The nuclear receptor mouse retinoid X receptor α (mRXRα) 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 RXRa isoform. Overexpression and UV activation of the stress-activated kinases, c-Jun NH2-terminal kinases 1 and 2 (JNK1 and JNK2), hyperphosphorylated RXRa, 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 RXRa 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 RXRa bound to its heterodimeric partners, retinoic acid receptors α and γ (RARα and RARγ). Hyperphosphorylation by JNKs did not affect the transactivation properties of either RXRa homodimers or RXRa/RARα 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, the vitamin D3 receptor, and the peroxisome proliferator-activated receptors (11–15). As members of the nuclear receptor family (22), RARs have been shown to be phosphoproteins (23–25). In mouse RXRa, a phosphorylated serine residue has been identified in region B; it is phosphorylated by Cdk7 associated with the general transcription factor TFIIH, and this phosphorylation has been shown to be crucial for AF-1 activity in transfected COS cells (26). In addition, mRXRa 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 RARγ. Most interestingly, phosphorylation of residues in the AF-1 and AF-2 domains of both RXRα and RXRγ 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 RXRa is also a phosphoprotein. As for RXRa, phosphorylation sites for proline-directed protein kinases are located in the A/B region of mouse RXRa1 and are constitutively phosphorylated in transfected COS-1 cells. In addition, we demonstrate that under stress conditions
Cellulose plates (27, 45). With trypsin. Phosphopeptides were resolved in two dimensions on WBD chemiluminescence (A). At serine 22.

In lane 2

sion vectors (5 m (lanes 1 and 2), 44 MAPK (lanes 3 and 4), or activated JNKs (lanes 5 and 6). After SDS-PAGE and electrotransfer, phosphopeptides were visualized by autoradiography (12P) and immunoblotting with RPRXαD (WB). The signal indicated by an asterisk corresponds to RXRα degraded in the A region. B, two-dimensional tryptic phosphopeptide map of RXRαWT phosphorylated by 32P Cdk1/cyclin B (panel 1) and 44 MAPK (panel 2) and of RXRαΔAB phosphorylated by 44 MAPK (panel 3).

such as UV irradiation, mouse RXRa is hyperphosphorylated by endogenous and/or overexpressed stress-activated protein kinases, such as the C-Jun NH2-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 RARα, hyperphosphorylation by JNKs does not appear to modulate the transcriptional properties of RXRa in cultured cells transfected with retinoic acid-responsive reporter genes.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructions**—The pSG5-based expression vectors for mRARα1 (16), mRXRa (53), and murine RXRa deleted for the A/B region (mRXRaΔAB) (16) were as described. For the construction of mRXRa1 mutants, mRXRa1 was first subcloned into the XhoI/EcoRI sites of pSG5-Cas (16) after polymerase chain reaction amplification of the A to E regions. The mRXRa1 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 XhoI/EcoRV fragment containing the appropriate mutation. The double mutant RXRaS75A/T87A was constructed according to the same protocol by introducing the T87A mutation into the RXRaS75A mutant. Similarly, the RXRaS61A/S75A/T87A expression vector was constructed by introducing the S61A mutation into the RXRaS75A/T87A double mutant. RXRaS265A was also constructed by double polymerase chain reaction amplification reaction, generating an EcoRV/BssHII fragment containing the mutation. The double mutant RXRaS22A/S265A was prepared by subcloning the EcoRI/EcoRV fragment containing the S22A mutation into the same sites of RXRaS265A. The same strategy was followed for constructing the RXRaS61A/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 mRARα2-CAT have been previously described (16). The expression vectors for dominant active Rα (RαVal-12) and dominant negative Rα (RαAm-17) were gifts from B. Wasylyk (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).
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 RPRXa(A). Note that lanes 6–9 (upper panel) correspond to a shorter exposure than lanes 1–5. B, COS-1 cells transfected with RXXaWT expression vector (5 µg) in the absence (lanes 1–5) or presence (lanes 6–11) of JNK1 (1 µg) vector, were UV-irradiated (40 J/m²) 5, 10, 30, 60, or 120 min before harvesting. WCEs (15 µg) were resolved by SDS-PAGE and immunoblotted with RPRXa(A). C, COS-1 cells were irradiated as in B, and cytosols (100 µg) 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 RXXa 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 RPRXa(A). Control immunoprecipitates are shown in lanes 1, 5, and 9. E, COS-1 cells were transfected with mRXRα expression vector (5 µg) either alone (lanes 1 and 5) or in the presence of JNK2 (1 µg; lanes 2 and 3) or JNK1 (1 µg; lane 4) vectors and UV-irradiated where indicated. WCEs were immunoprecipitated with mAb 9αA6 and processed as in Fig. 2A for autoradiography and immunoblotting with RPα(F).

Fig. 4. Overexpressed and UV-activated JNKs hyperphosphorylate mRXRα concomitantly with an upward shift in its electrophoretic mobility. A, COS-1 cells were transfected with RXXaWT expression vector (5 µg) either alone (lanes 1, 4, 6, and 7) or in the presence of Ras WT (1 µg, lane 2), Ras G12V (1 µg, lane 3), Cdk7 (0.5 µg, lane 5), or JNK1 expression vector. 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 RPRXa(A). Note that lanes 6–9 (upper panel) correspond to a shorter exposure than lanes 1–5. B, COS-1 cells transfected with RXXaWT expression vector (5 µg) in the absence (lanes 1–5) or presence (lanes 6–11) of JNK1 (1 µg) vector, were UV-irradiated (40 J/m²) 5, 10, 30, 60, or 120 min before harvesting. WCEs (15 µg) were resolved by SDS-PAGE and immunoblotted with RPRXa(A). C, COS-1 cells were irradiated as in B, and cytosols (100 µg) 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 RXXa 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 RPRXa(A). Control immunoprecipitates are shown in lanes 1, 5, and 9. E, COS-1 cells were transfected with mRXRα expression vector (5 µg) either alone (lanes 1 and 5) or in the presence of JNK2 (1 µg; lanes 2 and 3) or JNK1 (1 µg; lane 4) vectors and UV-irradiated where indicated. WCEs were immunoprecipitated with mAb 9αA6 and processed as in Fig. 2A for autoradiography and immunoblotting with RPα(F).

Overexpressed recombinant RARα1WT, RARαΔAB, RARγ1WT, RXXaWT, and RXXaΔAB overexpressed in Escherichia coli were gifts from H. Gronemeyer.

Antibodies—Mouse monoclonal antibodies against the DE regions (monoclonal antibody (mAb) 4RX3A2) of RXXa and rabbit polyclonal antibodies against the A (RPRXa(A)) and D (RPRXa(D)) regions of RXXa have been described by Rochette-Egly et al. (41). Mouse monoclonal and rabbit polyclonal antibodies against the F region of RARα, mAb 9αF and RPRF(F), respectively, and mouse monoclonal antibodies against the A1 region of RARγ1 (mAb LY1(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.

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 Na3VO4, 0.125 µM 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 × g for 20 s, supernatants were supplemented with NaCl to 120 mM and clarified (16,000 × 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 RXXa1 (1 µg), either using 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, phosphorylated proteins were resolved by SDS-10% PAGE, electrophoresed onto nitrocellulose membranes, and detected by immunoblotting and chemiluminescence according to the manufacturer’s protocol (Amersham Pharmacia Biotech).
prepared, immunoprecipitated, and resolved by SDS-PAGE, and after
electrotransfer, the phosphorylated proteins were revealed by autora-
diography and immunopробing (26, 27).

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

**RESULTS**

**RXRa Overexpressed in COS-1 Cells Is Phosphorylated in Its
NH₂-terminal A/B Region**—To determine whether the nuclear
RXRa is a phosphoprotein, COS-1 cells were transfected with
RXRaWT (33) expression vector and labeled with [32P]orthophos-
phate. RXRAs 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
RXRaWT 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 phosphoi-
lation and phosphopeptide maps were not significantly different
within 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 RXRa1 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 mu-
tants were expressed in COS-1 cells. Their level of phosphory-
ation and the phosphopeptide maps were not significantly different
from those of RXRaWT except for RXRaS22A, which lacked phosphopeptides a and a’ (Fig. 2C, panel 3, and data not
shown). The observation that a single mutation (S22A) abro-
gated two phosphorylated spots (a and a’) lying on a diagonal
suggests that they may correspond to interdependent phosphory-
lation of adjacent serines (at positions 17–19), with the slow-
est 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 phosphoserides present in peptide x, which
could be possibly located outside of the A/B region, re-
mains to be identified.

**In Vitro Phosphorylation of RXRas by Cyclin-dependent
Kinases (CDKs) and Mitogen-activated Protein Kinases
(MAPKs)**—Serine 22 belongs to a conserved motif for proline-
directed Ser/Thr kinases, such as the CDKs (46) and the MAPK
family. The latter includes extracellular signal-regulated ki-

![Fig. 5. Serine 265 located in the E region of RXRa is hyper-
phosphorylated upon overexpression and activation of JNKs. A. COS-1 cells were transfected with RXRaWT (lanes 1 and 2), RXRaS265A (lanes 3 and 4), or RXRaΔAB (lanes 5 and 6) expression vectors (5 µg) in the absence (lanes 1, 3, and 5) or presence (lanes 2, 4, and 6) of JNK1 (1 µg) vector. Cells were labeled with [32P]orthophos-
phate, 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 RPrXRalpha(D). B, two-
dimensional tryptic phosphopeptide map of [32P]labeled immunoprecipi-
tated RXRaWT, ΔAB, and S265A, with or without cotransfected JNK1
and UV irradiation, as indicated.

The purified bacterially expressed RXRa1WT was used as a sub-
strate for these kinases in an in vitro phosphorylation assay.
RXRa 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 phosphopeptide maps identical to
those of RXRa phosphorylated in transfected COS-1 cells (Fig.
3B, panel 1), whereas RXRa phosphorylated with p44 MAPK
or JNKs yielded a distinct tryptic phosphopeptide map; all of the
phosphopeptides generated from RXRa phosphorylated in
transfected COS cells were missing, while novel peptides (y1, y2, and z) were present (Fig. 3B, panel 2, and data not shown).

RXRa deleted for the A/B region was not phosphorylated by
p34 Cdk1/cyclin B (Fig. 3A, lane 2). However, RXRaΔAB 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 RXRa1 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 re-
maining protein.
Overexpression of Activated JNKs Increases the Phosphorylation of RXRa in COS-1 Cells, whereas ERKs and CDKs Are Inefficient—The above in vitro results suggest that RXRa 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 RARα (26), had no effect on the level of RXRa 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 RXRa in COS-1 cells remains to be investigated.

Since RXRa was a substrate for p44 MAPK in vitro, we also examined whether stimulation of the MAPK pathways could affect the phosphorylation of RXRa 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 RXRa was not affected by epidermal growth factor treatment or by overexpression of either an activated Ras (RasVal12 (35)) or a dominant negative Ras (RasAsn-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 RXRa phosphorylation in COS cells.

In contrast, overexpression and UV activation (40J/m²) of the stress-activated protein kinase JNK1 increased the level of RXRa phosphorylation concomitantly with a marked decrease in its electrophoretic mobility that is characteristic of a hyperphosphorylated form (Fig. 4A, compare lanes 6 and 9). This upward shift of RXRa, which was visible both by immunoblotting and by incorporation of ^32P (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 RXRa (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-ACTIVE™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 RXRa 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 RXRoWT is inducibly hyperphosphorylated by activated JNKs, whereas under the same conditions, there is no hyperphosphorylation ofRARα (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 RXRo1—Two-dimensional trypsin phosphopeptide mapping was used to determine which RXRo residues were phosphorylated in transfected COS cells upon activation of JNKs. Several novel RXRo 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 RXRo 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, RXRoΔAB 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 NH2-terminal end of the RXRo E region at position 265 (Fig. 1), we mutated the serine residue at this site into alanine (RXRoS265A). 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 RXRo 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 phospho-residues contained in spot z. Note that the presence of spot z was associated with the upward shift of RXRo, since RXRoΔAB, 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 RXRo (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 RXRo induced by activated JNKs.

This latter possibility was corroborated by [32P]orthophosphate labeling and subsequent trypsin phosphopeptide mapping. Indeed, phosphopeptide z was lacking in RXRoS61A and decreased in RXRoS75A and RXRoT87A upon overexpression and activation of JNKs (Fig. 6B, compare panels 3 and 7, and data not shown). As expected, peptides y and y1–y3 were all lacking from the trypsin digest of the quadruple mutant (RXRoS61A/S75A/T87A/S265A) (Fig. 6B, panel 8).

Altogether, our results demonstrate that serine 265 is phosphorylated 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 RXRo induced by activated JNKs.

JNKs Do Not Bind RXRo but Bind RARα and RARγ in Vitro—Binding assays between RXRo and JNKs were performed in vitro with purified recombinant proteins to investigate whether RXRo and JNKs could stably interact with each other. Purified bacterially expressed RXRo was mixed with GST-JNK2 (also named GST-SAPKα (48)) fusion protein attached to glutathione-Sepharose beads, and bound protein was revealed by immunoblotting. No significant binding was detected between RXRo and JNK2, either in the absence or presence of 9cRA (1 μM) (Fig. 7A, lane 3, and data not shown). In contrast, the GST-JNK2 beads retained purified bacterially expressed RARγ and RARα (Fig. 7A, lanes 6; Fig. 7B, lane 2; and data not shown) in a ligand-independent manner. Neither RARγ nor RARα was detected on control GST beads (Fig. 7A, lane 5, and Fig. 7B, lane 3). RARβ also interacted with the GST-JNK3 fusion protein (JNK3 is also known as SAPKβ (48); data not shown), and purified bacterially expressed RARαΔAB also interacted with either GST-JNK2 or GST-JNK3 fusion proteins (Fig. 7B, lanes 4–7).
FIG. 8. Transactivation by RXRa homodimers and by RXRa/RARa heterodimers is not modulated by activated JNK1. A, COS-1 cells were cotransfected with the DR1G-tk-CAT (1 μg) reporter gene without (lane 1) or with RXRaWT (lanes 2–9) or RXRaΔAB (lanes 10–13) expression vector (0.5 μg) and treated with 9cRA (10⁻⁷ M). Cells were also cotransfected with MAPK kinase (0.4 μg, lane 3), CL100 (0.02 μg, lane 4), Ras²⁶⁵-¹² (0.5 μg, lanes 5 and 12), Ras⁶¹² (0.5 μg, lane 6) or JNK1 (0.5 μg, 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 RXRaWT, S265A, S61A/S75A/T87A, or S61A/S75A/T87A/S265A expression vectors (0.5 μg) 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 mRARβ2-CAT reporter gene (5 μg) without (lanes 1–4) or with RARα or RXRa expression vectors (0.1 μg), 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 RARα1 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 RXRα—** The ability of activated JNKs to influence RXRα-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 mRARβ2 promoter that is preferentially activated by RXRα/RARα heterodimers or the synthetic DR1G-tk promoter that is preferentially activated by RXRα homodimers (16).

In the presence of 9cRA (10−7 M), DR1G-tk CAT expression was stimulated by RXRα1WT (Fig. 8A, lane 2, and Fig. 8B, lane 2). Deletion of the A/B region increased transcriptional activation by RXRα (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 RXRα (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 RXRα was not modified by coexpressing in COS cells either an activated Ras (RasVal−12), a dominant negative Ras (RasAsn−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 under UV irradiation (Fig. 8A, lanes 9 and 11, and Fig. 8B, lanes 10–13).

Similar transfection experiments were then performed using the mRARβ2/CAT reporter gene. RARα and RXRα activated transcription in the presence of their respective ligand (Fig. 8C, lanes 5 and 9), and a further increase was observed upon cotransfection of RARα and RXRα 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 transcriptional properties of RXRα, whether it was overexpressed alone (Fig. 8D, lanes 2–5, and data not shown) or in association with RARα (Fig. 8D, lanes 6–9). Again, JNK1 or JNK2 overexpression and activation by UV irradiation did not affect the transcriptional properties of either RARα (Fig. 8C, lanes 9–12) or RXRα/RARα 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 RARα/RXRα 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 mCRABPII promoter (16) (data not shown).

The effect of activated JNKs on transactivation by RXRαs and RARαs was also studied in F9 cells stably transduced with a lacZ reporter gene under the control of the murine RARβ2 promoter (43). As observed with transiently transfected COS cells, activation of endogenous JNKs by UV irradiation and subsequent hyperphosphorylation of endogenous RXRα,3 had no effect either on the increase of β-galactosidase activity upon tRA and 9cRA treatment (Fig. 8F, compare lanes 9–12).

**DISCUSSION**

**RXRα Overexpressed in COS-1 Cells Is “Constitutively” Phosphorylated—** We have shown here that the α1 isoform of mRXRα, like other nuclear receptors, is a phosphoprotein when overexpressed in COS-1 cells. RXRα is phosphorylated in the absence of 9cRAA, and no changes occur upon ligand binding. As other nuclear hormone receptors (22, 26), RXRα 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 α1 isoform of the RXRα isoform (Fig. 1 and Ref. 8), while the location of the others remains to be identified. This is in contrast to the case of RARα1, 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 RXRα 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 RXRα phosphorylation, while the latter increased the phosphorylation of RARαs (26). Whether other cyclin-dependent kinases or another proline-dependent kinase could be involved in this constitutive phosphorylation of RXRα1 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 RXRα1, although it was also an in vitro target for ERKs that belong to the MAPK family.

**RXRα Is Hyperphosphorylated by “Activated” SAPKs—** In contrast to ERKs that could not phosphorylate RXRα1 in vivo, we demonstrated here that other kinases belonging to the MAPK family, the c-Jun NH2-terminal kinase, and the MAPKs (for reviews, see Refs. 30–32 and references therein). SAPKs are efficiently and preferentially activated by environmental stresses (heat shock), inflammatory cytokines (TNFα and IL-1β), DNA dam-

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3 S. Adam-Stitah, L. Penna, P. Chambon, and C. Rochette-Egly, unpublished results.
aging, and apoptotic agents (UV-, γ-irradiation, 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). ATF4a (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 RXRα 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, RXRα was unable to interact stably with JNKs, suggesting that a labile and transient interaction between JNKs and RXRα is sufficient for its phosphorylation. RXRα 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 (TPTT) that is present in ATF4a and ATF2 proteins (55). Thus, serine 75 and threonine 87 might be instrumental in serine 61 hyperphosphorylation through their involvement in a labile RXRα-JNK interaction.

What Could Be the Function of Hyperphosphorylated RXRα?—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 RXRα homodimers. We have shown here that RARα and RARγ, the heterodimeric partners of RXRα, are able to bind JNKs, although they are not efficient substrates for JNKs in COS cells, thus suggesting that the presence of RARα or RARγ may enhance the phosphorylation of RXRα by activated JNKs. However, cotransfection of RARα along with RXRα did not affect either the phosphorylation of RXRα by activated JNKsα nor the transcriptional activity of RXRα/RARα heterodimers using a reporter gene with a promoter containing a DR5 RARE (the natural mRARβ2 promoter). The same observations were made with endogenous RARα, RXRα, and JNKs in an F9 reporter cell line containing the RARβ2 promoter coupled to lacZ. Therefore, JNK-mediated RXRα hyperphosphorylation does not seem to be involved in the transcriptional synergy of RXRα 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 RXRα hyperphosphorylation is involved in the transactivation of other genes in other cell types.

What could then be the function of hyperphosphorylated RXRα? One possibility might be that JNK-mediated phosphorylation stabilizes RXRα by protecting it from ubiquitination and subsequent proteolytic degradation as previously reported for c-Jun and p53 (for a review, see Ref. 62). Alternatively, RXRα 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, RXRα 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 RXRα hyperphosphorylation is actually involved in apoptosis.

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