SDP1 is a peroxisome-proliferator-activated receptor γ2 co-activator that binds through its SCAN domain

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Peroxisome-proliferator-activated receptors (PPARs), members of the nuclear hormone receptor superfamily, play an important role in the regulation of lipid metabolism and energy homoeostasis. In a yeast two-hybrid experiment using the zinc-finger transcription factor ZNF202 as bait, we previously identified the SCAN-domain-containing protein SDP1. SDP1 shares a high degree of amino acid sequence identity with PGC-2, a previously identified PPARγ2 co-activator from the mouse. Here we show that SDP1 and PGC-2 interact with PPARγ2 through their SCAN domains, even though PPARγ2 does not contain a SCAN domain. Similar to PGC-2, SDP1 enhanced PPARγ2-dependent gene transcription in transiently transfected cells but did not alter the affinity of PPARγ2 for agonists. Although the SCAN domain was necessary for binding to PPARγ2, it was not sufficient for co-activation in cells, suggesting that other features of SDP1 are responsible for transcriptional co-activation. The ability of SDP1 to interact with two different transcription factors that regulate genes involved in lipid metabolism, ZNF202 and PPARγ2, suggests that SDP1 may be an important co-regulator of such genes.

Key words: peroxisome-proliferator-activated receptor, transcription, zinc finger, ZNF202.

INTRODUCTION

Nuclear hormone receptors (NHRs) are ligand-dependent transcription factors that regulate gene expression upon the binding of small molecules or hormones, thus co-ordinating a variety of physiological stimuli. The peroxisome-proliferator-activated receptors (PPARs), of which there are three genes, PPARα (NR1C1), PPARδ (NR1C2), and PPARγ (NR1C3), are members of the NHR superfamily that regulate genes involved in lipid metabolism and energy homoeostasis [1,2].

Similar to many transcription factors, PPARs are modular in function, with separate domains involved in DNA-binding and transcriptional activation. The N-terminal A/B domain is poorly conserved among PPAR family members and contains a ligand-independent transcriptional activation domain, activation function-1 (AF-1). Within PPARγ2, a PPARγ splice variant expressed primarily in adipose tissue, the AF-1 domain contains a mitogen-activated protein kinase phosphorylation site, which upon phosphorylation decreases PPARγ2 transcriptional activity [3–5]. Mutations in the AF-1 domain have been shown to affect phosphorylation and to alter the affinity of PPARγ2 for ligands [6,7]. The centrally located DNA-binding domain (DBD) is composed of two zinc-finger type DNA-binding modules that show a high degree of sequence conservation among PPAR family members. PPARγ2, like all PPAR family members, binds to DNA response elements containing two repeats of the consensus sequence AGGTCA separated by a single nucleotide, known as DR-1 elements, as a heterodimer with the 9-*cis* retinoic acid receptor (RXRα) [8–11].

The C-terminal half of the receptor contains the ligand-binding domain (LBD), which accounts for the pharmacologically distinct functions of the different PPAR family members [12]. Ligand binding induces a conformational change within the LBD of PPARγ2 that can lead to the recruitment of co-activator proteins, such as steroid receptor co-activator-1 (SRC-1) or p300/CBP, and subsequent transcriptional activation [13–15]. Ligand-dependent co-activators, such as SRC-1 or p300, interact with PPARγ2 through an LXXLL motif [16,17]. In contrast to SRC-1 or p300/CBP, PGC-2, a PPARγ2 co-activator from mouse, interacts with PPARγ2 in the absence of ligand and does not contain an LXXLL motif [18]. PGC-2 increases the transcriptional activity of PPARγ2 in transiently transfected COS-7 cells and functions as a differentiation cofactor, enhancing the ability of PPARγ2 to promote adipogenesis in 3T3-L1 cells [18].

Little else is known about PGC-2 except that it contains a nearly complete SCAN domain. The SCAN domain was originally identified in a subset of Krüppel-type zinc-finger proteins as a conserved region of approx. 60 amino acid residues enriched in leucine and glutamic acid amino acid residues [19,20]. Subsequently, the SCAN domain has been shown to mediate selective protein–protein interactions with other SCAN-domain-containing proteins [21–23].

In the present study, we show that SDP1 (SCAN-domain-containing protein 1; also termed RAZ1) [23], similar to PGC-2, enhances PPARγ2-dependent transcriptional activation in transiently transfected cells. Using a series of mutant proteins, we show that SDP1 binds the DNA-binding/hinge region of PPARγ2 through the SDP1 SCAN domain. However, the SDP1 SCAN domain is insufficient for co-activation *in vivo*, which suggests that sequences within the N-terminus of SDP1 contribute to its function as a PPARγ2 co-activator. Therefore the ability of the SDP1 SCAN domain to interact with both PPARγ2, a non-SCAN-domain-containing protein, and with SCAN-domain-containing zinc-finger transcription factors, suggests a new and broader role for SDP1 in the transcriptional regulation of genes involved in lipid metabolism and energy homoeostasis.

Abbreviations used: AF-1, activation function-1; DBD, DNA-binding domain; DMEM, Dulbecco's modified Eagle's medium; ER, oestrogen receptor; FBS, fetal bovine serum; GST, glutathione S-transferase; LBD, ligand-binding domain; LPL, lipoprotein lipase; NHR, nuclear hormone receptor; PGC-2, PPARγ co-activator-2; PPAR, peroxisome-proliferator-activated receptor; PPRE, PPAR response element; RXR, retinoic acid receptor; SDP1, SCAN-domain-containing protein 1; SRC-1, steroid receptor co-activator-1.

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MATERIALS AND METHODS

Construction of plasmids

pCMVβ was purchased from ClonTech Laboratories. The plasmids pCDNA3.1(+)ZNF202, pCDNA3.1(+)ZNF202m3 [24], pCDNA3.1(+)SDP1 and pGEX4T-SDP1 [22] have been described previously. PGC-2 coding sequences were amplified by PCR using the IMAGE consortium clone 407124 (Research Genetics, Huntsville, AL, U.S.A.) as a template and inserted into the *Xba*I and *Eco*RI sites of pCDNA3.1(+) and *Eco*RI site of pGEX4T-1 respectively. The Cyp4A6Z.SV40.luc reporter plasmid was created by inserting two copies of the Cyp4A6Z peroxisome-proliferator response element (ACACTGAACTAGGGCAAAGTTGAGGG) in a forward/reverse orientation into the *Xho*I site of the pGL3-promoter vector (Promega). The Gal4-SEAP reporter, pG5-SEAP, was constructed by inserting a *Kpn*I and *Nhe*I fragment from pG5-luc (ClonTech Laboratories), containing five tandem repeats of the Gal4 UAS, into the *Nhe*I and *Kpn*I sites of pSEAP2-promoter (ClonTech Laboratories).

The plasmid pCDNA3.1(+)KhPPARγ2 was constructed as follows. An *Asp*718I/*Xho*I fragment containing the entire PPARγ2 open-reading frame was inserted into the *Asp*718I/*Xho*I sites of pCDNA3.1(+), creating the plasmid pCDNA3.1(+)hPPARγ2. To create pCDNA3.1(+)KhPPARγ2, the N-terminal *Bam*HI fragment of hPPARγ2 was replaced with a PCR generated fragment containing a consensus Kozak sequence using the primers (5′-CGGGATCCACCATGGGTGAAACTCTGG-3′) and (3′-CGCCAACAGCTTCTCCT-5′). The plasmids pCDNA3.1(+)hPPARα, pGEX4T-1hPPARα, pCDNA3.1(+)hPPARδ, and pGEX4T-1hPPARδ were created by transferring the PPAR coding sequence as a *Bam*HI fragment from pSG5TetR-PPARα or pSG5TetR-PPARδ [25] to the *Bam*HI site of pCDNA3.1(+) or pGEX4T-1 respectively. The plasmid pCDNA3.1(+)hRXRα was created by inserting RXRα coding sequences into the *Eco*RI and *Xba*I sites of pCDNA3.1(+). The plasmid pCDNA3.1(+)ERα was created by inserting the oestrogen receptor (ER) α coding sequences from pGEX2TK-ERα (kindly provided by M. Brown, Dana Farber Cancer Institute, MA, U.S.A.) as a *Bam*HI and *Eco*RI fragment into the *Bam*HI and *Eco*RI sites of pCDNA3.1(+). The plasmid pGEX4T-1hPPARγ2 was generated by PCR amplification of the N-terminal 138 amino acids of hPPARγ2 using PCR primers containing *Eco*RI and *Bam*HI sites and inserted into the *Eco*RI and *Bam*HI sites of pGEX4T-1 to create pGEX4T-1hPPARγ2/1-138. Subsequently, a *Mun*I and *Xho*I fragment containing C-terminal PPARγ2 sequences from pCDNA3.1(+)KhPPARγ2 was cloned into the *Mun*I and *Xho*I sites of pGEX4T-1hPPARγ2/1-138 to create pGEX4T-1hPPARγ2.

The pCDNA3.1(+) PPARγ2 deletion constructs (PPAR$\gamma2_{139-505}$, PPAR$\gamma2_{1-310}$, PPAR$\gamma2_{1-138}$, PPAR$\gamma2_{139-310}$ and PPAR$\gamma2_{311-505}$), N-terminal SDP1 [pCDNA3.1(+)SDP1$_{1-102}$, pGEX4T-1SDP1$_{1-102}$] and C-terminal SDP1 [pCDNA3.1(+)SDP1$_{103-179}$, pGEX4T-1SDP1$_{103-179}$] and Gal4 fusion (pBIND-SDP1, pBIND-SDP1$_{103-179}$, pBIND-SDP1$_{103-179}$) expression constructs were generated by PCR using appropriate pairs of oligonucleotide primers, and inserted into pCDNA3.1(+), pGEX4T-1 (Amersham Biosciences) or pBIND (Promega). All constructs were verified by DNA sequence analysis.

Protein expression

The glutathione S-transferase (GST) fusion proteins used in protein–protein interaction assays were produced as described previously [26]. Briefly, 100 ml cultures of transformed strains of *Escherichia coli* (BL21) DE3 were grown at 37 °C to an attenuance (D_{600}) of approx. 0.6 and induced with 0.5 mM isopropyl β-D-thiogalactoside (IPTG). After 4 h of induction, cells were harvested by centrifugation and purified as described previously [26]. Bound proteins were analysed by SDS/PAGE followed by Coomassie staining to determine protein integrity and purity. [^{35}S]Methionine-labelled proteins were produced *in vitro* from pCDNA3.1(+) constructs using the TNT T7 Quick coupled transcription/translation system (Promega) according to the manufacturer's recommendations.

Protein–protein binding assays

Purified GST-fusion proteins (approx. 1.0 μg) bound to glutathione–Sepharose beads (Pharmacia) were incubated with 6 μl of [^{35}S]methionine-labelled proteins and 25 μl of HEGMN buffer {25 mM HEPES (pH 7.5), 100 mM KCl, 12.5 mM $MgCl_2$, 10 % (v/v) glycerol, 0.1 mM EDTA and 0.1 % Nonidet P40 [27]} containing 0.15 M KCl, in a total volume of 50 μl for 2 h at room temperature. Bound proteins were recovered as described previously [28] and analysed by SDS/PAGE and autoradiography.

Cell culture

All cell lines were obtained from American Type Culture Collection (Manassas, VA, U.S.A.). HeLa and HEK293 cells were maintained in high glucose Dulbecco's Modified Eagle's Medium (DMEM; Invitrogen) containing 10 % (v/v) fetal bovine serum (FBS; Gemini Bio-products, Woodland, CA, U.S.A.). HepG2 cells were maintained in Minimum Essential Medium alpha (Invitrogen) containing 10 % (v/v) FBS.

Reporter assays

Approx. 24 h prior to transfection, 1×10^5 cells per well (HeLa, HEK293, or HepG2) were plated in a 12-well plate in 1 ml of Phenol-Red-free, low-glucose DMEM (Invitrogen) containing 4 mM Glutamax I (Invitrogen) and 10 % (w/v) charcoal-treated FBS. Cells were maintained in Phenol-Red-free medium containing 10 % (w/v) charcoal-treated FBS throughout the course of the reporter gene assay. FBS was depleted of lipids by charcoal treatment as follows: 5 g of Norit-A charcoal (Sigma) and 0.5 g Dextran T70 (Pharmacia) were added to 500 ml of 1 × PBS and stirred slowly overnight at room temperature. The following day, the suspension was centrifuged at 3000 ***g*** for 15 min. After discarding the supernatant, the pellet was resuspended in 500 ml of FBS and incubated for 30 min at 55 °C in a water bath with slow stirring. Following centrifugation as described above, the supernatant was sterile filtered and stored at −20 °C in 50 ml aliquots for no more than 1 year.

We performed pilot experiments in HeLa, HepG2, and HEK293 cells to determine the linear range for reporter gene activation by PPARγ2 plasmid for each cell type. To assay reporter gene activation, cells were transfected with 250 ng of reporter plasmid, 50 ng of pCMV-β internal control plasmid, 250 ng of pCDNA3.1(+)SDP1 or its derivatives along with the amount of pCDNA3.1(+)PPARγ2 expression plasmid determined in the pilot experiments to give 50 % maximal activation (10 ng for HeLa and HEK293 cells or 5 ng for HepG2 cells) using FuGENE (Roche) diluted in serum-free optiMEM media (Invitrogen; according to manufacturer's recommended conditions). Fresh medium, containing compounds or solvent, was added to the cells 6 h after transfection. The following day the medium was changed as before. Approx. 24 h later (40–48 h post-transfection), cells were washed twice with 1 × PBS and

lysed with 200 μl of lysis buffer (Tropix, Bedford, MA, U.S.A.). Luciferase and β-galactosidase (Dual-light assay kit, Tropix) or SEAP (secreted alkaline phosphatase; Tropix) activities were analysed by chemiluminescent detection (according to manufacturer's recommendations). Luminescent activities were determined in triplicate in 96-well plates by luminometry (EG&G Berthold, Boston, MA, U.S.A.).

RESULTS

We had previously cloned a SCAN-domain-containing protein, SDP1, in a yeast two-hybrid screen to identify human proteins that interact with the N-terminal SCAN domain of the zinc-finger transcription factor ZNF202 [22]. To elucidate a possible function for SDP1 *in vivo*, we performed a database search to identify proteins that were similar to SDP1. As shown in Figure 1(A), SDP1 shares striking similarity with the SCAN-domain-containing protein PGC-2, a PPARγ2 co-activator previously identified from mouse [18]. Overall, SDP1 and PGC-2 are 59 % identical at the amino acid level, and the SDP1 SCAN domain (amino acids 103–179) is nearly identical (96 %) with that of PGC-2.

Owing to the strong sequence similarity between the two proteins, particularly within the SCAN domain, we suspected that SDP1 was the human orthologue of PGC-2. To determine whether SDP1 functions as a PPARγ2 co-activator in cells, we transiently transfected HEK293 cells with a reporter construct containing two copies of a PPAR response element (PPRE) from the Cyp4A6 gene and a human PPARγ2-expression plasmid, either alone or in the presence of PGC-2 or SDP1 expression plasmids. As shown in Figure 1(B), addition of the PPARγ-specific ligand BRL 49653 failed to stimulate reporter gene expression, possibly due to low levels of endogenous PPARγ2 in HEK293 cells, while overexpression of PPARγ2 resulted in a 3-fold ligand-dependent induction of reporter gene expression. Co-expression of PGC-2 with PPARγ2 resulted in a further 2-fold

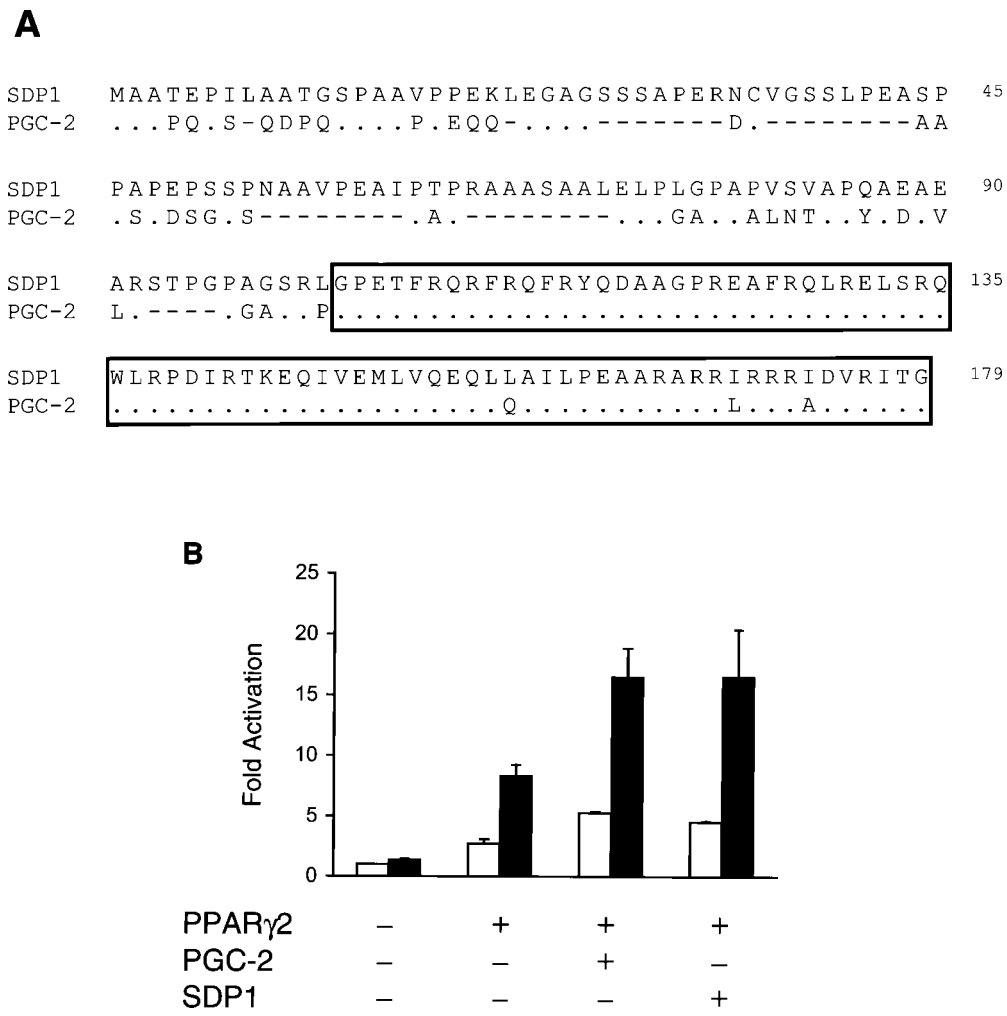

Figure 1 SDP1 is a PPARγ2 transcriptional co-activator

(**A**) Amino acid sequence alignment of SDP1 and PGC-2. Amino acids are represented by their one-letter abbreviation. Gaps in the alignment are indicated by dashes (—), identical amino acids in PGC-2 are indicated by dots (.), and the SCAN domain is boxed. Amino acids are numbered according to their position in SDP1. (**B**) SDP1 and PGC-2 co-activate PPARγ2 in transiently transfected cells. HEK293 cells (1×10^5) were transiently transfected with a luciferase reporter construct containing two copies of a PPRE from the Cyp4A6 gene along with either PPARγ2, PGC-2, or SDP1 expression plasmids in the presence (black bars) or absence (white bars) of the PPARγ-specific ligand BRL 49653 (10 μM). The presence or absence of PPARγ2, PGC-2 and SDP1 expression plasmids is indicated at the bottom of the figure. Cell lysates were prepared 48 h after transfection and luciferase activity was measured in a luminometric assay and normalized to control β-galactosidase activity as described in the Materials and Methods section. The data shown are the average of three independent transfections with S.E. indicated.

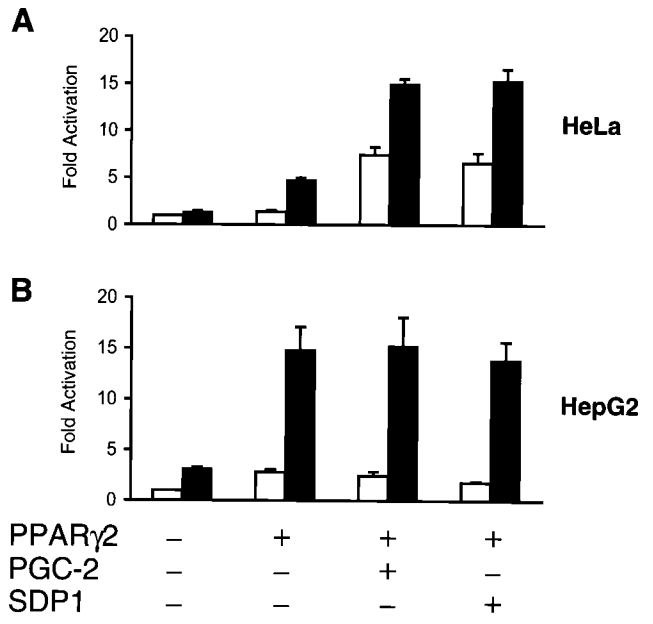

Figure 2 SDP1 and PGC-2 fail to co-activate PPARγ2 in HepG2 cells

HeLa (**A**) or HepG2 (**B**) cells were transiently transfected with a PPRE reporter plasmid and PPARγ2, SDP1 and PGC-2 expression plasmids in the presence (black bars) or absence (white bars) of the PPARγ2-specific ligand BRL 49653 (10 μM), as described in Figure 1.The presence or absence of PPARγ2, PGC-2 and SDP1 expression plasmids is indicated at the bottom of the figure. The data shown are the average of three independent transfections with S.E. indicated.

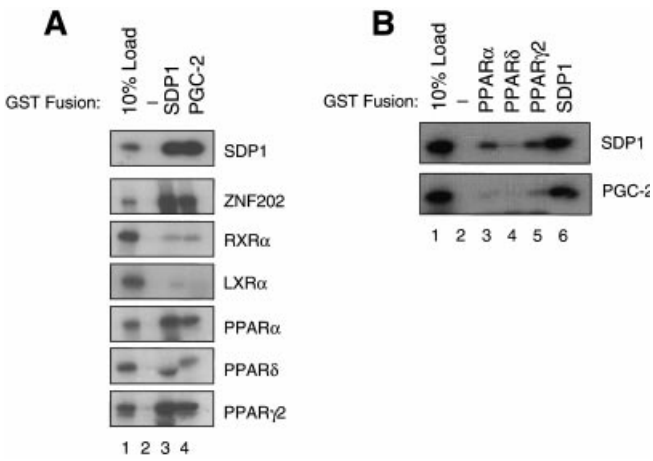

Figure 3 *In vitro* binding of SDP1 and PGC-2 to NHR family members

(**A**) SDP1 and PGC-2 interact with members of the NHR superfamily. GST alone (lane 2) or fused to SDP1 or PGC-2 (lanes 3 and 4 respectively) was incubated in the presence of *in vitro* translated and [^{35}S]methionine-labelled proteins, indicated at the right of each panel. (**B**) GST alone (lane 2) or fused to PPARα (lane 3), PPARδ (lane 4), PPARγ2 (lane 5) or SDP1 (lane 6) was incubated in the presence of *in vitro* translated and [^{35}S]methionine-labelled SDP1 (upper panel) or PGC-2 (lower panel). Lane 1 shows 10% of the input protein. Recovered proteins were analysed by SDS/PAGE and autoradiography.

increase in ligand-dependent reporter gene expression, consistent with previous results [18], and also increased reporter gene expression in the absence of ligand. Expression of SDP1 increased reporter gene expression to similar levels, suggesting that, at the level of transcriptional activation, SDP1 is a functional orthologue of the PPARγ2 transcriptional co-activator PGC-2.

Northern blot analysis showed that both SDP1 and PGC-2 are ubiquitously expressed, suggesting that they function as co-activators in a variety of cell and tissue types [18,22,23]. Therefore we tested the co-activator function of SDP1 and PGC-2 in two different human cell lines, HeLa (human cervical carcinoma) and HepG2 (human hepatoma) cells, to determine whether they display cell-type specific transcriptional activation properties. As shown in Figure 2, SDP1 and PGC-2 show strong levels of co-activator function in HeLa cells (Figure 2A), increasing PPARγ2 reporter gene expression approx. 3-fold, both in the presence and absence of ligand. Surprisingly, SDP1 and PGC-2 exhibited no co-activation properties in HepG2 cells (Figure 2B), failing to stimulate PPARγ2 reporter gene expression. Western blot analysis of HepG2 cell extracts failed to show SDP1 protein in untransfected cells, suggesting that the lack of co-activation does not result from high endogenous levels of SDP1 protein (results not shown). Thus HepG2 cells may harbour other PPARγ co-activator proteins that preclude co-activation by SDP1 (or PGC-2). Alternatively, SDP1 may display cell-type specific co-activation properties.

In vitro, PGC-2 has been shown to interact with PPARγ2 (and ERα), but not with other PPAR family members [18]. To determine the binding specificity of SDP1, we tested the binding of an *E. coli*-expressed and purified GST-SDP1 fusion protein to *in vitro* translated PPARγ2 and several other NHRs. As a positive control for protein–protein binding, we included ZNF202 and SDP1, both of which contain SCAN domains and have been shown to interact with GST-SDP1 in a similar assay [22]. As shown in Figure 3(A), compared with GST alone (lane 2), GST-SDP1 (lane 3) binds both ZNF202 (100% of input recovered) and SDP1 (22%). GST-SDP1 (and GST-PGC-2, lane 4) also bound PPARγ2 (21%) and ERα (7%; results not shown) and surprisingly, bound PPARα (14%), PPARδ (11%), although at weaker levels compared with PPARγ2. In contrast, RXRα (< 1%), the heterodimeric partner of PPAR family members, and LXRα (< 1%) failed to bind GST-SDP1.

To confirm our results, we tested the binding of *E. coli*-expressed and purified GST-PPAR fusion proteins to *in vitro* translated SDP1 or PGC-2 in a reciprocal protein–protein interaction assay. As shown in Figure 3(B), GST-PPARα and GST-PPARγ2 (lanes 3 and 5) bound *in vitro* translated SDP1 (upper panel) effectively, whereas GST-PPARδ (lane 4) bound weakly. PGC-2 showed a similar, albeit weaker, pattern of binding to the GST-PPAR fusions (lower panel). Overall, Figures 3(A) and 3(B) show that SDP1 and PGC-2 display similar specificity in their ability to interact with PPAR family members, as would be expected for human/mouse orthologues.

PGC-2 was identified by its ability to bind to an N-terminal fragment of PPARγ2 (amino acids 1–138) in a yeast two-hybrid assay, and this N-terminal fragment was suggested to be sufficient for PGC-2 binding [18]. To determine which regions of PPARγ2 are necessary and sufficient for SDP1 binding, we created a set of PPARγ2 deletions, as illustrated in Figure 4(A). As shown in Figure 4(A), removal of the PPARγ2 LBD (PPARγ2$_{1–310}$) did not disrupt GST-SDP1 binding as compared with full-length PPARγ2 (18% versus 18%). Unexpectedly, removal of the AF-1 domain (PPARγ2$_{139–505}$) only modestly decreased binding to GST-SDP1. Consistent with this observation, neither the AF-1 domain (PPARγ2$_{1–138}$) nor the LBD (PPARγ2$_{310–505}$) effectively bound GST-SDP1 (5% versus 5% respectively), whereas the PPARγ2 DNA-binding/hinge region (PPARγ2$_{139–310}$) did bind GST-SDP1 (20%) to the same degree as full-length PPARγ2. A similar pattern of binding was seen with PGC-2 (results not

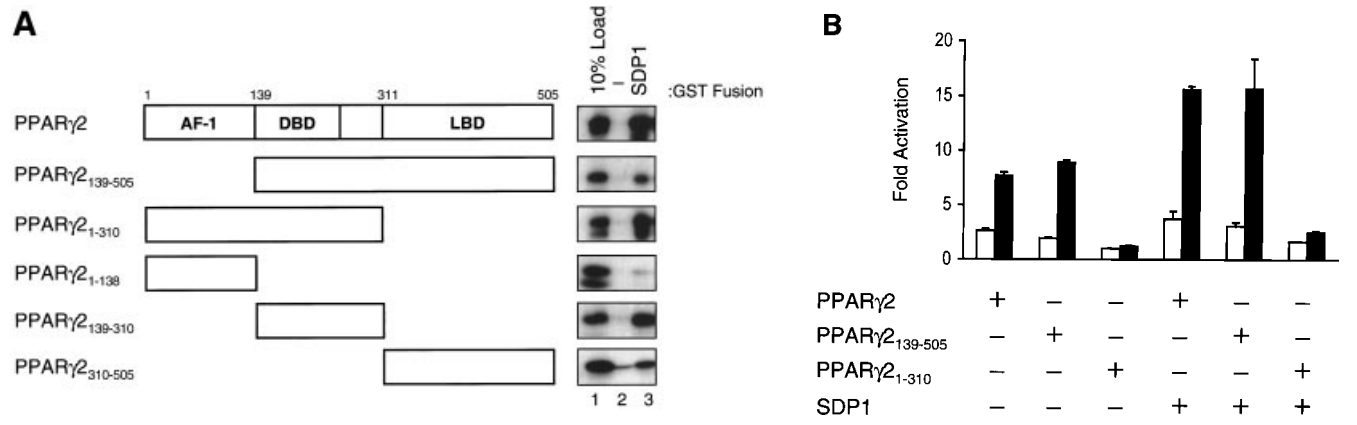

Figure 4 SDP1 interacts with the DNA binding domain or hinge region of PPARγ2

(**A**) GST alone (lane 2) or fused to SDP1 (lane 3) was incubated in the presence of *in vitro* translated and [^{35}S]methionine-labelled proteins, indicated at the left of each panel. Lane 1 shows 10% of the input protein. Recovered proteins were analysed by SDS/PAGE and autoradiography. (**B**) HEK293 cells were transiently transfected with a PPRE reporter plasmid, SDP1 expression plasmid and either wild-type or mutant PPARγ2 and expression plasmids in the presence (black bars) or absence (white bars) of the PPARγ2-specific ligand BRL 49653 (10 μM) as described in Figure 1. The data shown is the average of three independent transfections with S.E. indicated.

shown). These results show that the PPARγ DNA-binding/hinge region is sufficient for binding between PPARγ2 and SDP1.

We next tested the ability of SDP1 to co-activate PPARγ2 deletion constructs that lack either the N-terminal AF-1 region or the LBD. As shown in Figure 4(B), deletion of the AF-1 domain (PPAR$\gamma 2_{139-505}$) displayed near wild-type levels of ligand-dependent transcriptional activation, as expected [29], and consistent with the *in vitro* binding studies, co-expression of SDP1 enhanced the transcriptional activity of PPAR$\gamma 2_{139-505}$. In contrast, deletion of the PPARγ2 LBD (PPAR$\gamma 2_{1-310}$) removed essentially all of the PPARγ2 transcriptional activity. SDP1 failed to co-activate this mutant even though SDP1 was able to bind it *in vitro*. Overall, these data indicate that SDP1 interacts with the DNA-binding/hinge region of PPARγ2.

Mutational studies within the PPARγ2 AF-1 domain have been shown to alter the affinity of PPARγ2 for its ligand [7]. Because SDP1 shows some interaction with the AF-1 domain, in addition to the DBD, we reasoned that SDP1 could function by altering the affinity of PPARγ2 for agonists. To determine whether expression of SDP1 alters the affinity of PPARγ2 for its agonists, we transiently transfected HeLa cells and generated a dose–response curve, either in the absence or presence of SDP1, for two synthetic agonists; the PPARγ-specific ligand, BRL 49653 (rosiglitazone) (Figure 5A), and the PPARα/PPARγ dual-specificity ligand KRP 297 [30] (Figure 5B). As shown in Figure 5(A), the half-maximal concentration (EC_{50}) of BRL 49653 needed for PPARγ2 activation was not significantly altered by the presence of SDP1 (32 nM versus 26 nM). Similar results were seen with the dual-specificity ligand KRP 297 (290 nM versus 290 nM; Figure 5B). Thus under conditions that co-activate PPARγ2, SDP1 did not significantly alter the affinity of PPARγ2 for two distinct PPARγ agonists.

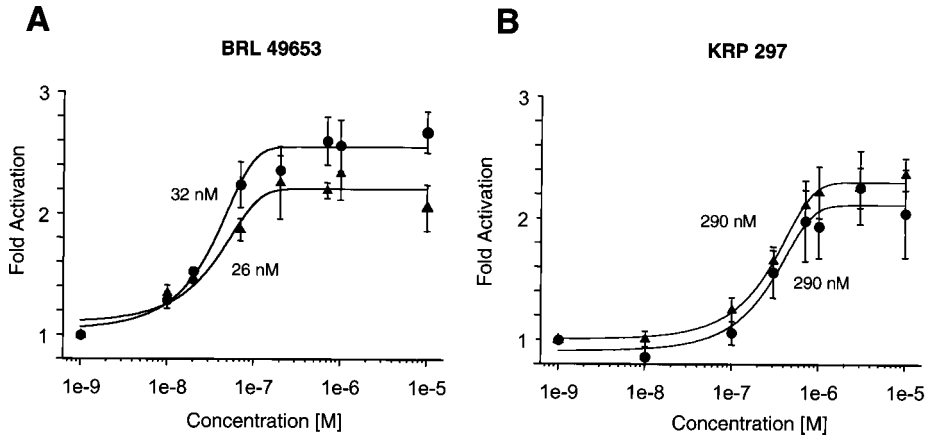

Figure 5 SDP1 does not alter the affinity of PPARγ2 for its ligand

Dose–response curve of PPARγ2 for two synthetic agonists. HeLa cells were transiently transfected as described in Figure 1, either in the absence (●) or presence (▲) of SDP1. The concentration of the PPARγ-specific ligand BRL 49653 (**A**) or the PPARα/γ dual specificity ligand KRP 297 (**B**) is indicated on the x-axis and is shown in logarithmic scale. The level or transcriptional activation in the absence of ligand was arbitrarily set at 1. The EC_{50} of each ligand for PPARγ2, in either the absence or presence or SDP1, is indicated next to each curve. Curves were generated using Sigma Plot (v.5) and EC_{50} values were determined using XL_{fit}. The data shown are the averages of three independent transfections with S.E. indicated.

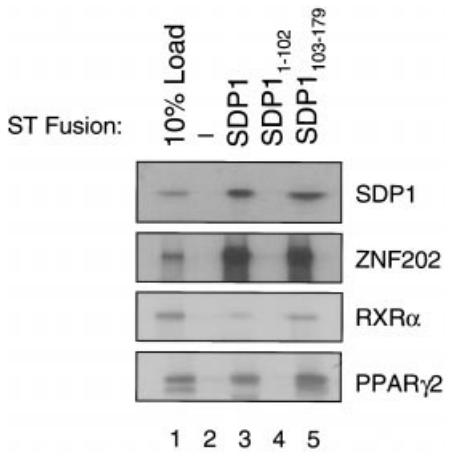

Figure 6 The SDP1 SCAN domain interacts with PPARγ2

In vitro binding of N- and C-terminal halves of SDP1 to PPARγ2. GST alone (lane 2) or fused to SDP1, $SDP1_{1–102}$ or $SDP1_{103–179}$ (lanes 3, 4 and 5 respectively) was incubated in the presence of *in vitro* translated and [^{35}S]methionine-labelled proteins, indicated at the right of each panel. Lane 1 shows 10% of the input protein. Recovered proteins were analysed by SDS/PAGE and autoradiography.

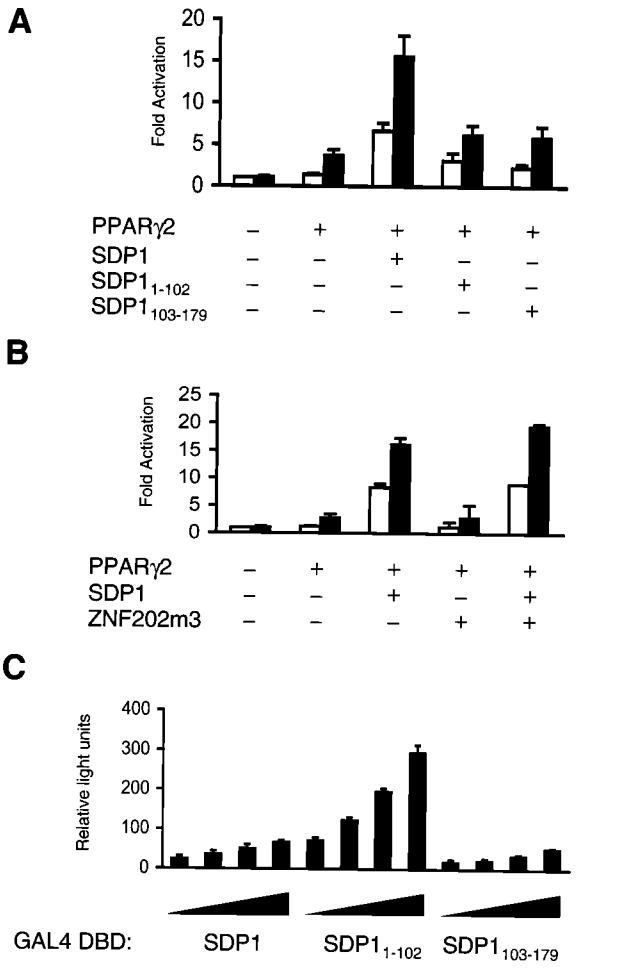

Figure 7 The SCAN domain fails to co-activate PPARγ2 in transiently transfected cells

(**A**) The SDP1 SCAN domain is not sufficient to co-activate PPARγ2 in transiently transfected cells. HeLa cells were transiently transfected with PPRE reporter plasmid and PPARγ2 expression plasmid with either full-length SDP1, N-terminal ($SDP1_{1–102}$) or C-terminal SCAN-domain-containing ($SDP1_{103–179}$) halves of SDP1, as described in Figure 1.The presence or absence of the PPARγ2-specific ligand BRL49653 (10 μM) is indicated by black or white bars respectively. (**B**) The ZNF202m3 splice variant does not co-activate PPARγ2. HeLa cells were transiently transfected in the presence (black bars) or absence (white bars) of the PPARγ2-specific ligand BRL49653 (10 μM) with PPARγ2 and SDP1 or ZNF202m3 expression plasmids, either alone or in combination, as described in Figure 1. (**C**) The SDP1 N-terminus possesses weak transcriptional activating properties. HeLa cells were transiently transfected with Gal4 reporter plasmid and increasing amounts (25 ng, 100 ng, 250 ng and 500 ng) of various Gal4-DBD SDP1 fusion constructs as indicated. The data shown is the average of three independent transfections with S.E. indicated.

Previous studies of SCAN-domain-containing proteins have shown that they display specificity in their interaction with other SCAN-domain-containing proteins [21–23]. To determine which region of SDP1 interacts with PPARγ2, we compared the ability of the SDP1 N-terminus ($SDP1_{1–102}$) with the C-terminal SCAN-domain-containing half of SDP1 ($SDP1_{103–179}$) to bind PPARγ2 as shown in Figure 6. As expected, the SDP1 SCAN domain ($SDP1_{103–179}$, lane 5), but not $SDP1_{1–102}$ (lane 4), bound the SCAN-domain-containing proteins SDP1 and ZNF202 to an extent similar to full-length SDP1 (compare lanes 3 and 5). The SDP1 SCAN domain failed to bind RXRα, yet bound PPARγ2 as effectively as full-length SDP1. The isolated SDP1 SCAN domain also bound PPARα, PPARδ and ERα (results not shown). Similar results were seen using the N-terminal and SCAN-domain-containing sequences of PGC-2 (results not shown). Overall, these results reveal that the SDP1 SCAN domain interacts with PPARγ2, a protein that lacks any obvious SCAN domain.

Because SDP1 interacts with PPARγ2 through its SCAN domain, we tested whether the SDP1 SCAN domain is sufficient for PPARγ2 co-activation in transiently transfected cells. As shown in Figure 7(A), although the SDP1 SCAN domain is sufficient for PPARγ2 binding *in vitro*, neither the SCAN domain ($SDP1_{103–179}$) nor the SDP1 N-terminus ($SDP1_{1–102}$) is sufficient for full co-activation in this assay. We also tested another SCAN-domain-containing protein, ZNF202m3 [24], a SCAN-only splice variant of ZNF202 that lacks zinc-finger domains, to see whether it could co-activate PPARγ2. As shown in Figure 7(B), ZNF202m3 did not co-activate PPARγ2 and also did not affect SDP1 co-activation of PPARγ2. Thus not all SCAN-domain-containing proteins function as PPARγ2 co-activators in cells.

Removal of the SDP1 N-terminus creates a protein that still retains the ability to interact with PPARγ2 but fails to function as a co-activator. This suggested that the transcriptional co-activating properties of SDP1 may reside in its N-terminus. To determine whether SDP1 contains any intrinsic transcriptional activation properties, we fused either full-length SDP1 or its N- or C-terminal halves to the GAL4 DBD and tested their transcriptional activation properties in transiently transfected HeLa cells. As shown in Figure 7(C), increasing amounts of Gal4-SDP1 plasmid resulted in modest activation of the reporter, similar to observations with PGC-2 [18]. The SDP1 SCAN domain ($SDP1_{103–179}$) alone also failed to stimulate the reporter. In contrast, the SDP1 N-terminus ($SDP1_{1–102}$) activated reporter gene transcription in this assay. These results suggest that the SDP1 N-terminus contains a transcriptional activation domain and that the presence of the SDP1 SCAN domain masks this activating function.

DISCUSSION

In the present study we analysed the properties of the SCAN-domain-containing protein SDP1. The results show that SDP1 bound and functioned as a PPARγ2 co-activator in PPARγ2 reporter gene assays. Based on these observations and the strong sequence similarity, we surmised that SDP1 is the human orthologue of the PPARγ2 co-regulator PGC-2. SDP1 interacted with the DNA-binding/hinge region of PPARγ2 through the SDP1 SCAN domain. However, the SDP1 SCAN domain was not sufficient for PPARγ2 co-activation in transiently transfected cells.

SDP1 and PGC-2 are unique among SCAN-domain-containing genes because they lack any associated zinc-finger domains [31]. SCAN domains, originally identified as a conserved domain in zinc-finger proteins, mediate the selective homo- and hetero-dimerization of SCAN containing proteins [19,21–23]. When we searched for proteins that are similar to SDP1 (http://www.ncbi.nlm.nih.gov/BLAST), the mouse protein PGC-2 displayed the strongest similarity with SDP1 (59% overall identity), with nearly identical SCAN domains. SDP1 has been mapped to human chromosome 20q11.1-q11.23. Sequencing of the mouse genome has provisionally placed PGC-2 on mouse chromosome 2 in a syntenic region to SDP1 (http://www.informatics.jax.org/reports/homologymap/mouse_human.shtml). Based on their functional similarity as PPARγ2 co-activators and the evidence for synteny, we believe SDP1 and PGC-2 to be orthologues.

Examination of the *in vitro* binding specificity of SDP1 showed that SDP1 bound PPARα, PPARδ and ERα, in addition to PPARγ2. Although SDP1 binding to PPARα and PPARδ was weaker than that to PPARγ2, this result was unanticipated, because studies by Castillo et al. [18] failed to demonstrate binding to either PPARα or PPARδ. We confirmed our results by repeating the assay using Castillo's conditions. Perhaps our use of human PPARs compared with their presumed use of murine PPAR proteins may explain the discrepancy in the results.

The *in vitro* binding studies showed that the SDP1 SCAN domain, and not the less conserved N-terminus, mediates binding to PPARγ2. This result was expected given that PGC-2 and SDP1 have greatest similarity in the SCAN domain. Previous studies established that SCAN domains bind other SCAN-domain-containing proteins, often displaying selectivity in binding [21–23]. The results presented here demonstrated that SCAN domains can also bind to non-SCAN-domain-containing proteins (e.g. PPARγ2), at levels similar to SCAN–SCAN interactions [Figure 3(A) compare SDP1 (22%) to PPARγ2 (21%)]. This binding of the SDP1 SCAN domain to a non-SCAN-domain-containing protein (e.g. PPARγ2) cannot be attributed to promiscuity by the SDP1 SCAN domain, as SDP1 failed to bind PPARγ2 heterodimeric partner RXRα.

Using PPARγ2 deletion mutants, we showed that SDP1 binds the PPARγ2 DNA-binding/hinge region, consistent with the ability of SDP1 to interact with PPARγ2 in the absence of ligand. In pulldown assays (Figure 4A), deletion of the AF-domain 1 (amino acid residues 1–138) appears to reduce the affinity of SDP1 for PPAR$\gamma_{139-505}$, but PPARγ_{1-138} on its own does not bind SDP1 appreciably. In the co-activation assays shown in Figure 4(B), SDP1 increased transactivation by PPARγ (wild-type) and by PPAR$\gamma_{139-505}$ to an identical degree, suggesting that the AF-1 domain makes little, if any, contribution to SDP1 recruitment. Thus consideration of both our *in vitro* and cellular data suggests that SDP1 interacts predominantly with the DNA-binding/hinge region of PPARγ. Several other co-regulator proteins, such as p/CAF, PSF-A, and PGC-1 have also been shown to interact with the DNA binding/hinge region of NHRs [32–35]. Our data expand upon previous experiments that showed that PGC-2 interacts with the A/B (AF-1) differentiation domain of PPARγ2 [18]. The studies presented here include a more extensive set of PPARγ2 deletions, as well as reporter gene assays which demonstrate that removal of the AF-1 domain has no effect on the ability of SDP1 to co-activate PPARγ2. The observation that SDP1 interacts with the PPARγ2 DBD, which is the most conserved region among PPAR family members, may explain the ability of SDP1 to interact with PPARα and PPARγ.

Fusion of SDP1, or PGC-2 [18], to the GAL4 DBD created a protein that neither activated nor repressed reporter gene expression. However, when the SDP1 SCAN domain was removed, the SDP1 N-terminus (SDP1$_{1-102}$) displayed transcriptional activation properties (Figure 7C). This suggests that the SDP1 SCAN domain masks a transcriptional activation domain within SDP1. Indeed, SDP1$_{103-179}$ showed slightly lower levels of activation compared with full-length SDP1. Similar observations of masked activation domains have also been observed with the PPARγ co-activators PGC-1 and PERC [36,37].

Although SDP1 interacted with the central region of PPARγ2, SDP1 failed to show strong levels of co-activation with a PPARγ2 construct that lacks the LBD (Figure 4B). Therefore recruitment of SDP1 alone is not sufficient for co-activation and suggests that the SDP1 N-terminus communicates with other parts of PPARγ2 (i.e. the LBD) for co-activation. In addition, this transcriptional activation function is conserved within the PGC-2 N-terminus. Perhaps the SDP1 N-terminus recruits other factors, which, in conjunction with the PPARγ2 LBD, activate transcription [18]. The presence of these other factors could also explain the cell-type specificity of SDP1 co-activation.

The state of SCAN domain oligomerization, such as the binding of SDP1 to ZNF202, may influence the activity of zinc-finger transcription factor function [22]. Indeed, SDP1 was shown to disrupt the binding of the transcriptional repressor KAP1 to ZNF202 *in vitro* [38]. Both the lipoprotein lipase (LPL) and ABCA1 genes, which play important roles in lipid homoeostasis and contribute to atherosclerosis [39–41], contain ZNF202 binding sites in their promoters [24,38]. PPAR family members and their ligands also regulate these genes, either directly or indirectly. For example, PPARγ2 increases ABCA1 mRNA levels by inducing expression of LXRα, a member of the nuclear receptor superfamily that positively regulates ABCA1 gene transcription [42–46]. ZNF202 was shown to repress expression of the ABCA1 promoter and the ZNF202 SCAN domain is crucial for this repression [38]. Thus SDP1, by enhancing PPARγ2 activity and disrupting KAP1 binding to ZNF202, could serve to increase levels of ABCA1 transcription.

LPL, which converts triacylglycerols found in lipoprotein particles into fatty acids and monoacylglycerol, also contains a PPARγ2 (-169 to -157) and a ZNF202 (-427 to -405) binding site within its promoter [24,47]. The presence of the PPARγ2 and ZNF202 binding sites within the LPL promoter could allow SDP1 to up-regulate LPL transcription through both of these transcription factors, similar to ABCA1. In addition, the close proximity of the two binding sites could theoretically allow for the formation of a ZNF202-SDP1-PPARγ2 transcriptional regulatory complex. This would depend on whether or not the amino acid residues in the SCAN domain that are important for SCAN–SCAN interactions are the same as those for SCAN-PPARγ2 binding. The binding of SDP1 as a dimer (or ZNF202-SDP1 heterodimer) to PPARγ2 could permit such a complex to form. Our results that showed failure of the ZNF202m3 splice form to compete with SDP1 for PPARγ2 co-

activation suggest that SDP1 may bind PPARγ2 on a different surface than is required for SCAN–SCAN binding. Nevertheless, the studies presented here indicate that the transcriptional co-activator SDP1 binds both SCAN (e.g. ZNF202) and non-SCAN (e.g. PPARγ2) domain-containing transcription factors through the SDP1 SCAN domain, whereas the non-SCAN sequences in SDP1 are important for transcriptional activation. Thus the interaction of SDP1 with two distinct transcriptional regulators allows SDP1 to impact multiple transcriptional regulatory networks and cellular processes.

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