Dysregulation of the Peroxisome Proliferator-Activated Receptor Target Genes by XPD Mutations

Emmanuel Compe, Pascal Drané, Camille Laurent, Karin Diderich, Cathy Braun, Jan H. J. Hoeijmakers, and Jean-Marc Egly

Institut de Génétique et de Biologie Moléculaire et Cellulaire, BP 10142, 67404 Illkirch Cedex, France, and MGC-Department of Cell Biology and Genetics, Centre for Biomedical Genetics, Erasmus Medical Center, 3000 DR Rotterdam, The Netherlands

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Mutations in the XPD subunit of TFIIH give rise to human genetic disorders initially defined as DNA repair syndromes. Nevertheless, xeroderma pigmentosum (XP) group D (XP-D) patients develop clinical features such as hypoplasia of the adipose tissue, implying a putative transcriptional defect. Knowing that peroxisome proliferator-activated receptors (PPARs) are implicated in lipid metabolism, we investigated the expression of PPAR target genes in the adipose tissues and the livers of XPD-deficient mice and found that (i) some genes are abnormally overexpressed in a ligand-independent manner which parallels an increase in the recruitment of RNA polymerase (pol) II but not PPARs on their promoter and (ii) upon treatment with PPAR ligands, other genes are much less induced compared to the wild type, which is due to a lower recruitment of both PPARs and RNA pol II. The defect in transactivation by PPARs is likely attributable to their weaker phosphorylation by the cdk7 kinase of TFIIH. Having identified the phosphorylated residues in PPAR isotypes, we demonstrate how their transactivation defect in XPD-deficient cells can be circumvented by overexpression of either a wild-type XPD or a constitutively phosphorylated PPAR S/E. This work emphasizes that underphosphorylation of PPARs affects their transactivation and consequently the expression of PPAR target genes, thus contributing in part to the XP-D phenotype.

XPD mutations cause the rare autosomal recessive disorder xeroderma pigmentosum (XP), primarily defined as a DNA repair defect, which is sometimes associated with Cockayne syndrome, as well as trichothiodystrophy (TTD) (29, 47). Besides the potential for carcinogenesis caused by photosensitivity, these patients exhibit a large spectrum of clinical abnormalities, such as immature sexual development, mental retardation, skeletal abnormalities, and dwarfism. The principal hallmark of TTD patients is brittle hair and nails, caused by a reduction in cysteine-rich matrix proteins. Some TTD patients have ichthyotic skin and an unusual face that appears aged due to the lack of subcutaneous fatty tissue (15, 21). In women, the breast tissue may be completely absent in spite of normal development of the nipples. Obviously, these various clinical features cannot solely be explained by DNA repair defects (5). While one cannot exclude the biological effects due to the damage itself (that can in some cases prevent one of the ongoing cellular mechanisms such as replication or transcription), it however has to be pointed out that the product of the XPD gene belongs to the TFIIH complex, which is involved in the DNA repair as well as in the transcription process.

TFIIH can be resolved into two subcomplexes: the core-TFIIH (containing in particular the XPB helicase) and the cdk-activating kinase (CAK) complex containing the cyclin-dependent kinase cdk7 (40). The core-TFIIH and CAK subcomplexes are bridged by the XPB helicase subunit, which interacts with p44 on the one side and MAT1 on the other side (9, 41). In the nucleotide excision reaction, TFIIH, once recruited on the damaged DNA already recognized by the XPC-HR23B factor, unwinds the DNA around the lesion upon addition of ATP to allow the recruitment of XPA, RPA, XPG, and XPF/ERCC1 repair factors (37). When XPD is mutated in the C-terminal end, its interaction with its regulatory partner p44 is weakened (9). In that case, the XPD helicase is no longer upregulated by p44 and cannot optimally operate in the nucleotide excision repair process (49). In the transcription process, TFIIH together with TFIIA, -B, -D, -E, and -F basal transcription factors and RNA polymerase (pol) II, participates in the initiation of RNA synthesis by opening the promoter around the start site via its XPB helicase (18) and by phosphorylating via its cdk7 kinase the carboxy-terminal domain of the largest subunit of RNA pol II and some transcriptional activators including nuclear receptors (7, 12, 25, 32, 38).

The peroxisome proliferator-activated receptors PPARα, -γ, and -δ are ligand-activated nuclear receptors which form heterodimers with the retinoid X receptor and bind to PPAR-responsive elements of numerous target genes (26). These transcription factors regulate lipid metabolism (3, 28) and are activated by fatty acids, their derivatives, and/or synthetic compounds (11). PPARα enhances fatty acid combustion in the liver by upregulating genes encoding enzymes in the β-oxidation pathway and hence mediates the hypolipidemic effects of fibrates (35). In contrast, PPARγ serves as an essential regulator of adipocyte differentiation and promotes lipid storage in mature adipocytes (39). Two PPARγ isoforms (PPARγ1 and -2) are produced by the differential use of three promoters and alternative splicing (reviewed in reference 11). Lastly, PPARδ regulates fat burning in peripheral tissues by coordinating fatty...
acid oxidation and energy expenditure (48). Besides their function in lipid metabolism, many potential roles for PPARs have been named in pathological states observed in XP group D (XP-D) patients, such as infertility and cancers (4). In order to evaluate whether a transcriptional defect takes part in the XP-D phenotype, we focused on a putative connection between the hypoplasia of adipose tissue observed in XP-D patients and a defect in transactivation by PPARs. Contrary to what was expected, we observed that the expression of PPARγ target genes is differently affected in the adipose tissues from XPD/TTD-deficient mice. Indeed, whereas some of these genes are abnormally overexpressed in a ligand-independent manner, we also observed a lower induction of PPARγ target genes after treatment with a specific ligand. We found that the defect in transactivation by PPARs in XPD-deficient cells (isolated from both TTD mice and XP-D patients) is associated with a weaker phosphorylation of these nuclear receptors by cdk7. Having identified the phosphorylated residues in the PPAR isoforms, we demonstrate how their transactivation defect in XP-D-deficient cells can be circumvented upon the overexpression of either wild-type (WT) XPD or PPAR S/E, in which the glutamic acid mimics a constitutively phosphorylated residue.

MATERIALS AND METHODS

Animals and morphological studies. The generation of the TTD mouse line R722W has been described previously (10). Mice were fed standard chow with a 5% (wt/wt) fat content (R03 breeding diet; UAR, Villeneuve, France). Fragments of adipose tissues and liver were fixed in 4% formaldehyde for 48 h prior to paraffin embedding. Hematoxylin-and-eosin (H&E)-stained sections were 5 μm thick, while frozen sections for oil red-O staining were 15 μm thick. Apoptosis was assessed by the terminal deoxynucleotidyltransferase-mediated dUTP-biotin nick end labeling (TUNEL) method using the ApopTag kit (Chemicon Int.). Briefly, liver sections were deparaffinized, hydrated, and incubated for 1 min at room temperature in 20 mg/ml proteinase K. After being thoroughly washed in distilled water and immersed in terminal deoxynucleotidyltransferase (TDT) buffer for 10 min at room temperature, the sections were incubated in TDT buffer, digoxigenin-dUTP, and TDT for 60 min in a humidified chamber at 37°C. The reaction was visualized by an immunohistochemical technique using anti-digoxigenin-peroxidase antibodies. Cell regeneration was evaluated by immunohistochemical studies using antibody against the cell proliferation marker Ki67 according to the manufacturer’s instructions (Novocastra). Fragments of various tissues were fixed in 3.7% formaldehyde for RNA and protein extraction. Three-month-old mice were treated by oral gavage with rosiglitazone (10 mg/kg body weight for 3 days; GlaxoSmithKline, WY14643 (100 mg/kg body weight for 20 h; Calbiochem), or the corresponding vehicle (ethanol at 3 ml/kg or dimethyl sulfoxide at 3 ml/kg, respectively).

Retrotranscription and real-time quantitative PCR. RNAs were prepared with TRIzol (GIBCO-BRL) and treated with RNasefree DNase (Promega) according to the manufacturer’s instructions. For reverse transcribe PCR, total RNA (2 μg) was reverse transcribed with Moloney murine leukemia virus reverse transcriptase (Invitrogen) using random hexanucleotides. Real-time quantitative PCR was done using the FastStart DNA Master SYBR Green kit and the LightCycler apparatus (Roche Diagnostic). The primer sequences and the PCR conditions for each target gene are available upon request.

Chromatin immunoprecipitation (ChIP). Hepatic fragments were fixed by paraformaldehyde for 1 h at 4°C. The fragments were harvested by aspiration, resuspended in lysis buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate [SDS], 0.1% Na-deoxycholate containing protease inhibitors. Fragments were homogenized, and cell lysates were then sheared extensively by sonication on ice to obtain fragments of 200 to 600 bp (as revealed by ethidium bromide staining of aliquots run on agarose gels). Samples were centrifuged to pellet debris, and an aliquot was taken for gel analysis and inputs. Soluble chromatin were pre-treated for 2 h at 4°C with protein G (previously saturated with 1 mg/ml of sonicated salmon sperm DNA and 1 mg/ml of bovine serum albumin). Samples were then incubated overnight at 4°C with antibodies of interest; protein G (previously saturated) was then added, and the mixture was incubated for 2 h at 4°C. Sepharose beads were washed twice for 10 min at 4°C with wash 1 (sonicate buffer with 50 mM NaCl) and then with wash 2 (0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 20 mM Tris-HCl [pH 8.0]), and finally with Tris-EDTA (TE; pH 8.0). Immune complexes were eluted from the beads with 1% SDS in TE (pH 8.0), and protein-DNA cross-links were reversed by adding 200 mM NaCl and heating for 5 h at 65°C. After treatment with proteinase K (2 h at 42°C), the samples were purified by phenol-chloroform-isooamyl alcohol extraction and precipitated with ethanol. One-fifteenth of the immunoprecipitated DNA and 1% of the input DNA were quantified by real-time quantitative PCR (see above). Results are expressed relative to the amount of input RNA per ChIP.

Antibodies. Monoclonal antibodies against the TFIHII subunits, TATA box binding protein (TBP), and RNA pol II were produced by the Institut de Génétique et de Biologie Moléculaire et Cellulaire facility. Polyclonal antibodies against VDR (sc-9164; Santa Cruz), PPARα (H-98; Santa Cruz), PPARγ (PA1-820; ABR), and PPAR δ (K-20; Santa Cruz) and monoclonal antibodies raised against PPARγ E-8 (for Western blotting [Santa Cruz], AB12409 for immunoprecipitation [AB Cam LTD]) or phosphorylated S84/S112 PPARγ (1F5; Euromedex) were purchased.

Coimmunoprecipitation assays. PPARα and PPARγ were inserted into the pVL1392 vector, and the resulting vectors were recombined with baculovirus DNA (Baculogold; Pharmingen) as previously described (13). Sf9 cells were infected with combinations of baculoviruses encoding the TFIHII (45) and either VDR (31), PPARα, or PPARγ. Whole-cell extracts were incubated with an antibody directed against either the p44 subunit of TFIHII, VDR, or the corresponding PPAR isoform. After extensive washing (300 mM KC1), bound proteins were resolved by SDS-polyacrylamide gel electrophoresis (PAGE) and revealed by immunoblotting.

Plasmids and construction of PPAR mutants. Specific PCR products including the truncated coding sequence, the truncated coding sequence without the A/B domain, or the A/B domain alone for each PPAR isoform were inserted into the NdeI cloning site of the prokaryotic pET15b expression vector (Novagen). Serine residue changes to alanine or glutamic acid were introduced using the QuikChange site-directed mutagenesis kit (Stratagene).

In vitro kinase assays. His fusion proteins were produced into Escherichia coli strain BL21 and purified on Ni-nitrilotriacetic acid agarose (Qiagen). Equal amounts (1 μg) of recombinant proteins were incubated with purified recombinant CCAK complex (40) or highly purified TFIHII in the presence of [γ-32P]ATP (0.14 μM).

Mass spectrometry. PPAR A/B domains were in vitro phosphorylated by CAK, purified by SDS-PAGE, and in gel digested with Staphylococcus aureus V8 protease (for PPARα and γ) or Asp-N endoprotease (for PPARγ). Peptide mixtures (1 μl) were mixed with equal volume of bicine buffer with 500 mM NaCl) and then with wash 2 (0.25 M LiCl, 0.5% NP-40, 0.5% sodium deoxycholate, 1 mM EDTA, 20 mM Tris-HCl [pH 8.0]), and finally with Tris-EDTA (TE; pH 8.0). Immune complexes were eluted from the beads with 1% SDS in TE (pH 8.0), and protein-DNA cross-links were reversed by adding 200 mM NaCl and heating for 5 h at 65°C. After treatment with proteinase K (2 h at 42°C), the samples were purified by phenol-chloroform-isooamyl alcohol extraction and precipitated with ethanol. One-fifteenth of the immunoprecipitated DNA and 1% of the input DNA were quantified by real-time quantitative PCR (see above). Results are expressed relative to the amount of input RNA per ChIP.

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FIG. 1. Lean phenotype in TTD mice. (A) Photographs of representative 3-month-old XPD+/+, XPD<sup>R722W/R722W</sup>, and XPD<sup>R722W/R722W</sup> mice. (B) Body weights of male WT (n = 6) and TTD (n = 6) mice were monitored for 18 months. (C) Weights of the liver (Liv), intra-peritoneal WAT, and interscapular BAT were normalized to body weight for 3- and 6-month-old male TTD mice (n = 6). The values are percentages relative to those observed for WT mice.

cells were treated with red phenol-free medium containing 10% charcoal-treated FCS–40 μg/ml gentamicin. After 12 h of incubation, the appropriate ligand for each PPAR isotype was added and the mixture was incubated for 24 h. Fibroblasts extracts were analyzed for luciferase and β-galactosidase activities as previously described (25). The results are the mean of at least three independent experiments performed in triplicate.

Statistical analysis. The number of mice for each group used in experiments is indicated in the figure legends. Values are presented as the mean ± the standard error of the mean. A two-tailed Student test was used to calculate P values.

RESULTS

TTD mice exhibit hypoplasia of the adipose tissues. TTD mice bearing the XPD point mutation R722W were used in our study. These mice develop features similar to those observed in XP-D patients, such as brittle hair, developmental defects, skeletal abnormalities, and cachexia (Fig. 1A; reviewed in reference 10). Indeed, weight differences between WT and TTD mice appear and become more pronounced a few months after birth (TTD mice have about 20% lower body weights at 3 months of age; Fig. 1B). The weight loss is not due to disruption of the daily food intake (3.8 ± 0.2 and 4.2 ± 0.2 g/animal/day for WT and TTD mice, respectively). The appearance and fat composition of the feces of TTD mice are normal (data not shown), arguing against malabsorption as the cause of weight loss.

The weights of various tissues were then analyzed at different ages (Fig. 1C). Whereas the TTD liver exhibits a weight similar to the WT, we observed a progressive mass reduction for the perivisceral WAT (48% and 32% of that observed in 3- and 6-month-old WT mice, respectively; Fig. 1C and 2A, parts 1 and 2, arrows) and the interscapular BAT (67% and 52% of that observed in 3- and 6-month-old WT mice, respectively; Fig. 1C). Interestingly, the perivisceral WAT and the interscapular BAT are almost nonexistent in 18-month-old TTD mice (Fig. 2A). Histological analysis after standard H&E staining revealed that the adipocytes in the intraperitoneal WAT of 3-month-old TTD mice are reduced and heterogeneous in size, whereas they are homogeneous and uniform in the WAT of control littermates (Fig. 2B, parts 1 and 2). In parallel, oil red-O staining of TTD BAT cells reveals a decrease in the accumulation of lipid droplets (Fig. 2B, parts 5 and 6), which explains the decrease in the intracytoplasmic vacuoles observed in the H&E staining (parts 3 and 4). Similar histological observations were found in TTD mice at different ages (data not shown).

Dysregulation of PPARγ-responsive genes in the adipose tissues. We were wondering whether the morphological alterations of the TTD mouse adipose tissues could be associated with dysregulation of some genes. Knowing that PPARs play a preponderant role in these tissues (46, 48), we analyzed the expression of genes known to be controlled by PPARs and/or involved in various pathways of lipid metabolism (Fig. 2C).

WT and TTD mice were thus treated by gavage with rosiglitazone (10 mg/kg body weight for 3 days), a specific ligand of PPARγ (30). In WT BAT, the rosiglitazone ligand stimulated the expression of UCP-1 and -3 (uncoupling proteins 1 and 3, involved in the uncoupling of oxidative phosphorylation), mCPT1 (the muscle form of carnitine palmitoyltransferase 1, involved in fatty acid β-oxidation), and aP2 (a fatty acid binding protein) (Fig. 2C). More importantly, we observed that the induction of these genes was 50% lower in TTD BAT than in WT BAT. A similar reduction was observed by transfection assays in XPD-deficient cells (see Fig. 5B and C). We also found in TTD BAT an unexpected ligand-independent over-expression of UCP-1 (10-fold), mCPT1 (3.5-fold) (two genes usually expressed in BAT), and aP2 (4.5-fold). Note that PGC1α, a coactivator that regulates the expression of genes involved in biological programs linked to energy homeostasis (such as thermal regulation), is also overexpressed in both TTD BAT and WAT (about twofold). Finally, under both standard and gavage conditions, no significant changes between WT and TTD mice were observed in the expression of some genes, such as PPARγ, PPARα, the very long chain acyl coenzyme A (CoA) synthetase (involved in fatty acid β-oxidation), UCP-2, the lipoprotein lipase (involved in triglyceride hydrolysis), Spot 14 (involved in lipogenesis), and the acyl-CoA binding protein (involved in fatty acid trafficking) (Fig. 2C and data not shown).

To understand whether such dysregulations are due to PPARγ, we set up ChIP assays. The genomic DNA fragments bound to either PPARγ or RNA pol II were immunoprecipitated with the corresponding antibodies and further analyzed by quantitative PCR. Upon rosiglitazone treatment, PPARγ was much less recruited on the promoters of UCP-1, UCP-3, mCPT1, and aP2 in TTD BAT (Fig. 2C). Therefore, it is not surprising that the occupancy on the respective promoters by RNA pol II parallels that of PPARγ, thus explaining the lower
RNA synthesis level. In the case of the abnormal overexpression of aP2 in TTD WAT, we found that mRNA synthesis perfectly parallels RNA pol II recruitment whereas PPARγ/H9253 is no longer recruited (Fig. 2C). We next hardly detected PPARγ recruitment on the promoters of UCP-1 and mCPT1 (masked in the background; data not shown).

Altogether, our data underline a profound dysregulation of PPARγ-dependent genes in TTD adipose tissues. Whereas the
basal dysregulation observed for some of these genes might occur in a PPARγ (and ligand)-independent manner, the lower response of genes to a specific PPARγ treatment is associated with lower PPARγ recruitment and consequently of the transcription apparatus on their promoters.

**Dysregulation of PPARα-responsive genes in the livers of TTD mice.** We next evaluated whether the transactivity of the transcription apparatus on their promoters.

We next analyzed the aspecific and isolated status of these necroses. Although macroscopic and histological analyses did not reveal strong alterations of the TTD liver, we next analyzed the aspecific and isolated status of these necroses. Weak alterations have been observed, our results led us to suggest the presence of apoptosis in the 3-month-old TTD liver was assessed by the TUNEL method (Fig. 3, parts 9 and 10). We observed a very slight (5%) increase in apoptosis in the livers of TTD mice compared to their control littermates. More importantly, the apoptotic areas were not colocalized to the necrotic areas. Immunodetection of the cell proliferation marker Ki67 revealed a slight increase in cell regeneration in the livers of WT mice compared to TTD mice (9%, parts 11 and 12). Since neither necrosis accumulation nor other hepatic lesions have been observed, our results led us to suggest the aspecific and isolated status of these necroses.

Although macroscopic and histological analyses did not reveal strong alterations of the TTD liver, we next analyzed the expression of hepatic genes known to be regulated by PPARα. Three-month-old WT and TTD mice were treated for 20 h with VY14643, a PPARα agonist, in order to measure the induction of hepatic genes known to be highly and quickly activated by PPARα (8). Under those conditions, the PPARα mRNA level remained unchanged in both WT and TTD livers (Fig. 3B). We also noted that the cellular concentrations of PPARα were similar in WT and TTD mice (see Fig. 5A, right part). Moreover, the expression of CD36 (encoding a glycoprotein involved in the uptake of long-chain fatty acids), the acyl-CoA oxidase (the rate-limiting enzyme in peroxisome β-oxidation of fatty acids), or the mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (involved in ketogenesis) was similarly induced in WT and TTD mice (Fig. 3B and data not shown). On the contrary, pACOTH (peroxisomal acyl-CoA oxidase), involved in the hydrolysis of acyl-CoAs and CYP4A1 (cytochrome P450 4A1, involved in the ω-oxidation of fatty acids) are much less overexpressed in TTD liver, while its recruitment on the CD36 promoter is similar in both livers (Fig. 3B). As expected, upon VY14643 treatment, we found that the recruitment of RNA pol II was much lower on the CYP4A1 promoter in TTD liver. Lastly, no significant difference was observed between WT and TTD mice in the recruitment of TFIIH on the promoters of the CD36 and CYP4A1 genes (Fig. 3B).

Finally, PPARs are phosphorylated by TFIIH via cdk7. Knowing that some nuclear receptors cooperate with TFIIH (7, 25, 38), we examined the physical and enzymatic connections between the PPAR nuclear receptors and TFIIH transcription factor. Extracts from Sf9 insect cells coinfected with baculoviruses encoding the subunits of TFIIH and either PPARγ2 or PPARα were incubated with Ab-p44, a monoclonal antibody directed against the p44 subunit of TFIIH (20). Ab-p44 immunoprecipitates either PPARγ2 or PPARα in addition to TFIIH (Fig. 4A, left and right parts, lanes 8), while it does not retain PPARs in the absence of TFIIH (lanes 6). The presence of a specific ligand does not affect the interaction between TFIIH and PPARs (data not shown). As a control, we observed that Ab-C, an unrelated antibody, does not immunoadsorb either of these components (lanes 9). As previously stated (12), no interaction has been observed between TFIIH and the vitamin D nuclear receptor (VDR), demonstrating the specificity of the PPAR-TFIIH interactions (Fig. 4A, middle part, lane 8). To further determine which TFIIH subunit is targeted by PPARs, Sf9 insect cell extracts coinfected with baculoviruses encoding PPARα or PPARγ2 and either one of the subunits of TFIIH were incubated with the corresponding antibodies (Fig. 4B). Both PPARγ2 and PPARα immunoprecipitated with XPB, p62, p44, and MAT1.

We also investigated whether PPARs can be used as substrates by cdk7. In vitro kinase assays show that both CAK and TFIIH phosphorylate recombinant PPARγ2 and PPARα (Fig. 4D, left and right parts, lanes 1 to 3) and more precisely their A/B domain (both parts, lanes 7 to 9). Indeed, PPAR A/B, the truncated forms that lack the A/B domain, are not phosphorylated (both parts, lanes 4 to 6). Moreover, the A/B PPARs were not phosphorylated by rIIH-Cmut, a recombinant TFIIH in which cdk7 was mutated in its ATP binding site (both parts, lanes 10 to 12). The A/B PPAR motifs were next phosphorylated in vitro by CAK, resolved by SDS-PAGE, and digested for mass spectrometry analysis. Accordingly, and knowing that cdks are serine/threonine kinases, each serine or threonine contained in the putative phosphorylated regions of PPARγ2 and PPARα was mutated to alanine. The mutated A/B proteins were produced in *E. coli* and further purified on a nickel chelate column before being tested in vitro kinase assays. Having observed that some mutations of serine to alanine prevent phosphorylation by either CAK or the entire TFIIH, we demonstrate that residue S112 of PPARγ2 (corresponding to serine 84 of PPARγ1) is a target for cdk7 (Fig. 4E, left part, lanes 4 to 6 and 10 to 12). We also found that the A/B domain of PPARα contains two phosphorylation sites at positions S12 and S21 (Fig. 4E, right part). Indeed, A/B PPARα-S12/S21, in which serines 12 and 21 were changed to alanine, is no longer phosphorylated by TFIIH (Fig. 4E, right part, lanes 13 to 16), whereas A/B PPARα-S12A or A/B
FIG. 3. Expression of PPARα target genes in the TTD liver. (A) Macroscopic view (parts 1 and 2) of 3-month-old WT and TTD mouse livers. Parts 3 to 6: H&E-stained liver sections. Arrows indicate focal lesions corresponding to hepatic necrosis. Parts 7 and 8: oil red-O-dyed liver sections. Parts 9 and 10: in situ apoptosis detection in liver sections by TUNEL assay (see arrows). Parts 11 and 12: immunoperoxidase staining of liver sections for detection of the Ki67 antigen. Arrows indicate Ki67-positive nuclei. PV and CV, portal and centrolobular veins. Magnifications were 60× and 100× for all images.
PPARα-S21A is less phosphorylated (lanes 5 to 12), suggesting a synergistic effect of the phosphorylation of both serines 12 and 21. Additionally, preliminary mass spectral data strongly suggest that the PPARα A/B domain is phosphorylated by cdk7 between residues 44 and 55 (DLSQNSSPSSLL; data not shown).

Phosphorylation of PPARα is crucial for their transactivation. We next questioned whether a default in the phosphor-

FIG. 4. Interaction between TFIIH and PPARs and determination of their cdk7 phosphorylation sites. (A) Sf9 cells were coinfected with baculoviruses encoding the subunits of TFIIH (rIIH wt) and PPARγ2 (left part), VDR (middle part), or PPARα (right part). Immunoprecipitation (IP) was done using an antibody directed against the TFIIH/p44 subunit (Ab-p44, lanes 5 to 8) or a control antibody (Ab-C, lanes 9). The bound proteins were analyzed by Western blotting using antibodies against subunits of TFIIH (XPD, p62 and cdk7), PPARγ2, VDR, or PPARα. (B) Sf9 cell extracts overexpressing each TFIIH subunit alone (−) or in combination with PPARγ2 or PPARα (as indicated) were incubated with antibodies directed against the corresponding PPAR isotype. Immunoprecipitated proteins were analyzed by Western blotting using antibodies against each subunit of TFIIH. The input lanes (IN) represent 10% of the total volume of extracts used in each immunoprecipitation. (C) Schematic representation of the truncated PPAR proteins with a histidine tag (dark box). The different domains (A to F) of PPARγ2 and PPARα and the cdk7 phosphorylation sites are depicted. Serine 112 in PPARγ2 corresponds to serine 84 in the PPARα isoform. (D) Purified PPAR, PPARαA/B, and A/B PPAR were incubated in the absence (lanes 1, 4, 7, and 10) or presence of either free CAK (lanes 2, 5, and 8), TFIIH isolated from HeLa cells (lanes 3, 6, and 9), recombinant TFIIH (rIIH wt, lanes 11), or recombinant TFIIH mutated in the cdk7 ATP binding site (rIIH CKmut, lanes 12). Coomassie blue-stained gels (top parts) and autoradiography (Autoradio; bottom parts) of the incubated fractions are shown. (E) Left part: A/B PPARγ1-wt, A/B PPARγ2-wt, A/B PPARγ1 S84A, and A/B PPARγ2 S112A were incubated with CAK or TFIIH in the presence of 0.14 μM [γ-32P]ATP. Right part: purified A/B PPARα-wt, S12A, S21A, and S12A/S21A were incubated with CAK and increasing concentrations of [γ-32P]ATP (0.07, 0.14, 0.42, and 0.70 μM).
ylation of PPARs might explain their lower ability to transactivate their target genes in XPD-mutated cells. Nuclear extracts of WAT, BAT, and liver isolated from 3-month-old WT and TTD mice were resolved by SDS-PAGE, followed by Western blot analysis (Fig. 5A). We found that the cellular concentrations of PPARγ/1/2 and PPARγ/1/2 are similar in both WT and TTD mice (left and right parts). Nevertheless, using an antibody specifically designed for either phosphorylated S84/S112 of PPARγ/1/2 (Ab PPARγ/1/2-P) or phosphorylated S12 of PPARγ (Ab PPARγ/2-S12), and TBP (Ab TBP) in hepatic nuclear extracts (25 μg) from 3-month-old WT and TTD mice. (B) WT (dark boxes) and TTD (open boxes) mouse fibroblasts were cotransfected with pPPAR-RE-Luc (1 μg), pCH110 (1 μg), and either PPARγ/1-wt, PPARγ/2-wt, PPARγ/1S84A, PPARγ/1S84E, PPARγ/2S112A, PPARγ/2S112E, pSG5-PPARγ-wt, PPARγ S12A/S21A, or PPARγS12E/S21E (100 ng). The cells were next treated with a specific ligand for PPARγ/1/2 (rosiglitazone, 0.5 μM) or PPARγ/1 (WY14643, 1 μM). Luciferase (Luc) activity was measured 24 h later and normalized relative to β-galactosidase activity. Note that the β-galactosidase values were similar in WT and TTD fibroblasts. (C) HeLa (dark boxes) and HD2 (open boxes) cells were transfected with pPPAR-RE-Luc (1 μg), pCH110 (1 μg), pSG5-PPARγ/1 (100 ng), pSG5-PPARγ/2 (100 ng), pSG5-PPARγ/1 (100 ng), and either pcDNA-XPDwt (XPDwt) or empty pcDNA. Cells were then treated with the corresponding ligand, as mentioned for panel B. The values are presented as percentages, 100% being the level of transactivation obtained in HeLa cells overexpressing each PPAR isotype in the presence of the corresponding ligand. Rosi, rosiglitazone.

FIG. 5. Phosphorylation of PPARs is crucial for their transactivation. (A) Left part: Western blotting analysis of PPARγ/1 and -2 (Ab PPARγ) and their phosphorylated status on serines 84 and 112 (Ab PPARγ-P) in WAT (10 μg, lanes 1 and 2) and BAT (25 μg, lanes 3 and 4) nuclear extracts from 3-month-old WT and TTD mice. TBP (Ab TBP) was used as an internal control. Middle part: detection of PPARγ/2 (PPARγ), its phosphorylated status on serine 112 (Ab PPARγ-P) and XPD (Ab XPD) in crude extracts (100 μg) from HeLa and HD2 (bearing XPD point mutation R683W) cells overexpressing PPARγ/2 (lanes 1 to 4) and WT XPD (Ab XPD, lanes 3 and 4). TBP (Ab TBP) was used as an internal control. Right part: detection of PPARα (Ab PPARα), phosphorylated S12 PPARα (Ab PPARα/S12), and TBP (Ab TBP) in hepatic nuclear extracts (25 μg) from 3-month-old WT and TTD mice. (B) WT (dark boxes) and TTD (open boxes) mouse fibroblasts were cotransfected with pPPAR-RE-Luc (1 μg), pCH110 (1 μg), and either PPARγ/1-wt, PPARγ/2-wt, PPARγ/1S84A, PPARγ/1S84E, PPARγ/2S112A, PPARγ/2S112E, pSG5-PPARγ-wt, PPARα S12A/S21A, or PPARαS12E/S21E (100 ng). The cells were next treated with a specific ligand for PPARγ/1/2 (rosiglitazone, 0.5 μM) or PPARγ/1 (WY14643, 1 μM). Luciferase (Luc) activity was measured 24 h later and normalized relative to β-galactosidase activity. Note that the β-galactosidase values were similar in WT and TTD fibroblasts. (C) HeLa (dark boxes) and HD2 (open boxes) cells were transfected with pPPAR-RE-Luc (1 μg), pCH110 (1 μg), pSG5-PPARγ/1 (100 ng), pSG5-PPARγ/2 (100 ng), pSG5-PPARγ/1 (100 ng), and either pcDNA-XPDwt (XPDwt) or empty pcDNA. Cells were then treated with the corresponding ligand, as mentioned for panel B. The values are presented as percentages, 100% being the level of transactivation obtained in HeLa cells overexpressing each PPAR isotype in the presence of the corresponding ligand. Rosi, rosiglitazone.

ylation of PPARs might explain their lower ability to transactivate their target genes in XPD-mutated cells. Nuclear extracts of WAT, BAT, and liver isolated from 3-month-old WT and TTD mice were resolved by SDS-PAGE, followed by Western blot (Fig. 5A). We found that the cellular concentrations of PPARγ/1/2 and PPARα are similar in both WT and TTD mice (left and right parts). Nevertheless, using an antibody specifically designed for either phosphorylated S84/S112 of PPARγ/1/2 (Ab PPARγ-P) or phosphorylated S12 of PPARα (Ab PPARα/S12), we observed that PPARγ/1/2 and PPARα are significantly less phosphorylated in the adipose tissues and liver isolated from TTD mice.

We next designed PPAR expression vectors in which the serine residues phosphorylated by cdk7 were changed to either alanine or glutamic acid, which mimics a constitutively phosphorylated serine residue. Wild-type and TTD mouse fibroblasts were cotransfected with these expression vectors in association with pPPAR-RE-Luc (Fig. 5B). First, we observed that luciferase gene expression was stimulated in WT fibroblasts by PPARγ/1/2 (3.3-fold), PPARγ/1/2 (4.8-fold), and PPARα (4.4-fold) following the addition of their corresponding ligands (Fig. 5B, each part, lanes 3 and 4, dark boxes). In contrast, the transactivation directed by PPARγ/1/2, PPARγ/1/2, or PPARα was approximately 50% lower in TTD fibroblasts than in WT cells (lanes 3 and 4, open boxes). Control experiments showed that reporter gene activation requires both PPAR overexpression...
TABLE 1. Transactivation by PPARs in XPD-deficient human fibroblasts

<table>
<thead>
<tr>
<th>Cell line</th>
<th>XPD mutation</th>
<th>Phenotype</th>
<th>PPARγ1</th>
<th>PPARγ2</th>
<th>PPARα</th>
</tr>
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<tbody>
<tr>
<td>TTD8PV</td>
<td>R112H</td>
<td>TTD</td>
<td>100 ± 8</td>
<td>97 ± 5</td>
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<td>XPICLO</td>
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<td>64 ± 3</td>
<td>65 ± 7</td>
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</tr>
<tr>
<td>TTD12PV</td>
<td>R722W</td>
<td>TTD</td>
<td>63 ± 5</td>
<td>66 ± 3</td>
<td>63 ± 1</td>
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* TTD8PV, XPICLO, and TTD12PV, human primary fibroblasts were transfected with pPPAR- RE-Luc and the expression vector pSG5 PPARα, pSG5 PPARγ1, or pSG5 PPARγ2-wt (Table 1). In the presence of ligand, we observed that luciferase gene expression was reduced in cells bearing XPD C-terminal mutations R683W and R722W, while it was normal in TTD8PV cells harboring the N-terminal mutation R112H. These results demonstrate that transactivation by PPARs is altered in fibroblasts from XP or TTD patients and suggest that this deficiency is associated with the position of the XPD mutation rather than with the nature of the disorder.

DISCUSSION

This work is part of a study aiming to better understand the involvement of TFIIH in the transactivation process. We analyzed how a mutation in its XPD subunit affects this process, which in fine might explain the emergence of some clinical features observed in XP-D patients. We thus found that the adipose tissues and livers of TTD mice (bearing the XPD R722W mutation) have a defect in the regulation of their PPAR-responsive genes. We demonstrate that lower PPAR phosphorylation by the cdk7 kinase of TFIIH contributes to the dysregulation of these genes.

PPAR phosphorylation by cdk7. Our results demonstrate that the activity of PPAR nuclear receptors depends on their ability to be phosphorylated by the DNA repair-transcription factor TFIIH. Mass spectral analysis and mutagenesis show that the phosphorylation sites are located in their A/B domain, also called the AF1 domain, in a serine/proline consensus site, namely, at positions S12 and S21 for PPARα and S84 and S112 for PPARγ1 and -2, respectively. Interestingly, the sites of PPAR phosphorylation by the cdk7 kinase are also targeted by the mitogen-activated protein (MAP) kinases (19, 23). This observation is not restricted to PPARs, since other nuclear receptors are also phosphorylated on the same residue by cdk7 and/or MAP kinases, such as retinoic acid receptor α (38) and estrogen receptor α (7, 24). How do we reconcile that the same residue could be phosphorylated by different kinases? MAP kinases and cdk7 are involved in different signaling pathways, and their actions might occur at different times in cellular life. Whereas PPARs (and in extenso other nuclear receptors) would be phosphorylated in response to some mitogenic signal that activates the MAP kinase pathway, their phosphorylation by cdk7 would occur together with the formation of the transcription complex that puts TFIIH (within the preinitiation complex) and PPARs in very close proximity. Consequently, the same phosphorylation performed by various kinases differently affects the activity of the target factor. Along this line, it was demonstrated that serine 5 from the carboxyl-terminal domain of RNA pol II could be phosphorylated by both cdk7 and cdk8 kinases with adverse consequences for the transcription process. While phosphorylation by cdk8 (which occurs outside the preinitiation complex) prevents the recruitment of RNA pol II on the promoter, modification of serine 5 by cdk7 (which occurs inside the preinitiation complex) promotes initiation of transcription by facilitating promoter escape (2, 17, 36).

Interestingly, PPAR nuclear receptors are much less phosphorylated by cdk7 in cells bearing an XPD C-terminal muta-
tion, as shown in cells isolated from XP-D R683W patients, as well as in tissues of TTD R722W mice. In these cells, the PPAR underphosphorylation is due to the XPD mutation itself, since overexpression of WT XPD restores PPAR phosphorylation to the normal level. How can one explain that any mutation in the C-terminal end of XPD affects PPAR phosphorylation? XPD C-terminal mutations weaken the interaction between XPD and the p44 subunit, and consequently the anchoring of the CAK to the core-TFIIH (9). This might explain why these mutations not only prevent the regulation of the XPD helicase by p44 to allow elimination of DNA damage (13) but also the ability of cdk7 to phosphorylate the nuclear receptors, such as PPARs.

Incidence of PPAR underphosphorylation. In the adipose tissues and livers of TTD mice, the XPD mutation R722W affects the phosphorylation of PPARγ1/2 and PPARα and consequently the expression of PPAR target genes. In some cases, the transactivation by underphosphorylated PPARs is maintained after treatment with a specific ligand, although the PPAR-responsive genes are induced to a much lower level than in the WT ones, as shown for aP2 (in WAT), UCP-1, UCP-3 and mCPT1 (in BAT), and CYP4A1 and pACOTH (in the liver) (Fig. 2C and 3B). Additional ChIP experiments clearly demonstrate that the slight induction of these genes in TTD tissues is consistent with the lower recruitment of PPARs, as well as RNA pol II, on their promoters. Their expression defect is not due to a dysfunction of TFIIH in the basal transcription process, since (i) TFIIH is normally recruited on their promoters and (ii) a TFIIH complex that carries an XPD C-terminal end mutation is able to normally promote RNA synthesis in in vitro transcription assays (9, 25). The expression defect (that was also observed in our transient-transfection assays) is rather due to the inability of TFIIH to accurately phosphorylate PPARγ or PPARα. Indeed, overexpression of WT XPD restores not only transactivation by PPARs but also their phosphorylation in XPD-mutated cells (Fig. 5A, middle part, and C). Moreover, overexpression of the PPAR S-to-E-mutated forms (in which the glutamic acid mimics a constitutively phosphorylated residue) allows normal expression of PPAR-dependent genes even in XPD-mutated cells (Fig. 5B). It remains to be determined whether lower phosphorylation prevents the recruitment of accurate cofactors which are required for the PPAR transactivation process.

In other cases, the expression of PPAR target genes is unexpectedly increased in a tissue-specific manner, as observed for UCP-1, mCPT1, and aP2 in TTD WAT. Since such overexpression occurs independently of the presence of a specific ligand, it is not surprising that PPARs are no longer recruited on their corresponding promoters, while the recruitment of RNA pol II is consistent with the expression level (as illustrated for aP2 in Fig. 2C). Knowing that the expression of any gene requires a subtle combination of several DNA binding factors (14), a defect in the function of one of them might completely reprogram the transcription process. Since PPARs cannot be correctly phosphorylated, one cannot exclude the possibility that their transactivation function is diminished to such a level that they are supplanted by other tissue-specific factors. Further investigations should be undertaken to understand why underphosphorylated PPARs are no longer recruited, giving rise to other regulatory pathways. Altogether, our results show that PPAR target genes are specifically affected by PPAR underphosphorylation in a tissue specific manner. This differential effect can thus explain the apparent discrepancies between our results and data showing that the S112A mutation in PPARγ2 increases the expression of responsive genes (1, 19). In the latter case, it is likely that the nonphosphorylated PPAR S112A might be either assisted or supplanted by another transcriptional factor.

Contribution of PPAR defects to the TTD phenotype. The reduction of adipose mass in TTD mice may occur by a disruption of adipogenesis and/or elevated energy dissipation. Defects in adipogenesis typically result in irregular metabolic features, such as fatty liver, hyperglycemia, and insulin resistance (34, 43). However, in TTD mice, no apparent abnormal fat accumulation was observed in the liver, and the circulating glucose and total triglyceride levels are normal (data not shown). Subtle adipogenic defects cannot be excluded, since the adipose tissues disappear with age (Fig. 2A) and TTD mouse embryonic fibroblasts have a reduced capability to differentiate into adipocytes in the presence of prostaglandin PGJ2, a PPARγ ligand known to induce adipocyte differentiation (data not shown). Besides adipogenic dysfunction, we also observed in TTD mice elevated energy dissipation by indirect calorimetric analysis (1.4 ± 0.06 and 1.6 ± 0.07 kcal/h in WT and TTD mice, respectively; P < 0.05; data not shown), as well as the aberrant overexpression in TTD WAT of BAT-specific genes involved in fatty acid catabolism, such as PGC1α and UCP-1 (33, 42). The overexpression of these genes could contribute to the higher energy expenditure in TTD mice, as previously found in transgenic mice overexpressing UCP-1 in WAT (27).

Therefore, the hypoplasia of the adipose tissues observed within the TTD mice might be due at least partially to a dysfunction of the PPAR nuclear receptors, which are essential in the lipid metabolism and both differentiation (46) and in vivo survival of the adipocytes (16). However, it seems also clear that the overall pleiotropic nature of the XP-D phenotype might result from the defect of various transcriptional factors, such as other nuclear receptors (12, 25). By using diverse treatments, XPD-deficient mice could provide excellent models to elucidate the contribution of a defect of different nuclear receptors, such as the estrogen receptor or the thyroid receptor, in various physiological pathways.

Our in vivo results underline the crucial role of phosphorylation by TFIIH for the transactivation process. Moreover, we demonstrate that a defect in phosphorylating nuclear receptors might simply result in a drop in their transactivation capacity that could (as a function of the tissue and the environmental situation) lead to their being opportunistically replaced by other factors. The major challenge for the future will be to understand how phosphorylation by TFIIH directs the integration of nuclear receptors in the transactivation complex.

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