NRIP, a Novel Nuclear Receptor Interaction Protein, Enhances the Transcriptional Activity of Nuclear Receptors*

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Transcriptional regulation by members of the nuclear hormone receptor superfamily is a modular process requiring the mediation of distinct subclasses of coregulators. In this study, we isolated a novel WD40 repeat-containing gene, human nuclear receptor interaction protein (NRIP). We found NRIP interacts with either androgen or glucocorticoid receptors from in vitro and in vivo pulldown assays. Subsequently, transient transfection and luciferase activity assays suggested that NRIP was a ligand-dependent coactivator of steroid receptors (androgen and glucocorticoid) in distinct promoters. To further clarify the function of NRIP, we found an RNA interference-3-targeted NRIP gene sequence (5′-GATGATACAGCACGAGAAC-3′) that could efficiently and specifically knock down endogenous and exogenous NRIP gene expression and that significantly diminished cell proliferation in prostate (LNCaP) and cervical (C33A) cells. Therefore, NRIP may play a role in enhancing the transcriptional activity of nuclear receptors and may be a critical target for developing therapeutic agents against nuclear receptor-mediated progression of prostate and cervical cancers.

Steroid hormone receptors, such as androgen receptor (AR),1 estrogen receptor, progesterone receptor, and glucocorticoid receptor (GR) etc., are known as type I nuclear receptors (1). Nuclear receptor family members function as ligand-inducible transcription factors (2). The binding of growth hormone to nuclear receptor induces receptor dimerization, facilitating the ability of the nuclear receptor to bind to its cognate response element and recruit coregulators to promote the expression of target genes (3). In the past decade, several coactivators have been cloned and characterized that associate with steroid receptors and enhance their ability to transactivate target genes (4). Well studied coactivators include the p160 family proteins (SRC-1, TIF-2/GRIP-1, ACTR/CIP) (5), the p300/cAMP response element-binding protein-binding protein family (6), Ubc9 (7), ARA70 (8), ARA55 (9), TIP60 (10), and protein arginine methyltransferases (11). Some cofactors act through functional modification of other activators or coactivators (12), resulting in efficient recruitment of coactivators by the nuclear receptor to the target gene and/or the stabilization of general initiation factors that form pre-initiation complexes on common core promoter elements (13). Some coactivators contain intrinsic histone acetyltransferase or methyltransferase activities, suggesting that they modify chromatin and integrating stimuli into an appropriate transcription response at a wide variety of promoters (14–16). In addition, a class of coregulators serves as bridges mediating interactions between the coactivators and other general transcription factors at post-chromatin-remodeling steps (17).

In this study, we isolated a novel gene named nuclear receptor interaction protein (NRIP) by using the yeast two-hybrid system. From amino acid sequence comparisons, NRIP is identical to the unnamed protein product (GenBank™ accession number CAD 48617) that was deposited in NCBI on September 2002 with unknown function. Therefore, we characterized this human novel gene (NRIP) by assaying its subcellular location, interaction with nuclear receptors (such as AR and GR), and transactivation activity in distinct promoters. Our results indicate that NRIP contains 860 amino acids and its expression is restricted to the cell nucleus. Additionally, NRIP binds to both AR and GR and functions as a nuclear receptor coactivator. Therefore, NRIP may be a transcriptional cofactor of steroid receptors. To further define the roles of NRIP, short interference RNA (siRNA)-mediated NRIP gene silencing in mammalian cells was conducted. A specific NRIP siRNA targeting sequence was also found that could knock down endogenous and exogenous NRIP gene expression, resulting in significantly diminished cell proliferation in prostate (LNCaP) and cervical (C33A) cancer cells. Therefore, our data illustrate that the novel gene NRIP may function to enhance transcriptional activity of nuclear receptors and thus can be a therapeutic target for eliminating tumor growth.

EXPERIMENTAL PROCEDURES

Yeast Two-hybrid Screen—A pACT2-HeLa MATCHMAKER cDNA library (Clontech) that consists of the GAL4 activation domain (aa 788–881) fused with a human HeLa cDNA library was transformed into CG-1945 yeast strain (Clontech), along with a plasmid, pAS2–1/AR595–918, containing GAL4 DBD (aa 1–147) fused with the C-terminal domain of AR (aa 595–918). 5 × 10^6 yeast transformants were screened and selected on synthetic dropout (S.D.; Difco) medium lacking leucine, tryptophan, and histidine in the presence of 25 μM 3-aminol,2,4-triazole (3-AT; Sigma) and 10 μM dihydrotestosterone (DHT; Sigma). Colonies were tested for LacZ reporter gene activity in a β-galactosidase filter assay. Plasmid DNAs from positive clones were recovered from yeast, amplified in Escherichia coli, and confirmed by sequencing.

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† The nucleotide sequence(s) reported in this paper has been submitted to the DDBJ/GenBank™/EBI Data Bank with accession number(s) AY766164 and AAX09330.

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1 The abbreviations used are: AR, androgen receptor; NRIP, nuclear receptor interaction protein; GR, glucocorticoid receptor; MMTV, mouse mammary tumor virus; HPV, human papilloma virus; siRNA, short interference RNA; DHT, dihydrotestosterone; ARE, androgen response element; TRE, glucocorticoid response element; NLS, nuclear localization sequence; GFP, green fluorescent protein; EGFP, enhanced GFP; LCR, long control region; PSA, prostate-specific antigen; RNAi, RNA interference; RT, reverse transcription; wt, wild-type; mt, mutant; aa, amino acid; HA, hemagglutinin.

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5'-Rapid Amplification of cDNA Ends (RACE)-PCR—5'-RACE-PCR was used to obtain the remaining 5'-end sequence of the above isolated NRIP gene. The PCR amplification was performed using human Hela Mammalian-ready cDNA (Clontech) as a template. The first amplification was performed using the adaptor primer 1 and the gene-specific primer 5'-AGGTCATTTTTCTTCTCAGTGGAA-3' for 28 cycles followed by a final elongation of 10 min at 72 °C. Each cycle consisted of 15 s at 94 °C, 60 s at 58 °C, and 2 min at 72 °C. 1 µl of PCR product was used as a template for the second amplification with the adaptor primer 2 and the nested gene-specific primer 5'-ACTGTTGTACCTCCTCCCTGGTTTGCG-3' for 28 cycles, using the same conditions as those used for the first amplification. Thereafter, the PCR product was cloned into pGEM-T vector (Promega) and sequenced.

Plasmid Constructions—The full-length NRIP was cloned in the mammalian expression vector pcDNA3.1-HisC (Invitrogen) and named pcDNA3.1-His-C-NRIP, containing N-terminal Xpress and histidine epitope. The plasmid pEGFP-NRIP was generated by tagging EYFP at the 5'-end of the NRIP gene. Mutant NRIP was made by following two PCR-based approaches as described by Mikelaid and Sergeant (18). Wild-type NRIP was used as a template, and the primers sequences are as follows: 5'-TGGCAATTTTTCAGTGGGGCTCGTT- TACCCACAC-3' (primer 1); 5'-AGGTTGTACCTCCTCCCTGGTTTGCG-3' (primer 2); 5'-GACCGAAAGAAGCACGAGGGAGTTTTGCTTGGCTTTGC-3' (mutagenic primer 3); 5'-AGGTTGTACCTCCTCCCTGGTTTGCG-3' (mutagenic primer 4). Mutagenic primers 3 and 4 contained silent mutations (underlined) corresponding to the RNA interference (RNAi) 3-targeted position (Fig. 7A). Primer 1 and mutagenic primer 4 were used as a pair in one reaction; mutagenic primers 3 and 2 were used in a separate reaction in the first round of PCR. Amplified products were loaded on 1% agarose gels and purified. The second round of PCR, 20–50 ng of each purified fragment were mixed as a template and added to primers 1 and 2 containing EcoRI sites for PCR as described previously (18). The obtained mutant NRIP fragment was inserted into the pEGFP-C2 vector and named pEGFP-NRIP (mt). The plasmid pcDNA3.1-angioatin-HA was derived from the NotI fragment from pXK-angioatin-HA (19) subcloned into a pcDNA3.1 vector; therefore it contained cDNA of mouse angioatin (aa 1–466) and a HA tag at its C-terminal.

Northern Blotting Analysis—Human multiple tissue Northern blots were purchased from Clontech. A probe corresponding to the full-length cDNA of NRIP was 32P-labeled in vitro using a random prime labeling system (Amersham Biosciences) according to the manufacturer's protocol and hybridized overnight. After washing, the blot was exposed and autoradiographed. The relative amounts of total RNA treated with or without 10 µg of pEGFP-NRIP using the FuGENE 6 transfection reagent (Roche Applied Science) according to the manufacturer's instructions. Forty-eight hours after transfection, cells were fixed with 4% paraformaldehyde and stained with a nuclear counterstaining dye, 0.2 µg/ml DAPI (4,6-diamidino-2-phenylindole dihydrochloride, blue color; Sigma). Fluorescent GFP-NRIP was monitored by a ZEISS Axiovert 100 TK inverted confocal laser microscope.

Luciferase Reporter Plasmid DNA Constructions—The plasmid LCR-79 contained a 79-bp oligonucleotide covering the sequence from 7352 to 7430 of HPV-16 long control region (LCR) (GenBank™ accession number NC_001526) including the ARE/GRE sequence (5'-GCTA GAACCTGTCTTGAGTC-3'). The synthesized 79-bp oligonucleotides were cloned into a pGL3-Promoter vector (Promega) at KpnI and Xhol sites and named LCR-79 (Fig. 6A). The prostate-specific antigen reporter construct (PSA-Luc) was kindly provided by Dr. Mien-Chie Hung (M. D. Anderson Cancer Center, Houston, Texas) (20). MMTV-Luc plasmid was a kind gift from Dr. M. Danielsen, Georgetown University, Washington, D.C.

Cell Culture and Luciferase Activity Assays—293T and C33A cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum and 1% minimal essential medium non-essential amino acids solution (Invitrogen). LNCaP cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum and antibiotics. Prior to transfection, cells were seeded on 60-mm dishes at a density of 4 × 10^4 in Dulbecco's modified Eagle's medium with 10% dextan-coated charcoal-stripped fetal bovine serum (HyClone) for 24 h. Transient transfections were performed with FuGENE 6 (Roche Applied Science) or SuperFect (Qiagen) transfection reagents according to the manufacturer's instructions. DNA constructs as indicated were transfected, including 2 µg of internal control (pcHL110, β-galactosidase; Amersham Biosciences). The total amount of plasmid transfected per dish was kept constant by adding empty pcDNA3 vector as needed. 24 h after each transfection, the medium was changed and treated with or without 10 nM DHT or dexamethasone. After another 24 h, cells were harvested and the luciferase activity assayed by a Luminoskan TL Plus luminometer (Thermo Labsystems). The β-galactosidase activity of cell lysates was determined and used to normalize luciferase activity.

Cell Proliferation Assay—The Cell Titer 96® Aqueous One solution cell proliferation assay is a quantitative colorimetric method for determining mammalian cell survival and proliferation. Cells were seeded at 10^5 cells/well into 24-well plates and maintained in the absence of hormone-containing medium for 24 h. Cells were then transiently transfected with 2 µg of RNAi-3 plasmid DNA using the FuGENE 6 or SuperFect transfection reagent. 24 h post-transfection, cells were treated with or without 10 nM DHT for 0, 24, 48, and 72 h. 100 µl of Cell Titer 96 Aqueous One solution reagent (Promega) was added to each well, and the absorbance at 490 nm was read after incubation for 4 h at 37 °C by a PerkinElmer Lambda 40 UV/VIS spectrophotometer.

GenBank™ Accession Number—The human NRIP nucleotide and protein sequences have been submitted to the GenBank™ database with accession numbers AY766184 and AAx05390.
In this study, we isolated NRIP by using the androgen receptor (amino acids 595–918) as the bait to screen a human HeLa MATCHMAKER cDNA library in a yeast two-hybrid screening assay. Full-length NRIP cDNA (3085 bp) contained 93 bp of 5′-untranslated region, 409 bp of 3′-untranslated region, and 2583 bp of open reading frame (Fig. 1A) that encoded a protein of 860 amino acids (aa) with a calculated mass of 96 kDa (Fig. 1B). The flanking sequence of the start codon (ATG) of NRIP is GCCATGTCTCGG, which is similar to the Kozak sequences ((A/G)cc ATG Gat) (23). The amino acid sequence of NRIP was identical to the unnamed protein product (GenBank™ accession number CAD48617) reported in NCBI in September 2002 of unknown function. Based on SMART data base (smart.embl-heidelberg.de/) search results, NRIP was found to contain several important functional domains, including WD40 repeat domains and NLS motif, as shaded and underlined, respectively. The alignments are based on human NRIP sequences. The asterisk (*) indicates identical residues, and the dash (-) marks gaps introduced to maximize homology between human and mouse NRIP protein sequences.

To look for the evolutionary functions and developmental importance of NRIP in close species, we compared the deduced amino acid sequences with one putative mouse cDNA coding for an unnamed protein product and deposited it in GenBank™ with the accession number AK004618. As shown in Fig. 1B, amino acid sequence analysis of human and mouse NRIP proteins showed 86.3% homology. The mouse NRIP also possessed seven WD40 domains, and the amino acid sequence showed complete homology to human NRIP. The highly conserved protein sequences and, in particular, strong conservation of WD40 domains indicated NRIP might have developmental and evolutionary significance.

To determine the size of the NRIP protein, we transfected a plasmid encoding Xpress-tagged NRIP into 293T cells and performed an in vivo immunoprecipitation assay. Analysis of the cell extracts detected a protein with an apparent molecular mass of 160 kDa (Fig. 1D). This significantly higher mass than...
predicted (96 kDa) may have been due to aberrant electrophoretic mobility imparted by the highly charged amino acid content (40%) of the protein sequence.

To analyze the expression pattern of NRIP mRNA, Northern blot analysis of endogenous NRIP gene expression in human normal tissues was measured by using human multiple tissue Northern blots (Clontech). As shown in Fig. 2A, NRIP mRNA (4 kb) was highly expressed in skeletal muscle and testis and was expressed to a lesser degree in heart, prostate, and adrenal gland.

Human NRIP showed some specific features such as a nuclear localization sequence (NLS) motif and seven WD40 domains (Fig. 1B). The NLS sequence suggested that NRIP may be a nuclear protein. As shown in Fig. 3, subcellular compartmentalization of NRIP was restricted to the nucleus as seen by examining expression of green fluorescent protein (GFP)-tagged NRIP (panel A) under a fluorescence microscope; this was confirmed by DAPI nucleus staining (panel C). NRIP may be involved in protein-protein interactions, because WD40 repeats are thought to coordinate interactions with other proteins and/or small ligands (24, 25). Based on its nuclear localization and its association with AR in our yeast two-hybrid assay, we inferred that NRIP might play a role in regulating the transcriptional activity of nuclear receptors.

Nuclear receptors, such as AR and GR, play an important role in some hormone-responsive tumors (26). Before investigating whether NRIP was involved in hormone-related regulation, we first analyzed the gene expression of NRIP, AR, and GR in various cell lines (such as 293T, C33A, CaSki, HeLa, SiHa, MCF-7, SCM-1, PC-3, and LNCaP) by RT-PCR using total RNA from each cell line as described under “Experimental Procedures.” The expected 1427-, 357-, and 511-bp fragments, respectively, indicating that NRIP can increase AR-driven transcriptional activity with ligand present. In addition, we performed an in vivo assay by co-transfecting AR or GR expression constructs plus a GFP-tagged NRIP plasmid (pEGFP-NRIP) into 293T cells and treated with DHT (Fig. 4A, lane 7) on Dex (Fig. 4A, lane 8) ligand, respectively. The cell lysates were immunoprecipitated with antibodies to GFP, AR, or GR, and the precipitates were blotted using the indicated antibodies. As shown in Fig. 4B, lanes 4 and 6, AR associated with NRIP. Likewise, Fig. 4C, lanes 4 and 6, showed that GR also interacted with NRIP.

In summary, our data demonstrated that NRIP could directly interact with either AR or GR in the presence of its specific ligand under in vitro and in vivo conditions.

NRIP Enhances AR- or GR-mediated Transcriptional Activity of MMTV Promoter—Because NRIP could bind with either AR or GR (Fig. 4), we examined whether NRIP could regulate the nuclear receptors on a naturally occurring gene promoter. The MMTV promoter was chosen because the steroid hormone receptors (AR and GR) are known to regulate MMTV-driven gene expression in a ligand-dependent fashion (27). 293T and C33A cells were chosen for these assays because these two cell lines weakly express endogenous AR and GR (Fig. 2B). Therefore, 293T and C33A cells were co-transfected with an MMTV-Luc reporter construct (containing two copies of androgen and four copies of glucocorticoid receptor response elements fused upstream of a luciferase reporter gene) as well as a steroid receptor expression plasmid (either AR or GR), plus NRIP with or without ligand. As shown in Fig. 5, the ligand-free AR had only minimal reporter activity of MMTV-Luc in the presence or absence of NRIP in 293T (Fig. 5A) and C33A cells (Fig. 5B). However, addition of 10 nM 5α-dihydrotestosterone (DHT) resulted in 5.2- and 4.5-fold increases in the transcriptional activities of the MMTV-Luc promoter co-transfected with AR and NRIP in 293T (Fig. 5A) and C33A (Fig. 5B) cells, respectively, indicating that NRIP can increase AR-driven transcriptional activity with ligand present.

When NRIP was added to cells containing MMTV-Luc and GR
in the absence of ligand, negligible GR transcriptional activity was seen in 293T and C33A cells (Fig. 5, C and D). Addition of 10 nM dexamethasone to cells containing GR and MMTV-Luc, but no NRIP, resulted in 5.7- and 3.1-fold increases of GR transcriptional activity in 293T (Fig. 5C) and C33A (Fig. 5D) cells, respectively. An even greater increase in GR transcriptional activity was seen, however, in cells with NRIP present, 18.2- and 21.9-fold, respectively, in 293T (Fig. 5C) and C33A (Fig. 5D) cells. The above data indicated NRIP could enhance AR- or GR-mediated transcription activity in the presence of AR or GR ligands, implying that NRIP functions as a ligand-dependent coactivator of nuclear receptors by enhancing their transcriptional activity.

FIG. 3. Sublocalization of NRIP in the nuclei of 293T cells. Cells were transiently transfected with pEGFP-NRIP plasmid. Forty-eight hours after transfection, cells were fixed with 4% paraformaldehyde and stained with DAPI. A, green signal represents the localization of NRIP fusion protein. B, phase-contrast image. C, the blue signal represents DAPI-stained nucleus.

FIG. 4. Co-immunoprecipitation of NRIP with AR and GR. A, in vitro co-immunoprecipitation of each 35S-labeled protein as indicated precipitated with an anti-Xpress antibody for NRIP (lanes 5–9) or a HA-tagged antibody for angiostatin (lane 10) as described under “Experimental Procedures.” B, in vivo interaction between NRIP and AR. 293T cells were co-transfected with 15 μg of pcDNA3.0-AR along with 15 μg of pEGFP-NRIP or pEGFP (control) for each 100-mm dish transfection using the calcium phosphate precipitation method. Thirty-six hours after transfection, the medium was changed and 10 nM DHT added. After another 12 h, cell extracts were harvested and immunoprecipitated with GFP or AR antibody and then immunoblotted with GFP or AR antibody as indicated. Lanes 1, 3, 5, and 7, AR + EGFP; lanes 2, 4, 6, and 8, AR + NRIP. C, in vivo interaction between NRIP and GR. The same experimental procedures shown in panel B were followed, except that the BSVT7-GR plasmid and dexamethasone were used instead of pcDNA3.0-AR and its specific ligand. Lanes 1, 3, 5, and 7, GR + EGFP; lanes 2, 4, 6, and 8, GR + NRIP. B and C, lanes 1 and 2, IP and WB with the same GFP antibody; lanes 3 and 4, IP with AR or GR antibody, WB with GFP antibody; lanes 5 and 6, IP with GFP antibody, WB with AR or GR antibody; lanes 7 and 8, IP and WB with AR or GR antibody. IP, immunoprecipitation; WB, Western blot.
The Effects of NRIP on the Transcriptional Activities of PSA and HPV-16 Promoter—To determine the relevance of NRIP in cancers, we investigated two hormone-related diseases, prostate cancer and cervical cancer. Prostate cancer is the second leading cause of cancer death of men in the United States (28). Prostate-specific antigen (PSA) is a 33-kDa glycoprotein, elevated levels of which occur in sera from prostate cancer patients (29). Hence, PSA is a sensitive indicator of tumor burden. AR is responsible for the transactivation of PSA through its binding to a steroid receptor binding consensus sequence in PSA promoter/enhancer regions. To investigate the role of NRIP in prostate cancers, transient transfections were carried out in AR-expressing prostate cancer cells (LNCaP) (Fig. 2B) with reporter construct PSA-Luc, generated by subcloning a genomic DNA (1.5 kb) containing the PSA promoter (640 bp) and enhancer (820 bp) linked into the luciferase expression vector, and have throughout been used to analyze AR-mediated transcriptional activity (20). Our data indicated that PSA promoter activity was significantly activated by AR plus NRIP in a ligand-dependent manner in LNCaP cells (Fig. 6A).

Human papillomavirus type 16 (HPV-16) is the most frequently found virus in cervical cancers and the most extensively studied for the effect of hormones on viral gene expression (30). Concurrently, steroid hormones are proposed to act as cofactors with HPV receptors to induce viral transcription by glucocorticoids (30). One of the GREs at nucleotide position (7385-GCTACAtccTGTTT-7399) is highly homologous to the AR binding consensus sequence (5'-GGTACAnnnTGTTCT-3') and is named ARE/GRE. Therefore, a construct (LCR-79) containing a 79-nucleotide fragment from sequence 7352 to 7430 covering the ARE/GRE sequence fused upstream of a TATA promoter and luciferase reporter gene was used to assay HPV-16 promoter activity (Fig. 6B). We investigated whether NRIP functions as a cofactor of nuclear receptors in C33A cells, which are human cervical carcinoma cells and negative for human papillomavirus DNA and RNA. As shown in Fig. 6B, NRIP could activate both AR- and GR-driven gene transcription in C33A cells with the respective ligands. Therefore, these findings further confirmed that NRIP functions as a transcriptional cofactor in regulating AR- and GR-mediated gene activation in a ligand-dependent pathway.

Identification of siRNA to NRIP—RNAi has proven to be a powerful tool to silence gene expression in a sequence-specific manner. Recent advances in the understanding of RNAi have provided practical tools to knock down gene expression in mammalian cells, thus making it possible to quickly generate gene knock-out models to investigate the functions of NRIP genes on nuclear receptor transactivation. The pSUPER vector-based siRNA system (21) was used in this study. Short 19-nt stem-loop structures of the various siRNAs were designed to corre-
Response to the NRIP nucleotide positions shown in Fig. 7A. Because a NRIP-specific antibody was not available, we ectopically expressed an NRIP fusion protein tagged with EGFP at its 5′-end and used a commercial GFP tag-specific antibody to quantify the amount of protein expressed. Therefore, quantitation of exogenous EGFP-NRIP protein was analyzed after cells had been treated with the various siRNA constructs. Results showed the RNAi-3 construct could efficiently diminish exogenous EGFP-NRIP fusion protein expression both in 293T (Fig. 7B, upper panel) and C33A (lower panel) cells. To further determine whether the NRIP-3 construct could specifically knock down exogenous NRIP gene expression, we generated the mutant plasmid pEGFP-NRIP (mt), in which the sequence corresponding to the RNAi-3-targeted sequence (position 943–961) was mutated to 5′-GACGACACGGCCCGG-GAGC-3′. After co-transfection of wt or mt NRIP plasmid with RNAi-3 into 293T (Fig. 7C, upper panel) or C33A (lower panel) cells, Western blot analysis showed that RNAi-3 only inhibited wild-type protein expression (lane 2), but not mutant type expression (lane 4), in both 293T and C33A cells. The results of Fig. 7C were also confirmed by fluorescence microscopy (Fig. 7D). EGFP-NRIP green fluorescence diminished only in the presence of RNAi-3 when pEGFP-NRIP (wt) was co-transfected (Fig. 7D, left lower), but not in the control vector (pSuper) with pEGFP-NRIP (wt) (Fig. 7D, left upper) or the control plasmid with pEGFP-NRIP (mt) (right upper) or RNAi-3 plus pEGFP-NRIP (mt) (right lower). Therefore, RNAi-3 efficiently inhibited exogenous NRIP gene expression.

Moreover, to investigate whether RNAi-3 could silence endogenous NRIP gene expression, an increased dose of RNAi-3 was introduced into the cells and analyzed by RT-PCR. As shown in Fig. 7E, RNAi gene expression was proportionally diminished by the addition of RNAi-3 to 293T (Fig. 7E, upper panel) and C33A (lower panel) cells. Taken together, the designed sequence of RNAi-3 could target the NRIP gene and diminish its gene expression.

RNAi-3-mediated Silencing of Endogenous NRIP Gene Expression Affects Cell Growth—To determine the biological effect of RNAi-3 inhibition of NRIP gene expression, we measured cell proliferation rates in RNAi-3-transfected 293T and C33A cells (Fig. 8A); it caused a reduction in cell proliferation in these two tested cells. To further investigate the physiological role of NRIP in prostate cancer, we measured RNAi-3 effects on LNCaP cells with or without DHT ligand with regard to cell growth rate. As shown in Fig. 8B, RNAi-3 diminished the growth rate of LNCaP cells in the presence or absence of ligand. Next, we determined the effect of RNAi-3 on PSA promoter-driven gene expression. The results showed that RNAi-3-reduced PSA promoter-mediated gene expression was dose dependent (Fig. 8C). Investigation of whether the decreased growth of LNCaP cells by RNAi-3 (Fig. 8B) was correlated with the endogenous PSA gene expression revealed that the decrease in endogenous NRIP expression caused by RNAi-3 affected PSA gene expression (Fig. 8D, lanes 3 and 4), but not AR and β-actin expression, in LNCaP cells treated with or without ligand (Fig. 8D). More strikingly, we found that NRIP gene

![Figure 6](http://www.jbc.org/)

**Fig. 6.** NRIP enhances AR- and GR-driven transcription of the PSA and HPV-16 promoters, respectively. A, AR-mediated transcription of the PSA-Luc promoter. LNCaP cells were transiently transfected with 3 μg of PSA-Luc reporter, 2 μg of pcDNA3.0-AR, 1 μg of pcDNA3.1-HisC-NRIP, and 2 μg of pCH110. The total amount of plasmid was adjusted with empty pcDNA3 vector to 11 μg for each 60-mm dish transfection using SuperFect transfection reagent. Twenty-four hours after transfection, cells were treated with or without 10 nM DHT for another 24 h. Relative luciferase activity was determined as described in Fig. 5. **B,** schematic diagram of the LCR-79 reporter plasmid sequence (top). The LCR-79 reporter plasmid contained the HPV-16 sequence 7352–7430 covering the ARE/GRE binding consensus sequence linked to a TATA box. Relative luciferase activity was determined as described in Fig. 5. All values represent an average of at least three independent experiments.
expression could be induced by the ligand-treated cells. The evidence showed that NRIP gene expression increased in DHT ligand-treated LNCaP cells (Fig. 8 lane 2) in comparison to non-ligand-treated cells (lane 1). Additionally, the cell growth rate of RNAi-3-transfecting LNCaP cells was slightly higher in the presence of ligand than in the absence of ligand (Fig. 8B). This suggests that NRIP may be an androgen-responsive gene. In sum, many of these experiments confirmed the specificity of the effect of RNAi-3 on the NRIP down-regulation of PSA promoter activity and PSA gene expression. The implication is that NRIP may play an important role in the development or progression of prostate cancer.

**DISCUSSION**

**NRIP Is a Cofactor of Nuclear Receptors and Contains WD40 Domains**—In this study, we found a novel human gene (NRIP) containing seven WD40 domains and one NES motif. The NES sequence implied that NRIP was a nuclear protein, which was confirmed by fluorescence microscopy (Fig. 3). NRIP could interact with either AR or GR in *in vitro* and *in vivo* pulldown assays (Fig. 4) and increased AR- and GR-mediated transcriptional activity of MMTV, HPV-16, and PSA promoters in a ligand-dependent manner (Figs. 5 and 6). This study illustrates that human NRIP is a transcriptional cofactor of both AR or GR. Recently, a number of steroid receptor coactivators have been identified as being able to modulate steroid hormone receptor transactivation. Most of these factors have been identified through their physical interactions with nuclear receptors and can enhance nuclear receptor-mediated transcription *in vivo* (4). The shared protein-protein interaction domain would thus be the most important characteristic of transcription cofactors. To date, it has been reported that coactivators of steroid nuclear receptors such as SRC-1 (33), TIF2/GRIP1 (34, 35), and ACTR/AIB1/RAC3 (36–38) contain a basic helix-loop-helix domain and Per-AhR-Sim domain for protein-protein interactions. Cofactors such as FLH2 (39) and ARA55 (9), however, have an LIM motif, which is cysteine-rich and also
involved in protein-protein interactions (40, 41). The cofactor ARA70 contains an LXXLL motif (where X is any amino acid) involved in the interaction with the AF-2 of nuclear receptors (42). ARA267 contains SET and PHD domains (43, 44). SNURF contains a C3HC4-type zinc finger (a RING finger motif) governing protein-protein interactions (45). NRIP identified in this study is a WD40 repeat-containing protein. WD40 domain-containing proteins are reported to be present in all eukaryotes, but not in prokaryotes (25). WD40 domain-containing proteins have been implicated in protein-protein interactions and regulate a wide variety of cellular functions, including chromatin remodeling and transcription, cell division, cell fate determination, transmembrane signaling, mRNA modification, and vesicle fusion (24, 46, 47). The role of WD40 domains in NRIP remains to be elucidated. We are in the process of deleting the WD40 domain and mapping its interaction with AR or GR.

NRIP Is a Ligand-dependent Coactivator of Nuclear Receptors

RNAi-3 Knock Down NRIP Gene Expression, Resulting in the Inhibition of Cell Proliferation—NRIP was expressed in all of our tested cancer cells, including cervical, prostate, and breast cancer cells (Fig. 2B). Prostate and cervical cancers are both hormone-related diseases. In cervical cancer, steroid hormones act as cofactors with HPV-16 (30). Progesterone and glucocorticoid hormones have been reported to induce the expression of HPV-16 via its three glucocorticoid response elements in the viral regulatory region (48). Oral contraceptives, which contain progestin and estrogen steroid hormones as active ingredients, are epidemiologically associated with an increased risk of cervical cancer (49–51). In addition, the increased level of progesterone during pregnancy is a significant risk factor for malignant conversion (21, 52). This implies that the oncogenic potential of HPV is associated with the responses of steroid hormones to their regulatory regions. Our study demonstrated that NRIP could induce AR- and GR-driven gene expression of the HPV-16 promoter in the presence of its cognate ligand (Fig. 6B). When decreasing NRIP gene expression, the E7 gene expression of HPV-16 was reduced in CaSki cells, which contains ~600 copies of HPV-16 genome/cell (53). Consequently, it inhibited the cell growth of CaSki cells. In addition, C33A is a HPV-negative cervical cancer cell, the growth rate of which was inhibited by knockdown NRIP gene expression (Fig. 8A). Therefore, aside from the effect on HPV gene expression by NRIP (Fig. 6B), NRIP may also influence the other hormone-related genes involved in the progression of cervical cancer. In sum, NRIP

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FIG. 8. RNAi-3-mediated silencing of endogenous NRIP gene expression affects cell growth. A, RNAi-3 inhibits the growth rate of 293T and C33A cells. Cell proliferation assays were performed as described under “Experimental Procedures.” All experiments were performed three times with triplicate samples. The results show the mean of all data. Error bars indicate S.D. B, RNAi-3 suppresses the growth of LNCap cells in the presence or absence of ligand. C, RNAi-3 represses AR-mediated transactivation of PSA promoter activity. LNCap cells were transiently transfected with 3 μg of PSA-Luc reporter, along with increasing amounts of RNAi-3 plasmid construct as indicated and 2 μg of pCH110 expression plasmid. The total amount of plasmid was adjusted with empty pSuper vector to 11 μg. Relative luciferase activity was determined as described above in Fig. 5. The luciferase activity of cells transfected with the PSA promoter plus DHT ligand was set as 100%. D, RNAi-3 suppresses endogenous PSA expression in LNCap cells. Cells were seeded into 60-mm dishes in the absence of hormone-containing medium for 24 h, followed by transient transfection of 4 μg of RNAi-3 construct DNA/dish. The total amount of plasmid was adjusted with empty pSuper vector to 10 μg. Twenty-four hours post-transfection, cells were treated with or without 10 nM DHT for an additional 6 h. Total RNA was isolated and endogenous NRIP, AR, and PSA gene expression measured by RT-PCR analysis. The expected NRIP, AR, and PSA products were 1427-, 357-, and 786-bp fragments, respectively. β-actin was used as a control to verify equivalent RNA loading.

\[ T. C. Tsai, Y. L. Lee, Y. P. Tsao, and S. L. Chen, unpublished data. \]
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may play a role in the development of cervical cancer and is worth further investigation.

As to prostate cancers, the AR is a member of the steroid receptor superfamily and plays an important role in male sexual differentiation and prostate cell proliferation (54). Several cofactors of AR have currently been identified to facilitate the major promoter activity of prostate tumor growth via androgen-dependent pathway. In this study, we demonstrated that NRIP could activate an AR-driven PSA promoter via ligand-dependent pathway in prostate cancer cells (LNCaP). To illustrate the significance of NRIP in prostate cancer, we found an NRIP interference sequence (RNAi-3) that could demolish the in vivo pathway in prostate cancer cells (LNCaP). NRIP functions as a transcriptional coactivator in nuclear receptor-driven gene expression and has a role in cell growth, indicating that NRIP may be a critical target for developing therapeutic agents against nuclear receptor-mediated progression of prostate and cervical cancers.

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