Phosphorylation of Retinoid X Receptor α at Serine 260 Impairs Its Metabolism and Function in Human Hepatocellular Carcinoma

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ABSTRACT

Retinoids induce apoptosis and differentiation of hepatocellular carcinoma (HCC) cells and are used clinically in the chemoprevention of HCC. We have shown previously that hepatocarcinogenesis is accompanied by accumulation of full-length retinoid X receptor α (RXRα), although the underlying mechanisms and biological implications have remained unclear. The present studies were based on the finding that the accumulated full-length RXRα was phosphorylated at serine/threonine residues both in all HCC tissues examined and in human HCC-derived HuH7 cells. Phosphorylation at serine 260 of RXRα, a consensus site of mitogen-activated protein kinase, was closely linked to its retarded degradation, low transactivating activity, and the promotion of cancer cell growth. There was no genomic mutation in the RXRα gene, and abrogation of phosphorylation by mitogen-activated protein kinase-specific inhibitors restored the degradation of RXRα in an RXR ligand-dependent manner. These results suggest that phosphorylation of RXRα may interfere with its metabolism and signaling in human HCC, which could lead to growth promotion of these tumors.

INTRODUCTION

HCC associated with chronic viral hepatitis or cirrhosis is a major worldwide problem that is increasing in incidence (1–4). Hepatocarcinogenesis is closely related to impaired signaling of retinoids (vitamin A and its derivatives). For example, hepatic retinoid content decreases at an early phase of hepatocarcinogenesis (5), and supplementation with a retinoid analogue prevents the occurrence of second primary tumors after curative treatment of the first tumor (6, 7).

Retinoids transduce their signals primarily through two families of nuclear receptors, the RARs and RXRs (8). These receptors are ligand-dependent manner. These results suggest that phosphorylation of RXRα may interfere with its metabolism and signaling in human HCC, which could lead to growth promotion of these tumors.

MATERIALS AND METHODS

Materials. 9cRA, STS, SB203580 (p38 MAPK inhibitor), KN-93 (calmodulin kinase II inhibitor), monoclonal antibodies against phosphoserine (PSR-45) and phosphothreonine (PTR-8), and BrdUrd were purchased from Sigma Chemical Co. (St. Louis, MO). PD98059 (MEK or MAPK kinase inhibitor) was obtained from Research Biochemicals International (Natick, MA).32 P i and Hi-Trap NHS-activated Sepharose came from Amersham-Pharmacutica Bio-tech, Ltd. (Buckinghamshire, United Kingdom). Polyclonal anti-RXRα (AN 197), anti-RXRβ (C-20), anti-RXRγ (Y-20), and anti-p34 cdc2 kinase (H-297) antibodies and polyclonal anti-phospho p44/p42 MAPK (ERK1/ERK2) antibody were from Santa Cruz Biotechnology, Inc. (Santa Cruz Biotechnology, CA) and Cell Signaling Technology (Beverly, MA), respectively. Rhodamine-labeled antirabbit IgG polyclonal antibody and fluorescein isothiocyanate-
labeled anti-BrdUrd monoclonal antibody were from ICN Pharmaceuticals, Inc. (Aurora, OH) and Progen Biotechnik (Heidelberg, Germany), respectively. Ro25-7386 (RXRα-selective agonist), Ch55 (pan-RAR-selective agonist), and constitutively active MEK1 cDNA were generous gifts from Drs. M. Klaus (F. Hoffmann-La Roche, Basel, Switzerland), K. Shudo (The University of Tokyo, Tokyo, Japan), and Dr. N. G. Ahn (Howard Hughes Medical Institute, University of Colorado, Boulder, CO; Ref. 18), respectively.

Site-directed Mutagenesis in RXRα. Human RXRα expression vector, pRSRiRXRα (19), was kindly provided by Dr. R. M. Evans (The Salk Institute, La Jolla, CA). Human RXRα mutants (threonine 81 and 82→alanine (T82A), serine 259 and 260→alanine (S260A), and their combination (T82D/S260D)) as well as threonine 81 and 82→aspartate (T82D), serine 259 and 260→aspartate (S260D), and their combination (T82D/S260D) were constructed using the Mutan-Super Express kit supplied by TAKARA Biomedicals (Tokyo, Japan). Briefly, RXRα cDNA was subcloned into the EcoRI site of pJK188 plasmid vector containing dual amber mutations on the kanamycin-resistant gene. PCR was performed using a combination of sense primers containing the desired mutations and an anti-sense primer to revert the amber mutations. Sense primers used were as follows (the alanine or aspartate mutations are underlined): T82A: 5′-CCACTCTCAT-GTGGTGCCCCGCAACCACCATGGTC-3′; S260A: 5′-GAACCC-GCGCCGGGAAACCCGTTCACCAACATTGG-3′; T82D: 5′-CCACTCTCAT-GTGGTGCCCCGCAACCACCATGGTC-3′; and S260D: 5′-GAACCCCGAAGCCGAACCGTTCACCAACATTGG-3′. With this PCR, amplified mutagenic-sequence DNA functioned as a PCR primer, yielding nicked double-stranded plasmid. When this nicked DNA was transformed into Escherichia coli MV1184, the nick was repaired, and only transformants containing a desired site-specific mutation were grown in the presence of kanamycin. T82AS260A and T82DS260D double mutants were constructed inside the fragment of the Scal-BstHII fragment of S260A or S260D mutant with that of T82A or T82D mutant, respectively. The ΔA/B mutant lacking the A/B domain was constructed by PCR, using sense primer 5′-AAAGGATCTCAGATGAC-CACACGACATCTGCGTCC-3′ and antisense primer 5′-AAAGAATTCGCCGCAATCACCATGCTCC-3′. Sequences of mutants were verified by sequencing. The mutant hRXRα cDNAs were cloned into the EcoRI-XhoI site of pcDNA3.1/Myc-Hist (+) mammalian expression vector (Invitrogen Corp., Carlsbad, CA), which contains an oligonucleotide encoding a polyhistidine (His6) metal-binding peptide at the COOH terminus of the desired protein.

Tissue Specimens. HCC and its surrounding noncancerous tissues were obtained by surgical resection from 10 patients infected with hepatitis viruses B (3 cases) or C (7 cases). In all of the cases, tumor and its surrounding tissues were classified histologically as moderately differentiated HCC and liver cirrhosis, respectively. A histologically normal liver specimen was obtained from a patient with hypercitrullinemia who underwent liver transplantation. The study was approved by Gifu University School of Medicine Ethics Committee, and all of the patients gave informed consent.

Cell Culture and Treatment. HuH7 cells were obtained from the Japanese Cancer Research Resources Bank (Tokyo, Japan) and maintained in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD) supplemented with 1% FCS (Life Technologies, Inc.) and 0.2% lactalbumin hydrolysate (Sigma Chemical Co.). Hc cells, normal human hepatocytes, were purchased from Applied Cell Biology Research Institute (Kirkland, WA) and maintained in the attached CS-C complete medium. Hc cells were synchronized in G0-G1 phase by incubating with isoleucine-free RPMI 1640 containing 3% dialyzed FCS for 24 h in 1% FCS-containing isoleucine-free RPMI 1640 (Hc cells) or 3% FCS-containing lactalbumin-containing RPMI 1640 (Hc cells). Thereafter, cell lysates were prepared, and luciferase activity of each cell lysate was measured using the LumiCount microplate luminometer (Packard Instrument Co., Meriden, CT). Changes in firefly luciferase activity were calculated and assessed after normalization with changes in Renilla luciferase activity in the same sample.

Immunocytochemistry. After 1-h incubation with 20 μM BrdUrd, cells were fixed with 10% formalin in PBS for 10 min at room temperature, washed twice with PBS, and permeabilized with 0.3% Triton X-100 in PBS for 10 min at room temperature. Cells were treated with 1 N HCl for 1 h at room temperature and neutralized with 0.1 N boric acid buffer (pH 8.5). After blocking with nonimmune rabbit IgG (final, 100 μg/ml) for 1 h, samples were incubated with anti-RXRα antibodies (ΔN 197; final, 1:50 dilution) overnight at 4°C. After washing with PBS-0.1% Tween 20, the cells were incubated at room temperature with rhodamine-labeled second antibody (1:100) for 90 min and then visualized using an Amersham-Pharmacia ECL system. Densitometric analysis was performed using the NIH image version 1.61 software.

RESULTS

Accumulation of Phosphorylated Full-length RXRα in Human HCC Tissues. Fig. 1A shows the result of Western blot analyses of nuclear extracts prepared from HCC, its noncancerous surrounding tissue, and normal liver with an anti-RXRα antibody that recognizes a ligand binding sequence in the E-domain and thus reacts with both full-length M44,000 and fragmented M47,000 and M44,000 RXRαs (10). Although ΔN 197 could recognize all three subtypes of XRα, Western blotting of the same sample with specific antibody to RXRβ and RXRγ did not reveal any bands (data not shown), suggesting that the visualized bands represented RXRα. A major species at M44,000 plus minor M47,000 and M44,000 species were detected in HCC tissues (Fig. 1A, Lane 1). In contrast, in noncancerous surrounding and normal liver tissues, M44,000 bands were faintly (Fig. 1A, Lane 2) and barely (Fig. 1A, Lane 3) detected, respectively, whereas fragmented M44,000
bands were predominant in these tissues. A control experiment using anti-p34 cdc2 kinase antibody confirmed a constant amount of nuclear proteins among samples (data not shown). Similar observations were made in all 10 cases examined (Fig. 1D), suggesting that the accumulation of full-length RXRα was limited to cancerous liver tissues.

Under this condition, phosphorylated RXRα was identified with both anti-phosphoserine (Fig. 1B) and anti-phosphothreonine (Fig. 1C) antibodies after immunoprecipitation with ΔN 197-immobilized beads. Strong bands were expressed in tumor tissues only at M, 54,000 but not at M, 47,000 or M, 44,000 (Fig. 1, B and C, Lanes 1), indicating that full-length RXRα was exclusively phosphorylated at both serine and threonine residues in human HCC. No band was detected when proteins precipitated with nonimmunized rabbit antibody-immobilized beads were analyzed (data not shown). Much weaker expression was seen in noncancerous adjacent tissues (Fig. 1, B and C, Lanes 2), and the protein was barely detectable in a histologically normal liver sample (Fig. 1, B and C, Lanes 3). Phosphorylated bands were not detected with anti-phosphotyrosine antibody (PY-20; Wako Pure Chemicals; data not shown). Similar observations were made in 10 cases examined (Fig. 1, E and F), suggesting a potential link between retarded degradation and phosphorylation of RXRα in human HCC.

Accumulation of Phosphorylated Full-length RXRα in Human HCC Cells and Its Degradation by RXRα-selective Ligands. To study the relationship between phosphorylation and degradation of RXRα as well as its biological implications, we used cultures of a human HCC cell line, HuH7 cells, and normal human hepatocytes, Hc cells. We first tested whether in vivo results were reproduced in cultured cells. By Western blot (Fig. 2A), HuH7 expressed RXRα, but not RXRβ or RXRγ (Fig. 2A, Lanes 1–3), most of which was the M, 54,000 full-length form (Fig. 2A, Lane 1), whereas the majority of RXRα in Hc cells was the M, 44,000 truncated form (Fig. 2A, Lane 5), establishing the utility of HuH7 and Hc cells as representatives of clinical HCC and normal liver tissues, respectively. HuH7 cells transfected with plasmid encoding a full-length RXRα cDNA expressed an increased amount of M, 54,000 RXRα with reduced M, 47,000 fragment (Fig. 2A, Lane 4), whereas Hc cells expressed only an increased amount of M, 44,000 fragmented RXRα (Fig. 2A, Lane 6), suggesting that RXRα mRNA was alternatively spliced in Hc cells and/or that RXRα protein translated from the same mRNA was differentially metabolized between the two cell types. The latter idea was supported by the in vivo data that only phosphorylated RXRα escaped degradation. We did not detect any mutations in the full-length RXRα cDNA derived from either human HCC tissues or HuH7 cells (data not shown).

To determine whether full-length RXRα was constitutively phosphorylated, we performed metabolic labeling experiments. After treatment of the cells under the indicated conditions, half of each culture was radiolabeled, and phosphorylated RXRα was immunoprecipitated and detected by SDS-PAGE/autoradiography (Fig. 2B), and the other half was used to monitor the levels of both M, 54,000 and M, 44,000 RXRα by Western blot analyses (Fig. 2C). As seen in Fig. 2B, Lane 1 (inset), M, 54,000 full-length RXRα was phosphorylated. Radioactive bands were detected only at M, 54,000 but not at M, 44,000, suggesting that the M, 44,000 truncated RXRα peptide was minimally or not phosphorylated. Next, a correlation between phosphorylation and degradation of RXRα was assessed by inhibiting phosphorylation with a broad-spectrum protein kinase inhibitor, STS, and/or a ligand for RXRα, 9cRA, which has been reported to attenuate phosphorylation via induction of phosphatase activity (22). The amounts of both M, 54,000 and M, 44,000 RXRα were almost unchanged when phosphorylation was inhibited by 30% with STS alone (Fig. 2, B and C, Column 2 and Lane 2, each inset). 9cRA alone did not affect either phosphorylation or degradation (Fig. 2, B and C, Column 3 and Lane 3, each inset). However, a combination of these reduced phosphorylation by 70% and reduced the amount of M, 54,000 RXRα by 50%, with emergence of the M, 44,000 fragment (Fig. 2, B and C, compare Column 4 and Lane 4, each inset), suggesting that a combination of dephosphorylation and ligand-binding may induce degradation of RXRα. In contrast, Hc cells did not contain any detectable levels of phosphorylated RXRα (Fig. 2B, Column 5 and Lane 5, inset) and expressed mainly M, 44,000 RXRα (Fig. 2C, Column 5 and Lane 5, inset).

Next, we examined the ligand specificity of RXRα degradation using receptor-selective retinoids, because 9cRA can bind to both RXR and RAR. As shown in Fig. 2D, both 9cRA (pan-RAR and RXR agonist) and Ro25-7386 (RXRα-selective agonist) enhanced RXRα degradation in the presence of STS (Fig. 2D, Columns 3 and 5), but not in the presence of Ch55 (pan-RAR-selective agonist, Column 7). 9cRA induced the degradation of RXRα in a dose-dependent manner (ED50, 60 μM) in the presence of a low concentration (50 μM) of STS, suggesting that this effect is physiologically relevant (data not shown).

Involvement of MAPK in the Reduced Degradation of RXRα. Because STS suppresses several kinase activities (23), we examined the effect of kinase-selective inhibitors to identify which protein...
kinase(s) might be involved. HuH7 cells were treated with a combination of 9cRA and one of four different protein kinase inhibitors, and the amount of M, 54,000 RXRα was assessed semiquantitatively by Western blotting. As shown in Fig. 3A, degradation was induced to almost the same extent by STS (Column 3) as well as SB203580 (p38 MAPK inhibitor; Column 5) and PD98059 (MEK inhibitor; Column 7) in the presence of 9cRA. PD98059 also induced the degradation in the absence of 9cRA (Column 6). However, KN-93 (calmodulin kinase II inhibitor; Column 9) did not induce the degradation even at a concentration as high as 10 μM. These results suggest that the current system in HuH7 cells may be controlled, at least, by the MAPKs. In support of this conclusion, HuH7 cells expressed high levels of phosphorylated (activated) p42 MAPK (ERK2), whereas Hc normal hepatocytes did not (Fig. 3B, compare Lanes 1 and 2). When Hc cells were transiently transfected with constitutively active MEK1 cDNA, they expressed high levels of ERK1/ERK2 (Fig. 3B, Lane 3), and the degradation of RXRα was attenuated (Fig. 3B, compare Lanes 4 and 5).

Regulation of RXRα Degradation by Phosphorylation at Serine 260. To confirm that MAPKs were involved in RXRα phosphorylation and its reduced degradation, we constructed plasmids encoding mutant RXRα in which threonine 82 and/or serine 260, the putative phosphorylation sites by MAPKs (14, 16), were mutated to either alanine (unphosphorylated form) or aspartate (phosphomimic). HuH7 and Hc cells were transfected, and the amount of full-length M, 54,000 RXRα was semiquantitatively by Western blotting after treatment with 9cRA, which was required for degradation. The antigenicities of these mutant RXRαs were similar to the wild-type RXRα (data not shown). As depicted in Fig. 4A, whereas native and overexpressed wild-type RXRαs were not degraded in HuH7 cells (Columns 2 and 4, respectively), the alanine 260-mutant (S260A) as well as alanine 82-mutant/alanine 260-mutant (T82A/S260A) mimicked the unphosphorylated type and restored the degradation after treatment with 9cRA (Columns

Fig. 2. Phosphorylated full-length RXRα in the HCC cells. A, Western blot analyses of endogenous and overexpressed RXRαs in HuH7 (Lanes 1–4) and Hc (Lanes 5 and 6) cells. Electroporation was performed with full-length human RXRα cDNA. Nuclear extracts were prepared from respective Hc and electroporated cells (Lanes 4 and 6) 48 h after transfection. Western blotting was performed using AN 197 (Lanes 1 and 4–6), anti-RXRβ antibody (Lane 2), and anti-RXRγ antibody (Lane 3) as before. Representative results from three different experiments with similar results are shown. B and C, parallel reduction in phosphorylation and degradation of endogenous RXRα in HuH7 cells after treatment with a combination of STS and 9cRA. After phosphate starvation for 24 h, HuH7 cells were incubated with 32P for 24 h in the absence (Lane and Column 1) or presence of 50 μM STS (Lane and Column 2) or 1 μM 9cRA (Lane and Column 3), or their combination (Lane and Column 4) in phosphate-free RPMI 1640 containing 1% FCS. Hc cells synchronized to G0–G1 were incubated with 32P similarly to HuH7 cells in isoleucine- and phosphate-free RPMI 1640 containing 3% FCS in the absence of any compounds (Lane and Column 5). In a parallel experiment, both cell types were incubated under the same condition except metabolic labeling. Nuclear extracts were prepared, and RXRαs were immunoprecipitated using AN 197, separated by SDS-PAGE, and visualized by autoradiography (B, inset). Western blot analyses of RXRαs in each extract was detected by anti-RXRα antibody (C, inset). Intensities of each band were analyzed by densitometry, and the ratios of M, 54,000 RXRα bands to M, 54,000 plus M, 44,000 and M, 47,000 bands either in the autoradiogram (B) or in Western blot (C) were calculated and plotted as the percentage of those obtained from nontreated control HuH7 cells. Values are the means (n = 5); bars, SD. *P < 0.01, significantly different from control cells (B) or from control, STS-treated cells, and 9cRA-treated cells (C). Representative results from five independent experiments with similar results are shown. D, degradation of endogenous RXRαs in HuH7 cells after treatment with a combination of STS and ligand-selective retinoids. HuH7 cells were left untreated (Column 1) or treated for 24 h with 1 μM 9cRA (Columns 2 and 3), Ro25-7386 (Columns 4 and 5), or Ch55 (Columns 6 and 7) in the absence (Columns 2, 4, and 6) and the presence (Columns 3, 5, and 7) of 50 μM STS in RPMI 1640 containing 1% FCS. Nuclear extracts were prepared, Western blot for the detection of RXRα protein in each extract was performed, and the densities of full-length RXRαs were determined as before. Relative intensities of the RXRα bands were expressed as percentage of those obtained from untreated control cells. Values are the means (n = 5); bars, SD. *P < 0.01, obtained from a comparison between the cells with and without STS treatment for each retinoid. +, P < 0.01, significantly different from control cells (Columns 1). Representative results from three different experiments with similar results are shown.
obtained from a comparison between plus/minus 9cRA treatment for each kinase inhibitor. were prepared from HuH7 cell cultures (p42/p44 MAPKs (ERK1/ERK2) and RXR \( \beta \)) and MEK1-transfected (1

analyzed by densitometry, and the ratios of shown. and

from untreated control cells. Values are the means (\( n \), bars, SD. \( +, P < 0.01 \), obtained from a comparison between plus/minus 9cRA treatment for each kinase inhibitor. +, \( P < 0.01 \), significantly different from control cells (Column 1). B, whole cell extracts were prepared from HuH7 cell cultures (Lane 1) and the cultures of C\(_6\)G\(_7\)G\(_7\) synchronized (Lane 2) and MEK1-transfected (1 \( \mu \)g/100 mm dish; Lane 3) Hc cells. Phosphorylated p42/p44 MAPKs (ERK1/ERK2) and RXR\( \beta \) were detected by Western blot using specific antibody to the activated state of ERK1/ERK2 and RXR\( \beta \) (AN 197), respectively. For A and B, representative results from three different experiments with similar results are shown.

8 and 10, respectively). Mutation of threonine 82 to alanine (T82A) did not affect degradation (Fig. 4A, Column 6). A similar result was obtained when protein synthesis was inhibited by treatment with cycloheximide during incubation with 9cRA (Fig. 4B), confirming the degradation of S260A mutant RXR\( \alpha \). Because comparable expression was observed between wild-type and S260A mutant RXR\( \alpha \) (data not shown), possible interference with transcription and/or translation of the mutant genes was not likely. These results suggested that phosphorylation at serine 260 by MAPKs may render RXR\( \alpha \) resistant to degradation, whereas phosphorylation at threonine 82 may be an important determinant of RXR\( \alpha \) degradation. Mutant RXR\( \alpha \)s with aspartate 82 and/or aspartate 260 (T82D, S260D, and T82D/S260D) mimicked the phosphorylated type and did not degrade into smaller peptides in HuH7 cells when overexpressed (data not shown).

In contrast, when S260D and T82D/S260D mutant RXR\( \alpha \)s were overexpressed in Hc cells, these phosphorylated RXR\( \alpha \) mimics were found to be resistant to proteolysis (Fig. 4C, Columns 4 and 5, respectively). On the other hand, both wild-type and T82D mutant RXR\( \alpha \)s decayed (Columns 2 and 3, respectively), as in the cases of T82A, S260A, and T82A/S260A mutant RXR\( \alpha \)s (data not shown). A similar result was also obtained in the presence of cycloheximide (Fig. 4D).

Inactivation of RXR\( \alpha \) by Phosphorylation at Threonine 82 and Serine 260. We assessed the effect of phosphorylation as well as degradation of RXR\( \alpha \) on its transactivation activity via RXRE using the RXR\( \alpha \)-responsive reporter, CRBPII-RXRE-luciferase (Fig. 5). Hc cells expressed \( \sim 2 \)-fold higher transactivation activity than HuH7 cells. 9cRA treatment enhanced the activity \( \sim 2 \)-fold in both cell types (Fig. 5, A and B, Columns 2), suggesting that Hc cells possess 2-fold higher ligand-dependent transactivation activity than HuH7 cells. Overexpression of wild-type RXR\( \alpha \)s increased the ligand-dependent activity in both cell types. However, the effect was more potent in Hc cells (7-fold increase; Fig. 5B, Column 4) than in HuH7 cells (5-fold increase; Fig. 5A, Column 4), implying that phosphorylation of RXR\( \alpha \) in HuH7 cells might interfere with its transactivation activity. Overexpression of T82A mutant RXR\( \alpha \) in HuH7 cells resulted in a further (6-fold) potentiation (Fig. 5A, Column 6), whereas overexpression of T82D mutant RXR\( \alpha \) in Hc cells led to a significantly smaller (2.5-fold) increase (Fig. 5B, Column 6), implying that phosphorylation of threonine 82 might reduce ligand-dependent transactivation activity. On the other hand, overexpression of S260A or T82A/S260A mutant RXR\( \alpha \) in HuH7 cells resulted in 3–3.5-fold increases (Fig. 5A, Columns 8 and 10, respectively), whereas overexpression of S260D and T82D/S260D mutant RXR\( \alpha \)s in Hc cells suppressed ligand-dependent activities almost completely (Fig. 5B, Columns 8 and 10, respectively). Overexpression of T82D, S260D, and T82D/S260D mutant RXR\( \alpha \)s in HuH7 cells did not enhance ligand-dependent transactivation activity, whereas overexpression of T82A, S260A, and T82A/S260A mutant RXR\( \alpha \)s in Hc cells resulted in almost similar levels of ligand-dependent transactivation activity, as detected with wild-type RXR\( \alpha \) (data not shown). Columns 11 and 12 in Fig. 5A display the result when the \( \Delta \)A/B mutant lacking the A/B domain, a mimic of M, 44,000 protein, was overexpressed in HuH7 cells. The truncated RXR\( \alpha \)s did not display ligand-induced transactivation activity. Similar results were obtained using a luciferase reporter gene, DR-5 Luc (21), the transactivation of which is driven via RARE by the RAR/RXR heterodimer (data not shown).

A Link between Enhanced Cellular Proliferation and Phosphorylation at Serine 260 of RXR\( \alpha \). Finally, we examined the influence of RXR\( \alpha \) phosphorylation on cellular proliferation by determining the BrdUrd labeling index (Fig. 6). HuH7 and Hc cells were transfected with wild-type or various mutant RXR\( \alpha \) cDNAs and, after treatment with 9cRA, BrdUrd labeling indexes were determined. Because transfection efficiency was relatively low (approximately 5–7\% for each cDNA in both types of cells), there was concern that the effect of overexpressed RXR\( \alpha \) mutants would be diluted by the influence of high levels of endogenous RXR\( \alpha \) expression in HuH7. To surmount this problem, we performed BrdUrd and RXR\( \alpha \) double staining and estimated the proliferation only of transfected RXR\( \alpha \)-overexpressing cells. We analyzed RXR\( \alpha \)-transfected cells, where nuclear RXR\( \alpha \)s was more than twice as great as endogenous RXR\( \alpha \) in corresponding empty vector-transfected cells, and then determined the ratio between the number of BrdUrd double-positive cells against the total RXR\( \alpha \)-positive cell number. In HuH7 cells (Fig. 6A), overexpression of T82A (Column 2), S260A (Column 3), or T82A/S260A (Column 4) mutant RXR\( \alpha \)s resulted in a significant (22–44\%) reduction in the BrdUrd-positive cell numbers compared with wild-type RXR\( \alpha \) transfected cells (Column 1). In contrast, in Hc cells (Fig. 6B), overexpression of biologically inactive T82D (Column 2), S260D (Column 3), and T82D/S260D (Column 4) mutant RXR\( \alpha \)s significantly increased the number of BrdUrd-positive cells.

DISCUSSION

We present new evidence of RXR\( \alpha \) phosphorylation in human HCC. RXR\( \alpha \)s was phosphorylated in human HCC tissue samples (Fig. 1) as well as in a human HCC cell line (Fig. 2B). Phosphorylated
RXRα was resistant to proteolytic degradation (Figs. 1, 2, and 4), lacked transactivation activity (Fig. 5), and exerted dominant-negative inhibition (Fig. 5), which was likely linked to the promotion of HCC cell growth (Fig. 6). These findings are summarized in Fig. 7. These changes were triggered by MAPK induction in HCC cells (Fig. 3), as has been reported in vivo (24), and not attributable to a mutation in the RXRα gene (data not shown). Only full-length RXRα (M, 54,000) but not the truncated receptor (M, 44,000) was phosphorylated (Figs. 1 and 2) at serine 260 (Figs. 3 and 4). Dephosphorylation at this site by site-directed mutagenesis restored the degradation (Fig. 4) as well as transactivation activities (Fig. 5) and suppressed growth of the cancer cells (Fig. 6), particularly in the presence of the ligand. Suppression of cell growth was also observed when phosphorylation was modified. Accumulation of full-length RXRα may represent the presence of the phosphorylated inactive receptor in HCC.

Normal liver tissues and Hc hepatocyte cultures, both of which were arrested in the G0-G1 phase, did not contain phosphorylated RXRα (Figs. 1 and 2B, respectively), probably because normal hepatocytes did not have detectable levels of active MAPKs (Fig. 3B). When active MAPKs were induced in Hc cells by transfecting constitutively active MEK1 cDNA, RXRα degradation was inhibited, as has been observed for HuH7 cells (Fig. 3B), suggesting that MAPKs play a role in phosphorylation and retarded degradation of RXRα. Although M, 54,000 RXRα in noncancerous surrounding liver was slightly phosphorylated (Fig. 1), this might be explained by the presence of cirrhosis in these tissues, in which hepatocytes proliferate in a regenerative response to inflammation and necrosis. Similarly, normal Hc hepatocytes in growth phase also expressed a slight amount of phosphorylated M, 54,000 RXRα (data not shown). Full-length M, 54,000 RXRα overexpressed in Hc cells was detected in the experiment in Fig. 4C, but not in Fig. 2A, for unclear reasons. However, this might be related to the difference in the method of transfection [electroporation (Fig. 2A) versus lipofection (Fig. 4C)].

As shown in Fig. 5A, if transactivation activity in HuH7 cells is solely regulated by phosphorylation state at both threonine 82 and serine 260, all T82A, S260A, and T82A/S260A mutants should have higher activity compared with wild-type receptor. However, only the T82A mutant showed higher activity than the wild-type RXRα. The ΔA/B mutant, which mimics the cleaved M, 44,000 form of RXRα (12, 13), loses ligand-induced transactivation activity, consistent with the previous report (25). Accordingly, we speculate that loss of transactivation activity upon cleavage of the A/B domain explains why the S260A and T82A/S260A mutants did not enhance transactivation activity more than the wild-type. In supporting this hypothesis, the T82A mutant showed higher activity than the wild-type RXRα, i.e., that the T82A mutant restored the transactivating activity due to dephosphorylation at threonine 82, which was sustained due to its relative resistance to proteolysis, whereas the S260A mutant restored the transactivating activity to a lesser extent, which decayed as a result of its degradation to the A/B domain truncated form. In contrast, Hc cells possess high transactivation activity of RXRα, although most of it exists as a degraded form in this cell type. Therefore, it might be possible that phosphorylation and degradation of RXRα and their effects on transactivation activity may be cell type context dependent.

Controversial results have been reported regarding biological consequences of phosphorylation of RXR. Treatment of COS and CVI cells with a protein phosphatase inhibitor leads to an increase in transactivation of RXR-responsive reporter genes (26, 27). On the other hand, hyper-
Representative results from three different experiments with similar results are shown. 9cRA (serine 260, the putative consensus sites of the MAPK family (14, 16). We have demonstrated that RXRα ligands are required for dephosphorylation (Fig. 2B) and/or degradation (Figs. 2–4) of RXRα as well as for its transactivation activity (Fig. 5). In normal bronchial epithelial cells, RA inhibits both JNK and ERK1/ERK2 activities by inducing the dual-specificity MAPK phosphatase 1 (22). Inhibition of phosphorylation with kinase inhibitors might restore the function of RXRα in part and thereby, in the presence of the ligand, stimulate the expression of MAPK phosphatase 1, which would in turn inhibit phosphorylation of the receptor. The results in Fig. 4D underscore the importance of ligand binding to RXRα for its metabolism. On the basis of a previous study (30), ligand-

Fig. 5. Transactivation activities of endogenous as well as overexpressed wild-type and mutant RXRαs in HuH7 and Hc cells. HuH7 cells were cotransfected with CRBPII-RXRE-luciferase reporter gene, δ-CRBPII-Luc, plus either empty (Columns 1 and 2), wild-type RXRα-expressing (Columns 3 and 4), its alanine mutant (T82A (Columns 5 and 6), S260A (Columns 7 and 8), T82A/S260A (Columns 9 and 10))-expressing, or A/B domain-truncated ΔA/B mutant-expressing (Columns 11 and 12) vectors along with pRL-CMV (Renilla luciferase) as an internal standard using lipofection (A). Similarly, Hc cells were cotransfected with δ-CRBP/Luc plus either empty (Columns 1 and 2), wild-type RXRα-expressing (Columns 3 and 4), or its aspartate mutants (T82D (Columns 5 and 6), S260D (Columns 7 and 8), and T82D/S260D (Columns 9 and 10))-expressing vectors (B). On the next day after transfection, cells were left untreated (odd-numbered samples) or were treated (even-numbered samples) for another 24 h with 1 μM 9cRA. Thereafter, luciferase activity in cell lysates was measured and plotted as fold induction compared with the activity in empty vector-transfected, 9cRA-untreated each cell (Column 1) after normalized to Renilla luciferase activity. Values are the means (n = 5); bars, SD. *, P < 0.01, significant difference obtained by a comparison between indicated pairs. Representative results from three different experiments with similar results are shown.

 phosphorylation by JNKs does not affect the transactivation properties of either RXRα homodimers or RXRα/RARα heterodimers in COS-1 cells (16). In contrast, phosphorylation of human RXRα at serine 260 can interfere with the vitamin D3 receptor/RXR-dependent as well as RXR/ RXR-dependent signaling pathways and thereby confer a hormone-resistant growth advantage in ras-transformed human keratinocytes (14). Repression in the activity of phosphorylated RXRα by MKK4/SEK1 and cAMP-dependent protein kinase has also been reported in COS cells (15, 28). Thus, regulation of RXR function by phosphorylation appears to be cell context dependent. Most steroid and nuclear receptors are phosphoproteins (17, 29). It is important to assess the effect of the phosphorylation of RXRα on transactivation through other nuclear hormone receptors/RXR heterodimers. We found that overexpression of the S260D mutant in Hc cells inhibited transactivation through an RARE and peroxisome proliferator-activated receptor-responsive element (data not shown). Because RXRα forms heterodimers with other nuclear receptors, alterations in the phosphorylation of RXRα may affect other nuclear receptor pathways as well. Moreover, it will be intriguing to determine whether other members of nuclear receptors might be phosphorylated in HCC tissues.

Divergent biological consequences of RXR signaling might be also dependent on which amino acids are phosphorylated. We investigated here the influence of phosphorylation at threonine 82 and/or serine 260, the putative consensus sites of the MAPK family (14, 16). We cannot rule out the possibility that phosphorylation at other sites might also affect transactivation activity and cell growth. It has been reported that in addition to threonine 82 (threonine 87 in mouse) and serine 260 (serine 265 in mouse), mouse RXRα is phosphorylated by JNK1 and JNK2 at serine 61 (serine 56 in human) and serine 75 (serine 70 in human; Ref. 16). Furthermore, serine 27 can be phosphorylated by a cAMP-dependent protein kinase (28). We are now investigating whether phosphorylation at these amino acid residues might affect degradation and function of RXRα.

Fig. 6. Effect of overexpressing wild-type and mutant RXRαs on the proliferation of HuH7 and Hc cells. HuH7 (4 × 104 cells/35-mm dish) and Hc cells (B; 2 × 104 cells/35-mm dish) were transfected with various plasmid vectors expressing wild-type (Column 1) or mutant RXRα cDNAs (Columns 2–4). Twenty-four h after transfection, cells were treated with 1 μM 9cRA for another 24 h, and thereafter, BrdUrd labeling indexes and expression of RXRα were measured by immunocytochemical methods as described in “Materials and Methods.” The data represent the percentage of BrdUrd and RXRα-positive cells. Values are the means (n = 6); bars, SD. *, P < 0.01, significantly different from the cells transfected with wild-type RXRα. Representative results from three independent experiments with similar results are shown.

Fig. 7. Working model of RXR phosphorylation in HCC. In HCC, highly activated MAPK family (3) phosphorylates RXRα at least at serine 260 (22), which renders the receptor resistant to proteolysis (8), which may be linked to the uncontrolled growth of the cancer cells (8).
binding to RXRα promotes its degradation via the ubiquitin-proteasome pathway. Similarly, we found recently that S260A and T82A/S260A mutant RXRαs undergo rapid ubiquitination and subsequent degradation by the proteasome in HuH7 cells upon 9cRA-treatment, implying that phosphorylation at serine 260 might interfere with ubiquitination and thereby prevent proteasomal degradation. Finally, ligand binding is essential for recruiting coactivators and exerting transactivation activity (8, 9). Thus, RXRα ligands appear to function indirectly to inhibit kinases, thereby promoting degradation and transactivation. It will also be important to assess the effect of phosphorylation of RXR on homo- and heterodimerization.

Despite recent advances in diagnosis and treatment of HCC, the 5-year survival rate barely reaches 50% because of the high incidence of second primary tumors (31). The impaired function of phosphorylated RXRα and its improvement by RXR ligands may partly explain the close relationship between hepatocarcinogenesis and a reduction in hepatic retinoid content at an early phase of hepatocarcinogenesis (5). Restoration of RXRα function may therefore be a potential approach to treating HCC. In this regard, we have demonstrated a chemopreventive effect of a retinoid analogue, a ligand to RXRα, on the occurrence of second primary HCC (6, 7). This compound, named acyclic retinoid, induced apoptosis in HuH7 cells (32) via induction of an apoptosis-inducing enzyme, tissue transglutaminase (33–36), the transcription of which is governed by RAR-RXR heterodimers (37). Recently, we have found that the acyclic retinoid restored the activity of RXRα by inducing dephosphorylation of the receptor, even in the absence of kinase inhibitor as well as by functioning as a ligand in HuH7 cells. This might well be linked to its apoptotic effect via tissue transglutaminase in HCC cells.

ACKNOWLEDGMENTS

We thank Drs. Y. Shidoji (Siegob University of Nagasaki, Nagasaki, Japan) and Y. Okano (Gifu University, Gifu, Japan) for fruitful discussion, and Y. Suzuki, J. Shimada, K. Todokoro, Y. Nagata, and C. Iijima (RIKEN) for technical assistance and useful discussions.

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Phosphorylation of Retinoid X Receptor α at Serine 260 Impairs Its Metabolism and Function in Human Hepatocellular Carcinoma


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