

# The proliferating cell nuclear antigen regulates retinoic acid receptor transcriptional activity through direct protein–protein interaction

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## ABSTRACT

**Retinoic acid receptors (RARs) interact, in a ligand-dependent fashion, with many coregulators that participate in a wide spectrum of biological responses, ranging from embryonic development to cellular growth control. The transactivating function of these ligand-inducible transcription factors reside mainly, but not exclusively, in their ligand-binding domain (AF2), which recruits or dismiss coregulators in a ligand-dependent fashion. However, little is known about AF2-independent function(s) of RARs. We have isolated the proliferating cell nuclear antigen (PCNA) as a repressor of RAR transcriptional activity, able to interact with an AF2-crippled RAR. The N-terminus of PCNA interacts directly with the DNA-binding domain of RAR, and PCNA is recruited to a retinoid-regulated promoter in intact cells. This interaction affects the transcriptional response to retinoic acid in a promoter-specific manner, conferring an unanticipated role to PCNA in transcriptional regulation. Our findings also suggest a role for RAR as a factor coordinating DNA transcription and repair.**

## INTRODUCTION

The modulation of gene expression by ligand-regulated nuclear receptors (NRs) is carefully controlled to achieve a precise spacial and temporal expression of proteins involved in crucial cellular processes. Several mechanisms leading to such a restricted expression have been identified, among which the availability of the cognate ligand, the tissue-specific expression of NRs themselves or of their coregulators have been documented. These different parameters condition the biological responses to a given ligand and, therefore, will affect

major biological processes, such as differentiation, proliferation or apoptosis.

While elegant studies in yeast have characterized the role of network of transcription factors in the control of the cell cycle (1), relatively little is known on how cell cycling affects transcription factors activity. Nevertheless, D-type cyclins, which regulate the progression through the G<sub>1</sub> phase of the cell cycle, have been shown to interact physically with transcription factors and to regulate their activities. Notably, cyclin D1 interacts with a number of transcription factors, such as the general transcription factor TAF<sub>II</sub>250 (2), STAT3 (3), several NRs [estrogen receptor (ER), androgen receptor (AR) and thyroid hormone receptor (TR) (4–6)] and some of their coregulators [SRC1/NCoA1, GRIP1/NCoA2, AIB1/NCoA3 and pCAF; reviewed in (7)]. Similarly, the protein phosphatase Cdc25B, which activates cyclin-dependent kinases, acts as a coactivator for several NRs [ER, AR, glucocorticoid receptor (GR) and progesterone receptor (PR) (8)]. While the interaction of cyclin D1 and of Cdc25B with NRs has a different outcome on their transcriptional activity, these observations, however, hint at a regulation of NRs activity during cell cycle progression. Indeed, responsiveness to glucocorticoids, which activate GR, is observed in G<sub>0</sub> and S phases, but not in the G<sub>2</sub> phase (9), and the AR losses its transcriptional activity at the G<sub>1</sub>/S transition (10).

Retinoic acid receptors (RARs) belong to the superfamily of NRs and bind to specific retinoic acid response elements (RAREs) as heterodimers with retinoic X receptors (RXRs). The transcriptional activation of these heterodimers is triggered upon binding of all-*trans* retinoic acid (atRA) to RAR [reviewed in (11)]. atRA plays a fundamental role in embryonic development and homeostasis of vertebrates through its ability to directly control the transcription of target genes involved in the control of proliferation, differentiation and apoptosis (12). Binding of atRA to RAR induces conformational changes in the ligand-binding domain (LBD), which contains the activating function 2 (AF-2), and notably induces the repositioning of the C-terminal helix H12 (or AF2-activating

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domain), resulting in the creation of a charge clamp required for coactivator recruitment (13), and subsequent transcriptional activation (14). Among these coactivators are proteins of the p160 family [steroid receptor coactivators 1, 2 and 3 (SRC-1, 2, 3 or NCoA1, 2, 3)] and CBP/p300, which possess protein acetyl transferase activity, and secondary coactivators, such as CARM1 or PRMT1, which harbor protein methyltransferase activity (11). These cofactors allow chromatin modification and recruitment of the mediator complex [TRAP/DRIP (15,16)], which stimulates phosphorylation of the largest subunit of Pol II by TFIIF (17).

While a detailed understanding of the ligand-dependent activation of RARs has been achieved by structural and functional studies, little is known about the ligand-independent regulation of RAR transcriptional activity. Nevertheless, it has been demonstrated that post-translational modifications alter RAR activity independently of ligand (18–20). We therefore undertook a two-hybrid screen in yeast using an AF2-inactivated human RAR $\alpha$  (hRAR $\alpha$ ) as a bait to identify proteins potentially able to regulate RAR functions in a ligand-independent manner. Among several proteins, the proliferating cell nuclear antigen (PCNA) was identified as an RAR $\alpha$  interacting protein.

PCNA is the homolog of the  $\beta$  subunit of the prokaryotic DNA polymerase and is well known as a processivity factor of eukaryotic DNA polymerases  $\delta$  and  $\epsilon$  (21). PCNA is an essential component of the eukaryotic chromosomal DNA replisome and clamps onto DNA as a trimeric ring, sliding along it during replication (22). Further studies have also demonstrated the interaction of PCNA with multiple proteins involved in DNA repair (23), cell cycle regulation (24) and chromatin structure remodeling (25).

In this paper, we describe for the first time the physical, direct interaction of PCNA with RAR $\alpha$ . We explored the functional consequences of this interaction on retinoid-regulated transcription by cell-based transfection and gene knockdown experiments.

## MATERIALS AND METHODS

### Materials

atRA was obtained from Sigma. DNA restriction and modification enzymes were from Promega (Charbonnières, France). Polyethyleneimine (ExGen 500) was from Euromedex (Souffelweyersheim, France), and Lipofectamine and Lipofectamine 2000 were from Invitrogen Life Technologies (Carlsbad, CA). [<sup>35</sup>S]methionine was purchased from GE Healthcare (Les Ulis, France).

### Plasmids

The yeast expression plasmid pLex12-hRARK244A-K262A was generated by insertion of the hRAR $\alpha$ K244A-K262A cDNA into the BglII/XbaI sites of pLex12, a LexA DBD fusion vector. pGL3-DR5tk-Luc, p800tk-Luc (containing 800 bp of the human RAR $\beta$ 2 promoter), pGL3tk-Luc, pSG5-hRXR $\alpha$ , pSG5-hRAR $\alpha$ , pSG5-hRAR $\alpha$  K244A-K262A, pSG5-hRAR $\alpha$  AF1, pSG5-hRAR $\alpha$  AF2, pSG5-hRAR $\Delta$ 403, pSG5-hRAR $\beta$ , pSG5-hRAR $\gamma$ , pSG5-hVDR and pSG5-hPPAR $\gamma$  were described elsewhere (26–31). pET23-PCNA

and pGEX4T-PCNA were gifts from Z. Jonsson and K. Kohno, respectively. The pCR3.1-PCNA plasmid was obtained by TA cloning (TA cloning kit; Invitrogen Life Technologies). pSG5-hRAR $\alpha$ , pET23-PCNA, pCR3.1-PCNA, pM-PCNA and pGEX4T-PCNA mutants were generated using the QuickChange Site-Directed Mutagenesis Kit (Stratagene). All constructs were checked by automatic sequencing.

### Yeast two-hybrid library screen

A HeLa cDNA library (in pACT2 vector; Clontech) was screened using the L40a yeast strain transformed with the pLex12-hRARK244A-K262A vector, essentially as described previously (32).

### Cell culture and transfections

HeLa Tet-On cells were cultured as monolayers in DMEM supplemented with 10% fetal calf serum. Cells were transfected with expression vectors and reporter vectors when indicated and were then treated when indicated for 16 h with 10<sup>-6</sup> M atRA. Transfections were carried out as described previously (27). The luciferase assay was performed with the Bright-Glo Luciferase assay system from Promega, and data points represent the average of at least three independent experiments performed with triplicate samples.

### GST pull-down experiments

The GST vectors were transformed into the *Escherichia coli* strain BL21. <sup>35</sup>S-labeled proteins were synthesized using the Quick T7 TnT kit (Promega). Experiments were carried out essentially as described previously (27). Resin-bound proteins were resolved by 10% SDS-PAGE and quantified with a Storm 860 PhosphorImager (Molecular Dynamics). At least three independent experiments were carried out with two different bacterial extracts.

### Coimmunoprecipitation assays

HeLa cells were first transfected with the pSG5-hRAR $\alpha$  expression vector, then HeLa and MCF7 cells were treated for 16 h with 1  $\mu$ M atRA or left untreated. Cell lysates were prepared using the lysis buffer (10 mM Tris-EDTA, pH 8.0, 240 mM NaCl and 0.1% NP40) and were first incubated for 2 h with equilibrated protein G-Sepharose (preclearing). The pre-cleared lysate was incubated overnight at 4°C with 2  $\mu$ g of anti-RAR $\alpha$  antiserum (sc-551; Santa Cruz Biotechnology) or non-specific IgG. Immune complexes were then incubated with 50  $\mu$ l of protein G-Sepharose for 2 h at 4°C and beads were washed three times with lysis buffer. Immuno-adsorbed proteins were analyzed by western blotting with an anti-PCNA monoclonal antibody or an anti-RAR $\alpha$  antiserum (sc-56 and sc-551, respectively; Santa Cruz Biotechnology). A similar protocol was followed to characterize PCNA and its acetylated forms, using an antiacetylated (epsilon acetylated lysines) protein antibody (Novus Biologicals, Littleton, CO).

### siRNA transfection experiments

Human PCNA siRNAs were provided by Dharmacon. siRNAs were transfected in HeLa and MCF7 cells with Lipofectamine 2000 according to the manufacturer's protocol.

### RNA preparation and RT-PCR

RNA extractions and RT-PCR were carried out as described previously (33). PCRs were carried out as follows: 94°C for 1 min, 58°C for 1 min and 72°C for 1 min. The number of cycles was adjusted for each transcript to ensure that amplification was in a linear range. Primers were as follows: GAPDH primers, 5'-CCATCACCATCTTCCAGGAG-3' and 5'-CCTGCTTACCACCTTCTTG-3'; RAR $\alpha$ 1 primers, 5'-GCCAGGCGCTCTGACCACTC-3' and 5'-AGCCCTTGCAGCCCTCACAG-3'; RAR $\alpha$ 2 primers, 5'-CCACCCCTAATCCCTTCTA-3' and 5'-AGCCCTTGCAGCCCTCACAG-3'; RAR $\beta$ 2 primers, 5'-AACGCGAGCGATCCGAGCAG-3' and 5'-ATTGTCTGCTGGCAGACGAAGCA-3'; and CRABP2 primers, 5'-GTGGAGATCAAACAGGAGGGAG-3' and 5'-CATGGTCAGGATCAGTTCCCC-3'.

### DNA and chromatin immunoprecipitation (ChIP) assays

A total of  $5 \times 10^6$  HeLa cells were transfected as described above with pGL3-DR5-tk Luc, pSG5-hRAR $\alpha$  and pSG5-hRXR $\alpha$  vectors. Non-transfected P19 cells or transfected HeLa cells were treated with 1  $\mu$ M atRA overnight. DNA immunoprecipitations in HeLa cells, ChIP of the RAR $\beta$ 2 promoter in P19 cells and PCR amplification of the promoter region of the gene of interest were carried out as described previously (30,34).

### Statistical analysis

All incubations or assays were performed at least in triplicate. Measured values were used to calculate mean  $\pm$  SEM. Calculations were carried out using Prism software (GraphPAD Inc., San Diego, CA).

## RESULTS

The N-terminus of PCNA interacts with an AF-2 inactivated RAR $\alpha$  and represses wild-type RAR transcriptional activity.

To investigate novel, AF2-independent function(s) of RAR $\alpha$ , a yeast two-hybrid screen was used to select proteins able to interact with an AF-2 inactivated hRAR $\alpha$ . AF-2 inactivation was obtained by mutating K244 and K262 into alanine, in order to prevent RAR $\alpha$ -helix 12 interaction with the LBD, and thus to impede coactivator recruitment. The K244A-K262A (termed RAR2K hereafter) mutant displays a dramatically decreased transcriptional response to retinoids (27), heterodimerizes with RXR (28) but is unable to interact with SRC1 *in vitro* (32). About  $10^6$  colonies from a HeLa cell cDNA library were screened with the RAR2K bait. Two out of the nineteen positive clones encoded the N-terminal fragment of PCNA (1–127), which includes the N-terminal domain of PCNA and half of the interdomain connecting loop [(35) and Figure 1A]. The secondary screening in yeast confirmed this interaction, which was not affected in the presence of 1  $\mu$ M atRA (Figure 1B). To assess the ability of PCNA to interfere with RAR $\alpha$  transcriptional activity, both proteins were overexpressed in HeLa cells, and RAR $\alpha$  transcriptional activity was monitored using a chimeric reporter gene whose promoter is driven by three DR5 RAREs. Full-length PCNA was able to significantly decrease RAR $\alpha$  transcriptional activity, as well as the isolated PCNA N-terminal domain, albeit to a lesser

extent (Figure 1C). In contrast, the isolated PCNA C-terminal domain (which displays significant structural homology with, but strongly diverges from the N-terminal domain when considering its amino acid sequence) did not affect RAR $\alpha$  transcriptional activity. Moreover, PCNA overexpression did not affect the luciferase activity of a control reporter gene without DR5 (tk-Luc), showing that this repression is occurring through the RAREs.

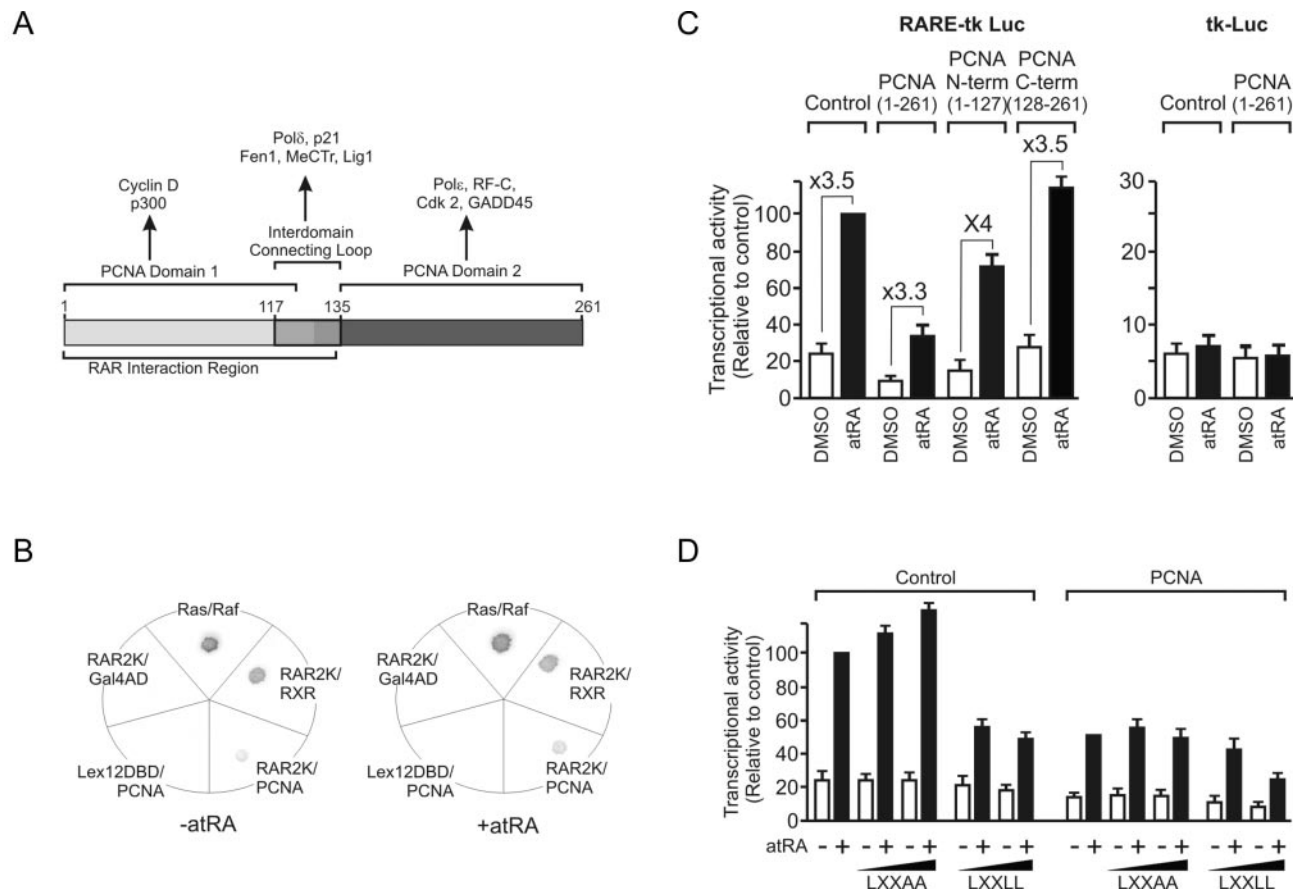
Although PCNA was isolated with a transcriptionally inactive RAR $\alpha$ , this does not exclude a possible interference with the ligand-dependent coactivator loading onto RAR $\alpha$ . We explored this possibility by overexpression of the DRIP205 NR1 and NR2 boxes, together or not with PCNA. DRIP205, a subunit of the DRIP/TRAP complex, interacts directly with NRs. DRIP205 NR boxes contain two LXXLL sequences required for NR interaction and exert, when overexpressed, a dominant negative effect on vitamin D3 receptor (31) and on RAR $\alpha$  (Figure 1D) transcriptional activities. Coexpression of PCNA could therefore either increase DRIP NR boxes inhibitory effect, or have no effect. Preventing coactivator loading by overexpression of the DRIP NR boxes did not affect PCNA inhibitory effect (Figure 1D). Overexpression of the mutant LXXAA motifs, which are unable to interact with NRs, had no statistically significant effect on our system. These results are thus consistent with the hypothesis that PCNA does not target AF2 function.

### PCNA associates with RAR $\alpha$ in HeLa cells and in MCF7 cells and is recruited to an atRA-regulated promoter

To investigate whether PCNA participates in RAR $\alpha$  transcriptional repression through physical interaction in intact mammalian cells, we carried out immunoprecipitation assays with an anti-RAR $\alpha$  antibody (Figure 2). Western blot analysis of the immunoprecipitates from HeLa cells, in which hRAR $\alpha$  has been overexpressed, revealed that the anti-RAR $\alpha$  antibody was able to immunoprecipitate endogenous PCNA (Figure 2A). Similar experiments were carried out in MCF7 cells, which express a significant amount of endogenous RAR $\alpha$ . Endogenous RAR $\alpha$  was immunoprecipitated, and analysis of the immunoprecipitate also showed that RAR $\alpha$  coimmunoprecipitated with PCNA (Figure 2B). Thus, in both cell lines, atRA could significantly increase wtRAR interaction with endogenous PCNA, in contrast with results obtained in yeast.

To study the role of the PCNA-RAR interaction in transcriptional repression, we next determined whether PCNA could interact with DNA-bound RAR-RXR heterodimers. We first used an *in vitro* GST pull-down assay in which the ability of PCNA to interact with DNA-bound heterodimers was assessed (Figure 2C). hRAR $\alpha$ /hRXR $\alpha$  heterodimers were assembled on DNA [DR5 RARE, (27)], incubated or not with 1  $\mu$ M atRA and adsorbed on a GST-PCNA matrix. Only monomeric RAR $\alpha$  could interact with PCNA, whereas monomeric RXR $\alpha$  was unable to engage such an interaction. When the PCNA matrix was incubated in the presence of RXR $\alpha$  and RAR $\alpha$ , a slightly increased amount of RXR $\alpha$  could be detected in the matrix-bound fraction. The PCNA/RAR $\alpha$ /RXR $\alpha$  interaction was increased when heterodimers were assembled onto the DR5 RARE, and became clearly influenced by the ligand. Similar experiments were carried





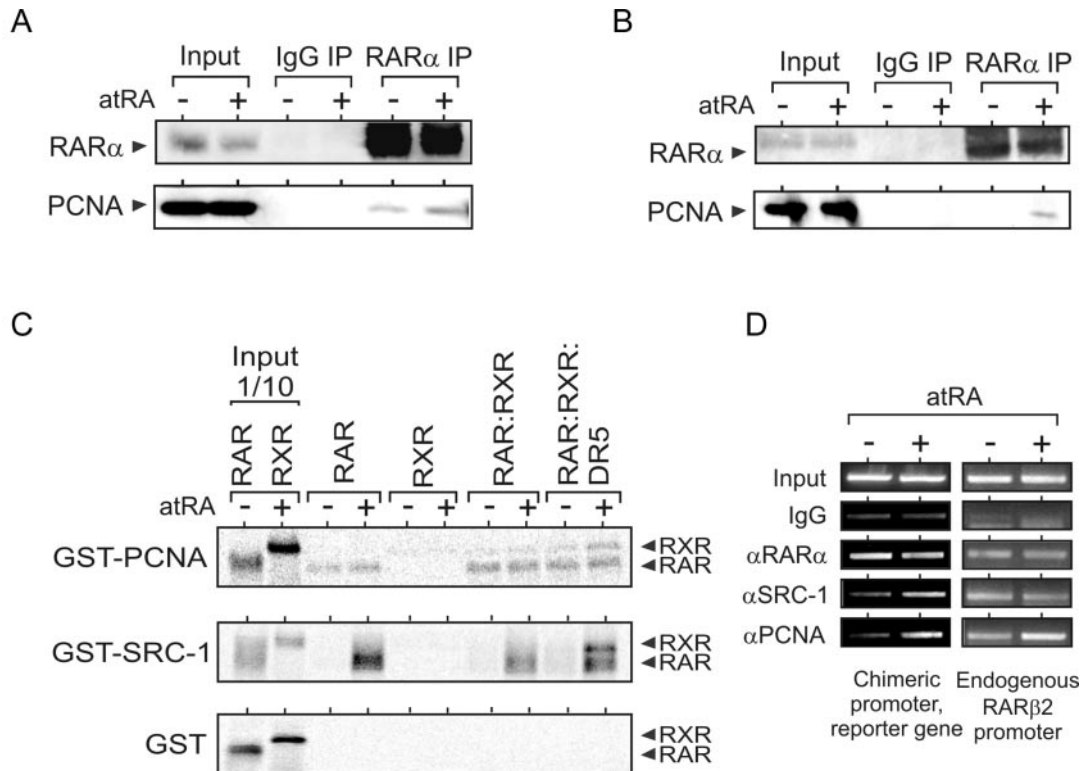
**Figure 1.** A yeast two-hybrid screen identify PCNA as a partner of human RAR $\alpha$ . (A) Main features of the PCNA molecule. Domains of PCNA and some of its most prominent partners are indicated. (B) The AF2-inactivated RAR mutant (RAR2K) interacts with PCNA in a ligand-independent manner. Yeasts were transformed with various pLex12 expression vectors (empty vector, Ras fusion, RAR2K fusion) and baits fused to the Gal4 activation domain (empty vector, Raf, RXR DBD, PCNA). The  $\beta$ -galactosidase activity resulting from the activation of the LacZ gene hooked to a LexA response element was detected 24 h after retinoic acid challenge. The Ras/Raf system was used as a positive control. (C) PCNA overexpression inhibits retinoid-induced transcription. HeLa cells were cotransfected in 6-wells cluster ( $\sim 1.5 \times 10^5$  cells) with a retinoid-inducible reporter gene pGL3(RARE) $_3$ tkLuc (500 ng) designated as DR5tk-Luc hereafter, or a tk-driven luciferase reporter gene (500 ng, tk-Luc, see Materials and Methods), RAR $\alpha$  and RXR $\alpha$  expression vectors (50 ng of each), and a 10-fold stoichiometric excess of a PCNA expression vector. Twenty-four hours after transfection, cells were challenged, or not, overnight with 1  $\mu$ M atRA and luciferase activity was assayed. Results are expressed as the mean of three independent experiments performed in triplicate. (D) Preventing coactivator binding site occupancy does not block PCNA repressive effect. HeLa cells were transfected as above with a reporter gene, RAR $\alpha$  and RXR $\alpha$  expression vectors, with or without PCNA (1  $\mu$ g) and increasing amounts (0.25, 0.5 and 1  $\mu$ g) of an expression vector coding for the NR box of DRIP205 (LXXLL) or its mutated inactive version (LXXAA). Luciferase activity was assayed as described above.

out using a GST-SRC1 matrix as a control. Here again, only RAR $\alpha$  could interact with SRC1 under its monomeric form. DNA-bound heterodimers recruited efficiently SRC1 in the presence of ligand, demonstrating that our assay reflects the DNA- and ligand-dependent formation of the ternary complex RXR/RAR/SRC1.

Taken together, these results suggest that PCNA is able to interact with stable RXR/RAR heterodimers, and that this DNA-bound configuration confers a ligand dependency to the interaction.

We then determined whether PCNA is indeed recruited to DNA-bound RXR/RAR heterodimers in intact cells (Figure 2D). First, HeLa cells were transfected with the DR5 tk-Luc reporter gene, RAR $\alpha$  and RXR $\alpha$  expression vectors, and subjected to a DNA immunoprecipitation assay to assess for the interaction of RAR $\alpha$ , PCNA and SRC1 on the chimeric atRA-regulated promoter. RAR $\alpha$  was recruited to the promoter, irrespective of the presence of atRA, in agreement with previous results, and SRC1 was recruited in a

ligand-dependent manner. The PCNA recruitment profile was very similar to that of SRC1, with a barely detectable interaction in the absence of ligand, which was significantly increased upon atRA treatment of target cells. P19 cells are highly sensitive to retinoids and the RAR $\beta$ 2 promoter is in this cell line highly responsive to atRA treatment. Moreover, they express a high amount of RAR $\alpha$ , allowing an efficient immunoprecipitation of RAR $\alpha$ -associated chromatin, in opposition to HeLa cells. ChIP assays were therefore carried out to assess for the presence of PCNA on the promoter of this retinoid-regulated gene. As shown in Figure 2D (right panel), RAR $\alpha$  was recruited to the RAR $\beta$ 2 promoter in a ligand-independent manner. PCNA was also recruited to the promoter, and again atRA treatment increased the interaction of PCNA with this promoter, thus presumably with RAR $\alpha$ . The coactivator SRC1, in sharp contrast, was recruited in a ligand-independent manner, an unexpected behavior which is probably related to the specific chromatin organization of this promoter (S. Flajollet and B. Lefebvre, personal communication). Indeed, our



**Figure 2.** PCNA interacts with RAR $\alpha$  in intact cells and *in vitro*. (A) PCNA coimmunoprecipitates with RAR $\alpha$  in HeLa cells. HeLa cells were transfected with a RAR $\alpha$  expression vector and total extracts were immunoprecipitated with Sepharose-conjugated anti-RAR or PCNA antibodies (or mouse IgG as a control), respectively, after retinoid challenge. Immune complexes were analyzed by western blots for the presence of RAR $\alpha$  and PCNA. (B) PCNA coimmunoprecipitates with RAR $\alpha$  in MCF7 cells. MCF7 total extracts were immunoprecipitated and analyzed as above. (C) The RXR-RAR heterodimer interacts with PCNA *in vitro*. RXR and/or RAR were labeled by *in vitro* translation and incubated with a Glutathione-Sepharose matrix coupled to either GST-PCNA or the receptor interacting domain of SRC1 fused to GST or GST alone. When indicated, a DR5 RARE was added to the binding mixture. Receptors interacting with the matrix were visualized by autoradiography. (D) PCNA associates with a retinoid-induced promoter in intact cells. HeLa cells were cotransfected with PCNA, RXR and RAR expression vectors together with DR5tk-Luc retinoid-inducible reporter gene. A ChIP assay was carried out with a mouse IgG (control), an anti-RAR $\alpha$ , an anti-SRC1 or an anti-PCNA antibody. The precipitated DNA was amplified by PCR and analyzed on an agarose gel. Naive P19 cells were used for a similar assay to quantify the amount of endogenous RAR $\beta$ 2 promoter DNA associated to PCNA.

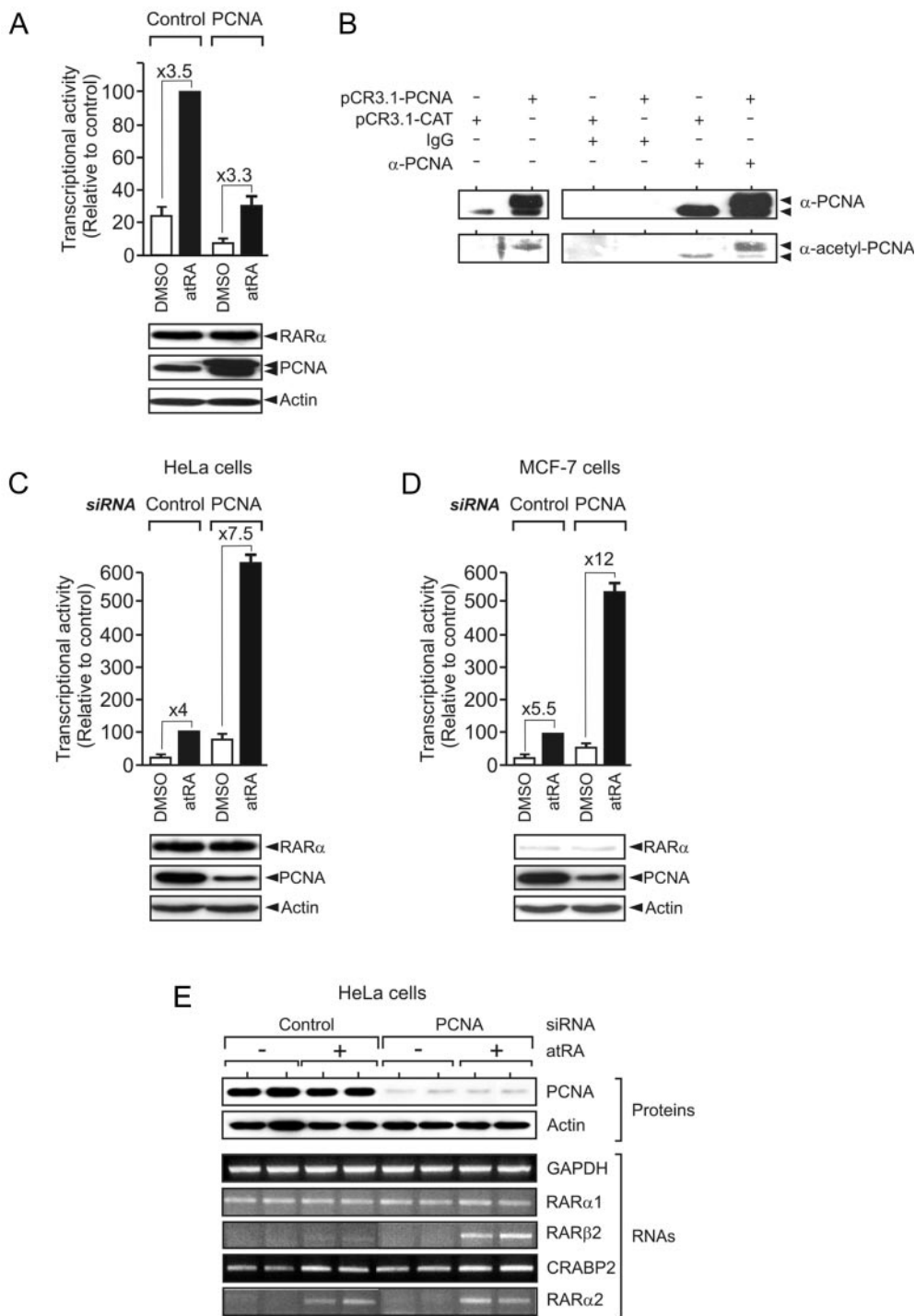
previous reports (26,34) as well as a recent publication from Reinberg and co-workers (36) demonstrate that the endogenous RAR $\beta$ 2 promoter is constitutively associated to RXR/RAR heterodimers, the Mediator complex and RNA polymerase II. Taken collectively, these data suggest that PCNA is recruited to promoter-bound heterodimers in a ligand-dependent manner, on both chimeric, unchromatinized and endogenous, chromatinized DNA templates. This interaction did not turned out to be cell-specific, since it was detected in several cell types (HeLa, MCF7 and P19 cells).

#### PCNA level affects the activity of RA-regulated chimeric and endogenous promoters

Full-length PCNA was overexpressed in HeLa cells together with RAR $\alpha$  and RXR $\alpha$ . The transcriptional activity of the RXR/RAR heterodimer was monitored by assaying the luciferase activity from a synthetic RA-responsive reporter gene (Figure 3A, DR5tk-Luc). In control conditions, atRA induced a 3.5-fold activation of the reporter gene activity, whereas in the presence of overexpressed PCNA, the basal activity of the reporter gene was decreased. However, the inducibility of the promoter by atRA was not significantly altered. This repression did not result from decreased steady-state levels in RAR $\alpha$

(Figure 3A, lower panel). Interestingly, PCNA overexpression resulted in the presence of an additional PCNA form, which migrated more slowly during SDS-PAGE. PCNA was thus immunoprecipitated with an anti-PCNA antibody and probed by western blotting using an anti-acetylated lysine antibody. These experiments revealed that this slowly migrating species resulted from hyperacetylation of PCNA (Figure 3B), a post-translational modification that targets PCNA to the chromatin, nuclear matrix and nucleoplasm (37).

We next explored the effect of PCNA depletion in HeLa cells. HeLa cells were treated with equal amounts of control small interfering RNA (siRNA) or anti-PCNA siRNA, and the transcriptional activity of RXR $\alpha$ /RAR $\alpha$  heterodimers was assayed 3 days after siRNA treatment (Figure 3C) As shown in Figure 3C and D (lower panels), PCNA expression level was decreased by 50% in these conditions, whereas RAR $\alpha$  and actin levels were not affected. Knocking down PCNA expression resulted in a strong increase of the overall transcriptional activity of the reporter gene, showing an increased basal level and an increased sensitivity to atRA (4-fold versus 7.5-fold induction). We next assessed the transcriptional activity of endogenously expressed RAR $\alpha$  in response to PCNA knockdown (Figure 3D). MCF7 cells were transfected as above, using a more sensitive reporter



**Figure 3.** The level of expression of PCNA affects transcriptional activation by RAR $\alpha$ . (A) HeLa cells were transfected with a retinoid-inducible reporter gene, RAR $\alpha$ , RXR $\alpha$ , and/or PCNA expression vectors. The luciferase activity was assayed after treatment with 1  $\mu$ M atRA overnight. RAR $\alpha$ , PCNA and actin levels were assayed by western blot (lower panel). (B) Overexpressed PCNA is hyperacetylated. HeLa cells were transfected either with an expression vector coding for PCNA or chloramphenicol acetyltransferase as a control. Forty-eight hours later, nuclear extracts were prepared and incubated with a mouse IgG (control) or with an anti-PCNA antibody. Immune complexes were immunoprecipitated with protein G-Sepharose beads and analyzed by western blot using an anti-PCNA antibody (upper panel) or an anti-acetylated lysine antibody. The left panel corresponds to one tenth of the input in the immunoprecipitation assay. (C) PCNA knockdown increases atRA responsiveness in HeLa cells. HeLa cells were transfected with a retinoid-inducible reporter gene, RAR $\alpha$  and RXR $\alpha$  expression vector after a 24 h treatment with anti-PCNA siRNA or control siRNA. atRA treatment and luciferase assays were as in (A). (D) PCNA knockdown increases atRA responsiveness in the presence of physiological levels of hRAR $\alpha$ . MCF7 cells were treated with control or anti-PCNA siRNA and transfected with a retinoid-inducible reporter gene, p800-tkLuc (see Materials and Methods). atRA responsiveness of the system was monitored as above. (E) Effect of PCNA concentration on atRA responsiveness of endogenous genes. HeLa cells were treated as above with control or anti-PCNA siRNA, and mRNA levels were assessed by semi-quantitative RT-PCR after an overnight challenge with 1  $\mu$ M atRA (RNAs). A representative experiment is shown; similar results were obtained twice in independent experiments. PCNA and actin levels were assayed by western blot (proteins).

gene containing a 800 bp fragment from the RAR $\beta$ 2 promoter (p800 tk Luc) and omitting the RAR $\alpha$  expression vector (Figure 3D). Note that this reporter gene (which contains a single DR5 RARE) behaved in all instances similar to the DR5tk-Luc reporter gene (which contains three repeats of a DR5 RARE) with respect to PCNA overexpression (data not shown). As shown by western blot analysis (Figure 3D, lower panel), the level of expression of RAR $\alpha$  in MCF7 cells was lower than in transfected HeLa cells. In conditions for which PCNA was significantly down-regulated, we noted again that the basal activity of the reporter gene was increased, and that atRA responsiveness was much improved when compared with cells harboring unaltered PCNA levels [5.5-fold versus 12-fold (Figure 3D)].

An analogous strategy was followed to assess the effect of PCNA down-regulation on the activity of atRA-sensitive endogenous genes. The RAR $\alpha$ 1, RAR $\alpha$ 2, RAR $\beta$ 2, and CRABP-II genes are regulated by DR5 [RAR $\alpha$ 1&2, RAR $\beta$ 2 (38–40)], DR2 and DR1 types RAREs [CRABP-II (41)]. In HeLa cells, RAR $\alpha$ 1 and RAR $\beta$ 2 promoters were not sensitive to atRA. CRABP-II was weakly inducible by this ligand in HeLa cells. RAR $\alpha$ 2 expression was strongly increased in response to atRA challenge (Figure 3E). PCNA knockdown strongly modified the pattern of expression of the RAR $\beta$ 2 gene. Indeed, this promoter gained full atRA responsiveness with no detectable increase of the basal level of expression. A weak increase of the CRABP-II gene expression was also consistently noticed in HeLa cells, although this stemmed from an increased basal level. Thus, our finding points to an important role of PCNA as a promoter-specific repressor of RA-regulated genes.

### PCNA interacts physically with the DNA-binding domain of RAR $\alpha$

We next investigated the domain(s) of PCNA that interacted directly with RAR $\alpha$  by performing GST pull-down experiments, using purified GST-PCNA or GST-RAR $\alpha$  derivatives. The ability of isolated domains from RAR $\alpha$  to interact with PCNA was first assayed (Figure 4A). The RAR $\alpha$  DNA-binding domain (DBD) interacted strongly with PCNA, whereas the isolated LBD did not display detectable interaction with PCNA.

Several RAR $\alpha$  mutants, truncated either from the N- or the C-terminus, were also tested for the interaction with full-length PCNA (Figure 4B). Full-length, wild-type hRAR $\alpha$  and RAR2K interacted in a ligand-independent manner with PCNA, as well as a mutant deleted from the A and B domains [(hRAR $\alpha$ )AB], excluding a contribution from the N-terminus of hRAR $\alpha$ . Further deletions within the RAR $\alpha$  DBD were introduced to define more precisely the RAR-PCNA interaction domain (Figure 4B). As predicted from the results shown above, the deletion of the DBD within the context of the full-length RAR abolished the interaction with PCNA. A shorter deletion, removing the first zinc finger involved in direct DNA-RAR interaction, did not affect PCNA binding. In contrast, removing the linker peptide (amino acids 109–123) between the first and second zinc finger regions severely affected this interaction. Deletion of the second zinc finger, which is involved in receptor dimerization (42,43), also generated a RAR $\alpha$  mutant with very low binding to PCNA. Since the linker peptide does not contain any consensus PCNA binding

sequence [QXX(I/L/M)XX(F/Y)(F/Y) (44)], we carried out an Ala scan on this region. No mutation altered the binding of RAR $\alpha$  to PCNA (data not shown), suggesting that the RAR interacting domain is a complex structure.

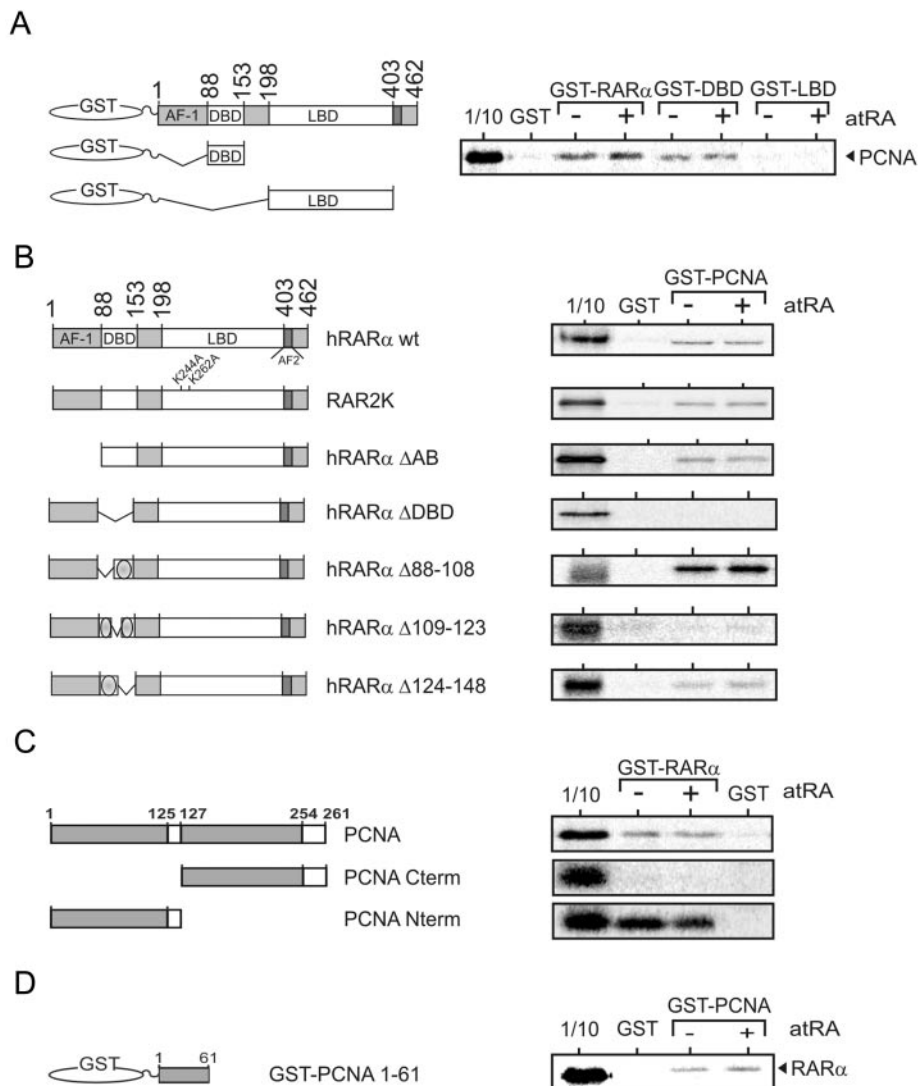
A similar strategy was applied to PCNA (Figure 4C and D). As expected from previous results, only the N-terminus of PCNA interacted significantly with RAR $\alpha$ . A further truncation of the N-terminus of PCNA showed that RAR interacted strongly with the 1–61 region of PCNA. Taken together, these domain mapping experiments show that RAR interacts with the N-terminus of PCNA through the C-terminal part of the RAR $\alpha$  DBD.

The specificity of the PCNA-RAR interaction was further characterized (Figure 5). As it could be predicted from the high homology of the DBD with RAR $\beta$  and RAR $\gamma$ , we found that PCNA interacted equally well with these two RAR isotypes. As shown in Figure 2C, the dimerization partner of RAR, RXR (RXR $\alpha$  in our assay) did not interact with PCNA (Figure 5A). Two other NRs heterodimerizing with RXR, the vitamin D receptor (VDR) and PPAR $\gamma$ , were unable to engage physical interactions with PCNA. In agreement with this, the transcriptional activity of RAR $\beta$  and RAR $\gamma$  heterodimerized to RXR $\alpha$  was blunted in the presence of PCNA (Figure 5B), whereas the transcriptional activity of a DR1-driven reporter gene in the presence of RXR alone or of a combination of RXR $\alpha$  and PPAR $\gamma$  was not sensitive to PCNA overexpression. Similarly, a DR3-driven reporter gene was not sensitive to PCNA overexpression in the presence of RXR $\alpha$  and VDR (Figure 5C).

## DISCUSSION

NRs are subjected to a variety of controls that modulate their transcriptional activity. They interact with CoAs or CoRs in a ligand-dependent manner, are modified post-translationally, mostly by protein kinases and/or degraded through the proteasome pathway. Most of protein-protein interactions known to affect RAR transcriptional activity occur within the LBD, and they have been extensively dissected from both a structural and a mechanistic point of view. This allowed to propose a very refined picture of ligand-mediated transcriptional activation of RXR:RAR heterodimers, in which C-terminal helix 12 (H12) repositioning plays a crucial role to generate CoR or CoA interaction interfaces. Much less is known on H12-independent mechanism(s) that may affect RAR activity. To gain some insight into these processes, we undertook a two-hybrid screen aiming at identifying proteins interacting with RAR in a AF-2/H12 independent manner. Among several candidates, including RXR, the obligate dimerization partner of RAR, we isolated PCNA, originally identified as a DNA sliding clamp enabling DNA polymerases to replicate DNA. However, PCNA functions extend well beyond DNA replication, and it participates through multiple protein-protein interaction to DNA methylation, chromatin remodeling and cell cycling (45). Using several approaches, we demonstrate that (i) PCNA can interact directly with RAR in a ligand-independent manner in an acellular assay and in yeast, (ii) PCNA interacts with RAR in intact mammalian cells, (iii) the PCNA-RAR interaction becomes ligand-dependent when the interaction takes place within the context of a DNA-bound RXR-RAR heterodimer *in vitro*, (iv) this





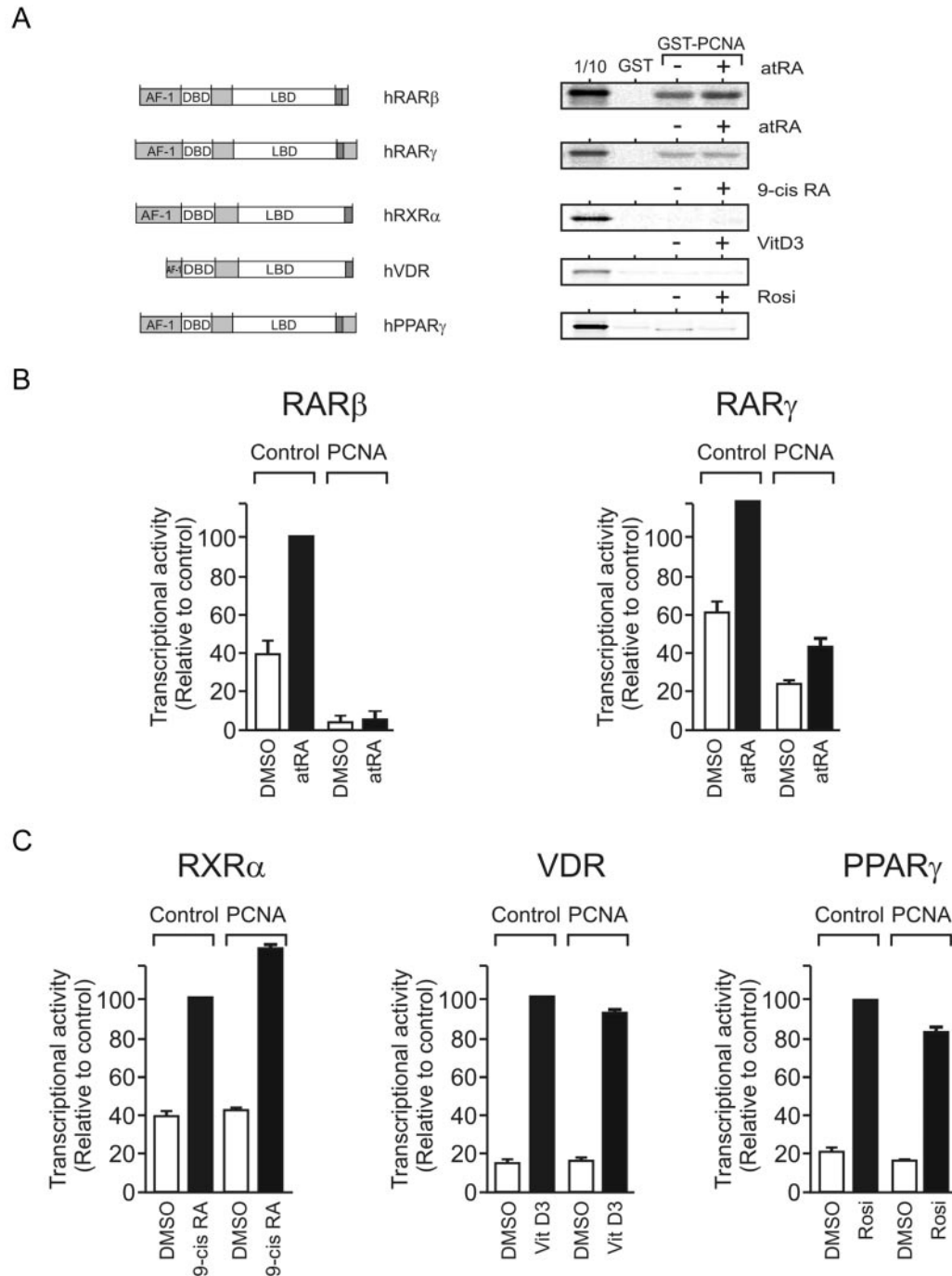
**Figure 4.** Domain mapping of the RAR $\alpha$ -PCNA interaction. (A) The RAR $\alpha$ DBD is sufficient for RAR-PCNA interaction. Full-length RAR $\alpha$ , or the isolated DBD or LBD fused to GST were incubated with labeled PCNA and incubated with GST alone or GST-RAR bound to Sepharose beads. After extensive washing, bound material was analyzed by SDS-PAGE and bound PCNA was visualized by autoradiography. (B) GST pull-down assay with full-length PCNA. The indicated RAR mutants were translated and labeled *in vitro* and incubated with a Sepharose-glutathione/GST-PCNA slurry. The interaction was analyzed as in (A). A similar assay was carried out with RXR $\alpha$ . (C) Mapping of the RAR interaction region within PCNA. Various PCNA deletion mutants were labeled and incubated with the full-length RAR $\alpha$ -GST fusion protein. Bound PCNA was analyzed as above. (D) Minimal PCNA-RAR interaction domain. A Sepharose-bound GST fused to amino acids 1-61 from PCNA was incubated with labeled, full-length RAR $\alpha$ . Bound material was assayed as above.

interaction occurs on both chimeric and endogenous retinoid-regulated promoters in intact cells and (v) this interaction represses RAR transcriptional activity in a promoter- and cell type-specific manner. Our results therefore support a role for PCNA in regulating RAR transcriptional activity and hints at a cell cycle-dependent regulation of RAR-mediated transcription. An yet unresolved issue is why the RAR-PCNA interaction is ligand-dependent in intact cells. Our *in vitro* data suggest that the assembly of RXR-RAR heterodimers on DNA confers the ligand dependence to this interaction, alternatively, this may result from a considerably stabilized RAR-RXR interaction through DNA binding. A RAR $\alpha$  mutant, unable to bind to RAREs, also interacts with PCNA, albeit in a ligand-independent manner. This could indicate, although this remains purely speculative,

that RAR $\alpha$  engages interaction either with PCNA when bound to DNA as a heterodimer with RXR, or as a monomer with DNA-bound PCNA.

Our discovery that PCNA interacts with RAR $\alpha$  was unexpected, since RAR $\alpha$  contains none of the PCNA interaction motifs, the PIP box [QXX(L/I/M)XX(Y/F)(Y/F)] or the KA box [(A/L/Q)XX(L/V)]. Moreover, PCNA has been shown to interact mostly with factors having a low affinity for DNA and limited DNA sequence specificity. It is not clear at this point how PCNA exerts its repressive effect on RAR. We have observed that *in vitro* DNA binding of RXR/RAR heterodimers is not affected by increasing concentrations of PCNA (data not shown), excluding a steric hindrance of RAR $\alpha$  DBD upon PCNA binding. This is further strengthened by the fact that PCNA is detected on chimeric and endogenous





**Figure 5.** (A) PCNA interacts specifically with RARs. hRAR $\beta$ , hRAR $\gamma$ , hRXR $\alpha$ , hVDR and hPPAR $\gamma$  receptors were translated and labeled *in vitro* and incubated with a Sepharose–glutathione/GST–PCNA slurry. The interaction was analyzed as described in (Figure 4A). (B) PCNA overexpression inhibits transcription induced par RARs isotypes. HeLa cells were cotransfected as described elsewhere in 6-wells cluster ( $\sim 1.5 \times 10^5$  cells) with a retinoid-inducible reporter gene pGL3(RAR-E)<sub>3</sub>tkLuc (500 ng), RAR $\beta$  or RAR $\gamma$  and RXR $\alpha$  expression vectors (50 ng of each), and a 10-fold stoichiometric excess of a PCNA expression vector. Twenty-four hours after transfection, cells were challenged, or not, overnight with 1  $\mu$ M atRA and luciferase activity was assayed. Results are expressed as the mean of three independent experiments performed in triplicate. (C) RXR $\alpha$ , VDR and PPAR $\gamma$  induced transcriptional activities are not sensitive to PCNA overexpression. HeLa cells were cotransfected as in B) with a DR1-driven or a DR3-driven reporter genes, RXR $\alpha$  and/or VDR or PPAR $\gamma$  expression vectors. Twenty-four hours after transfection, cells were challenged, or not, overnight with 1  $\mu$ M 9-*cis* RA, Vit D3 or rosiglitazone, respectively and luciferase activity was assayed.

retinoid-regulated promoter in intact cells (Figure 2). PCNA interacts with a wide array of proteins, with diverse biological activities, including cyclin-dependent kinase (cdk)-cyclin E or -cyclin D complexes. D- and E-type cyclins have been shown to

act as CoA and CoR for several NRs, including RAR (6,46–51). PCNA also acts by recruiting p300 and inhibiting its acetyl transferase activity (25,52), thus behaving as a selective inhibitor of p300-mediated transcription. In light of these

results, it is tempting to speculate that the selective effect of PCNA expression knockdown on endogenous RAR $\beta$ 2 expression might reflect a requirement for p300 for RAR $\beta$ 2 promoter activation. However, this would reflect an AF2-dependent mechanism, and we showed that PCNA interaction is AF2-independent. Moreover, p300 acetyl transferase activity is not required for retinoid-induced transcription (53); on the contrary, pCAF acetyl transferase activity is necessary for retinoid-induced transcription, and its interaction with the RAR DBD has been documented (54). An alternative mechanism to coactivator displacement could be the tethering of CoR. Histone desacetylase (HDAC) activity has been shown to be an integral component of the repressive activity of unliganded RAR, with HDAC1 and HDAC3 interacting indirectly with RAR through corepressors molecules, such as SMRT and NCoR. Recently, PCNA has been shown to interact directly with HDAC1 (55), rising the possibility that RAR-bound PCNA targets an additional HDAC molecule to further repress RAR-controlled transcriptional mechanisms. Here, it is worth noting that these various partners may engage interactions, which were not detected in our initial screen, and may contribute to establish a ligand dependency to the RAR-PCNA interaction in intact cells. At this point, these mechanisms remain purely speculative and additional experiments are required to fully characterize the PCNA-mediated repression mechanism. Since PCNA is a cyclin, whose expression is highly upregulated in S phase (56), it would also be of interest to identify retinoid-controlled genes, which undergo a cell cycle-dependent, PCNA-mediated repression.

Besides a 'simple' cell cycle-dependent repression of retinoid-controlled genes, can we envision another role for RAR-PCNA interaction? PCNA is intimately linked to the nucleotide excision repair (NER) process through its association with XPG (57) and its involvement in DNA resynthesis. NER can be divided into two types of activities: global genome repair, which is a slow, non-targeted process, and the transcription-coupled repair (TCR), which relies on the targeted recruitment of the NER machinery to elongating RNA polymerase II [see (58) and references therein]. Some NER factors participate equally to DNA repair and transcription activation: TFIIH, which is part of the NER complex, binds, phosphorylates and activates RAR in an AF2-independent manner (20). A role for RAR in TCR has thus been hypothesized and demonstrated (58), which could be exerted through chromatin remodeling and/or the recruitment of DNA repair factors. Here, we provide data suggesting that PCNA could participate to transcriptional control and DNA repair in a fashion analogous to that of TFIIH, by interacting with RAR. It would be of interest to determine the actual role of RAR in TCR and DNA synthesis, if any.

In summary, our data are consistent with a role of PCNA as a promoter- and cell-specific repressor of RAR transcriptional activity, and suggest, although this remains to be formally established, that the RAR-PCNA interaction may participate in coupling transcription to DNA repair.

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