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The Phosphorylation Site Located in the A Region of Retinoic X Receptor α Is Required for the Antiproliferative Effect of Retinoic Acid (RA) and the Activation of RA Target Genes in F9 Cells*

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Julie Bastien‡, Sylvie Adam-Stitah‡, Jean-Luc Plassat, Pierre Chambon,
and Cécile Rochette-Egly§

From the Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP/Collège de France,
BP 163, 67404 Illkirch Cedex, France

Mouse F9 embryocarcinoma cells constitute a well established cell autonomous model system for investigating retinoic acid (RA) signaling *in vitro*. RA induces the differentiation of F9 cells grown as monolayers into endodermal-like cells and decreases their rate of proliferation. Knock-out of the retinoic X receptor α (RXR α) gene abolishes endodermal differentiation and the induction of several endogenous RA-responsive genes. RXR α null cells are also drastically impaired in their antiproliferative response to RA. The role of the RXR α phosphorylation site located in the N-terminal A region (Ser²²) has been investigated here by establishing cell lines re-expressing RXR α either wild type or mutated at the phosphorylation site (RXR α S22A) in a RXR α -null background. We show that Ser²² is dispensable for RA-induced endodermal differentiation but is crucial for the expression of several RA-responsive genes. Ser²² is also indispensable for the antiproliferative effect of RA and necessary for the RA-induced down-regulation of p21^{CIP} and p27^{KIP} CKI proteins that are known to be involved in the control of cell cycle progression.

autonomous model system for investigating retinoid signaling *in vitro* (for review see Ref. 8 and references therein). Upon RA treatment, and depending on culture conditions, F9 cells differentiate into three distinct cell types resembling primitive, parietal, and visceral endodermal extraembryonic cells (9). This RA-induced differentiation is also accompanied by a decrease in the rate of proliferation and the induction of expression of a number of genes. F9 cells express all RAR and RXR isoforms, with RXR α 1, RAR α 1, and RAR γ 2 being the main isoforms. Two strategies have been used to investigate their roles in the response of F9 embryonal carcinoma cells to RA treatment. Firstly, F9 cells lacking one or several RARs or RXRs were engineered through homologous recombination (10–15). Secondly, wild type (WT) and mutant F9 cells were treated with pan-RXR and RAR isotype (α , β , or γ)-selective retinoids (12, 13, 16–18). These studies demonstrated that RAR γ 2/RXR α heterodimers are the functional units transducing most RA-induced events (*e.g.* primitive and visceral differentiation, growth arrest, and activation of expression of a number of genes), whereas RAR α /RXR α heterodimers mediate some other events such as parietal differentiation.

Retinoic acid (RA),¹ the most potent biologically active metabolite of vitamin A, plays crucial roles in a wide variety of biological processes and influences the proliferation and differentiation of a variety of cell types (for reviews, see Refs. 1–4). RA exerts its effects through two families of nuclear ligand-dependent transcriptional regulators, the retinoic acid receptors (RARs) and the retinoid X receptors (RXRs). There are three RAR (α , β , and γ) and three RXR isotypes (α , β , and γ), and for each isotype, there are at least two main isoforms that are generated by differential promoter usage and alternative splicing and that differ only in their N-terminal A region (Refs. 5–7 and references therein).

F9 murine embryonal carcinoma cells provide a powerful cell

RARs and RXRs possess two transcriptional activation functions (AFs): AF-1 located in the N-terminal A/B region (19, 20) and AF-2 associated with the ligand-binding domain and activated by the ligand (Refs. 6 and 21 and references therein). The AF-1 domain of RARs is phosphorylated at conserved residues that belong to consensus sites for proline-directed kinases, which include the cyclin-dependent kinases and the mitogen-activated protein kinases (for review see Refs. 22 and 23). In RAR α 1 and RAR γ 2, the phosphorylated residues have been identified and found to be located in the conserved B region (24, 25). RXR α 1 is also phosphorylated, but the phosphorylation site (serine 22) is located in the RXR α 1-specific A region (26).

Because the various RA-responses of F9 cells can be restored upon re-expression of WT RAR γ in RAR γ -null cells (27), the role of the activation functions AF-2 and AF-1 and that of the phosphorylation of RAR γ 2 in RA-induced events have been studied by re-expressing a variety of RAR γ 2 mutants in these cells (RAR γ Δ AF-2, RAR γ Δ AF-1, and RAR γ S66/68A “rescue” lines). This strategy allowed us to demonstrate that RAR γ 2 needs the integrity of both its AF-1 and AF-2 domains to efficiently transduce the RA signal (18, 28). RAR γ 2 further requires the phosphorylation site of its AF-1 domain for inducing RA target gene and F9 cell differentiation (18). Phosphorylation is also necessary for the RA-induced degradation of RAR γ 2 by the ubiquitin-proteasome pathway (29).

By contrast, little is known about the mechanisms through which RXR exerts its transcriptional activity. *In vitro* studies demonstrated that liganded RXR is not active unless its RAR

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§ To whom correspondence should be addressed: IGBMC, BP 163, 67 404 Illkirch Cedex, CU de Strasbourg, France. Tel.: 33-3-88-65-34-59; Fax: 33-3-88-65-32-01; E-mail: cegly@igbmc.u-strasbg.fr.

¹ The abbreviations used are: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; WT, wild type; AF, activation function; WCE, whole cell extract; RT, reverse transcription; CKI, cyclin-dependent kinase inhibitor.

partner is itself liganded (16–18, 30, 31). Phenotypic analysis of mice expressing RXR α with its N-terminal A/B region deleted indicated that the RXR α AF-1 domain is functionally important for efficiently transducing the retinoid signal during embryonic development (32). However, little is known about the mechanisms through which the N-terminal A region and its phosphorylation site participate in the global activity of RXR α under physiological conditions.

Because RXR α -null F9 cells are drastically impaired in primitive and parietal endodermal differentiation as well as in their antiproliferative response to RA (14), we functionally dissected the role of RXR α Ser²² in these processes by establishing a rescue line expressing RXR α S22A in a RXR α -null background. Our results demonstrate that in F9 cells Ser²² is dispensable for primitive and subsequent parietal endodermal differentiation but is required for the induction of several RA-responsive genes. This phosphorylation site is also crucial for the antiproliferative effect of RA. In that context, Ser²² is necessary for the RA-induced decrease in the levels of p21^{CIP} and p27^{KIP} proteins that are involved in the control of cell cycle progression.

EXPERIMENTAL PROCEDURES

Plasmid Constructs—The mouse full-length cDNA of RXR α 1 was cloned into the pD402A vector (a gift of D. Lohnes), which is driven by the PGK promoter (33). RXR α S22A in pD402A was constructed by subcloning the *Xho*I-*Sac*I fragment containing the mutation from the pSG5-mRXR α construct (26) into the same sites of pD402A RXR α WT.

Cell Culture and Establishment of Stable Rescue Lines—F9 cells were cultured as monolayers on gelatinized surfaces as described previously (10). For differentiation studies, 10⁵ cells were cultured in 10-cm dishes and treated with tRA (100 nM) alone or in combination with 250 μ M dibutyryl-cAMP (Sigma) for 96 h with a medium change after 48 h. The control cells were treated with vehicle (final ethanol concentration, 0.1%). To establish the rescue lines, RXR α ^{-/-} cells (4.5 \times 10⁶ cells) were electroporated with the constructs indicated in Fig. 1A linearized with *Aat*II, along with a *Xho*I-linearized plasmid conferring resistance to hygromycin in a ratio of 10:1. After 24 h, the cells were selected with hygromycin (400 μ g/ml) for 10 days (27) and analyzed for the presence and expression of the transgene by Southern and Western blotting.

Antibodies—Rabbit polyclonal antibodies raised against the A region of RXR α 1, RPRX α (A), were as described (34). Those against p21^{CIP} (C-19) and p27^{KIP} (Ab-2) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA) and NeoMarkers (Lab Vision Corp.), respectively. Goat polyclonal antibodies raised against β -actin were from Santa Cruz Biotechnology, Inc.

Cell Extracts and Immunoblotting—Whole cell extracts (WCEs) were prepared as described previously (35). The proteins (40 μ g) were resolved by SDS-PAGE (12% acrylamide), electrotransferred onto nitrocellulose filters, immunoprobed, and detected by chemiluminescence according to the manufacturer's protocol (Amersham Biosciences).

RNA Isolation and RT-PCR—Total RNAs were isolated using the guanidinium thiocyanate method (36), and the aliquots (500 ng) were subjected to real time quantitative RT-PCR, using the SYBR Green Light cycler detection system (Roche Molecular Biochemicals). The transcripts levels were normalized according to 36B4 transcripts, which are unresponsive to retinoids treatment. The RT-PCR oligonucleotides for 36B4, Collagen IV, Laminin B1, HNF3 α , and HNF1 β were as described (14, 18). The primers were: Stra6, 5'-CTGCAGACCAGCTA-CTCCGA-3' and 5'-ACAGTAGGCCACGCTCAC-3'; Hoxa-1, 5'-GAGCTGGAGAAGGAGTTCCA-3' and 5'-CAGAGTTGGGCTGGAGTAGC-3'; Hoxb-1, 5'-CTCGAAGACTTCCCAAACCTCAC-3' and 5'-TCTCTAAGCTCAAAGGCACTGAAC-3'; CRABP1, 5'-AACCTCCACCAGCTGTCCGAA-3' and 5'-AGGCAGTCTTGGACCCGTA-3'; p21^{CIP}, 5'-GCCGTGATTGCGATGCGCTC-3' and 5'-CTCCTGACCCACAGCAGAAG-3'; and p27^{KIP}, 5'-GAGTCAGCGCAAGTGAATTT-3' and 5'-GCC-TGTAGTAGAACTCGGGCA-3'.

Cell Growth Analysis—Cell counting experiments were performed in triplicate with untreated and RA-treated cells as follows. The cells were plated at identical densities (2.5 \times 10³ cells/well) in 6-well plates and fed with fresh medium containing either vehicle or RA (100 nM) every 2 days. At days 3 and 5, the remaining adherent cells were trypsinized and counted with a Coulter particle counter (Coultronics France, SA). The percentage of growth inhibition by RA was calculated as described previously (14).

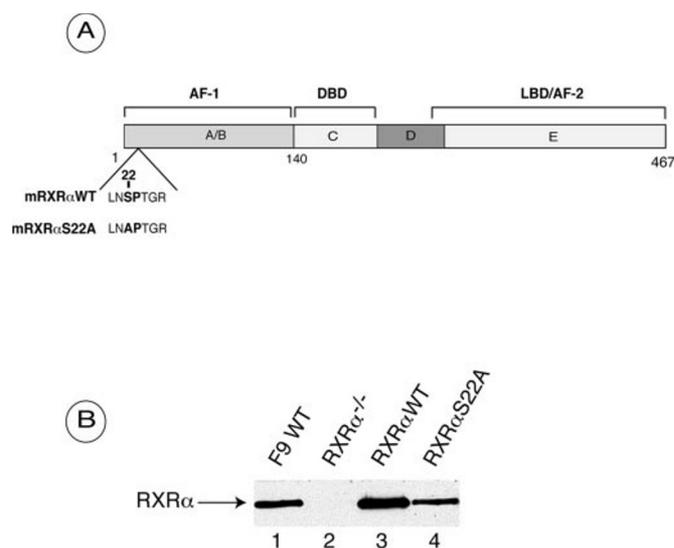


FIG. 1. Generation of stable rescue lines re-expressing RXR α WT or RXR α S22A. A, schematic representation of the constructs used to generate RXR α rescue lines in RXR α -null cells. Mouse RXR α 1 with the DNA-binding domain (DBD) and the AF-1 and AF-2 activation domains, which lie in the A/B and E regions, respectively, are schematically represented (not to scale). The target sequence for phosphorylation by proline-dependent kinases in the A region of RXR α 1 is shown, and the serine residue, which has been mutated to alanine (Ser²²) is indicated. B, RXR α protein in rescue lines. WCEs were prepared from WT F9 cells, RXR α ^{-/-} cells, and the two rescue lines (RXR α WT and RXR α S22A). The proteins were resolved by SDS-PAGE, and RXR α was detected by Western blotting with a specific rabbit polyclonal antibody, RPRX(A). The presented results correspond to a representative experiment of three.

The cell cycle profiles of F9 WT, RXR α ^{-/-}, RXR α WT, and RXR α S22A cells were determined by cell cycle flow cytometry based on cellular DNA content analysis using a FACScan (Beckton Dickinson, Inc.). Subconfluent cultures of control or RA-treated cells were trypsinized and combined with their culture supernatants, pelleted, resuspended in 500 μ l of hypotonic buffer (0.1% Triton X-100, 0.1% sodium citrate) containing 50 μ g/ml propidium iodide, and incubated for 15 h in the dark at 4 $^{\circ}$ C. The percentage of cells in the different phases of the cell cycle was determined using the Cell Quest software.

Statistical Analysis—The data are expressed as the means \pm S.E. of three independent experiments unless otherwise indicated. Statistical analysis was performed using the analysis of variance followed by 2 \times 2 comparisons based on the Newman-Keul's test.

RESULTS

Generation of Rescue Lines Expressing RXR α —We have previously shown that RXR α is “constitutively” (*i.e.* in the absence of ligand) phosphorylated at serine 22 in COS-1 cells (26) and also in F9 cells.² To investigate whether this phosphorylation of RXR α is involved in primitive and parietal endodermal differentiation of F9 cells, as well as in their antiproliferative response to RA, rescue lines re-expressing wild type RXR α (RXR α WT line) or RXR α mutated at the phosphorylation site (RXR α S22A line) were derived from RXR α -null cells (Fig. 1A). Two clones were obtained for the RXR α WT rescue transgene and one clone for the RXR α S22A rescue transgene. The presence of the S22A mutation was verified by sequencing cDNA fragments amplified by RT-PCR from total RNA of the RXR α S22A rescue line (data not shown). The expression level of RXR α WT and RXR α S22A in the derived cell lines was compared with the expression of endogenous RXR α in F9 WT cells after immunoblotting. RXR α S22A was expressed in the corresponding rescue line at levels similar to that of RXR α in F9 WT

² J. Bastien, S. Adam-Stitah, and C. Rochette-Egly, unpublished results.

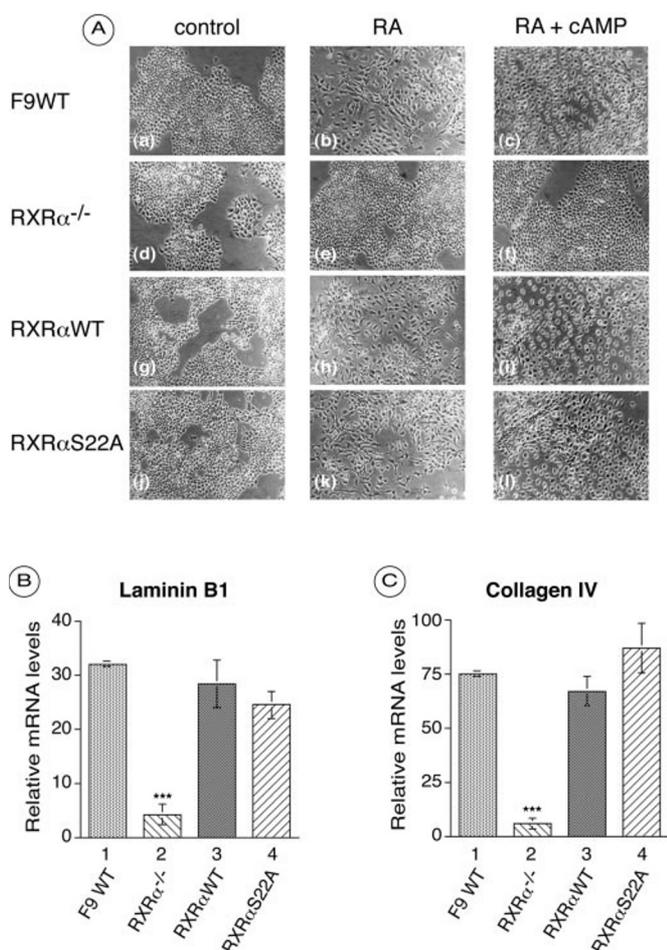


FIG. 2. The phosphorylation site located in the N-terminal A region of RXR α (Ser²²) is dispensable for rescuing primitive endodermal differentiation. A, morphological differentiation of F9 WT, RXR $\alpha^{-/-}$, RXR α WT, and RXR α S22A cells (as indicated) grown for 96 h in presence of 100 nM RA alone or combined with 250 μ M cAMP, as viewed under phase contrast microscopy. The control cells treated with 0.1% ethanol (vehicle) or with cAMP alone remained undifferentiated. B and C, relative expression of the differentiation markers laminin B1 and collagen IV(α 1). Total RNA (500 ng) from F9 WT, RXR $\alpha^{-/-}$, RXR α WT, and RXR α S22A cells treated as in A was subjected to quantitative RT-PCR analysis for collagen IV and laminin B1 (see “Experimental Procedures”). The values correspond to the fold induction relative to the amount of RNA transcripts present in ethanol-treated cells. ***, statistically significant differences between WT cells and the other cell lines ($p < 0.001$).

cells (Fig. 1B, lane 4). The RXR α WT rescue lines slightly over-expressed the RXR α protein relative to endogenous RXR α . Because they yielded similar results in the studies described thereafter, one was selected (Fig. 1B, lane 3).

The RA-induced Endodermal Differentiation of F9 Cells Does Not Require the Phosphorylation Site Located in the A Region of RXR α —When grown as monolayers in the presence of RA for 96 h, F9 WT cells differentiate into primitive endoderm-like cells (37) exhibiting a characteristic flat triangular morphology (Fig. 2A, panel b). The addition of cAMP along with RA results in the formation of parietal endoderm-like cells (38), which have a rounded and refractile appearance (Fig. 2A, panel c). These two types of differentiation are drastically impaired in RXR $\alpha^{-/-}$ cells (14) (Fig. 2A, compare panels e and f with panels b and c), and re-expression of RXR α WT (RXR α WT rescue line) restores the RA responsiveness (Fig. 2A, panels g–i). Similarly, the RXR α S22A rescue line differentiates upon treatment with RA alone or with RA plus cAMP (Fig. 2A, panels j–l), indicating that RXR α can efficiently mediate the induction of primitive

and parietal endoderm differentiation in the absence of Ser²².

The differentiation of the various rescue lines was further analyzed by determining the expression of two markers of primitive endodermal differentiation, laminin B1 and collagen IV(α 1), using quantitative RT-PCR. RA-induced expression of laminin B1 and collagen IV (Fig. 2, B and C, columns 1), which was impaired in RXR $\alpha^{-/-}$ cells (14) (Fig. 2, B and C, columns 2), was restored in the RXR α WT rescue line to levels similar to those achieved in F9 WT cells (Fig. 2, B and C, columns 3). In agreement with the morphological differentiation, the expression of these two markers was completely restored in the RXR α S22A rescue line (Fig. 2, B and C, columns 4). Altogether, these results indicate that Ser²², located in the N-terminal A region of RXR α , is dispensable for RA-induced endodermal differentiation of F9 cells. Similar results were obtained concerning parietal endodermal differentiation as assessed by the expression of a specific marker, thrombomodulin (data not shown) (39).

Role of RXR α Ser²² on the Expression of Several RA-responsive Genes—Knock-out of the RXR α gene in F9 cells results in a drastic reduction of the expression of several RA-responsive genes (13, 14), such as Stra6, Hoxa-1, HNF3 α , CRABP1, Hoxb-1, and HNF1 β (Fig. 3, in each panel, compare lanes 1 and 2; $p < 0.001$). We investigated the ability of RXR α WT and RXR α S22A to rescue the expression of these RA target genes, using quantitative real time RT-PCR after treatment of the different cell lines with 100 nM RA for 24 h. Re-expression of RXR α WT restored the expression of all genes tested to levels significantly similar to those achieved in F9 WT cells (Fig. 3, compare lanes 1 and 3 in each panel). RXR α S22A also restored the expression of Stra6 and Hoxa-1 with the same efficiency as RXR α WT (Fig. 3, A and B, compare lanes 1 and 4). However, RXR α S22A did not restore the expression of HNF3 α , CRABP1, Hoxb-1, nor HNF1 β to the levels achieved in WT cells (Fig. 3, C–F, compare lanes 1 and 4; $p < 0.001$). No responsiveness was observed for up to 96 h of RA treatment, (data not shown), indicating that the RXR α S22A mutant does not lead to a delayed activation of these RA target genes.

Collectively, our results indicate that Ser²² is crucial for the induction of several RA target genes expression and that this process is promoter context-dependent. Note that a difference in the stability of the mutant receptor is ruled out, because RXR α WT and RXR α S22A levels are not affected within 48 h of RA treatment.²

The Antiproliferative Effect of RA Requires the RXR α Phosphorylation Site Located in the A Region—RA-induced differentiation of F9 cells is also accompanied by a marked decrease in their proliferation rate as determined by counting of the adherent cells with 58 and 84% growth inhibition at 3 and 5 days of RA treatment, respectively (Fig. 4). This antiproliferative response to RA is significantly reduced in RXR $\alpha^{-/-}$ cells (14), which exhibit only 32 and 54% growth inhibition upon 3 and 5 days of RA treatment, respectively (Fig. 4). Re-expression of RXR α WT restored the antiproliferative response to RA with a growth inhibition similar to that observed in F9 WT cells (Fig. 4). In contrast, the RXR α S22A rescue line depicted a different behavior. Indeed, at 3 days of RA treatment, the RXR α S22A rescue line retained an antiproliferative response that was not significantly different from that of RXR $\alpha^{-/-}$ cells (Fig. 4A; $p > 0.05$). However, after 5 days of RA treatment, the growth inhibition was slightly rescued but was still significantly different from that of WT cells (Fig. 4B; $p < 0.05$). Thus, RXR α appears to mediate part of the antiproliferative effect of RA through the Ser²² phosphorylation site.

Previous studies have shown that RA treatment of F9 cells also results in the lengthening of the G₁ portion of the cell cycle

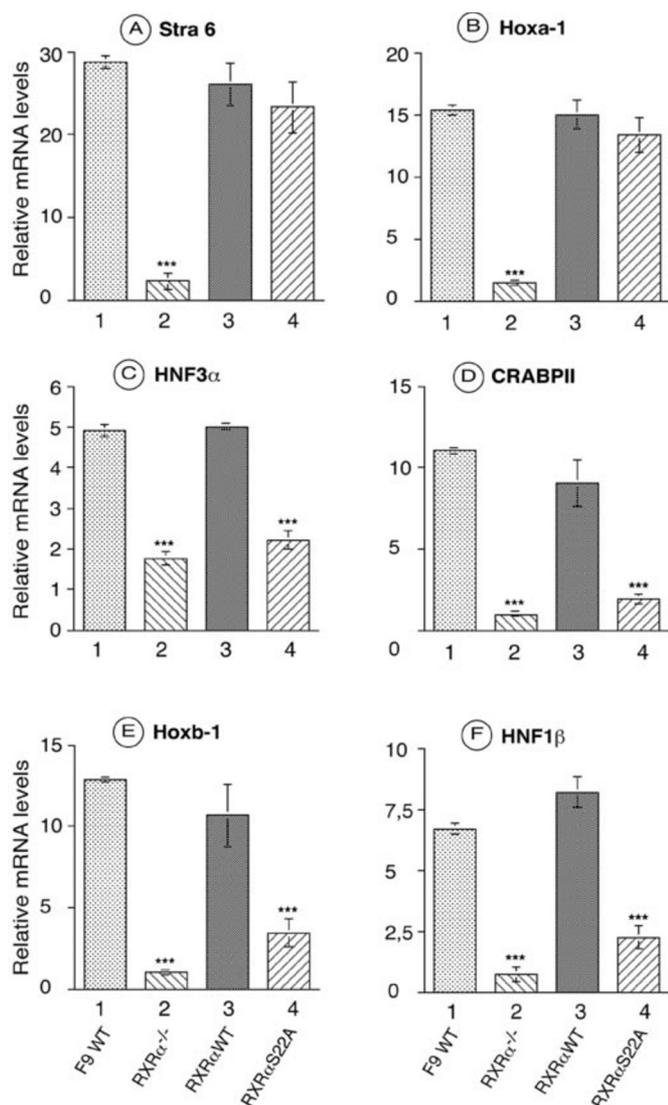


FIG. 3. The phosphorylation site of RXR γ (Ser²²) is required for the induction of several RA-responsive genes. The differential RA inducibility of the various RA-responsive genes in WT, RXR α ^{-/-}, RXR α WT, and RXR α S22A F9 cells grown in the presence of RA (100 nM) for 24 h was analyzed by quantitative RT-PCR as in Fig. 2. ***, statistically significant differences between the WT cells and the other cell lines ($p < 0.001$).

and that this effect is less pronounced for RXR α ^{-/-} cells (14). Therefore, we investigated by cell cycle flow cytometry (see “Experimental Procedures”) whether RXR α Ser²² is also involved in the RA-induced accumulation in the G₁ phase. The untreated WT, RXR α WT, or RXR α S22A cell lines exhibited similar cell cycle profiles, with some insignificant fluctuations reflecting variations in the basal proliferation rate (Table I). Note that the RXR α ^{-/-} line depicting a lower proportion of cells in the G₁ phase may be due to a slight higher basal proliferation rate (14).

RA treatment of F9 WT cells for 5 days resulted in an accumulation of the cells in the G₁ phase of the cell cycle from 36 ± 0.58 to $53 \pm 0.88\%$ (Table I). This accumulation was significantly decreased in RXR α ^{-/-} cells (14) with $36 \pm 1.2\%$ of cells in G₁ instead of 53% for WT cells (Table I, $p < 0.001$). The RXR α WT rescue cell line recovered a proportion of cells in the G₁ phase that was similar to that observed with WT F9 cells (Table I). In contrast, in RA-treated RXR α S22A cells, the proportion of cells in the G₁ phase remained statistically the same as that of RXR α ^{-/-} cells (Table I). Collectively, these results

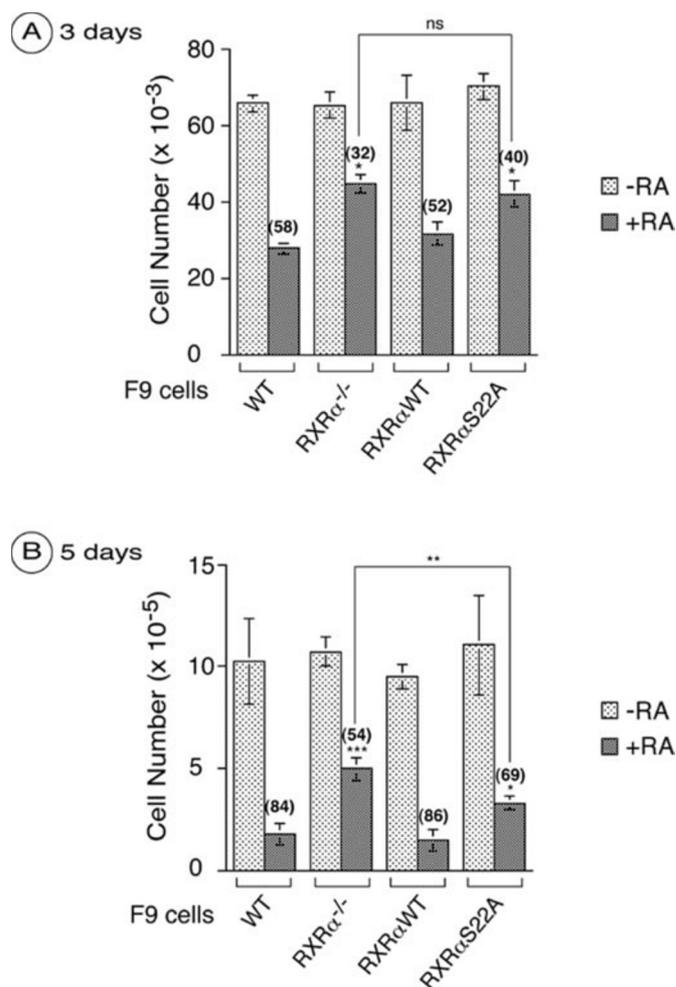


FIG. 4. The phosphorylation site of RXR α (Ser²²) is required for the antiproliferative effect of RA. WT F9 cells, RXR α ^{-/-} cells, and rescue cells were plated in triplicate in 6-well plates at an identical density of 2.5×10^3 cells/well and counted after 3 (A) and 5 (B) days of culture in the absence or presence of RA (100 nM). The number of cells after 3 or 5 days of culture in the presence or absence of RA is indicated. The asterisks indicate statistically significant differences between the RA-treated cell lines (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$). ns, not significant ($p > 0.05$). The percentages of growth inhibition are indicated in parentheses.

indicate that RXR α Ser²² plays a crucial role in the antiproliferative effect of RA.

RXR α Ser²² Is Required for the RA-induced Down-regulation of the CKI Proteins, p21^{CIP} and p27^{KIP}—To corroborate the role of RXR α Ser²² in the antiproliferative effect of RA, we investigated its contribution to the regulation of some G₁ phase-associated molecules that have been shown to be targets for RA action (40–45). We focused upon the cyclin-dependent kinase inhibitors p21^{CIP} and p27^{KIP}.

The expression of p27^{KIP} and p21^{CIP} transcripts did not vary significantly upon RA treatment of F9 WT cells up to 96 h (data not shown). However, p27^{KIP} and p21^{CIP} protein levels were strongly decreased within 48 and 72 h, respectively (Fig. 5), indicating that in F9WT cells, the antiproliferative effect of RA correlates with a down-regulation of these CKIs. Interestingly, in RXR α -null cells, the RA-induced down-regulation of p27^{KIP} was delayed and occurred at 96 h instead of 48 h (Fig. 5A), whereas that of p21^{CIP} was completely abolished (Fig. 5B). The down-regulation of both CKIs was fully restored in the RXR α WT rescue line (Fig. 5). However, in the RXR α S22A line, the decrease in p27^{KIP} was not rescued (Fig. 5A), whereas that in p21^{CIP} was restored with a delay (Fig. 5B). Altogether, these

TABLE I

The phosphorylation site of RXR α (Ser²²) is indispensable for the RA-induced accumulation of F9 cells into the G₁ phase of the cell cycle

F9 wild type RXR $\alpha^{-/-}$, RXR α WT, and RXR α S22A cells were grown for 5 days in the presence of 100 nM RA. The percentages of cells in the different phases of the cell cycle were analyzed with an fluorescence-activated cell sorter as described under "Experimental Procedures." The values correspond to the percentages of cells in the G₀/G₁, S, and G₂/M phases.

Day 5	Cell cycle phase	F9 cells			
		Wild type	RXR $\alpha^{-/-}$	RXR α WT	RXR α S22A
-RA	G ₀ /G ₁	36 ± 0.58	28 ± 1.53	33 ± 1.16	31 ± 0.33
	S	35	39	38	39
	G ₂ /M	29	33	31	30
+RA	G ₀ /G ₁	53 ± 0.88	36 ± 1.2 ^a	50 ± 1.21	37 ± 1.45 ^a
	S	26	34	18	36
	G ₂ /M	18	30	26	25

^a These differences are statistically significant between WT cells and the different cell lines, upon RA treatment ($p < 0.001$).

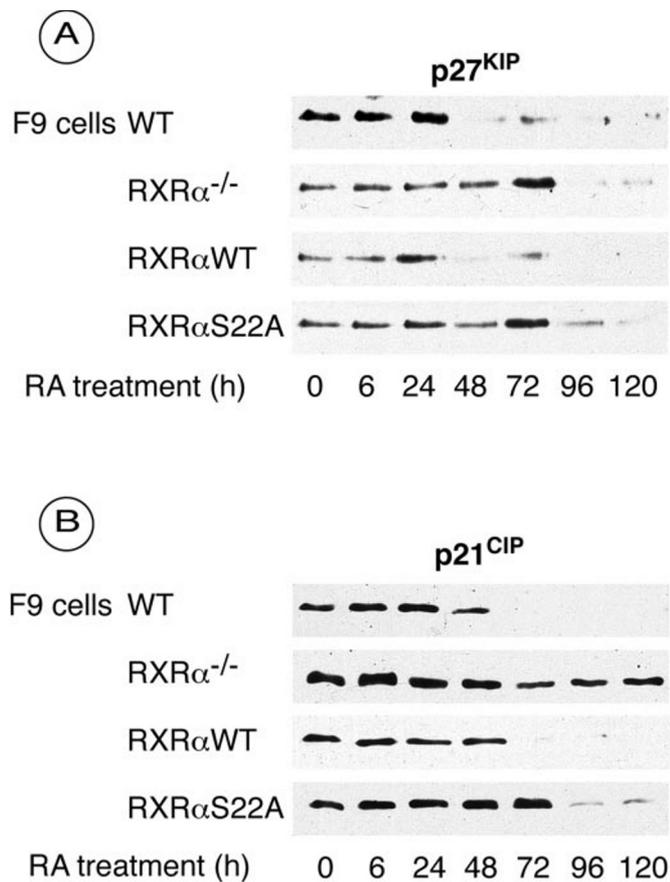


FIG. 5. The phosphorylation site of RXR α (Ser²²) is required for the RA-induced down-regulation of p27^{KIP} and p21^{CIP} proteins. WCEs were prepared from F9 WT, RXR $\alpha^{-/-}$, RXR α WT, and RXR α S22A cells left untreated or treated with RA for the indicated times. Equal amounts of WCEs, as assessed by immunoblotting with actin antibodies (data not shown) were resolved by SDS-PAGE and p27^{KIP} (A) and p21^{CIP} (B) proteins were detected by immunoblotting with specific rabbit polyclonal antibodies. The presented results correspond to a similar representative experiment of three.

data indicate that, in F9 cells, the RA-induced down-regulation of p27^{KIP} and to a lesser extent of p21^{CIP} requires the phosphorylation site located in the A region of RXR α .

Because p21^{CIP} and p27^{KIP} proteins are essentially regulated post-transcriptionally by the ubiquitin-proteasome pathway (46–51), we investigated whether in F9 cells, the RA-induced down-regulation of these CKIs involves the activation of this pathway. Treatment of control cells with the proteasome inhibitor MG132 did not significantly affect p21^{CIP} protein levels but markedly increased p27^{KIP} (Fig. 6A), suggesting that in F9 cells, the proteasome-dependent pathway is involved in the turnover of this CKI. In contrast, in RA-treated F9 cells,

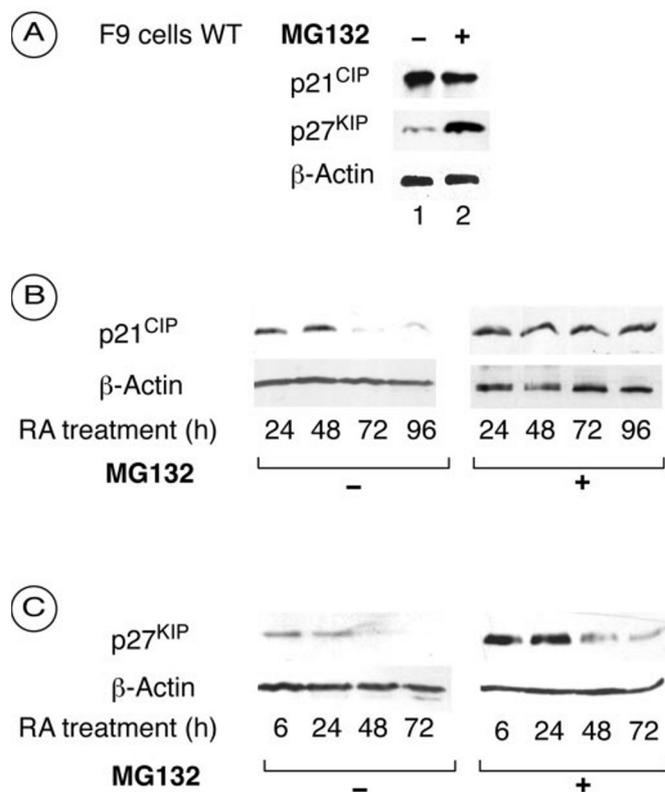


FIG. 6. The proteasome and p21^{CIP} and p27^{KIP} protein levels. A, control F9 WT cells were incubated or not with MG132 (40 μ M) 15 h before harvesting. Equal WCE amounts, as checked by immunoblotting with actin antibodies, were immunoblotted with p21^{CIP} or p27^{KIP} antibodies. B and C, same as A, but F9 WT cells were treated with RA for the indicated times and left untreated or incubated with MG132 15 h before harvesting. WCEs were immunoblotted with actin, p21^{CIP} (B), or p27^{KIP} (C) antibodies.

MG132 abrogated the decrease in p21^{CIP} levels (Fig. 6B) but not that of p27^{KIP} (Fig. 6C). Altogether, these results suggest that the down-regulation of p21^{CIP} induced by RA involves the proteasome pathway, whereas that of p27^{KIP} may occur through an other mechanism.

DISCUSSION

The present investigation was designed to analyze the contribution of the constitutively phosphorylated serine residue located in the N-terminal A region of RXR α (Ser²²), in the control of F9 cells differentiation and growth arrest, and in the expression of RA target genes. To that end, we used rescue RXR α -null F9 cells re-expressing RXR α either WT or mutated at Ser²². Analysis of the RA response of these cell lines allows us to draw the following conclusions.

First, we demonstrate that the phosphorylation site located

in the N-terminal A region of RXR α is dispensable for the RA-induced differentiation of F9 cells, because the line rescued with RXR α S22A is able to differentiate into primitive endoderm-like cells and subsequently into parietal endodermal cells.

Second, RXR α Ser²² is necessary for the induction of certain RA target genes. In F9 cells, the expression of most RA-responsive genes such as Hoxa-1, HNF3 α , HNF1 β , Stra6, and CRABP-II, is known to be controlled by RAR γ /RXR heterodimers, whereas the induction of Hoxb-1 can be mediated by either RAR γ /RXR or RAR α /RXR heterodimers (8). The activation of these genes is strongly decreased or abrogated in RXR α -null cells (13, 14). Our results demonstrate that the N-terminal phosphorylation site of RXR α is necessary for the RA-induced expression of some of these genes, such as HNF3 α , HNF1 β , CRABP-II, and Hoxb-1, because RXR α S22A is inefficient in restoring their inducibility. This may result from distinct steric conformations of the AF-1 domain of RXR α bound to different promoters and therefore from different interactions with putative AF-1 coactivators that could be differentially modulated by phosphorylation. In this respect, we note that the phosphorylation of the A/B domain of some nuclear receptors has been shown to modulate their interaction with coactivators or their ligand affinity. For example, phosphorylation of the estrogen receptor A/B domain promotes recruitment of the SRC-1 coactivator (52), whereas phosphorylation of the PPAR γ A/B domain reduces the ligand binding affinity of the receptor, thus negatively regulating its transcriptional activity (53, 54).

Third, RXR α Ser²² is required for the antiproliferative response to RA and the accumulation in the G₁ phase, which are severely altered in RXR α -null cells (14). In several cell lines, the growth inhibitory effect of RA has been correlated to the expression level of RAR β 2 (15, 55, 56). However, our results are not consistent with such a mechanism, because RAR β 2 is similarly induced in F9 WT cells, RXR α -null cells (13, 14), and the different rescue lines (data not shown). In fact, progression through the cell cycle is ensured by a number of factors including cyclins, cyclin-dependent kinases, and CKIs (22, 57, 58). Although considerable advances have been made in understanding the role of these factors in G₁ progression, how RA controls the coordinated action of these molecules in F9 cells is not completely elucidated. However, according to a number of reports, the antiproliferative effect of RA has been associated with variations in the expression of the CKIs p21^{CIP} and p27^{KIP} (40–44). Initially considered as inhibitors of proliferation, increasing evidence now suggests that CKIs play a complex role and may be also associated with cell cycle progression (59–61). Accordingly, depending on the cell system, either increases or decreases in CKIs levels have been associated with the antiproliferative effect of RA. In the present study performed with F9 cells, we found that RA down-regulates p21^{CIP} and p27^{KIP} levels. The mechanism of this down-regulation remains to be investigated. Similarly, how this down-regulation participates in the antiproliferative effect of RA is still unknown. Nevertheless, the important point of the present investigation is that the phosphorylation site localized in the N-terminal region of RXR α 1, which is involved in the antiproliferative effect of RA, is also required for the RA-induced variations in the levels of some proteins engaged in G₁ progression. The identification of the RA-responsive genes specifically involved in the regulation of cell cycle progression would provide new insights for understanding cell cycle regulation and the role of RXR α in RA signaling.

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