

Mitogen-activated protein kinase inhibits 1,25-dihydroxyvitamin D₃-dependent signal transduction by phosphorylating human retinoid X receptor α

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Human retinoid X receptor α (hRXR α) is a member of the nuclear receptor family of transcriptional regulators. It regulates transcription through its association with several heterodimeric partners, including the vitamin D₃ receptor (VDR). Signaling through the VDR is essential for normal calcium homeostasis and has been shown to inhibit the proliferation of cancer cells derived from a number of tissues. Here we show that phosphorylation of hRXR α in *ras*-transformed human keratinocytes through the activated Ras-Raf-mitogen-activated protein kinase (Ras-Raf-MAP kinase) pathway results in attenuated transactivation by the VDR and resistance to the growth inhibitory action of 1,25 dihydroxyvitamin D₃ [1,25(OH)₂D₃] and RXR-specific agonist LG1069 (4-[1-(5,6,7,8-tetrahydro-3,5,5,8,8-pentamethyl-2-naphthalenyl) ethenyl]-benzoic acid). Phosphorylation of hRXR α occurs at serine 260, a consensus MAP kinase site. Inhibition of MAP kinase activity or point mutagenesis of serine 260 of hRXR α reverses the observed resistance to 1,25(OH)₂D₃ and LG1069. Thus, hRXR α is a downstream target of MAP kinase, and its phosphorylation may play an important role in malignant transformation.

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Introduction

Resistance to anti-tumor agents is often a limiting factor in cancer therapy (1). Activation of *ras*, the most common oncogene implicated in human cancer, has been detected in a wide variety of tumors, including carcinomas of the pancreas and squamous tumors of the colon, lung, skin, and breast (2). Activation of the Ras-Raf-mitogen-activated protein kinase (Ras-Raf-MAP kinase) pathway leads to phosphorylation of numerous downstream targets (3) and is a potential mechanism for the control of nuclear receptor activation and function (4).

The biologically active form of vitamin D₃, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃], directly modulates the transcription of several target genes by binding to the vitamin D₃ receptor (VDR), a member of the nuclear receptor family of transcriptional regulators. The ligand-bound VDR functions as a heterodimer by interacting with members of the retinoid X receptor (RXR) family of nuclear receptors (5). An extensive body of literature links the function of 1,25(OH)₂D₃ to the inhibition of cancer cell growth and stimulation of cellular differentiation. Administration of 1,25(OH)₂D₃ prolonged the life expectancy of leukemic mice (6), inhibited the formation of preneoplastic mammary lesions in rats, and inhibited the proliferation of cancer cell lines derived from many tissues, including breast (7), endometrium (8), head and neck (9), lung (10), prostate (11), and hematopoietic lineages (12). However, several cancer cell lines have been identified as resistant to the growth inhibitory action of 1,25(OH)₂D₃, including squamous cell carcinomas derived from human keratinocytes (13), human pancreatic cancer cells (14), and human breast carcinoma cells

(15). An understanding of tumor cells' mechanism of resistance to 1,25(OH)₂D₃ could lead to new methods for controlling the deregulated growth of these cells.

The transformed human keratinocyte cell line HPK1A ras overexpresses H-*ras* and forms squamous tumors in nude mice (16). Unlike the parental HPK1A cell line and normal human keratinocytes, HPK1A ras cells are partially resistant to growth inhibition by 1,25(OH)₂D₃ (13). Human retinoid X receptor α (hRXR α) is the predominant RXR expressed in these cells (17). Interestingly, VDR/RXR complexes formed on vitamin D response element (VDRE) oligonucleotides can be supershifted with an antibody that recognizes the hRXR α ligand-binding domain (LBD) in extracts of normal human keratinocytes and HPK1A cells but not *ras*-transformed HPK1A ras cells (17). The LBD of hRXR α possesses an interface for interaction with heterodimerization partners such as the VDR, thyroid hormone receptors (TRs), and retinoic acid receptors (RARs) (18). Here we show that the LBD of hRXR α is a nuclear target of MAP kinase in HPK1A ras cells, which affects its normal function through phosphorylation at a specific site.

Methods

Cell growth assays and transfections. The HPK1A and HPK1A ras cell lines have been described (16, 13, 19). HPK1A cells are nontumorigenic, whereas HPK1A ras cells form colonies in soft agar and produce invasive squamous cell carcinomas when transplanted into nude mice. For assessment of cell growth, HPK1A and HPK1A ras cells were seeded in 24-well plates at a density of 4×10^3 cells per well and grown in DMEM (GIBCO BRL, Burlington, Ontario, Canada) supplemented with 10% FBS. When the cells reached 40% confluence, the medium was replaced with serum-free DMEM for 24 hours to synchronize the cells. At time 0, the medi-

um was replaced with DMEM supplemented with 5% charcoal-stripped FBS in the presence of increasing concentrations of 1,25(OH)₂D₃ (10⁻¹⁰ to 10⁻⁷ M) or LG1069 (10⁻¹⁰ to 10⁻⁶ M) (a kind gift of E. Allegretto, Ligand Pharmaceuticals, San Diego, California, USA). In some experiments, cells were treated with 25 μM of the mitogen-activated protein kinase (MAPKK) inhibitor PD098059 (Research Biochemical International, Natick, Massachusetts, USA). Cells were trypsinized after 96 hours and counted with a Coulter counter (Coulter Electronics, Luton, United Kingdom). Cell activity was also assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) formazan assay. Briefly, this procedure assesses cellular growth based on the intensity of a colorimetric reaction resulting from the reduction of a tetrazolium reagent (Promega Corp., Madison, Wisconsin, USA) to a soluble formazan salt, with absorbance being directly proportional to viable cell density. Cells were seeded with 2 × 10³ cells per well in 96-well plates and treated with LG1069 as above. This assay was performed as described previously (13). For the transfection studies, cells were grown in 6-well plates seeded with 4 × 10⁵ cells per well. At 60% confluence, cells were transfected with 5 μg of the 1,25(OH)₂D₃-sensitive chloramphenicol acetyl transferase (CAT) reporter plasmid mOP3 (20) and 0.1 μg of a human growth hormone (hGH) reporter plasmid (22) as an internal control for transfection efficiency (17). Transfections were performed by incubating plasmid DNA (5 μg) with Lipofectamine (GIBCO BRL) (15 μg) for 20 hours in Opti-MEM (GIBCO BRL) and then replacing the medium with fresh DMEM containing 10% FBS with varying concentrations of 1,25(OH)₂D₃ or LG1069 in the absence or presence of 25 μM PD098059 for 24 hours. The medium was retained for the hGH assay. The cells were trypsinized, washed in PBS, resuspended in 0.25 M Tris-HCl (pH 8.0), and lysed by 5 freeze-thaw cycles. The cell lysate was centrifuged, and aliquots of cell extracts were used for the CAT assays. Assays were performed using a CAT ELISA kit (5 Prime-3 Prime, Boulder, Colorado, USA). Human growth hormone assays were performed using an hGH ELISA kit (Boehringer Mannheim, Laval, Quebec, Canada).

Nuclear extracts. Nuclear extracts were prepared from HPK1A and HPK1A_{ras} cells as described previously (17). Nuclear extracts were also prepared from HPK1A and HPK1A_{ras} cells that had been treated with 25 μM PD098059 for 48 hours prior to extraction.

Gel mobility shift analysis. Nuclear extracts (2 μg) were incubated for 20 minutes on ice with 10⁻⁷ M 1,25(OH)₂D₃ and 1 μg poly dI.dC in a binding buffer (25 mM Tris-HCl [pH 8.0], 5% glycerol,

0.5 mM DTT). Five femtomoles of the ³²P-labeled mouse osteopontin VDRE (mOP VDRE) (5'-GTACAAGGTTTCACGAG-GTTTCACGTCTTA-3') was added and incubated for 20 minutes at room temperature. When required, specific antibodies were added to the incubation. The anti-RXR antibody 4X1D12 recognizes the COOH-terminal domain of RXR and produces a supershift (a kind gift of P. Chambon, College de France, Illkirch, France). The anti-VDR antibody recognizes the LBD of VDR and produces a supershift (Affinity BioReagents Inc., Neshanic Station, New Jersey, USA). The anti-RXR antibody that recognizes the NH₂-terminal domain of RXR close to the DNA-binding domain inhibits formation of the complex (Santa Cruz Biotechnology Inc., Santa Cruz, California, USA) (23). The samples were electrophoresed on 5% nondenaturing polyacrylamide gels, dried, and exposed to Kodak XAR-5 film.

Immunoprecipitation. Serine and threonine phosphorylation of hRXRα from HPK1A and HPK1A_{ras} cells was determined by immunoprecipitating hRXRα from 100 μg of total protein from the nuclear extracts through a 2-hour incubation at 4°C with 1 μg of a polyclonal anti-RXRα antibody that recognizes the LBD of hRXRα (Santa Cruz Biotechnology Inc.) or an anti-hemagglutinin (HA) antibody (Babco, Berkeley, CA). Protein content was determined with a protein assay kit (Bio-Rad Laboratories, Mississauga, Ontario, Canada). The mixture was incubated overnight with protein G-Sepharose beads (Pharmacia Biotech, Baie d'Urfé, Quebec, Canada). Immunoprecipitates were washed 4 times in radioimmunoprecipitation assay (RIPA) buffer (0.1% SDS, 1% NP-40, 0.5% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl [pH 7.4], 1 mM EDTA, 0.25 mM phenylmethylsulfonyl fluoride), boiled for 5 minutes, resolved on 10% SDS-PAGE, and transferred to PVDF membranes (Bio-Rad Laboratories Inc., Hercules, California, USA). Blots were probed with monoclonal anti-phosphoserine or anti-phosphothreonine antibodies (diluted to 1:3,000) (Sigma Chemical Co., St. Louis, Missouri, USA). After incubation with a horseradish peroxidase-conjugated secondary antibody (Bio-Rad Laboratories), the complexes were viewed by enhanced chemiluminescence (ECL; Amersham Life Sciences Inc., Arlington Heights, Illinois, USA).

Site-directed mutagenesis. The ala260 hRXRα mutant was constructed through a double PCR (24) using an hRXRα expression plasmid (a kind gift of R. Evans, The Salk Institute of Biological Sciences, La Jolla, California, USA) (17) and was subcloned into the EcoRI site of pGEM-7Zf(+) (Promega Corp.).

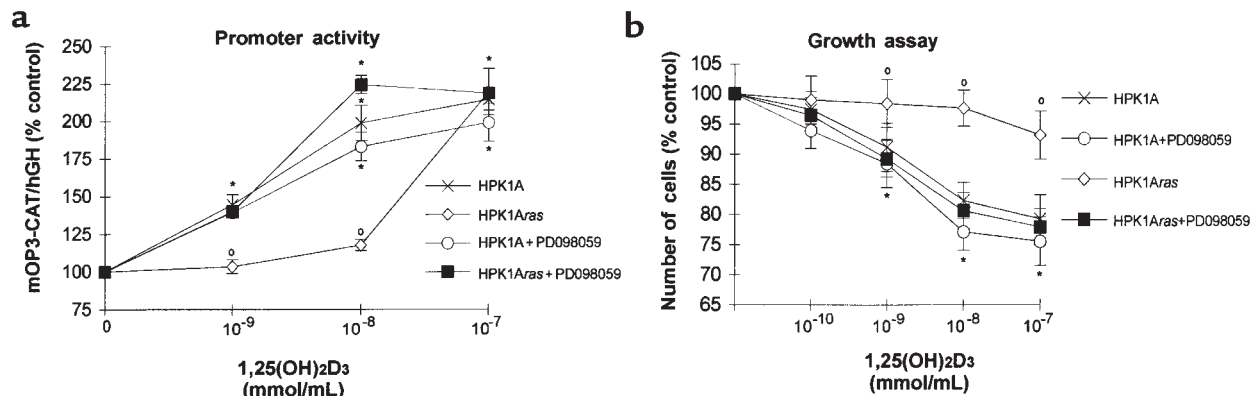


Figure 1

Effect of 1,25(OH)₂D₃ and PD098059 on cell growth in HPK1A and HPK1A_{ras} cells. Cells were transfected with the mOP3 reporter plasmid (20) and the growth hormone expression plasmid pLTR-GH, and then were treated with increasing concentrations of 1,25(OH)₂D₃ and 5% charcoal-stripped FBS in the absence or presence of 25 μM PD098059. (a) CAT activity was assayed after 24 hours and normalized for transfection efficiency by the corresponding hGH activity. (b) Cells were treated as above, and cell numbers were expressed as percent of vehicle-treated control cell numbers after 96 hours. Each value represents the mean ± SD of 3 determinations and is representative of 3 different experiments. Asterisks indicate a significant difference from vehicle-treated control values, whereas open circles indicate a significant difference between HPK1A and HPK1A_{ras} cells in the absence of PD098059 at the 1,25(OH)₂D₃ concentration indicated.

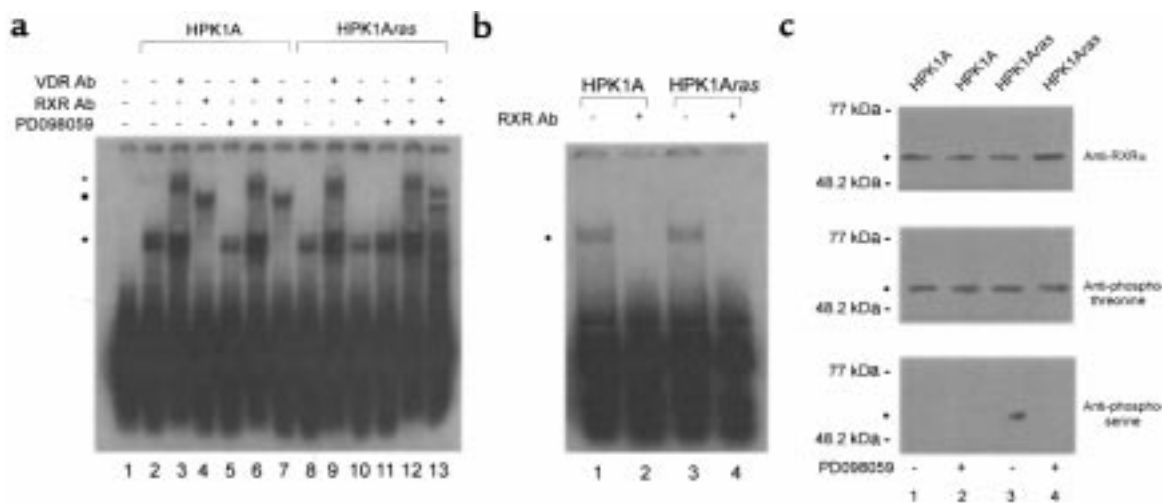


Figure 2

Effect of $1,25(\text{OH})_2\text{D}_3$ and PD098059 on VDR/RXR complex formation in HPK1A and HPK1A $_{\text{ras}}$ cells. (a) A mOP VDRE was ^{32}P labeled and incubated with nuclear extracts from HPK1A cells (lanes 2–4), PD098059-treated HPK1A cells (lanes 5–7), HPK1A $_{\text{ras}}$ cells (lanes 8–10), or PD098059-treated HPK1A $_{\text{ras}}$ cells (lanes 11–13), each in the presence or absence of either an anti-VDR antibody that recognizes the COOH-terminal domain of VDR (VDR Ab) or an anti-RXR antibody that recognizes the LBD of RXR (RXR Ab). The diamond indicates the presence of putative VDR/RXR complexes, the circle indicates supershifted complexes containing RXR, and the asterisk indicates supershifted complexes containing VDR. In lane 1, no nuclear extracts were incubated with the probe. (b) A mOP VDRE was ^{32}P labeled and incubated with nuclear extracts from HPK1A cells (lanes 1 and 2) or HPK1A $_{\text{ras}}$ cells (lanes 3 and 4) in the absence or presence of an anti-RXR antibody that recognizes the NH $_2$ -terminal domain of hRXR α (RXR Ab) and inhibits VDR/RXR complex formation on the VDRE. The diamond indicates the presence of putative VDR/RXR complexes. (c) Nuclear extracts prepared from HPK1A and HPK1A $_{\text{ras}}$ cells were immunoprecipitated with an anti-RXR α antibody, followed by SDS-PAGE and Western blotting. The blots were treated with an anti-phosphoserine antibody, anti-phosphothreonine antibody, or an anti-RXR α antibody to confirm equal loading of the protein as indicated. The diamond indicates the presence of the hRXR α protein.

The external oligonucleotides used were: downstream, hRXR α (5'-CTCCAAGGACGCATAGAC-3'), and upstream, hRXR α (5'-GGAACGAGAATGAGGTGG-3'), encompassing the BssHII and Sall sites. Internal oligonucleotides were as follows (with the alanine mutations underlined): upstream, 5'-GAACCCCGCCGCGCCGAACGACCCTGTACCAACATTTGC-3'; and downstream, 5'-TTCGGCGCGGGGGTTCAGCCCCATGTTTG-3'. The BssHII-Sall fragment was then ligated into the hRXR α plasmid, and the mutant hRXR α was subcloned into the plasmid pcDNA3HA (a kind gift of H. Imataka, McGill University, Montreal, Quebec, Canada), which contains a synthetic oligonucleotide encoding the HA epitope inserted into the HindIII site of pcDNA3 (Invitrogen Corp., Carlsbad, California, USA). Sequence of the ala260 hRXR α mutant was verified by chain-termination sequencing.

In vitro kinase assay. COS-7 cells were grown in DMEM supplemented with 10% FBS. Transfections were performed by incubating 5 μg of plasmid DNA expressing the human vitamin D receptor (hVDR) (a kind gift of M. Haussler, University of Arizona, Tucson, Arizona, USA) and 5 μg of plasmid DNA encoding either wild-type hRXR α or the ala260 hRXR α mutant with 15 μg Lipofectamine, as described above. Transiently transfected cells were harvested for gel retardation assays 48 hours after transfection by washing the cells twice in PBS and then collecting them in 1 mL PBS. Cells were centrifuged at 500 g for 10 minutes at 4°C, and the pellets were resuspended in 1 mL of high-salt buffer (25 mM Tris [pH 7.5], 0.3 mM DTT, 0.1 M KCl, 20% glycerol). Cells were lysed by 3 freeze-thaw cycles and then centrifuged at 10,000 g for 15 minutes at 4°C. Cell extract (2 μg) was incubated in 1 \times MAP kinase buffer (New England Biolabs Inc., Beverly, Massachusetts, USA), 5 mM ATP, and activated recombinant MAP kinase (Calbiochem-Novabiochem Corp., Hornby, Ontario, Canada) at 30°C for 15 minutes. MAP kinase-treated cell extracts were then subjected to gel mobility shift analysis as described above.

Statistical analysis. Statistical significance was determined by

one-way ANOVA or by Student's *t* test. A probability value of *P* < 0.05 was considered to be significant.

Results

Inhibition of MAP kinase activity reverses the phenotypic resistance of ras-transformed keratinocytes to $1,25(\text{OH})_2\text{D}_3$. The role of MAP kinase activation in partial resistance of HPK1A $_{\text{ras}}$ cells to growth inhibition by $1,25(\text{OH})_2\text{D}_3$ was first analyzed using PD098059, which is a selective inhibitor of MAPKK, the enzyme directly responsible for the activation of MAP kinase (21). The effect of PD098059 on $1,25(\text{OH})_2\text{D}_3$ -dependent transactivation was assessed by transfecting HPK1A $_{\text{ras}}$ cells with the $1,25(\text{OH})_2\text{D}_3$ -sensitive CAT reporter plasmid mOP3 (20). PD098059 treatment of HPK1A $_{\text{ras}}$ cells completely reversed partial resistance to $1,25(\text{OH})_2\text{D}_3$ -dependent transactivation (Figure 1a). Treatment with PD098059 also reversed partial $1,25(\text{OH})_2\text{D}_3$ resistance of HPK1A $_{\text{ras}}$ cells observed in growth curves (Figure 1b), as well as in thymidine incorporation and formazan assays (data not shown).

Human RXR α is phosphorylated on serine 260 in ras-transformed keratinocytes. Complexes of identical mobility containing the VDR were formed in electrophoretic mobility shift assays (EMSA) using a mOP VDRE oligonucleotide and extracts of HPK1A and HPK1A $_{\text{ras}}$ cells (Figure 2a). The supershifting VDR antibody (Figure 2a, lanes 3, 6, 9, and 12) did not shift the entire complex, suggesting the presence of other heterodimers containing RXR at this site. However, a different monoclonal VDR antibody that inhibits formation of a VDR/RXR complex on the mOP VDRE has been used previously (17) and is able to inhibit formation of this

complex in its entirety, indicating that this complex contains only heterodimers of VDR/RXR. However, unlike the complexes formed in extracts of HPK1A cells (Figure 2a, lanes 4 and 7), those formed in *ras*-transformed cells could be shifted with an antibody recognizing the LBD of hRXR α only if the cells were pretreated with PD098059 (Figure 2a, lane 13). Identical results were obtained in EMSAs performed using a human osteocalcin VDRE (data not shown). Furthermore, experiments performed using a different antibody recognizing the NH₂-terminal domain of hRXR α inhibited complex formation in both HPK1A and HPK1A*ras* cell extracts,

revealing that hRXR α was indeed present in these heterodimers (Figure 2b). These results raised the possibility that hRXR α in HPK1A*ras* cells was phosphorylated by the activated MAP kinase pathway. Western analysis of extracts immunoprecipitated with an antibody recognizing the NH₂-terminus of hRXR α showed that similar levels of hRXR α protein were expressed in HPK1A and HPK1A*ras* cells (Figure 2c, top). Unlike the VDR, which contains no MAP kinase consensus sequences, hRXR α contains 2 MAP kinase sites, one at threonine 82 and another at serine 260 (ser260). Human RXR α was phosphorylated to similar degrees on threonine residues in HPK1A and HPK1A*ras* cells (Figure 2c, middle). In contrast, phosphorylation on serine residues was observed only in HPK1A*ras* cells. Threonine phosphorylation was not affected by treatment of cells with PD098059, whereas serine phosphorylation in HPK1A*ras* cells was completely eliminated (Figure 2c, lane 4, middle and bottom). Western analysis with anti-phosphoserine and anti-phosphothreonine antibodies did not detect any differences in VDR phosphorylation in HPK1A and HPK1A*ras* cells (data not shown).

Further evidence for the role of MAP kinase-dependent phosphorylation of hRXR α in modulating 1,25(OH)₂D₃-dependent transactivation came from experiments in HPK1A cells transiently transfected with a constitutively active MAPKK expression vector (Figure 3a). VDR-dependent transactivation was observed only at high concentrations of 1,25(OH)₂D₃ in HPK1A cells transfected with constitutively active MAPKK, whereas transactivation in cells transfected with an inactive MAPKK control was unaffected. Moreover, expression of constitutively active MAPKK in HPK1A cells induced phosphorylation on serine of hRXR α (Figure 3b, lane 2, top).

Mutation of ser260 of hRXR α abolishes partial resistance to 1,25(OH)₂D₃ in ras-transformed keratinocytes. A mutant hRXR α was constructed with the putative phosphorylation site, ser260, mutated to an alanine (ala260). Gene transfer experiments were performed in which HPK1A*ras* cells were transfected with vectors expressing either the wild-type or the ala260 mutant hRXR α . Whereas HPK1A*ras* cells overexpressing the wild-type hRXR α remained resistant to 1,25(OH)₂D₃, this resistance was abolished in HPK1A*ras* cells overexpressing the mutant construct (Figure 4a). The mutant was tagged by cloning it into a plasmid, pCDNA3HA, which possesses a synthetic oligonucleotide that encodes the HA epitope tag. Nuclear extracts were prepared from the HPK1A*ras* cells expressing the tagged mutant and immunoprecipitated with an antibody to the HA epitope. Blots probed with anti-phosphoserine antibody revealed that the mutant hRXR α protein was not phosphorylated on serine, unlike wild-type hRXR α (Figure 4b), and firmly established that ser260 was the target site of MAP kinase in the hRXR α .

In vitro phosphorylation of hRXR α by MAP kinase prevents the supershift of the VDR/RXR complex on gel retardation assays. An *in vitro* kinase assay was performed and revealed that extracts of COS-7 cells transiently transfected with wild-type hRXR α and treated with recombinant MAP kinase formed complexes on a VDRE that could not be supershifted with the anti-RXR antibody (Figure 4c, lane 3).

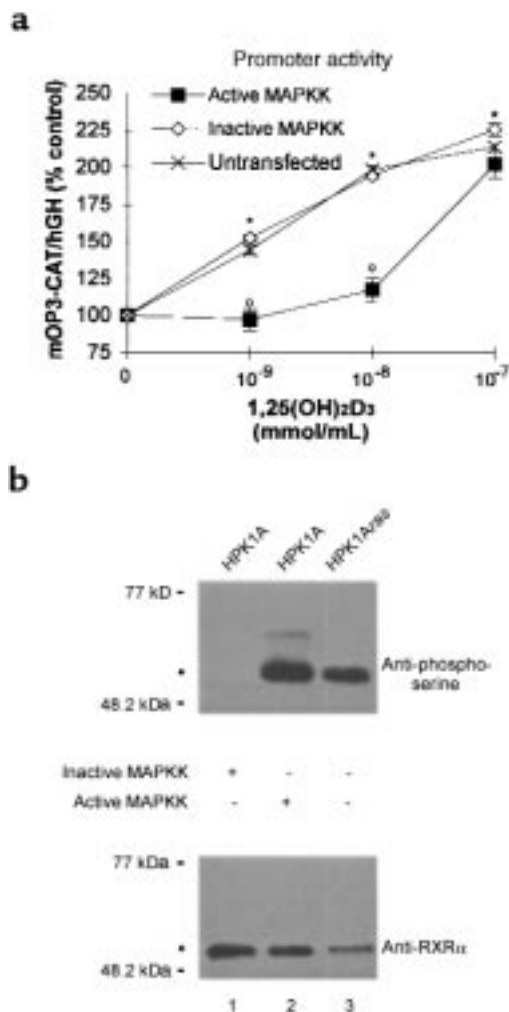


Figure 3 MAP kinase-dependent phosphorylation of hRXR α in HPK1A and HPK1A*ras* cells. (a) HPK1A cells were transfected with activated or inactivated MAPKK expression plasmids and were treated with increasing concentrations of 1,25(OH)₂D₃ and 5% charcoal-stripped FBS. CAT activity was assayed after 24 hours, as in Figure 1a. Asterisks indicate a significant difference from vehicle-treated control values, whereas open circles indicate a significant difference between cells overexpressing active and inactive MAPKK. (b) Nuclear extracts prepared from these cells were immunoprecipitated with an anti-RXR α antibody, followed by SDS-PAGE and Western blotting. The blots were treated with an anti-phosphoserine antibody or an anti-RXR α antibody to confirm equal loading of the protein as indicated. The diamond indicates the presence of the hRXR α protein.

Figure 4

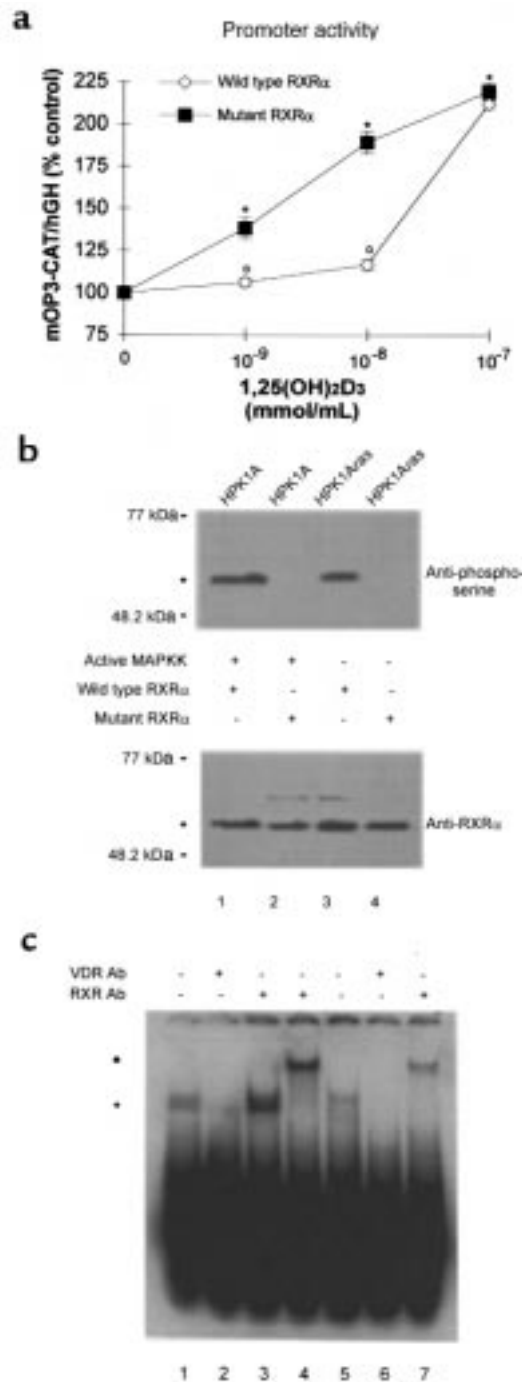
Mutation of hRXR α at ser260 abrogates MAP kinase-dependent phosphorylation and eliminates partial resistance to 1,25(OH) $_2$ D $_3$. (a) HPK1*Aras* cells were transfected with vectors expressing either wild-type hRXR α or hRXR α containing the ala260 mutation. Cells were treated with 5% charcoal-stripped FBS and increasing concentrations of 1,25(OH) $_2$ D $_3$ as indicated. CAT activity was assayed after 24 hours as in Figure 1a. Asterisks indicate a significant difference from vehicle-treated control values, whereas open circles indicate a significant difference between cells overexpressing wild-type and mutant hRXR α . (b) HPK1A cells transfected with HA-tagged wild-type (lane 1) or mutant (lane 2) hRXR α and the constitutively active MAPKK expression plasmid, and HPK1*Aras* cells transfected with HA-tagged wild-type (lane 3) or mutant (lane 4) hRXR α , were extracted and immunoprecipitated with an anti-HA antibody. The immunoprecipitates were analyzed by Western blotting. The membrane was incubated with an anti-phosphoserine antibody or with an anti-RXR α antibody as a control for hRXR α levels. The diamond indicates the presence of the hRXR α protein. (c) A mOP VDRE was 32 P labeled and incubated with COS-7 cell extracts from cells transfected with wild-type hRXR α and hVDR and treated (lanes 1-3) or not (lane 4) with recombinant MAP kinase, or extracts from cells transfected with the ala260 hRXR α mutant and hVDR, and treated with recombinant MAP kinase (lanes 5-7). The diamond indicates the presence of putative VDR/RXR complexes, and the circle indicates supershifted complexes containing RXR.

Furthermore, extracts of COS-7 cells transfected with the ala260 hRXR α mutant and treated with recombinant MAP kinase formed complexes in gel retardation assays that could be shifted in the presence of the anti-RXR antibody (Figure 4c, lane 7). This result firmly established that phosphorylation of hRXR α at ser260 by MAP kinase renders the VDR/RXR complex unshiftable by the anti-RXR antibody on gel retardation assays.

Ras-transformed keratinocytes also exhibit a resistance to the RXR-specific ligand LG1069. LG1069 specifically binds RXR and promotes RXR/RXR homodimers. This ligand was used to determine if the phosphorylation event observed in *ras*-transformed keratinocytes affected other signaling pathways involving RXR, in addition to vitamin D signaling. Growth of the HPK1A cells, as determined by cell count or formazan assay, was inhibited by LG1069, whereas the HPK1*Aras* cells were completely resistant to the growth inhibitory action of LG1069 (Figure 5, a and b). This resistance phenomenon of the *ras*-transformed cells to LG1069 was completely reversed following treatment with the MAPKK inhibitor PD098059. Hence, phosphorylation of hRXR α by MAP kinase does influence signal transduction pathways in the *ras*-transformed keratinocytes involving nuclear receptors other than the VDR.

Discussion

Several cell lines derived from *in vivo* tumors have demonstrated resistance to the growth inhibitory influences of 1,25(OH) $_2$ D $_3$ (13-15, 17). It is not uncommon to observe the spontaneous development of resistance to a growth inhibitory substance in human cancer. The occurrence of 1,25(OH) $_2$ D $_3$ resistance reported in this study is not associated with a genetic alteration of the VDR (17). In addition, Western analyses showed that hRXR α is expressed at similar levels in HPK1A and HPK1*Aras* cells, suggesting that partial resistance to 1,25(OH) $_2$ D $_3$ in HPK1*Aras* cells

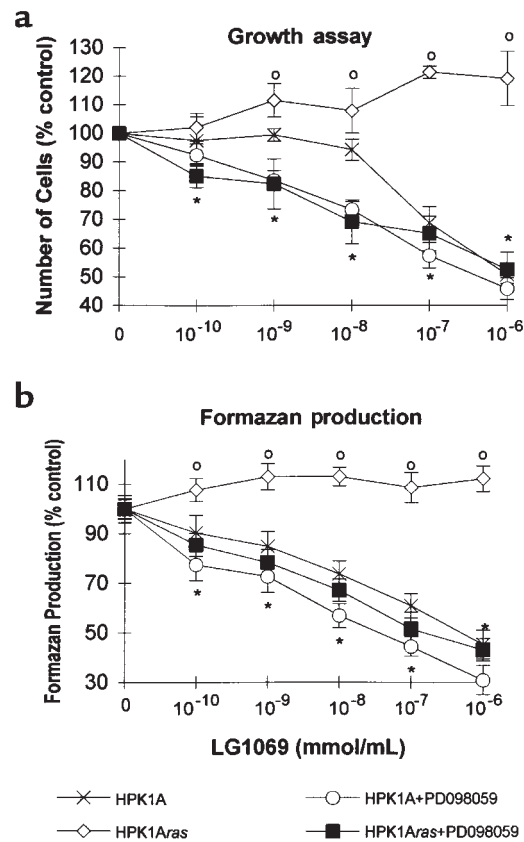


does not arise from increased degradation of hRXR α . Instead, this study indicates that transcriptional activation by the VDR is attenuated through phosphorylation of the heterodimeric partner hRXR α by MAP kinase. This post-translational modification is mediated through overexpression of the *ras* oncoprotein, which induces the Ras-Raf-MAP kinase cascade in HPK1*Aras* cells and stimulates phosphorylation of hRXR α on ser260.

The resistance phenomenon observed in HPK1*Aras* cells not only affects cell growth, but also affects endogenous genes known to be important in the control of cell growth and differentiation (13, 25). In par-

Figure 5

Effect of an RXR-specific ligand, LG1069, on HPK1A and HPK1A*ras* cells. (a) Cells were treated with increasing concentrations of LG1069 (10^{-10} to 10^{-6} M) in the absence or presence of 25 μ M PD098059 and 5% charcoal-stripped FBS. Cell numbers were expressed as percent of DMSO vehicle-treated control cell numbers after 96 hours. Each value represents the mean \pm SD of 3 determinations. Asterisks indicate a significant difference from vehicle-treated control values, whereas open circles indicate a significant difference between HPK1A and HPK1A*ras* cells at the LG1069 concentration indicated. (b) MTT-Microculture Tetrazolium Assay for Cell Growth (Promega Corp.). Cells were treated as in a, and formazan production was monitored by absorbance as described in Methods. Each value represents the mean \pm SD of 4 determinations. Asterisks indicate a significant difference from vehicle-treated control values, whereas open circles indicate a significant difference between HPK1A and HPK1A*ras* cells at the LG1069 concentration indicated.



ticular, the observed resistance to parathyroid hormone-related peptide (PTHrP) expression by HPK1A*ras* cells detected previously (25) is especially relevant because PTHrP was found to be antiproliferative (26) and prodifferentiative (26) in HPK1A cells.

Although our present study defines a specific cellular model, the potential ramifications of these findings are extensive. $1,25(\text{OH})_2\text{D}_3$ inhibits the growth of tumors derived from a variety of tissues (27). Given the high frequency of *ras* activation in human cancers, phosphorylation of hRXR α may be a common mechanism by which cancer cells escape the growth inhibitory effects of the endogenously produced hormone $1,25(\text{OH})_2\text{D}_3$. Moreover, hRXR α functions in a wide variety of transcriptional responses through its heterodimerization with several nuclear receptors in addition to the VDR, including thyroid hormone receptors (TRs), retinoic acid receptors (RARs), peroxisome proliferator-activated receptors (PPARs), and several orphan receptors (18). Signaling through heterodimeric partners of RXRs other than the VDR can modulate cellular growth and differentiation. For example, PPAR α promotes differentiation of adipocytes (28) and has recently been shown to inhibit growth of colon cancer cell lines (29). Consequently, disruption of signal transduction by members of this superfamily of receptors could have multiple consequences for the control of cell growth and differentiation.

Through studies with the RXR-specific ligand LG1069, we have demonstrated that this MAP kinase phosphorylation of hRXR α in *ras*-transformed keratinocytes affects another signaling pathway that relies on the RXR. The *ras*-transformed cells are not only resistant to the growth

inhibitory action of $1,25(\text{OH})_2\text{D}_3$ but are also resistant to the growth inhibitory action of LG1069. Furthermore, we have shown previously in gel retardation assays a similar phenomenon in thyroid hormone signaling: the RXR antibody also fails to shift TR/hRXR α heterodimers formed on thyroid hormone response elements (TREs) (17).

Targeting hRXR α confers a growth advantage to cells that have been transformed by *ras* by affecting several signaling pathways that depend on RXR. Because $1,25(\text{OH})_2\text{D}_3$ is a powerful inhibitor of cell growth, it is beneficial for a cancer cell to resist this inhibitory pathway. In addition, by affecting the common link among many nuclear receptor pathways, cells transformed by *ras* are able to influence signal transduction in a much broader sense. Although this study focused on $1,25(\text{OH})_2\text{D}_3$ action, we have demonstrated that phosphorylation of hRXR α on ser260 also affects RXR/RXR signaling, indicating that this is a common mechanism used by *ras*-transformed keratinocytes to interfere with hormone signaling.

Gel retardation assays performed in the presence of specific antibodies raise the possibility that phosphorylation of hRXR α on ser260 induces a conformational change within the receptor that causes attenuation of ligand-dependent transactivation. An anti-RXR antibody with an epitope spanning ser260 was unable to recognize and therefore supershift the VDR/RXR complex from the *ras*-transformed keratinocytes (Figure 2a), whereas an anti-RXR antibody possessing an epitope in the NH₂-terminal domain of hRXR α remote from ser260 was able to recognize and therefore inhibit the formation of a VDR/RXR complex on the ³²P-labeled VDRE (Figure 2b). Phosphorylation of ser260 occurs at a critical site close to regions of

potential coactivator interaction with the RXR (30, 31). Taken together, these results indicate that a conformational change within the LBD of hRXR α probably occurs following phosphorylation of ser260.

Indeed, several adaptor proteins that can act as coactivators for nuclear receptors have been identified recently (32), including the coactivator SRC-1 (steroid receptor coactivator-1), which can interact with the LBD of steroid receptors to increase ligand-dependent transcriptional activation (33). We cannot exclude the possibility that phosphorylation of ser260 of hRXR α may disrupt the interaction of this heterodimeric partner with SRC-1 or other coactivators and thus attenuate ligand-dependent transactivation. Irrespective of the exact consequences of RXR phosphorylation on RXR conformation, our study has identified a new target of *ras* activation that may play a critical role in malignant transformation.

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- McClay, E.F., Albright, K.D., Jones, J.A., Christen, R.D., and Howell, S.B. 1994. Tamoxifen delays the development of resistance to cisplatin in human melanoma and ovarian cancer cell lines. *Br. J. Cancer*. **70**:449-452.
- Zachos, G., and Spandidos, G.A. 1997. Expression of *ras* proto-oncogenes: regulation and implications in the development of human tumors. *Crit. Rev. Oncol. Hematol.* **26**:65-75.
- Davis, R.J. 1993. The mitogen-activated protein kinase signal transduction pathway. *J. Biol. Chem.* **268**:14553-14556.
- Weigel, N.L. 1994. Receptor phosphorylation in molecular biology intelligence unit. In *Mechanism of steroid hormone regulation of gene transcription*. M-J. Tsai, editor. R.G. Landes Co. Austin, Texas. 93-110.
- Haussler, M.R., et al. 1998. The nuclear vitamin D receptor: biological and molecular regulatory properties revealed. *J. Bone Miner. Res.* **13**:325-349.
- Zhou, J.Y., et al. 1990. 1,25-dihydroxy-16-ene-23-yne-vitamin D₃ prolongs survival time of leukemic mice. *Proc. Natl. Acad. Sci. USA.* **87**:3929-3932.
- Buras, R.R., et al. 1994. Vitamin D receptors in breast cancer cells. *Breast Cancer Res. Treat.* **31**:191-202.
- Yabushita, H., Hirata, M., Noguchi, M., and Nakanishi, M. 1996. Vitamin D receptor in endometrial carcinoma and the differentiation-inducing effect of 1,25-dihydroxyvitamin D₃ on endometrial carcinoma cell lines. *J. Obstet. Gynaecol. Res.* **22**:529-539.
- Kornfehl, J., Formanek, M., Temmel, A., Knerer, B., and Willheim, M. 1996. Antiproliferative effects of the biologically active metabolite of vitamin D₃ on head and neck squamous cell carcinoma cell lines. *Eur. Arch. Otorhinolaryngol.* **253**:341-344.
- Higashimoto, Y., et al. 1996. 1- α ,25-dihydroxyvitamin D₃ and all-trans retinoic acid inhibit the growth of a lung cancer cell line. *Anticancer Res.* **16**:2653-2659.
- Hedlund, T.E., Moffat, K.A., and Miller, G.J. 1996. Vitamin D receptor expression is required for growth modulation by 1- α , 25-dihydroxyvitamin D₃ in the human prostatic carcinoma cell line ALVA-31. *J. Steroid Biochem. Mol. Biol.* **58**:277-288.
- Botling, J., et al. 1996. Vitamin D₃ and retinoic acid-induced monocytic differentiation: interactions between the endogenous vitamin D₃ receptor, retinoic acid receptors, and retinoid X receptors in U-937 cells. *Cell Growth Differ.* **7**:1239-1249.
- Sebag, M., Henderson, J., Rhim, J., and Kremer, R. 1992. Relative resistance to 1,25-dihydroxyvitamin D₃ in a keratinocyte model of tumor progression. *J. Biol. Chem.* **267**:12162-12167.
- Zugmaier, G., et al. 1996. Growth inhibitory effects of vitamin D analogues and retinoids on human pancreatic cancer cells. *Br. J. Cancer.* **73**:1341-1346.
- Narvaez, C.J., Vanweelden, K., Byrne, I., and Welsh, J. 1996. Characterization of a vitamin D₃-resistant MCF-7 cell line. *Endocrinology.* **137**:400-409.
- Dürst, M., Gallahan, D., Jay, G., and Rhim, J.S. 1989. Glucocorticoid-enhanced neoplastic transformation of human keratinocytes by human papilloma virus type 16 and an activated *ras* oncogene. *Virology.* **173**:767-771.
- Solomon, C., Sebag, M., White, J.H., Rhim, J., and Kremer, R. 1998. Disruption of vitamin D receptor-retinoid X receptor heterodimer formation following *ras* transformation of human keratinocytes. *J. Biol. Chem.* **273**:17573-17578.
- Mangelsdorf, D.J., and Evans, R.M. 1995. The RXR heterodimers and orphan receptors. *Cell.* **83**:841-850.
- Dürst, M., Dzarlieva-Petrusevska, R.T., Boukamp, P., Fusenig, N.E., and Gissman, L. 1987. Molecular and cytogenetic analysis of immortalized human primary keratinocytes obtained after transfection with human papilloma virus type 16 DNA. *Oncogene.* **1**:251-256.
- Ferrara, J., McCuaig, K.A., Hendy, G.N., Uskokovic, M., and White, J.H. 1994. Highly potent transcriptional activation by 16-ene derivatives of 1,25-dihydroxyvitamin D₃. *J. Biol. Chem.* **269**:2971-2981.
- Dudley, D.T., Pang, L., Decker, S.J., Bridges, A.J., and Saltiel, A.R. 1995. A synthetic inhibitor of the mitogen activated protein kinase cascade. *Proc. Natl. Acad. Sci. USA.* **92**:7686-7689.
- Banville, D., and Boie, Y. 1990. Retroviral long terminal repeat is the promoter of the gene encoding the tumor-associated calcium-binding protein oncomodulin in the rat. *J. Mol. Biol.* **207**:481.
- Kozzewski, N., Reinhardt, T., and Horst, R. 1996. Vitamin D receptor interactions with the murine osteopontin response element. *J. Steroid Biochem.* **59**:377-388.
- Ho, S.N., Hunt, H.D., Horton, R.M., Pullen, J.K., and Pease, L.R. 1989. Site directed mutagenesis by overlap extension using the polymerase chain reaction. *Gene.* **77**:51-59.
- Henderson, J., Sebag, M., Rhim, J., and Kremer, R. 1991. Dysregulation of parathyroid hormone-like peptide expression and secretion in a keratinocyte model of tumor progression. *Cancer Res.* **51**:6521-6528.
- Kaiser, S.M., Sebag, M., Rhim, J.S., Kremer, R., and Goltzman, D. 1994. Antisense-mediated inhibition of parathyroid hormone-related peptide production in a keratinocyte cell line impedes differentiation. *Mol. Endocrinol.* **8**:139-147.
- Niles, R.M. 1995. Use of vitamins A and D in chemoprevention and therapy of cancer: control of nuclear receptor expression and function. *Adv. Exp. Med. Biol.* **375**:1-15.
- Kliwer, S.A., et al. 1995. A prostaglandin J₂ metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation. *Cell.* **83**:813-819.
- Sarraf, P., et al. 1998. Differentiation and reversal of malignant changes in colon cancer through PPAR γ . *Nat. Med.* **4**:1046-1052.
- Westin, S., et al. 1998. Interactions controlling the assembly of nuclear-receptor heterodimers and co-activators. *Nature.* **395**:199-202.
- Henttu, P.M., Kalkoven, E., and Parker, M.G. 1997. AF-2 activity and recruitment of steroid receptor coactivator 1 to the estrogen receptor depend on a lysine residue conserved in nuclear receptors. *Mol. Cell. Biol.* **17**:1832-1839.
- Meyer, M.E., et al. 1989. Steroid hormone receptors compete for factors that mediate their enhancer function. *Cell.* **57**:433-442.
- Nolte, R.T., et al. 1998. Ligand binding and coactivator assembly of the peroxisome proliferator-activated receptor- γ . *Nature.* **395**:137-143.