Components of the CCR4-NOT Complex Function as Nuclear Hormone Receptor Coactivators via Association with the NRC-interacting Factor NIF-1*

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CCR4-NOT is an evolutionarily conserved, multicomponent complex known to be involved in transcription as well as mRNA degradation. Various subunits (e.g. CNOT1 and CNOT7/CAF1) have been reported to be involved in influencing nuclear hormone receptor activities. Here, we show that CCR4/CNOT6 and RCD1/CNOT9, members of the CCR4-NOT complex, potentiate nuclear receptor activity. RCD1 interacts in vivo and in vitro with NIF-1 (NRC-interacting factor), a previously characterized nuclear receptor coactivator that activates nuclear receptors via its interaction with NRC. As with NIF-1, RCD1 and CCR4 do not directly associate with nuclear receptors; however, they enhance ligand-dependent transcriptional activation by nuclear hormone receptors. CCR4 mediates its effect through the ligand binding domain of nuclear receptors and small interference RNA-mediated silencing of endogenous CCR4 results in a marked decrease in nuclear receptor activation. Furthermore, knockdown of CCR4 results in an attenuated stimulation of nuclear receptor activation. These observations are consistent with the reported interaction of CCR4 with NIF-1. In addition, the CCR4-enhanced transcriptional activation by nuclear receptors is dependent on NIF-1. The small interference RNA-mediated knockdown of NIF-1 blocks the ligand-dependent potentiating effect of CCR4. Our results suggest that CCR4 plays a role in the regulation of certain endogenous NRAR target genes and that RCD1 and CCR4 might mediate their function through their interaction with NIF-1.

The nuclear receptor superfamily consists of ligand-regulated transcription factors that orchestrate the coordinated expression of gene networks in diverse physiological, developmental, and metabolic processes (1, 2). These include thyroid hormone receptors (TRs),2 retinoic acid receptors (RARs), retinoid X receptors (RXRs), vitamin D receptor, glucocorticoid receptor (GR), progesterone receptor, estrogen receptors (ERs), peroxisome proliferator-activated receptors, among others. Nuclear receptors are characterized by a common modular structure, an N-terminal “A/B” domain, a central zinc-finger-containing DNA binding “C” domain (DBD), a “D” hinge region, and a C-terminal “EF” ligand binding domain (LBD) (3, 4). Most receptors harbor at least two activation domains: 1) a ligand-independent activation function AF-1, which generally resides in the A/B domain, and 2) a second activation domain AF-2 that is ligand-dependent and localized in the C-terminal LBD (5).

Certain unliganded receptors (e.g. TRs and RARs) associate with corepressor complexes such as N-CoR and SMRT, which have been shown to associate with histone deacetylases and repress transcription (6–8). Ligand binding induces a conformational change leading to the release of the corepressor complexes, in turn facilitating the recruitment of coactivator complexes (9, 10). In addition, liganded receptors direct the disruption of the nucleosomal structure around target genes by recruiting chromatin-modifying factors such as the ATP-dependent SWI/SNF complex (11).

Well studied coactivators include members of the p160 family (SRC-1, SRC-2, and SRC-3) (12–18), the CBP/p300 family (19–21), p/CAF (22, 23), NRC/NCaA6/ASC-2/RAP250/TRBP/PRIP/AIB3 (24–30), NRIF3 (31, 32), PGC-1 (33, 34), NIF-1 (35), and CARMA1 and PRMT1 (36, 37). Another set of coactivators recruited to ligand-bound nuclear hormone receptors are the TRAP/DRIP/SMCC/Mediator complexes (38–40). Many nuclear receptors, including TRs, ERs, vitamin D receptor, and peroxisome proliferator-activated receptors, interact with the TRAP/DRIP complex through the DRIP205/TRAP220/MEDI1 component in a ligand-dependent manner (41–44). The TRAP/DRIP complex is implicated in the direct communication between nuclear receptors and the general transcriptional machinery. Several components interacting with RNA polymerase II, including NUT2, SRB7, SRB10, and SRB11, were identified as part of the TRAP/DRIP complex (38, 45, 46). A recent study (47) indicates that a DRIP205/TRAP220/MEDI1-containing complex is tightly associated with the general transcription machinery.

*This work was supported by National Institutes of Health Grant DK 16636 and a grant from the Entertainment Industries Foundation. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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2 The abbreviations used are: TR, thyroid hormone receptor; RAR, retinoic acid receptor; RA, retinoic acid; RXR, retinoid X receptor; GR, glucocorticoid receptor; ER, estrogen receptor; PPAR, peroxisome proliferator-activated receptor; LBD, ligand binding domain; DBD, DNA binding domain; CBP, CREB-binding protein; CCR4, carbon catabolite repressor 4; CAF1, CCR4-associated factor 1; NIF-1, NRC-interacting factor; X-gal, 5-bromo-4-chloro-3-indoyl-β-D-galactopyranoside; HA, hemagglutinin; T_{\alpha}, triiodothyronine; E_{\beta}, estradiol; CAT, chloramphenicol acetyltransferase; GST, glutathione S-transferase; siRNA, small interference RNA; RT, reverse transcription; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
with a near stoichiometric level of RNA polymerase II along with other pre-initiation factors such as TBP, SRB7, and TFIIH. The various distinct coactivator complexes cooperate with one another temporally at target gene promoters. Studies have indicated a sequential recruitment of coactivators in vivo, with chromatin modification (Swi/Snf and histone acetyltransferases) preceding the recruitment of TRAP/DRIP complexes (48–50).

The CCR4-NOT complex is a multicompartment, evolutionarily conserved global regulator of gene expression. The yeast CCR4-NOT complex exists in two forms of 1.0 and 1.9 MDa, the smaller complex consisting of nine core proteins: CCR4 (carbon catabolite repressor 4), NOT1–5, CAF1 (CCR4-associated factor 1), CAF40, and CAF130 (51, 52). CCR4 was first identified in Saccharomyces cerevisiae as being necessary for non-fermentative gene expression (53). CCR4 and CAF1 have also been shown to be components of the major cytoplasmic mRNA deadenylase, with CCR4 as the catalytic component (54, 55). The NOT proteins were isolated as repressors of transcription from promoters lacking a canonical TATA sequence (56). The NOT proteins play an important role in transcriptional regulation through their interaction with TFIIID and other components of the Mediator complex (57, 58).

Several human orthologs of subunits of the CCR4-NOT complex have been identified (59, 60). The composition of the mammalian CCR4-NOT complex and the functionality of several components have also been conserved. For instance, CNOT2 (hNOT2) functions as a repressor of reporter gene activity, with a suggestion of the involvement of a histone deacetylase activity (61). A recent study identified CNOT1 as a ligand-dependent repressor of nuclear hormone receptor activity (62). CNOT6 (hCCR4) and the newly identified CNOT6L (another homolog of yCCR4) have both been shown to possess ligand-dependent transcriptional activation by nuclear hormone receptors through their interaction with TFIID and other components of the Mediator complex (57, 58).

In this study, we report that NIF-1 associates with RCD1, and RCD1 as well as CAF40 function as nuclear hormone receptor coactivators. CCR4 is an AF-2-dependent coactivator and appears to play a vital and specific role in the regulation of certain RARα target genes. Although these factors do not directly associate with nuclear receptors, our findings are consistent with a model that suggests that they mediate their effects through a NIF-1 coactivator complex.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid cDNA Library from GH4C1 Cells—** Poly(A⁺) RNA isolated from GH4C1 cells was used for the synthesis of cDNA with a Stratagene cDNA synthesis system. The cDNA was size-fractionated using Sephacryl S200. Fractions above 0.4 kb were pooled, precipitated with ethanol, and ligated with EcoRI-Xhol-digested pEG202, which constitutively expresses the cDNA as a fusion with the LexA DNA binding domain in S. cerevisiae. The pEG202-cDNA library was transformed into Sure bacteria (Stratagene) by electroporation. This cDNA library contains ~1.2 × 10⁷ independent transformants, and the average insert size was estimated to be 1.5 kb.

**Yeast Two-hybrid Screen—** Full-length NIF-1 was cloned into the EcoRI site of pJGM vector (35) and was used as bait in a yeast two-hybrid screen. pJGM was derived from the parent vector pJG4–5, as described earlier (24). All methods and transformation procedures have been described previously (24). The yeast strain EGY48 harboring the LacZ reporter pSH18–34 and pJGM-NIF-1 was transformed with the GH4C1 pEG202 cDNA library. Transformants were directly screened on 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) synthetic defined minimal medium (SD)-galactose-raffinose plates lacking Trp, Ura, His, and Leu. The putative positive clones were then verified by replica plating each clone on Trp−, Ura−, His−, and Leu− X-gal SD-galactose-raffinose and Trp−, Ura−, His−, and Leu− X-gal SD-dextrose plates. Yeast clones exhibiting a positive LacZ response on galactose-raffinose plates, but not on dextrose plates, were considered to be potential NIF-1-interacting clones. The putative cDNAs were then isolated, sequenced, and subjected to size determination and further analysis.

**Plasmids—** Expression plasmids for nuclear receptors and various reporters have been described previously (24). The cloning of pCDNA3-NIF-1 and pJGM-NIF-1 has also been described (35). The plasmid IRES-RCD1 (66) was generously provided by Dr. Hiroto Okayama (University of Tokyo). pGEX-RCD1 was cloned by inserting full-length RCD1 into the EcoRI site of pGEX-4T-1. The pLP vector (which introduces a FLAG and an HA tag into the cloning sequence) was kindly provided by Dr. Jeffrey Ye. pLPC-RCD1 was generated by first introducing a 5′-BamHI and 3′-EcoRI restriction enzyme site into the coding sequence of RCD1. The BamHI-EcoRI fragment was then cloned in-frame into the pLP vector. Primers: 5′-BamHI, CTACCAAGATGGATCCCAGGCGAAGCCTGCGC; 3′-EcoRI, CTACCAAGATGATTCTCACCAGGGGACAGGGGATACC. The plasmid pSG5-FLAG-CCR4 (67) was a kind gift from Dr. Laura Corbo (Unité INSERM, France). The yeast expression vectors for nuclear receptors have been described earlier (24, 35). The yeast expression plasmid pEG202ΔLPRCD1 was constructed by cloning the EcoRI insert from IRES-RCD1 into the pEG202ΔL vector. The plasmid pEG202ΔLPRCD1 was cloned by inserting the BamHI-Sall fragment of pSG5-CCR4 in the pEG202ΔL multiple cloning site.

**Cell Culture and DNA Transfection—** HeLa cells were routinely maintained in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum supplemented with glutamine.
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and antibiotics. MCF-7 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal calf serum supplemented with glutamine and antibiotics. For the reporter studies, HeLa cells were seeded in 6-well plates at 150,000 cells per well, and the medium was replaced with Dulbecco's modified Eagle's medium containing 10% hormone-depleted bovine calf serum (68) before transfection. Transfections were performed using calcium phosphate coprecipitation. Various ligands such as T₃ for TRα, 9-cis-RA for RARα and RXRα, E₂ for ERα, and deoxy cortisolone for GR were used at concentrations of 1 μM. Yeast colonies were first grown exponentially in Ura⁻/HIS⁻/LYS⁻ medium for 12 h followed by quantitation of OD₆₀₀ and OD₅₀₀, respectively.

Yeast β-Galactosidase Assays—Various ligands, such as T₃ for TRα, 9-cis-RA for RARα and RXRα, E₂ for ERα, and deoxy cortisolone for GR were used at concentrations of 1 μM. Yeast colonies were first grown exponentially in Ura⁻, His⁻, and Trp⁻ SD-dextrose medium, washed, diluted to the appropriate density, and incubated in Ura⁻, His⁻, and Trp⁻ SD-galactose-raffinose medium for 12 h followed by quantitation of β-galactosidase as described earlier (35). β-Galactosidase units are expressed as (A₄₂₀ × 1000)/(minutes of incubation × A₆₆₀ of the yeast suspension), where A₄₂₀ and A₆₆₀ are optical density at 420 and 600 nm, respectively.

In Vitro Binding Assay—GST-RCD1 and GST-NRC-15 were expressed in SG1117 Escherichia coli by induction with isopropyl-1-thiogalactopyranoside and then purified and immobilized to glutathione-agarose, as described previously (35, 69). NIF-1 was labeled by in vitro transcription-translation with [l-³⁵S]methionine using rabbit reticulocyte lysate system (Promega, Madison, WI). Typically, 300–500 ng of GST protein bound to glutathione agarose was used per assay. ³⁵S-Labeled NIF-1 was mixed with GST, GST-RCD1, or GST-NRC-15 beads. The samples were incubated for 15 min at 25 °C followed by 2 h at 4 °C in binding buffer (20 mM Hepes, pH 7.4, 1 mM MgCl₂, 100 mM KCl, 0.5 μM ZnCl₂, 1 mM dithiothreitol, 10% glycerol, 0.05% Triton X-100, 10 μg/ml ovalbumin). The samples were then washed with same binding buffer, and the bound ³⁵S-labeled protein was analyzed by SDS-gel electrophoresis followed by autoradiography.

Antibodies and Coimmunoprecipitation—Rabbit polyclonal antibody against RCD1 was a kind gift from Dr. Hiroto Okayama (University of Tokyo). Rabbit polyclonal antibody against CCR4 was a kind gift from Dr. Laura Corbo (Unité INSERM, France). The anti-NIF-1 (BL2739) and the anti-Sox9 (AB5533) antibodies were from Bethyl Laboratories and Chemicon International, respectively. The anti-Cyclin D1 antibody was a gift from Dr. Michele Pagano (New York University School of Medicine). The antibody against β-actin was from Abcam. The anti-HA antibody was from Roche Applied Sciences. For coimmunoprecipitation, pLPC-RCD1 (HA-tagged) and full-length NIF-1 were transfected into HeLa cells. The cells were lysed with buffer (50 mM Tris-HCl, pH 7.4, 1% Nonidet P-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 1 mM NaF, and protease inhibitors), and the lysate was incubated with an anti-HA affinity matrix (Roche Applied Science). The immunoprecipitated proteins were then eluted from the beads with an HA peptide (Covance Inc.) and then analyzed by Western blotting using anti-NIF-1 antibody. To study the interaction between CCR4 and NIF-1, pSG5-CCR4 (FLAG-tagged) and full-length NIF-1 were transfected into 293T cells. Whole cell lysates were prepared in lysis buffer (20 mM HEPES, pH 7.9, 150 mM NaCl, 20 mM KCl, 0.2 mM EDTA, 10 μM ZnCl₂, 1 mM MgCl₂, 0.1% Triton X-100, and protease inhibitors) as described previously (67). Equal amounts of the lysates were incubated with anti-FLAG affinity matrix (Sigma). The immunoprecipitated proteins were eluted with FLAG peptide (Sigma) and then analyzed by Western blotting using anti-NIF-1 antibody.

siRNA Transfection—siRNAs directed against RCD1, CCR4, NIF-1, and a non-targeting control (siControl) were obtained from Dharmacon. HeLa and MCF-7 cells were transfected with the siRNAs (final concentration, 25 nM and 40 nM, respectively) using Hiperfect siRNA transfection reagent from Qiagen. For the receptor reporter assays, the appropriate DNA constructs were transfected into HeLa cells (Lipofectamine® 24 h after the siRNA transfection. The CAT assay was performed 64 h after the initial siRNA transfection. To study the effect of the siRNA-mediated knockdown on endogenous target genes, 40 nM of the siRNAs was transfected into MCF-7 cells using Hiperfect. The cells were lysed after appropriate incubation with ligand. Equal amounts of protein were then analyzed by SDS-gel electrophoresis, followed by Western blotting for either Sox9 or cyclin D1.

RT-PCR Analysis—Total RNA was extracted using TRIzol® (Invitrogen). For semi-quantitative RT-PCR, 100 ng of the RNA samples was reverse-transcribed and analyzed using the C. Therm polymerase One-step RT-PCR system (Roche Applied Science). The following primers were used: RCD1, 5'-GCTG-GAGCTAAGTAAAGAGCCGAGAATC-3' and 5'-GGGAGA-AACGCTCATACTGTTGAC-3'; CCR4, 5'-CAGAACAATCGG-AAACATGGTATCACTC-3' and 5'-GATCCACAGTAGCC-GTATAACTGC-3'; and GAPDH, 5'-TGAAGGTCGGAGTGTAACGG-3' and 5'-CATGTGGCCCATGAGTCGAT-CACCACAC-3'. For quantitative PCR, 1 μg of total RNA was reverse-transcribed using Superscript III® Reverse Transcriptase (Invitrogen). 1 μl of cDNA was used for real-time PCR using SYBR Green (Sigma) on a Roche LightCycler® 2.0 system. The values were normalized to an internal GAPDH control. The following primers were used: HoxA1, 5'-CAGCTTCACTACCAAGCGACG-3' and 5'-GACTCTTCGTAAGTTCTCG-3'; Sox9, 5'-CTCAAAGGCTACGACTGAG-3' and 5'-CTCGTTCAAGTCTCAGACG-3'; and GAPDH, 5'-CTCTACGAGACTCTGTT-3' and 5'-CCCTGTGGCTGTAGCAGGAAT-3'.

RESULTS

Identification of RCD1 as an NIF-1 Interactor—A LexA-tagged NIF-1 was found to be constitutively active in S. cerevisiae and could not be used to identify potential NIF-1 interactors from the previously described cDNA library (35). Therefore, a full-length NIF-1 expressed as a B42 fusion protein was used as bait against a GH4Cl LexA cDNA library in a yeast
NIF-1 Interacts with RCD1 in Vitro and in Vivo—To confirm the interaction between RCD1 and NIF-1 in vitro, a full-length GST-RCD1 fusion was expressed in E. coli and purified with glutathione-agarose beads. The 35S-labeled NIF-1 was incubated with GST or GST-RCD1, and the samples were analyzed for binding as described under “Experimental Procedures.” As shown in Fig. 1A, 35S-NIF1 binds to GST-RCD1 but not to GST alone. The interaction between NRC-15 (fragment of NRC encompassing the NIF-1-interacting domain and the nuclear receptor-interacting domain), and NIF-1 has been well characterized and was used as a positive control (35). A yeast two-hybrid interaction assay further confirmed the association between RCD1 and NIF-1. A LexA-tagged RCD1 and B42-tagged NIF-1 were re-introduced into S. cerevisiae. The interaction between RCD1 and full-length NIF-1 was determined by measuring the activity of β-galactosidase in yeast extracts. As shown in Fig. 1B, a specific interaction between NIF-1 and RCD1 was confirmed. To determine whether RCD1 and NIF-1 interact in vivo, an influenza hemagglutinin (HA) epitope-tagged RCD1 was coexpressed with NIF-1 in HeLa cells. The lysates were immunoprecipitated with HA antibody affinity matrix and eluted with an HA peptide, followed by SDS-gel electrophoresis and Western blotting with an antibody against NIF-1 (Fig. 1C). These results confirm the specific interaction between RCD1 and NIF-1 in mammalian cells.

RCD1 Enhances Ligand-dependent Transcriptional Activity of Nuclear Hormone Receptors—Our results in Fig. 1 indicate an interaction between RCD1 and NIF-1. To determine if RCD1 could affect nuclear receptor activity, we carried out an initial experiment by expressing TRα and its appropriate CAT reporter ΔMTV-IR-CAT in HeLa cells along with increasing amounts of RCD1. RCD1 enhanced the ligand-dependent activation of TRα in a dose-dependent manner (Fig. 2A). NIF-1 has been previously described as a potent cotransducer of nuclear receptors and was therefore used as a positive control (35).

RCD1 has been shown to be part of a RA-dependent complex that also contains the RARs, although a direct interaction between RARα and RCD1 has not been confirmed (66). We sought to examine the effect of RCD1 expression on the ligand-dependent activation of RARα, RXRα, ERα, and GR (Fig. 2, B and C). Expression vectors for RCD1, nuclear receptors, and the respective CAT reporters were introduced into HeLa cells. RCD1 enhanced activation by both RARα and RXRα in a ligand-dependent manner. The effect of RCD1 expression on the activation by GR was comparatively less than the other receptors (TRα, RARα, and RXRα) studied. No effect on ERα was observed. These results suggest that expression of RCD1 enhances ligand-dependent nuclear receptor activation, but with a preference for certain receptors.

CCR4, a Component of the CCR4-NOT Complex, Is a Nuclear Hormone Receptor Coactivator—hCCR4 protein, also termed as CNOT6, is an integral component of the eukaryotic CCR4-NOT complex (63). CCR4 and its associated factor, CAF1 have been implicated in transcription regulation in yeast and mammalian cells. Both CAF1 and CCR4 have been shown to enhance the activity of ERα in transient transfection studies (67, 72). We decided to study the effect of CCR4 on ligand-dependent nuclear hormone receptor activation. Various nuclear receptors and their respective CAT reporters were expressed in HeLa cells along with CCR4. CCR4 enhanced the activation by TRα, RARα, RXRα, and GR in a ligand-de-
pendent manner (Fig. 3). As noted previously, ERα activation was also increased by CCR4 expression.

**CCR4 and RCD1 Do Not Directly Interact with Nuclear Hormone Receptors**—A direct interaction between CCR4/RCD1 and nuclear hormone receptors could explain the increased activation as shown above (Figs. 2 and 3). To explore this possibility, we set up a yeast two-hybrid interaction assay between LexA fusions of nuclear receptors and B42-RCD1 or B42-CCR4. A B42 fusion of NRC-15 has been shown previously to interact directly with receptors and was therefore used as a positive control (24). RCD1, CCR4, and NRC-15 were found to be expressed at similar levels as assessed by Western blotting with an anti-HA antibody. However, as shown in Fig. 4, there was no interaction detected between RXRα and either RCD1 or CCR4. Additional interaction studies between RCD1 or CCR4 and TRα-LBD, full-length TRα, ERα-LBD, full-length ERα, RARα-LBD, and GR-LBD also showed no evidence for any direct interaction (data not shown).

**CCR4 Functions through the Ligand-binding Domain of Nuclear Hormone Receptors**—The LBDs of nuclear hormone receptors encompass a ligand-dependent activation function 2 (AF2 domain) through which a number of coactivators (e.g. p160/SRCs and NRC/NCoA6) mediate their effects (5). Our results show that CCR4 is a strong coactivator of nuclear hormone receptors. To determine if CCR4 functions through the ligand binding domain of the receptors, the LBDs fused to the Gal4 DNA binding domain were used in lieu of full-length receptors in reporter assays. Various Gal4 DBD–LBD constructs were expressed in HeLa cells along with a Gal4-responsive CAT reporter. Expression of CCR4 resulted in enhanced ligand-dependent activation of the LBD constructs (Fig. 5A). Although the expression of RCD1 enhanced the ligand-dependent activation of TRα, RARα, and RXRα, its effect on the Gal4-LBD constructs of these receptors was less pronounced than that found with CCR4 (Fig. 5B).

**Down-regulation of CCR4 Expression Decreases Nuclear Hormone Receptor Activation**—Overexpression of RCD1 and CCR4 enhances ligand-dependent activation of nuclear hormone receptors (Figs. 2 and 3). To better explore the physiological role of endogenous RCD1 and CCR4 in nuclear receptor activation, an RNA interference approach was used. HeLa cells were transfected with siRNAs designed specifically against RCD1 and CCR4 mRNAs. The decrease in the protein levels of RCD1 and CCR4 was analyzed 60 h later by Western blotting (Fig. 6A). To study the effect of knockdown of RCD1 and CCR4 on nuclear receptor activation, expression vectors for various nuclear receptors and their corresponding CAT reporters were introduced into HeLa cells 24 h post siRNA transfection, and the CAT activity was subsequently analyzed 40 h later.

The transcriptional activation of TRα, RARα, and RXRα was significantly inhibited by the knockdown of endogenous CCR4, whereas ERα and GR activation was affected to a lesser degree (Fig. 6, B and C). This suggests a selective role for CCR4 in nuclear hormone receptor activation. The decrease in RCD1 expression using RCD1-directed siRNA had less of an effect on nuclear receptor activation than that observed with the knockdown of CCR4. The ligand-dependent transcriptional activation of the respective reporter genes by TRα, RARα, RXRα,
ERα, and GR was reduced, but by a lesser degree (Fig. 7A and data not shown). The siRNA-mediated knockdown of RCD1 also did not significantly affect the CCR4-dependent enhancement of ERα and GR transcriptional activity (as shown in Fig. 7B).

NIF-1 Knockdown Results in Decreased Nuclear Hormone Receptor Activation—RCD1 has been shown to interact with NIF-1 (Fig. 1). To further study the effect of endogenous NIF-1 on nuclear receptor activation, we utilized an siRNA against NIF-1 mRNA to markedly reduce endogenous NIF-1 protein levels in HeLa cells (Fig. 8A). The effect of NIF-1 silencing on ligand-dependent activation of TRα, RARα, and GR was subsequently analyzed. Nuclear hormone receptors and their reporter constructs were expressed in HeLa cells in which NIF-1 expression had been attenuated. The results suggest that the transcriptional activity of the receptors was considerably inhibited when NIF-1 was knocked down (Fig. 8B). These results concur with the ability of NIF-1 to enhance ligand-dependent activation of nuclear receptors as shown previously (35).

Effect of Silencing of RCD1 and CCR4 on Endogenous Nuclear Receptor Target Genes—Although the transfection results (Figs. 2–4) indicate that RCD1 and CCR4 enhance nuclear receptor activation, the effect of the knockdown of CCR4 and RCD1 on endogenous target genes of nuclear hormone receptors would more clearly elucidate their physiological role. MCF-7 cells have been well documented to be both ER- and RAR-responsive (73, 74). We therefore examined the effect of silencing of RCD1 and CCR4 on the expression of Sox9 (an RA receptor target gene) and Cyclin D1 (an ERα target gene) in MCF-7 cells.

The siRNA-mediated silencing of CCR4 and NIF-1 specifically attenuated the levels of endogenous Sox9 protein but had no detectable effect on the levels of endogenous β-actin (Fig. 9A). The knockdown of RCD1 also reduced Sox9 protein levels but to a lesser degree. The effect of siRNA-mediated silencing of CCR4 and RCD1 on Cyclin D1, an endogenous ERα target gene, was also analyzed. As noted above, the knockdown of CCR4 and RCD1 had a somewhat lesser effect on the ligand-dependent activation of ERα in transient transfection experiments, as compared with the other receptors studied (Fig. 6 and data not shown). Consistent with these results, the expression of cyclin D1 was not affected by the siRNA-mediated knockdown of either RCD1 or CCR4 (as shown in Fig. 9B) in the E2-responsive MCF-7 cells.

Another well documented RARα-responsive gene is HoxA1 (75). Because there are no antibodies against HoxA1 available for Western blotting, we examined the effect of silencing of RCD1, CCR4, and NIF-1 on HoxA1 expression by a quantitative RT-PCR method. The decrease in RCD1 and CCR4 mRNA levels was first confirmed by semi-quantitative PCR (Fig. 10A). Western blotting confirmed the knockdown of NIF-1 in MCF-7 cells (Fig. 10A). The HoxA1 mRNA levels were initially analyzed by semi-quantitative RT-PCR to determine the relative importance of RCD1, CCR4, and NIF-1 (data not shown). These results were further confirmed and quantitated by real-time RT-PCR analysis (Fig. 10B, left panel). The silencing of both CCR4 as well as NIF-1 resulted in a decrease in the induction of HoxA1 mRNA in MCF-7 cells. In contrast, RCD1 does not appear to have as significant a role in the expression of endogenous HoxA1 by RARα. We also analyzed the endogenous Sox9 mRNA expression, both by semi-quantitative RT-PCR (data not shown) and real-time RT-PCR analysis (Fig. 10B, right panel), and validated the effect of the knockdown of CCR4 and NIF-1.

CCR4 Associates with NIF-1 in Vivo in an RCD1-independent Manner—Our results indicate that both CCR4 and NIF-1 play a role in the regulation of Sox9 and HoxA1 (RARα target genes) (Fig. 10). This raised the possibility of an association between...
CCR4 and NIF-1. To address this question, FLAG-tagged CCR4 was coexpressed with NIF-1 in 293T cells. The protein lysates were initially immunoprecipitated with FLAG antibody affinity matrix. The FLAG-tagged CCR4 and its associated proteins were then eluted from the affinity matrix with a FLAG peptide. The samples were subjected to SDS-gel electrophoresis followed by Western blotting with an antibody against NIF-1. As shown in Fig. 11A, a specific interaction between CCR4 and NIF-1 was determined. The eluates were simultaneously Western blotted with an anti-FLAG antibody to confirm comparable expression levels of CCR4.

Because CCR4 and RCD1 are components of the CCR4-NOT complex and RCD1 has been shown to interact directly with NIF-1 (Fig. 1), we sought to determine if the association between CCR4 and NIF-1 occurs in an RCD1-dependent manner. 293T cells were transfected with either a control siRNA or an siRNA against RCD1 mRNA. The expression vectors for FLAG-tagged CCR4 and NIF-1 were then introduced into 293T cells 24 h post siRNA transfection. The whole cell lysates were immunoprecipitated and eluted as above, followed by SDS-gel electrophoresis and Western blotting with an anti-NIF-1 antibody. Our results indicate that the association between CCR4 and NIF-1 is independent of RCD1 (data not shown).

**CCR4-enhanced Activation by RARα Is NIF-1-dependent**—An in vivo interaction between CCR4 and NIF-1 (Fig. 11A) and a comparable affect on the RARα target genes studied (Sox9 and HoxA1) (Fig. 10) prompted us to further examine the association between CCR4 and NIF-1. MCF-7 cells were transfected with either an siRNA against NIF-1 or a corresponding control siRNA. An expression vector for CCR4 along with a RARα-responsive CAT reporter was introduced 24 h after siRNA transfection and the CAT activity was assayed 40 h post-DNA transfection. Error bars represent ± S.D. C, similar to B except that the expression vectors for GR and ERα were transfected along with their respective reporter constructs.
DISCUSSION

The evolutionarily conserved CCR4-NOT complex controls gene expression both at the level of transcription initiation and of mRNA degradation. The mammalian CCR4-NOT complex contains the orthologs of yeast NOT proteins (CNOT1-CNOT4) and also CNOT6 (hCCR4), CNOT7 (hCAF1), CNOT8 (hCalif), CNOT9/RCD1 (hCAF40), and CNOT10 (hCAF130) proteins (63). Certain NOT proteins have been reported to function as repressors of transcription. A repressor domain called the NOT-box has been identified in both CNOT2 as well as CNOT3 (61). The SMRT/NCoR-HDAC3 corepressor complex is thought to function as a cofactor for transcriptional repression by CNOT2 (76). The CCR4-NOT complex is also involved in mRNA decay. CNOT6 (hCCR4) has been shown to play a role in mRNA deadenylation (64).

Various components of the mammalian CCR4-NOT complex have been reported to be involved in transcriptional activation by nuclear hormone receptors. mCAF1 was found to be an essential factor for spermatogenesis and has been shown to be a regulator for RXRβ (77, 78). CNOT6 (hCCR4) and CNOT7 (hCAF1) were previously shown to significantly enhance the ERα-mediated activation of a luciferase reporter gene (67, 72). Our results here show that CCR4 augments the ligand-dependent transcriptional activation of a wide variety of nuclear receptors, including ERα.
NIF-1 was previously characterized, through its interaction with the coactivator NRC, as a factor that enhances nuclear receptor activation (25, 35). Because NIF-1 does not directly interact with nuclear receptors, the modulation of ligand-bound nuclear hormone receptors by NIF-1 appears to occur through the direct interaction between NRC and nuclear hormone receptors. In this study, we identified RCD1 as an NIF-1-interacting factor through a yeast two-hybrid screen. RCD1 is highly conserved across species and has been shown to be critical for RA-induced differentiation of F9 cells. RCD1 was identified as a component of the DRF transcriptional complex in these cells, which consists of at least p300/CBP, ATF-2, and the interactor factor through a yeast two-hybrid screen. RCD1 is much more abundant than CCR4, the effect of RCD1 on nuclear receptors is less than that observed for RCD1. This could be explained by differences in the relative abundance of these factors. Thus, if RCD1 acts through the CCR4-NOT complex and RCD1 is much more abundant than CCR4, the effect of RCD1 expression or knockdown will be less than that found with CCR4. Alternatively, it is possible that RCD1 and CCR4 function independently through other yet to be defined pathways in addition to the CCR4-NOT complex. It must be noted here that the CCR4 knockdown resulted in a greater decrease in the ligand-dependent activation of TRα, RARα, and RXRα compared with ERα and GR. This selective function of CCR4 was further elucidated with studies on endogenous hormone responsive target genes. The knockdown of CCR4 resulted in a decrease in RAR-mediated stimulation of Sox9 protein levels but did not have an effect on the ER- and GR-mediated stimulation of cyclin D1. However, estrogen also initiates many non-nuclear signaling events, including the activation of several signal transduction pathways (e.g. phosphatidylinositol 3-kinase/Akt, protein kinase C, p38/mitogen-activated protein kinase, and Janus tyrosine kinase/signal transducers and activators of transcription) (80). It has been shown previously that estrogen rapidly stimulates phosphatidylinositol 3-kinase and Akt in MCF-7 cells leading to an Src-dependent induction of cyclin D1 expression (81, 82). Therefore, it is possible that this non-genomic expression of cyclin D1 is independent of RCD1 and CCR4 and thus is not affected by the siRNA-mediated knockdown of either of the two factors.

3 S. Garapaty, M. A. Mahajan, and H. H. Samuels, unpublished results.
Quantitative PCR analysis confirmed the effect of CCR4 silencing on Sox9 mRNA levels. HoxA1, another RARα-responsive gene in MCF-7 cells was also affected by the siRNA-mediated knockdown of CCR4. The silencing of RCD1 did not influence the mRNA levels of HoxA1, consistent with the results observed with Sox9. As mentioned previously, NIF-1 expression enhances ligand-dependent nuclear receptor activation (35). In keeping with the coactivator function of NIF-1, Sox9 and HoxA1 mRNA levels were both attenuated by siRNA-mediated silencing of NIF-1. This decrease in the RAR-mediated stimulation of Sox9 and HoxA1 by the knockdown of NIF-1 was comparable to that observed with the silencing of CCR4.

Although neither RCD1 nor CCR4 directly interact with nuclear receptors, they potentiate their ligand-dependent activities. CCR4 mediates this function through the LBDs of nuclear receptors. This suggests that the RCD1-CCR4 complex tethers to the promoters of target genes through the interaction with another factor(s) that bind to the liganded nuclear recep-
tor LBD. One such model would involve the interaction of a CCR4-NOT-RCD1 complex with NIF-1, which in turn interacts with NRC, which has been shown to bind directly to the liganded LBD of nuclear receptors (35). Our results indicate an in vivo association between CCR4 and NIF-1, which occurs independent of the presence of RCD1. Consistent with this result, the silencing of RCD1 did not affect CCR4-dependent enhancement of nuclear receptor activation (Fig. 7B). In a similar vein, we show that NIF-1 is necessary for the coactivator function of CCR4, providing an additional link between the two factors. In further support of this model, a recent study suggested that the knockdown of NRC leads to a marked reduction of RAR-mediated stimulation of Sox9 in MCF-7 cells (83).

In summary, we report that RCD1 and in particular CCR4 function as nuclear receptor coactivators. RCD1 was isolated as an interactor of NIF-1, which has been shown to enhance nuclear receptor activation. Our results provide the first indication that CCR4 enhances the activity of a variety of nuclear receptors, other than ERα. Our findings on the effect of CCR4 on HoxA1 expression, along with the previously reported role of RCD1 in RA-mediated differentiation (66), suggest a link between components of the CCR4-NOT complex and the growth and differentiation of mammalian tissues.

Acknowledgments—We thank Hiroto Okayama for providing us with pRES-RCD1 and the anti-RCD1 antibody, Michele Pagano for the anti-cyclin D1 antibody. We thank Susan Logan and Derek Mitchell for their help with the real-time PCR analysis.
