The DLK gene is a transcriptional target of PPARγ

Jean-Philippe COUTURE and Richard BLOUIN

Département de Biologie, Faculté des sciences, Université de Sherbrooke, Sherbrooke, QC, Canada, J1K 2R1

INTRODUCTION

PPARγ (peroxisome-proliferator-activated receptor γ) is a type II nuclear receptor found in several tissues, including brain, lower intestine and WAT (white adipose tissue), which act on diverse metabolic processes by modulating either positively or negatively the expression of its target genes. Different promoter usage and alternative splicing yield two distinct isoforms of this protein, namely PPARγ1 and PPARγ2, the latter bearing an additional 30 amino acid residues at the N-terminus and being exclusively found in WAT [1]. In order to bind to a PPRE (PPAR response element) in the promoter region of its target genes, PPARγ absolutely requires the heterodimerization with RXR (retinoid X receptor), another member of the nuclear receptor family. Activation or repression of genes targeted by PPARγ is then determined by the binding of a specific ligand into its ligand-binding domain, rather than by the sole recognition of DNA by the transcription factor [2]. In the absence of a ligand, the DNA-bound PPARγ–RXR heterodimer recruits co-repressors, such as N-CoR (nuclear receptor co-repressor) or SMRTs (silencing mediator of retinoid and thyroid receptors), as part of multiprotein complexes that prevent accurate gene transcription [3]. Conversely, the binding of a ligand to PPARγ induces a conformational change that lowers the affinity of the heterodimer for the co-repressors, promotes the recruitment of co-activator complexes such as SRC1 (steroid receptor co-activator 1)/CBP [CREB (cAMP-response-element-binding protein)-binding protein]-binding protein and TRAP (thymus and activation-regulated transcription activator protein)–interacting protein–ARC (activator-recruited cofactor) and results in the activation of gene transcription [4].

Depending on its tissue localization, PPARγ fulfils many different roles in key metabolic processes. It is indeed considered as the master regulator of adipocyte differentiation and, as such, plays a crucial role in the maintenance of glucose homeostasis and adipocyte-related metabolism [5,6]. In obese patients with Type 2 diabetes, activation of PPARγ by synthetic agonists of the TZD (thiazolidinedione) family effectively results in an increase in insulin sensitivity and thus in glucose clearance [7]. Moreover, a higher number of small adipocytes were detected in TZD-treated obese Zucker rats than in untreated littermates [8]. In macrophages, TZD activation of PPARγ rather leads to the repression of several pro-inflammatory genes, such as iNOS (inducible nitric oxide synthase), TNFα (tumour necrosis factor α) and MMP9 (matrix metalloproteinase 9) [9]. There is also increasing evidence that PPARγ plays critical roles in a number of central nervous system diseases and that it may therefore be a highly interesting therapeutic target for the treatment of those illnesses (reviewed in [10]). Thus systemic treatments with a single PPARγ agonist can lead to several distinct responses, depending on target genes and/or cell types.

Of particular interest in this context is the recent observation that the PPARγ agonist rosiglitazone stimulates the accumulation of DLK (dual leucine zipper-bearing kinase) in 3T3-L1 pre-adipocytes induced to undergo differentiation [11]. Originally identified in a screen for protein kinases that are differentially expressed during retinoic acid-induced differentiation of human NT2 teratocarcinoma cells [12], DLK is a MAPKKK [MAPK (mitogen-activated protein kinase) kinase] kinase that emerges as a pivotal component of the MAPK pathways in mammals. Its expression is highest in brain, kidney, skin and adipose tissue,

Abbreviations used: CBP, CREB (cAMP-response-element-binding protein)-binding protein; ChIP, chromatin immunoprecipitation; DLK, dual leucine zipper-bearing kinase; DMEM, Dulbecco’s modified Eagle’s medium; EMSA, electrophoretic mobility-shift assay; FBS, fetal bovine serum; GADPH, glyceraldehyde-3-phosphate dehydrogenase; HEK-293T cell, human embryonic kidney 293 cells expressing the large T-antigen of SV40 (simian virus 40); IBMX, 3-isobutyl-1-methylxanthine; MAPK, mitogen-activated protein kinase; N2a cell, Neuro2a cell; PPARγ, peroxisome-proliferator-activated receptor γ; PPRE, PPAR response element; RT–QPCR, reverse transcription–quantitative PCR; RXR, retinoid X receptor; shRNA, small-hairpin RNA; Sp, specificity protein; TBS-T, Tris-buffered saline/Tween 20; TZD, thiazolidinedione; WAT, white adipose tissue; WT, wild-type.

1 To whom correspondence should be addressed (email Richard.Blouin@USherbrooke.ca).
and targeted deletion of the DLK gene in mice causes perinatal death [13], indicating that this protein has a fundamental role in vivo. Embryos lacking DLK also display abnormal brain development, characterized by defects in axon growth and radial migration of neocortical pyramidal neurons [13,14]. Furthermore, mice with a gene-trap mutation of DLK demonstrated axonal preservation following sciatic nerve injury in vivo, suggesting that this enzyme has a central role in the neuronal response to injury [15]. Additionally, studies by us and others using overexpression and knockdown approaches have shown that DLK is also involved in the regulation of apoptosis [16–18], CREB transcriptional activity [19], terminal differentiation of human epidermal keratinocytes [20], adipocyte differentiation [11], PDGF (platelet-derived growth factor)-stimulated Akt (also known as protein kinase B) kinase and ERK (extracellular-signal-regulated kinase) activation [21], and axonal growth [22]. Altogether, these findings demonstrate a key role for DLK in controlling various fundamental biological processes and illustrate the importance of understanding how DLK is regulated.

Although accumulating evidence suggests that DLK activity and/or abundance in mammals is subjected to regulation by phosphorylation, interactions with different protein partners and ubiquitin-mediated degradation [23], there is still limited knowledge about the molecular mechanisms involved in the transcriptional control of DLK gene expression. To date, the only reported transcription factor that has been shown to be involved in the transcriptional control of DLK is Sp (specificity protein) 3 [24], a ubiquitously expressed member of the Sp family of transcription factors [25]. Because it seemed unlikely that DLK gene expression could be only mediated by Sp3, we evaluated the possibility that PPARγ acts as transcription factor for DLK based on the observation that rosiglitazone increased protein levels of DLK in differentiating 3T3-L1 adipocytes [11]. Our data indicate that PPARγ regulates DLK expression in both 3T3-L1 adipocytes and neuroblastoma Neuro-2a cells, and that DLK is a direct PPARγ target gene with functional PPREs within its proximal promoter.

MATERIALS AND METHODS

Chemicals and antibodies

Dexamethasone, GW9662, IBMX (3-isobutyl-1-methyl-xanthine), insulin, protease inhibitors, a mouse monoclonal antibody raised against α-tubulin and all other common reagents were purchased from Sigma–Aldrich. Rosiglitazone and recombinant PPARγ were purchased from Cayman Chemicals. Recombinant RXRα was obtained from ProteinOne. The rabbit polyclonal antibody against DLK used in the experiments with adipocytes was obtained from Abgent. The rabbit polyclonal antibody against DLKSh (short-hairpin RNA) vector (clone TRCN0000000999; Open Biosystems) as a control or the pLKO.1-based lentiviral vector (Addgene), the pLKO.1-based lentiviral human DLK shRNA (short-hairpin RNA) vector (clone TRCN0000001656, TRCN0000001657 (used in adipocytes), TRCN0000001658, TRCN0000001659 or TRCN0000001660, Open Biosystems) using FuGENETM 6 reagent (Roche Diagnostics). At 72 h post-transfection, the culture medium containing lentiviruses was harvested, treated with polybrene (8 μg/ml) and filtered. For infections, 3T3-L1 (2.5 × 10⁴) and N2a (5 × 10⁴) cells were seeded in 100-mm dishes and infected 24 h later with 1 ml of viral stock diluted in 1 ml of culture medium. After a 1 h incubation at 37°C, 8 ml of fresh culture medium was added, and the cells were either directly subjected (N2a) to selection with 2 μg/ml puromycin or tetracytrinized and replated (3T3-L1) into a medium supplemented with puromycin. The medium was changed every 2 days and, when the cells reached 100% (3T3-L1) or 60–70% confluence (N2a), they were induced to differentiate as described above.

Preparation of cell or tissue lysates and immunoblotting

Cells were lysed for 60 min at 4°C in lysis buffer (50 mM Tris/HCl, pH 7.4, 1% Triton X-100, 150 mM NaCl, 5 mM EDTA, 0.2 mM sodium orthovanadate, 0.2 mM sodium fluoride, 1 mM PMSF, 1 mg/ml leupeptin and 1 mg/ml aprotinin). Lysates were clarified by centrifugation (12 000 × g) and the supernatant was used for the modified Bradford protein assay (Bio-Rad Laboratories). For the preparation of the homogenates, the tissues preserved in liquid nitrogen were ground to a fine powder with a
mortar and a pestle. The tissue powder was then resuspended in the cell lysis buffer described above, incubated for 60 min at 4 °C and clarified by centrifugation. Quantification of the tissue lysates was also performed using the modified Bradford protein assay. For immunoblotting, equal amounts of proteins were fractionated by SDS/PAGE and transferred on to PVDF membranes (Roche Diagnostics) using a semi-dry transfer apparatus (Bio-Rad Laboratories). Membranes were incubated overnight on a rotating plate at 4 °C in a TBS-T (Tris-buffered saline/Tween 20) solution: 20 mM Tris/HCl (pH 7.5), 150 mM NaCl and 0.1% Tween 20 supplemented with 5% (w/v) non-fat dried skimmed milk powder and the primary antibody. Membranes were washed twice in TBS-T before incubation in a solution containing TBS-T, 5% (w/v) non-fat dried skimmed milk powder and the secondary horseradish peroxidase-conjugated antibodies on a rotating plate for 1 h at room temperature. Membranes were washed two more times in TBS-T before immunoreactive bands were detected by using enhanced chemiluminescence (ECL® Plus Western blotting kit; GE Healthcare). The bands from the Western blots were quantified using Genetools® software (Syngene).

**EMSA (electrophoretic mobility-shift assay)**

Oligonucleotides corresponding to the two putative PPARγ−RXR-binding sites in the DLK promoter (−611 bp: 5'-TTCC-TCTTACCTCTAACCCTA TTCCTTTC-3' and −767 bp: 5'-AGGCTTTGGAGAGCCAAGGTAATCGTGA-3') or oligonucleotides incorporating mutations within the core sequence of these sites (−611 bp mut: 5'-TTCCCTGTCAAGGGAATTTCTATCTTC-3' and −767 bp mut: 5'-AGGGTTTGAGGACATTGCC-AAGCGTGCCTTA-3') were labelled with [γ-32P]ATP using T4 polynucleotide kinase for 1 h at 37°C. After gel purification, labelled oligonucleotides were annealed with their respective complementary sequences by heating at 95°C for 5 min, followed by a brief incubation at 37°C. Then, 4000 c.p.m. of each labelled double-stranded probe were incubated with or without 250 ng of recombinant PPARγ−RXR heterodimer and either excess unlabelled WT (wild-type) oligonucleotide or unlabelled mutant oligonucleotide for 15 min at room temperature in binding buffer [4% glycerol, 1 mM MgCl2, 0.5 mM EDTA, 0.5 mM DTT (dithiothreitol), 50 mM NaCl, 10 mM Tris/HCl, pH 7.5, and 1 μg of poly[dI/dC]]. The shifted bands were visualized by autoradiography after non-denaturing electrophoresis in polyacrylamide gels.

**ChIP (chromatin immunoprecipitation)**

Each experiment was performed with a confluent 100 mm dish of 3T3-L1 cells differentiated for the indicated period. Briefly, cells were cross-linked for 10 min at room temperature with 1% formaldehyde in PBS. Cells were then washed in PBS, resuspended in 200 μl of ChIP lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris/HCl, pH 8.0, and protease inhibitors) and sonicated (60% maximum output for 15 s with 3 min pause, four cycles; Branson sonifier type 450 with microtip) in an ice bath. The chromatin solution was diluted 10-fold in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris/HCl, pH 8.1, 16.7 mM NaCl and protease inhibitors). A 5% portion of the lysate was used for purification of total DNA. Each sample was preclarified by incubating with 0.2 μg of salmon sperm DNA−Protein A−agarose, 50% gel slurry (Roche Diagnostics) for 2 h at 4°C. A 10 μg aliquot of indicated antibody (or no antibody for the control) was added and immunoprecipitated at 4°C overnight. The immunoprecipitate was collected using salmon sperm DNA−Protein A−agarose and washed sequentially with the following buffers: low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.1, and 150 mM NaCl); high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris/HCl, pH 8.1, and 500 mM NaCl); LiCl wash buffer (0.25 M LiCl, 1% Nonidet P40, 1% sodium deoxycholate, 1 mM EDTA and 10 mM Tris/HCl, pH 8.1); and TE solution (10 mM Tris/HCl, pH 8.0, and 1 mM EDTA). DNA−protein cross-links were reversed by incubation at 65°C overnight, followed by Proteinase K treatment. DNA was recovered by purification with the Qiaqiuq PCR purification column (Qiagen). Results were analysed by real-time PCR with primers spanning the PPARγ-binding site in the promoter of the DLK gene (forward: 5'-GGTCCCTTTGTCTACCCGACATC-3'; reverse: 5'-GAAAACGAGCGATCCATCTAC-3'), a site located 1.6 kb upstream of the DLK transcription start site (forward: 5'-CGTAAAGGCTCTGCTTCTCCGAGA-3'; reverse: 5'-GCCA-AGTGACAAATCGTCA-3') or, as a negative control, a portion of the osteocalcin gene promoter (forward: 5'-TTCT-GC-ACCCCTCAGCTCCCA-3'; reverse: 5'-TTGCCCCAGGACGCC-GCAATC-3').

**RNA extraction and RT–QPCR (reverse transcription–quantitative PCR) analysis**

Total RNA from differentiated 3T3-L1 cells was recovered using the RNEasy kit from Qiagen. An optional on-column DNase digestion was carried out at room temperature for 15 min to eliminate residual genomic DNA from the samples. Reverse transcription was performed using the MMLV (Moroney murine leukaemia virus)−RT system from Promega (Madison, WI, U.S.A.), random hexamers and 0.25 μg of RNA for 1 h at 37°C. Then, quantitative real-time PCR analysis was carried out with primers specific to DLK pre-mRNA (forward: 5'-CC-TGTCACCCCTCTCTGCTG-3'; reverse: 5'-ATAGGGAGTTGGCCAGGTCGC-3') and PPARγ mRNA (forward: 5'-GCC-CAGGGTTGCTGAACGTGAAG-3' and reverse: 5'-ACAGTGTCTCGTGAGTCACCGT-3').

**In silico promoter analysis**

The proximal promoter sequence of the murine DLK gene (1 kb upstream of the transcriptional start site) was derived from the Ensembl database (http://www.ensembl.org) and screened in silico for putative PPARγ-binding sites using the net-based program MatInspector® of the Genomatix database (http://www.genomatix.de).

**RESULTS**

PPARγ is required for DLK expression in differentiating adipocytes

DLK is expressed in mouse adipose tissue and 3T3-L1 cells induced to undergo adipocyte differentiation [11]. As previously reported, its expression can be increased further in these cells upon stimulation with the PPARγ agonist rosiglitazone (Figure 1A), suggesting the potential involvement of PPARγ in this response. To assess the contribution of PPARγ to DLK expression, we first looked at the effects of pharmacological inhibition of PPARγ with GW9662 on DLK abundance in 3T3-L1 preadipocytes induced to differentiate for 2, 4 or 6 days. The results show that GW9662 completely blocked the accumulation of DLK during the 6-day differentiation period when compared with cells exposed to DMSO (vehicle) or rosiglitazone (Figure 1A). Western blot analysis with an anti-PPARγ antibody also revealed that rosiglitazone and GW9662 have opposite effects on PPARγ in differentiating 3T3-L1 cells, the former stimulating and the latter inhibiting its expression.
To ensure that the effects seen on pharmacological inhibition of PPARγ were not mediated by unspecific targets, we silenced endogenous PPARγ expression by RNA interference in 3T3-L1 cells and assessed DLK levels by immunoblotting. PPARγ knockdown was accomplished by infecting 3T3-L1 cells at the pre-adipocyte stage with a lentivirus encoding puromycin resistance and an shRNA that targets mouse PPARγ mRNA, followed by selection with puromycin and growth in differentiation medium. As a control, cells were also infected with an empty lentivector or a lentivector expressing mouse PPARγ, which express barely detectable levels of DLK (results not shown). Similarly, no alteration of DLK abundance was observed in differentiating adipocytes infected with an empty lentivirus or a lentivirus expressing mouse PPARγ-shRNA were induced to differentiate for 4 days, after which total RNA was extracted and subjected to real-time quantitative PCR analysis with primers specific to DLK pre-mRNA and PPARγ mRNA. Results are representative of several independent experiments. *Statistical significance as determined by the Student’s t test (P < 0.05).

Rosiglitazone treatment increases DLK expression in vivo

Transcriptional analysis of human and mouse tissues has previously revealed that DLK mRNA is mainly expressed in the brain and kidney [12,27]. On the basis of Western blot analysis of mouse organs, high levels of DLK protein are also present in both mesenteric WAT and brown adipose tissue [11]. Because treatment with rosiglitazone causes changes in gene expression in adipose tissue [28], we next examined its effects on DLK abundance in vivo. To do so, female CD-1 mice were treated daily with rosiglitazone with a dose of 3 mg/kg of body weight for 6 days and changes in DLK protein levels were monitored by immunoblotting. As shown in Figures 2(A), 2(B) and 2(D), rosiglitazone treatment promoted a significant increase in DLK protein levels in mesenteric WAT and, to a lesser extent, in brown adipose tissue. In contrast, there was no significant change in gonadal, retroperitoneal, omental and inguinal WAT depots, which express barely detectable levels of DLK (results not shown). Similarly, no alteration of DLK abundance was observed in the heart after rosiglitazone administration (Figures 2C and 2D), which was also without effect on PPARγ expression in any of the tissues examined. Thus these results indicate that DLK expression can be positively modulated by the action of rosiglitazone in vivo and that rosiglitazone-mediated effects on DLK are tissue-specific rather than systemic.

The PPARγ–RXRα heterodimer binds to putative PPRE sequences in the proximal promoter of the DLK gene

Since very little information is known about the genetic control of DLK expression in mammals, we analysed the DNA sequence of the DLK promoter with the MatInspector™ algorithm to identify putative binding sites for PPARγ–RXRα heterodimers. This analysis led to the observation that the DLK promoter contains two potential PPREs located at positions −611 and −767 relative to the transcription start site (Figure 3A). To find out whether
Rosiglitazone treatment increases DLK expression in vivo

Figure 2 Rosiglitazone treatment increases DLK expression in vivo

Figure 2 Rosiglitazone treatment increases DLK expression in vivo

(A–C) Female CD-1 mice were treated daily with either DMSO or rosiglitazone (Rosi; 3 mg/kg of body weight) and were killed 6 days later for tissue collection. Tissues were ground to a fine powder in liquid nitrogen, solubilized in lysis buffer and analysed by immunoblotting with antibodies directed against DLK, PPARγ, and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as the loading control. Histograms represent quantification of DLK abundance, expressed as a fold increase relative to mouse 1. (D) Means ± S.D. of the DLK levels in DMSO compared with rosiglitazone-treated mice reported in (A) (mesenteric adipose tissue), (B) (brown adipose tissue) and (C) (heart). BAT, brown adipose tissue; Mesent., mesenteric adipose tissue; WB, western blot. *Statistical difference as determined by the Student’s t test (P < 0.05). #Non-significant statistical difference (P > 0.05).

these sequences could be recognized by the PPARγ–RXRα heterodimer, we performed EMSA with recombinant PPARγ, recombinant RXRα and 32P-labelled oligonucleotides containing WT or mutated PPRE motifs. As depicted in Figure 3(B), oligonucleotides with WT PPARγ binding sites shifted upon the addition of the protein complex (lanes 2 and 7), whereas mutations in these sites eliminated the DNA–protein association (lanes 3 and 8). Moreover, the formation of the DNA–protein complex was efficiently competed by molar excess of unlabeled WT PPRE oligonucleotides (lanes 4 and 9), but not by the mutant ones (lanes 5 and 10). Taken together, these results show that the PPARγ–RXRα heterodimer can bind to both PPREs in the DLK promoter.

PPARγ, RXR and RNA polymerase II bind to the promoter of DLK in differentiating adipocytes

Because the above in vitro results support a possible role for PPARγ in the regulation of DLK transcription, we verified by ChIP the direct association of PPARγ, RXRα and RNA polymerase II with the DLK promoter in 3T3-L1 cells induced to differentiate for 0, 2, 4 or 6 days. As shown in Figure 4(A), ChIP assays using specific antibodies and primers that span a region between position −649 and −541 relative to the DLK gene transcription start site (highlighted in light grey in Figure 3A) demonstrated a gradual increase in PPARγ, RXRα and RNA polymerase II recruitment over the first four days of differentiation, followed by a decline thereafter. Similar results were obtained with primers spanning the PPRE motif located at position −767 (results not shown). This association was specific, since only background binding for both PPARγ and RXRα was detected at a control site located 1.6 kb upstream of the DLK transcription start site (highlighted in dark grey in Figure 3A). Because the binding of RNA polymerase II to this control region followed a pattern similar to that observed on the −611 PPRE motif, we tested by ChIP the association of RNA polymerase II with the promoter of the osteoblast-specific gene marker, osteocalcin, which was expected to be silent in adipocytes. Our results indicate that there was no significant recruitment of RNA polymerase II to the osteocalcin promoter as differentiation proceeds (Figure 4A), thereby eliminating the possibility of non-specific binding in our experimental conditions.

We also attempted to verify the binding of RNA polymerase II at the transcription start site of the DLK promoter by ChIP but did not succeed, presumably due to the high GC content in this region.

To extend these findings further, we next examined the impact of the PPARγ agonist rosiglitazone and the PPARγ antagonist GW9662 on PPARγ, RXRα and RNA polymerase II binding to the DLK promoter in 4-day-differentiated 3T3-L1 cells. We found
that PPARγ activation or inhibition had either no effect or only a partial but significant stimulatory effect on PPARγ and RXRα binding to the DLK PPRE sequence (Figure 4B). In contrast, the binding of RNA polymerase II to the DLK promoter increased substantially after treatment with rosiglitazone, and it was slightly reduced by GW9662 treatment (Figure 4B), suggesting that these compounds modulate the ability of PPARγ to interact with the transcriptional machinery and consequently regulate DLK gene transcription. Although interesting, this hypothesis remains to be tested extensively because, despite the fact that CBP is a known positive PPARγ cofactor [4], we have not observed an increase or a decrease in its binding to the DLK PPRE motif in response to rosiglitazone or GW9662 (Figure 4B).

To assess the necessity of PPARγ in the recruitment of RXRα and RNA polymerase II to the DLK gene promoter, we silenced its expression in 3T3-L1 cells by RNA interference and assayed its requirement for RXRα and RNA polymerase II binding to the DLK PPRE motif by ChIP 4 days after induction of differentiation. As expected, depletion of PPARγ resulted in reduced PPARγ binding to the DLK promoter and this reduction was paralleled by a marked decrease in the binding of RXRα and RNA polymerase II when compared with control cells (Figure 4C). Taken together, our results demonstrate that the PPARγ–RXRα heterodimer binds to the PPRE sequence of DLK in differentiated 3T3-L1 adipocytes and that PPARγ activation correlates with RNA polymerase II occupancy of the DLK promoter.

### PPARγ regulates DLK gene expression in neuronal cells

Because DLK and PPARγ were both reported to be expressed in neurons [29,30], we verified whether PPARγ also controls DLK gene expression in these cells using the mouse neuroblastoma N2a cell model. To address this, parallel N2a cultures were grown in low-serum medium supplemented with DMSO (control), rosiglitazone or GW9662 for 2 or 4 days and analysed by immunoblot analysis for DLK and PPARγ levels. As shown in Figure 5(A), our results indicate that both DLK and PPARγ proteins were expressed at barely detectable levels in normal culture conditions and accumulated in response to serum deprivation, a known stimulus for neuronal differentiation of N2a cells [31]. In addition, cell extracts from rosiglitazone-treated cells showed a similar accumulation of DLK (Figure 5A), whereas GW9662 antagonized the positive regulatory effects of serum deprivation on DLK and PPARγ levels, suggesting that PPARγ is involved in the transcriptional regulation of DLK. In support of this hypothesis, we found that DLK induction in response to serum deprivation was impaired when PPARγ was depleted by RNA-mediated interference in N2a cells (Figure 5B).

### DISCUSSION

DLK has a tissue-specific distribution in both mice and humans [12,27], implying the existence of a finely tuned regulatory mechanism for DLK gene expression. To date, our understanding
of the key elements conferring tissue-specific expression of DLK is very limited, although it appears that the ubiquitous transcription factor Sp3 drives basal promoter activity [24]. On the basis of recent findings showing that the PPARγ agonist rosiglitazone stimulates the accumulation of DLK in differentiating 3T3-L1 adipocytes [11], we hypothesized that PPARγ regulates DLK expression. Consistent with this hypothesis, the present study shows that DLK protein levels are positively correlated with PPARγ levels in two distinct cell models, namely 3T3-L1 adipocytes and N2a neuroblastoma cells.
Interestingly, PPARγ seems indispensable for DLK expression in these cells as inhibition of its activity or expression by pharmacological and RNA interference approaches completely abolished DLK accumulation in response to differentiation. Moreover, two putative PPREs, located between positions −800 and −600 relative to the transcription start site, were identified in the mouse DLK proximal promoter by in silico analysis and EMSA, together with ChIP-confirmed binding of PPARγ and RXRα to them. Thus, although these results do not invalidate or confirm the participation of Sp3 in DLK gene transcription, they demonstrate an obligatory role for PPARγ in regulating DLK expression in both adipocytes and neurons. In addition, taken together with our previous report showing that DLK-depleted 3T3-L1 adipocytes express lower PPAR levels [11], these findings suggest a model whereby DLK regulates the expression of PPARγ, which in turn binds to the promoter of DLK to activate its transcription.

As stated above, PPARγ is a critical transcription factor for adipocyte differentiation [5]. It regulates genes involved in lipid and glucose metabolism, and its expression is essential for the development of adipose tissue in vivo. Recent studies have also shown that PPARγ is expressed in neurons where it has an essential protective function against damage caused by ischaemia and oxidative stress [32]. This cytoprotective role of PPARγ is probably attributable to the activation of genes involved in cell survival. PPARγ transcriptional activity is dependent on ligand binding to the C-terminal portion of the receptor and the ligands for PPARγ include fatty acids and their derivatives as well as synthetic molecules such as rosiglitazone [2]. In addition to its effects in 3T3-L1 cells, rosiglitazone was also found to stimulate significant DLK accumulation in mesenteric adipose tissue of all treated mice when compared with their control littermates. This finding is particularly interesting since rosiglitazone (Avandia®) is an oral drug used for treating patients with Type 2 diabetes that has, in addition to its beneficial therapeutic effect on this disease, considerable toxicity. For instance, it is known to cause or worsen heart failure [33] and increase the risk of fractures in postmenopausal women [34]. Whether DLK has any impact on these responses to rosiglitazone is unknown at present, but will be an important question to address in the future. Very recently, the American Heart Association and American College of Cardiology Foundation recommended to the pharmaceutical industry to immediately initiate appropriately designed clinical trials of currently approved glucose-lowering agents to determine their effects on cardiovascular events and asked the U.S. Food and Drug Administration to require that such trials be included as part of the initial or ongoing evaluation of new glucose-lowering agents [35]. Given the side effects associated with rosiglitazone and other TZDs, a better knowledge of the mechanisms of PPARγ activation and action would be useful for the rational design of new agonists effective against Type 2 diabetes. As a possible clue, a recent paper has reported that PPARγ is phosphorylated at Ser273 by cyclin-dependent kinase 5 in mice fed on a high-fat diet and that this post-translational modification represses expression of the insulin-sensitizing adipokine adiponectin without affecting the adipogenic capacity of PPARγ [36]. Thus, antagonizing such insulin-resistance-associated post-translational modifications might represent an interesting anti-diabetic strategy.

Together with the observations that the DLK gene is a target for PPARγ-mediated transcriptional activation in adipocytes, our results revealed a function for PPARγ in the regulation of DLK in neurons. Because DLK is involved in brain development and neuronal response to injury [13–15,37], this finding may have important implications for understanding its precise functional role in neurons. In contrast with the effects observed in mesenteric adipose tissue, the expression of DLK was not up-regulated in the brain of mice treated with rosiglitazone (results not shown). The reason for this is not clear but it may relate to the fact that the blood–brain barrier prevents rosiglitazone from entering the brain [38,39]. As PPARγ activators such as prostaglandins or TZDs have been recognized for their neuroprotective properties [10], it will be worth studying what effects they have on neuronal expression of DLK and whether DLK contributes to these PPARγ-mediated responses.

In conclusion, our results indicate that the DLK gene is a novel transcriptional target of PPARγ in adipocytes and neuronal cells, with functional PPREs in its promoter. Since DLK is known to be involved in a wide range of biological events, including development, differentiation and apoptosis, these results provide important insights into the mechanism of DLK regulation.

**Figure 5** PPARγ is required for DLK expression in neuronal cells

(A) N2a cells treated for 0, 2 and 4 days with DMSO, rosiglitazone (Rosi) or GW9662 were lysed and subjected to immunoblot analysis with antibodies against DLK and PPARγ, and GAPDH as the loading control. (B) N2a cells were infected with an empty lentiviral vector (EV) or with lentiviruses expressing mouse PPARγ shRNAs (numbered 1–5). Subsequently, cells were lysed and subjected to immunoblot analysis with specific antibodies against DLK and PPARγ. As a control for protein loading, immunoblots were probed in parallel with an antibody targeting GAPDH. Histograms represent quantification of DLK abundance, expressed as a fold increase ± S.D. relative to control cells. Results are representative of three experiments. WB, Western blot.

**AUTHOR CONTRIBUTION**

Jean-Philippe Couture conceived the study with Richard Blouin and performed the experiments. Jean-Philippe Couture and Richard Blouin analysed the data and wrote the manuscript.
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