RXRα Inhibits the NRF2-ARE Signaling Pathway through a Direct Interaction with the Neh7 Domain of NRF2

Hongyan Wang1,2, Kaihua Liu2, Miao Geng1, Peng Gao2, Xiaoyuan Wu1, Yan Hai1, Yangxia Li1, Yulong Li1, Lin Luo2, John D. Hayes3, Xiujun Wang2, and Xiwen Tang1

Abstract

The transcription factor NRF2 (NFE2L2) is a pivotal activator of genes encoding cytoprotective and detoxifying enzymes that limit the action of cytotoxic therapies in cancer. NRF2 acts by binding antioxidant response elements (ARE) in its target genes, but there is relatively limited knowledge about how it is negatively controlled. Here, we report that retinoic X receptor alpha (RXRα) is a hitherto unrecognized repressor of NRF2. RNAi-mediated knockdown of RXRα increased basal ARE-driven gene expression and induction of ARE-driven genes by the NRF2 activator tert-butylhydroquinone (tBHQ). Conversely, overexpression of RXRα decreased ARE-driven gene expression. Biochemical investigations showed that RXRα interacts physically with NRF2 in cancer cells and in murine small intestine and liver tissues. Furthermore, RXRα bound to ARE sequences in the promoters of NRF2-regulated genes. RXRα loading onto AREs was concomitant with the presence of NRF2, supporting the hypothesis that a direct interaction between the two proteins on gene promoters accounts for the antagonism of ARE-driven gene expression. Mutation analyses revealed that interaction between the two transcription factors involves the DNA-binding domain of RXRα and a region comprising amino acids 209-316 in human NRF2 that had not been defined functionally, but that we now designate as the NRF2-ECH homology (Neh) 7 domain. In non–small cell lung cancer cells where NRF2 levels are elevated, RXRα expression downregulated NRF2 and sensitized cells to the cytotoxic effects of therapeutic drugs. In summary, our findings show that RXRα diminishes cytoprotection by NRF2 by binding directly to the newly defined Neh7 domain in NRF2. Cancer Res; 73(10); 1–12. ©2013 AACR.

Introduction

The human body is continuously threatened by reactive oxygen species (ROS) and electrophiles that are generated by metabolism and by environmental agents. The NF-E2 p45-related factor 2 (NRF2) is a cap’n’collar (CNC) basic-region leucine zipper (bZIP) transcription factor, which plays a major role in protecting cells from prooxidants and electrophiles because it regulates basal and inducible expression of genes that contain antioxidant response element (ARE) sequences in their promoter regions. NRF2-target genes include those encoding antioxidant and detoxification enzymes such as aldo-keto reductase (AKR), heme oxygenase-1 (HO-1), glutathione S-transferase (GST), glutamate-cysteine ligase, and NADP(H):quinone oxidoreductase-1 (NQO1; refs. 1–3).

The ubiquitin ligase substrate adaptor kelch-like ECH-associated protein 1 (KEAP1) is a major repressor of NRF2. Under normal conditions, NRF2 is constantly degraded via the ubiquitin-proteasome pathway in a KEAP1-dependent manner. Under stressed conditions, ROS or electrophiles modify cysteine residues in KEAP1 causing loss of its adaptor activity, and in turn failure to ubiquitylate NRF2. Upon inactivation of KEAP1, NRF2 accumulates in the nucleus where it heterodimerizes with small Maf proteins and activates ARE-driven genes (4, 5). Recent studies have shown that KEAP1-dependent ubiquitylation of NRF2 can be prevented by protein-protein interactions: these include the binding of p21 to NRF2 or the binding of p62/sequestosome-1 to KEAP1 (6, 7). In contrast to the wealth of knowledge about the activation of NRF2, far less is known about the mechanisms by which cells turn off or downregulate NRF2 once it has been activated. Importantly, several transcriptional repressors of NRF2 have been identified, such as Bach1, P53, activating transcription factor 3 (ATF3), and estrogen-related receptor beta (ERRβ; refs. 8–11), suggesting that NRF2 activity is strictly regulated even when KEAP1 is inactivated. Nonetheless, constitutive upregulation of NRF2 has been observed...
in various tumors, including non–small cell lung cancer (NSCLC), and those of breast, head and neck, and gallbladder (12–14). Indeed, deregulation of NRF2 has been shown to contribute to tumorigenesis and drug resistance (14–18). Thus, NRF2 is emerging as a new molecular target for the treatment of certain cancers. It is therefore important to understand the molecular mechanisms by which NRF2 activity can be suppressed because this might provide novel strategies for therapeutic intervention.

In a previous study, we reported that retinoic acid receptor alpha (RARα) antagonizes NRF2 activity (19). Retinoic X receptor (RXR) is the obligatory heterodimerization partner for RARα (20, 21), but its role in regulating the function of NRF2 has not been investigated to date. In the present study, we discovered that RXRα can inhibit the transcriptional activity of NRF2 through a physical interaction between the 2 factors. A RXRα-binding region, which is located between the NRF2-ECH homology (Neh) 5 and Neh6 domains of human NRF2, has been identified. Mutation analyses have revealed that the DNA-binding domain (DBD) of RXRα is required for the interaction with NRF2. Moreover, we have provided evidence that RXRα is capable of interacting with NRF2 on ARE sites in gene promoters, showing a previously unrecognized mechanism by which the CNC-bZIP factor can be inhibited. Downregulation of NRF2 via forced expression of RXRα in NSCLC A549 cells, where the CNC-bZIP factor is constitutively active, increased sensitivity to therapeutic drugs. Thus, our data show a novel mechanism by which RXRα can suppress drug resistance.

Materials and Methods

Chemicals and cell culture

Unless otherwise stated, all chemicals were from Sigma-Aldrich Co., Ltd., and all antibodies were from Santa Cruz Biotechnology. Antibody against mouse Nrf2 (H300) (sc-13032, Santa Cruz) was used for this study. Actin and β-tubulin antibodies were purchased from Sigma. Alexa Fluor 488 goat anti-rabbit IgG(H+L) was obtained from Invitrogen. HEK293 (human embryonic kidney-293), MCF7 (human breast carcinoma), Caco2 (human colon cancer), and NSCLC A549 cell lines were from the American Type Culture Collection.

Animals

Five-week-old male C57BL/6 male mice were used in this study. The mice (n = 8) were given butylated hydroxyanisole (BHA) by intragastric gavage (i.g.) at 200 mg/kg daily for 3 days. The equivalent volume of corn oil (vehicle) was given to the control mice (n = 8). Mice were sacrificed and tissues were processed as described previously (22). All animal procedures were performed in accordance with the approval of the Laboratory Animals Ethics Committee of Zhejiang University (Zhejiang, China).

Plasmids

Plasmids encoding mouse (m) Nrf2 were provided by Dr Mike McMahon (Medical Research Institute, University of Dundee, Scotland, United Kingdom): these included pcDNA3.1/V5-mNrf2 (full-length), pcDNA3.1/V5-mNrf2DADIDLD (lacking amino acids 79-82) and pcDNA3.1/V5-mNrf2ADIDLDG (lacking amino acids 17-32; ref. 23). pHyg-EGF-hNRF2 encoding EGFP tagged full-length human (h) NRF2 was kindly provided by Dr. Masayuki Yamamoto and Dr. Ken Itoh (Institute of Basic Medical Sciences, University of Tsukuba, Japan). pSG5-mRXRα encoding full-length mRXRα was generously provided by Dr. Pierre Chambon (Institut de Génétique et de Biologie Moleculaire et Cellulaire, CNRS/INSERM/ULP, France). We generated a series of plasmids expressing tagged hNRF2, mNrf2, or mRXRα wild-type or mutants (see Supplementary Methods): as shown in Fig. 2C, 6 plasmids expressing GST-tagged hNRF2 mutants and 7 plasmids encoding GFP-tagged hNRF2 or mNrf2 mutants were created; as shown in Fig. 3A, 4 plasmids expressing GST-tagged mRXRα mutants were generated. All plasmids were verified by DNA sequencing.

Transfections and luciferase reporter gene activity

Lipofectamine 2000 (Invitrogen) was used for transfection (24). The siRNA against hRXRα (RXRα-siRNA) or nontargeting negative control siRNA (scrambled-siRNA) were synthesized by Takara Biotechnology. The sequences for RXRα-siRNA were 5′-GGAGAUGCACUAUUUAAATT-3′ (forward) and 5′-UUUAUAUGAUGCACUUCCCTG-3′ (reverse). Empty vectors were used as negative controls for transfection experiments with plasmids. Twenty-four or 48 hours after transfection, the transfected cells were treated with xenobiotics for 6 hours to 24 hours before being harvested for further analysis. The ARE-luciferase reporter plasmid pGL-GST/2:41bp-ARE was used and the dual luciferase activities were determined as described elsewhere (25). Stable cell lines A549-mRXRα and A549-EGFP overexpressing GFP-mRXRα and GFP, respectively, were generated as described in Supplementary Methods.

Real-time quantitative PCR

Total RNA isolation and real-time (RT)-PCR was conducted as described previously (24).

Western blot analysis, GST pull-down assay, and immunoprecipitation

Preparation of protein samples, SDS-PAGE gels and immunoblotting was carried out using standard protocols (24). Immunoblotting with antibody against actin or β-tubulin was conducted to confirm equal loading for whole-cell extracts and nuclear extracts, respectively. GST pull-down assay and immunoprecipitation was conducted to detect the interaction between NRF2 and mRXRα mutant GST or GFP fusion proteins. The procedures are provided in Supplementary Methods.

Fluorescently tagged proteins and immunofluorescence

Cells were seeded on glass coverslips, and transiently transfected with the indicated fluorescently labeled proteins. Forty-eight hours later, cells were fixed, processed, and examined as described previously (4, 5). Anti-RXRα antibody was used to detect endogenous RXRα, followed by staining with Texas Red goat anti-rabbit IgG(H+L). Counterstaining with 4,6-diamidino-2-phenylindole (DAPI) was used to verify the...
location and integrity of nuclei. The fluorescence images were observed with a Zeiss LSM510 Meta laser-scanning confocal microscope (Carl Zeiss, Inc.).

**Chromatin immunoprecipitation assay**

Chromatin immunoprecipitation (ChIP) assays were conducted as described previously (24). The relative binding of NRF2 or RXRα to ARE sites were calculated by quantification of band intensity with an Odyssey Infrared Imaging System (LI-COR Biosciences) normalized to that of the input.

**Biotinylated ARE-binding assay**

The preparation of nuclear extracts and the Bio-ARE pull-down assay was conducted as described previously (19). A double-stranded 5′-biotinylated ARE probe, representing the 41 bp of nucleotides -682 to -722 in the rat GSTA2 gene promoter, was synthesized by TaKaRa Biotechnology. NRF2 predepletion was conducted by 1 hour incubation of cell lysates with antibody against RXRα before the Bio-ARE pull-down procedures. The pulled down mixture was analyzed by SDS–PAGE followed by immunoblotting with antibody against RXRα or NRF2.

**Cytotoxicity assay**

Cytotoxicity was determined as described previously (24). The IC₅₀ and the combination index for determining synergism were calculated as described elsewhere (24).

**Statistical analysis**

Statistical comparisons were conducted by unpaired Student t tests. P < 0.05 was considered statistically significant.

**Results**

**RXRα inhibits basal and inducible ARE-driven gene expression**

To evaluate the effect of RXRα on NRF2 and ARE-driven gene expression, we transfected MCF7 cells with siRNA to knockdown RXRα; immunoblotting confirmed successful knockdown of RXRα in these cells (Fig. 1A, top). In MCF7 cells cotransfected with the ARE-driven reporter plasmid pGL3–GSTA2/41bp-ARE, knockdown of RXRα was found to increase basal luciferase reporter activity about 1.5-fold, and increased induction of the reporter gene activity by 20 μmol/L tert-butyldihydroquinone (tBHQ) from 4- to 6-fold. To test whether this increase in ARE-driven gene expression was a general effect, we also examined colon cancer Caco2 cells (Fig. 1B, top). Knockdown of RXRα in Caco2 cells resulted in a 2-fold increase in the basal levels of mRNA for endogenous AKR1C1 and HO-1, both of which are NRF2-target genes. Treatment of Caco2 cells with 20 μmol/L tBHQ in which RXRα had been knocked down further increased AKR1C1 mRNA from 10- to 14-fold, and HO-1 mRNA from 5- to 7-fold (Fig. 1B, bottom). These data indicate that loss of RXRα increases NRF2 activity.

We next tested whether overexpression of RXRα might suppress ARE-driven gene expression in Caco2 cells using the pEGFP-C1-mRXRα expression vector; transient expression of exogenous GFP-mRXRα was confirmed by immunoblotting with a specific antibody against RXRα (Fig. 1C). As anticipated, both basal and inducible NRF2-target gene expression were inhibited by forced overexpression of RXRα: the basal AKR1C1 and HO-1 mRNA levels were reduced by 20% and the induction of AKR1C1 and HO-1 mRNA levels by tBHQ was decreased from 11-fold to just 2- and 4-fold, respectively (Fig. 1D). These data show that NRF2-regulated genes are the targets of RXRα-mediated repression, and RXRα can suppress the expression of ARE-driven genes in a ligand-independent manner.

**Antagonism of ARE-driven gene expression by RXRα is independent of KEAP1**

It is well established that KEAP1 is a major repressor of NRF2 activity (5). To investigate whether KEAP1 plays any role in the antagonism of NRF2 by RXRα, we carried out a further study in the A549 NSCLC cell line, which contains a loss-of-function mutation in KEAP1 (14). RXRα-siRNA was transfected into A549 cells, and knockdown of RXRα was confirmed by immunoblotting (Fig. 1E, left). Consistent with our observations in MCF7 and Caco2 cells, knockdown of RXRα in A549 cells increased AKR1C1 mRNA 2-fold and HO-1 mRNA 4-fold (Fig. 1E, right). It also increased AKR1C1 and HO-1 protein levels significantly (Fig. 1E, left panel). These findings suggest that RXRα inhibition of ARE-driven transcription occurs independently of KEAP1.

**RXRα and NRF2 physically interact in vitro**

To investigate the mechanism by which RXRα represses NRF2, we examined the localization of the CNC-bZIP transcription factor and its abundance after overexpression of RXRα. Using Caco2 and A549 cells, we found that RXRα altered neither the nuclear accumulation of NRF2 nor its abundance (Fig. 1C & Fig. 6A). We next considered whether RXRα-mediated repression of NRF2 might be a consequence of a direct interaction between the 2 proteins. To test this possibility, we generated a GST-tagged NRF2 construct, and conducted GST-pull-down experiments that tested its ability to interact with RXRα (Fig. 2A and B). The recombinant full-length NRF2 (GST-hNRF2) interacted strongly with His-tagged full-length RXRα protein (Fig. 2B, lane 1). In contrast, the GST control did not bind specifically to RXRα (lane 2). An inverse GST-pull-down assay with recombinant GST-RXRα and His-tagged NRF2 confirmed that the 2 proteins interact specifically (Fig. 3B, lane 2). Thus, our data indicate that NRF2 and RXRα can form a complex in vitro.

To determine the region of NRF2 that is required to interact with RXRα, a series of NRF2 truncated proteins tagged with GST (see Fig. 2C) were expressed, and their abilities to interact with purified recombinant His-RXRα were tested by GST-pull-down assay. We found RXRα interacted with the N-terminal NRF217–338 protein (Fig. 2D, lane 2). In contrast, RXRα failed to interact with the C-terminal NRF2339–660 protein (Fig. 2D, lane 7), suggesting that the Neh6, Neh1, and Neh3 domains of NRF2 are not required for the interaction between the 2 factors. In addition, RXRα failed to interact with the NRF217–110 or NRF2109–239 proteins, which contain Neh2 or Neh4 plus Neh5, respectively (Fig. 2D, lanes 4 and 5), suggesting that these individual domains are not sufficient to enable NRF2 to bind
Remarkably, RXRa interacted with NRF2109-338 and NRF2209-316 (lanes 3 and 6), whereas deletion of the amino acids 209-316 completely abolished the interaction (Supplementary Fig. S1A, lanes 2 and 3). These results indicate residues 209-316 of NRF2 are sufficient to support an interaction with RXRa in vitro.

To further confirm that amino acids 209-316 of NRF2 are required for the interaction with RXRa, we created a series of

Figure 1. RXRa inhibits ARE-driven gene expression. A, knockdown of RXRa in MCF7 cells enhanced both the basal and tBHQ-induced ARE-luciferase activity. MCF7 cells were transiently transfected with RXRa-siRNA, the ARE reporter plasmid pGL-GSTA2.41bp-ARE and the plasmid pRL-TK. Forty-eight hours later, the cells were treated with 20 μmol/L tBHQ for 6 hours before they were harvested. The level of RXRa protein in the lysed cells was determined by immunoblotting (top). Actin was used as a loading control. The relative luciferase activity in lysates was determined as described in Materials and Methods (bottom). The value for cells transfected with scrambled siRNA and treated with DMSO was used as control and set at 1. B, knockdown of RXRa enhanced both the basal and tBHQ-induced AKR1C1 and HO-1 mRNA levels in Caco2 cells. The Caco2 cells were transfected with RXRa-siRNA. Forty-eight hours later, cells were treated with 20 μmol/L tBHQ, and after 6-hour incubation they were harvested. The AKR1C1 and HO-1 mRNA levels were determined by RT-PCR. The level of 18S rRNA was used as an internal standard. The value for the same gene from the cells that had been transfected with scrambled siRNA and treated with DMSO was used as a control and set at 1. C and D, overexpression of RXRa reduced AKR1C1 and HO-1 mRNA levels in Caco2 cells. The Caco2 cells were transiently transfected with pEGFP-mRXRa. After 24 hr recovery, the cells were treated for 6 hr with 20 μM tBHQ. After lysis, the whole-cell extracts were subjected to SDS-PAGE analysis, and the protein levels of GFP-RXRa and the endogenous RXRa were measured by Western blotting with antibody against RXRa. The level of NRF2 protein was measured by Western blotting. Actin was used as a loading control. Also, AKR1C1 and HO-1 mRNA levels were determined by RT-PCR (D). The value for the same gene from cells that had been transfected with pEGFP and treated with DMSO was used as control and set at 1. E, knockdown of RXRa in A549 cells increased the expression of AKR1C1 and HO-1. Forty-eight hours after A549 cells were transfected with RXRa-siRNA, they were harvested. The levels of RXRa, HO-1, and AKR1C proteins were determined by immunoblotting with individual antibodies. Actin was used as a loading control (left). The AKR1C1 and HO-1 mRNA levels were determined by RT-PCR (right). The amount of 18S rRNA was used as an internal standard. The value for the same gene from the cells transfected with scrambled siRNA was used as control and set at 1. Values shown are mean ± SD. *, P < 0.05; **, P < 0.005. The results are from 3 separate experiments.
expression vectors encoding GFP-tagged NRF2 mutants (Fig. 2C). The analyses were conducted using purified His-RXRα to coimmunoprecipitate material from lysates of HEK293 cells that had been previously transiently transfected with the various NRF2-mutant expression plasmids. Consistent with the GST-pull-down assay results, an antibody against RXRα could coimmunoprecipitate the mNrf2^{D71E}, NRF2^{17-316}, and NRF2^{109-316} mutant proteins, all of which contain amino acids 209-316 of NRF2 (Fig. 2E, lanes 1, 2, and 6). The deletion of amino acids 209-316 abolished this interaction (Supplementary Fig. S1B, lane 3). Moreover, residues 201-329 of mNrf2, which are orthologous to residues 209-316 of hNRF2, could also be immunoprecipitated from the cell extracts (Fig. 2E, lane 7). As a further control, we tested HEK293 cell extracts that expressed GFP alone, and showed that RXRα could not coimmunoprecipitate GFP (Fig. 3D and E). Collectively, these results

Figure 2. Mapping of the domain in NRF2 required for its interaction with RXRα. A, NRF2 was expressed in E. coli as a GST fusion protein, and purified on glutathione (GSH)-Sepharose beads. The purified proteins were resolved on a 10% SDS-PAGE gel and visualized by staining with Coomassie brilliant blue. B, NRF2 and RXRα physically interact in vitro. Association of GST-NRF2 fusion protein with His-RXRα was evaluated in a GST-pulldown assay. The same amounts of GST protein or GST-NRF2 fusion protein, shown in A, were incubated with his-RXRα. The proteins bound to GST-Sepharose were eluted, separated by SDS-PAGE and subjected to immunoblotting using antibody against either RXRα or GST. The input control represents 10% of the total amount used for GST-pulldown. C, schematic illustration of the GST- or GFP-tagged NRF2 mutants and their interactions with RXRα. In the cartoon, the regions within NRF2 that are of interest are indicated by bars, and the amino acid residues involved are indicated by the polypeptide designations. D, pull-down of his-RXRα with the indicated mutant hNRF2 proteins fused at their N-termini with GST. The GST-Sepharose-bound proteins were separated by SDS-PAGE and subjected to immunoblotting using antibodies against either RXRα or GST; both GST and GST-luc were used as negative controls. The input represents 10% of the total amount of his-RXRα used for the GST pulldown assay. E, HEK293T cells were transfected with various pEGFPC1 plasmids encoding GFP tagged to truncated forms of NRF2 as depicted in C. Twenty-four hours after transfection, the whole-cell extracts were incubated with purified His-RXRα. The mixtures were subjected to immunoprecipitation using antibody specific to RXRα. The immunoprecipitates were separated by SDS-PAGE and immunoblotted using antibodies against GFP or RXRα. The input represents 10% of the total amount of cell lysate used for immunoprecipitation. GST-luc, GST-luciferase. NRF2-mutant proteins are indicated by /C, respectively. The molecular mass in kDa is indicated. IB, immunoblot. n.s., nonspecific bands. The results presented are typical examples from at least 3 separate experiments.
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Figure 3. Interaction of RXRα with NRF2 in vitro. A, schematic of GST-tagged mRXRα mutants and their interactions with NRF2. In the cartoon, the regions within RXRα that are of interest are indicated by bars, and the specific amino acid residues are indicated by the polypeptide designations. B, GST pull-down assay of his-NRF2 with GST–mRXRα fusion proteins. GST–mRXRα fusion proteins, as depicted in A, were incubated with GST–Sepharose beads. The bound GST–mRXRα (b) was incubated with purified His-NRF2 (c). The bound NRF2 was detected by immunoblotting with antibody against NRF2 (a). The input represents 10% of the total amount of his-NRF2 used for the GST pull-down assay. GST and GST-luc were used as negative controls. GST-luc, GST-luciferase. RXRα mutant proteins are indicated by “Δ”, respectively. The molecular mass in kDa is indicated. IB, immunoblot. The results shown are from at least 3 separate experiments.

indicate that amino acids 209–316 of human NRF2 are necessary for its interaction with RXRα in vitro.

The RXRα DBD is required for interaction with NRF2
RXRα comprises 3 major domains: the N-terminal AF-1 domain, the well-conserved DNA-binding domain (DBD), and the ligand-binding domain (LBD) that is responsible for dimerization. It also contains the relatively small AF-2 region with a ligand-dependent transactivation function (refs. 26–28; Fig. 3A). To delineate which region of RXRα is necessary for interaction with NRF2, 4 recombinant GST–RXRα mutants (GST–mRXRα) were expressed and purified, and their abilities to interact with purified His-NRF2 were tested by GST-pull-down assay (Fig. 3A). Mutant RXRαΔ1-139 and RXRαΔ140–205, which contains the C-terminal ligand binding and dimerization domains, failed to interact with NRF2 (Fig. 3B, lane 4). Likewise, NRF2 was unable to bind to RXRαΔ1-139, which represents the AF-1 region (lane 6). In contrast, mRXRαΔ1-229, representing the N-terminal amino acid half of RXRα, interacted well with NRF2 (lane 3). Importantly, the mutant RXRαΔ1-229 protein that comprises the DBD domain interacted strongly with NRF2 (lane 5). Accordingly, the GST-luc and GST controls did not bind NRF2 (lanes 1 and 7). Taken together, our data indicate that the DBD is sufficient to interact with NRF2.

RXRα and NRF2 directly interact in vivo
To show whether a physical interaction occurs between RXRα and NRF2 in vivo, we examined whether both proteins colocalize in cells. Plasmids encoding GFP-tagged NRF2 and RFP-tagged mRXRα were transfected into HEK293 cells. Images obtained by confocal laser scanning microscopy revealed that singly expressed GFP-NRF2 and RFP-RXRα were predominately localized in the nucleus of the transfected cells (Fig. 4A, image a–d). As a control, we monitored the cellular localization of the GFP and RFP proteins, both of which were distributed uniformly in the cytoplasm and the nucleus (Supplementary Fig. S2). When GFP-NRF2 and RFP-RXRα were coexpressed, a substantial portion of both proteins coexisted in small foci-like structures within the nucleus (Fig. 4A, image e–g). To confirm these observations, we next tested whether the ectopically expressed GFP-NRF2 could localize with endogenous RXRα. After pHyg-GFP-hNrf2 was transiently transfected into A549 cells alone, the cellular localization of endogenous RXRα was examined using indirect immunofluorescence. Confocal laser scanning microscopy showed a similar nuclear colocalization of the 2 proteins (Fig. 4B, image c). Thus, these results support our contention that NRF2 interacts with RXRα in the nucleus. Moreover, we conducted coimmunoprecipitation experiments with total lysates prepared from COS7 cells transfected with the expression vector for V5-tagged mouse Nr2f2 with a deletion of residues 17–32 in its Neh2 domain (mNrf2Δ17-32; V5). When precipitated with an anti-RXRα antibody, a strong mNrf2 band was detected that suggested both transcription factors coexist in an immunoprecipitable complex (Supplementary Fig. S3, top blot, lane 4).

To examine whether an interaction occurs between endogenous NRF2 and endogenous RXRα, we exposed MCF7 cells to 20 μmol/L tBHQ for 24 hours. Immunoprecipitation using antibodies against NRF2 and RXRα revealed the presence of an immunocomplex in the MCF7 cell lysates between endogenous NRF2 and RXRα, which could be increased further by tBHQ treatment [Fig. 4C (a) and 4C (b), lanes 3 and 4]. The abundance of NRF2 (Fig. 4C, lane 2) and the expression of its target gene AKR1C (Supplementary Fig. S4, lane 2) was increased as reported previously (25). We next carried out similar studies with cell extracts prepared from mouse tissues.
Strikingly, we found that the 2 transcription factors interacted strongly in both small intestine and liver [Fig. 4D (a) and 4D (b), lane 3]. When the mice were treated with BHA, expression of the Nrf2-target genes Nqo1 and Gstm1 was increased in the small intestine and liver (Supplementary Fig. S5A and S5B, lane 2) as expected (1, 22, 29, 30). Again, the interaction between Nrf2 and RXRα was increased by BHA [Fig. 4D (a) and 4D (b), lane 4], suggesting that RXRα is important in regulating the function of Nrf2 in these tissues. Taken together, these results establish that RXRα and Nrf2 interact directly in vivo.

RXRα is recruited to the ARE in a Nrf2-dependent manner

To assess whether the interaction between RXRα and Nrf2 occurs when the factors are bound to DNA, we conducted a ChIP assay with antibody against RXRα or NRF2. As expected, increased NRF2 bound to ARE sequences in the promoters of HO-1 and AKR1C1 after MCF7 cells had been exposed to 20 μmol/L tBHQ for 6 hour (Fig. 5A, lanes 3 and 4). Interestingly, the ChIP assays revealed that RXRα was able to associate with ARE sites (Fig. 5A, lanes 5 and 6), though it was observed that the DNA bands representing the transcription factors were not as strong as those for NRF2. This suggests that RXRα is recruited to the ARE in a NRF2-dependent manner.
RXRα associated with ARE sequences were much weaker than those representing NRF2. Upon exposure to tBHQ, the amount of RXRα that associated with ARE sequences increased approximately 1.5-fold (Fig. 5A, lanes 5 and 6), correlating with the increase in NRF2 on ARE sites (Fig. 5A, lanes 3 and 4); this occurred in spite of the fact that the abundance of RXRα protein remained unchanged, as seen in MCF7 cells (Fig. 5B, bottom blot, lanes 1 and 2). These results are specific to ARE-containing gene promoters; a DNA sequence in the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter was not detected in complexes immunoprecipitated with either antibody (Fig. 5A).
suggested that the association of RXRα with ARE sequences possibly requires the presence of NRF2, which in turn is able to recruit RXRα to the cis-element.

To further evaluate whether RXRα requires NRF2 to recognize AREs, we carried out a biotinylated-ARE (Bio-ARE) oligonucleotide pull-down assay on nuclear extracts from MCF7 cells treated with 20 μmol/L tBHQ. Although tBHQ did not influence the nuclear levels of RXRα (Fig. 5B, bottom blot, lanes 1 and 2), only RXRα from tBHQ-treated MCF7 cells bound strongly to Bio-ARE beads (Fig. 5B, bottom blot, lane 8). Such binding was concomitant with nuclear accumulation of NRF2 and its increased binding to Bio-ARE beads (Fig. 5B, top blot, lanes 2 and 8). Importantly, depletion of NRF2 from the nuclear extracts after preincubation with an antibody against NRF2 (Fig. 5C, lane 3), not only abolished the binding of NRF2 to Bio-ARE as expected (Fig. 5C, top blot, lanes 8 and 9), but also significantly reduced the binding of RXRα to Bio-ARE (Fig. 5C, bottom blot, lanes 8 and 9). As a negative control, an unrelated biotin-labeled double-stranded oligonucleotide failed to pull down NRF2 or RXRα from the nuclear extracts (Fig. 5B, lane 16; 5C, lane 7). These results suggest that RXRα can be tethered onto DNA by forming a heteromeric protein-protein complex with NRF2.

To examine whether RXRα might form inactive complexes with NRF2 on ARE sequences, we generated a stable MCF7 cell line, designated MCF7-RXRα, which overexpresses RXRα from a pEGFP-mRXRα plasmid. Immunoprecipitation confirmed that both the endogenous RXRα and exogenous derived GFP-mRXRα associated with NRF2 in these stably transfected cells (Supplementary Fig. S6, lane 4). In agreement with the observation following transient transfection of RXRα (Fig. 1D), induction of HO-1 by tBHQ (20 μmol/L) was nearly completely blocked in MCF7-RXRα cells (Fig. 5D, lanes 3 and 4). We found comparable levels of nuclear NRF2 and its binding to ARE sequences stimulated by tBHQ (Fig. 5D and Supplementary Fig. S7, lanes 5–8). However, a ChIP assay revealed that the loading of CREB (cAMP response element binding protein) binding protein (CBP) and RNA polymerase II (RNA Pol II) onto ARE-sites upon tBHQ treatment were markedly attenuated in MCF7-RXRα cells (Fig. 5E, lanes 7, 8, 11 and 12). This is in contrast to the situation in MCF7 cells, where the levels of CBP and RNA Pol II on the ARE site were dramatically increased under the same conditions (Fig. 5E, lanes 5, 6, 9, and 10). Taken together, our data suggest that the lack of transcriptional activity of NRF2 caused by RXRα overexpression is likely due to direct negative interference by RXRα on ARE-sites, leading to the disruption of the recruitment of CBP and RNA Pol II to the promoters.

Overexpression of RXRα in A549 cells downregulates NRF2 and increases sensitivity to anticancer drugs

Recent studies have provided evidence that high constitutive expression of NRF2 occurs in many cancer cells (31), and RNAi knockdown of the CNC-bZIP factor can sensitize such cells to chemotherapeutic drugs (24, 32, 33). To test whether the repression of NRF2 by RXRα has similar biologic consequences, we generated a cell line named A549-RXRα in which RXRα is stably expressed. As A549 cells carry a somatic KEAP1 mutation, it contains supranormal levels of NRF2 and its target genes are constitutively overexpressed, which in turn increases cell proliferation and resistance to anticancer drugs (14, 32). Immunoblotting confirmed transgene expression (Fig. 6A, B, C).
As expected, expression of the NRF2-target gene HO-1 was diminished in A549-RXRα cells. Cytotoxicity revealed that the IC_{50} of A549-RXRα to the anticancer drug oxaliplatin was about 40 μmol/L, compared with an IC_{50} of 100 μmol/L in A549 cells that stably expressed GFP, named A549-GFP (Fig. 6B). The A549-RXRα cells also displayed increased sensitivity to doxorubicin (Fig. 6C). Our results therefore suggest that the downregulation of cytoprotective genes regulated by NRF2 contributes to the increased sensitivity of A549 cells to these drugs by overexpression of RXRα.

Discussion

Regulation of NRF2 by KEAP1 has been a subject of intense study and it is relatively well characterized. However, little is known about other mechanisms by which NRF2 activity is controlled. Herein, we have presented the first evidence that RXRα functions as a repressor of ARE-driven gene expression. We found RXRα-mediated repression of ARE-driven genes is ligand- and retinoic acid-dependent, and requires the presence of NRF2 to recruit RXRα to the promoters of target genes. Importantly, we have discovered that amino acids 209–316 of human NRF2 are necessary for its interaction with RXRα, and as this region has not previously been shown to possess functional importance we have designated it the Neh7 domain. Repression of NRF2 by RXRα was observed in 5 different cell types, as well as mouse small intestine and liver, thereby indicating its general significance. To our knowledge, it has not been reported previously that RXRα attenuates ARE-driven gene transcription by directly targeting NRF2.

RXRs play an essential role in the regulation of multiple nuclear hormone-signaling pathways through their ability to dimerize with other nuclear receptors. RXRs mediate retinoid signalling through forming a heterodimer with RAR and by forming a homodimer (21, 34). In addition, RXRs form heterodimers with many other members of the subfamily of nuclear receptors, including peroxisome proliferator-activated receptor, liver X receptor (LXR), pregnane X receptor (PXR) and constitutive androstane receptor (CAR; refs. 21, 34). Heterodimerization of RXR with its partners dramatically enhances its DNA-binding activity (21, 34). Upon binding DNA, some nuclear receptors repress transcription of target genes through their interaction with transcriptional co-repressors in the absence of ligands. Furthermore, ligand binding by a transcriptional agonist causes conformational changes in corepressors, allowing dissociation of transcriptional co-repressors and association of transcriptional coactivators (35). Herein we found that RXRα did not influence nuclear translocation of NRF2 or its binding to ARE sequences. Instead, RXRα associated with ARE-bound NRF2, suggesting that inhibition of NRF2 by RXRα is likely due to the direct interference of recruitment by the CNC-hZIP factor of coactivators to gene promoters. Significantly, we found that an RXRα mutant lacking its ligand binding and heterodimerization domains was as efficient at interacting with NRF2 as the wild-type protein, showing that the nuclear receptor is able to repress NRF2 in a ligand-independent manner.

Previously, we reported that RARα mediates inhibition of NRF2 by all trans retinoic acid through an undefined protein–protein interaction (19). Herein, we have described physical and functional interactions between RXRα and NRF2. Although RXRα and RARα can heterodimerize, our in vitro GST-pull-down study indicates that RXRα binds NRF2 directly and that RARα is not necessary for the interaction between RXRα and NRF2. Specifically, the physical interaction involves the DBD of RXRα and the Neh7 domain of NRF2, and we failed to detect any interaction between RXRα and the CNC-hZIP Neh1 domain of NRF2. This is distinct from other interactions between nuclear hormone receptors and bZIP proteins such as jun and BZLF1 (36, 37) in which the zinc finger DNA-binding domain of the receptor interacts with the bZIP domain of the transcription factor. Thus, our studies show that both RARα and RXRα are repressors of NRF2 activity and presumably act via distinct mechanisms.

Previous work has shown NRF2 contains 6 functional domains, named Neh1-Neh6 (5). It is well known that the stability of NRF2 is controlled through protein–protein interactions between its Neh2 domain and KEAP1. The stability of NRF2 is also controlled by its Neh6 domain, and recently it has been found that this involves β-TrCP-mediated ubiquitylation and phosphorylation of residues in Neh6 by GSK-3 (23, 38). In contrast, the Neh4 and Neh5 regions, which lie adjacent to each other, were found by Kato and colleagues to act together as a transactivation domain (39): Neh4 and Neh5, both individually and cooperatively, bind CBP, and are indispensable for maximal NRF2 transactivation. Subsequently, Zhang and colleagues (40) reported that the Neh4 and Neh5 domains of NRF2 recruit BRG1, a catalytic subunit of SWI2/SNF2-like chromatin-remodeling complexes, to HO-1 enhancers for transcription initiation. In the present study, we identified a RXRα interaction domain in NRF2 (now called Neh7), which abuts the Neh5 domain of NRF2. We found overexpression of RXRα reduced the loading of CBP and RNA Pol II onto ARE sites, suggesting that the binding of RXRα may disrupt the binding of CBP to the Neh4 and Neh5 domains of NRF2, thereby suppressing transcriptional initiation. We therefore propose a model in which protein–protein contact between RXRα and NRF2 prevents a productive interaction between the transactivation domains of NRF2 and the basal transcription machinery.

In adult mammalian liver, RXRα is the most abundant among the 3 RXR isoforms (i.e., RXRα, -β, and -γ) and is an obligatory partner of two major xenobiotic receptors, CAR and PXR (41). Previous studies have revealed that Nrf2 is also highly expressed in the liver (22), and plays a key role in regulating the expression of phase II drug-metabolizing enzymes and drug-efflux pumps (30, 33, 42, 43). In the present study, we showed that Nrf2 and RXRα interact in vivo. In agreement with our observation, Dai and colleagues (44) reported that in hepatocyte-specific RXRα knockout mice, the expression of Gsta1 and/or Gsta2, Gstm1 and/or Gstm3, Gstm2 and Gstm4, all of which are regulated by NRF2 (29, 30, 44), were increased compared with their levels in the liver of wild-type mice, presumably due to loss of NRF2 suppression. Moreover, the expression of these Gst subunits from hepatocyte-specific RXRα knockout mice was further enhanced by acetaminophen, whose hepatotoxic...
metabolite was able to directly activate the KEAP1-NRF2 pathway (45), showing RXRα represses NRF2/ARE signalling pathway in vivo. Taken together, we hypothesize that RXRα plays a key role in linking xenobiotic metabolism with the NRF2-ARE cytoprotective signaling pathway. Furthermore, several nuclear receptors besides RXRα repress NRF2 (11, 46, 47). It is interesting to speculate that antagonism of NRF2 by RXRα occurs because at some level the CNC-bZIP factor protein and nuclear receptors are functionally incompatible, and that it might be advantageous to attenuate NRF2 activity.

Recent studies have revealed that NRF2 exhibits abnormal increased activity in several types of tumor due to oncogene activation, or somatic mutation of KEAP1 or NRF2 (15, 17). The overactivation of NRF2-ARE signaling in cancer cells promotes drug resistance and cell proliferation (31, 48). It has been reported that RXRα is downregulated in many tumors (49–51). On the basis of our study, it seems plausible that a reduction of RXRα will up-regulate the NRF2-ARE signalling pathway, thereby contributing to tumorigenesis and drug resistance. In this study, we have shown that forced expression of RXRα in NSCLC A549 cells downregulated the NRF2/ARE cytoprotective pathway and sensitized them to anticancer drugs, suggesting that transcriptional repression by RXRα may be used as a mechanism to attain an appropriate level of gene expression in NRF2 overactive cell types. Here, we describe the Neh7 domain in Nrf2 as a potential target by which the CNC-bZIP factor can be inhibited.

In summary, in this report we describe the interaction of NRF2 with RXRα introducing a new dimension to our understanding of the transcriptional hierarchy in ARE-driven gene regulation. Our studies, therefore, suggest a novel, and as yet unrecognized, function of RXRα as a putative transcriptional co-repressor of NRF2.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H. Wang, K. Liu, P. Gao, Y. Hai, Y. Li, J.D. Hayes, X.J. Wang, X. Tang
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H. Wang, K. Liu, M. Geng, P. Gao, X. Wu, Y. Hai, Y. Li, J. L. Luo, X.J. Wang
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H. Wang, K. Liu, M. Geng, P. Gao, X. Wu, Y. Hai, Y. Li, X.J. Wang, X. Tang
Writing, review, and/or revision of the manuscript: H. Wang, J.D. Hayes, X.J. Wang, X. Tang
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): H. Wang, K. Liu, M. Geng, X. Wu, Y. Li, J. L. Luo, X. J. Wang
Study supervision: H. Wang, K. Liu, Y. Li, X.J. Wang, X. Tang

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Direct Interaction with the Neh7 Domain of NRF2 Inhibits the NRF2-ARE Signaling Pathway through a αRXR αRXR

Hongyan Wang, Kaihua Liu, Miao Geng, et al.

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