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Hyperphosphorylation of the Retinoid X Receptor α by Activated c-Jun NH₂-terminal Kinases*

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Sylvie Adam-Stitah[‡], Lucia Penna, 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, CU de Strasbourg, France

The nuclear receptor mouse retinoid X receptor α (mRXR α) was shown to be constitutively phosphorylated in its NH₂-terminal A/B region, which contains potential phosphorylation sites for proline-directed Ser/Thr kinases. Mutants for each putative site were generated and overexpressed in transfected COS-1 cells. Constitutively phosphorylated residues identified by tryptic phosphopeptide mapping included serine 22 located in the A1 region that is specific to the RXR α 1 isoform. Overexpression and UV activation of the stress-activated kinases, c-Jun NH₂-terminal kinases 1 and 2 (JNK1 and JNK2), hyperphosphorylated RXR α , resulting in a marked decrease in its electrophoretic mobility. This inducible hyperphosphorylation involved three residues (serines 61 and 75 and threonine 87) in the B region of RXR α and one residue (serine 265) in the ligand binding domain (E region). Binding assays performed *in vitro* with purified recombinant proteins demonstrated that JNKs did not interact with RXR α but bound to its heterodimeric partners, retinoic acid receptors α and γ (RAR α and RAR γ). Hyperphosphorylation by JNKs did not affect the transactivation properties of either RXR α homodimers or RXR α /RAR α heterodimers in transfected cultured cells.

Retinoids are derivatives of vitamin A that play key roles in a variety of biological processes ranging from pattern formation and organogenesis during embryogenesis to maintenance of homeostasis in the adult (1–4). Retinoids exert their pleiotropic effects through two classes of nuclear receptors acting as ligand-dependent transcriptional regulators, the retinoic acid receptors (RARs)¹ and the retinoid X receptors (RXRs) (4–7). RARs are activated by both all-*trans*-retinoic acid (tRA) and 9-*cis*-retinoic acid (9cRA), whereas RXRs are activated exclusively by 9cRA. There are three RAR isotypes and three RXR isotypes (α , β , and γ), encoded by distinct genes, and for each isotype, there

are at least two main isoforms, which differ in their NH₂-terminal A regions and are generated by differential promoter usage and/or alternative splicing (5, 6, 8). Several lines of evidence support the conclusion that RAR/RXR heterodimers are the functional units transducing the retinoid signal *in vivo* (Refs. 4–6, 9, and 10 and references therein). However, RXRs are also able to heterodimerize with other members of the nuclear receptor superfamily, such as the thyroid hormone receptors, the vitamin D₃ receptor, and the peroxisome proliferator activated receptors (11–15).

As other members of the nuclear steroid/thyroid hormone receptor superfamily, RARs and RXRs exhibit a conserved modular structure with six variably conserved functional regions (A to F) (Fig. 1 and Refs. 5 and 6). The amino-terminal A/B region of RARs and RXRs contains a ligand-independent transactivation function AF-1 (16, 17), while the highly conserved C region is included in the DNA-binding domain. The E region is more complex, since, in addition to the ligand-binding domain, it contains a dimerization surface and a ligand-dependent transcriptional activation function AF-2 (6, 16–19). In the COOH-terminal part of the E region, there is a well conserved amphipathic helix (the AF-2 AD core, helix 12 of the ligand-binding domain), which has been shown to be an essential element of the AF-2 function. Upon ligand binding, there is a major transconformational change of the ligand-binding domain that involves the folding back of helix 12 and the formation of a new surface required for interaction with coactivators that relay the AF-2 activity to the transcriptional machinery and/or to the chromatin template (6, 19–21).

As most members of the nuclear receptor family (22), RARs have been shown to be phosphoproteins (23–25). In mouse RAR α , a phosphorylated serine residue has been identified in region B; it is phosphorylated by Cdk7 associated with the general transcription factor TFIIF, and this phosphorylation has been shown to be crucial for AF-1 activity in transfected COS cells (26). In addition, mRAR α is phosphorylated by protein kinase A at a serine residue located in the ligand-binding domain/AF-2 domain (27). These serine residues that are conserved among RARs were also found to be phosphorylated in RAR γ .² Most interestingly, phosphorylation of residues in the AF-1 and AF-2 domains of both RAR α and RAR γ has been shown to be indispensable for differentiation of embryonal carcinoma F9 cells upon retinoic acid and cyclic AMP treatment (28).

In the present study, we report that RXR α is also a phosphoprotein. As for RAR α , phosphorylation sites for proline-directed protein kinases are located in the A/B region of mouse RXR α 1 and are constitutively phosphorylated in transfected COS-1 cells. In addition, we demonstrate that under stress conditions

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[§] To whom correspondence should be addressed. 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: RAR, retinoic acid receptor; mRAR, murine RAR; RXR, retinoid X receptor; mRXR, murine RXR; tRA, all-*trans*-retinoic acid; 9cRA, 9-*cis*-retinoic acid; JNK, c-Jun NH₂-terminal kinase; CAT, chloramphenicol acetyltransferase; PAGE, polyacrylamide gel electrophoresis; GST, glutathione S-transferase; MAPK, mitogen-activated protein kinase; SAPK, stress-activated protein kinase; CDK, cyclin-dependent kinase; ERK, extracellular signal-regulated kinase; WCE, whole cell extract; mAb, monoclonal antibody.

² S. Adam-Stitah, L. Penna, P. Chambon, and C. Rochette-Egly, unpublished data.

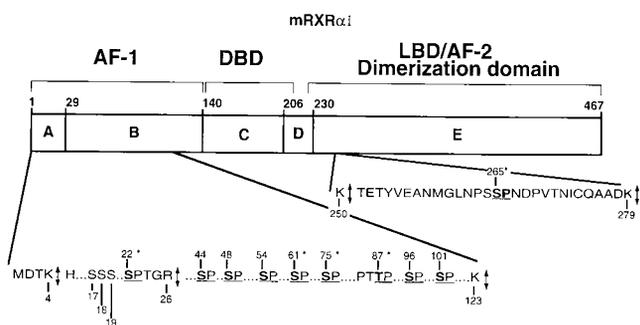


FIG. 1. Schematic representation of the different mRXR α 1 regions. The putative phosphorylation sites are underlined, and the known phosphorylated residues are indicated by an *asterisk*. All of them belong to consensus proline-directed protein kinases sites. The *arrows* show the trypsin cleavage sites.

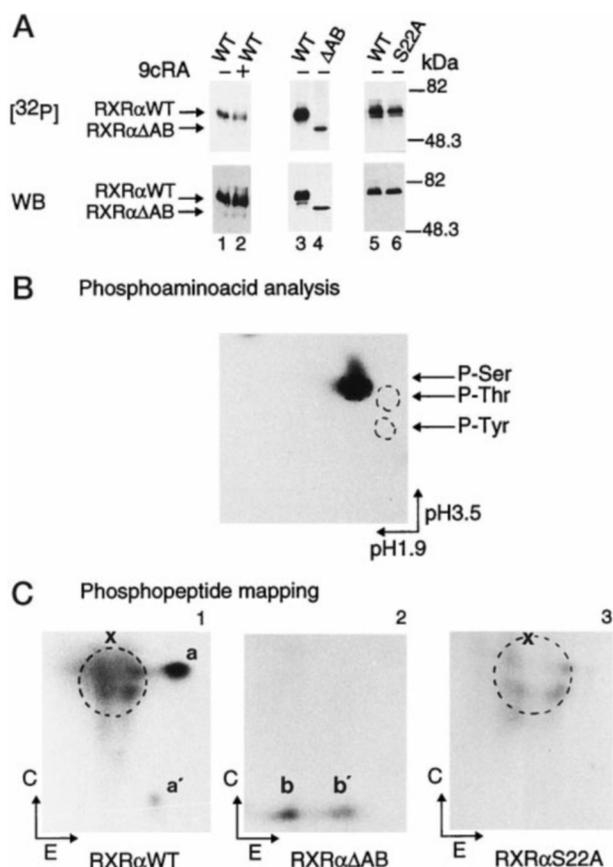


FIG. 2. mRXR α 1 overexpressed in COS-1 cells is phosphorylated at serine 22. **A**, COS-1 cells were transfected with RXR α WT (lanes 1, 2, 3, and 5), RXR α Δ AB (lane 4), or RXR α S22A (lane 6) expression vectors (5 μ g) and subsequently labeled with [32 P]orthophosphate. In lane 2, cells were treated with 10^{-7} M 9cRA for 1 h. Whole cell extracts (WCEs) were immunoprecipitated with mAb 4RX3A2, resolved by SDS-PAGE, and electrotransferred onto nitrocellulose membranes. Phosphorylated RXR α was detected by autoradiography (32 P) and chemiluminescence (WB) after immunoreaction with RPRX α (D). **B**, phosphorylated and immunoprecipitated RXR α was resolved by SDS-PAGE, electrotransferred onto Immobilon membranes, and subjected to acid hydrolysis. Phosphoamino acids were separated by two-dimensional cellulose thin layer electrophoresis (27, 45) and visualized by autoradiography. The dotted lines correspond to the position of the ninhydrin-stained phosphoamino acid standards superimposed on the autoradiograms. **C**, two-dimensional tryptic phosphopeptide map of RXR α WT, Δ AB, and S22A. 32 P-Labeled and immunoprecipitated RXR α was electrotransferred onto nitrocellulose filters as in **A** and digested with trypsin. Phosphopeptides were resolved in two dimensions on cellulose plates (27, 45).

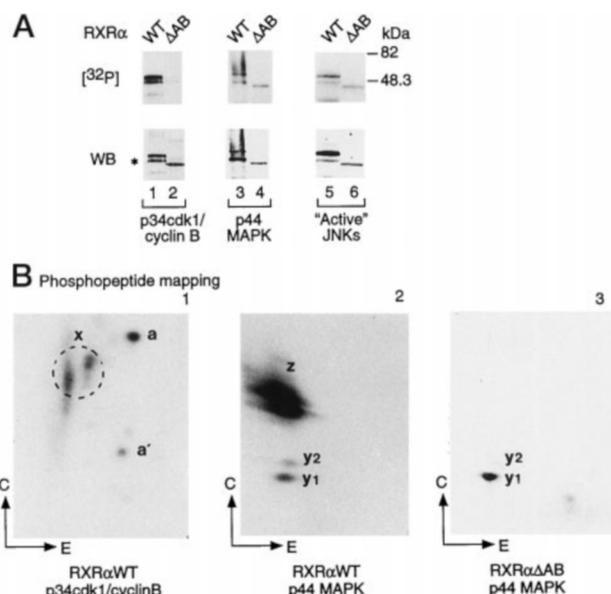


FIG. 3. *In vitro* phosphorylation of RXR α 1 by p34 Cdk1/cyclin B, p44 MAPK, and activated JNKs. **A**, purified bacterially expressed RXR α WT (lanes 1, 3, and 5) or RXR α Δ AB (lanes 2, 4, and 6) were phosphorylated with p34 Cdk1/cyclin B (lanes 1 and 2), p44 MAPK (lanes 3 and 4), or activated JNKs (lanes 5 and 6). After SDS-PAGE and electrotransfer, phosphorylated proteins were visualized by autoradiography (32 P) and immunoblotting with RPRX α (D) (WB). The signal indicated by an *asterisk* corresponds to RXR α degraded in the A region. **B**, two-dimensional tryptic phosphopeptide map of RXR α WT phosphorylated by p34 Cdk1/cyclin B (panel 1) and p44 MAPK (panel 2) and of RXR α Δ AB phosphorylated by p44 MAPK (panel 3).

such as UV irradiation, mouse RXR α 1 is hyperphosphorylated by endogenous and/or overexpressed stress-activated protein kinases, such as the c-Jun NH $_2$ -terminal kinases, JNK1 and JNK2 (29–32). This hyperphosphorylation involves serines 61 and 75 and threonine 87 that are located in the B region and serine 265 in the E region. However, in contrast to RAR α , hyperphosphorylation by JNKs does not appear to modulate the transcriptional properties of RXR α in cultured cells transfected with retinoic acid-responsive reporter genes.

EXPERIMENTAL PROCEDURES

Plasmid Constructions—The pSG5-based expression vectors for mRAR α 1 (16), mRXR α 1 (33), and murine RXR α deleted for the A/B region (mRXR α Δ AB) (16) were as described. For the construction of mRXR α 1 mutants, mRXR α 1 was first subcloned into the *Xho*I/*Bgl*II sites of pSG5-Cas (16) after polymerase chain reaction amplification of the A to E regions. The mRXR α 1 S22A, S44A, S48A, S54A, S61A, S75A, T87A, S96A, and S101A expression vectors were constructed by double polymerase chain reaction amplification reactions (27), according to Ho *et al.* (34), generating a *Xho*I/*Eco*RV fragment containing the appropriate mutation. The double mutant RXR α S75A/T87A was constructed according to the same protocol by introducing the T87A mutation into the RXR α S75A mutant. Similarly, the RXR α S61A/S75A/T87A expression vector was constructed by introducing the S61A mutation into the RXR α S75A/T87A double mutant. RXR α S265A was also constructed by double polymerase chain reaction amplification reaction, generating an *Eco*RV/*Bam*HI fragment containing the mutation. The double mutant RXR α S22A/S265A was prepared by subcloning the *Eco*RI/*Eco*RV fragment containing the S22A mutation into the same sites of RXR α S265A. The same strategy was followed for constructing the RXR α S61A/S75A/T87A/S265A mutant. All plasmids were verified by automated DNA sequencing. Additional details of constructions and oligonucleotide sequences are available upon request.

The reporter genes DR1G-tk-CAT and mRAR β 2-CAT have been previously described (16). The expression vectors for dominant active Ras (Ras^{Val-12}) and dominant negative Ras (Ras^{Asn-17}) were gifts from B. Wasyluk (35) and G. M. Cooper (36, 37), respectively. Those for human JNK1 and JNK2 were gifts from M. Karin (38, 39), and that of the Cdk7 expression vector was as described (26). Dominant active Cdk1 (A14F15) expression vector was a gift from P. Nurse (40).

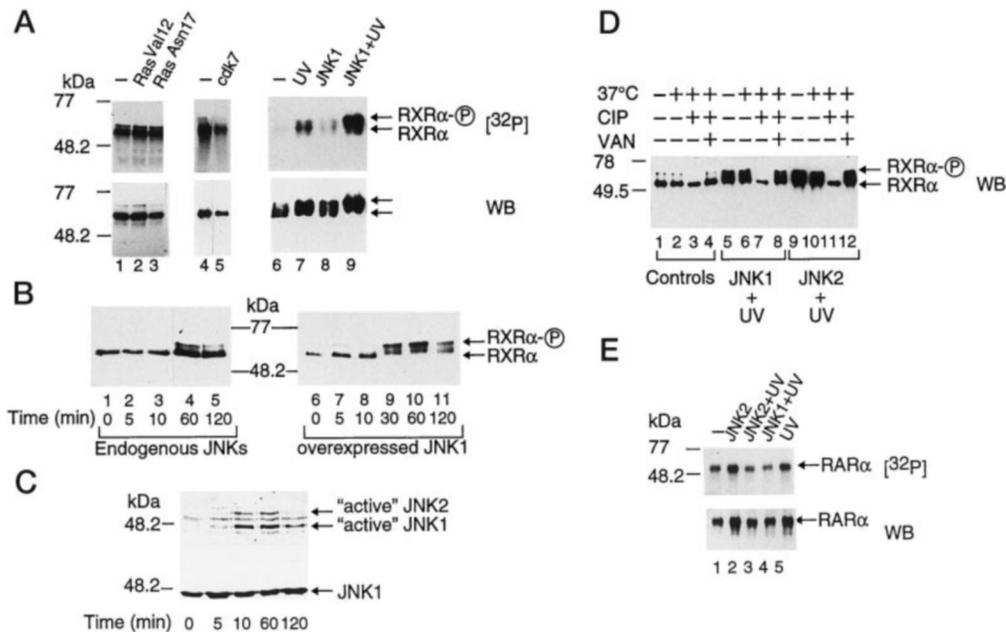


FIG. 4. Overexpressed and UV-activated JNKs hyperphosphorylate mRXR α 1 concomitantly with an upward shift in its electrophoretic mobility. *A*, COS-1 cells were transfected with RXR α WT expression vector (5 μ g) either alone (lanes 1, 4, 6, and 7) or in the presence of Ras^{Val-12} (1 μ g, lane 2), Ras^{Asn-17} (1 μ g, lane 3), Cdk7 (0.5 μ g, lane 5), or JNK1 (1 μ g, lanes 8 and 9) vectors. Where indicated, cells were UV-irradiated (40 J/m²) 1 h before harvesting. WCEs were immunoprecipitated with mAb 4RX3A2 and processed as in Fig. 2A for autoradiography and immunoblotting with RPRX α (A). Note that lanes 6–9 (upper panel) correspond to a shorter exposure than lanes 1–5. *B*, COS-1 cells transfected with RXR α WT expression vector (5 μ g) in the absence (lanes 1–5) or presence (lanes 6–11) of JNK1 (1 μ g) vector, were UV-irradiated (40 J/m²) 5, 10, 30, 60, or 120 min before harvesting. WCEs (15 μ g) were resolved by SDS-PAGE and immunoblotted with RPRX α (A). *C*, COS-1 cells were irradiated as in *B*, and cytosols (100 μ g) were resolved by SDS-PAGE and immunoblotted with either JNK1 monoclonal antibody (bottom) or with anti-ACTIVETM JNK polyclonal antibody (top). *D*, COS-1 cells transfected with RXR α either alone (lanes 1–4) or in the presence of JNK1 (lanes 5–8) or JNK2 (lanes 9–12) expression vector, were UV-irradiated (40 J/m²) 1 h before harvesting, and WCEs were immunoprecipitated with mAb 4RX3A2. Immunoprecipitates were incubated for 3 h at 37 °C in the absence (lanes 2, 6, and 10) or in the presence of calf intestinal alkaline phosphatase (CIP), without (lanes 3, 7, and 11) or with sodium orthovanadate (lanes 4, 8, and 12) and then processed for immunoblotting with RPRX α (A). Control immunoprecipitates are shown in lanes 1, 5, and 9. *E*, COS-1 cells were transfected with mRAR α 1 expression vector (5 μ g) either alone (lanes 1 and 5) or in the presence of JNK2 (1 μ g; lanes 2 and 3) or JNK1 (1 μ g; lane 4) vectors and UV-irradiated where indicated. WCEs were immunoprecipitated with mAb 9 α 9A6 and processed as in Fig. 2A for autoradiography and immunoblotting with RP α (F).

Purified recombinant RAR α 1WT, RAR α Δ AB, RAR γ 1WT, RXR α 1WT, and RXR α Δ AB overexpressed in *Escherichia coli* were gifts from H. Gronemeyer.

Antibodies—Mouse monoclonal antibodies against the DE regions (monoclonal antibody (mAb) 4RX3A2) of RXR α and rabbit polyclonal antibodies against the A (RPRX α (A)) and D (RPRX α (D)) regions of RXR α 1 have been described by Rochette-Egly *et al.* (41). Mouse monoclonal and rabbit polyclonal antibodies against the F region of RAR α , mAb 9 α (F) and RP α (F), respectively, and mouse monoclonal antibodies against the A1 region of RAR γ 1 (mAb 1 γ (A1)) were as described (23, 25). Purified mouse anti-human JNK1 monoclonal antibodies were purchased from Pharmingen (San Diego, CA), and anti-JNK1 polyclonal antibodies as agarose conjugates were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Rabbit anti-ACTIVETM JNK polyclonal antibodies were from Promega.

Cells, Transfections, and Chloramphenicol Acetyltransferase (CAT) Assays—COS-1 cells maintained in Eagle's modified Dulbecco's medium supplemented with 5% fetal calf serum were transiently transfected using the calcium phosphate precipitation technique (25). In addition to the expression vectors or reporters described in each figure legend, all transfections contained the β -galactosidase expression vector pCH110 (1 μ g) to correct for variations in transfection efficiency and Bluescript DNA as a carrier. After a 20-h incubation with calcium phosphate-precipitated DNA, the cells were washed, maintained for 8 h in the appropriate medium, and incubated for another 20 h in medium containing 0.5% charcoal-treated serum along with the ligand (10⁻⁷ M 9cRA). Where mentioned, cells were UV-irradiated (40 J/m²) 4 h before harvesting (42). CAT assays were performed using the enzyme-linked immunosorbent assay method (CAT ELISA, Roche Molecular Biochemicals). Results were expressed as pg of CAT/unit of β -galactosidase.

The F9–1.8 reporter cell line stably transfected with the mRAR β 2 promoter coupled to the *lacZ* gene was maintained in Dulbecco's modified Eagle's medium containing 7.5% fetal calf serum as described by Maden *et al.* (43).

Immunoprecipitations and CIP Treatment of the Immunoprecipitates—Whole cell extracts were prepared from transfected COS as de-

scribed (26) and incubated with Protein A-Sepharose beads cross-linked with the indicated monoclonal antibodies in IP buffer (50 mM Tris-HCl, pH 7.5, 10% glycerol, 0.1 mM EDTA, 150 mM KCl, 5 mM MgCl₂, and 0.1% Nonidet P-40) for 2 h at 4 °C. The beads were washed in IP buffer; resuspended in 100 μ l of phosphatase reaction buffer (100 mM Tris-HCl, pH 9.8, 1 mM MgCl₂, 0.1 mM ZnCl₂) containing 20 units of calf intestinal alkaline phosphatase (Roche Molecular Biochemicals) in the absence or presence of sodium orthovanadate (50 μ M) and incubated at 37 °C for 3 h. After washing, the immunoprecipitated proteins were resolved by SDS-10% PAGE, electrotransferred onto nitrocellulose membranes, and detected by immunoblotting and chemiluminescence according to the manufacturer's protocol (Amersham Pharmacia Biotech).

Cytosolic Extracts for Detection and Isolation of Activated JNKs—Cells were washed and lysed as described by Sadowski and Gilman (44) in ice-cold hypotonic buffer (20 mM HEPES, pH 7.9, 20 mM NaF, 1 mM Na₂VO₄, 0.125 μ M okadaic acid, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and a mixture of protease inhibitors) containing 0.2% Nonidet P-40. After centrifugation at 16,000 \times g for 20 s, supernatants were supplemented with NaCl to 120 mM and clarified (16,000 \times g for 20 min), and glycerol was added to 10%.

In Vitro and in Vivo Phosphorylation—*In vitro* phosphorylation reactions were performed as described by Rochette-Egly *et al.* (27) with purified bacterially expressed RXR α 1 (1 μ g), using either p44 mitogen-activated protein kinase (20 ng) or p34 Cdk1/cyclin B (20 ng) (Upstate Biotechnology, Inc., Lake Placid, NY). In the case of JNKs, activated JNKs were first isolated from cytosols of UV-irradiated COS cells by immunoprecipitation with JNK1 antibodies conjugated to agarose beads (Santa Cruz Biotechnology), and the reaction was initiated by the addition of purified RXR α 1 to the beads. Phosphorylated proteins were resolved by SDS-PAGE, electrotransferred onto nitrocellulose membranes, and visualized by autoradiography and immunoblotting.

For in vivo phosphorylation, COS-1 cells were transfected with wild type or mutated mRXR α 1 expression vectors (5 μ g) and labeled with [³²P]orthophosphate as described (26, 27). Where mentioned, cells were UV-irradiated (40 J/m²) 1 h before harvesting. Whole cell extracts were

prepared, immunoprecipitated, and resolved by SDS-PAGE, and after electrotransfer, the phosphorylated proteins were revealed by autoradiography and immunoprobings (26, 27).

Two-dimensional phosphoamino acid analysis and tryptic phosphopeptide mapping were carried out on thin layer cellulose plates using the Hunter thin-layer electrophoresis system as described (27, 45).

GST Pull-down Assays—Purified glutathione *S*-transferase-stress-activated protein kinase α (GST-SAPK α) and GST-SAPK β fusion proteins (2.5 μ g) (Upstate Biotechnology) were bound to glutathione-agarose beads (Amersham Pharmacia Biotech) and incubated with 500 ng of either RAR γ 1WT, RAR α 1WT, RAR α Δ AB, or RXR α 1WT proteins for 4 h at 4 $^{\circ}$ C in a 500- μ l final volume of binding buffer (50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM MgCl $_2$, 0.3 mM dithiothreitol, 5–10% glycerol, 0.1% Nonidet P-40). After four washes with the same buffer, the beads were resuspended in 30 μ l of Laemmli buffer, and after boiling, the proteins were resolved by SDS-PAGE and analyzed by immunoblotting.

RESULTS

RXR α Overexpressed in COS-1 Cells Is Phosphorylated in Its NH $_2$ -terminal A/B Region—To determine whether the nuclear RXR α is a phosphoprotein, COS-1 cells were transfected with RXR α WT (33) expression vector and labeled with [32 P]orthophosphate. RXR α was phosphorylated irrespective of the addition of 9cRA (10 $^{-7}$ M) to the culture medium (Fig. 2A, lanes 1 and 2). Phosphoamino acid analysis indicated that serine residues were phosphorylated (Fig. 2B). Tryptic phosphopeptide mapping of RXR α WT yielded two main phosphopeptides named a and a' and an array of additional peptides, named x, lying on two parallel diagonals (Fig. 2C, panel 1). It must be stressed that, depending on the experiments, these x phosphopeptides were variably distinct, suggesting that they may be partial digestion products or phosphoisomers (45). Identical phosphopeptide patterns were obtained whether or not COS-1 cells were treated with 9cRA (data not shown).

To characterize the phosphorylated regions, COS-1 cells were transfected with RXR α Δ AB and subsequently labeled with [32 P]orthophosphate. The level of phosphorylation was not significantly affected in RXR α Δ AB (Fig. 2A, compare lanes 3 and 4). However, the phosphopeptides observed in RXR α WT were lacking in RXR α Δ AB (Fig. 2C, panel 2), and two additional phosphopeptides (b and b'), which were not detectable in RXR α WT, appeared. These results indicate that RXR α was phosphorylated mainly in the A/B region and also suggest that the A/B region may prevent the phosphorylation of sites located elsewhere in the protein.

There are nine potential phosphorylation sites for proline-directed kinases in the RXR α 1 A/B region (see Fig. 1). The serine and threonine residues of these putative sites (serines 22, 44, 48, 54, 61, 75, 96, and 101 and threonine 87) were individually mutated to alanine, and the corresponding mutants were expressed in COS-1 cells. Their level of phosphorylation and phosphopeptide maps were not significantly different from those of RXR α WT except for RXR α S22A, which lacked phosphopeptides a and a' (Fig. 2C, panel 3, and data not shown). The observation that a single mutation (S22A) abrogated two phosphorylated spots (a and a') lying on a diagonal suggests that they may correspond to interdependent phosphorylation of adjacent serines (at positions 17–19), with the slowest migrating peptide toward the anode (peptide a) containing only a single phosphate (45). Note that a third spot situated on the same diagonal was sometimes observed (see Fig. 3B, panel 1). The nature of the phosphoresidues present in peptides x, which could be possibly located outside of the A/B region, remains to be identified.

In Vitro Phosphorylation of RXR α by Cyclin-dependent Kinases (CDKs) and Mitogen-activated Protein Kinases (MAPKs)—Serine 22 belongs to a conserved motif for proline-

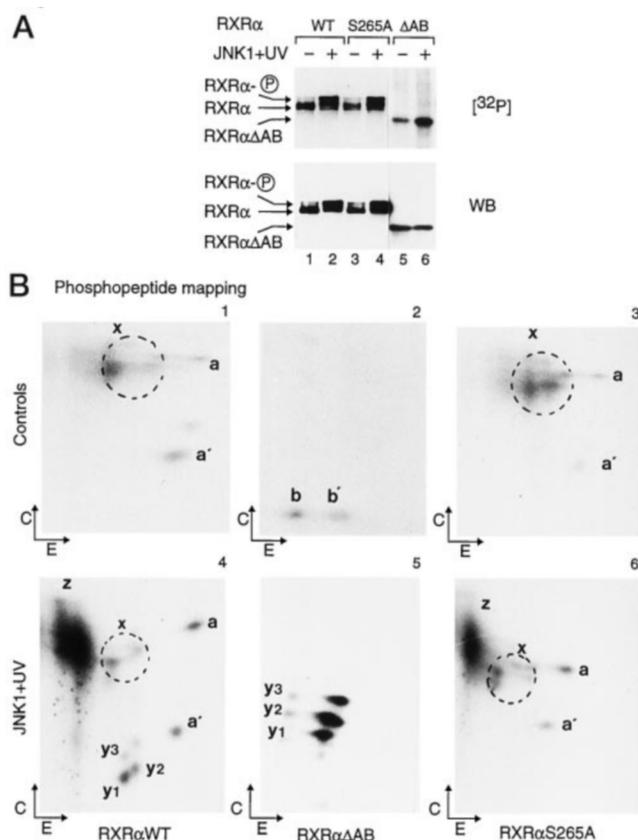


FIG. 5. Serine 265 located in the E region of RXR α is hyperphosphorylated upon overexpression and activation of JNKs. A, COS-1 cells were transfected with RXR α WT (lanes 1 and 2), RXR α S265A (lanes 3 and 4), or RXR α Δ AB (lanes 5 and 6) expression vectors (5 μ g) in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of JNK1 (1 μ g) vector. Cells were labeled with [32 P]orthophosphate, and JNK1-cotransfected cells were UV-irradiated 1 h before harvesting. WCEs were immunoprecipitated and processed as in Fig. 2A for autoradiography and immunoblotting with RPRX α (D). B, two-dimensional tryptic phosphopeptide map of [32 P]-labeled immunoprecipitated RXR α WT, Δ AB, and S265A, with or without cotransfected JNK1 and UV irradiation, as indicated.

directed Ser/Thr kinases, such as the CDKs (46) and the MAPK family. The latter includes extracellular signal-regulated kinases (ERKs), as well as SAPKs, such as the JNKs (29–32).

Purified bacterially expressed RXR α 1WT was used as a substrate for these kinases in an *in vitro* phosphorylation assay. RXR α was strongly phosphorylated *in vitro* by p34 Cdk1/cyclin B, p44 MAPK (also named ERK1 (29)), or activated JNKs (Fig. 3A, lanes 1, 3, and 5). Interestingly, phosphorylation by p34 Cdk1/cyclin B generated tryptic phosphopeptides identical to those of RXR α phosphorylated in transfected COS-1 cells (Fig. 3B, panel 1), whereas RXR α phosphorylated with p44 MAPK or JNKs yielded a distinct tryptic phosphopeptide map; all of the phosphopeptides generated from RXR α phosphorylated in transfected COS cells were missing, while novel peptides (y1, y2, and z) were present (Fig. 3B, panel 2, and data not shown).

RXR α deleted for the A/B region was not phosphorylated by p34 Cdk1/cyclin B (Fig. 3A, lane 2). However, RXR α Δ AB was phosphorylated by p44 MAPK and JNKs (Fig. 3A, lanes 4 and 6) and yielded only phosphopeptides y1 and y2 (Fig. 3B, panel 3, and data not shown).

Thus, the phosphorylation pattern of RXR α 1 overexpressed in COS cells is similar to that observed *in vitro* with p34 Cdk1/cyclin B, while ERKs and JNKs appear to phosphorylate different residues located both in the A/B region and the remaining protein.

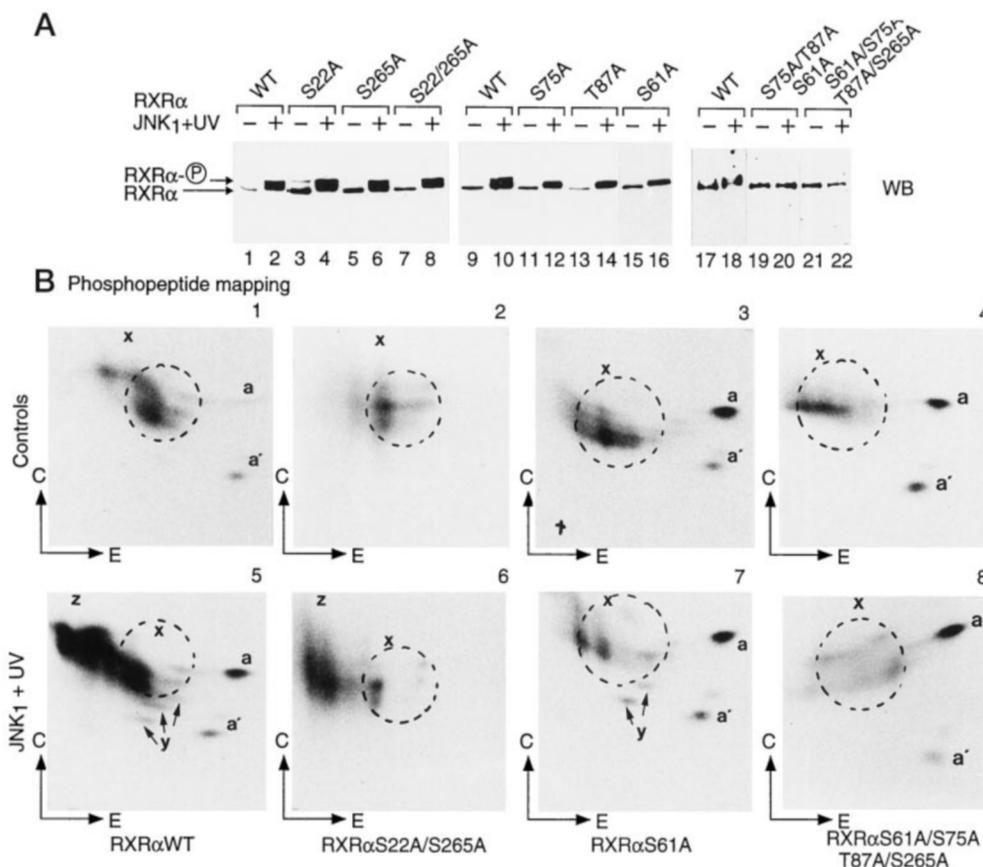


FIG. 6. Residues located in the B region are involved in the upward shift of mRXR α 1 electrophoretic mobility. A, COS-1 cells were transfected with wild type (WT) (lanes 1, 2, 9, 10, 17, and 18), S22A (lanes 3 and 4), S265A (lanes 5 and 6), S22A/S265A (lanes 7 and 8), S75A (lanes 11 and 12), T87A (lanes 13 and 14), S61A (lanes 15 and 16), S61A/S75A/T87A (lanes 19 and 20), or S61A/S75A/T87A/S265A (lanes 21 and 22) RXR α expression vectors in the absence (lanes 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, and 21) or presence (lanes 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, and 22) of JNK1 vector. Where indicated, cells were UV-irradiated 1 h before harvesting. WCEs were immunoprecipitated with mAb 4RX3A2 and processed for immunoblotting with RPRX α (A). B, two-dimensional tryptic phosphopeptide map of 32 P-labeled immunoprecipitated RXR α WT, RXR α S22A/S265A, RXR α S61A, and RXR α S61A/S75A/T87A/S265A, with or without cotransfected JNK1 and UV irradiation, as indicated.

Overexpression of Activated JNKs Increases the Phosphorylation of RXR α in COS-1 Cells, whereas ERKs and CDKs Are Inefficient—The above *in vitro* results suggest that RXR α could be a target for CDKs. However, overexpression of a dominant active Cdk1 (40) or of Cdk7, which was previously shown to increase the phosphorylation of RAR α (26), had no effect on the level of RXR α phosphorylation and on its phosphopeptide maps (Fig. 4A, compare lanes 4 and 5, and data not shown). Whether CDKs other than those tested here could be implicated in the basal phosphorylation of RXR α in COS-1 cells remains to be investigated.

Since RXR α was a substrate for p44 MAPK *in vitro*, we also examined whether stimulation of the MAPK pathways could affect the phosphorylation of RXR α in transfected COS-1 cells. The ERK pathway is stimulated in response to growth factors through Ras activation, while JNKs are activated by stress stimuli or UV irradiation. Activation of ERKs and JNKs involves their own phosphorylation by other kinases located further upstream in the specific signaling cascade (for reviews, see Refs. 29–32, 47, 48, and references therein). The phosphopeptide map of RXR α was not affected by epidermal growth factor treatment or by overexpression of either an activated Ras (Ras^{Val-12} (35)) or a dominant negative Ras (Ras^{Asn-17} (36, 37)) (Fig. 4A, lanes 1–3, and data not shown). Similar results were obtained by overexpressing MAPK kinase or the MAPK kinase-specific phosphatase, CL100 (49) (data not shown). Thus, the Ras-ERK cascade of the growth factor receptor tyrosine kinase signaling pathway does not appear to be involved in RXR α phosphorylation in COS cells.

In contrast, overexpression and UV activation (40J/m²) of the stress-activated protein kinase JNK1 increased the level of RXR α phosphorylation concomitantly with a marked decrease in its electrophoretic mobility that is characteristic of a hyperphosphorylation (Fig. 4A, compare lanes 6 and 9). This upward shift of RXR α , which was visible both by immunoblotting and by incorporation of 32 P (Fig. 4A, compare lanes 6 and 9, upper and lower panels), could be detected within 5 min after UV irradiation and persisted for at least 2 h (Fig. 4B, lanes 6–11). Overexpression of JNK2, another c-Jun NH₂-terminal kinase, had the same effect (Fig. 4D, lane 9). Overexpression of JNK1 without UV irradiation did not cause this mobility shift (Fig. 4A, lane 8). In contrast, UV irradiation in the absence of cotransfected JNK expression vector induced within 1 h a slight but significant reduction of the electrophoretic mobility of RXR α (Fig. 4A, lane 7, and Fig. 4B, lanes 1–5). Treatment of cell extracts with calf intestinal alkaline phosphatase in the absence of vanadate (a phosphatase inhibitor) abrogated the upward shift induced by either JNK1 or JNK2 overexpression and UV treatment (Fig. 4D, compare lanes 7 and 11 with lanes 5 and 9, respectively). Note that, as expected, both overexpressed JNK1 and JNK2 (46 and 54 kDa, respectively) were activated by UV irradiation, as determined by Western blot analysis with anti-ACTIVETM JNK antibodies that recognize the phosphorylated form of JNKs (Fig. 4C, upper panel), while the JNK protein content was not affected (Fig. 4C, bottom, and data not shown). Furthermore, serum starvation (4 h) that is also known to activate JNKs (50) similarly induced an upward shift in RXR α electrophoretic mobility (data not shown). In contrast,

no upward shift was seen upon retinoic acid treatment (either tRA or 9cRA at 10^{-7} M) for up to 24 h (data not shown).

Altogether, these results demonstrate that RXR α WT is inducibly hyperphosphorylated by activated JNKs, whereas under the same conditions, there is no hyperphosphorylation of RAR α (Fig. 4E), and its phosphorylation pattern is not affected (data not shown).

Activated JNKs Phosphorylate Serine Residues Located in both the B and E Regions of RXR α 1—Two-dimensional tryptic phosphopeptide mapping was used to determine which RXR α residues were phosphorylated in transfected COS cells upon activation of JNKs. Several novel RXR α phosphopeptides (y1, y2, and z) were generated (Fig. 5B, panel 4) in addition to those obtained from control COS cells (Fig. 5B, panel 1); they were similar to those derived from RXR α phosphorylated *in vitro* with ERKs and JNKs (see Fig. 3B, panel 2). Note that a third y spot (y3) was often observed and that similar phosphopeptide maps were obtained whether JNKs were activated by UV irradiation or serum deprivation (4 h, data not shown). Interestingly, RXR α Δ AB did not yield phosphopeptide z while phosphopeptides y1–y3 were still present (Fig. 5B, panel 5), thus suggesting that peptide z was generated from the A/B region, whereas peptides y1–y3 originated from elsewhere in the protein.

Since there is a consensus phosphorylation site for proline-directed kinases in the NH $_2$ -terminal end of the RXR α E region at position 265 (Fig. 1), we mutated the serine residue at this site into alanine (RXR α S265A). This mutation resulted in the loss of phosphopeptides y1–y3, indicating that serine 265 is a target for activated JNKs (Fig. 5B, panel 6) and that phosphopeptides y1–y3 may correspond to partial digestion products (containing serine 265) and/or to phosphoisomers (45) resulting from the interdependent phosphorylation of the adjacent serine at position 264. The S265A mutation did not suppress the upward shift of RXR α upon hyperphosphorylation induced by activated JNKs (Fig. 5A, lanes 3 and 4), thus indicating that phosphorylation of this residue is not sufficient for that process.

The next set of experiments was aimed at identifying the phosphoresidues contained in spot z. Note that the presence of spot z was associated with the upward shift of RXR α , since RXR α Δ AB, which did not yield spot z (Fig. 5B, panel 5), was not upward shifted (Fig. 5A, lanes 5 and 6). The mutation of six sites (serines 22, 44, 48, 54, 96, and 101) among the nine potential phosphorylation sites present in the A/B region, either individually or in association with mutation of serine 265, had no apparent effect on the upward shift of RXR α (Fig. 6A, lanes 1–8, and data not shown) and did not affect the presence of phosphopeptide z (Fig. 6B, compare panels 2 and 6, and data not shown). In fact, the upward shift was decreased when serine 61, serine 75, or threonine 87 was individually mutated to alanine (Fig. 6A, lanes 9–16), whereas it was abrogated upon simultaneous mutation of the three residues (Fig. 6A, lanes 19 and 20), irrespective of mutation of serine 265 (Fig. 6A, lanes 21 and 22). Thus, our results suggest that serine 61, serine 75, and threonine 87 are involved in the electrophoretic upward shift of RXR α induced by activated JNKs.

This latter possibility was corroborated by [32 P]orthophosphate labeling and subsequent tryptic phosphopeptide mapping. Indeed, phosphopeptide z was lacking in RXR α S61A and decreased in RXR α S75A and RXR α T87A upon overexpression and activation of JNKs (Fig. 6B, compare panels 3 and 7, and data not shown). As expected, peptides z and y1–y3 were all lacking from the tryptic digest of the quadruple mutant (RXR α S61A/S75A/T87A/S265A) (Fig. 6B, panel 8).

Altogether, our results demonstrate that serine 265 is phos-

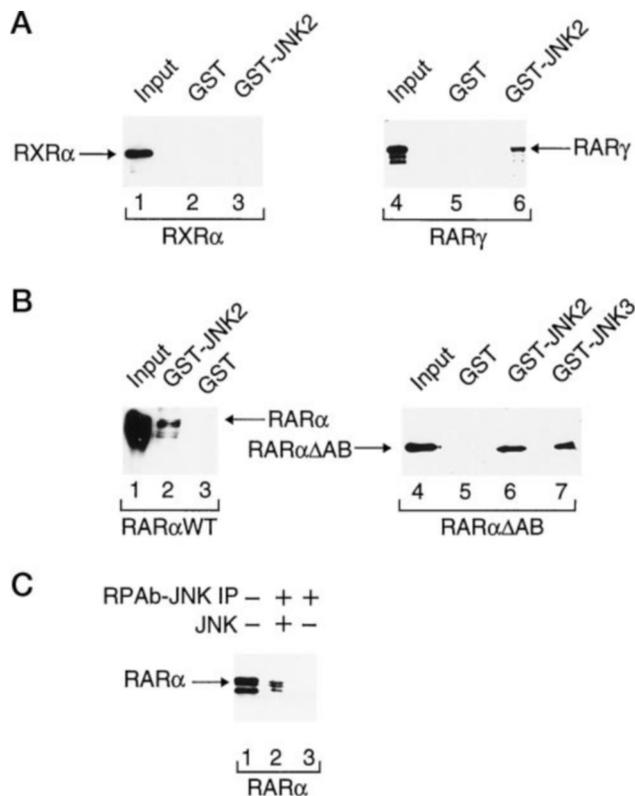


FIG. 7. RAR α and RAR γ , but not RXR α , interact with JNKs. *A*, purified bacterially expressed (0.5 μ g) RXR α WT (lanes 2 and 3) and RAR γ 1WT (lanes 5 and 6) were incubated with control GST (lanes 2 and 5) or GST-JNK2 (lanes 3 and 6) fusion proteins bound to glutathione-Sepharose beads. Bound RXR α and RAR γ were detected by immunoblotting with RPRX α (A) (lanes 1–3) and mAb 1 γ (A1) (lanes 4–6), respectively. Lanes 1 and 4 correspond to 50% of the input RXR α WT and RAR γ proteins. *B*, purified bacterially expressed (0.5 μ g) RAR α 1WT (lanes 2 and 3) or Δ AB (lanes 5–7) were incubated with control GST (lanes 3 and 5), GST-JNK2 (lanes 2 and 6), or GST-JNK3 (lane 7) fusion proteins bound to glutathione beads. Bound RAR α was detected by immunoblotting with RP α (F). Lanes 1 and 4 correspond to 30 and 20%, respectively, of the input RAR α WT and Δ AB proteins. *C*, purified bacterially expressed RAR α 1WT (0.5 μ g) was incubated with recombinant JNK1 (0.4 μ g) (Calbiochem) immunoadsorbed on agarose beads cross-linked with JNK1 antibodies. After SDS-PAGE, bound RAR α was detected by immunoblotting with RP α (F).

phorylated by activated JNKs and is contained in spots y. In addition, serine 61 in association with two other residues located in the B region (serine 75 and threonine 87) is involved in the appearance of phosphopeptide z and the upward shift of RXR α induced by activated JNKs.

JNKs Do Not Bind RXR α but Bind RAR α and RAR γ *In Vitro*—Binding assays between RXR α and JNKs were performed *in vitro* with purified recombinant proteins to investigate whether RXR α and JNKs could stably interact with each other. Purified bacterially expressed RXR α was mixed with GST-JNK2 (also named GST-SAPK α (48)) fusion protein attached to glutathione-Sepharose beads, and bound protein was revealed by immunoblotting. No significant binding was detected between RXR α and JNK2, either in the absence or presence of 9cRA (1 μ M) (Fig. 7A, lane 3, and data not shown). In contrast, the GST-JNK2 beads retained purified bacterially expressed RAR γ and RAR α (Fig. 7A, lane 6; Fig. 7B, lane 2; and data not shown) in a ligand-independent manner. Neither RAR γ 1 nor RAR α 1 was detected on control GST beads (Fig. 7A, lane 5, and Fig. 7B, lane 3). RAR α 1 also interacted with the GST-JNK3 fusion protein (JNK3 is also known as SAPK β (48)) (data not shown), and purified bacterially expressed RAR α Δ AB also interacted with either GST-JNK2 or GST-JNK3 fusion

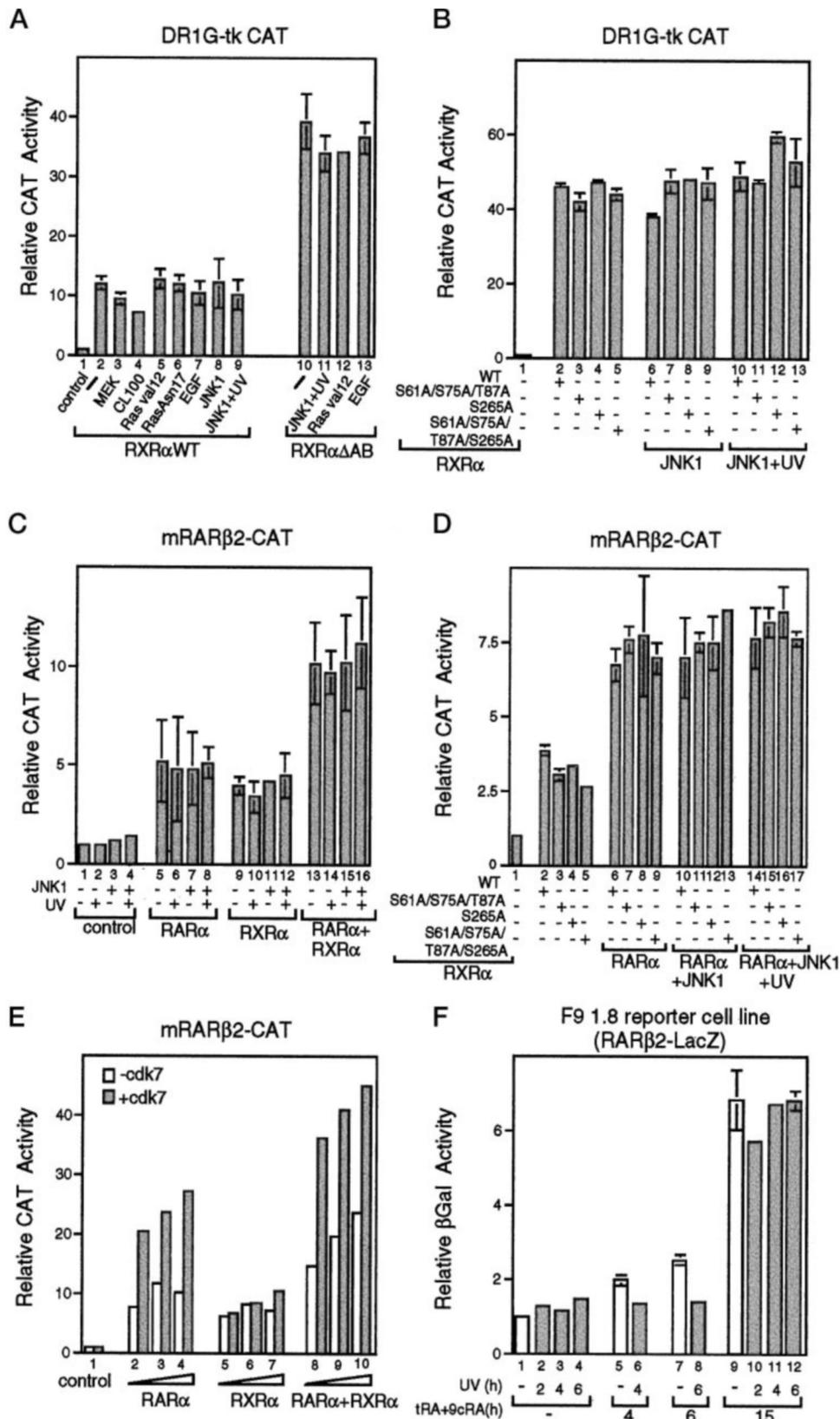


FIG. 8. Transactivation by RXR α homodimers and by RXR α /RAR α heterodimers is not modulated by activated JNK1. A, COS-1 cells were cotransfected with the DR1G-tk-CAT (1 μ g) reporter gene without (lane 1) or with RXR α WT (lanes 2–9) or RXR α Δ AB (lanes 10–13) expression vector (0.5 μ g) and treated with 9cRA (10^{-7} M). Cells were also cotransfected with MAPK kinase (0.4 μ g, lane 3), CL100 (0.02 μ g, lane 4), Ras^{Val12} (0.5 μ g, lanes 5 and 12), Ras^{Asn17} (0.5 μ g, lane 6) or JNK1 (0.5 μ g, lanes 8, 9, and 11) vectors. Where indicated, cells were treated with epidermal growth factor (lanes 7 and 13) or UV-irradiated (lanes 9 and 11) 4 h before harvesting. The results are expressed as relative CAT activity, taking the increase in expression of the reporter gene in the presence of ligand but in the absence of receptor expression vector as 1. B, COS-1 cells were cotransfected with the DR1G-tk-CAT reporter gene without (lane 1) or with RXR α WT, S265A, S61A/S75A/T87A, or S61A/S75A/T87A/S265A expression vectors (0.5 μ g) as indicated and were treated with 9cRA (10^{-7} M). When mentioned, cells were cotransfected with JNK1 expression vector and UV-irradiated as in A. C, COS-1 cells were cotransfected with the mRAR β 2-CAT reporter gene (5 μ g) without (lanes 1–4) or with RAR α 1 or RXR α expression vectors (0.1 μ g), either individually (lanes 5–8 and 9–12, respectively) or in combination (lanes 13–16). Cells were treated with

proteins, irrespective of the presence of tRA (Fig. 7B, lanes 6 and 7, and data not shown). In addition, purified RAR α 1 was retained by JNK1 immobilized onto agarose beads cross-linked with JNK1 polyclonal antibodies (Fig. 7C, lane 2), thus corroborating the above results.

Phosphorylation by Activated JNKs Does Not Affect Transactivation by RXR α —The ability of activated JNKs to influence RXR α -mediated activation of transcription was analyzed. COS-1 cells were cotransfected with a reporter construct containing the CAT gene under the control of a retinoic acid-inducible promoter, the natural mRAR β 2 promoter that is preferentially activated by RXR α /RAR α heterodimers or the synthetic DR1G-tk promoter that is preferentially activated by RXR homodimers (16).

In the presence of 9cRA (10^{-7} M), DR1G-tk CAT expression was stimulated by RXR α 1WT (Fig. 8A, lane 2, and Fig. 8B, lane 2). Deletion of the A/B region increased transcriptional activation by RXR α (Fig. 8A, lane 10) as described previously (16). However, the alanine mutation of serine 22, which is constitutively phosphorylated in COS cells, did not affect the transcriptional activity of RXR α (data not shown). Mutation of the residues hyperphosphorylated by JNKs (serine 61, serine 75, threonine 87, and serine 265), either individually or in combination, had no effect either (Fig. 8B, lanes 2–5, and data not shown).

As expected, the transcriptional activity of RXR α was not modified by coexpressing in COS cells either an activated Ras (Ras^{Val-12}), a dominant negative Ras (Ras^{Asn-17}), MAPK kinase, or the MAPK phosphatase CL100 (Fig. 8A, lanes 3–6 and 12). Treatment of COS cells with epidermal growth factor had no effect either (Fig. 8A, lanes 7 and 13). Overexpressed JNK1 or JNK2 was also without effect (Fig. 8A, lane 8; Fig. 8B, lanes 6–9; and data not shown) even after UV irradiation (Fig. 8A, lanes 9 and 11, and Fig. 8B, lanes 10–13).

Similar transfection experiments were then performed using the mRAR β 2-CAT reporter gene. RAR α and RXR α activated transcription in the presence of their respective ligand (Fig. 8C, lanes 5 and 9), and a further increase was observed upon cotransfection of RAR α and RXR α and the addition of both tRA and 9cRA (Fig. 8C, lane 13) (18). As described above with the DR1G-tk-CAT reporter gene, the S61A, S75A, T87A, and S265A mutations, individually or in association, did not affect significantly the transactivation properties of RXR α , whether it was overexpressed alone (Fig. 8D, lanes 2–5, and data not shown) or in association with RAR α (Fig. 8D, lanes 6–9). Again, JNK1 or JNK2 overexpression and activation by UV irradiation did not affect the transcriptional properties of either RXR α (Fig. 8C, lanes 9–12) or RXR α /RAR α heterodimers (Fig. 8C, lanes 13–16, and Fig. 8D, lanes 10–17) in the absence (data not shown) or in the presence of ligand (10^{-7} M tRA and 9cRA). UV irradiation was also without effect on its own (Fig. 8C, lanes 2, 6, 10, and 14). Note that although unaffected by overexpressed and activated JNKs (Fig. 8C, lanes 5–8 and 13–16), stimulation of transcription by RAR α /RXR α heterodimers was enhanced (2–3-fold) by overexpressed Cdk7 as previously reported (26)

(Fig. 8E, lanes 2–4 and 8–10). Similar results were observed with another CAT reporter gene under the control of the mCRABP2 promoter (16) (data not shown).

The effect of activated JNKs on transactivation by RXRs and RARs was also studied in F9 cells stably transfected with a lacZ reporter gene under the control of the murine RAR β 2 promoter (43). As observed with transiently transfected COS cells, activation of endogenous JNKs by UV irradiation and subsequent hyperphosphorylation of endogenous RXR α ,³ had no effect either on the increase of β -galactosidase activity upon tRA and 9cRA treatment (Fig. 8F, compare lanes 9–12).

DISCUSSION

RXR α Overexpressed in COS-1 Cells Is “Constitutively” Phosphorylated—We have shown here that the α 1 isoform of mRXR α , like other nuclear receptors, is a phosphoprotein when overexpressed in COS-1 cells. RXR α is phosphorylated in the absence of 9cRA, and no changes occur upon ligand binding. As other nuclear hormone receptors (22, 26), RXR α is phosphorylated at several residues located in the A/B region that contains the AF-1 transactivation domain. Interestingly, one of the phosphorylated residues has been identified as serine 22, which is unique to the A1 region of the RXR α 1 isoform (Fig. 1 and Ref. 8), while the location of the others remains to be identified. This is in contrast to the case of RAR α 1, for which no phosphorylation in the isoform-specific A1 region has been found (26).

Serine 22, which is followed by a proline residue, is in a favorable context for phosphorylation by proline-directed kinases that includes CDKs and MAPKs. Although RXR α could be phosphorylated *in vitro* by either of these protein kinases, only CDKs yielded a pattern of phosphorylated peptides identical to that obtained in transfected COS-1 cells. However, in COS cells, coexpression of Cdk1 or Cdk7 did not affect RXR α phosphorylation, while the latter increased the phosphorylation of RAR α (26). Whether other cyclin-dependent kinases or another proline-dependent kinase could be involved in this constitutive phosphorylation of RXR α 1 needs further investigation. Furthermore, we found no evidence supporting an *in vivo* involvement of the tyrosine kinase receptor/Ras/Raf/ERK cascade in the phosphorylation of RXR α 1, although it was also an *in vitro* target for ERKs that belong to the MAPK family.

RXR α Is Hyperphosphorylated by “Activated” SAPKs—In contrast to ERKs that could not phosphorylate RXR α 1 *in vivo*, we demonstrated here that other kinases belonging to the MAPK family, the c-Jun NH₂-terminal kinases, also referred to as SAPKs, are able to hyperphosphorylate RXR α . There are three main JNK members, JNK1 (SAPK γ , 46 kDa), JNK2 (SAPK α , 55 kDa), and JNK3 (SAPK β , 48 kDa) (for reviews, see Refs. 30–32 and references therein). JNKs are efficiently and preferentially activated by environmental stresses (heat shock), inflammatory cytokines (TNF α and IL-1 β), DNA dam-

³ S. Adam-Stitah, L. Penna, P. Chambon, and C. Rochette-Egly, unpublished results.

10^{-7} M tRA (lanes 5–8), 10^{-7} M 9cRA (lanes 9–12), or both ligands (lanes 13–16). When mentioned, cells were cotransfected with JNK1 vector and UV-irradiated. D, COS-1 cells were cotransfected with the mRAR β 2-CAT reporter gene without (lane 1) or with RXR α WT, S265A, S61A/S75A/T87A, or S61A/S75A/T87A/S265A expression vectors (0.1 μ g) either alone (lanes 2–5) or in association with RAR α 1 (0.1 μ g, lanes 6–17) and treated with both tRA and 9cRA (10^{-7} M each). JNK1 vector was also cotransfected (lanes 10–17) without (lanes 10–13) or with (lanes 14–17) UV irradiation. E, COS-1 cells were cotransfected with the mRAR β 2-CAT reporter gene without (lane 1) or with increasing amounts (0.1, 0.2, and 0.5 μ g) of RAR α 1 (lanes 2–4), RXR α (lanes 5–7), or both RAR α 1 and RXR α (lanes 8–10) expression vectors. The Cdk7 vector was also cotransfected (0.5 μ g; dark bars). Cells were treated with 10^{-7} M tRA (lanes 2–4), 10^{-7} M 9cRA (lanes 5–7), or both ligands (lanes 8–10). F, F9–1.8 reporter cells were treated with both tRA and 9cRA (10^{-7} M each) for 4 h (lanes 5 and 6), 6 h (lanes 7 and 8), or 15 h (lanes 9–12) or left untreated (lanes 1–4). Where mentioned, cells were UV-irradiated 2 h (lanes 2 and 10), 4 h (lanes 3, 6, and 11), or 6 h (lanes 4, 8, and 12) before harvesting. Cells were scraped and lysed in 0.25 M Tris buffer (pH 7.5) by four freeze-thaw cycles, and β -galactosidase activity was determined as in Ref. 43. The results are expressed as -fold induction compared with the β -galactosidase activity in control cells. All of the presented results are the means of 2–4 independent experiments.

aging, and apoptotic agents (UV-, γ -radiation, cisplatin) through a sequential protein kinase pathway similar to that of the ERK members of the MAPK family (for reviews, see Refs. 30, 31, 47, and 48). Once activated, JNKs phosphorylate and activate different transcription factors, including c-Jun, ATF2, NFAT4, and the Ets domain of Elk1 and Sap1 (for reviews, see Refs. 29–32, 51, and 52), as well as p53 (53), ATF α (54, 55), and the glucocorticoid receptor (56). Efficient phosphorylation of JNK substrates, such as c-Jun and ATF2 requires a direct and bipartite interaction between the two proteins involving both an effective docking site and a favorable phosphoacceptor region (31, 38, 54, 55, 57, 58).

In the present study, we have shown that RXR α is hyperphosphorylated by either JNK1 or JNK2 upon activation by UV irradiation, resulting in an upward shift in its electrophoretic mobility. Whether JNK3 has a similar effect remains to be seen. In contrast to c-Jun, RXR α was unable to interact stably with JNKs, suggesting that a labile and transient interaction between JNKs and RXR α is sufficient for its phosphorylation. RXR α hyperphosphorylation involves residues that are distinct from those that are involved in constitutive phosphorylation; one of these residues (serine 265) is located at the NH₂-terminal end of the E region, while the three others (serines 61 and 75 and threonine 87) are located in the B region. It is interesting to note that serine 265 corresponds to a readily accessible phosphoacceptor site, since it is exposed outside the ligand-binding domain α -helical sandwich, within the Ω loop between α helices H2 and H3 (20). Among the three residues located in the B region, serine 61 was clearly phosphorylated by activated JNKs, since its mutation results in the disappearance of phosphopeptide z (Fig. 6B), whereas the mutation of serine 75 and threonine 87 only decreased the intensity of its ³²P labeling. Interestingly, these residues belong to a conserved element involved in JNK binding (TPTPT) that is present in ATF α and ATF2 proteins (55). Thus, serine 75 and threonine 87 might be instrumental in serine 61 hyperphosphorylation through their involvement in a labile RXR α -JNK interaction.

What Could Be the Function of Hyperphosphorylated RXR α ?—Phosphorylation is an essential prerequisite for the transcriptional activity of various transcription factors such as c-Jun, ATF2, and RARs (Refs. 26, 27, 31, 52, and references therein). However, hyperphosphorylation by activated JNKs did not increase the transcriptional activity of RXR α homodimers. We have shown here that RAR α and RAR γ , the heterodimeric partners of RXR α , are able to bind JNKs, although they are not efficient substrates for JNKs in COS cells, thus suggesting that the presence of RAR α or RAR γ may enhance the phosphorylation of RXR α by activated JNKs. However, cotransfection of RAR α along with RXR α did affect neither the phosphorylation of RXR α by activated JNKs³ nor the transcriptional activity of RXR α /RAR α heterodimers using a reporter gene with a promoter containing a DR5 RARE (the natural mRAR β 2 promoter). The same observations were made with endogenous RAR α , RXR α , and JNKs in an F9 reporter cell line containing the RAR β 2 promoter coupled to *lacZ*. Therefore, JNK-mediated RXR α hyperphosphorylation does not seem to be involved in the transcriptional synergy of RXR α and RARs (59–61). However, due to the promoter and cell context specificity of the transcriptional functions of RARs and RXRs, the possibility cannot be excluded that RXR α hyperphosphorylation is involved in the transactivation of other genes in other cell types.

What could then be the function of hyperphosphorylated RXR α ? One possibility might be that JNK-mediated phosphorylation stabilizes RXR α by protecting it from ubiquitination and subsequent proteolytic degradation as previously reported

for c-Jun and p53 (for a review, see Ref. 62). Alternatively, RXR α hyperphosphorylation may play a role in apoptosis. UV radiations, as well as other stress agents, in addition to being JNK activators, are known to be DNA-damaging agents and to induce apoptosis (for a review, see Ref. 31). Interestingly, RXR α has been shown to be essential for the induction of apoptosis in F9 embryocarcinoma cells in response to retinoids (60). Thus, our data suggest the existence of cross-talks between the stress-activated kinases and the RA signaling pathways, both leading to apoptosis. Studies are in progress to investigate whether RXR α hyperphosphorylation is actually involved in apoptosis.

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