Stress Pathway Activation Induces Phosphorylation of Retinoid X Receptor*

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Cellular stresses inhibit retinoid signaling, but the molecular basis for this phenomenon has not been revealed. Here, we present evidence that retinoid X receptor (RXR) is a substrate for both mitogen-activated protein kinase kinase-4 (MKK4/SEK1) and its downstream mediator c-Jun N-terminal kinase (JNK). MKK4/SEK1 and JNK recognized distinct features on RXR in the DE and AB regions, respectively. Phosphorylation by MKK4/SEK1 had profound effects on the biochemical properties of RXR, inhibiting the expression of genes activated by RXR-retinoic acid receptor complexes. Tyr-249 in the RXR DE region was required for the inhibitory effect of MKK4/SEK1. These effects were significantly reduced in MKK4/SEK1-null cells, indicating that MKK4/SEK1 is required for the suppression of retinoid signaling by stress. Findings presented here demonstrate that MKK4/SEK1 can directly modulate transcription by phosphorylating RXR, a novel MKK4/SEK1 substrate.

Retinoids are ligands for nuclear hormone receptors, including retinoid X receptors (RXRa, β, γ) and retinoic acid receptors (RARa, β, γ), which are members of the steroid/thyroid receptor superfamily (1). Of the naturally occurring retinoids, 9-cis retinoic acid is a ligand for RARs and RXRs, and all-trans-retinoic acid (t-RA) is a ligand for RARs. These receptors form RXR homodimers and RXR-RAR heterodimers, which activate gene transcription directly by binding to specific retinoic acid response elements (RAREs) in gene promoter regions. RXR homodimers and RXR-RAR heterodimers bind to distinct RAREs, thus activating different signal transduction pathways. Disruption of genetic loci associated with specific RARs and RXRs has revealed the importance of retinoid receptors in mediating the biologic effects of retinoids (2, 3).

Retinoid receptor transcriptional activity is regulated by factors both intrinsic and extrinsic to the receptor complex. In the unliganded state, retinoid receptors are bound to co-repressors, including silencing mediator for retinoid and thyroid hormone receptors and nuclear receptor co-repressor (4). These co-repressors form complexes with histone deacetylases to induce chromatin condensation and transcriptional repression. Ligand binding causes retinoid receptors to dissociate from co-repressors and bind to co-activators, including CREB-binding protein, p300/CBP-interacting protein, and members of the p160/SRC family of co-activators, such as steroid receptor co-activator-1 (5). Co-activators form multiprotein complexes that possess intrinsic histone acetyltransferase activity, which is required for retinoid receptor transcriptional activation. Although ligand binding is thought to be the primary means of activation, retinoid signaling is also modulated by cellular stresses. For example, ultraviolet (UV) irradiation decreases intracellular RARγ and RXRα protein levels and inhibits ligand-induced transcription of RAR-RAR target genes (6). Peptide growth factors also decrease retinoid-induced expression of RAR-RAR target genes and block the biological effects of retinoids on cells (7, 8). However, the mechanism by which these cellular stresses inhibit retinoid signaling pathways has not been defined.

Stress-activated signaling pathways include the c-Jun N-terminal kinases (JNK1–3), also known as stress-activated protein kinases (SAPKs) (9). These MAP kinases can be activated directly by MAP kinase kinases such as MKK4/SEK1 and MKK7 (10, 11). Although MAP kinases have been shown to phosphorylate a number of transcription factors, including nuclear receptors (12–17), no substrates other than MAP kinases have been identified for MKKs.

Because stress inhibits retinoid signaling, we tested the hypothesis that stress-activated protein kinases mediate these effects by acting on nuclear retinoid receptors. Here we present evidence that RXR is a substrate of the stress-activated protein kinases JNK and MKK4/SEK1. MKK4/SEK1 directly phosphorylates RXR, inhibiting ligand-induced transactivation of RAREs, effects that are greatly diminished in MKK4/SEK1-null cells. The functional effects of MKK4/SEK1-mediated phosphorylation support the conclusion that this kinase, though it is dedicated to the phosphorylation of stress-sensitive MAP kinases (18, 19), has other substrates that bypass the rest of the downstream MAP kinase signaling cascade.
Experimental Procedures

Plasmid Constructs—This study involved the use of the following expression plasmids: human GST-tagged constitutively active MKK4/SEK1 (ST to ED mutant; S287E, T291D) cDNA (MKK4 E-D) (a gift from Dr. John M. Kyriakis, Harvard University, Boston, MA) (20); human hemagglutinin-tagged constitutively active MKK1 (R4F mutant) cDNA (a gift from Dr. Natalie Ahn, University of Colorado, Boulder, CO) (21); wild-type human MKK7 (22); dominant-negative mutants of human JNK1 and JNK2 cDNAs (a gift from Dr. Bing Su, M.D. Anderson Cancer Center) (23); human skp1 and cul1 (a gift from Dr. J. Wade Harper, Baylor College of Medicine, Houston, TX) (24); human GST-RARα and GST-TRβ expression plasmids (gifts from Dr. William W. Lamph, Ligand Pharmaceuticals, San Diego, CA); luciferase reporter plasmids containing RAREs, which include DR5 (direct repeats of AGGTCA separated by 5 nucleotides in the context of a heterologous TK promoter), and βRARE (the human RAR-β gene promoter from −1470 to +163, containing a DR5 RARE from position −55 to −35) (25); luciferase reporter plasmids containing the canonical AP-1 response element TGAAGTCACA in the context of a minimal c-fos promoter with no identifiable regulatory elements except a TATA box (26).

The following human RXRα deletion mutants were constructed: AB (amino acids 1–135), ABC (amino acids 1–200), and DE (amino acids 216–463). Flag-tagged deletion constructs were created by PCR amplification using the following primers: B region, sense (5′-GCC GAT GTG CTT GGT-3′); C region, antisense (5′-CCG AAT TCA GCC CAT CAC GCA-3′) and sense (5′-TAG GTC CCA TGG ACT ACA AGG AGC AGG ACA TCC GCG CTC GGG AGG ACA TTG-3′); E region, antisense (5′-CCG AAT TCT AAG ACA TTT GGT GGC-3′) and sense (5′-CAA GAT CTG GAA GGA GAA TGC GAA GTC TCT-3′). Primers used to create His-tagged cDNAs were the following: A region, sense (5′-GCA GAT CTG GAC ACC AAA CAT TTC-3′); C region, sense (5′-CAA GAT CTG GCC ATC TGC GGG GA-3′); D region, sense (5′-AAA GAT CTG AAC GAG ATG GGTG-3′). PCR products were subcloned into pCMX and pET32a (Invitrogen) to create Flag- and His-tagged cDNAs, respectively. These GST- and His-tagged RXR constructs were expressed in transfected 293T cells and purified using glutathione-Sepharose beads (Amersham Pharmacia Biotech) and nickel column (Qiagen), respectively.

Site-directed mutagenesis was performed on the full-length RXR cDNA to create Y169F, Y249F, and Y397F mutant cDNAs by PCR amplification using the following primers: Y169F, sense (5′-GCC GAT GTG CTT GGT-3′); Y249F, sense (5′-GAAG ACC TTC ACC GCC GGC AAC AA-3′) and antisense (5′-TTG TCG CCG CAG GTG AAG CTC GGC GAA-3′); Y397F, sense (5′-GTA GGG AGA AGG TTC TCT GTG CCT TCT-3′) and antisense (5′-AAC GAC GAC CAA GTC ACC TTC TCT CTC-3′).

Antibodies—Polyclonal antibodies against histidine repeats, c-Jun phosphorylated on serine 63, ERK-1 and -2, RAR and laminin B1 (Polyclonal Antibody Catalog, The Upstate Company), and monoclonal antibodies against Flag (M2) was purchased (I-BioKodak).

Cell Lines—COS-7 cells, RXRα-null F9 cells, RXRα-null F9 cells stably transfected with RXRα (Re7), and mouse embryo fibroblasts derived from wild type and MKK4/SEK1-null 129/J mice were maintained in Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum (Life Technologies). The following human RXR cDNA expression plasmids were expressed in transient transfection in 293T cells: RXRα, RXRα Y169F, RXRα Y249F, RXRα Y397F, RXRα Y169F Y249F Y397F, RXRα D199A, RXRα A535T, RXRα Y397F, and RXRα Y397F A535T.

Luciferase Assays—Cells were seeded in 24-well tissue culture plates and transfected with plasmids using LipofectAMINE (Life Technologies, Inc.). Total amount of plasmid DNA was adjusted to 1 μg/plate with vector DNA. The transfection solution was removed after 8 h of transfection, and the cells were cultured for 16 h in medium containing 0.1% serum. Cells were then treated with 1 μM t-RA overnight. When indicated, cells were cotreated with anisomycin at the indicated dose and time. Cells were subjected to luciferase assays as described previously (25). Luciferase activities were expressed as the means and standard deviations from three identical wells.

Radioactive Labeling of Intact Cells—Cells were seeded onto 100-mm plates and transfected with plasmid DNAs using LipofectAMINE. For co-transfections, total transfected plasmid DNA was kept constant (10 μg) using empty vector. After 8 h of transfection, cells were serum-starved by changing medium to Dulbecco’s modified Eagle’s medium plus 0.1% fetal calf serum. Twenty-four hours after transfection, cells were treated with t-RA (1 μM) for 16 h and labeled with carrier-free [3H]thymidine or [35S]methionine. Radioactive labeling was performed in the presence of t-RA (1 μM) to investigate ligand-dependent effects unless otherwise indicated. [3H]Labeled RXRα was immunoprecipitated with either RXRα-specific polyclonal antibody or Flag-specific monoclonal antibody M2. Immunoprecipitates were washed four times with lysis buffer, separated by SDS-PAGE, and analyzed by autoradiography. For subsequent phosphoamino acid analysis, the phosphopeptides localized by autoradiography were recovered and treated as described below. Western blotting autoradiographs were quantitated by scanning densitometry (MultiImage light cabinet, Alpha-Innotech Corp.).

Protein Kinase Assays—Following transfection of COS-7 cells with GST-tagged MKK4 E-D, MKK4/SEK1 was affinity-purified from whole cell extracts with glutathione-Sepharose beads (Amersham Pharmacia Biotech) by incubation for 3 h at 4 °C. Activated JNK1 was immunoprecipitated from COS-7 cells transiently co-transfected with MKK4 E-D and JNK1 expression vectors using anti-JNK1 antibodies (Santa Cruz Biotechnology). The JNK1 immune complexes were washed twice with lysis buffer and three times with kinase buffer (25 mM HEPES (pH 7.4), 25 mM β-glycerophosphate, 25 mM MgCl2, 0.5 mM EGTA, 2 mM dithiothreitol, 0.5 mM sodium orthovanadate) for use in immune complex kinase reactions. Kinase reactions were performed at 30 °C for 30 min in 30 μl of kinase buffer containing 1 μg of substrate and 10 μCi of [γ-32P]ATP. Kinase reactions were terminated by adding 10 μl of 4X SDS loading buffer. Samples were boiled and electrophoresed by SDS-PAGE, dried, and visualized by autoradiography.

Phosphoamino acid analysis was carried out on RXR phosphorylated by JNK1 and MKK4/SEK1 in vitro and in intact cells. The protein was transferred to polyvinylidene difluoride membrane (Bio-Rad), and phosphopeptides localized by autoradiography were excised, digested with 6 N HCl, and further dried. Pellets were dissolved in buffer (pH 1.9) containing 5 μg of unlabeled standard phosphoamino acids, and then
separated on 20 × 20-cm thin layer cellulose plate. The plates were then dried, sprayed with ninhydrin to visualize phosphoamino acid standards, and exposed to x-ray film.

RESULTS

Activation of Stress Signaling Inhibits the Biologic Effects of All-trans-retinoic Acid—We first investigated the effects of stress on retinoid signaling in cells that are biologically responsive to retinoid treatment. t-RA induces differentiation of F9 teratocarcinoma cells, a process marked by an increase in the transcription of several genes mediated directly by RXR/RAR heterodimeric complexes. Through this mechanism, the expression of RARβ and laminin B1 increases within the first 6 and 48 h, respectively (29, 30). We examined the effect of stress pathway activation on RARβ and laminin B1 expression. Drugs such as anisomycin are potent activators of the stress response. Northern analysis revealed that treatment with anisomycin or transfection with a constitutively active form of MKK4 E-D, wild type MKK7, or constitutively active MKK1 (R4F mutant) expression vectors, treated for 16 h with t-RA (10⁻⁶ m) and then labeled for 3 h with [³²P]orthophosphate in the presence or absence of anisomycin (5 µg/ml). B and C, cells were co-transfected with MKK4 E-D, wild type MKK7, or constitutively active MKK1 (R4F mutant) expression vectors, treated for 16 h with t-RA (10⁻⁶ m) and labeled for 3 h with [³²P]orthophosphate. Cells extracts were subjected to either immunoprecipitation (IP) with anti-RXRα, -RARα, or -Flag antibodies or Western analysis (W) with anti-RXR or -RAR antibodies. MKK7 and MKK1 induced phosphorylation of JNK and ERK, respectively, in cells labeled with [³²P]orthophosphate (data not shown). The total amount of plasmid DNA was adjusted to 10 µg/plate with vector DNA. The different relative densities of upper and lower bands observed in MKK4 E-D-transfected cells (B) by immunoprecipitation with anti-Flag and anti-RXR antibodies may relate to different affinities of antibodies for phosphorylated forms of RXR.

FIG. 3. Stress pathway activation induces phosphorylation of RXRα but not RARα. COS-7 cells were transiently transfected with Flag-RXRα or Flag-RARα. A, transfectants were treated with t-RA (10⁻⁶ m) for 16 h and then labeled for 3 h with [³²P]orthophosphate in the presence or absence of anisomycin (5 µg/ml). B and C, cells were co-transfected with MKK4 E-D, wild type MKK7, or constitutively active MKK1 (R4F mutant) expression vectors, treated for 16 h with t-RA (10⁻⁶ m), and labeled for 3 h with [³²P]orthophosphate. Cells extracts were subjected to either immunoprecipitation (IP) with anti-RXRα, -RARα, or -Flag antibodies or Western analysis (W) with anti-RXR or -RAR antibodies. MKK7 and MKK1 induced phosphorylation of JNK and ERK, respectively, in cells labeled with [³²P]orthophosphate (data not shown). The total amount of plasmid DNA was adjusted to 10 µg/plate with vector DNA. The different relative densities of upper and lower bands observed in MKK4 E-D-transfected cells (B) by immunoprecipitation with anti-Flag and anti-RXR antibodies may relate to different affinities of antibodies for phosphorylated forms of RXR.

clearly suppressed t-RA-induced RARβ and laminin B1 mRNA levels (Fig. 1). Importantly, these effects of stress were not observed in F9 cells that lack RXRα through genetic knockout (Fig. 1). Stably reintroducing RXRα into RXRα-null F9 cells (Rc7 cells) restored stress-induced suppression of t-RA-induced laminin B1 and RARβ mRNA (Fig. 1). These findings demonstrate an absolute requirement of RXRα in mediating stress-induced suppression of retinoid signaling in vivo.

Activation of Stress Signaling Inhibits Ligand-induced Transactivation of RXR-RAR Complexes—Based on these findings, we hypothesized that stress inhibits ligand-induced transcriptional activation of promoters containing RAREs. We investigated retinoid receptor transcriptional activity in COS-7 cells using reporters containing RAREs that bind RXR-RAR heterodimeric complexes in a complex of a TK heterologous promoter in the context of a TK heterologous promoter (DR5) or the RAR-β gene promoter (β-RARE). Cells were treated for 16 h with or without t-RA (10⁻⁶ m). To activate stress pathways, cells were either co-treated with anisomycin at the indicated concentrations during t-RA treatment or co-transfected with the indicated amounts of MKK4 E-D. The total amount of plasmid DNA was adjusted to 1 µg/well with vector DNA. Luciferase results are the means (± S.D.) of three identical wells.
scriptional activity, with effects ranging from 50% to 70% inhibition (Fig. 2). Co-transfection with constitutively active MKK1 (R4F mutant), an activator of ERK MAP kinases, activated an AP-1 response element but did not detectably alter ligand-induced RARE activity (data not shown), suggesting that transcriptional activity of the RXR–RAR complex is regu-

**Fig. 4.** JNK1 phosphorylates serine and threonine residues in the AB region of RXRα. A, Activated JNK1 was immunoprecipitated from COS-7 cells transiently transfected with MKK4 E-D, and immune complex kinase assays (KA) were performed to examine the effect of activated JNK on the phosphorylation of His-tagged full-length (RXR) and deletion mutant RXR, which are illustrated diagrammatically. Samples of full-length and deletion mutant RXRs were subjected to Western analysis (W) with anti-histidine antibodies to illustrate their expression and relative size (designated with arrow). B, phosphoamino acid analysis of the in vitro phosphorylated full-length RXR band was performed to examine the effect of activated JNK on the phosphorylation of serine (pS), threonine (pT), and tyrosine (pY).

**Fig. 5.** MKK4/SEK1 physically associates with RXRα and phospholylates serine, threonine, and tyrosine residues in the RXRα DE region. A, GST-MKK4 E-D was affinity-purified from COS-7 cells transiently transfected with GST-MKK4 E-D using glutathione-Sepharose beads, and kinase assays (KA) were performed to examine the effect of constitutively active MKK4/SEK1 on the phosphorylation of GST-RXRα, -RARα, -TRβ, and -JNK1 (as a positive control). The relative size of the bacterial GST fusion proteins is illustrated on a Coomassie-stained gel. B, COS-7 cells were transiently co-transfected with Flag-RXRα and GST-MKK4 E-D expression vectors, and GST-MKK4 E-D was affinity-purified with glutathione-Sepharose beads. Association of MKK4/SEK1 with RXR was examined by subjecting the GST complex to SDS-PAGE electrophoresis and Western blotting (W) with anti-RXRα antibodies. Cell lysates were subjected to Western analysis with anti-Flag antibodies to indicate the levels of RXR expressed in the cells. C, GST-MKK4 E-D was affinity-purified from COS-7 cells transiently transfected with GST-MKK4 E-D using glutathione-Sepharose beads, and kinase assays (KA) were performed to examine the effect of MKK4 E-D on the phosphorylation of His-tagged full-length (RXR) and deletion mutant RXR, which are illustrated diagrammatically. Samples of full-length and deletion mutant RXRs were subjected to Western analysis (W) with anti-histidine antibodies to illustrate their expression and relative size (designated with arrow). D, kinase reactions were subjected to phosphoamino acid analysis to examine the effect of constitutively active MKK4/SEK1 on the phosphorylation of serine (pS), threonine (pT), and tyrosine (pY) in the RXR D-E region (D-E) or full-length RXR (RXR).
Stress Kinases Inhibit RXR—We investigated whether the transcriptional effects we observed might be caused by phosphorylation of retinoid receptors. COS-7 cells were transiently transfected with Flag-RARα or Flag-RXRα, treated for 16 h with t-RA (10^{-6} M), and labeled with [35S]methionine (to demonstrate synthesis of the tagged protein) and subjected to immunoprecipitation (IP) with anti-Flag antibodies. Immune complex kinase assays (KA) were performed to examine the effect of MKK4 E-D on His-tagged wild type RXR (RXR) or the indicated RXR mutants. Aliquots of bacterial RXR were subjected to Western analysis (W) using anti-RXR antibodies to demonstrate their relative size. In these assays, we observed a reduction in the electrophoretic mobility of RXR, which is consistent with the conclusion that a significant fraction of RXR was phosphorylated on at least one residue. Co-transfection of Flag-RXRα with other MKKs (MKK7 or MKK1) did not detectably induce phosphorylation of RXR (Fig. 3C), demonstrating the specificity of RXR for MKK4/SEK1. Phosphoamino acid analysis of RXR immunoprecipitated from COS-7 cells radiolabeled with [32P]orthophosphate following transient co-transfection with MKK4 E-D and Flag-RXRα revealed phosphoserine, phosphothreonine, and phosphotyrosine (data not shown).

MKK4/SEK1 is an intermediate in a cascade involving an upstream MKK kinase and MAP kinases (including JNK/ SAPK). No MKK4/SEK1 substrates other than MAP kinase family members have been identified. Therefore, we hypothesized that RXR phosphorylation by MKK4/SEK1 is mediated through JNK/SAPK, which is known to phosphorylate other steroid receptors (12, 16). We tested the capacity of activated JNK1 and MKK4/SEK1 to phosphorylate RXR in vitro. JNK1 phosphorylated RXRα in vitro (Fig. 4A) as well as it did c-Jun (data not shown). The region of RXR phosphorylated by JNK was defined using RXRα deletion mutants. JNK phosphorylated RXRα fragments lacking the DE region but not those lacking the ABC region (Fig. 4A). Phosphoamino acid analysis of the RXRα band revealed phosphoserine and phosphothreonine (Fig. 4B). In the AB region, there are more than 10 potential JNK phosphorylation sites as defined by the minimal consensus site (S/TP). Surprisingly, RXRα was also phosphorylated by MKK4 E-D in an in vitro kinase reaction (Fig. 5A). MKK4 E-D minimally phosphorylated GST-RARα or GST-thyroid receptor (TR) (Fig. 5A), demonstrating the specificity of MKK4/SEK1 for RXR. We tested the possibility that MKK4/SEK1 bound stably to RXR. COS-7 cells were co-transfected with plasmids encoding GST-MKK4 E-D and Flag-RXRα. MKK4 E-D was affinity purified from cell lysates and immunoblotted to RXRα antibodies. Stable binding between MKK4 E-D and RXR was observed (Fig. 5B).

To determine the region of RXR phosphorylated by MKK4/ SEK1, in vitro kinase assays were performed on RXRα deletion mutants. MKK4 E-D phosphorylated a mutant lacking the DE region but not those lacking the ABC region (Fig. 4A). Phosphoamino acid analysis of the RXR DE region revealed phosphoserine, phosphothreonine, and phosphotyrosine (Fig. 5D), which is consistent with the dual-specificity kinase activity of MKK4/SEK1 (10, 31). Tyrosine phosphorylation was more prominent relative to serine in the context of full-length RXR protein than in the D-E region (Fig. 5D), suggesting that conformational effects influenced relative rates of phosphorylation of different sites on RXR. This type of conformational effect has been observed previously for steroid...
fibroblasts. RXR

expression of retinoid signaling by stress, we tested the effect of steady, RXR is a direct substrate of MKK4/SEK1. In findings are consistent with the conclusion that JNK activation effect on RXR phosphorylation (Fig. 7). Taken together, these E-D-induced phosphorylation of c-Jun but had no detectable

structures. Dominant negative JNK-1 and -2 suppressed MKK4 antibodies to examine dominant negative activity of JNK constructs (dominant negative JNK-2 was more effective than dominant negative JNK1 in inhibiting c-Jun phosphorylation). Relative to control (Flag-RXR alone), the density of pJun bands from cells transfected with MKK4 (E-D) alone or co-transfected with MKK4 (E-D) and dominant negative JNK-1 or -2 were 1.2, 0.88, and 0.57, respectively.

Based on the known specificity of MKK4/SEK1 for phosphorylating tyrosine residues nearby to serine or threonine, we mutated tyrosine residues with that feature in the D-E region (Tyr-249) and -397) and in the C (DNA-binding) region (Tyr-169) to identify potential MKK4/SEK1 phosphorylation sites in RXR. Phosphorylation of these RXR mutants by MKK4 E-D was examined in COS-7 cells labeled with [32P]orthophosphate and in vitro (Fig. 6A). RXR phosphorylation by MKK4 E-D was reduced by mutation of either tyrosine 397 (Y397F) or 249 (Y249F). Mutation of tyrosine 169 (Y169F) had no effect on RXR phosphorylation by MKK4 E-D, which is consistent with the result that the RXR C region was not phosphorylated by MKK4/SEK1. We investigated the necessity of RXR phosphorylation in the suppression of RARE transactivation by MKK4/SEK1. Transient co-transfection assays in COS-7 cells revealed that the suppression of RARE activity by MKK4 E-D was abrogated by mutation of RXR at Tyr-249 (Y249F) but not Tyr-397 (Y397F) or Tyr-169 (Y169F) (Fig. 6B), indicating that RXR phosphorylation on Tyr-249 is critical for the effects of MKK4/SEK1 on RARE transactivation.

Our findings demonstrate that MKK4/SEK1 phosphorylated RXR domains distinct from those phosphorylated by JNK1. To confirm that MKK4/SEK1 phosphorylated RXR through a JNK-independent mechanism, COS-7 cells were co-transfected with MKK4 E-D, a dominant negative JNK construct (JNK-1 or -2) to block JNK activation, and Flag-RXR. The transfectants were labeled with [32P]orthophosphate in the presence of t-RA (10^{-6} M) and subjected to immunoprecipitation (IP) with anti-Flag antibodies to examine RXR phosphorylation and Western analysis (W) with anti-phospho-c-Jun antibodies to examine dominant negative activity of JNK constructs (dominant negative JNK-2 was more effective than dominant negative JNK1 in inhibiting c-Jun phosphorylation). Relative to control (Flag-RXR alone), the density of pJun bands from cells transfected with MKK4 (E-D) alone or co-transfected with MKK4 (E-D) and dominant negative JNK-1 or -2 were 1.2, 0.88, and 0.57, respectively.

Phosphorylation was examined by immunoprecipitation or Western analysis (Fig. 6B). The transfectants were treated for 16 h with t-RA (10^{-6} M) and subjected to immunoprecipitation (IP) or Western analysis (W) with anti-RXR antibodies. MKK4/SEK1-null cells were also transiently co-transfected with constitutively active MKK4/SEK1 (MKK4 E-D), treated for 16 h with t-RA (10^{-6} M), and labeled for 3 h with [32P]orthophosphate. Cells were lysed, and extracts were subjected to either immunoprecipitation (IP) or Western analysis (W) with anti-RXR antibodies. B, stress-induced changes in RARE transcriptional activity were examined. Wild type and MKK4/SEK1-null cells were transiently transfected with reporter plasmids containing DR5 RAREs in the context of a TK heterologous promoter. Transfectants were treated for 16 h with t-RA (10^{-6} M) and with or without anisomycin (1 µg/ml) for 3 h. MKK4/SEK1-null cells were also transiently co-transfected with the indicated amounts of MKK4 E-D and t-RA (10^{-6} M) for 3 h with [32P]orthophosphate. The total amount of plasmid DNA was adjusted to 1 µg/well with vector DNA. Illustrates the luciferase activity of cells treated with anisomycin or transfected with MKK4 E-D relative to that of cells treated with t-RA alone. Results are the means (± S.D.) of three identical wells.

**FIG.7.** Dominant negative JNK1 and -2 do not alter RXR phosphorylation by MKK4/SEK1. COS-7 cells were transiently co-transfected with Flag-RXRα, MKK4 E-D, and dominant negative JNK-1 or -2 (dnJNK). Transfectants were treated for 16 h with t-RA (10^{-6} M) and then labeled for 3 h with [32P]orthophosphate. Cells were subjected to immunoprecipitation (IP) with anti-Flag antibodies to examine RXR phosphorylation or Western analysis (W) with anti-phospho-c-Jun antibodies to examine dominant negative activity of JNK constructs (dominant negative JNK-2 was more effective than dominant negative JNK1 in inhibiting c-Jun phosphorylation). Relative to control (Flag-RXR alone), the density of pJun bands from cells transfected with MKK4 (E-D) alone or co-transfected with MKK4 (E-D) and dominant negative JNK-1 or -2 were 1.2, 0.88, and 0.57, respectively.

**FIG.8.** MKK4/SEK1 contributes to RXR regulation by stress. Wild type (+/+ ) and MKK4/SEK1-null (-/- ) mouse embryo fibroblasts were treated for 16 h with t-RA (10^{-6} M). The cells were then labeled for 3 h with [32P]orthophosphate (A) in the presence or absence of anisomycin (1 µg/ml). MKK4/SEK1-null cells were also transiently transfected with constitutively active MKK4/SEK1 (MKK4 E-D), treated for 16 h with t-RA (10^{-6} M), and labeled for 3 h with [32P]orthophosphate. Cells were lysed, and extracts were subjected to either immunoprecipitation (IP) or Western analysis (W) with anti-RXR antibodies. B, stress-induced changes in RARE transcriptional activity were examined. Wild type and MKK4/SEK1-null cells were transiently transfected with reporter plasmids containing DR5 RAREs in the context of a TK heterologous promoter. Transfectants were treated for 16 h with t-RA (10^{-6} M) and with or without anisomycin (1 µg/ml) for 3 h. MKK4/SEK1-null cells were also transiently co-transfected with the indicated amounts of MKK4 E-D and t-RA (10^{-6} M) for 3 h with [32P]orthophosphate. The total amount of plasmid DNA was adjusted to 1 µg/well with vector DNA. Illustrates the luciferase activity of cells treated with anisomycin or transfected with MKK4 E-D relative to that of cells treated with t-RA alone. Results are the means (± S.D.) of three identical wells.

**DISCUSSION**

Stress signaling is activated in response to a variety of environmental stimuli, including starvation, changes in osmolality, heat shock, proinflammatory cytokines, UV light, and DNA damaging agents (33). Potential biologic responses to these stimuli include growth, apoptosis, inflammation, and differentiation. The mechanisms by which diverse biologic outcomes
can result from stress pathway activation have not been elucidated. Toward this end, substrates of stress-activated protein kinases have been identified, including a number of nuclear receptors (12–17). Here we provide the first evidence that MKK4/SEK1 can directly modulate transcription by phosphorylating RXR, a novel MKK4/SEK1 substrate. The consequence of RXR phosphorylation by MKK4/SEK1 is a marked inhibition of retinoid-mediated transcriptional signaling. Based on the lack of any other known substrates for MKK4/SEK1, it has been presumed that MKK4/SEK1 acted only upon stress-activated kinases JNK/SAPKs. However, several lines of evidence presented here indicate that MKK4/SEK1 can directly phosphorylate RXR in vivo, independent of its downstream mediator JNK. First, anisomycin enhanced RXR phosphorylation in wild type but not MKK4/SEK1-null cells, supporting MKK4/SEK1 as an essential mediator of stress-induced RXR phosphorylation. Second, MKK4/SEK1 was shown to directly phosphorylate RXR in vitro on serine, threonine, and tyrosine residues, all of which were phosphorylated in intact cells. Third, MKK4/SEK1 and JNK phosphorylated distinct RXR domains, indicating that these kinases recognize different features of RXR. Fourth, neither dominant-negative JNK1 nor JNK2 blocked RXR phosphorylation by MKK4/SEK1 in intact cells, suggesting that JNK activation was not required. Phosphorylation by MKK4/SEK1 appears to be specific, since neither MKK7 nor MKK1 phosphorylated RXR, nor did MKK4/SEK1 phosphorylate RXR other nuclear receptors, RAR or TR. These findings demonstrate a new paradigm in MAP kinase signaling by revealing a novel substrate of MKK4/SEK1, previously believed to act only as an intermediate in a kinase cascade.

Our findings in MKK4/SEK1-null fibroblasts indicate that MKK4/SEK1 is required for RXR phosphorylation by anisomycin. Further, mutation of Tyr-249 decreased RXR phosphorylation and abrogated the suppression of RARE transcriptional activity by MKK4/SEK1. Together, these findings support a role for RXR phosphorylation by MKK4/SEK1 in stress-induced inhibition of retinoid signaling. However, suppression of RARE transcriptional activity by anisomycin was only partially abrogated in MKK4/SEK1-null cells, suggesting that other anisomycin-sensitive kinases regulate the activity of RXR-RAR heterodimers. In this study, MKK7 overexpression had no effect on RXR phosphorylation. As reported previously (34), we found that JNK1 phosphorylated RXR, but this has been reported to have no effect on the transactivation properties of RXR-RAR heterodimers (34). Additional studies will be required to fully understand the pathways through which stress regulates the activity of RXR-RAR heterodimers.

What may be the physiologic consequence of stress-mediated inhibition of RXR function? Chronic exposure of human skin to UV light, a potent activator of stress pathways, induces tumor suppressor properties (36, 37), and loss of specific retinoid receptors is thought to be an important event in carcinogenesis of the skin, lung, breast, esophagus, and cervix (38–43). Data presented here indicate that MKK4/SEK1 can result from stress pathway activation and inhibit the expression of RARα, RXRs, and RAR-RXR target genes (6, 35). Retinoid receptors have tumor suppressor properties (36, 37), and loss of specific retinoid receptors is thought to be an important event in carcinogenesis of the skin, lung, breast, esophagus, and cervix (38–43). Data presented here offer a potential mechanism by which environmental stress could suppress retinoid signaling in epithelial tissues. Supporting a role for RXR phosphorylation in malignant transformation, RXR is phosphorylated by MAP kinase in Ras-transformed cells, which results in suppression of vitamin D receptor:RXR transactivation and attenuation of the growth inhibitory effects of 1,25-dihydroxyvitamin D3 (44). Future studies will be required to determine the importance of MKK4/SEK1 as a physiologic regulator of retinoid signaling and the role of stress-activated kinases in the pathogenesis of diseases induced by environmental stress.

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