Phosphorylation of the Human Retinoid X Receptor α at Serine 260 Impairs Coactivator(s) Recruitment and Induces Hormone Resistance to Multiple Ligands*

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The retinoid X receptor α (RXRα) is a member of the nuclear receptor superfamily that regulates transcription of target genes through heterodimerization with several partners, including peroxisome proliferator-activated receptor, retinoic acid receptor, thyroid receptor, and vitamin D receptor (VDR). We have shown previously that signaling through VDR-RXRα heterodimers was attenuated in ras-transformed keratinocytes due to phosphorylation of serine 260 of the RXRα via the activated Ras-Raf-MAPK cascade in these cells. In this study we demonstrate that phosphorylation at serine 260, a site located in the omega loop-AF-2 interacting domain of RXRα, inhibits signaling through several heterodimeric partners of the RXRα. The inhibition of signaling results in reduced transactivational response to ligand presentation and the reduced physiological response of growth inhibition not only of 1,25-dihydroxyvitamin D3 but also of retinoic acid receptor α ligands and LG1069 (an RXRα ligand). This partial resistance to ligands could be reversed by inhibition of MAPK activity or by overexpression of a non-phosphorylatable RXRα mutant at serine 260 (RXRα Ser260 → Ala). Importantly, phosphorylation of RXRα at serine 260 impaired the recruitment of DRIP205 and other coactivators to the VDR-RXRα complex. Chromatin immunoprecipitation and pulldown assays further demonstrated that coactivator recruitment to the VDR-RXRα complex could be restored by treatment with a MAPK inhibitor. Our data suggest that phosphorylation at serine 260 plays a critical role in inducing hormone resistance of RXRα-mediated signaling likely through structural changes in the H1-H3 omega loop-AF2 coactivator(s) interacting domain.

Ras activation has been detected in numerous cancers, including hepatocellular carcinoma, colorectal carcinomas, breast tumors, leukemias, and squamous tumors of the head and neck (1). Activation of the Ras-Raf-mitogen-activated protein kinase (MAPK-ERK)2 signaling cascade leads to phosphorylation of downstream targets, including some nuclear receptors, resulting in a mechanism for receptor control (2). Signaling through nuclear receptors is required in many aspects of cellular functions (3). Several nuclear receptors require heterodimerization with the retinoid X receptor (RXR) to fulfill their signaling functions (4). Upon dimerization, the receptors recognize and bind bipartite regions of promoters of target genes, known as response elements, involving a discrete DNA binding domain within the receptors (5).

HPK1Aras, a ras-transformed keratinocyte cell line, is resistant to the growth-inhibitory effects of 1,25-dihydroxyvitamin D3 (1,25(OH)2D3) (6), as are several pancreatic (7) and breast carcinoma cell lines (8). The phosphorylation of RXRα at serine 260 caused the resistance of the HPK1Aras cell line to the anti-proliferative effects of 1,25(OH)2D3 (9) as well as resistance to the antiproliferative effect of all-trans-retinoic acid (ATRA) in hepatocellular carcinoma cells (10) This serine lies within a PSSP MAPK recognition sequence and the phosphorylation is MAPK kinase (MAPKK)-dependent (9). The latter study also raised the possibility that RXR phosphorylation on serine 260 induces conformational changes of the VDR-RXR complex. Ser-260 is located at a critical site in the omega loop between H1 and H3 helices of the ligand binding domain of RXRα (11) and in close spatial proximity to regions of potential coactivator-corepressor interactions with the RXR (12). In the present study we show that RXRα phosphorylation at Ser-260 affects several signaling pathways involving recruitment of partners other than the VDR. Furthermore, we demonstrate that coactivator recruitment to the VDR-RXR complex is disrupted in HPK1Aras cells and can be restored by treatment with a MAPKK inhibitor or overexpression of a non-phosphorylatable human RXRα mutant at serine 260.

MATERIALS AND METHODS

Cell Culture and Transfections—HPK1A and HPK1Aras cell lines were described previously (13, 14). The HPK1A cells are

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2 The abbreviations used are: MAPK, mitogen-activated protein kinase; ERK, extracellular signal-regulated kinase; RXR, retinoid X receptor; 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; ATRA, all-trans-retinoic acid; MAPK, MAPK kinase; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; CAT, chloramphenicol acetyl transferase; HA, hemagglutinin;RAR, retinoic acid receptor; PPAR, peroxisome proliferator-activated receptor; RXRE, RXR element; CHIP, chromatin immunoprecipitation; VDRE, vitamin D receptor element; WT, wild type; SRC1, steroid receptor coactivator 1; TTNPB, 1-[2-(5,6,7,8-tetrahydro-5,5,8,8-tetramethyl-2-naphthyl)yl]-1-propenyl] benzoic acid; LBD, ligand binding domain; mOP, mouse osteopontin.
non-tumor forming, whereas HPK1Aras cells result in squamous cell carcinomas upon transplantation into nude mice (14). Cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) (Invitrogen) supplemented with 10% fetal bovine serum (FBS). Cells were plated at a density of 4 × 10^3 cells per well in 24-well plates for growth curves, grown to 60% confluency in 6-well plates for chloramphenicol acetyl transferase (CAT) and β-galactosidase reporter assays, and grown to 60% confluency in 100-mm² plates for cell and nuclear extracts. In some experiments cells in 100-mm² plates were transfected with the non-phosphorylatable RXRα Ser-260 → Ala (9) or vector alone (pCDNA 3.1). Human RXRα Ser-260 → Ala (9) was subcloned into the EcoRI site of pCDNA 3.1 expression vector (Invitrogen). In some experiments wild-type and human RXRα Ser-260 → Ala mutants were subcloned into the plasmid pCDNA3HA, which contains a plasmid encoding the HA epitope (9). Transfections were performed by incubating 5 μg of plasmid DNA with 15 μl of FuGene 6 transfection reagent (Roche Applied Science) in fresh serum-free Opti-MEM medium (Invitrogen) and adding 10% charcoal-stripped FBS after 6 h of incubation. After 24 h of transfection the medium was replaced with fresh DMEM containing 5% charcoal-stripped FBS and 1,25(OH)₂D₃ at the concentrations indicated or with vehicle.

Cellular Extracts—24 h later, cellular or nuclear extracts were prepared as described below. Cellular extracts were prepared by lysing HPK1A and HPK1Aras cells using a triple detergent lysis buffer (50 mM Tris/HCl, pH 8.5, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% sodium deoxycholate, Complete protease inhibitor tablet (Roche Applied Science)).

Nuclear Extracts—Nuclear extracts were prepared by scraping the cells in phosphate-buffered saline, washing the cells with phosphate-buffered saline, and then resuspending the cells in 500 μl of lysis buffer (10 mM Tris (pH 7.9), 10 mM KCl, 0.1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, a complete protease inhibitor tablet (Roche Applied Science), 1 mM sodium orthovanadate, 20 mM NaF) and incubation on ice for 15 min. 25 μl of 10% Nonidet P-40 was added, and the samples were vortexed for 15 s. The samples were spun, and the pellets were resuspended in a nuclear buffer (20 mM Tris (pH 7.9), 400 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM dithiothreitol, a mini Complete protease inhibitor tablet, 1 mM sodium orthovanadate, 20 mM NaF). The samples were placed in a microcentrifuge tube shaker for 1 h at 4 °C, then spun, and the supernatant was aliquoted and stored at −80 °C. In some experiments cells were transfected with the RXRα mutant RXRα Ser-260 → Ala or the pCDNA vector alone driven by a cytomegalovirus promoter as described above. In other experiments when HPK1A or HPK1Aras cells in 100-mm² plates reached 60% confluency, medium was replaced with fresh DMEM containing 10% charcoal-stripped FBS and 1,25(OH)₂D₃ (10⁻⁷ M) or vehicle (ethanol) was added to cells for another 24 h. MAPKK inhibitors PD098059 (Sigma-Aldrich) or UO126 (Fisher Scientific, Mississauga, Ontario, Canada) or vehicle (Me₂SO) were added 30 min prior to 1,25(OH)₂D₃ addition and incubated for 24 h prior to extraction as described above.

**Synthetic Oligonucleotides Used**—The oligonucleotides were as follows: mOP VDRE: 5’-GTACAAGGGTTCAAGGAAGTTCAAGGTCTCTTA-3’; TRE (DR-4 consensus oligonucleotide, Santa Cruz Biotechnologies, Santa Cruz, CA) 5’-AGCTCTAGGTACACAGGAGTCAGAGCT-3’; RARE (DR-5 wild-type oligonucleotide, Geneka Biotech Inc., Montreal, Quebec, Canada) 5’-TGAAGTCAGAGGAGTTCATCGC-3’; PARE (wild-type oligonucleotide, Geneka) 5’-GGAATGTTCAAAGGTCTACCTCCCCT-3’; and RXRE (DR1) 5’-TCGACTGTCAAGGTACAGGTCAACATGTTCA-3’.

**Antibodies**—The RXRα, VDR C terminus (C-20), RXRα N terminus, PPARγ, TRβ, RIP140, DRIP205, ACTR, SRCI, GRIP1, p21<sup>WAF/CIP<sub>1</sub></sup>, Rb (C-15), and HA-probe (sc-805) antibodies were purchased from Santa Cruz Biotechnologies. The RXRα LBD–recognizing antibody 4X1D12 was a kind gift from Pierre Chambon (College de France, Illkirch, France).

**Western Blotting**—Protein extracts were resolved by SDS-PAGE (6–10% acrylamide), electrotransferred onto polyvinylidene difluoride membrane (Bio-Rad, Toronto, Ontario, Canada), immunoprobed, and detected using Lumiglo chemiluminescence (KPL Laboratories, Washington, D. C.). The membranes were exposed to Kodak XAR-5 film.

**Growth Assays**—At 40% confluency, cells were placed under serum-free conditions to synchronize the cells. The medium was then replaced with DMEM 5% charcoal-stripped FBS in the presence or absence of increasing concentrations of 1,25(OH)₂D₃, LG1069 (Ligand Pharmaceuticals, a kind gift of E. Allegreto) or ATRA (Sigma-Aldrich) or TTNPB (Sigma). The cells were also pretreated for 1 h with vehicle or 25 μM PD098059 (Sigma-Aldrich) and then treated with 25 μM PD098059 or vehicle during the ligand treatment. Media was replaced after 48 h. After 96 h, cells were trypsinized and counted with a coulter counter (Coulter Electronics, Lufton, United Kingdom).

**Reporter Assays**—Cells were transfected using Fugene transfection reagent (Roche Applied Science) and 4.5 μg of reporter plasmid, and 0.5 μg of β-galactosidase plasmid as an internal control for transfection efficiency in 6-well plates. Cells were treated with increasing amounts of ligand with or without 25 μM PD098059. The concentration of the MAPKK inhibitor used is consistent with its Kᵢ for the enzyme reported elsewhere (15). For all assays the cells were scraped after 24 h and washed with phosphate-buffered saline and lysed in lysis buffer (Roche Applied Science). The retinoic acid response element (RARE) reporter and the retinoid X response element (RXRE) consisted of three copies of the RARα RARE or RXRα RXRE in front of a thymidine kinase reporter driving the expression of a CAT gene (Fisher Scientific, Nepean, Ontario, Canada), and assays were performed using a CAT enzyme-linked immunosorbent assay kit (Roche Applied Science). β-Galactosidase was measured in a β-galactosidase colorimetric assay.

**Electrophoretic Mobility Gel Shift Assay**—These assays were performed as previously described (9). Briefly, nuclear extracts (5 μg) were incubated for 20 min on ice with 10⁻⁷ M ligand and 1 μg of poly(dIdC) in a binding buffer (25 mM Tris·HCl, pH 8, 5% glycerol, and 0.5 mM dithiothreitol). 5 fmol of ³²P-labeled response element oligonucleotide was added followed by 20 min of incubation at room temperature. In some cases, antibodies were added, and the mix was incubated for another 20 min at room temperature. The samples were resolved on a 5%
TABLE 1

| Promoter                                      | Annealing temperature | Location         | Primer sequence          |
|-----------------------------------------------|-----------------------|------------------|--------------------------|
| Cyclin-dependent kinase inhibitor (P21ras)     |                        |                  | 5'-CACCACTGAGCTCTCTCAC-3'|
| Cyclin C                                      |                        |                  | 5'-CAGTCAGCCTTGGAGGAAC-3'|
| Vitamin D 24 hydroxylase (CYP24)              | 66                    | -64 to -480      | 5'-GTCCAGGTCGGTACTCTG-3'  |
| Calmin (calponin-like, transmembrane) (CLMN)  | 60                    | -1522 to -1828   | 5'-AACACAGGAAATGTCTTCTCTGCC-3'|

FIGURE 1. Western blot analysis of heterodimeric partners in HPK1A and HPK1Aras cells (A) and coactivators (B). Whole cell extracts of HPK1A and HPK1Aras cells were analyzed by Western blotting. A, membranes were incubated with an antibody specific to the vitamin D receptor (lanes 1 and 2), the retinoic X receptor α (lanes 3 and 4), the retinoic acid receptor α (lanes 5 and 6), thyroid receptor β (lanes 7 and 8), and the peroxisomes proliferators-activated receptor γ (lanes 9 and 10). B, membranes were incubated with an antibody specific for DRIP205 (lanes 1 and 2), RIP140 (lanes 3 and 4), ACTR (lanes 5 and 6), SRC1 (lanes 7 and 8), and GRIP1 (lanes 9 and 10). The arrow represents the position of the receptor, coactivator, or β-tubulin.

The pulldown assay was performed by incubating nuclear extracts from cells treated with 1,25(OH)₂D₃ (10⁻⁷ M) or 1,25(OH)₂D₃ (10⁻⁸ M) or both and prepared as described above under nuclear extracts preparation. Nuclear extracts (1000 μg) were incubated with 2 μg of poly(dIdC) and 1 μg of double-stranded biotinylated mOP adjusted to a final volume of 500 μl with HKMG buffer (10 mm Hepes, pH 7.9, 100 mm KCl, 5 mm MgCl₂, 10% glycerol, 1 mm dithiothreitol, 1.5% Nodinet P-40, 1 mm sodium orthovanadate, 20 mm NaF with a complete protease inhibitor tablet (Roche Applied Science) for 1 h at 4 °C and then further incubated with 150 μl of 4% streptavidin-agarose beads (Sigma-Aldrich) for another 3 h at 4 °C. The optimal concentration of the biotinylated probe was assessed by first using increasing concentrations of the probe to reach maximal binding. All subsequent experiments were done using this probe concentration.
At the end of incubation the tube was centrifuged at 5000 × g in a microcentrifuge for 30 s. The supernatant was removed, and the pellet washed four times with 1 ml of iced phosphate-buffered saline. The pellet was then resuspended in 500 μl of Laemmli buffer and boiled for 5 min to elute protein and centrifuge at 5000 × g in a microcentrifuge for 30 s. The sample was
placed on a 6–10% SDS-PAGE gel for further separation on a polyvinylidene difluoride nitrocellulose membrane (Bio-Rad) and immunoblotting using specific antibodies. Following incubation with horseradish peroxidase-conjugated secondary antibody, the proteins were viewed using Lumiglo chemiluminescence (KPL Laboratories, Washington D. C.). The membranes were exposed to Kodak XAR-5 film.

Chromatin Immunoprecipitation Assays—ChIP assays were performed using the Upstate Biotechnology Assay (Charlottesville, VA) according to the manufacturer’s instructions. HPK1Aras cells were cultured in DMEM supplemented with 10% FBS to 40–50% confluency. Cells were synchronized in serum-free DMEM overnight. Medium was then replaced with DMEM supplemented with 5% charcoal-stripped FBS and treated with 10−6 M U0126 (Promega, Madison, WI) or vehicle (Me2SO), before addition of 10−7 M 1,25(OH)2D3 for 4 h. The concentration of U0126 used is consistent with its KD for the enzyme reported elsewhere (15).

Genomic DNA was then cross-linked by adding 37% formaldehyde directly to the medium to a final concentration of 1% and the incubation continued at 37 °C for 15 min. Cells were washed with ice-cold phosphate-buffered saline containing a protease inhibitor (mini complete protease inhibitor tablet, Roche Applied Science), collected, and lysed in SDS lysis buffer containing a protease inhibitor. The bulk chromatin was then sonicated to obtain DNA fragments between 200 and 500 bp. Sonicated chromatin was then pre-treated with protein A-agarose/salmon sperm DNA (50% vol/vol) for 1 h with 10−6 M U0126 (Promega, Madison, WI) or vehicle (Me2SO), before addition of 10−7 M 1,25(OH)2D3 for 4 h. The concentration of U0126 used is consistent with its KD for the enzyme reported elsewhere (15).

FIGURE 2. Electrophoretic mobility shift assays of RXRα-homodimeric and heterodimeric partner complex formation and response element binding. A, a mOP VDR was 32P-labeled and incubated with 6 μg of nuclear extracts from HPK1A cells (lanes 1–6), HPK1Aras cells (lanes 7–12), or HPK1Aras cells transfected with a mutated RXRα, RXRα Ser-260 → Ala (lanes 13–14). An open circle indicates the formation of a VDR/RXRα complex in the presence of 1,25(OH)2D3 (lanes 1–14). Addition of an antibody specific for VDR was used to identify the presence of VDR in the complex (lanes 2 and 8). A closed circle indicates a supershift in the presence of a VDR (C-20) antibody. A closed square indicates a supershift in the presence of the RXRα N-terminal antibody, and a closed circle indicates a supershift in the presence of the RXRα LBD antibody. B, a retinoic X receptor response element (RXRE) was 32P-labeled and incubated with HPK1A (lanes 1–5) and HPK1Aras (lanes 6–10) extracts in the presence of the RXRα specific ligand LG1069. An open circle indicates the presence of the RXRα-homodimeric complex RXRα-RXRα (lanes 1–12). A closed diamond indicates a supershift in the presence of an RXRα N-terminal antibody, and a closed square indicates a supershift in the presence of an RXRα LBD antibody. C, an RARE was incubated with HPK1A and HPK1Aras extracts in the presence of the RAR-specific ligand ATRA. An open circle indicates the presence of the heterodimeric complex RARα-RXRα (lanes 1–14). Addition of an antibody specific for RARα was used to identify the presence of RARα in the complex formed (lanes 2 and 8). A closed circle indicates the supershift in the presence of an RARα antibody. A closed circle indicates a supershift in the presence of RXRα N-terminal antibody, and a closed circle indicates a supershift in the presence of an RXRα LBD antibody. D, a thyroid hormone receptor response element element was incubated with extracts from HPK1A and HPK1Aras cells in the presence of thyroid hormone. An open circle indicates the presence of the heterodimeric complex TRβ/RXRα (lanes 1–14). Addition of an antibody specific for TRβ was used to identify the presence of TRβ in the complex formed (lanes 2 and 8). Note that the addition of the TRβ antibody inhibits complex formation in both HPK1A (lane 2) and HPK1Aras (lane 8) as indicated by the open circle. A closed diamond indicates a supershift in the presence of the RXRα N-terminal antibody, and a closed square a supershift in the presence of the RXRα LBD antibody. E, a PPARY was incubated with extracts from HPK1A and HPK1Aras cells in the presence of the specific PPARY and coactivators. An open circle indicates the presence of the heterodimeric complex PPARY-RXRα (lanes 1–14). Addition of an antibody specific for PPARY was used to identify the presence of PPARY in the complex formed (lanes 2 and 8). A closed circle indicates the supershift in the presence of a PPARY antibody. A closed circle indicates a supershift in the presence of the RXRα N-terminal antibody, and a closed square a supershift in the presence of an RXRα LBD antibody.

RESULTS

Expression of Heterodimeric Partners of RXRαs and Coactivators in HPK1A and HPK1Aras Keratinocytes—In this study, we performed Western blotting of whole cell extracts to identify the heterodimeric partners of RXRαs and coactivators expressed in the keratinocyte cell lines (Fig. 1). In addition to VDR (panel A, lanes 1 and 2) and RXRα (panel A, lanes 3 and 4), RARα (panel A, lanes 5 and 6), TRβ (panel A, lanes 7 and 8), and PPARY (panel A, lanes 9 and 10) were expressed in these cells. TRα and PPARY protein expression were not found in the cell lines (data not shown). We next examined the nuclear expression of coactivators using specific antibodies and found that DRIP205 and RIP140 were expressed at high levels (panel B, lanes 1–4), whereas ACTR (SRC3/AIB1/TRAM1) was expressed at low levels (panel B, lanes 5 and 6) and SRC1 (panel B, lanes 7 and 8) and GRIP1 (SRC2/TIF2) (panel B, lanes 9 and 10) could not be detected. Several bands of higher and lower molecular weight were observed for some of the receptors or cofactors. In some cases (RIP140) a band of higher molecular weight...
was observed in HPK1Aras but not in HPK1A cells. However, reprobing of these blots with only an anti-IgG antibody indicated that these bands were nonspecific. β-Tubulin expression of whole cell extracts (Abcam Inc., Cambridge, MA) was used as an indicator of protein loading.

**Gel Mobility Shift Analysis of RXR-Heterodimeric Partner Binding**—Our previous studies suggested that phosphorylation of RXRα on serine 260 induces a conformational change within the receptor (9), because the LBD antibody, which spans serine 260, was unable to supershift the VDR-RXRα complex, whereas an antibody directed at the NH2-terminal domain of RXRα could recognize this complex. In contrast an antibody directed against the VDR consistently recognized the VDR-RXR complex by an antibody against the C-terminal antibody of the LBD domain indicating that these conformational changes are reversible.

We next examined the formation of the homodimeric complex RXRα-RXRα (Fig. 2B). Nuclear extracts from HPK1A and HPK1Aras cells formed a complex with a 32P-labeled RXRE double-stranded oligonucleotide in the presence of the RXRα ligand LG1069 (Fig. 2B, lanes 1 and 6). This complex could be shifted by the addition of an antibody directed at the NH2-terminal portion of the RXRs with both HPK1A and HPK1Aras extracts (Fig. 2B, lanes 2 and 7). In contrast, and similar to the observation with the VDR-RXRα complex formation, the antibody directed at the LBD of the RXRs shifted the RXRα-RXRα complex in HPK1A extracts (Fig. 2B, lane 3) but not in HPK1Aras extracts (Fig. 2B, lane 8). The supershift could then be restored in HPK1Aras cells by either transient transfection with the mutant RXRa Ser-260 → Ala (Fig. 2B, lane 12) or pre-treatment of HPK1Aras cells with the MAPKK inhibitor PD098059 (Fig. 2B, lane 10) as compared with vector alone (Fig. 2B, lane 8). As controls addition of PD098059 to HPK1A cells had no effect on either RXRα-RXRα complex formation (Fig. 2B, lane 4) or its normal supershift (Fig. 2B, lane 5). These data therefore indicate that Ser-260 phosphorylation in HPK1Aras extracts do not affect homodimerization of the RXR-RXR complex but alters its recognition by a specific antibody raised against the LBD.

The next set of experiments was aimed at determining the consequences of RXRα phosphorylation at Ser-260 on the other heterodimeric partners of the RXRα. Similar experiments were performed with specific response elements and antibodies directed at the RARα, TRβ, and PPARγ. Addition of specific ligands to the RARα (ATRA), TRβ (Tamoxifen), and PPARγ (troglitazone) resulted in complex formation recognized by 32P-labeled RARE (Fig. 2C, lanes 1 and 7), 32P-labeled thyroid hormone response element (Fig. 2D, lanes 1 and 7), and 32P-labeled PPRE (Fig. 2E, lanes 1 and 7). The specificity of the complexes was determined by addition of specific antibodies directed at RARα (Fig. 2C, lanes 2 and 8), TRβ (Fig. 2D, lanes 2 and 8), and PPARγ (Fig. 2D, lanes 2 and 8). Note that TRβ recognizes the receptor A/B domain and abrogates the binding of the complex with TRE. In contrast, the RARα and PPARγ antibodies induce a supershift of the heterodimeric complex. Similar to the observations in HPK1A and HPK1Aras extracts using an NH2-terminal antibody against the RXRs, a supershift of the RARα-RXRα complex (Fig. 2C, lanes 3 and 9), TRβ-RXRα (Fig. 2D, lanes 3 and 9), and the PPARγ-RXRα (Fig. 2E, lanes 3 and 9) complexes was observed. In contrast the antibody directed against the LBD of RXRs could shift the complexes in HPK1A extracts but was unable to do so in HPK1Aras extracts: RARα-RXRα (Fig. 2C, lanes 4 and 10) TRβ-RXRα (Fig. 2D, lanes 4 and 10), and PPARγ-RXRα (Fig. 2E, lanes 4 and 10). Transient transfection of the non-phosphorylatable RXRα Ser-260 → Ala mutant or treatment with the MAPKK inhibitor PD098059 of HPK1Aras cells restored the RARα-RXRα (Fig. 2C, lanes 14 and 12), TRβ-RXRα (Fig. 2D, lanes 14 and 12), and PPARγ-RXRα (Fig. 2E, lanes 14 and 12) complexes. Similar to the effects on the VDR-RXR and RXR-RXR complexes, phosphorylation at Ser-260 does not affect heterodimerization of RXR with either RAR, TR, or PPAR but alters its recognition by a specific antibody raised against the LBD.

**Inhibition of MAPKK Activity Results in a Reversal of the Partial Resistance of Ras-transformed Keratinocytes to the Growth**
Inhibitory Effects of Both Vitamin D and Retinoids—We previously demonstrated that the ras-transformed keratinocyte cell line, HPK1Aras, is partially resistant to the growth inhibitory effects of 1,25(OH)\(_2\)D\(_3\) (6) and LG1069 (9) compared with the control cell line HPK1A. We further demonstrated that the MAPKK inhibitor PD098059 reversed this partial resistance on cell growth with a maximal effect observed at 96 h (9). Here we extended these observations by examining the growth inhibitory effect of ATRA (the natural ligand of RAR, Fig. 3C) and TTNPB (a highly specific synthetic ligand of RAR, Fig. 3D) as compared with LG1069 (Fig. 3B) and 1,25(OH)\(_2\)D\(_3\) (Fig. 3A) using HPK1A and HPK1Aras cells. A significant and dose-dependent growth inhibition was observed in HPK1A cells with both ATRA and TTNPB similar to the effect of 1,25(OH)\(_2\)D\(_3\) and LG1069. In contrast HPK1Aras cells were partially resistant to ATRA and TTNPB growth-inhibitory effects and required ~100-fold higher concentrations of the ligands to achieve the same effect. Addition of the MAPKK inhibitor PD098059 reversed the partial resistance to both ATRA and TTNPB in HPK1Aras cells restoring their growth-inhibitory effect similar to the one observed in HPK1A cells. For comparison the effect of 1,25(OH)\(_2\)D\(_3\) and LG1069 on HPK1Aras cells in the absence and presence of PD098059 are shown in panels A and B. These data therefore indicate that phosphorylation of RXR\(\alpha\) in HPK1Aras cells by Ras Raf MAPK activation results in resistance to the growth inhibitory effects of vitamin D and retinoids and that this resistance can be reversed by inhibiting the MAPK pathway.

Transfection of the Non-phosphorylatable hRXR\(\alpha\) Mutant Reverses Partial Resistance to 1,25(OH)\(_2\)D\(_3\)-induced Growth Inhibition in Ras-transformed Keratinocytes—The HA-tagged wild-type (WT) hRXR\(\alpha\), or HA-tagged Ser-260 mutant (Ala-260) or vector alone (pCDNA3.1) were transfected into HPK1Aras cells. HPK1Aras cells transfected with the WT hRXR\(\alpha\) remained resistant to 1,25(OH)\(_2\)D\(_3\) similar to control cells (vector alone). In contrast HPK1Aras cells transfected with the mutant construct had increased sensitivity to 1,25(OH)\(_2\)D\(_3\) (Fig. 4A). Nuclear extracts of HPK1Aras cells transfected with the HA-tagged WT hRXR\(\alpha\), HA-tagged hRXR\(\alpha\) mutant or vector alone (pCDNA3.1) were analyzed by Western blotting with an anti-hRXR\(\alpha\) antibody, anti-HA antibody, and an anti-(total) Rb antibody (loading control) (Fig. 4B). Blots probed with the antiRXR\(\alpha\) antibody indicated that transient transfection of the hRXR\(\alpha\) mutant...
RXRα Phosphorylation at Ser-260 Impairs Coactivator Recruitment

A

Cell growth assay

B

Western blots analysis

FIGURE 4. Transfection of the non-phosphorylable hRXRα mutant reverses partial resistance to 1,25(OH)₂D₃-induced growth inhibition in HPK1A ras cells. A, HPK1A ras cells were transfected with either HA-tagged wild-type (WT) hRXRα or hRXRα containing the Ala-260 mutation or vector alone (pCDNA3). Cells were treated with 5% charcoal-stripped FBS and increasing concentrations of 1,25(OH)₂D₃ as indicated. Cell numbers were expressed as percent of vehicle-treated control cell numbers after 48 h. Each value represents the mean ± S.D. of three determinations and is representative of three different experiments. Asterisks indicate a significant difference from vehicle-treated control values (no 1,25(OH)₂D₃ added), whereas open circles indicate a significant difference between cells transfected with mutant hRXRα and cells transfected with either vector alone or WT hRXRα at the concentrations indicated. B, HPK1A ras cells were transfected with either vector alone (1), HA-tagged wild-type hRXRα (2), or containing the Ala-260 mutation (3). 15 μg of nuclear extracts prepared from HPK1A ras cells was denatured followed by SDS-PAGE and Western blotting. The blots were treated with an anti-RXRα antibody, stripped with a Re-Blot Plus Mild solution (Chemicon), and reprobed with an anti-HA antibody to verify transfection efficiency and relative expression of exogenous hRXRα wild type and mutant. The blots were stripped and probed with an anti-Rb antibody (for total Rb) to verify equal loading. Immunoreactive bands were visualized by enhanced chemiluminescence.

expression of the transfected exogenous wild-type and mutant hRXRα. HPK1A ras extract transfected with pCDNA3 alone is shown as a negative control. Hence, expression of the non-phosphorylable mutant (but not WT hRXRα) to levels approximately equal to endogenous hRXRα restores HPK1A ras sensitivity to growth inhibition by 1,25(OH)₂D₃.

Inhibition of MAPKK Activity Results in a Reversal of the Partial Resistance of HPK1A ras Cells to the Transactivation Potential of Retinoids—We have shown that the transactivation of mOP by 1,25(OH)₂D₃ is partially inhibited in the MAPKK-activated HPK1A ras cells compared with that of HPK1A cells (18). Furthermore, this inhibition can be reversed by selectively inhibiting the MAPKK signaling pathway or expression of a non-phosphorylable mutant Ser-260 → Ala mutant. mOP3 activity increased ~120% above basal levels similar to the effect of 1,25(OH)₂D₃ seen in HPK1A cells (9). By transfecting a reporter plasmid containing three repeats of the RARE β cloned in front of a thymidine kinase-driven chloramphenicol acetyltransferase (CAT) gene, we now demonstrate that the resistance to the transactivation potential of increasing amounts of ATRA in HPK1A ras was reversed by the addition of 25 μM PD098059, a MAPKK inhibitor (Fig. 5A). In addition, by transfecting a reporter plasmid containing three repeats of the RXRE cloned in front of a thymidine kinase-driven CAT gene, we determined that the resistance to the transactivation potential of increasing amounts of LG1069 existing within HPK1A ras cells was reversed by the addition of 25 μM PD098059 (Fig. 5B). Similar results were observed following transfection of the non-phosphorylable Ser-260 → Ala mutant of RXRα (Fig. 5, A and B). These data indicate that phosphorylation of RXRα in HPK1A ras cells through activation of the Ras/Raf/MAPK pathway results in resistance to the transcriptional activation of retinoids and that this resistance can be reversed by inhibiting the MAPK pathway or specifically blocking phosphorylation on Ser-260.

Inhibition of MAPKK Activity Enhances the Effect of 1,25(OH)₂D₃ on Cell Cycle Regulatory Proteins—p21WAF1/CIP1, a cyclin-dependent kinase inhibitor, is a key target of 1,25(OH)₂D₃ antiproliferative effect (19) which directly up-regulates its expression. Here we demonstrate that in addition to reversing the resistance to 1,25(OH)₂D₃ on cell growth, inhibition of MAPKK activity had significant effects on the expression of the cell cycle regulated proteins p21WAF1/CIP1. As shown in Fig. 6A pre-treatment of HPK1A ras cells with the MAPKK inhibitor UO126 enhanced the stimulatory effect of 1,25(OH)₂D₃ on p21WAF1/CIP1 expression (lanes 4 versus 2) in comparison to basal (lane 1). UO126 alone moderately increased p21 expression as compared with basal (lane 3).

Inhibition of MAPKK Activity Enhances the Interaction of the VDR-RXR Complex with 1,25(OH)₂D₃-responsive Promoter Regions as Well as Coactivator Recruitment to the Complex—We screened several promoters for their interactions with the VDR-RXR complex and DRIP205 by ChIP assays using an antibody against either the VDR or DRIP205 following stimulation with 10⁻⁷ M 1,25(OH)₂D₃. Representative agarose gels of the PCR products are shown in Fig. 6B. Comparable chromatin content of the samples was measured by amplification of PCR products prior to immunoprecipitation (input lane).
RXRα Phosphorylation at Ser-260 Impairs Coactivator Recruitment

Treatment with 1,25(OH)₂D₃ of HPK1Aras cells in the absence of UO126 resulted in ~2-fold induction of the VDR-RXR complex binding to the designated promoter regions as compared with basal (lanes 4 versus 3). However, pre-treatment with 10⁻⁶ M UO126 of HPK1Aras cells in the presence of 1,25(OH)₂D₃ further induced by ~2 fold the VDR-RXR complex binding to several promoter regions including p21, p21CA, and the c-Fos/c-Jun transmembrane as compared with cells treated with 1,25(OH)₂D₃ alone (lane 2 versus 4). We next examined recruitment of DRIP205 to the VDR-RXR complex. No significant binding of DIRP 205 was observed in the absence of 125(OH)₂D₃ and UO126 (lane 3). Addition of 1,25(OH)₂D₃ significantly increased DRIP205 recruitment (lane 4) and to a lesser extent recruitment was enhanced with UO126 treatment in the absence of 1,25(OH)₂D₃ (lane 1). However, pre-treatment with UO126 in the presence of 1,25(OH)₂D₃ resulted in ~3- to 4-fold increase of DRIP205 recruitment as compared with cells treated with 1,25(OH)₂D₃ alone (lanes 2 versus 4). Taken together these data indicate that inactivation of MAPK significantly enhances the interaction of the VDR-RXR complex and its promoters as well as coactivator recruitment to the complex.

Disruption of Coactivators Interaction with the VDR-RXR Complex in HP1Aras Cells—Biotinylated mOP pulldown assays were used to assess VDR-RXR coactivators interaction. With this methodology it is possible to characterize the coactivator recruitment to the VDR-RXRα complex after pulldown of the protein complex with a biotinylated mOP VDRE probe and subsequently identify specific coactivators in the complex by Western blot analysis.

The strength of the interaction was assessed by comparing the intensity of the specific bands prior to and following treatment of HPK1A and HPK1Aras cells with 1,25(OH)₂D₃ (10⁻⁸ M) (Fig. 7). Addition of 1,25(OH)₂D₃ resulted in increased interaction of DRIP205 and RIP140 to the biotinylated mOP VDRE in both HPK1A and HPK1Aras cells. However, recruitment of DRIP205 and RIP140 to the VDR-RXRα complex was less intense in HPK1Aras as compared with HPK1A cells (panel A, lane 2 versus panel B, lane 2, and panel C, lane 2 versus panel D, lane 2). We next analyzed the recruitment of these coactivators after treatment of HPK1A and HPK1Aras cells with a MAPK inhibitor (UO126). As shown in Fig. 7 addition of the MAPK inhibitor did not alter the recruitment of RIP140 and DRIP205 in HPK1A cells in the presence of 1,25(OH)₂D₃ (panel B, lane 2 versus 4 and panel D, lane 2 versus 4). In contrast, pre-treatment of HPK1Aras cells with the MAPK inhibitor in the presence of 1,25(OH)₂D₃ restored the recruitment of both DRIP205 and RIP140 as indicated by a sharp increase in band intensity as compared with HPK1Aras cells treated with 1,25(OH)₂D₃ alone (panel A, lane 3 versus 4 and panel C, lane 3 versus 4). In each of the experiments equal amounts of proteins were loaded onto the gel (Western blot done with aliquots of HPK1A and HPK1Aras nuclear extracts prior to the pulldown assays is shown). There was no visible binding in pulldown assays when extracts were incubated with the non-relevant biotinylated probe (data not shown). Finally, we analyzed the specificity of serine 260 phosphorylation on the impairment of coactivator recruitment by expressing the non-phosphorylatable RXRα Ser-260 → Ala mutant or the empty vector in HPK1Aras cells and examining coactivator recruitment in the presence of 1,25(OH)₂D₃ (Fig. 8). As predicted, transfection of the RXRα Ser-260 → Ala mutant resulted in a sharp increase of coactivator recruitment (Fig. 8, lane 2 versus lane 1) in the presence of 1,25(OH)₂D₃ similar to the effect observed with the MAPK inhibitor (Fig. 7, panels A and C, lane 4). Furthermore, addition of the MAPK inhibitor UO126 to hRXRα Ser-260 → Ala transfected cells had no additive effect on coactivator recruitment (Fig. 8, lane 5 versus lane 2). These data therefore indicate that phosphorylation on Ser-260 of RXRα in HPK1Aras cells affect coactivator interaction with the VDR-RXR complex. Furthermore, when phosphorylation of Ser-260 is prevented by either MAPKK inhibition or introduction of a non-phosphorylatable Ala residue at position 260, the VDR-RXR complex interaction with coactivators is restored.

DISCUSSION

In the present study we have further defined the role of RXRα phosphorylation at serine 260. Our previous studies had demonstrated that ras-transformed keratinocytes are partially resistant to the growth inhibitory effect of 1,25(OH)₂D₃ (6). We then determined that the VDR-RXR complex was altered in...
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**A. Western blots analysis**

| Endogenous     | UO126 (1 µM) | 1,25(OH)2D3 (10^{-7} M) |
|----------------|--------------|------------------------|
| p21            | -            | +                      |
| GAPDH          | -            | -                      |

**B. ChIP assays**

- **Promoter**: Cyclin dependent kinase inhibitor (p21 (waf1/cip1))
- **VDREs**: -2156 tgtGGGCTTtagAGGTCT
- **Input 10%**:
  - **CHIP**:
    - VDR (C20)
    - DRIP205

**FIGURE 6. Effect of MAPKK inhibition on 1,25(OH)2D3-regulated cell cycle regulatory proteins and on 1,25(OH)2D3-mediated interaction between the VDR-RXR complex and the promoter region of target genes.** HPK1Aras cells were grown up to 40–50% confluency (see ChIP assays under “Materials and Methods”) and pre-treated with UO126 (10^{-7} M) or vehicle (Me2SO) for 1 h prior to addition of 1,25(OH)2D3 (10^{-7} M) or vehicle (ethanol). At 4 h cells were collected for either Western blot analysis (A) or ChIP assays (B). For Western blotting (A) membranes were incubated with antibodies against p21(waf1/cip1). Glyceraldehyde-3-phosphate dehydrogenase was used as a loading control. Binding of the VDR-RXR complex to response elements was done by ChIP assays (B). VDR-RXR complexes on 1,25(OH)2D3-responsive p21(waf1/cip1), CYP24, cyclin C, and CLMN promoter regions as well as recruitment of the coactivator DRIP205 to the complexes were analyzed. Chromatin was extracted from HPK1Aras cells and immunoprecipitated with an anti-VDR antibody (C-20) or an anti-DRIP205 antibody. Immunoprecipitation with IgG served as nonspecific controls. The relative change from cells incubated with 1,25(OH)2D3 alone (no UO126, plus 1,25(OH)2D3) is shown in the bar graph, which is representative of three different experiments (mean ± S.D.). * p < 0.05; indicates a significant increase from cells treated with 1,25(OH)2D3 alone.

**ras-transformed keratinocytes (18) due to phosphorylation of a MAPK consensus sequence in the C-terminal (E domain) of RXRa (9) on serine 260. The other potential MAPK consensus sequence located around threonine 82 (9) was not phosphorylated indicating that serine 260 was likely the major site of phosphorylation of the Ras-Raf-MAPK cascade in this model. Our studies also indicated that the Ras-Raf-MAPK cascade also affected the response to LG1069, a specific RXRa ligand (9, 20), raising the possibility that serine 260 phosphorylation of RXRa could also affect other partners of the RXRa such as RXRa/RXs, RXRa/RARα, RXRa/TRβ, and RXRa/PPAR. Indeed, subsequent studies by Matsushima-Nishiwaki et al. (10) indicated that vitamin A signaling through RARα-RXRα was also impaired by MAPK-dependent phosphorylation at serine 260 in hepatocellular carcinoma. The resistance to 1,25(OH)2D3 on cell growth inhibition is also observed at the transcriptional level (6, 9), including c-myc, an early response gene essential in cell cycle regulation (21, 22). In the present study we examined the effect of 1,25(OH)2D3 in ras transformed keratinocytes (HPK1Aras) pre-treated with MAPKK inhibitors on p21(waf1/cip1). p21(waf1/cip1) is a cyclin-dependent kinase inhibitor thought to play a major role in 1,25(OH)2D3-mediated growth inhibition (19). The promoter for p21 contains at least three VDREs (23), and p21(waf1/cip1) expression peaks around 2–4 h post-stimulation by 1,25(OH)2D3. In this study we demonstrate a significant increase of p21(waf1/cip1) 4 h post 1,25(OH)2D3 stimulation when cells were pre-treated with a MAPKK inhibitor indicating that phosphorylation of RXRa modulates 1,25(OH)2D3-responsive cell cycle regulators and that these changes precede the observed effects on cell growth. Finally, we analyzed 1,25(OH)2D3-induced growth inhibition in HPK1Aras cells expressing the non-phosphorylatable hRXRa Ser-260 → Ala mutant. Our data indicate that expression of this
transformed keratinocytes similar to our previous observation that restored 1,25(OH)₂D₃-dependent transactivation (9). Our data therefore indicate that phosphorylation at Ser-260 of RXRα by the Ras-Raf-MAPK cascade not only affects the vitamin D signaling pathway but also the retinoid signaling pathways in ras-transformed keratinocytes.

We next examined in detail the mechanisms of this resistance. Our previous observations indicate that serine 260 phosphorylation likely resulted in conformational changes of the RXRs but does not alter VDR-RXR heterodimerization (9). This hypothesis was based on our observation that RXRα phosphorylation altered the recognition of the VDR-RXRα complex by a specific antibody raised against the LBD (C-terminal domain) of the RXRα, which spans Ser-260 but not by a specific antibody raised against the N-terminal domain of RXRα. This C-terminal antibody failed to recognize the complex in gel shift analysis when HPK1Aras extracts were used but recognized the VDR-RXRα complex in HPK1A extracts, which are not phosphorylated at serine 260 (9, 18). In contrast, an antibody raised against regions of the NH₂-terminal domain of RXRα remote from serine 260 was able to recognize the VDR-RXRα complex of HPK1Aras extracts as well as HPK1A extracts indicating that phosphorylation at serine 260 (9, 18). In the present study we confirmed the critical location of serine 260 phosphorylation in altering the conformation of RXRα by overexpressing

exogenous mutant at levels comparable to the endogenous hRXRα is effective in re-establishing sensitivity to 1,25(OH)₂D₃-induced growth inhibition. Transfection of wild-type hRXRα induced similar changes in expression but had no effect on 1,25(OH)₂D₃ resistance indicating that the observed effects were not a consequence of artifactual overexpression of the exogenous receptor. We also analyzed retinoid signaling in response to specific ligands and found that Ras-Raf-MAPK activation inhibited both the growth inhibitory response and transactivation to these ligands. Transactivation activity in response to these ligands could be restored by overexpression of a non-phosphorylatable RXRα Ser-260 → Ala mutant in ras-

FIGURE 7. Recruitment of coactivators DRIP205 and RIP140 in nuclear extracts of HPK1A and HPK1Aras cells. Nuclear extracts from HPK1Aras (A and C) and HPK1A cells (B and D) treated without or with 1,25(OH)₂D₃ (10⁻¹⁰ M) (lanes 1 and 2) in the absence (lane 2) or presence (lane 4) of the MAPKK inhibitor UO126 (10⁻⁶ M). Immunoblot showing coactivator binding to biotinylated mOP VDRE and purified with streptavidin-agarose beads as described under "Materials and Methods" are shown in upper panels. The lower panels represent the densitometric analysis of the coactivator bands from three separate experiments (mean ± S.D.) and expressed as a percentage of basal activity in the absence of 1,25(OH)₂D₃ and UO126 (HPK1A and HPK1Aras cells incubated with vehicle alone, i.e. MeSO and ethanol). WB represents Western blots of an aliquot of nuclear extracts prior to pulldown with biotinylated mOP VDRE and probed for either DRIP205 or RIP140 to verify for coactivator loading. Equal protein loading was also assessed with the nuclear protein TBP18 (Abcam) (data not shown). An asterisk indicates a significant difference from basal (lane 1). An open circle indicates a significant difference between HPK1Aras cells treated with 1,25(OH)₂D₃ and UO126 versus HPK1A cells treated with 1,25(OH)₂D₃ alone. An open square indicates a significant difference between HPK1A cells and HPK1Aras cells treated with 1,25(OH)₂D₃ alone.

the RXRα Ser-260 → Ala mutant in HPK1Aras cells. Nuclear extracts from these cells were thus able to recognize this C-terminal antibody with an epitope spanning Ser-260 as indicated by a supershift of the VDR-RXRα complex (Fig. 2). Similarly, RXRα-RXRα, RARα-RXRα, and PPARγ-RXRα complexes in HPK1Aras nuclear extracts were recognized by this C-terminal antibody following overexpression of the RXRα Ser-260 → Ala mutant. These data indicate that serine 260 phosphorylation does not alter dimerization of the RXRα with its heterodimeric partners but likely results in conformational changes of the RXRα complexes not only with VDR but also with RARα, RXRα, as well as TRβ and PPARγ (Fig. 9). As a
general rule these receptors bind to their hormone-responsive elements in target genes as heterodimers rather than homodimers (24). It therefore raises the possibility that serine 260 is a key regulator of multiple signaling pathways mediated by the RXRα/H9251. However, this would not exclude the possibility that, within the same cells, ligands interact preferentially with homodimers to modulate their biological response. Further studies are needed to fully evaluate the biological consequences of Ser-260 phosphorylation of RXRα/H9251 on these other signaling pathways. In addition, more recent studies indicate that phosphorylation at serine 260 may affect cellular events unrelated to cancer biology. A recent study in HepG2 liver cells indicates that the acute phase response in liver cells initiated by the pro-inflammatory cytokine interleukin-1β (IL-1β) (25) directly phosphorylates RXRα/H9251 and may be responsible for the suppression of RXRα signaling. Interestingly, the resistance to 1,25(OH)_2D_3 in response to Ras-Raf-MAPK activation may be cell-dependent as reported in two osteoblastic cell lines MG-63 and MC3T3-E1 (26). In these studies MAPKK activation inhibited vitamin D signaling in MC3T3-E1 cells but not in MG-63 cells. Furthermore this “vitamin D resistance” was reversed by the MAPKK inhibitor UO126 in MC3T3-E1 cells. The critical importance of serine 260 of the human RXRα has also been reported with the mouse RXRα (27). In these studies F9 cells expressing phosphorylated wild-type RXRα on Ser-260 (Ser-265 of mouse RXRα is the equivalent of Ser-260 of human RXRα) were resistant to ATRA signaling but not F9 cells expressing the RXRα Ser-265 Ala mutant highlighting the importance of this residue in both mouse and human RXRα.

We next examined the consequences of MAPKK inhibition on the interaction between the VDR-RXR complex and the promoter regions of several 1,25(OH)_2D_3 target genes and DRIP205 recruitment to the complex by ChIP assays. As predicted pre-treatment of ras-transformed keratinocytes with the MAPKK inhibitor UO126 significantly enhanced these interactions in response to 1,25(OH)_2D_3. In addition to CYP24, we examined several genes implicated in the regulation of the cell cycle, including p21_Cip1/Waf1, cyclin C, and CLMN. In earlier studies it was demonstrated that p21_Cip1/Waf1 contains at least three VDREs, each of which show 1,25(OH)_2D_3-dependent recruitment of coactivator proteins (including DRIP205) to VDR-bound chromatin regions (23). A 2-fold increase in p21_Cip1/Waf1 binding between the VDR-RXR complex and VDREs and a 3-fold increase in DRIP205 recruitment was observed and suggested that vitamin D resistance is mediated at.
least in part by regulating this potent inhibitor of cell cycle progression. Similar results were obtained with cyclin C, a member of a cyclin protein superfamily activated by 1,25(OH)₂D₃, which controls cell cycle transition through activation of cyclin-dependent kinases (28). Our data therefore indicate that inhibition of the Ras-Raf-MAPK pathway reverses the resistance of 1,25(OH)₂D₃ at least in part by enhancing the binding of the VDR-RXR complex and coactivator recruitment to the promoter region of cell cycle-regulated genes. It is important to note that the observed effects on the VDR-RXR binding to the promoter and coactivator recruitment are rapid (within 4 h) and precede the changes observed in transcriptional activation and cell growth. These data strongly support a causal relationship between RXR phosphorylation-induced alteration of coactivator recruitment and downstream biological events. Although re-ChIP assays were not used in the present study, previous studies using re-ChIP assays demonstrated the simultaneous association of VDR with RXR and coactivators (23, 28) on both p21<sup>waft/cip1</sup> and cyclin C supporting the general model of coactivator recruitment on target genes (29).

Interestingly, UO126 binds with higher affinity to MAPKK as compared with PD98059 and has been shown to be effective in animal models in vivo in inhibiting MAPK activity without significant side effects (30). UO126 administration in vivo may therefore be useful in future cancer studies in combination with vitamin D compounds.

The importance of coactivator recruitment to steroid receptor signaling is now well established (31). Among these is steroid receptor coactivator 1 (SRC1), a major modulator of estrogen function (32) that belongs to the p160 family of coactivators and also includes SRC2 (GRIP1/TIF2) (33) and SRC3 (AIB1, ACTR, and TRAM1) (34). SRC1 and SRC2 have all been implicated in VDR-mediated transactivation (35, 36), whereas SRC3 has been implicated in TR signaling (37). We examined the expression of SRC1, -2, and -3 in both immortalized and ras-transformed keratinocytes and could not detect SRC1 or SRC2 (GRIP1), whereas SRC3 (activation of thyroid hormone receptors and retinoic acid receptors, ACTR) was detected at low levels in both cell lines (Fig. 1B). Previous studies also failed to detect SRC1 in human keratinocytes (38, 39), whereas SRC2 expression was reported in both normal human keratinocytes and a human squamous carcinoma cell line (38). It is possible that the expression of SRC-2 is cell line-specific. Low levels of expression of SRC-3 detected in our study (Fig. 1B) are in keeping with expression levels seen in human keratinocytes (38). We next examined the protein levels of DRIP205 using an antibody from Santa Cruz Biotechnology, which detects a major band at around 220 kDa. DRIP205 is an essential component of the DRIP complex critical for VDR activation (40), which binds to the LBD of VDR and initiates transcriptional activation by promoting interaction with vitamin D-responsive sequences (VDREs) on target genes. Using equal protein amount high and low expression was reported in both immortalized and ras-transformed keratinocytes (38). Finally, we analyzed expression of RIP140, a regulatory protein that acts as a co-repressor of the glucocorticoid receptor (41, 42) but also as a coactivator of vitamin D signaling (35). Our next objective was to analyze the recruitment of these coactivators to the VDR-RXRα complex. We focused our analyses to DRIP205 because of its importance in vitamin D signaling and on RIP140 because of its interesting dual activity and relatively high level of expression in our system as compared with coactivators of the SRC family. To quantitate coactivator recruitment we treated both immortalized and ras-transformed keratinocytes with 1,25(OH)₂D₃ and subsequently pulled down the VDR-RXR-coactivator complex from nuclear extracts using a biotinylated mOP VDRE. Proteins bound to mOP VDRE were then extracted with agarose beads and analyzed by Western using specific antibodies against the coactivators. Using equal amounts of proteins (as verified by simultaneous Western analysis of nuclear extracts prior to extraction with biotinylated mOP VDRE) we could compare recruitment of coactivators to the VDR-RXR complex in HPK1A cells versus HPK1A<sub>ras</sub> cells prior to and following treatment with 1,25(OH)₂D₃. Our study indicates that both DRIP205 and RIP140 are recruited to the complex; however, recruitment is significantly higher in HPK1A cells as compared with HPK1A<sub>ras</sub> cells (Fig. 7). Our next objective was then to determine the consequences of Ras-Raf-MAPK activation on coactivator recruitment. When ras-activated cells (HPK1A<sub>ras</sub>) were treated with the MAPK inhibitor UO126 in the presence of 1,25(OH)₂D₃ recruitment of coactivators at least doubled in HPK1A<sub>ras</sub> cells reaching levels comparable to 1,25(OH)₂D₃-treated HPK1A cells. Furthermore, UO126 had no effect on coactivator recruitment in HPK1A cells. These data clearly indicate that MAPK activation inhibits coactivator recruitment likely through phosphorylation of MAPK consensus sites on the VDR-RXR-coactivator complex. To address the importance and specificity of serine 260 phosphorylation of RXRα, we next expressed the non-phosphorylatable RXRα Ser-260 → Ala mutant into HPK1A<sub>ras</sub> cells treated or not with 1,25(OH)₂D₃. Our data indicate that expression of this mutant rescues coactivator recruitment to the complex (Fig. 8) similar to the effect observed with UO126. VDR is not a substrate for MAPK, but coactivators such as SRC1 and DRIP205 are possible targets (43, 44). Therefore we cannot exclude the possibility that MAPK phosphorylation of coactivators may play a role in their recruitment to the VDR-RXR complex, although this contribution is likely minimal because overexpression of the non-phosphorylatable RXRα Ser-260 → Ala mutant restores their recruitment fully and similarly to the effect observed with UO126. Interestingly, DRIP205 phosphorylation by MAPK-ERK increases intrinsic DRIP205 transcriptional activity as well as TR-dependent transcription (44) as opposed to the negative effect of MAPK-ERK on RXRα mediated transactivation found here. This would suggest that in our model MAPK-ERK-mediated inhibition of RXRα signaling predominates over a potential stimulatory effect of MAPK-ERK on DRIP205-mediated transactivation.

The one or more mechanisms by which phosphorylation of serine 260 of RXRα perturbs the recruitment of coactivator(s) to the VDR-RXR complex likely involve structural changes in the AF-2 domain, which appear to facilitate coactivator interaction (45, 46). Serine 260 is located in the omega loop, which contacts the AF-2 domain (11), and its phosphorylation likely
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alters the conformation of this region and accessibility of coactivators and subsequent transactivation (Fig. 9). Specific residues in the omega loop (Pro-264 at the start of H3 and Asn-262 in the loop between H2 and H3) bond with specific residues in the AF-2 domain (Phe-450 and Gln-453) (11). Mutations of these AF-2 residues in hRXRα or the equivalent sequences in mRXRα result in suppression of the transactivation activities of both RXR-RXR homodimers and RXR/VDR heterodimer (47) presumably by abolishing/reducing interaction between RXR and coactivators. Interestingly, phosphorylation of the omega loop at the equivalent position on Ser-265 in the mouse RXRα also inhibits transcription of retinoic acid target genes (27). The omega loop is located between helices H1 and H3 of the LBD (11), and its sequence is identical between mouse and human RXRα (NMGLNPSSPNDPVTN). Consequently, its phosphorylation potentially affects recruitment of coactivators on the various RXRα complexes across species. Further studies will be needed to explore these possibilities.

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