The RING Finger Protein, RNF8, Interacts with Retinoid X Receptor α and Enhances Its Transcription-stimulating Activity*

Received for publication, August 18, 2003, and in revised form, February 17, 2004
Published, JBC Papers in Press, February 23, 2004, DOI 10.1074/jbc.M309148200

Yukihiro Takano‡§, Seiji Adachi‡§, Masataka Okuno‡, Yoshinori Muto‡, Takashi Yoshioka‡, Rie Matsushima-Nishiwaki‡, Hisashi Tsurumi‡, Kenichi Ito‡, Scott L. Friedman**, Hisataka Moriwaki‡, Soichi Kojima‡‡, and Yukio Okano‡§§

From the §Department of Molecular Pathobiology, ¶First Department of Internal Medicine, and **Department of Basic Health Science and Fundamental Nursing, Gifu University School of Medicine, Gifu 500-8705, Japan, ‡Division of Liver Diseases, Mount Sinai Medical Center, New York, New York 10029-6574, and ‡‡Molecular Cellular Pathology Research Unit, RIKEN, Wako 351-0198, Japan

Retinoid X receptor α (RXRα) is a member of the steroid hormone receptor superfamily. Using yeast two-hybrid screening, β-galactosidase assays, and pull-down assays, we show that RNF8, a RING finger protein recently isolated as a protein binding to a ubiquitin-conjugating enzyme, binds to RXRα through the N-terminal regions of both proteins. In COS7 cells, overexpressed RNF8 colocalized and interacted with RXRα in the nucleus, as shown by fluorescence resonance energy transfer. A point mutation of RNF8, Cys-403 to Ser (C403S), which disrupts the RING finger structure, or deletion of the N-terminal region (ΔN) of RNF8 prevented localization of RNF8 to the nucleus without affecting nuclear localization of RXRα. Although transient overexpression of RNF8 had little effect on RXRα ubiquitination, RNF8 dose-dependently enhanced RXRα-mediated transactivation of the RXR-responsive element (RXRE)-bearing gene promoter without the addition of its ligand, 9-cis-retinoic acid (RA), and up-regulated the expression of the genes downstream of RXRE as well as an RA-response element. This transactivation-enhancing activity was not seen with either the C403S point mutant or the ΔN deletion mutant of RNF8. These results suggest a novel function of RNF8 as a regulator of RXRα-mediated transcriptional activity through interaction between their respective N-terminal regions.

Retinoic acid (RA), an active metabolite of vitamin A, has profound effects on the proliferation and differentiation of various cell types (1), mainly through two members of the nuclear receptor superfamily, retinoic acid receptor (RAR) and retinoid X receptor (RXR) (2, 3), which are ligand-dependent transcriptional regulators. Although all-trans-RA and 9-cis-RA (9cRA) both bind to the RAR, only 9cRA binds to and activates RXR (2).

Like other members of the nuclear receptor superfamily, RXR has a conserved structure made up of five domains, designated N terminus to C terminus as A to E. These domains have distinct functions and can act independently. The N-terminal region (domains A and B) contains an autonomously functioning region called activation function 1 (AF-1), which is involved in ligand-independent transcriptional transactivation and is not highly conserved between receptors. Domain C, which contains two zinc binding motifs (zinc finger-like), corresponds to the core of the DNA binding domain responsible for recognition of cognate response elements. Domain D (hinge region) is involved in ligand-induced functional changes and in the binding of receptors to co-repressors. Domain E, which is moderately conserved, is thought to be involved in ligand binding, contains ligand-dependent activation function 2 (AF-2), and provides a surface for dimerization (4).

Both the RAR and RXR have three subtypes, α, β, and γ, characterized by differences in the AF-1 domain (5). They usually form an RAR/RXR heterodimer and exert transcription-regulating activity by binding to a specific DNA sequence known as the RAR-responsive element in the promoter regions of target genes (6). RXR also forms heterodimers with several other members of the nuclear receptor superfamily and transactivates or inhibits target gene promoters by binding to a specific DNA sequence known as the hormone-responsive element (7). RXR can also form homodimers, which transduce signals by binding to a specific DNA sequence, known as the RXR-responsive element (RXRE), in the promoter regions of target genes (7–9). The transcriptional regulatory activity of these hetero- or homodimers is modulated by a variety of transcriptional co-activators and co-repressors, depending on the cell and tissue type involved (7). The ligand-dependent transcriptional activity is switched on by the binding of ligand to these retinoid receptors, which induces a conformational change, leading to either the dissociation of co-repressors from or the binding of co-activators to RAR/RXR or RXR/RXR dimers. The co-activators are components of multiprotein complexes with histone acetyltransferase activity that acetylate the N-terminal region of histones, leading to nucleosomal repulsion and chromatin decondensation, which is thought to be indispensable for transcriptional activation by the retinoid receptors (6). In contrast, little is known about ligand-independent transcriptional activation.

* This study was supported in part by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (to M. O., H. M., S. K., and Y. O.), NIDDK, National Institutes of Health Grant DK37340 of Education, Science, Sports, and Culture of Japan (to M. O., H. M., S. K., and Y. O.), NIDDK, National Institutes of Health Grant DK37340.

** This study was supported in part by grants-in-aid from the Ministry of Education, Science, Sports, and Culture of Japan (to M. O., H. M., S. K., and Y. O.), NIDDK, National Institutes of Health Grant DK37340.
Enhanced Transactivation via RXRα-RNF8 Interaction

The retinoid receptors play a pivotal role in preventing carcinogenic processes in various types of cancers (10–15). We have shown that normal functioning of RXRα is necessary to suppress aberrant growth and induce apoptosis in hepatocellular carcinoma cells (16–19). Phosphorylation of RXRα reduces RXRα-dependent transactivation and is closely related to hepatocarcinogenesis (20), and restoration of RXRα activity, therefore, provides a key target for the prevention of liver cancer (21).

We screened for proteins that bind to RXRα and found a really interesting new gene (RING) finger protein, RING finger protein 8 (RNF8), recently isolated as a protein of unknown physiological function, which binds to the ubiquitin (Ub)-conjugating enzyme, E2 (UBE2E2) (22). RNF8 contains the forkhead-associated domain, a phosphopeptide binding motif, in its N-terminal region and a RING finger domain in the C-terminal region (we searched the Conserved Domain Data base for conserved domain RNF8 at www.ncbi.nlm.nih.gov:80/Structure/cdd/cdd.shtml). RING finger proteins are involved in the ubiquitination of short-lived proteins (23–25) and in the modulation of transcriptional activation by certain nuclear receptors (26–28). Although RNF8 binds to Ube2E2, which promotes Ub/proteasome-mediated degradation of RXRα (29), here we have found that RNF8 does not participate in Ub/proteasome-mediated degradation of RXRα but, rather, as an enhancer of transactivation activity via RXRα.

EXPERIMENTAL PROCEDURES

Plasmid Construction—The wild-type (WT) human RXRα-expressing vector, pBS-RXRα, was kindly provided by Dr. R. M. Evans (Salk Institute, La Jolla, CA); the RXRα DNA was amplified by PCR and inserted into the pAS2–1 vector (Clontech, Palo Alto, CA) to generate pAS2–RXRα. The RXRα deletion mutants, indicated in Fig. 1A, were prepared by PCR using pAS2–RXRα as template and cloned into pAS2–1. The primer pairs used for ∆A/B (lacking the A and B domains) were 5′-AAAGAATTCGACAAGTCCAAGCAGATCTGCAGCCATC3′ and 5′-AAATTCTAGAGATCTTACGCGCAGCTCC3′, and those for ∆E terminating (lacking the terminal region of E domain) were 5′-AAAGATCTTCGAGCAAGTCAGACAAACAAAAT-3′ and 5′-AAATTCGAGCTCCTCAGGCGCTCCATCC3′. The sense primer used for the A/B/C, A/B, and A/B/C mutant (lacking the A, B, and C domains) were 5′-AAAGAATTCGACATCCGATCGAGGACTTGAGGTCG-3′, whereas the antisense primers were, respectively, 5′-AAAGATCTTCGAGCAAGTCAGACAAACAAAAT-3′ and 5′-AAATTCGAGCTCCTCAGGCGCTCCATCC3′. The primer pairs used for the ∆A/B/C mutant (lacking the A, B, and C domains) were prepared by deleting the fragment between the first and third NcoI sites (nucleotides 29–197) followed by self-ligation.

N-terminal polyhistidine His6 Xpress-tagged WT RNF8 and its deletion mutants and C403S point mutant (Cys-403 mutated to Ser), present in Fig. 1B, were constructed as described previously (22). The deletion and point mutations were confirmed by sequencing.

Expression vectors encoding fusion proteins consisting of the motif-binding protein (MBP) and WT RXRα or its ∆A/B mutant were constructed by subcloning cDNA encoding WT RXRα or the ∆A/B mutant, amplified by PCR using, respectively, pBS2-WT or ∆A/B RXRα as template, into the pMAL-p2X vector (New England Biolabs, Beverly, MA).

Enhanced green fluorescent protein (EGFP)-RXRα was constructed by subcloning PCR-amplified RXRα cDNA with BglII sites attached at both ends into the BglII/BamHI sites of the pEGFP-C1 vector (Clontech). Blue fluorescent protein (BFP)-RNF8 was generated by cloning the BglII-BglII fragment from pACT2-RNF8 into the BamHI-BamHI site of the pBQ500-C1 vector (Qiogene, Carlsbad, CA). The BFP-40-AE-EGFP plasmid encoding EGFP coupled to BFP by a 40-amino acid linker was prepared by ligation of EGFP cDNA into the BFP vector, placing the EGFP-coding sequence in the BFP-reading frame.

Yeast Two-hybrid Assay—A yeast two-hybrid library was screened to isolate proteins that interact with RXRα-binding proteins. A bait plasmid pACT2-RNF8 was constructed by subcloning the open reading frame of RNF8 into the pAS2–1 vector as a GAL4 DNA binding domain fusion (pAS2–RXRα), as described above, was used to identify binding proteins from a human liver cDNA activation domain fusion library in the pACT2 vector. The yeast strain Y363-2A was transformed with both plasmids. Positive clones were first selected on synthetic dropout (SD) medium in the absence of two nutrients (Leu and Trp). The colonies obtained were resuspended in water at equal densities, spotted onto SD medium in the absence of four nutrients (adename, His, Leu, and Trp), and scored for differential growth after 3–5 days. The results were confirmed by a quantitative assay testing for β-galactosidase activity in the yeast strain Y-187. The two-hybrid system was also used to assess the binding of WT RNF8 to various RXRα deletion mutants (∆AB, ∆E terminus, ∆A/B, ∆E, A/B, and ∆A/B/C) and of WT RXRα to various RNF8 deletion (1–440, 1–403, and 403–485) or point (C403S) mutants.

Quantitative β-Galactosidase Assay—β-Galactosidase activity in the yeast strain, Y-187, was determined by liquid culture as a quantitative assay. This assay was carried out using o-nitrophenyl-β-D-galactoside (Sigma) as the substrate. Briefly, 2 ml of cells grown in liquid SD selection medium was used to inoculate 8 ml of yeast complete medium. After 3–5 h of growth (A600nm = 0.5–0.8) in the absence or presence of 1 μM 9cRA, 1.5-ml aliquots of culture were placed in microcentrifuge tubes, and cells were collected by brief centrifugation. Each cell pellet was washed with 1.5 ml of Z buffer (60 mM NaHPO4, 40 mM Na2HPO4, 10 mM KCl, 1 mM MgSO4, pH 7) and resuspended in 150 μl of Z buffer. 100 μl of resuspended pellets was transferred in a new 1.5-ml microcentrifuge tube. Cells were then frozen in liquid nitrogen and thawed at 37 °C in a water bath 3 times to break open the yeast cells. Then 0.7 ml of Z buffer containing 0.27% (v/v) β-mercaptoethanol was added to the tube and mixed well followed by 160 μl of o-nitrophenyl-β-D-galactoside (0.1% w/v) and Z buffer (4 mM MgCl2, pH 7). The mixture was incubated at 30 °C until a yellow color developed; 0.4 ml of Na2CO3 was then added to stop the reaction, and the reaction time was recorded. The tubes were centrifuged for 10 min, and the absorbance of the supernatant at 420 nm was measured. The β-galactosidase activity was calculated by the equation, β-galactosidase units (u) = 1000 × [A600nm × V × A600/V], where t is elapsed time (in min) of incubation, V is 0.1 ml × concentration factor (10 in this experiment), and A600nm is absorbance at 600 nm of 1 ml of culture.

In Vitro Binding Assay—The MBP-WT RXRα and MBP-∆A/B RXRα fusion proteins were expressed in Escherichia coli and purified on amylose resin (New England BioLabs, Beverly, MA). COS7 cells overexpressing His- and Xpress-tagged WT or mutant RXRα deletion mutant (∆AR) were harvested and lysed in radiomimume precipitation assay buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Nonidet P-40, 0.1% SDS, 0.5% sodium deoxycholate, 1 mM phenylmethylsulfonyl fluoride, and 1 mM ZnCl2). Purified MBP-RXRα (500 ng) was added to the lysates, and the mixture was incubated for 2 h at 4 °C in the absence or presence of 1 μM 9cRA (Sigma), then amylose resin was added, and incubation was continued for 30 min at 4 °C. The resin was then washed with radiomimome precipitation assay buffer and boiled in SDS sample buffer, and the eluted proteins were separated by 8% SDS-PAGE and analyzed by Western blotting using anti-Xpress antibody (Invitrogen), which recognizes the DLYDDDXXD Xpress sequence adjacent to His.

Cell Culture and Transfection—COS7 cells were maintained in Dulbecco’s minimum essential medium (DMEM) containing penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% fetal calf serum (FCS). The human colorectal carcinoma-derived cells, Caco-2 (Japanese Cancer Research Resources Bank, Tokyo, Japan), were maintained in DMEM-M-12 (Invitrogen) containing penicillin (100 units/ml), streptomycin (100 μg/ml), and 10% FCS. Transfections were performed using LipofectAMINE Plus (Invitrogen) according to the manufacturer’s protocol. Briefly, before transfection Caco-2 cells were cultured for 24 h in phenol red- and FCS-free medium supplemented with ITS Supplement (Invitrogen) to give final concentrations of 10 μg/ml insulin, 5.5 mg/ml bovine serum albumin, and 6.7 μg/ml ITS supplement. The medium was then replaced to phenol red-free DMEM containing 10% charcoal-stripped FCS for 6 h, then the cells were transfected and harvested 48 h later. In some experiments, 9cRA (1 μM) or vehicle (ethanol, final concentration <0.05%) was added to the medium 24 h after transfection.

Immunostaining—COS7 cells, plated on coverslips in 6-mm dishes, were harvested with plasmaprecipitating WT RXRα (1 μg/dish) and WT or mutant (C403S or ∆AR) His-Xpress-RNF8 (2 μg/dish, respectively) as described above, then the cells were washed in phosphate-buffered saline (PBS), pH 7.4, fixed for 10 min in 4% paraformaldehyde in PBS, washed twice in PBS, and permeabilized for 3 min with 0.2% Triton X-100 in PBS. After blocking with 0.1% BSA in PBS, the coverslips were incubated for 2 h in PBS containing mouse mAb to RXRα (Invitrogen) and rabbit polyclonal anti-myc antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), washed with PBS, and then incubated for 2 h with Alexa 488-conjugated goat anti-mouse antibody and Alexa 546-conjugated goat anti-rabbit antibodies (Molecular
Table I

| Name       | Sequence                           | Nucleotide numbers | Product length |
|------------|------------------------------------|-------------------|----------------|
| RFN5 (U)   | 5'-AGCCTAAACTCTTCGCCATCAG-3'       | 705-729           | 610            |
| RFN5 (L)   | 5'-GGGCTCTATTCGCCATCCTAC-3'       | 1290-1314         | 3873           |
| RXRα (U)   | 5'-CCCTCGGAGCAGGCATATGGA-3'       | 425-444           | 528            |
| RXRα (L)   | 5'-TGAGCGTGGTGAGGAGCAGAAGG-3'     | 933-952           | 784            |
| CRBPII (U) | 5'-GGACCTGGGAGAGTGGAGTGT-3'       | 19-39             | 2118           |
| CRBPII (L) | 5'-TTTGCAACTGTGAGCAGCAGAC-3'      | 376-396           | 389            |
| RARα (U)   | 5'-CTGTAGTTACTCTAGCCGGAG-3'       | 46-65             | 387            |
| RARα (L)   | 5'-CCCTCGGAGCAGGCATATGGA-3'       | 415-434           | 393            |
| G3PDH (U)  | 5'-TGAAGCTGAGGTCAACCGTGTGGT-3'    | 11-36             | 983            |
| G3PDH (L)  | 5'-CATGTTGCGCATAGGTCACACC-3'      | 970-993           |                |

Enhanced Transactivation via RXRα-RNF8 Interaction

Probes, Eugene, OR, respectively. All procedures were carried out at room temperature. Images were acquired using an Olympus IX70 inverted microscope equipped with differential interference contrast (DIC) optics. Both the fluorescence and DIC images were captured as described previously (22) using a C5985 CCD camera with on-chip integration (Hamamatsu Photonics, Hamamatsu, Japan) and a Macintosh 8500 computer running C5985 imaging software. The fluorescence light source was a 75-W xenon arc lamp shuttered using a custom-made shutter unit so that cells were only illuminated during image acquisition.

Fluorescence Resonance Energy Transfer (FRET)—COS7 cells, grown on 35-mm glass-bottom culture dishes (Matsunami, Osaka, Japan), were transfected with plasmids encoding BFP, BFP-RNF8, EGFP-RXRα, or BFP-EGFP tandem, or a combination of EGFP-RXRα and either BFP-RNF8 or BFP. To obtain images of living cells, the culture dishes were observed using an I700 inverted microscope; the imaging system used was the same as that used for immunostaining experiments. The filter sets for discriminating between BFP and EGFP fluorescence were obtained from Omega Optical Inc. (Brattleboro, VT). For BFP detection, cells were viewed using a filter set with a 365/50 nm excitation filter, a 400-nm dichroic beam splitter, and a 535/45-nm emission filter (BFP channel). EGFP was detected using a filter set with a 475/40-nm excitation filter, a 505-nm dichroic beam splitter, and a 535/45-nm emission filter (EGFP channel). The filters used for FRET were a 365/50 nm excitation filter, a 400-nm dichroic beam splitter, and a 535/45-nm emission filter (FRET channel). The emission filters for the BFP channel and the FRET channel were switched using a filter changer (Lambda 10-2; Sutter Instrument, Novato, CA) coupled between the output port of the microscope and the C5985 CCD camera. To measure FRET, three images were acquired sequentially through the BFP, FRET, and EGFP channels. FRET quantification between the two channels was achieved using the following equation,

\[ N_{\text{FRET}} = \frac{I_{\text{FRET}} - I_{\text{EGFP}} \times a + I_{\text{BFP}} \times b}{\sqrt{I_{\text{EGFP}} \times I_{\text{BFP}}}} \] (Eq. 1)

where \( I_{\text{FRET}}, I_{\text{EGFP}}, \) and \( I_{\text{BFP}} \) correspond to background-subtracted images of cells co-expressing EGFP and BFP acquired through the FRET, EGFP, and BFP channels, respectively. Constants \( a \) and \( b \) represent the fraction of bleed-through of BFP and EGFP fluorescence, respectively, through the FRET channel and were determined using cells expressing either BFP-RNF8 or EGFP-RXRα. In our system ~24% of the EGFP signal and ~3% of the BFP signal were detected in the FRET channel. The FRET channel was presented in pseudocolor mode, according to a temperature-based lookup table, with blue (cold) indicating low values and red (hot) indicating high values. For quantitative comparison, the \( N_{\text{FRET}} \) for each pixel in nuclear areas was calculated, and the number of pixels with a given \( N_{\text{FRET}} \) value was counted and plotted as a distribution histogram. All calculations were performed using the public domain program, Image J Version 1.29 (developed at NIH), with plug-ins for Calculator Plus and SixteenBit Histogram.

In Vivo Ubiquitination Assays—Ubiquitination of proteins requires the covalent attachment of 8.6-kDa Ub to multiple lysine residues, forming polyUb chains bound to target proteins, and can be seen as a ladder of high molecular mass species on SDS-polyacrylamide gels (29). COS7 cells were transfected with various combinations of plasmids encoding RXRα (1 μg), HA-tagged Ub (kindly provided by Dr. D. Bohmann, European Molecular Biology Laboratory) (3 μg), or His-RNF8 (WT or C403S) (3 μg). MG132 (5 μM; Calbiochem), a potent proteasomal inhibitor, was added 24 h after transfection, and the cells were harvested 27–30 h after transfection. Cells were sonicated for 30 s in lysis buffer (100 mM sodium phosphate, pH 8.0, containing 6 M guanidine-HCl and 10 mM imidazole), then RXRα was immunoprecipitated using anti-RXRα antibodies (DN197; Santa Cruz Biotechnology) and subjected to SDS-PAGE, and ubiquitinated RXRα was detected by Western blotting using anti-HA antibodies (12CA5; Roche Applied Science) as described previously (29).

Luciferase Reporter Assays—A reporter plasmid, tk-CRBPII-Luc, which contains multiple copies of the DR1 type XXXX sequences within the rat cellular retinol-binding protein type II (CRBPII) promoter upstream of luciferase cDNA, was kindly provided by the late Dr. K. Umesono (Kyoto University, Kyoto, Japan). COS7 cells, seeded in 12-well plates, were transfected with WT RXRα-expressing plasmid (100 ng/well), WT, or mutant (C403S or ΔN) RNF8-expressing plasmid (10 or 100 ng/well), and tk-CRBPII-Luc reporter (350 ng/well) was transfected with pRL-CMV-Luc (Renilla luciferase, 50 ng/well, Promega, Madison, WI) as an internal standard to normalize transfection efficiency. After exposure of the cells for 24 h to the transfection mixture, the medium was replaced with DMEM containing 10% charcoal-stripped FCS in the absence or presence of 1 μM 9-cRA. Forty-eight hours after transfection, cell lysates were prepared, and the luciferase activity of each cell lysate was measured using a LumiCount microplate luminometer (Packard Instrument Co.). Changes in firefly luciferase activity were calculated and plotted after normalization to changes in Renilla luciferase activity in the same sample.

Reverse Transcription-PCR—Total RNA was extracted from Caco-2 cells using ISOGEN (WAKO Pure Chemical, Osaka, Japan) and isopropanol precipitation, then 1 μg was reverse-transcribed using a High Fidelity RNA PCR Kit (TAKARA Biomedicals, Tokyo, Japan) and an oligo(dT) primer according to the manufacturer’s protocol. mRNAs for RNF8, RXRα, CRBPII, RARα, and glyceraldehyde 3-phosphate dehydrogenase (G3PDH) were detected using the primer pairs derived from the human sequences shown in Table I. For RNF8, RXRα, RARα, and G3PDH, the PCR conditions were 1 cycle of 94°C for 5 min, 25 (for RARα and G3PDH) or 30 (for RNF8 and RXRα) cycles of denaturation at 94°C for 30 s, annealing at 56–60°C for 30 s, and extension at 72°C for 1 min, and 1 cycle of 72°C for 10 min; for CRBPII, conditions were 1 cycle of 94°C for 5 min, 22 cycles of denaturation at 94°C for 30 s, annealing at 56°C for 30 s, and extension at 72°C for 30 s, and 1 cycle of 72°C for 10 min. The PCR products were run on 2% agarose gels and visualized by ethidium bromide staining.

Results

Discovery of RNF8 as an RXRα-binding Protein by Screening a Human Liver cDNA Library—Using the yeast two-hybrid screening system and a human liver library, we picked out a cDNA encoding an RXRα-binding protein and identified it as the RING finger protein, RNF8, by comparing the sequence with the data base in the DNA Data Bank of Japan. RNF8 was originally isolated as a partner of the ubiquitin-conjugating enzyme, UBE2E2 (22). By testing the binding of various mu-
Enhanced Transactivation via RXRα-RNF8 Interaction

![Schematic illustrations of the various deletion and point mutants for RXRα (A) and RNF8 (B) are presented together with the results for the yeast two-hybrid screening and quantitative β-galactosidase assay.](image)

Fig. 1. Identification of binding sites on RXRα and RNF8 using the yeast two-hybrid screening and quantitative β-galactosidase assay. Schematic illustrations of the various deletion and point mutants for RXRα (A) and RNF8 (B) are presented together with the results for the yeast two-hybrid screening and β-galactosidase assay (right-hand side). WT (column 1) or mutant (columns 2–7) RXRα fused to a GAL4 DNA binding domain and WT RNF8 fused a GAL4 activation domain (A) or WT (column 8) or mutant (columns 9–13) RNF8 fused to a GAL4 DNA binding domain and WT RXRα fused to a GAL4 DNA-binding domain (B) were simultaneously overexpressed in the yeasts, and then the transformants were plated on SD media lacking Leu and Trp (SD−/Leu−/Trp). The colonies obtained were resuspended in water at equal densities, spotted on SD−/aducose−/His−/Leu−/Trp plates, and incubated at 30 °C for 3–5 days in the absence or presence of 1 μM 9cRA. The apparent strength of the binding was assessed by the degree of growth and is indicated as strong interaction (+++), intermediate interaction (+), weak interaction (+) no interaction (–). The binding of WT RNF8 to various RXRα deletion mutants (A) and of WT RXRα to various mutant RNF8 (B) was also assessed by quantitative β-galactosidase assay as described under “Experimental Procedures.” Values are the mean ± S.D. for three independent experiments. The deleted regions are shown as bars. The black box indicates the RING finger motif. C403S indicates the mutation of Cys-403 to Ser. The numbers under each construct indicate the positions of the amino acids. The experiments were repeated three times using colonies from separate transformations with reproducible results.

![Deletion mutants of RXRα](image)

**A** Deletion mutants of RXRα

| RXRα | 1 WT | 2 ΔA/B | 3 ΔE | 4 A/B terminal | 5 A/B/C | 6 1-28 | 7 ΔA/B/C |
|------|------|--------|-----|--------------|--------|-------|---------|
|      |      |        |     |              |        |       |         |
|      |      |        |     |              |        |       |         |

![Deletion mutants of RNF8](image)

**B** Deletion mutants of RNF8

| RNF8 | 8 WT | 9 1-440 | 10 1-403 | 11 ΔN | 12 403-485 | 13 C403S |
|------|------|---------|---------|-------|-----------|---------|
|      |      |         |         |       |           |         |
|      |      |         |         |       |           |         |

The fact that the binding of RXRα to RNF8 was mediated by the N-terminal region of each of the molecules was corroborated by pull-down experiments (Fig. 2). Purified WT or ΔA/B mutant RXRα-MBP fusion proteins or MBP from overexpressing bacteria were incubated with cell lysates prepared from His-Xpress-RNF8-overexpressing COS7 cells in the absence (Fig. 2A, odd lanes) or presence (even lanes) of 9cRA, and then proteins bound to MBP or MBP fusion protein were pulled down using amylose resins and analyzed by Western blots using anti-Xpress antibody (Fig. 2A, upper panel). His-Xpress-
of the His-Xpress-RXRα complex. Lower panel, input XRα proteins were stained with Coomassie Brilliant Blue. B, upper panel, cell lysates of COS7 cells overexpressing His-Xpress-WT RXRα (lanes 5 and 6) or ΔA/B RXRα (lanes 5 and 6) in the absence (odd lanes) or presence (even lanes) of 1 μM 9cRA, then the RXRα-RNF8 complex was pulled down using amylose resin, and RNF8 was detected by Western blotting using anti-Xpress antibody. Lower panel, input XRα proteins were stained with Coomassie Brilliant Blue. B, upper panel, cell lysates of COS7 cells overexpressing His-Xpress-WT RXRα (lanes 5 and 6) or ΔA/B RXRα (lanes 5 and 6) were incubated with purified MBP-WT RXRx and RNF8 was detected by Western blotting using anti-Xpress antibody. Lower panel, input XRα proteins were stained with Coomassie Brilliant Blue.

Subcellular localization of WT and mutant RNF8s in COS7 cells. COS7 cells were cotransfected with His-Xpress-tagged WT or mutant (C403S or ΔN) RNF8 and WT Myc-His-RXRα as described under “Experimental Procedures.” A, cell lysates were prepared, and the expression of WT (lane 1) and mutant (lane 2, C403S; lane 3, ΔN) RNF8 was detected by Western blotting using anti-Xpress antibody. B, cells were fixed in paraformaldehyde and immunostained using anti-Xpress antibody and Alexa 488-conjugated secondary antibody for RNF8 (green fluorescence, panels a, d, and g) as well as anti-c-Myc antibodies and Alexa 546-conjugated secondary antibodies for RXRα (orange fluorescence, panels b, e, and h). Fluorescence images (panels a, b, d, e, g, and h) and DIC images (panels c, f, and i) were acquired. Panels a–c, WT RNF8-transfected cells. Panels d–f, C403S RNF8-transfected cells. Panels g–i, ΔN RNF8-transfected cells. Bars, 10 μm. The micrographs presented are representative of three separate experiments.

Fig. 3. Subcellular localization of WT and mutant RNF8s in COS7 cells. COS7 cells were cotransfected with His-Xpress-tagged WT or mutant (C403S or ΔN) RNF8 and WT Myc-His-RXRα as described under “Experimental Procedures.” A, cell lysates were prepared, and the expression of WT (lane 1) and mutant (lane 2, C403S; lane 3, ΔN) RNF8 was detected by Western blotting using anti-Xpress antibody. B, cells were fixed in paraformaldehyde and immunostained using anti-Xpress antibody and Alexa 488-conjugated secondary antibody for RNF8 (green fluorescence, panels a, d, and g) as well as anti-c-Myc antibodies and Alexa 546-conjugated secondary antibodies for RXRα (orange fluorescence, panels b, e, and h). Fluorescence images (panels a, b, d, e, g, and h) and DIC images (panels c, f, and i) were acquired. Panels a–c, WT RNF8-transfected cells. Panels d–f, C403S RNF8-transfected cells. Panels g–i, ΔN RNF8-transfected cells. Bars, 10 μm. The micrographs presented are representative of three separate experiments.
Enhanced Transactivation via RXRα-RNF8 Interaction

Figure 4. Binding of RNF8 and RXRα in living COS7 cells as demonstrated by FRET. A, visualization of FRET in living cells. COS7 cells were transfected with plasmids expressing EGFP-WT RXRα and BFP-WT RNF8 (panels a–c), EGFP-WT RXRα and BFP (panels d–f), or EGFP-BFP tandem (panels g–i). Images were acquired using the acceptor filter set (EGFP filter) (panels a, d, and g) and the donor filter set (BFP filter) (panels b, e, and h). N_{FRET} values, calculated as described under “Experimental Procedures,” are shown as pseudocolor images (FRET signal) (panels c, f, and i). Bars, 10 μm. B, quantification of FRET between BFP-WT RNF8 and EGFP-WT RXRα. The normalized FRET (N_{FRET}) value for each pixel in nuclear areas was measured for four cells, and the number of pixels with a given N_{FRET} value plotted as a distribution histogram. Panel j, transfected cells expressing EGFP-WT RXRα and BFP-WT RNF8. Panel k, transfected cells expressing EGFP-WT RXRα and BFP. Panel l, transfected cells expressing EGFP-BFP tandem.

Figure 5. RNF8 has no effect on RXRα ubiquitination in COS7 cells. COS7 cells were transfected with plasmids encoding RXRα alone (lane 1) and HA-tagged Ub (lanes 2–5) together with control empty vector (ϕ, lane 3) or plasmids encoding His-Xpress-tagged WT RNF8 (lane 4) or His-Xpress-tagged C403S RNF8 (lane 5). Twenty-four hours later, the cells were treated for 3 h with the proteasome inhibitor MG132 (final 5 μM) and harvested under denaturing conditions as described under “Experimental Procedures.” RXRα was immunoprecipitated (IP) using anti-RXRα antibodies and subjected to SDS-PAGE followed by Western blot (WB) analysis using anti-HA antibody. The arrowhead shows the heavy chain of IgG. The results shown are representative of three independent experiments.

RNF8 Does Not Affect RXRα Ubiquitination—We previously reported that UBE2E2 acts as a Ub-conjugating enzyme during RXRα ubiquitination in hepatoma cells (29) and that RNF8 binds to UBE2E2 (22). We, therefore, examined the effects of RNF8 on RXRα ubiquitination in COS7 cells transfected with plasmids expressing RXRα- and HA-tagged Ub in the presence or absence of plasmids expressing His-Xpress-tagged WT or C403S RNF8 or control empty vector by immunoprecipitation of the lysate with anti-RXRα antibodies and analysis of the precipitate by SDS-PAGE and Western blotting using anti-HA antibody. As shown in Fig. 5, cells expressing RXRα and HA-tagged Ub showed multiple Ub conjugation to RXRα, seen as a ladder of high molecular mass species of ubiquitinated RXRα (Fig. 5, compare lanes 1 and 2). Unexpectedly, RXRα ubiquitination was neither enhanced by simultaneous overexpression of WT RNF8 (compare lanes 3 and 4) nor reduced by simultaneous overexpression of C403S RNF8 (compare lanes 3 and 5), showing that RNF8 was not involved in RXRα ubiquitination by endogenous UBE2E2.

RNF8 Enhances Promoter Transactivation via RXRα and Expression of Its Downstream Gene(s)—Because some RING finger proteins have been reported to modulate transactivation by certain nuclear receptors (26–28), we examined the effects of RNF8 on the RXRα-driven transactivation of the luciferase reporter gene, CRBPII-Luc, the promoter of which contains RXRE sequences. COS7 cells were cotransfected with WT RXRα, CRBPII-Luc, and increasing amounts of WT RNF8 expression vectors together with pRL-CMV-Luc as an internal control (Fig. 6). Transactivation of this reporter was dependent on both endogenous and exogenous RXR and 9cRA (Fig. 6A, compare column 1 and columns 4, 7, and 10). Interestingly, WT-RNF8 enhanced the transcription in a dose-dependent manner in the absence of 9cRA (Fig. 6A, columns 1–3), and the pixel distribution histograms (Fig. 4B). Most pixels from cells cotransfected with BFP-WT RNF8 and EGFP-WT RXRα (panel j) showed N_{FRET} values much higher than those in negative control cells (panel k), and the N_{FRET} value distribution in the BFP-RNF8/EGFP-RXRα-cotransfected cells overlapped with that for the positive control cells (panel l). These results showed efficient transfer of energy in the nucleus when both proteins were present, supporting direct binding between RNF8 and RXRα in the living cell nucleus.

Figure 4. Binding of RNF8 and RXRα in living COS7 cells as demonstrated by FRET. A, visualization of FRET in living cells. COS7 cells were transfected with plasmids expressing EGFP-WT RXRα and BFP-WT RNF8 (panels a–c), EGFP-WT RXRα and BFP (panels d–f), or EGFP-BFP tandem (panels g–i). Images were acquired using the acceptor filter set (EGFP filter) (panels a, d, and g) and the donor filter set (BFP filter) (panels b, e, and h). N_{FRET} values, calculated as described under “Experimental Procedures,” are shown as pseudocolor images (FRET signal) (panels c, f, and i). Bars, 10 μm. B, quantification of FRET between BFP-WT RNF8 and EGFP-WT RXRα. The normalized FRET (N_{FRET}) value for each pixel in nuclear areas was measured for four cells, and the number of pixels with a given N_{FRET} value plotted as a distribution histogram. Panel j, transfected cells expressing EGFP-WT RXRα and BFP-WT RNF8. Panel k, transfected cells expressing EGFP-WT RXRα and BFP. Panel l, transfected cells expressing EGFP-BFP tandem.

Quantitative analysis of the N_{FRET} values in the nucleus was performed on several cells, and the results are presented as
effect was more obvious when WT RXRα was overexpressed (columns 4–6). However, the enhancement of transactivation by 1 μM 9cRA in the presence of RNF8 was limited to ~2.5-fold compared with the level without the ligand, both in the absence (compare columns 3 and 9) and presence (compare columns 10 and 12) of exogenous RXRα, whereas in the absence of 9cRA up-regulated the CRBPII promoter activity by 4–5-fold both in the absence (compare columns 1 and 7) and presence (compare columns 4 and 10) of exogenous RXRα. C403S RNF8 or ΔN RNF8 did not increase RXRα-dependent transscription either in the absence (Fig. 6B columns 1–10) or presence (columns 11–20) of 9cRA, irrespective of cotransfection with WT RXRα (compare columns 1–5 and 6–10, and columns 11–15 and 16–20). These observations corroborate the result that C403S and ΔN RNF8 did not enter the nucleus (Fig. 3B).

Finally, we examined whether enhanced RNF8-mediated transactivation of the CRBPII promoter via the RXRE has biological relevance using colon carcinoma-derived Caco-2 cells that constitutively express CRBPII (34). Caco-2 cells also constitutively express endogenous RXRα and RNF8 (data not shown). Overexpression of WT 9cRA resulted in a significant 2.1-fold increase in CRBPII mRNA levels (Fig. 7A, lane 2), whereas overexpression of C403S or ΔN RNF8 had no effect (lanes 3 and 4, respectively). Moreover, because RXR forms heterodimers with RAR and is essential for the regulation of RXR/RAR-mediated gene expression (1–3), we also examined the effect of RNF8 on RXR/RAR-regulated expression of RARβ (Fig. 7B), which is endogenously expressed in Caco-2 cells. WT RNF8 up-regulated RARβ, which has typical RAR-responsive element in its promoter region (Fig. 7B, lane 6). Neither the C403S nor ΔN RNF8 affected the expression of RARβ (Fig. 7B, lanes 7 and 8, respectively). Similar results were obtained when the effects of WT and mutant RNF8 on the expression of CRBPII and RARβ were examined in the presence of 9cRA (data not shown). These results suggest that RNF8 may be an important factor promoting RXR-mediated as well as RXR/ RAR-mediated gene expression by physical interaction.

**DISCUSSION**

We demonstrated here that a novel RING finger protein, RNF8, colocalized with RXRα in the nucleus (Fig. 3B) and bound to RXRα (Fig. 4) through the N-terminal domain of each molecule (Figs. 1 and 2), leading to ligand-independent transactivation of the target genes (Figs. 6 and 7). A ligand for RXRα, 9cRA, did not affect the interaction between RNF8 and RXRα (Figs. 1 and 2), and localization of RNF8 in the nucleus required both a normal RING finger structure and the N-terminal region (Fig. 3B). Previous studies have demonstrated that the RING finger structure plays a crucial role in the nuclear localization of certain transcriptional regulators. For instance, both the tumor suppresser, BRCA1, and its associated protein, BARD1, contain N-terminal RING structures that are essential for their nuclear transport (35) and, thus, for their regulation of gene transcription (36). In addition, the N-terminal region of RNF8 contains the forkhead-associated domain that was originally identified as a phosphopeptide binding domain in a group of forkhead transcription factors but is now known to be present in a wide variety of proteins (37). Many forkhead-associated domain-containing proteins are found in the nucleus and are involved in DNA repair, cell cycle arrest, and pre-mRNA processing (37).

Because RNF8 is reported to bind to the Ub-conjugating
The RXR

The RXR

enzyme, UBE2E2 (22), which is involved in the ubiquitination of retinoid absorption. Moreover, RXRα ubiquitination by connecting the two molecules. However, this was found not to be the case, since no difference in the degree of RXRα ubiquitination by endogenous UBE2E2 was seen in control COS7 cells and RXRα-overexpressing cells (Fig. 5).

We found that RXRα promoted transactivation via RXRE of the CRBP II promoter (Fig. 6) and an increase in CRBP II mRNA levels (Fig. 7A) via the RXRE without the addition of the RXRα ligand, 9cRA. That CRBPII is a key protein in the absorption of retinoids by intestinal cells suggests possible involvement of RXRα in retinoid absorption. Moreover, RXRα also regulated the RXR/RAR heterodimer-mediated gene, RARβ (Fig. 7B), which is related to cell differentiation and growth suppression (38). Similar transactivation-promoting activity of RING finger proteins has been reported for ARA54/SNURF and TIF1β, which activate transcription of the androgen receptor and the glucocorticoid receptor, respectively (26–28).

The RXR contains two transcription activation domains, the ligand-independent AF-1 site, located in the N-terminal A/B domains, and the ligand-dependent AF-2 site, located in the C-terminal ligand binding domain (4). These domains participate in the transactivation of target genes, leading to the modulation of various physiological processes. Binding of 9cRA to RXRα causes an interaction between AF-1 and AF-2, leading to exposure of the AF-1 region due to a conformational change of RXRα (39). Such a conformational change is thought to be necessary for the recruitment of co-activators, including p300 and CBP (40–42). However, the effect of RXRα in increasing RXRE-mediated transcriptional activation was relatively weak (Fig. 6A) compared with that of the co-activators. In addition, the increment induced by RXRα appeared similar between in the absence and presence of ligand. These results suggest different mechanisms of action between a ligand, 9cRA, and RXRα. RXRα might cause some conformational change of RXRα that enhances transactivation independent of 9cRA. We are now examining whether p300 and CBP play a role in the RXRα-mediated enhancement of RXRE-dependent transactivation and testing the possibility that RXRα might link RXRα to these co-activators. In addition, it should be noted that we used cells expressing endogenous RXRα, and it is possible that cells lacking endogenous RXRα expression might show greater induction of RXRE-dependent transactivation on transfection with RXRα, a possibility we are now exploring.

In summary, we have described a novel function of RXRα, regulating RXRE-mediated gene expression, as a result of the direct binding of RXRα to RXRα. Because both RXRα and RXRα are reported to be expressed in a variety of tissues (43–45), we are now testing whether this interaction yields a similar biological result across these tissues.

Acknowledgments—We thank Dr. Y. Nozawa (Gifu International Institute of Biotechnology) for critical reading of the manuscript and Dr. N. Seki (Helix Research Institute, Kisarazu, Japan) for providing the plasmid encoding RXRα.

**REFERENCES**

1. Guasch, L. J., Sporn, M. B., and Roberts, A. B. (1994) in The Retinoids: Biology, Chemistry, and Medicine (Sporn, M. B., Roberts, A. B., and Goodman, D. S., eds) 2nd Ed., pp. 443–520, Raven Press, Ltd., New York.
2. Chambon, P. (1996) *FASEB J.* 10, 949–954.
3. Rastner, P., Mark, M., and Chambon, P. (1995) *Cell* 83, 859–869.
4. Parrado, A., Desponge, G., Kraiba, R., Le Fogh, C., Dupas, S., Choquette, M., Robledo, M., Larghero, J., Bui, H., Le Gall, I., Rochette-Egly, C., Chomienne, C., and Padua, R. A. (2001) *Nucleic Acids Res.* 29, 4981–4990.
5. Chiba, H., Cliford, J., Metzer, D., and Chambon, P. (1997) *J. Cell. Biol.* 139, 735–747.
6. Altucci, L., and Groneheimer, H. (2001) *Nat. Rev. Cancer* 1, 181–193.
7. Aranda, A., and Pascual, A. (2001) *Physiol. Rev.* 81, 1209–1304.
8. Lehrman, J. M., Jung, L., Fanjul, A., Cameron, J. F., Lu, X. P., Haefner, P., Dawson, M. I., and Pahl, M. (1992) *Science* 258, 1944–1946.
9. De Luca, L. M. (1991) *FASEB J.* 5, 2924–2933.
10. Lotan, R., Xu, X. C., Lippman, S. M., Ro, J. Y., Lee, J. S., Lee, J. J., and Hong, W. E. (1995) *Nature* 374, 1405–1410.
11. Berard, J., Labbene, F., Minkina, M., Massé, S., Kothyary, R., and Bradley, W. E. (1996) *FASEB J.* 10, 1091–1097.
12. Geisen, C., Denk, C., Gremm, B., Baust, C., Karger, A., Bollag, W., and

![Fig. 7. RNF8 enhances RXRα-mediated gene expression in Caco-2 cells.](image)
Enhanced Transactivation via RXRα-RNF8 Interaction

Schwarz, E. (1997) Cancer Res. 57, 1460–1467
13. Xu, X. C., Sneige, N., Liu, X., Nandagiri, R., Lee, J. J., Lukmanji, F., Hortobagyi, G., Lippman, S. M., Dingra, K., and Lotan, R. (1997) Cancer Res. 57, 4992–4996
14. Xu, X. C., Sozzi, G., Lee, J. S., Lee, J. J., Pastorino, U., Pilotti, S., Kurie, J. M., Hong, W. K., and Lotan, R. (1997) J. Natl. Cancer Inst. 89, 624–629
15. Xu, X. C., Liu, X., Tahara, E., Lippman, S. M., and Lotan, R. (1999) Cancer Res. 59, 2477–2483
16. Solomon, C., White, J. H., and Kremer, R. (1999) J. Clin. Invest. 103, 1729–1735
17. Benoit, G. R., Flexor, M., Besancon, F., Altucci, L., Rossin, A., Hillion, J., Balajthy, Z., Legres, L., Segal-Bendirdjian, E., Gronemeyer, H., and Lanotte, M. (2001) Mol. Endocrinol. 15, 1154–1169
18. Rochette-Egly, C., and Chambon, P. (2001) Histol. Histopathol. 16, 909–922
19. Nagy, L., Thomazy, V. A., Heyman, R. A., and Davies, P. J. (1998) Cell Death Differ. 5, 11–19
20. Matsushima-Nishiwaki, R., Okuno, M., Adachi, S., Sano, T., Akita, K., Mori, Y., Moriwaki, H., Friedman, S. L., and Moriwaki, H. (2001) Carcinogenesis 24, 1353–1359
21. Ito, K., Adachi, S., Iwakami, R., Yasuda, H., Muto, Y., Seki, N., and Okano, Y. (2001) Eur. J. Biochem. 286, 2725–2732
22. Lorick, K. L., Jensen, J. P., Fang, S., Ong, A. M., Hatakeyama, S., and Weissman, A. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 11364–11369
23. Freemont, P. S. (2000) Curr. Biol. 10, 84–87
24. Joazeiro, C. A., and Weissman, A. M. (2000) Cell 102, 549–552
25. Kang, H. Y., Yeh, S., Fujimoto, N., and Chang, C. (1999) J. Biol. Chem. 274, 8579–8576
26. Moilanen, A. M., Poukka, H., Karvonen, U., Hakli, M., Janne, O. A., and Palvimo, J. J. (1998) Mol. Cell. Biol. 18, 5128–5139
27. Chang, C. J., Chen, Y. L., and Lee, S. C. (1998) Mol. Cell. Biol. 18, 5880–5887
28. Seki, N., Hattori, A., Sugano, S., Suzuki, Y., Nakagawara, A., Ohhira, M., Muramatsu, M., Hori, T., and Saito, T. (1998) J. Hum. Genet. 43, 273–274
29. Mano, H., Ozawa, T., Takeyama, K., Yoshizawa, Y., Kojima, R., Kato, S., and Masushige, S. (1993) Biochem. Biophys. Res. Commun. 191, 943–949
The RING Finger Protein, RNF8, Interacts with Retinoid X Receptor α and Enhances Its Transcription-stimulating Activity
Yukihiko Takano, Seiji Adachi, Masataka Okuno, Yoshinori Muto, Takashi Yoshioka, Rie Matsushima-Nishiwaki, Hisashi Tsurumi, Kenichi Ito, Scott L. Friedman, Hisataka Moriwaki, Soichi Kojima and Yukio Okano

J. Biol. Chem. 2004, 279:18926-18934.
doi: 10.1074/jbc.M309148200 originally published online February 23, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M309148200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 44 references, 17 of which can be accessed free at http://www.jbc.org/content/279/18/18926.full.html#ref-list-1