed that PPARα transactivated this reporter gene depending on the ligand, but if the nucleotides in the motif were mutated, the transactivation was significantly reduced (Fig. 2A). This result indicates that this PPRE-like element is an active PPRE. Interestingly, PPARγ did not activate the reporter expression, indicating that the transactivating effect is specific for PPARα in this reporter gene construct.

By inspecting the mouse and human genome sequences, we were aware that the perilipin gene was located downstream of the PEX11α gene [25]. These genes were only

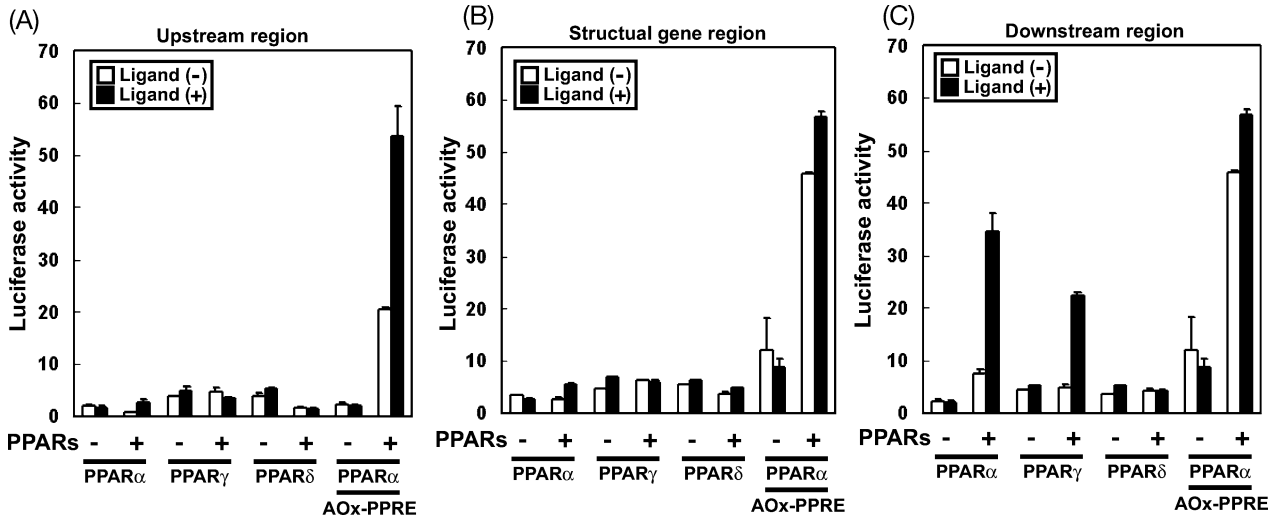

Fig. 1. An active PPRE is located in the downstream region of PEX11αgene. Reporter assays were carried out on: (A) the region upstream of the PEX11α gene promoter (between positions −2.2 kb and −274 relative to the cap site); (B) the structural gene region of PEX11α encompassing exon 1 through a most part of exon 3 (last exon), extending from positions +42 to +5.5 kb; and (C) the region encompassing the remaining part of exon 3 and the downstream sequence, extending positions +5.5 kb through +9.8 kb. The DNA fragments were inserted into the multicloning site of a luciferase vector, pGVP, carrying a SV40 promoter. pAOXPPREluc, containing the functional PPRE and promoter region of the rat acyl-CoA oxidase gene [44], was used as a positive control. To HeLa cells in 96-well plates, 0.1 μg of a reporter plasmid, 0.1 μg of a PPAR expression vector, and 0.175 μg of an empty vector (pCMVNot or pCMX) per well were co-transfected. After 4 h of transfection, the cells were cultured for 24 h in the presence or absence of ligands. Luciferase activity is shown in an arbitrary unit. We confirmed by a separate experiment that the region –273/+43 of PEX11α gene carried a basal promoter activity, but lacked a functional PPRE (data not shown).

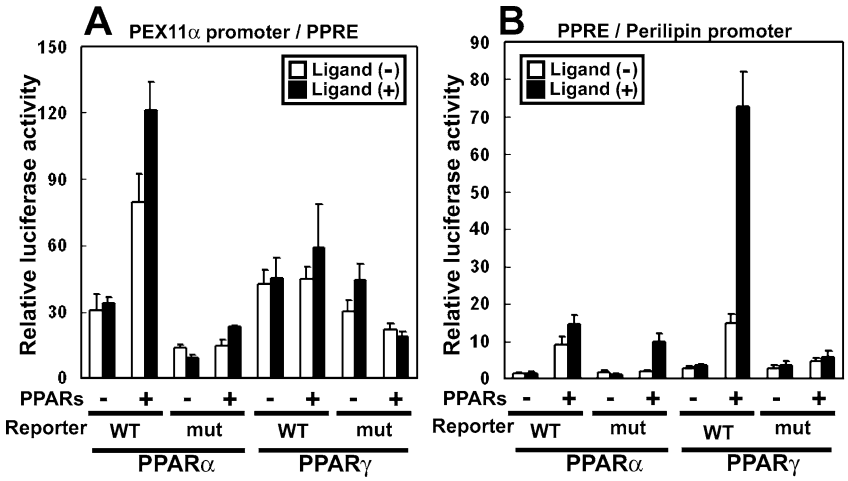

Fig. 2. PPARα and PPARγ2 selectively transactivate the PEX11α and perilipin genes, respectively, through the common PPRE. (A) Selective transactivation of the PEX11α gene by PPARα. The basal PEX11α promoter (positions –337 through +43) and the region between +3.0 kb and +9.7 kb containing the PPRE were inserted into a basal luciferase vector, pGVB, upstream and downstream of the luciferase gene, respectively. (B) Selective transactivation of the perilipin gene by PPARγ2. The region between positions –2877 and +56 relative to the perilipin gene cap site, containing the PPRE and the basal perilipin promoter, was inserted into pGVB. Luciferase activity is shown as a relative value, taking the activity of pGVB in the absence of both a ligand and a PPAR expression vector, as 1. WT, the wild-type construct; mut, a mutant construct in which the PPRE sequence was changed from TCACCTTTCACCC to TCACCTTTCgggC. Other experimental conditions were as in Fig. 1.

about 5 kb apart in the mouse genome, and the PEX11α PPRE was located 1.9 kb upstream of the cap site of the perilipin gene. Perilipin is an adipocyte-enriched protein, located on the surfaces of lipid droplets [30, 31]. Perilipin plays a key role in the regulation of lipolysis by restricting the access of hormone-sensitive lipase to the stored lipids at the basal state. Once the cells are stimulated by catecholamine, perilipin is phosphorylated and allows the lipase to access lipids [32, 33].

Perilipin is induced during the differentiation of 3T3-L1 cells into adipocytes [30], where PPARγ plays a central role. These observations suggest that the perilipin gene is a target of PPARγ, and raise a possibility that the PPRE downstream of the PEX11α gene is also an active PPRE for the perilipin gene.

To examine this possibility, we constructed a luciferase reporter plasmid containing the upstream region of mouse perilipin gene, containing the PEX11α PPRE and the perilipin gene promoter. PPARγ2 activated the expression of luciferase depending on the ligand, and the transactivation was canceled by a mutation of the PPRE (Fig. 2B). This result indicates that the PEX11α PPRE also serves as an active PPRE for the perilipin gene. On the other hand, only slight transactivation was observed with PPARα. This result suggests that the perilipin promoter was selectively activated by PPARγ through the PPRE, opposite to the case of PEX11α gene. We also observed significant reporter expression with this PPRE reporter construct in 3T3-L1 adipocytes, but not preadipocytes, depending on the PPARγ ligand [25]. Thus, this PPRE actively works with endogenous PPARγ in adipocytes.

Binding of PPARs to the PEX11α/perilipin PPRE in vitro *and* in vivo

We next examined the binding of PPARα and γ to the PPRE sequence by EMSA. When PPARα was mixed with RXRα, a shifted band appeared with the PEX11α/perilipin PPRE probe (Fig. 3). The binding was competed by the wild-type, but not the mutant oligonucleotide. Neither PPARα nor RXRα by itself exhibited binding to the probe (data not shown). With PPARγ, a shifted band appeared only when RXRα was added together, and the binding was competed with the wild-type but not the mutant PPRE. These results indicate that this PPRE sequence is recognized by both the PPARα and PPARγ in the presence of the heterodimer partner, RXR.

We also examined the binding of PPARα and PPARγ to the PPRE in vivo, by a ChIP assay using rat hepatoma H4IIEC3 cells and 3T3-L1 adipocytes. For the chromatin sample from the hepatoma cells, an anti-PPARα antibody immunoprecipitated the PPRE region of DNA (Fig. 4A). A control IgG did not precipitate this region, and a distal region was not recovered in the immunoprecipitate with any antibodies (data not shown). An anti-PPARγ antibody did not give a significant band. Thus, PPARα selectively bound to the PPRE in the hepatoma cells. As expected, RXR and a representative coactivator, CBP, also associated with the PPRE. On the other hand, in the differentiated adipocytes, PPARγ but not PPARα was found to bind to this PPRE, together with RXR and CBP (Fig. 4B). These results seem reasonable, because PPARα is

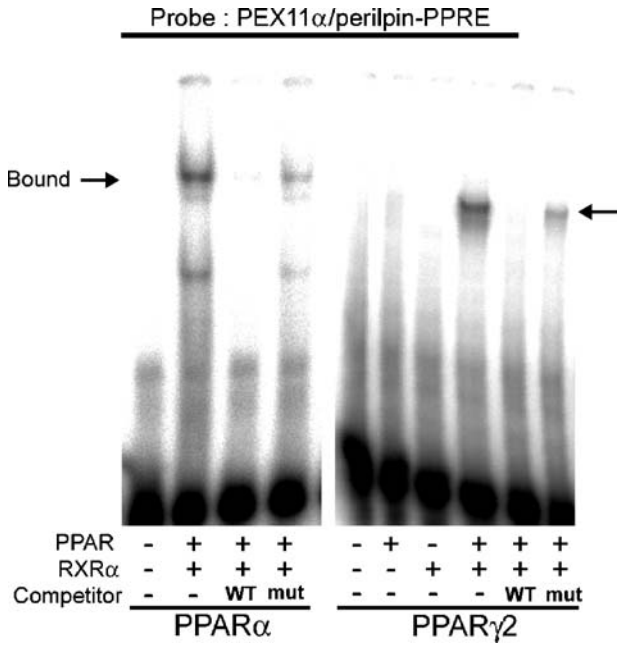

Fig. 3. PPARα and PPARγ2 bind to the PEX11α/perilipin PPRE *in vitro*. Specific binding of (A) PPARα/RXRα and (B) PPARγ/RXRα to the PPRE. For competition experiments, unlabeled oligonucleotides in 100-fold excess were used. Other experimental conditions and sequences of probes and competitors were as described in Materials and methods. WT, wild-type; mut, mutant.

the major PPAR subtype in the liver, while PPARγ is highly enriched in the adipose tissue.

Tissue selective activation of the PEX11α and perilipin genes by PPARα and γ through a common binding site

Based on the results above, we would propose a model for tissue-selective regulation of the rodent PEX11α and perilipin genes by the PPAR subtypes (Fig. 5). These genes are arranged in tandem on the genome with about a 5 kb distance, and a common PPRE is located in the spacer region between them. In the liver, PPARα binds to this PPRE and selectively activates the PEX11α gene, leading to the proliferation of peroxisomes. In the adipose tissue, in contrast, PPARγ binds to the PPRE and selectively activates the perilipin gene, which will in turn promote triglyceride accumulation by shielding lipid droplets.

AF-1 activity of PPARγ2 is enhanced by a mutation at a potential sumoylation site

Next we were interested in the regulatory mechanism of AF-1 of PPARγ. The amino-terminal A/B region of PPARγ

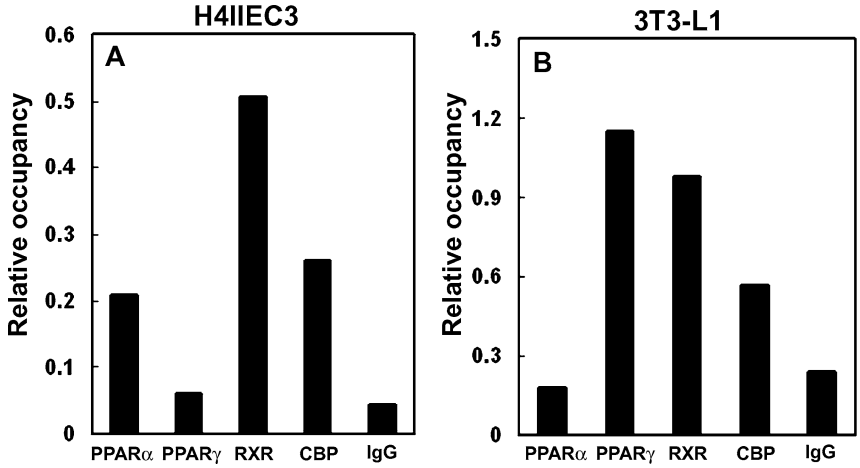

Fig. 4. PPARα and PPARγ2 bind to the PEX11α/perilipin PPRE tissue-selectively *in vivo*. (A) Selective binding of PPARα to the PPRE in the liver. H4IIEC3 cells were treated with 100 μM Wy14,643 for 2 days, and processed for the ChIP assay. (B) PPARγ selectively binds to the PPRE in the adipocytes. The ChIP assay was performed on the 3T3-L1 adipocytes. Relative occupancy of the PPRE by each protein was assessed by the amount of DNA recovered in the immunoprecipitate with each antibody, divided by the amount of input. The PCR primers used were: rat PEX11α/perilipin-PPRE, 5′-GAATGGCCAAGAGCCCTGCTC-3′ (positions +2282 to +2302; relative to the first residue of the putative polyadenylation signal of the PEX11α gene) and 5′-GCTCTGCTGACAAAGCTGGTC-3′ (+2462 to +2482); mouse PEX11α/perilipin-PPRE, 5′-GAGTGGTCAAGACCTCTGCTC-3′ (+2094 to +2114) and 5′-GCTCTGCTGACAAAGCCGGTC-3′ (+2265 to +2285); rat distal, 5′-ACCTATGCATGGATGACCACTA-3′ (−1737 to −1716) and 5′-CACAGCAATTAAACAGTGAC-3′ (−1543 to −1524); and mouse distal, 5′-CTGTGCATGAGTGACCACTCG-3′ (−1694 to −1674) and 5′-CTAAACAGTGACTAAGGAGTCATTA-3′ (−1500 to −1476). Other experimental conditions were as described in Materials and Methods.

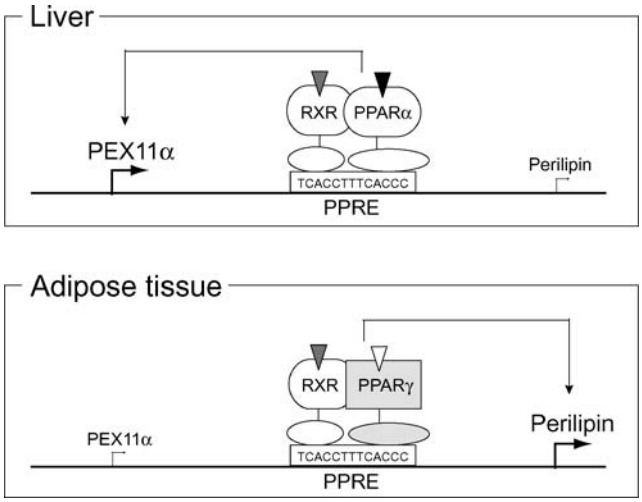

Fig. 5. Models of the tissue-selective regulation of PEX11α and perilipin genes.

corresponds to amino acid residues 1-108 for PPARγ1 and 1–138 for PPARγ2, respectively, γ1 lacking the initial 30 residues of γ2. AF-1 activity was demonstrated in the amino-terminal region, particularly for PPARγ2 [22, 23], and a result was also reported implying that the region, residues 100–138, had a negative effect on AF-1 activity [23]. We confirmed the AF-1 activity with NIH3T3 cells, using a plasmid construct in which residues 2–138 of PPARγ2 was connected to the yeast GAL4-BD (Fig. 6). We also confirmed that the construct containing only residues 2–99 of PPARγ2 exhibited a much higher transactivation than that containing the whole A/B region. In addition, the region of residues 100–138 conferred active repression, when separately fused to the GAL4-BD [26]. Thus, the amino-terminal region of PPARγ2 contains an activation domain (residues 2–99) and a repression domain (residues 100–138).

As described in Introduction, the repression domain contains a MAP kinase phosphorylation site (S112) as well as a potential sumoylation site (K107). SUMO is a group of ubiquitin-like small proteins conjugated to target proteins at specific lysine residues, with the assistance of conjugating enzymes including Ubc9 [34]. The major SUMO targets are transcriptional factors, and their transactivating functions are in most cases repressed by sumoylation [35, 36]. Accordingly, we examined the effects of mutations destroying the phosphorylation site and potential sumoylation site. K107R, a sumoylation site mutation, resulted in a significant enhancement of transactivation, comparable to the effect of deletion of the repression domain (Fig. 6, lanes 3 and 4). Although a phosphorylation site mutation, S112A, also enhanced the transactivating function, the effect was smaller than that of K107R (lane 5). K107R/S112A double mutant exhibited a higher level of transactivation than S112A, close to the K107R single mutant and the deletion construct lacking the repression domain (compare lanes 3, 4, and 6). Hence, residue K107 is even more important for

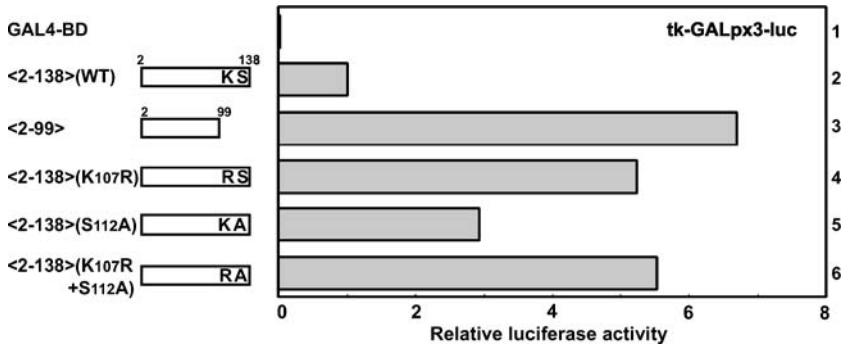

Fig. 6. K107R mutation enhances the AF-1 activity of PPARγ2. The fusion constructs indicated were transfected into NIH3T3 cells, together with a reporter plasmid, tk-GALpx3-luc. DNA/Lipofectamine Plus precipitates containing 0.04 μg of the expression vector of a PPARγ construct, 0.3 μg of the reporter plasmid, 0.04 μg of a β-galactosidase expression plasmid (pCMVβ) as a reference, and 0.02 μg of an empty vector (pCMX), were added to each well of a 24-well plate. The assay was performed in duplicate, and the averages are given as values relative to that of lane 2.

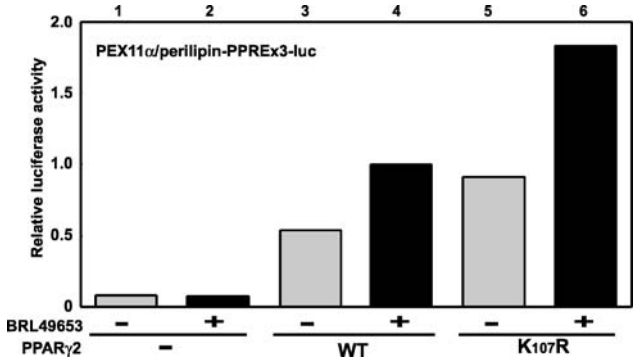

Fig. 7. K107R mutation enhances transactivation by PPARγ2. NIH3T3 cells were transfected with expression plasmids of WT or K107R of myc-PPARγ2 or pCMV5-myc (-) as indicated, together with PEX11α/perilipin-PPREx3-luc. Luciferase activities are shown as values relative to lane 4, which was taken as 1. Cells were cultured in the absence (gray bar) or presence (filled bar) of 1 μM BRL49,653. Other experimental conditions were as in Fig. 6.

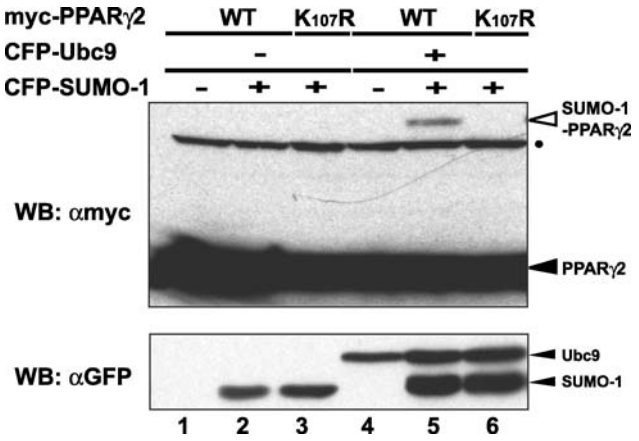

Fig. 8. PPARγ2 is sumoylated *in vivo* at K107. NIH3T3 cells stably expressing myc-PPARγ2 were transfected in 6-well plates with mixtures of expression plasmids, consisting of 1.6 μg of CFP-SUMO-1 (lanes 1–3) or 1.2 μg of CFP-SUMO-1 and 0.4 μg of CFP-Ubc9 (lanes 4–6). The lack of CFP-SUMO-1 constructs was balanced by the addition of pCMX (-). The cell lysates were analyzed 24 h after transfection, by Western blotting (WB) with anti-myc (Top) or anti-GFP (Bottom) antibodies. Open arrowhead indicates a sumoylated form of PPARγ2 and filled circle a non-specific band, respectively.

the function of repression domain, than the phosphorylation site, S112.

We also examined the significance of residue K107 in the whole PPARγ2 protein. In NIH3T3 cells, the K107R mutant of PPARγ2 exerted stronger transactivation than the wild-type through the PEX11α/perilipin PPRE, in both the presence and absence of the ligand (Fig. 7). Similar effect of the K107R mutation in the whole PPARγ2 was observed in HeLa cells, by employing a reporter plasmid containing a single or three copies of the same PPRE as well as another PPRE [26]. These results indicate that K107 is also important for the total transactivating function of PPARγ2.

PPARγ2 is sumoylated at K107

We asked if PPARγ2 was truly sumoylated. To answer this, we expressed CFP-fused SUMO-1, CFP-Ubc9 and myc-tagged PPARγ2 in NIH3T3 cells. By western blotting with an anti-myc antibody, we observed a band of PPARγ2 with an elevated size only when the wild-type CFP-SUMO-1 and CFP-Ubc9 were expressed (Fig. 8). This band seemed to be due to sumoylated PPARγ2, because a conjugation-defective mutant of SUMO did not yield this band (data not shown). A PPARγ ligand had no significant effect on the intensity of this band (data not shown). We obtained similar results with HeLa cells, and further confirmed that the larger form of PPARγ2 in fact represented K107-sumoylated PPARγ2, by immunoprecipitation with an anti-PPARγ antibody and Western blotting with an anti-SUMO-1 antibody [26]. These results indicate that PPARγ2 is sumoylated at K107.

Inverse correlation between transactivating competence and sumoylation level

We tried to further confirm that the stimulating effect of K107R mutation on the transactivating function is due to the lack of sumoylation. The sumoylation consensus is ΨKxE/D [34], where Ψ denotes a bulky hydrophobic amino acid. Accordingly, we examined the effects of mutations at these positions, I106M, I106A, and E109A, in addition to K107R, on the sumoylation and transactivation (Fig. 9A). When these mutants of PPARγ2 were co-expressed with CFP-SUMO-1, I106M was sumoylated more weakly than the wild-type, I106A even more weakly, and the sumoylation levels of K107R and E109A were negligible, being consistent with the consensus. In the reporter assay, on the other hand, I106M exhibited a stronger transactivation than the wild-type, I106A even stronger, and the highest activities were obtained with K107R and E109A. Thus, the transactivating function inversely correlated with the sumoylation level, supporting that sumoylation at K107 represses the transactivating function of PPARγ2.

The K107 sumoylation site and S112 phosphorylation site are at a close proximity in the repression domain. Accordingly, we asked if the sumoylation and phosphorylation are functionally linked. Other than K107R, S112A and K107R/S112A, we created a phosphorylation-mimicking mutant S112D and its double mutant with K107R, and P113Q, a mutation linked to obesity that would eliminate S112 phosphorylation [37]. We examined the sumoylation levels as well as transactivation functions for these mutants (Fig. 9B). The phosphorylation-defective mutants S112A and P113Q were both sumoylated significantly more weakly than the wild-type. Any mutants containing K107R mutation were not sumoylated. S112D mutation had no significant effect. This result suggests that phosphorylation at S112 promotes sumoylation at K107. The phosphorylation-defective mutants S112A and P113Q exhibited transactivation higher than the wild-type, whereas mutants containing K107R all had even higher activities, at similar levels to each other, irrespective of the mutation at S112. S112D mutation had no effect on transactivation under these conditions. These results further indicate the inverse correlation between the levels of sumoylation and transactivation, and suggest that K107 sumoylation is superior in the repressive function to S112 phosphorylation.

Negative regulation of transactivating function of PPARγ2 by sumoylation

We would present a model for the repressive function of sumoylation of PPARγ2 (Fig. 10). Sumoylation at

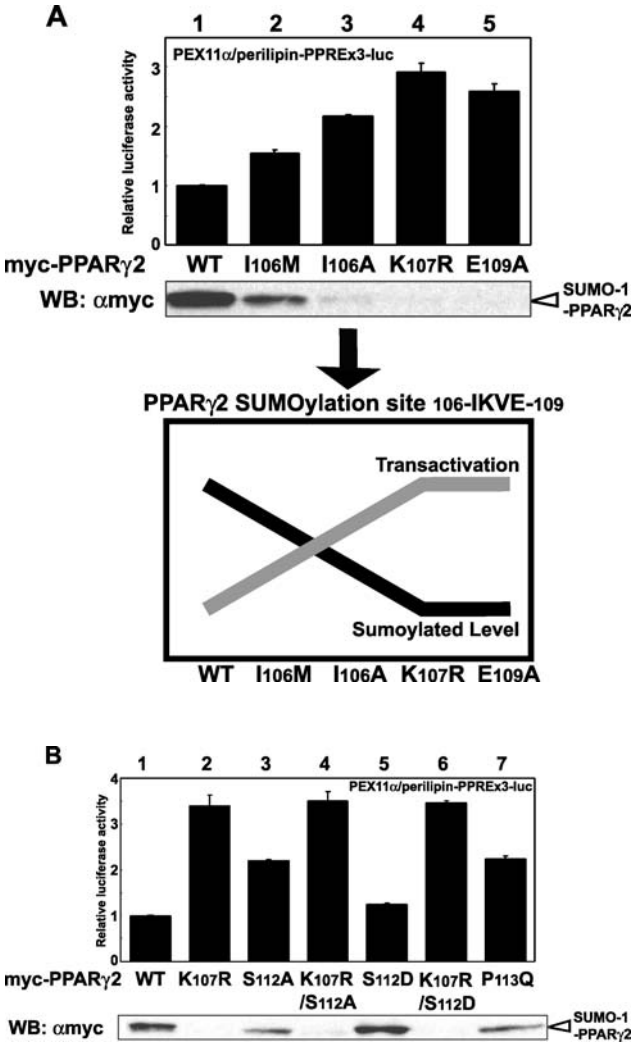

Fig. 9. Transactivation and sumoylation levels inversely correlate. (A) Effects of the amino acid substitutions in the sumoylation motif. (Top) HeLa cells in 24-well plates were transfected with 0.1 μg of expression plasmids of the wild-type or mutants of myc-PPARγ2, together with 0.75 μg of pPEX11α/perilipin-PPREx3-luc. The assay was performed in triplicate, and the averages are given as values relative to lane 1, together with standard deviations. (Middle) HeLa cells in 6 cm dishes were transfected with 6 μg of expression plasmids of WT or myc-PPARγ2 mutants and 6 μg of the CFP-SUMO-1 plasmid. Cell lysates were analyzed by Western blotting with an anti-myc antibody. (Bottom) A schematic view of the inverse correlation between the sumoylation level and the transactivating function of PPARγ2. (B) Phosphorylation at S112 correlates with K107 sumoylation. (Top) Sumoylation at K107 is superior to phosphorylation in the repression of PPARγ2 transactivating function. HeLa cells were transfected with the expression plasmids of WT or myc-PPARγ2 phosphorylation mutants, together with pPEX11α/perilipin-PPREx3-luc. Results are shown as relative values, taking the activity of lane 1 as 1. (Bottom) Decrease in sumoylation in phosphorylation-defective mutants. HeLa cells were transfected with the expression plasmids of WT or mutants of myc-PPARγ2, and the CFP-SUMO-1 expression plasmid. Cell lysates were analyzed by Western blotting with an anti-myc antibody. Other experimental conditions were as in Fig. 9A.

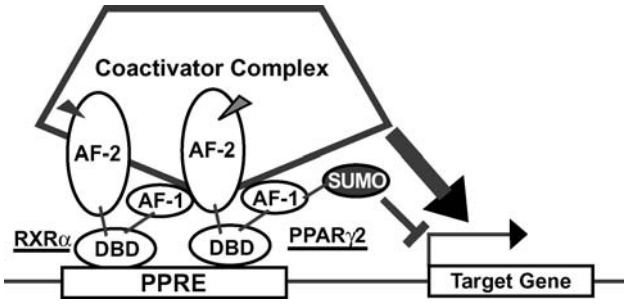

Fig. 10. Model of repression of PPARγ2 transactivating function by sumoylation.

K107 represses AF-1 of PPARγ2, probably by causing a conformational change affecting the interaction with coactivators, or by recruiting corepressors. The synergy between AF-1 and AF-2 would extend the effect of sumoylation to the total transactivating function of PPARγ2. We obtained similar results for PPARγ1, but not for PPARα and PPARδ [26]. Therefore, sumoylation is a regulatory mechanism unique to PPARγ among the PPAR subfamily.

Discussion

In this study, we demonstrated that the PEX11α and perilipin genes are transcriptionally regulated through a common PPRE located in between. The regulation is tissue-selective: In the liver, PPARα selectively binds to the PPRE and activate the PEX11α gene, whereas in the adipose tissue, PPARγ specifically binds to the element, leading to the activation of the perilipin gene. The selectivity of binding probably depends on the relative expression levels of PPARα and PPARγ in these organs, that is, higher level of expression of PPARα in the liver, whereas PPARγ in the adipose tissue. On the other hand, specific transactivation of the two genes by respective PPAR subtypes would be due to differential transactivating capacities on different promoters and/or different relative positions of PPRE to the promoters. Under the experimental conditions, where the plasmids were transiently transfected, epigenetic mechanisms such as differential chromatin condensation and insulation are less likely to be relevant.

To our knowledge, it is unique at present in higher animals that two adjacently positioned but functionally independent genes are regulated through a common cis-element. However, a recent analysis revealed that a number of human genes are arranged in close proximity with other genes on the genome [38]. Therefore, it is expected that similar regulatory mechanisms will be found for other gene pairs in future. In such cases, including the PEX11α/perilipin gene pair, higher order chromatin and nuclear structures would also be important in vivo for elaborate regulation of each gene.

Our present data suggest that sumoylation is a predominant mechanism of negative transcriptional regulation by the repression domain in PPARγ AF-1. MAP kinase-dependent phosphorylation at S112 has been ascribed as a mechanism of repression of PPARγ2 transactivating function through AF-1 [22–24]. Our results, however, indicate that the K107R mutation better enhanced the transactivating competence of PPARγ2 than the S112A and P113Q mutations, and the effect of K107R single mutation was indistinguishable with the double mutations K107R/S112A and K107R/S112D. This would mean that the K107R mutation cancels the effect of mutations affecting phosphorylation, rather than synergizing with them. Moreover, mutations lowering the phosphorylation level also lowered the sumoylation level. Based on these observations, it is tempting to hypothesize that phosphorylation at S112 of PPARγ2 promotes sumoylation at K107, and hence causes repression of the transactivating function. This possibility must be inspected in future, together with the mechanism of transcriptional repression by sumoylation.

Perilipin gene PPRE [39–41] and sumoylation of PPARγ [42, 43] were also reported recently by other groups.

References

1. Desvergne B, Wahli W: Peroxisome proliferator-activated receptors: Nuclear control of metabolism. Endocr Rev 20: 649–688, 1999
2. Issemann I, Green S: Activation of a member of the steroid hormone receptor superfamily by peroxisome proliferators. Nature 347: 645–650, 1990
3. Reddy JK, Hashimoto T: Peroxisomal β-oxidation and peroxisome proliferator-activated receptor α: an adaptive metabolic system. Annu Rev Nutr 21: 193–230, 2001
4. Kliewer SA, Umesono K, Noonan DJ, Heyman RA, Evans RM: Convergence of 9-cis retinoic acid and peroxisome proliferator signalling pathways through heterodimer formation of their receptors. Nature 358: 771–774, 1992
5. Tugwood JD, Issemann I, Anderson RG, Bundell KR, McPheat WL, Green S: The mouse peroxisome proliferator activated receptor recognizes a response element in the 5′ flanking sequence of the rat acyl CoA oxidase gene. EMBO J 11: 433–439, 1992
6. Palmer CN, Hsu MH, Griffin HJ, Johnson EF: Novel sequence determinants in peroxisome proliferator signaling. J Biol Chem 270: 16114–16121, 1995
7. Osada S, Tsukamoto T, Takiguchi M, Mori M, Osumi T: Identification of an extended half-site motif required for the function of peroxisome proliferator-activated receptor α. Genes Cells 2: 315–327, 1997
8. Juge-Aubry C, Pernin A, Favez T, Burger AG, Wahli W, Meier CA, Desvergne B: DNA binding properties of peroxisome proliferator-activated receptor subtypes on various natural peroxisome proliferator response elements. Importance of the 5′-flanking region. J Biol Chem 272: 25252–25259, 1997
9. Tontonoz P, Hu E, Spiegelman BM: Stimulation of adipogenesis in fibroblasts by PPARγ2, a lipid-activated transcription factor. Cell 79: 1147–1156, 1994
10. Peters JM, Lee SS, Li W, Ward JM, Gavrilova O, Everett C, Reitman ML, Hudson LD, Gonzalez FJ: Growth, adipose, brain, and skin alterations

resulting from targeted disruption of the mouse peroxisome proliferator-activated receptor $\beta(\delta)$. Mol Cell Biol 20: 5119–5128, 2000
11. Wang YX, Lee CH, Tiep S, Yu RT, Ham J, Kang H, Evans RM: Peroxisome-proliferator-activated receptor δ activates fat metabolism to prevent obesity. Cell 113: 159–170, 2003
12. Lemberger T, Desvergne B, Wahli W: Peroxisome proliferator-activated receptors: A nuclear receptor signaling pathway in lipid physiology. Annu Rev Cell Biol 12: 335–363, 1996
13. Berger J, Moller DE: The mechanisms of action of PPARs. Annu Rev Med 53: 409–435, 2002
14. Lehmann JM, Moore LB, Smith OT, Wilkison WO, Willson TM, Kliewer SA: An antidiabetic thiazolidinedione is a high affinity ligand for peroxisome proliferator-activated receptor γ (PPARγ). J Biol Chem 270: 12953–12956, 1995
15. Kliewer SA, Sundseth SS, Jones SA, Brown PJ, Wisely GB, Koble CS, Devchand P, Wahli W, Willson TM, Lenhard JM, Lehmann JM: Fatty acids and eicosanoids regulate gene expression through direct interactions with peroxisome proliferator-activated receptors α and γ. Proc Natl Acad Sci USA 94: 4318–4323, 1997
16. Forman BM, Chen J, Evans RM: Hypolipidemic drugs, polyunsaturated fatty acids, and eicosanoids are ligands for peroxisome proliferator-activated receptors α and δ. Proc Natl Acad Sci USA 94: 4312–4317, 1997
17. Distel B, Erdmann R, Gould SJ, Blobel G, Crane DI, Cregg JM, Dodt G, Fujiki Y, Goodman JM, Just WW, Kiel JA, Kunau WH, Lazarow PB, Mannaerts GP, Moser HW, Osumi T, Rachubinski RA, Roscher A, Subramani S, Tabak HF, Tsukamoto T, Valle D, van der Klei I, van Veldhoven PP, Veenhuis M: A unified nomenclature for peroxisome biogenesis factors. J Cell Biol 135: 1–3, 1996
18. Li X, Baumgart E, Dong GX, Morrell JC, Jimenez-Sanchez G, Valle D, Smith KD, Gould SJ: PEX11α is required for peroxisome proliferation in response to 4-phenylbutyrate but is dispensable for peroxisome proliferator-activated receptor α-mediated peroxisome proliferation. Mol Cell Biol 22: 8226–8240, 2002
19. Abe I, Okumoto K, Tamura S, Fujiki Y: Clofibrate-inducible, 28-kDa peroxisomal integral membrane protein is encoded by PEX11. FEBS Lett 431: 468–472, 1998
20. Schrader M, Reuber BE, Morrell JC, Jimenez-Sanchez G, Obie C, Stroh TA, Valle D, Schroer TA, Gould SJ: Expression of PEX11β mediates peroxisome proliferation in the absence of extracellular stimuli. J Biol Chem 273: 29607–29614, 1998
21. Passreiter M, Anton M, Lay D, Frank R, Harter C, Wieland FT, Gorgas K, Just WW: Peroxisome biogenesis: Involvement of ARF and coatomer. J Cell Biol 141: 373–383, 1998
22. Adams M, Reginato MJ, Shao D, Lazar MA, Chatterjee VK: Transcriptional activation by peroxisome proliferator-activated receptor γ is inhibited by phosphorylation at a consensus mitogen-activated protein kinase site. J Biol Chem 272: 5128–5132, 1997
23. Werman A, Hollenberg A, Solanes G, Bjørbæk C, Vidal-Puig AJ, Flier JS: Ligand-independent activation domain in the N terminus of peroxisome proliferator-activated receptor γ (PPARγ). Differential activity of PPARγ1 and -2 isoforms and influence of insulin. J Biol Chem 272: 20230–20235, 1997
24. Hu E, Kim JB, Sarraf P, Spiegelman BM: Inhibition of adipogenesis through MAP kinase-mediated phosphorylation of PPARγ. Science 274: 2100–2103, 1996
25. Shimizu M, Takeshita A, Tsukamoto T, Gonzalez FJ, Osumi T: Tissue-selective, bidirectional regulation of PEX11α and perilipin genes through a common peroxisome proliferator response element. Mol Cell Biol 24: 1313–1323, 2004
26. Yamashita D, Yamaguchi T, Shimizu M, Nakata N, Hirose F, Osumi T: The transactivating function of peroxisome proliferator-activated receptor γ is negatively regulated by SUMO conjugation in the amino-terminal domain. Genes Cells 9: 1017–1029, 2004
27. Hi R, Osada S, Yumoto N, Osumi T: Characterization of the amino-terminal activation domain of peroxisome proliferator-activated receptor α. Importance of α-helical structure in the transactivating function. J Biol Chem 274: 35152–35158, 1999
28. Sambrook J, Fritsch, EF, Maniatis T: Molecular cloning: A laboratory manual. Cold Spring Harbor, NY: cold Spring Harbor Laboratory Press. p. 16.32–16.36, 1989
29. Tominaga S, Morikawa M, Osumi T: Growth hormone has dual stage-specific effects on the differentiation of 3T3-L1 preadipocytes. J Biochem (Tokyo) 132: 881–889, 2002
30. Greenberg AS, Egan JJ, Wek SA, Garty NB, Blanchette-Mackie EJ, Londos C: Perilipin, a major hormonally regulated adipocyte-specific phosphoprotein associated with the periphery of lipid storage droplets. J Biol Chem 266: 11341–11346, 1991
31. Servetnick DA, Brasaemle DL, Gruia-Gray J, Kimmel AR, Wolff J, Londos C: Perilipins are associated with cholesteryl ester droplets in steroidogenic adrenal cortical and leydig cells. J Biol Chem 270: 16970–16973, 1995
32. Clifford GM, Londos C, Kraemer FB, Vernon RG, Yeaman SJ: Translocation of hormone-sensitive lipase and perilipin upon lipolytic stimulation of rat adipocytes. J Biol Chem 275: 5011–5015, 2000
33. Sztalryd C, Xu G, Dorward H, Tansey JT, Contreras JA, Kimmel AR, Londos C: Perilipin a is essential for the translocation of hormone-sensitive lipase during lipolytic activation. J Cell Biol 161: 1093–1103, 2003
34. Seeler JS, Dejean A: Nuclear and unclear functions of sumo. Nat Rev Mol Cell Biol 4: 690–699, 2003
35. Gill G: Post-translational modification by the small ubiquitin-related modifier sumo has big effects on transcription factor activity. Curr Opin Genet Dev 13: 108–113, 2003
36. Verger A, Perdomo J, Crossley M: Modification with SUMO. A role in transcriptional regulation. EMBO Rep 4: 137–142, 2003
37. Ristow M, Müller-Wieland D, Pfeiffer A, Krone W, Kahn CR: Obesity associated with a mutation in a genetic regulator of adipocyte differentiation. N Engl J Med 339: 953–959, 1998
38. Adachi N, Lieber MR: Bidirectional gene organization: a common architectural feature of the human genome. Cell 109: 807–809, 2002
39. Arimura N, Horiba T, Imagawa M, Shimizu M, Sato R: The peroxisome proliferator-activated receptor γ regulates expression of the perilipin gene in adipocytes. J Biol Chem 279: 10070–10076, 2004
40. Dalen KT, Schoonjans K, Ulven SM, Weedon-Fekjaer MS, Bentzen TG, Koutnikova H, Auwerx J, Nebb HI: Adipose tissue expression of the lipid droplet-associating proteins S3-12 and perilipin is controlled by peroxisome proliferator-activated receptor-γ. Diabetes 53: 1243–1252, 2004
41. Nagai S, Shimizu C, Umetsu M, Taniguchi S, Endo M, Miyoshi H, Yoshioka N, Kubo M, Koike T: Identification of a functional peroxisome proliferator-activated receptor responsive element within the murine perilipin gene. Endocrinology 145: 2346–2356, 2004
42. Ohshima T, Koga H, Shimotohno K: Transcriptional activity of peroxisome proliferator-activated receptor gamma is modulated by SUMO-1 modification. J Biol Chem 279: 29551–29557, 2004
43. Floyd ZE, Stephens JM: Control of peroxisome proliferator-activated receptor γ2 stability and activity by sumoylation. Obes Res 12: 921–928, 2004
44. Osumi T, Wen JK, Hashimoto T: Two cis-acting regulatory sequences in the peroxisome proliferator-responsive enhancer region of rat acyl-CoA oxidase gene. Biochem Biophys Res Commun 175: 866–871, 1991