Cloning and characterization of an androgen receptor N-terminal-interacting protein with ubiquitin–protein ligase activity

L K Beitel^{1,3}, Y A Elhaji^{1,2}, R Lumbroso¹, S S Wing³, V Panet-Raymond¹, B Gottlieb¹, L Pinsky^{1,2,3} and M A Trifiro^{1,2,3}

¹Lady Davis Institute for Medical Research, SMBD-Jewish General Hospital, 3755 Côte St Catherine Road, Montreal, Quebec, Canada H3T 1E2

²Department of Human Genetics, McGill University, Quebec, Canada H3A 1B1

³Department of Medicine, McGill University, Montreal, Quebec, Canada H3G 1Y6

(Requests for offprints should be addressed to L K Beitel; Email: lenore.beitel@mcgill.ca)

Abstract

The androgen receptor (AR) N-terminal domain plays a critical role in androgen-responsive gene regulation. A novel AR N-terminal-interacting protein (ARNIP) was isolated using the yeast two-hybrid system and its interaction with amino acids 11–172 of the normal or corresponding region of the polyglutamine-expanded human AR confirmed by glutathione S-transferase pulldown assays. ARNIP cDNAs cloned from NSC-34 (mouse neuroblastoma/spinal cord) or PC-3 (human prostate adenocarcinoma) mRNA encoded highly homologous 30 kDa (261 amino acids) cysteine-rich proteins with a RING-H2 (C3H2C3 zinc finger) domain; this motif is highly conserved in predicted ARNIP-homologous proteins from several other species. Expression of the ~1.7 kb ARNIP mRNA was detected in various tissues by Northern blotting, but was highest in mouse testes, kidney and several neuronal cell lines. In addition, the human ARNIP protein was found to be encoded by nine exons spanning 32 kb on chromosome 4q21. In COS-1 cells, coexpression of ARNIP and AR did not affect AR ligand-binding kinetics, nor did ARNIP act as a coactivator or corepressor in transactivation assays. However, AR N-terminal:C-terminal interaction was reduced in the presence of ARNIP. Intriguingly, ARNIP, and in particular its RING-H2 domain, functioned as a ubiquitin–protein ligase *in vitro* in the presence of a specific ubiquitin-conjugating enzyme, Ubc4-1. Mutation of a single cysteine residue in the ARNIP RING-H2 domain (Cys145Ala) abolished this E3 ubiquitin ligase activity. Fluorescent protein tagging studies revealed that AR–ARNIP interaction was hormone-independent in COS-1 cells, and suggest that colocalization of both AR and ARNIP to the nucleus upon androgen addition may allow ARNIP to play a role in nuclear processes. Thus, identification of a novel AR-interacting protein with ubiquitin ligase activity will stimulate further investigation into the role of ubiquitination and the ubiquitin–proteasome system in AR-mediated cellular functions.

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Introduction

The androgen receptor (AR), a member of the nuclear receptor superfamily, mediates androgen action by interacting with a number of coregulatory proteins, both coactivators and corepressors, to activate or repress transcription of androgen-responsive genes (Brinkmann *et al.* 1999, Edwards

1999, McKenna *et al.* 1999a). Tissue-specific expression of coregulators, promoter-specific protein interactions, and exposure to different androgens may also determine the specificity of AR action (MacLean *et al.* 1997). For certain nuclear receptors, ubiquitin-mediated degradation of receptors, their associated coactivators or transcription factors plays a role in regulating transactivation

(McKenna *et al.* 1999b, Lonard *et al.* 2000). The contribution of such processes to AR function is, however, largely undefined.

The AR has a structure typical of a steroid receptor: an N-terminal domain modulates transcriptional regulation, a central DNA-binding domain (DBD) binds androgen response elements, and a C-terminal ligand-binding domain (LBD) determines steroid specificity. These principal domains are not functionally autonomous. In fact, they each have a set of subsidiary functions including heat shock protein binding, nuclear localization, dimerization and transactivation, many of which reflect functional/structural interactions between the principal domains and/or their specific subdomains (MacLean *et al.* 1997, Gast *et al.* 1998). AR N-terminal:C-terminal (N-C terminal) interaction is androgen-dependent in yeast (Doesburg *et al.* 1997) and mammalian cells (Ikonen *et al.* 1997, Berrevoets *et al.* 1998, He *et al.* 1999) and forms a novel interaction interface for recruitment of p160 coactivators that influence AR transactivation (Berrevoets *et al.* 1998, Alen *et al.* 1999, Ma *et al.* 1999).

Although the DBD and LBD of the AR are closely related to those of the glucocorticoid, mineralocorticoid and progesterone receptors (Lubahn *et al.* 1988), the N-terminal domains of these steroid receptors are distinct (Thornton & Kelley 1998). Remarkably, the human AR (hAR) N-terminal contains a polymorphic polyglutamine (polyGln) tract, starting at amino acid 58, encoded by a (CAG)_n trinucleotide repeat; a similar polyGln repeat is found in the rat AR, albeit further towards the C-terminal (Thornton & Kelley 1998). Normally, the hAR polyGln tract consists of 9–36 Gln; variations in the polyGln tract length, even within the normal range, can increase the risk of certain conditions or diseases. Males with ≥28 Gln have a greater than 4-fold increased risk of infertility due to impaired spermatogenesis (Tut *et al.* 1997), and shorter Gln tracts (<20 Gln) have been associated with increased prostate cancer risk (Irvine *et al.* 2000 and references therein). Pathological expansion of the hAR polyGln tract to 40–62 Gln causes spinobulbar muscular atrophy (SBMA or Kennedy disease), with progressive muscle weakness and wasting due to motorneuronal loss and signs of mild androgen insensitivity such as infertility and gynecomastia (Pinsky *et al.* 2001).

Given the unique nature of the AR N-terminal domain, we postulated that hAR-specific N-terminal sequences could interact with specific proteins that affect AR-mediated gene regulation and/or AR-dependent disease processes. Employing the yeast two-hybrid system, we used the hAR N-terminal polyGln tract region (amino acids (aa) 11–172 according to Lubahn *et al.* (1988)) of the normal or polyGln-expanded hAR as bait to screen a cDNA library made from NSC-34 mRNA, a mouse neuroblastoma/spinal cord hybrid cell line (Cashman *et al.* 1992). We isolated a novel hAR N-terminal-interacting protein (ARNIP) that is differentially expressed and contains a highly conserved RING-H2 finger motif. The initial cloning of the mouse ARNIP (mARNIP) and human ARNIP (hARNIP) genes, the characterization of the ARNIP protein, and its function as an E2-dependent ubiquitin–protein ligase (E3) are described below.

Materials and methods

Preparation of NSC-34 cDNA library

Total RNA was prepared from NSC-34 cells (Cashman *et al.* 1992) as described (Beitel *et al.* 1994), and poly-A⁺ RNA (~7 µg) purified on a Oligotex column (Qiagen). The Two-Hybrid cDNA Library Construction kit (Clontech, Palo Alto, CA, USA) was used to generate an NSC-34 cDNA library in dephosphorylated EcoRI-digested pACT2 (Clontech). Approximately 8×10^5 independent clones were obtained following transformation of ElectroMAX DH10B cells (Gibco BRL, Life Technologies). After amplification, plasmid DNA was purified using the Plasmid Maxi kit (Qiagen).

Cloning and yeast two-hybrid screening

The AccI-AflIII fragment encoding aa 11–172 of the normal hAR (20 Gln; 20Q) (Lubahn *et al.* 1988) and the corresponding region of the polyGln-expanded hAR were excised from pSVhAR.BHEXE-20 or -50 Gln (Kazemi-Esfarjani *et al.* 1995), blunt-ended and ligated into dephosphorylated SmaI-digested pAS2-1 (Clontech) to form pAS2-hAR(11–172)20Q and 50Q. Longer constructs (pAS-hAR(11–341)20Q and 50Q and hAR(38–502)20Q and 50Q) independently activated the yeast β-galactosidase (β-gal)

reporter gene and therefore proved unsuitable for yeast two-hybrid screening. CG-1945 yeast were cotransformed with the pACT2 NSC-34 cDNA library and pAS2-hAR(11–172)20Q or 50Q plasmids according to Matchmaker Two-Hybrid System 2 kit protocols (Clontech). Colonies that grew on selective synthetic dropout (SD) plates lacking leucine, tryptophan and histidine and containing 5 mM 3-aminotriazole (Sigma) (SD – Leu – Trp – His + 3AT) were subjected to colony-lift filter assays to confirm β -gal activity.

Single isolates of β -gal-positive colonies were grown overnight in complete synthetic medium lacking leucine (Bio 101, Inc., Vista, CA, USA). The pACT2 plasmids were isolated from the yeast according to protocol (Clontech) and used to transform bacteria. Only pACT2 plasmids containing an insert were obtained, demonstrating that the recommended cycloheximide selection was unnecessary for isolation of pure candidate clones. Restriction enzyme analysis and detergent-enhanced sequencing (Hutchison & Dahl 1995) were used to classify candidate clones. After complete or partial digestion of the candidate pACT2 clones with HindIII, the fragments encoding the GAL4-AD/candidate fusion proteins were ligated into dephosphorylated HindIII-digested pcDNA3 (Invitrogen). Plasmid Maxi kits (Qiagen) were used for all large-scale plasmid preparations.

Small-scale cotransformations of CG-1945 yeast were performed using 300 ng of each of the different pAS-1 and pACT2 vectors. Transformed yeast was plated on SD lacking leucine and tryptophan (SD – Leu – Trp) to confirm cotransformation or SD – Leu – Trp – His + 3AT plates to assess growth under selective conditions. Liquid β -gal assays were performed according to Clontech protocols, in duplicate, on two distinct yeast colonies picked from SD – Leu – Trp plates.

Protein labeling and glutathione S-transferase (GST) pulldown assays

The GAL4-AD/candidate fusion proteins cloned in pcDNA3 were labeled with [35 S]Met and the full-length mARNIP labeled with [35 S]Cys using the TNT Coupled Reticulocyte Lysate System in 25 μ l reactions (Promega). NcoI-SalI fragments from pAS2-hAR(11–172)20Q or 50Q and pAS2-hAR(11–341)20Q were ligated into NcoI/XhoI-digested pGEX-KG (Guan & Dixon 1991) for

expression of the GST-hAR(11–172)20 or 50Q and GST-hAR(11–341)20Q fusion proteins. Overnight bacterial cultures were diluted 10-fold, grown $\sim$ 2.5 h and GST or GST-hAR fusion protein expression induced with 10 mM isopropyl β -D-thiogalactoside for 1.5 h. Bacteria were resuspended in bead-binding buffer (BBB; 50 mM potassium phosphate pH 7.5, 150 mM KCl, 1 mM MgCl₂, 10 μ l each of AEBSF (4-(2-aminoethyl)-benzenesulfonyl 1-fluoride), aprotinin, leupeptin and pepstatin), sonicated twice for 4 s, and Triton-X100 added to 1% w/v. Fifty microliters of 50% glutathione-agarose (Sigma) were added to the cleared lysate and rocked for 30 min at 4 °C. After three washes with BBB, the beads were rocked with the [35 S]Met-labeled proteins (4 μ l each) or [35 S]Cys-labeled mARNIP (3 μ l) in 100 μ l total BBB + 1% Triton-X100 + 0.5% skim milk for 1 h at 4 °C. Following three washes, proteins were eluted from the washed beads with 30–40 μ l 10 mM glutathione, and electrophoresed on replicate SDS-PAGE gels, which were exposed to X-ray film or stained with Coomassie Blue.

RT-PCR of ARNIP cDNAs

Following BLAST searches of expressed sequence tag (EST) databases in GenBank with the original pACT2-ARNIP clone (666 bp, nucleotides – 21 to 645 in Fig. 2A), the following primers were designed: ARNIP-START (5'GGGAATTCGGA GATGGCGGCGACGGCGCGGGAAG; EcoRI site underlined); ARNIP-3' (5'CTGCCTTAGA CATGACTCGGTAC), ARNIP-STOP-MUS (5'CTCGGGCCCTCACTGCTGATCCACTGGCAC TC; ApaI site underlined), and ARNIP-STOP HUM (5'CTCGGGCCCTCATTGCTGATCCA GTGAAATTC; ApaI site underlined). Expand Reverse Transcriptase (Boehringer-Mannheim, Dorval, Quebec, Canada) reactions were performed as directed using 2 μ g total NSC-34 or PC-3 RNA. ARNIP-START and ARNIP-STOP HUM (50 pmol each) primers were used to PCR amplify hARNIP from the PC-3 RT mix (2.5 μ l) using VENT DNA polymerase (New England Biolabs, Beverly, MA, USA), 1 $\times$ Thermopol buffer, 2 mM MgSO₄, and 200 mM dNTPs for 30 cycles at 95 °C for 30 s; 62 °C for 30 s; and 75 °C for 1 min. Sufficient DNA for cloning was obtained by re-amplifying the fragment obtained using the same PCR conditions. The 3' end of mARNIP cDNA was

amplified from NSC-34 mRNA using the ARNIP-3' and ARNIP-STOP-MUS primers and the above conditions. One-tenth of the reaction (10 µl) was added to 1 µg cDNA3-GAL4AD/ARNIP and 50 pmol ARNIP-START primer and ten PCR cycles performed. Fifty picomoles of ARNIP-STOP-MUS and 25 pmol ARNIP-START primer were added and 20 further cycles performed. The resulting ARNIP cDNAs were extracted with phenol:CHCl₃ and precipitated with ethanol.

Cloning of ARNIP cDNAs

mARNIP and hARNIP cDNAs were digested with EcoRI and ApaI and ligated into similarly digested

pcDNA3 (Invitrogen) and pS65T-C1 (pGFP; Clontech). An EcoRI-PstI fragment was isolated from pcDNA3-mARNIP, blunt-ended and ligated into EcoRI-digested, blunt-ended, dephosphorylated pGEX-KG to form pGEX-mARNIP(1–86). pGEX-mARNIP(1–86) was digested with XhoI, blunt-ended and redigested with SacII (cutting within the mARNIP cDNA) in order to insert either the SacII-HinfI(blunt) or SacII-SmaI fragment from pGFP-mARNIP and construct pGEX-mARNIP(1–129) or pGEX-mARNIP(1–261) respectively. pGEX-mARNIP(1–129) was digested with SacI, blunt-ended, then cut with BamHI. The BamHI-SacI(blunt) fragment was ligated into BamHI/EcoRV-digested pcDNA3 to form pcDNA3-mARNIP(1–129). pcDNA3-mARNIP(131–261) was formed by ligation of three fragments: NdeI-BstUI and TaqI(blunt)-ApaI fragments from pcDNA3-mARNIP and an ApaI-NdeI fragment from the pcDNA3 vector backbone. This construct effectively deleted aa 6–130 of mARNIP,

Figure 1 GST pull-down assay demonstrates specific interaction between human androgen receptor (hAR) and AR N-terminal-interacting protein (ARNIP). (A) Glutathione-s-transferase (GST) or GST-hAR fusion proteins encoding aa 11–172 or 11–341 of the normal hAR (according to Lubahn *et al.* (1988)) were expressed in bacteria, purified using glutathione-agarose beads, and incubated with [³⁵S]Met-labeled GAL4-AD/candidate fusion proteins (A, D, E, J, and S) in BBB plus 0.5% skim milk powder for 1 h at 4 °C. After washing, GST, GST-hAR(11–172)20Q, GST-hAR(11–341)20Q, and associated proteins were eluted from the beads with 10 mM glutathione. The autoradiogram of the dried SDS-PAGE gel loaded with 20 µl of eluate is shown. Fusion proteins J (ARNIP) and E (COXVb) interacted specifically with GST-hAR fusion proteins. In the GST-hAR(11–172)20Q lane, the migration of the band corresponding to the GAL4-AD-J (GAL4-AD-ARNIP) protein is altered by the large amount of GST-hAR protein present (predicted sizes of GAL4-AD-ARNIP and GST-hAR(11–172)20Q are 46 and 46.8 kDa respectively). (B, C) GST pull-down assays were performed with GST, GST-hAR(11–172)20Q, GST-hAR(11–172)50Q or extracts from uninduced bacteria (Unind.) and [³⁵S]Cys-labeled mARNIP as in (A). Parallel SDS-PAGE gels were loaded with 20 µl (B) or 5 µl (C) of eluate and exposed to X-ray film (B) or stained with Coomassie Blue (C). The 30 kDa mARNIP protein interacts specifically with the normal or polyGln-expanded GST-hAR fusion proteins but not GST. The input lanes show the relative molecular masses of the ³⁵S-labeled proteins used in the pull-down assays.

allowing for in-frame expression of aa 131–261 of mARNIP that includes the RING-H2 finger motif.

Northern blot analysis

Total RNA was isolated from cell lines, or frozen tissue ground to powder, using the guanidinium isothiocyanate method (Trifiro *et al.* 1991). Ten micrograms of poly(A)⁺-enriched RNA (Oligotex column; Qiagen) or 15 µg total RNA were fractionated on a 1.1% agarose-formaldehyde gels in MOPS buffer, and run at 5 V/cm at room temp according to the GeneScreen Plus protocol (Dupont Canada Inc., Mississauga, Ontario, Canada). Gels were stained with ethidium bromide and photographed to verify that equivalent amounts of RNA were loaded in each lane. RNA was transferred using an alkaline (0.05 M NaOH) downward capillary protocol onto GeneScreen Plus nylon membrane. Fifty nanograms of a 400 bp probe prepared from the N-terminal end of mARNIP (EcoRI-HinfI fragment from pcDNA3-mARNIP; nucleotide 1–387 Fig. 2A) were labeled with [α -³²P]dCTP (specific activity 3000 Ci/mmol; New England Nuclear, Boston, MA, USA) using the T7 Quick-Prime Kit (Amersham Pharmacia Biotech) and had a specific activity of 4.8×10^8 c.p.m./µg after passage on G-25 Sephadex. Blots were prehybridized at 42 °C for a minimum of 3 h with herring sperm DNA, hybridized at 42 °C overnight, washed as described (Huynh & Pollak 1993) and subjected to autoradiography with intensifying screen at –70 °C.

Transfection and expression studies

COS-1 and CV-1 cells were maintained in OptiMEM (Gibco BRL) supplemented with 5% fetal bovine serum (OptiMEM+5% FBS). COS-1 cells were electroporated as described (Kazemi-Esfarjani *et al.* 1993), omitting PBS washing and performing all steps at room temperature, with 1 µg pBHEXE-hAR plus 1, 3 or 9 µg pcDNA3-mARNIP or pcDNA3 for a total of 10 µg DNA/ 1×10^7 cells. Total specific androgen binding ($B_{\max}$) and dissociation rate constants (k) of hAR-mibolerone (MB) and hAR-methyltrienolone (MT; R1881) complexes were determined as described (Kazemi-Esfarjani *et al.* 1993).

Mammalian two-hybrid assays

The pGAL4-AR-LBD (pM-LBD or pM-hARLBD), pVP16AD-hARTAD (pVP16-TAD) plasmids, and the (17 m)5-E1bTATA-Luc (pGAL4-Luc) reporter vectors were constructed as described (Ghadessy *et al.* 1999). One day prior to transfection, 1.8×10^6 CV-1 cells were plated in a T25 flask. For each transfection 1 µg pM-LBD, 1 µg pVP16-TAD, 4 µg pGAL4-Luc, 800 ng pCMV- β -gal and 3 µg pcDNA3 or the pcDNA3-ARNIP constructs were combined with 30 µl Lipofectamine in 400 µl serum-free OptiMEM (sfm). After 30 min, 0.66 ml sfm was added to the Lipofectamine mixture, then the mixture added to sfm-rinsed cells. After 5–6 h the medium was changed to OptiMEM+5% FBS. The following day the cells were trypsinized and replated in six-well plates with or without 3 nM MB. Forty-eight hours later, cell extracts were prepared using 200 µl of $1 \times$ Reporter Lysis buffer (Promega) per well. Luciferase activity was measured using the Luciferase Assay System (Promega) and β -gal activity was determined using the recommended $2 \times$ Assay Buffer. Relative luciferase activity (luciferase/ β -gal activity) was determined for duplicate samples in the absence and presence of MB.

Ubiquitination assays

The pGEX-mARNIP(131–261) vector containing the ARNIP RING-H2 domain was constructed by inserting a blunt-ended TaqI-ApaI fragment from pcDNA3-mARNIP into the EcoRI-digested, blunt-ended and dephosphorylated pGEX vector. GST and GST-ARNIP fusion proteins were purified from *E. coli* using standard techniques (Guan & Dixon 1991). Reagents for the ubiquitin assays (E1, the E2 Ubc4-1 and [¹²⁵I]-labeled ubiquitin) were prepared as previously described (Wing & Jain 1995). (Note that the Ubc4-1 isoform (GenBank accession number U13176) was previously referred to as the 2E isoform of E2_{17 kD}, but has been renamed Ubc4-1 to conform with more commonly used nomenclature. The Ubc8A isoform (GenBank accession number U56407) has been designated Ubc4-testis.) The reactions contained E1 (50 nM), E2 (250 nM; Ubc4-1 (Wing & Jain 1995), Ubc4-testis (Rajapurohitam *et al.* 1999) or Ubc2 (Wing *et al.* 1992), as indicated), GST fusion proteins (1 µM), [¹²⁵I]-labeled ubiquitin (5 µM),

and AMP-PNP (5'-adenylylimidodiphosphate lithium; 2 mM) in ubiquitin buffer (50 mM Tris–HCl (pH 7.5), 2.5 mM MgCl₂ and 0.5 mM dithiothreitol (DTT)). Samples were incubated at 37 °C for the length of time indicated and stopped by addition of 6 × SDS sample buffer containing 10.3% SDS and 0.6 M DTT. Reactions were electrophoresed on a 10% SDS-PAGE gel, and the dried gels subjected to autoradiography. The ubiquitination activity of GST-ARNIP(1–261) relative to that of GST-ARNIP(131–261) was quantitated using the Storm 860 Gel and Blot imaging system (Amersham Pharmacia Biotech).

PCR mutagenesis

The overlap extension method was used to alter two bases in the ARNIP cDNA resulting in substitution of an alanine for the first cysteine of ARNIP-RING finger (Cys145Ala) and introduction of a BsmI restriction site. Two overlapping fragments were amplified using primers; 422A (starting at nucleotide 422) (5'CCCGGCAGAAATgcTCCAA TATGCTTGG3') and ARNIP-STOP-MUS or ARNIP-START and 451B (starting at nucleotide 451) (5'CCTCCAAGCATATTGGAgcATTCTG CC3'). One microliter of each product was used for the second PCR: an extension step with no primers for five cycles followed by 25 cycles with ARNIP-START and ARNIP-STOP-MUS primers to produce the full-length mARNIP(Cys145Ala) cDNA. The purified PCR product was digested with EcoRI and ApaI and ligated to similarly digested myc-pcDNA3.1 vector. The pGEX-ARNIP(131–261 Cys145Ala) vector containing the mutated RING-H2 domain was constructed by inserting a blunt-ended TaqI-ApaI fragment from pcDNA3.1-mARNIP(Cys145 Ala) into the EcoRI-digested, blunt-ended and dephosphorylated pGEX vector.

Fluorescent protein-tagging studies

COS-1 cells were plated (2 × 10⁵ cells/well) in six-well plates containing sterilized glass cover slips and transfected the following day with 2 µg pGFP, pGFP-mARNIP or a 3:1 ratio of pGFP-mARNIP:pBFP-hAR20Q or 50Q (hAR labeled C-terminally with blue fluorescent protein (BFP) (Panet-Raymond *et al.* 2001)) using 10 µl Lipofectamine (Gibco BRL). After 72 h cells were

incubated in the presence or absence of 100 nM MB for 2 h where indicated, fixed in 4% paraformaldehyde according to protocol (Clontech), stained (where noted) with 0.1 mg/ml 4',6-diamidino-2-phenylindole (DAPI) (Sigma) in PBS for 2 min, mounted on microscope slides, and viewed at 400 × magnification using a Leitz Aristoplan fluorescence microscope equipped with A and I3 filter blocks to visualize BFP and green fluorescent proteins (GFP) respectively.

Results

Isolation of ARNIP, an AR N-terminal-interacting protein

To identify novel proteins interacting with the N-terminal domain of hAR, we initiated yeast two-hybrid screening of a mouse neuroblastoma/spinal cord hybrid cell (NSC-34, Cashman *et al.* (1992)) cDNA library in pACT2 using pAS2-hAR(11–172)20Q and pAS2-hAR(11–172)50Q constructs as bait. In the initial screens with pAS2-hAR(11–172)20Q (~3 × 10⁵ transformants), 12 colonies were picked, of which seven grew on triple dropout plates (SD – Leu – Trp – His + 3AT) and were positive for β-gal reporter gene activity upon filter lift assays. Two of the 16 colonies picked from the 4 × 10⁴ pAS2-hAR(11–172)50Q transformants grew on SD – Leu – Trp – His + 3AT plates and were β-gal-positive. Five distinct AR-interacting candidate proteins were identified following isolation of the pACT plasmids, restriction mapping and sequencing, including candidate clone J, which was isolated initially with both pAS2-hAR(11–172)20Q and 50Q as bait, and subsequently in a second screen with pAS2-hAR(11–172)20Q.

To confirm specific interaction between the GAL4 activation domain (GAL4-AD) fusion proteins encoded by pACT2-J and the GAL4 DBD (GAL4-DBD)-hAR fusion proteins encoded by the pAS2 plasmids, further yeast two-hybrid assays were performed (Table 1). Yeast transformed with pACT2-J and either of the normal or polyGln-expanded hAR baits grew vigorously on the selective (SD – Leu – Trp – His + 3AT) plates, as did those cotransformed with pVA3-1 and pTD-1, known positive controls for protein interaction. In liquid β-gal assays, pAS2-hAR(11–172)20Q+ pACT2-J and pAS2-hAR(11–172)50Q+ pACT2-J

Table 1 Results of yeast two-hybrid assays

Expression vectors	Growth on SD–Leu–Trp–His+3AT	Relative β-gal activity ^a
pAS2-1 ^b +pACT2	–	0.2, 0 (<1)
pAS2-1+pACT2-J(ARNIP)	+/-	4, 53 (28)
pAS2-hAR(11–172)20Q+pACT2-J(ARNIP)	++	65, 108 (86)
pAS2-hAR(11–172)50Q+pACT2-J(ARNIP)	++	37, 99 (68)
pLAM5'-1+pACT2-J(ARNIP)	–	nd, 9
pVA3-1+pACT2-J(ARNIP)	–	nd, 1
pVA3-1+pTD1-1	++	100

^aThe results of liquid β-gal assays from two separate experiments are shown, with the mean in brackets, relative to that of the pVA3-1+pTD1-1-positive control.

^bpAS2-1 encodes the GAL4-DNA-binding domain; pACT2, the GAL4-activation domain; pLAM5'-1, the human lamin C_(66–230) protein in pAS2-1; pVA3-1, the murine p53_(72–390) in pAS2-1; and pTD1-1, the SV40 large T-antigen_(84–708) in pACT2.

cotransformants exhibited β-gal activity at least twice that of the pAS2-1+pACT2-J cotransformants, while little or no activity was seen when pACT2-J was cotransformed with the pLAM5'-1 or pVA3-1 plasmids encoding human lamin C_(66–230) or murine p53_(72–390) GAL4-DBD fusion proteins respectively. Thus, the protein encoded by pACT2 clone J appeared to interact specifically with the hAR N-terminal domain in yeast two-hybrid assays.

Specific interaction between ARNIP and AR

Physical interaction between hAR and the candidate clones was further verified using a GST pulldown assay. GAL4-AD fusion proteins from clones A, D, E, J and S were subcloned from pACT2 into pcDNA3 and the proteins labeled with [³⁵S]Met using the TNT coupled *in vitro* transcription/translation system. The candidate proteins were incubated with purified GST, GST-hAR(11–172)20Q or GST-hAR(11–341)20Q proteins on glutathione-agarose beads, and the bead-bound GST proteins and associated proteins eluted with glutathione, run on a SDS-PAGE gel and exposed to X-ray film (Fig. 1A). Clone J, renamed ARNIP (AR N-terminal-interacting protein), and clone E (COXVb, cytochrome c oxidase subunit Vb (Beauchemin *et al.* 2001)) bound to GST-hAR(11–172)20Q or GST-hAR(11–341)20Q but not to GST alone; several other [³⁵S]Met-labeled candidate proteins in the same reaction (clones A, D and S) failed to bind GST-hAR fusion proteins. In addition, GST pulldown assays were

performed subsequent to cloning the complete mARNIP cDNA. As shown in the autoradiogram (Fig. 1B), mARNIP specifically interacted with the glutathione-agarose beads preincubated with the GST-hAR(11–172)20Q or 50Q fusion proteins, but not GST alone. Thus, of the candidate AR-interacting proteins we isolated through yeast two-hybrid screening, only two, ARNIP and COXVb (Beauchemin *et al.* 2001), interacted specifically with the normal or polyGln-expanded GST-hAR N-terminal (aa 11–172) fusion proteins in GST pulldown assays.

Cloning of mARNIP and hARNIP open reading frames (ORFs)

The original mouse pACT2-ARNIP (mARNIP) clone contained an ORF of 222 amino acids (nucleotides –21 to 645 in Fig. 2A). At that time, no sequences highly homologous to mARNIP were found in GenBank or protein databases except within the EST database (dbEST). Further analysis of the homologous ESTs led to a strategy for cloning the complete mARNIP and hARNIP ORFs. When human ESTs homologous to the 5' end of mARNIP (GenBank accession numbers AA424427, AA443416, and AA370904), but containing additional 5' sequences, were aligned with the mARNIP sequence, an in-frame translation stop codon was found six codons upstream of the ATG corresponding to the ATG at base +1 of the mARNIP cDNA. This ATG also matched the Kozak consensus sequence for an initiator methionine or start codon, defining the start of the

Figure 2 Nucleotide sequence and deduced amino acid sequence of the cloned murine ARNIP cDNA and comparison with AR-interacting proteins containing RING finger motifs. (A) The cDNA sequence of mARNIP (GenBank accession number AF071222), as determined from clones isolated using the yeast two-hybrid system and RT-PCR, is shown with the predicted amino acid sequence below. The nucleotide sequence is numbered on the right and the A of the ATG start codon is designated as +1. Cysteine residues are marked by circles and aa 145–186 constituting the RING-H2 finger motif are boxed. The cysteine and histidine residues predicted to coordinate zinc ions are shown within black circles and squares respectively. (B) The RING-H2 finger of ARNIP was compared pairwise to the RING finger regions of AR-interacting proteins ARA54 and SNURF. The consensus cysteine and histidine residues are shown within black bars. Two dots (:) and one dot (.) indicate identical and similar amino acids respectively, relative to ARNIP.

ARNIP ORF. When the mouse and human ESTs that extended 3' of the mARNIP sequence (GenBank accession numbers AA547213, W01424 and AA524525) were compared, the sequences diverged after a TGA stop codon, signaling the end of the ARNIP ORF. Primers incorporating the predicted start or stop codons and appropriate restrictions sites were designed and used to clone the 786 bp mARNIP and hARNIP ORFs by performing RT-PCR on mRNA from NSC-34 or PC-3 (human prostate adenocarcinoma) cells.

Sequence analysis of the mARNIP cDNA predicted a cysteine-rich (11% Cys) 261 amino acid protein with a molecular mass of 30 kDa (Fig. 2A). Comparison of the mARNIP protein to the Pfam

database showed the most notable feature was a RING finger domain (Pfam zf-C3HC4 family PF00097, zinc finger, C3HC4 (RING finger) (Bateman *et al.* 1999)). mARNIP contains a RING-H2 finger, a variant of a C3HC4 zinc finger, where a histidine replaces the fourth cysteine to give a C3H2C3 pattern of residues capable of coordinating zinc ions. No obvious nuclear localization signal, coiled-coil regions or LXXLL receptor-interaction motifs were present. Several predicted protein kinase C and casein kinase II phosphorylation sites were found; their biological significance is unknown. Other regions of mARNIP exhibited no obvious homology to proteins in existing databases. Alignment of ARNIP

with two other known AR-interacting proteins containing a RING finger domain, SNURF (Moilanen *et al.* 1998) and ARA54 (Kang *et al.* 1999) (Fig. 2B), showed that the three proteins shared little homology apart from the amino acids forming the RING finger consensus sequence.

Intron–exon organization and cross-species conservation

Human genomic DNA sequences homologous to hARNIP were identified by BLAST search of the high through-put genomic sequence (HTGS) database. The homologous genomic sequence (GenBank accession number AC022059) originated from human chromosome 4, confirming the chromosomal location of the hARNIP gene, previously mapped to a single locus at 4q21 by fluorescent *in situ* hybridization (FISH; data not shown). Southern blot analysis with different regions of mARNIP confirmed that mARNIP is a single copy gene (data not shown).

Alignment of the hARNIP cDNA and genomic sequences showed that the coding region of hARNIP is divided among nine exons (Fig. 3A and Table 2) spanning 32 kb. All exon–intron junctions conformed to the GT/AG rule. Interestingly, the RING-H2 domain was distributed among several exons in hARNIP (exons 6, 7 and parts of exons 5 and 8). Sequencing mARNIP-homologous BAC clones revealed that the exon–intron boundaries for all hARNIP and mARNIP exons were absolutely conserved, although the introns differed slightly in size (Table 2). Contrarily, analysis of *Drosophila* genomic sequence (GenBank accession number AE003613) showed that the RING-H2 domain of the *Drosophila* ARNIP homologue was encoded within a single exon (Fig. 3B).

The hARNIP and mARNIP cDNAs are 88% identical and the predicted amino acid sequences 90.4% identical. Searches of the dbEST identified ARNIP-homologous cDNA sequences from several other species. The deduced protein sequences from rat, pig, bovine, chicken and zebrafish ESTs and *Drosophila* genomic sequence (CG16947 gene product) were aligned with hARNIP and mARNIP proteins using the MultAlin program (Corpet 1988). A highly conserved region, corresponding to aa 99–193 of hARNIP, was found in the ARNIP homologues (Fig. 3B), bracketing the RING-H2 domain from aa 145 to 186. The hARNIP,

mARNIP and rat ARNIP RING-H2 domains are 100% identical. The porcine, bovine, chicken and *Drosophila* RING-H2 domains are 97, 88, 93 and 57% identical to the hARNIP RING-H2 domain respectively. In particular, all the cysteine and histidine residues in the C3H2C3 RING motif are conserved. Pairwise comparison using the BLASTP 2·2·1 parameters in the BLAST 2 SEQUENCE program showed that, overall, hARNIP and the predicted rat, porcine, bovine, chicken, zebrafish and *Drosophila* ARNIP protein sequences were 93, 95, 85, 87, 71 and 50% identical respectively. Thus, the amino acid sequence of ARNIP homologues from distant species is highly conserved.

Differential expression of ARNIP mRNA

Northern blot analysis showed that ARNIP is differentially expressed in various tissues and cell lines. A predominant ~1.7 kb mRNA was found in NSC-34 and PC-12 (rat adrenal pheochromocytoma) cells, mouse testis, TM-4 (mouse Sertoli cells), MCF-7 (human breast adenocarcinoma), and AR24 (motoneuron-like cells stably expressing hAR) cells (Brooks *et al.* 1997) (Fig. 4A). In rat PC-12 and mouse TM-4 cells an additional smaller (~1.3 kb) mRNA species was seen. These mRNA species were not detected in PC-3 (human prostate adenocarcinoma), A2780 (human ovarian carcinoma) or GSF (genital skin fibroblast) cells. Additional Northern blot analysis (Fig. 4B) using total RNA from a range of mouse tissues showed mARNIP expression was highest in testis, slightly lower in kidney and NSC-34 cells, and lowest in brain, heart, lung, liver, ovary and uterus. Little or no ARNIP mRNA was expressed by rat spinal cord, CHO (Chinese hamster ovary), and LNCaP (human lymph node metastatic prostate adenocarcinoma) cells (Fig. 4B); COS-1 cells also lacked ARNIP expression (data not shown). Equivalent amounts of mRNA or total RNA were loaded in each lane in Fig. 4A and B as verified by ethidium bromide staining of the gels before transfer. The smaller ARNIP mRNA species observed could be the result of alternative splicing or use of tissue-specific promoters or polyadenylation signals.

Periodic searches of the EST databases provided additional information on ARNIP expression. ESTs that were 94–100% homologous to mARNIP and hARNIP were identified from a variety of

Figure 3 Intron/exon structure of the hARNIP gene and homology to predicted ARNIP proteins from other species. (A) The structure of the hARNIP gene, as predicted by comparison of the hARNIP cDNA with human chromosome 4 nucleotide sequence (GenBank accession number AC022059), is shown. Coding exons are represented by black boxes and the UTRs and introns, by a line. The sizes of the introns are indicated; the larger ones (>4 kb) are not to scale. The protein-coding regions are shown adjoined in the ARNIP protein and the RING-H2 motif is hatched. The RING-H2 finger is encoded by multiple exons (exons 6, 7 and parts of 5 and 8). (B) The amino acid sequences of the cloned human (*Homo sapiens*, HUM), mouse (*Mus musculus*, MUS) ARNIP proteins, and those predicted from EST or genomic sequences for rat (*Rattus norvegicus*, RAT; AW918714), pig (*Sus scrofa*, SUS; B119086 and B1400143), bovine (*Bos taurus*, BOS; AW464045 and AV610812), chicken (*Gallus gallus*, GAL; A1979768 and B1065536), zebrafish (*Danio rerio*, DAN; A1437075) and *Drosophila* (*Drosophila melanogaster*, DRO; AAF52385, aa 228–496) ARNIP homologues were aligned using the MultAlin Program and BLOSUM62 scoring matrix (Corpet 1988). Amino acids that differ from those in hARNIP are shown for each species; identical amino acids are indicated by a dash and deletions by a dot. The exon boundaries are indicated for hARNIP and its *Drosophila* homologue by filled and empty triangles respectively. The RING-H2 finger motif is boxed and the absolutely conserved cysteine and histidine residues are outlined by black bars.

Table 2 Exon–intron organization of the hARNIP and mARNIP genes

		Exon–intron junction			
	Size (bp)	5' splice sequence		Intron size (bp)	3' splice sequence
Exon					
1	90 ^a	TC	CTA AAG gtgacgcc	4900 (h) ^b	ggtgttag GCA CCT TG
		TC	CTC AAG gtgacgac	>4611 (m)	ggcgtcag GCA CCT TG
2	120	TT	CAA CAT gtaagatt	241 (h)	tttttttag GCC CAA CA
		TC	CAA CAT gtaaggcc	227 (m)	cttttcag GCC CAG CA
3	116	ATT	TGT AG gtactgta	14640 (h)	tttgctag G ATT GGT C
		ATT	TGT AG gtactgta	1921 (m)	tttggttag G ATT GGT C
4	79	GA	CAC AAG gtatataa	2167 (h)	ttaaacag TGT ATT GA
		AA	CAC AAG gtacatta	2276 (m)	cttttcag TGT ATT GA
5	45	GT	TTG GAG gtaggctt	106 (h)	atttacag GAC ATT CA
		GC	TTG GAG gtgagtg	102 (m)	cctcgtag GAC ATT CA
6	59	TTA	CAT AG gtaaagta	86 (h)	tctttcag A ACG TGT T
		CTA	CAT AG gtaagcag	85 (m)	tctttcag A ACG TGT T
7	27	TTG	AAA GA gtgagtg	909 (h)	tctttcag A GGC TAC A
		TTG	AAA GA gtgagttt	999 (m)	tcttgcag A GGT TAC A
8	121	CT	GTG GAT gtgagtag	7914 (h)	cttttcag ATT CTC TG
		CT	GTT GAC gtaagtag	1956 (m)	ttttcag ATT CTC TG
9	126 ^a	AG	CAA TGA cgagc . . .	–	–
		AG	CAG TGA gaagc . . .	–	–

^aNot including 5' or 3' UTRs or stop codon.^bLocation and sizes of introns were determined by comparison of the hARNIP cDNA to *Homo sapiens* chromosome 4 clone RP11-567N4 in the HTGS database (GenBank accession number AC022059) and alignment of the mARNIP cDNA with sequences from a mouse BAC clone (mBAC 531 O21) and the Arachne_Nov30 database available through the National Center for Biotechnology Information site for BLAST analysis of the mouse genome.

tissues including mouse embryo, kidney, liver, and mammary gland, human fetus, infant and adult brain and lung, colon, kidney, hNT neurons (Stratagene, La Jolla, CA, USA) and prostate. As well, an uncharacterized sequence from a human uterus cDNA library (GenBank accession number AL050144) was found to contain an hARNIP-homologous sequence. The reported sequence of this clone starts at nucleotide 98 of hARNIP sequence, and is identical to the hARNIP nucleotide sequence, with exception of a 2 bp deletion in a CACA repeat (nucleotides 402–403 in hARNIP) that results in a frameshift. The predicted 12.2 kDa protein is truncated before the RING-H2 finger motif; this may represent a cloning or sequencing artifact. However, this cDNA clone extends 732 bp 3' of the hARNIP cDNA clone, has a sequence identical to the human chromosome 4 genomic DNA downstream of hARNIP exon 9, and contains a poly(A)⁺ addition site and an A-rich tail. This human uterus cDNA clone therefore defines the complete 3' untranslated region (UTR) of at least one species of hARNIP mRNA. Similarly, a recently reported 1729 bp colon cDNA

clone from the RIKEN full-length enriched mouse cDNA library (GenBank accession number AK018488) may represent a full-length mARNIP cDNA as its length is in agreement with our estimates from Northern blot analyses.

Coexpression of ARNIP does not affect hAR–androgen binding

In order to assess the effect of ARNIP coexpression on hAR function, the ability of ARNIP to influence hAR ligand-binding kinetics was investigated. When coexpressed with hAR in COS-1 cells, mARNIP did not overtly affect the amount of androgen bound ($B_{\max}$) or the rate of dissociation of androgen from AR. For instance, COS-1 cells cotransfected with hAR (1 µg) and increasing amounts of mARNIP (0, 1, 3 or 9 µg) expression vectors bound comparable amounts of the synthetic androgens MB (848, 1034, 1059 and 977 fmol/mg protein respectively) and MT (291, 327, 349, 297 fmol/mg protein respectively). The MB or MT dissociation rate constants (k) were comparable regardless of the presence of ARNIP. COS-1 cells

Figure 4 Northern blot analysis of ARNIP expression. (A) A Northern blot containing 10 µg poly(A)⁺ mRNA per lane from the sources indicated was probed with ³²P-labeled mARNIP cDNA encoding aa 1–129 (nucleotides 1 to 387, Fig. 2A). The predominant ~1.7 kb mRNA is indicated; a smaller (~1.3 kb) mRNA isoform was expressed in certain cells (marked by arrows). (B) A Northern blot containing 15 µg total RNA from the tissues and cell lines indicated was probed as in (A). mARNIP was highly expressed in mouse testis and kidney, and significant expression was found in several neuronal cell lines, including NSC-34 and PC-12 cells.

transfected with pBHEXE-hAR alone or a 1:1, 1:3 or 1:9 ratio of pBEXE-hAR:pcDNA3-mARNIP had *k* values of 3.6, 3.6, 3.0 and 3.7×10^{-3} /min with MB, and 14.1, 15.2, 15.8 and 14.5×10^{-3} /min with MT respectively.

hAR N-C terminal interaction is inhibited by ARNIP

As coexpression of ARNIP and hAR did not appear to affect the hAR LBD, and direct hAR–ARNIP interactions were not measurable using our mammalian two-hybrid system (Beauchemin *et al.* 2001), further AR functional assays were performed. hAR N-C terminal interaction in the presence of the mARNIP or hARNIP, or selected subregions of mARNIP, was assayed using the mammalian two-hybrid system (Ghadessy *et al.* 1999). Vectors expressing the hAR C-terminal LBD (pM-hARLBD) and the N-terminal transactivation domain (pVP16-hARTAD) were cotransfected with ARNIP expres-

Figure 5 hAR N-C terminal interaction is decreased in the presence of ARNIP or its RING-H2 finger domain. (A) The ARNIP fragments shown were cloned into the pcDNA expression vector and hAR N-C terminal interaction was assayed using the mammalian two-hybrid system. Cells were transfected with pVP16-hARTAD (hAR N-terminal or transactivation domain), pM-hARLBD (hAR C-terminal domain or LBD), the pGAL4-Luc reporter construct, the pCMV-β-gal control plasmid and the pcDNA3 constructs shown. (B) Interaction of hARTAD and hARLBD results in ligand-dependent activation of the luciferase (Luc) reporter gene. Luciferase activities were corrected for transfection efficiency and the relative luciferase activity upon incubation with 3 nM mibolerone (MB) in the presence of the pcDNA3 was normalized to 100%. Expression of hARNIP or mARNIP, but not mARNIP (1–129), decreased relative luciferase activity by at least 30%. Expression of the RING-H2 finger portion of mARNIP, ARNIP(131–261), decreased relative luciferase activity by at least 50% suggesting that ARNIP, and in particular its RING-H2 domain, interferes with hAR N-C terminal interaction. Data shown are the mean of three separate experiments performed in duplicate ± s.e.

sion plasmids (Fig. 5A) into CV-1 cells along with luciferase reporter gene and β-gal control plasmids. Expression of hARNIP and mARNIP decreased

relative luciferase activity by at least 30% (Fig. 5B). Coexpression of the N-terminal half of mARNIP(1–129) did not significantly influence hAR N-C terminal interaction. Expression of the mARNIP RING finger domain, however, decreased luciferase activity in this assay by at least 50% (Fig. 5B). Similar results were noted when the data were analyzed in terms of the fold-induction of luciferase activity. The RING-H2 region of mARNIP significantly reduced the maximum induction in the presence of hormone from 28.0 ± 2.3 - to 13.1 ± 2.5 -fold ($P < 0.01$). These data show that the presence of ARNIP, and in particular the RING-H2 finger domain, can decrease the magnitude of hAR N-C terminal interaction. These results were not due to ARNIP-mediated interference with GAL4-DBD-binding activity, as coexpression of ARNIP, or fragments thereof, did not significantly decrease the transcriptional activity of GAL4 DBD-hAR(38–502)20Q or 50Q fusion proteins on the pGAL4-Luc reporter in mammalian one-hybrid assays (results not shown).

In order to determine if ARNIP could function as an AR coregulator, AR transactivation assays were performed in several different cell lines (COS-1, CV-1, NSC-34) using various androgen responsive reporter constructs (MMTV LTR, prostate-specific antigen or probasin promoters controlling expression of a growth hormone reporter gene). Although in certain experiments the coexpression of mARNIP, hARNIP or the RING-H2 finger domain appeared to reduce AR-mediated transactivation to a limited degree, overall, ARNIP did not significantly up- or downregulate AR transcriptional activation (results not shown). From these results we conclude that, under our experimental conditions, ARNIP does not act as a typical AR coactivator or corepressor.

ARNIP is a ubiquitin–protein ligase

Given that a number of RING and RING-H2 domain-containing proteins have ubiquitin–protein ligase (E3) activity (Freemont 2000), we investigated whether the RING-H2 domain of ARNIP could function in a similar fashion. The ubiquitin–protein ligase activity of the ARNIP RING-H2 domain was confirmed using an *in vitro* ubiquitination assay (Fig. 6A). [125 I]-ubiquitin–protein conjugates, appearing as high molecular weight (MW) radioactive

material in the well and lower MW [125 I]-ubiquitin-labeled proteins, increased with time in reactions containing GST-ARNIP(131–261), but not in those containing GST, even after extended incubation (Fig. 6A). Subsequently, we assessed ubiquitin ligase activity of several bacterially expressed GST-ARNIP fusion proteins including the full-length GST-ARNIP(1–261), the N-terminal region (GST-ARNIP(1–129)), the C-terminal RING-H2 domain (GST-ARNIP(131–261)), or a mutated RING domain (GST-ARNIP(130–261 Cys145Ala)). Ubiquitin–protein ligation reactions (Wing & Jain 1995) were performed in the presence or absence of the ubiquitin-conjugating enzyme Ubc4-1 (Rajapurohitam *et al.* 1999); rabbit ubiquitin-activating enzyme (E1), AMP-PNP, and [125 I]-ubiquitin were included in all reactions. (See note in Materials and methods/Ubiquitination assays about naming of Ubc4-1.) Following a 90 min incubation at 37 °C, only the GST-ARNIP RING fusion protein, and to a much lesser extent, the full-length GST-ARNIP fusion protein, were capable of promoting ubiquitin conjugation as determined by the appearance of high MW [125 I]-ubiquitin–protein conjugates (Fig. 6B, E2+ lanes). No ubiquitinated products were observed in the presence of GST, the N-terminal portion of ARNIP lacking the RING domain or the RING domain in which the first cysteine of the RING domain had been mutated to alanine. These results confirm previous reports that mutation of a cation-coordinating cysteine within the RING domain abolishes ubiquitin ligase activity (Lorick *et al.* 1999). Upon PhosphorImager analysis the E3 ligase activity of GST-ARNIP(1–261) was found to be 1–3% that of GST-ARNIP(131–261). It is possible that the full-length ARNIP GST-fusion protein may lack the conformation or modifications needed for full E3 activity. Alternatively, the N-terminal region of ARNIP may act as an inhibitory domain, or more likely, impose substrate specificity on the reaction. The ubiquitin–protein ligase activity of ARNIP was absolutely dependent on the presence of E2; in the absence of Ubc4-1, no ligation of [125 I]-ubiquitin to proteins in the reaction was observed (Fig. 6B, E2– lanes). Further experiments demonstrated that the E3 ligase activity of ARNIP was E2-specific. Ubc4-testis (Rajapurohitam *et al.* 1999) or Ubc2 (Wing *et al.* 1992) could not substitute for Ubc4-1 in the ubiquitination reaction (Fig. 6C). Significantly, these

experiments demonstrate that ARNIP functions as an E2-dependent ubiquitin–protein ligase and that the first cysteine of the RING-H2 domain is essential for this activity.

Colocalization of fluorescent protein-tagged AR and ARNIP

In order to determine if ARNIP and AR could interact within a cellular environment, COS-1 cells were transfected with GFP, GFP-tagged mARNIP,

BFP or BFP-tagged normal hAR alone or in various combinations and photographed using a fluorescent microscope. COS-1 cells transfected with GFP alone displayed a diffuse green fluorescence (Fig. 7A), while cells expressing GFP-mARNIP exhibited a punctate pattern of fluorescence (Fig. 7B). Counterstaining with DAPI, a blue fluorescent nuclear stain, showed that GFP-mARNIP localized to discrete cytoplasmic or perinuclear regions (Fig. 7B, merged). COS-1 cells transfected with both GFP-ARNIP and BFP exhibited the distinctive green punctate fluorescence due to GFP-ARNIP expression in a background of diffuse blue fluorescence (Fig. 7C). Furthermore, androgen treatment of GFP-mARNIP transfected cells did not change the pattern of discrete fluorescent foci (results not shown).

Cells were then cotransfected with both GFP-mARNIP and BFP-hAR (Fig. 7D and E) and photographed with appropriate filters to record green or blue fluorescence. Cells shown in Fig. 7D were not treated with hormone while cells in Fig. 7E were exposed to 100 nM MB (a non-metabolizable androgen) prior to fixation.

Figure 6 The RING-H2 domain of ARNIP functions as an E2-dependent ubiquitin–protein ligase. (A) Ubiquitination reactions were performed with GST or the RING finger domain of ARNIP (GST-ARNIP(131–261)) in the presence of E1, E2 (mammalian Ubc4-1), AMP-PNP, and [¹²⁵I]-ubiquitin (¹²⁵I-Ub). Samples were removed at the times indicated, separated on a 10% SDS-PAGE gel under reducing conditions and ¹²⁵I-Ub–protein conjugates (indicated by a bracket) visualized by autoradiography. GST-ARNIP, but not GST, catalyzed the increased formation of high MW ¹²⁵I-Ub–protein conjugates with time. The positions of the molecular mass markers are indicated. (B) Ubiquitination reactions with GST, full-length GST-ARNIP(1–261), GST-ARNIP(131–261), or the mutated RING domain (GST-ARNIP(131–261 Cys145 Ala); two distinct clones designated as (C145A-1) and (C145A-2)) fusion proteins indicated were carried out as in (A) in the absence (–) or presence (+) of Ubc4-1 and terminated after 90 min at 37 °C. Mutation of the first cysteine in the ARNIP RING finger abolished the UBC4-1-dependent ubiquitin ligase activity. (C) One of three different E2s (Ubc2, Ubc4-testis (Ubc4-T) or Ubc4-1) was included in reactions containing the GST or GST-ARNIP fusion proteins indicated. The E3 ubiquitin ligase activity of GST-ARNIP and GST-ARNIP(131–261) are absolutely dependent on the presence of a specific E2, the ubiquitin-conjugating enzyme Ubc4-1.

Figure 7 GFP-ARNIP relocates upon coexpression of BFP-hAR. COS-1 cells were transfected with GFP, BFP, GFP-ARNIP or BFP-hAR expression vectors as indicated. Photographs were taken at 400× magnification using a Leitz Aristoplan fluorescent microscope equipped to visualize green (GFP or GFP-ARNIP) or blue fluorescence (BFP, BFP-hAR or DAPI). In GFP-transfected cells (A) diffuse green fluorescence is seen. In contrast, GFP-ARNIP transfected cells (B) exhibit punctate green fluorescence located in cytoplasmic or perinuclear regions, as shown by DAPI-counterstaining to visualize the nuclei. In cells cotransfected with GFP-ARNIP and BFP (C), GFP-ARNIP remained in a punctate pattern. Cotransfection of BFP-hAR with GFP-ARNIP (D, E), however, resulted in redistribution of GFP-ARNIP; the diffuse green fluorescence of GFP-ARNIP matched the blue fluorescence of BFP-ARNIP in both the absence (D) and presence (E) of MB, a synthetic non-metabolizable androgen. Coexpression of BFP-hAR thus changes the distribution of GFP-ARNIP fluorescence from a punctate cytoplasmic/perinuclear pattern to a diffuse pattern corresponding to that of BFP-hAR, strongly suggesting that hAR and mARNIP interact *in vivo*.

Strikingly, the intracellular distribution of GFP-mARNIP changed in the presence of BFP-hAR; GFP-mARNIP fluorescence became diffuse and appeared identical in pattern to the blue fluorescence due to BFP-hAR in both hormone-free and androgen-treated cells (Figs 7D and E). Redistribution of GFP-mARNIP was also observed upon coexpression of BFP-tagged polyGln-expanded hAR (results not shown). Fluorescent protein-tagged hAR is known to be distributed throughout cells in the absence of androgen and to relocalize to the nucleus in the presence of androgen (Georget *et al.* 1997, Tyagi *et al.* 2000). Thus, our results strongly suggest that interaction of ARNIP and hAR occurs in a hormone-independent fashion *in vivo*, but as a result of the hormone-dependent nuclear localization of AR, ARNIP can be relocalized to the nucleus where it could potentially influence hAR-mediated events.

mARNIP and hARNIP GenBank accession numbers

mARNIP and hARNIP sequences have been submitted to the GenBank database under accession numbers AF071222 (mARNIP) and AF247041 (hARNIP).

Discussion

Numerous proteins interacting with the LBD and DBD of nuclear receptors have been identified (Edwards 1999, McKenna *et al.* 1999a, Robyr *et al.* 2000); fewer N-terminal domain-interacting factors are known. We used a subregion of the hAR N-terminal domain (aa 11–172) as bait in a yeast two-hybrid screen to isolate a novel hAR-interacting protein, designated ARNIP. When initially cloned, ARNIP was not represented in protein databases; its nucleotide sequence was, however, homologous to entries in EST databases, uncharacterized GenBank clones and to human and *Drosophila* genomic sequences. In addition, ARNIP is distinct from other known AR N-terminal-interacting proteins: AES (amino-terminal enhancer of split; Yu *et al.* (2001)); ARA24 (Ran, a nuclear Ras-related G-protein; Hsiao *et al.* (1999)); ARA160 (HIV-1 TATA element modulatory factor, TMF; Hsiao & Chang (1999)); caveolin-1

(Lu *et al.* 2001); CAK (cdk activating kinase; Lee *et al.* (2000)); FHL2 (four and half LIM domain protein 2; Müller *et al.* (2000)); glyceraldehyde-3-phosphate dehydrogenase (Koshy *et al.* 1996); PITALRE (a kinase subunit of positive elongation factor b, P-TEF-b; Lee *et al.* (2001)); PQBP-1 (polyGln-protein-1; Iwamoto *et al.* (2000)); RB (retinoblastoma; Yeh *et al.* (1998)); SHP (short heterodimer partner; Gobinet *et al.* (2001)) and TR4 (testicular orphan receptor-4; Lee *et al.* (1999)).

Cloning of hARNIP by RT-PCR revealed that the mARNIP and hARNIP proteins are highly homologous. Rat, pig, cow, chicken, zebrafish or *Drosophila* proteins predicted from ESTs or genomic sequences were also found to be homologous to hARNIP and were noted to contain a highly conserved RING-H2 domain. The AR, and in particular, regions of the AR N-terminal, have also been conserved during evolution (Thornton & Kelley 1998). Indeed, Borden (2000) proposes that a proline-rich (P-X-L/V-X-P-A/S-X-P) sequence mediates interaction with RING proteins. The hAR and mouse and rat ARs contain a P-X-Q-X-P-A-X-P sequence that matches this consensus at five out of six positions. Interestingly, this motif is within the hAR region used to isolate ARNIP and thus its role in AR–ARNIP interaction deserves further investigation.

Northern blot analysis demonstrated that ARNIP is differentially expressed. The expression pattern of ARNIP is distinct from, but overlaps with, several AR coactivators: ARIP3 is expressed predominantly in testis (Moilanen *et al.* 1999), ARA54 in testis, PC-3 and LNCaP cells (Kang *et al.* 1999) and ARA55 in HeLa and PC-3 cells, but not MCF, CHO or PC-12 cells (Fujimoto *et al.* 1999). ARNIP expression, unlike ARA54, was not detected in LNCaP cells by Northern blot analysis. Provocatively, hARNIP was cloned by RT-PCR from PC-3 total mRNA, suggesting that ARNIP may be actually expressed at low levels in prostate tissue. ARNIP expression was highest in testis, a testis-derived cell line (TM-4), kidney, and several neuronal cell lines (NSC-34, PC-12, AR24). ARNIP may thus play a role in AR-mediated processes in these tissues.

Nuclear receptor function requires interaction between different domains (Ikonen *et al.* 1997, Gast *et al.* 1998, McKenna *et al.* 1999b). Two regions of the AR N-terminal were shown to interact with the

LBD, aa 14–36 and aa 370–494 (Berrevoets *et al.* 1998), later refined to FXXLF (aa 23–27) and WXXLF (aa 433–437) motifs by He *et al.* (2000). ARNIP interacts with aa 11–172 of hAR, which includes the FXXLF motif. AR N-terminal–LBD interaction is required for association of p160 coactivators with the AR N-terminal domain (Alen *et al.* 1999b, Ma *et al.* 1999). In a mammalian two-hybrid assay, AR N-terminal–LBD interaction decreased in the presence of ARNIP, and in particular, its RING-H2 domain. ARNIP may thus bind to, and compete for, the site(s) necessary for interaction of the AR N-terminal with the C-terminal LBD. Alternatively, ARNIP could have an indirect effect by blocking the activity of a protein needed for hAR N–C terminal interaction. Reduced AR N–C terminal interactions in the presence of ARNIP could potentially influence AR–coactivator interaction and modulate transactivation. Interestingly, however, AR coregulators which can inhibit AR N–C terminal interactions can either repress AR-mediated transactivation, as in the case of p53 (Shenk *et al.* 2001) and SRC-1 (Ikonen *et al.* 1997) with the hAR and rat AR respectively, or potentiate rat AR transactivation as does RIP140 (Ikonen *et al.* 1997). ARNIP itself, however, did not act as an AR coactivator or corepressor under our experimental conditions.

The outstanding feature of the ARNIP protein is the RING-H2 finger domain. The RING finger, a zinc-binding motif defined by a conserved pattern of cysteine and histidine residues, is found in a variety of proteins (>700 members in the Version 6.6 zf-C3HC4 family, Pfam accession number PF00097 (Bateman *et al.* 1999)). The canonical RING motif comprises seven cysteine residues and one histidine residue (C3HC4). The variant C3H2C3 pattern, or RING-H2 finger (Saurin *et al.* 1996), is present in ARNIP. Two other RING finger AR-interacting proteins, SNURF (Moilanen *et al.* 1998) and ARA54 (Kang *et al.* 1999), were not homologous to ARNIP, except for specific residues in the RING finger motif.

RING and RING-H2 domains have been shown to mediate protein–protein interaction (Saurin *et al.* 1996, Meza *et al.* 1999, Borden 2000) and, more recently, to play a role in ubiquitination (Lorick *et al.* 1999, Freemont 2000). Ubiquitin-mediated degradation of regulatory proteins is critical to cellular processes such as cell-cycle progression, signal transduction, transcriptional regulation,

receptor downregulation and endocytosis (Hershko & Ciechanover 1998). Moreover, ubiquitination has been shown to act as a signal for protein trafficking, activation of transcription factors and kinases and other non-proteolytic processes (Hicke 2001, Pickart 2001, Weissman 2001). An enzymatic cascade involving a ubiquitin-activating enzyme (Uba or E1), a ubiquitin-conjugating enzyme (Ubc or E2) and, in most cases, a ubiquitin–protein ligase (E3), links ubiquitin to the substrate protein, determining the level or activity of that particular protein (Hershko & Ciechanover 1998, Joazeiro 2000, Hicke 2001, Pickart 2001, Weissman 2001).

A number of otherwise unrelated RING finger proteins are able to facilitate E2-dependent ubiquitination (Freemont 2000). The bacterially expressed RING finger domain of ARNIP did indeed exhibit high E3 ubiquitin ligase activity, and mutation of a single critical cysteine eliminated the ability of the ARNIP RING domain to promote polyubiquitination *in vitro*. RING-mediated ubiquitination may modulate the level of RING-containing proteins, or their associated proteins, through regulated proteasomal degradation (Lorick *et al.* 1999). Recent studies suggest that the ubiquitin–proteasome pathway plays a role in nuclear receptor-mediated gene activation. The ligand-mediated downregulation of estrogen receptor- α is dependent on E1 and E2 enzymes as well as the 26S proteasome (Nawaz *et al.* 1999a), while proteasome inhibitors increase AR levels in LNCaP and HepG2 cells (Sheflin *et al.* 2000). Nuclear receptor coactivators such as SRC-1A, TIF2, GRIP1 and CBP are also targets of the ubiquitin–proteasome system (Lonard 2000, Baumann *et al.* 2001). Furthermore, components of ubiquitin or ubiquitin-like systems have been identified as nuclear receptor-interacting proteins. For example, SUG1/TRIP1, an ATPase subunit of the 26S proteasome complex, associates with and modulates nuclear receptor function (Lee *et al.* 1995, vom Baur *et al.* 1996, Masuyama & MacDonald 1998), the E2-like Ubc9 interacts with the glucocorticoid receptor (Gottlicher *et al.* 1996) and androgen receptors (Poukka *et al.* 1999) and enhances AR-dependent transactivation, while E6-AP (Nawaz *et al.* 1999) and RSP5/RPF1 (Imhof & McDonnell 1996) act both as coactivators for the nuclear hormone receptor superfamily and as E3s. The identification of ARNIP, as both an AR-interacting protein and a ubiquitin–protein ligase,

serves as an additional example of the link between nuclear receptors and the ubiquitination system.

The interaction of ARNIP and hAR (aa 11–172) was initially detected using the yeast two-hybrid system, and subsequently confirmed by GST pulldown assays *in vitro*. In addition, fluorescent protein tagging studies strongly suggest the ARNIP–hAR interaction occurs *in vivo*, as has been demonstrated for other nuclear receptors and their associated proteins (Roderick *et al.* 1997, Kumar *et al.* 1999). Strikingly, in GFP-ARNIP/BFP-hAR cotransfection studies in COS-1 cells, three populations of cells were noted: (i) cells with only punctate green fluorescence due to GFP-ARNIP expression, which never exhibited blue fluorescence; (ii) cells with the typical blue fluorescence seen upon BFP-hAR expression (Panet-Raymond *et al.* 2001); and (iii) cells with a diffuse green fluorescence pattern that corresponded to the blue fluorescence of BFP-hAR. These results indicate that hAR expression caused redistribution of ARNIP within the doubly transfected cells and strongly suggest that hAR and ARNIP interact, either directly or indirectly, *in vivo*. Androgen addition is known to promote the nuclear translocation of AR (Georget *et al.* 1997, Tyagi *et al.* 2000). The localization of ARNIP to the nucleus, as part of an AR–androgen complex, would permit ARNIP to participate in the formation of coregulatory complexes and thus interact with, and possibly ubiquitinate, the proteins therein.

The substrate specificity of a ubiquitination reaction is largely determined by the E3 (either a single protein or a multimeric E3 complex) which, in conjunction with specific E2s, recognizes specific protein substrates or a subset of substrates containing similar but not identical structural motifs (Joazeiro 2000, Weissman 2001). The ubiquitination reaction catalyzed by ARNIP *in vitro* demonstrates exquisite E2 specificity, as two other E2s (Ubc2 and a spermatid-specific E2, Ubc4-testis) could not substitute for Ubc4-1. The basis of this specificity deserves investigation as Ubc4-1 and Ubc4-testis are 93% identical, differing in only 11 of 147 amino acids (Oughtred *et al.* 1998). At this point, however, the specific ubiquitination target(s) of ARNIP or ARNIP-containing complexes in a cellular environment remain to be identified.

Ubiquitination of nuclear receptors and their coregulators can determine the fate and function of these proteins. However, the molecular

mechanisms and factors that perform these modifications are not well characterized. We have isolated a novel AR N-terminal interacting protein, ARNIP, which contains a conserved RING-H2 domain that functions as a specific E2-recruitment module and can mediate ubiquitination. Future investigations into ARNIP are likely to contribute to understanding of the ubiquitination system in general, and those pathways involving nuclear receptors in particular. The N-terminal region of hAR used to isolate ARNIP contains both the polyGln repeat and LBD-interacting sequences, and is thus critical to AR function. Shortening of the AR N-terminal polyGln tract is associated with increased risk of prostate cancer (Irvine *et al.* 2000) and lengthening with male infertility (Tut *et al.* 1997); pathological expansion of the polyGln tract causes SBMA (Pinsky *et al.* 2001). It will be interesting to determine if ARNIP, through its interaction with the N-terminal domain of the normal or polyGln-expanded AR, and its ubiquitin–protein ligase activity, plays a significant role in the cellular processes that contribute to initiation or progression of these AR-dependent conditions.

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