An Androgen Receptor NH$_2$-terminal Conserved Motif Interacts with the COOH Terminus of the Hsp70-interacting Protein (CHIP)*

Received for publication, March 19, 2004, and in revised form, April 21, 2004
Published, JBC Papers in Press, April 23, 2004, DOI 10.1074/jbc.M403117200

Bin He‡§‡, Suxia Bai‡§, Andrew T. Hnat‡§, Rebecca I. Kalman‡§, John T. Minges‡§, Cam Patterson‡, and Elizabeth M. Wilson‡§‡‡

From the ‡Laboratories for Reproductive Biology and the Departments of §Pediatrics, ¶Medicine, and **Biochemistry and Biophysics, University of North Carolina, Chapel Hill, North Carolina 27599

Steroid receptors depend on multiple domains for their function as ligand-dependent transcriptional activators. The DNA- and ligand-binding domains have been studied extensively and have a high degree of structural and functional conservation. In contrast, the largely unstructured NH$_2$-terminal domains (1–3) are highly variable in size and sequence, and the molecular mechanisms that contribute to transactivation are not well understood. The size of the NH$_2$-terminal region of steroid receptors increases with evolutionary expansion (4, 5), from estrogen receptor-α (185 amino acid residues) to the glucocorticoid receptor (GR$^1$; 420 residues), androgen receptor (AR; 558 residues), progesterone receptor (B form; 566 residues), and mineralocorticoid receptor (602 residues). In contrast, transcription factors such as p53, NF-κB, and VP16 typically have transcriptional activation domains of <100 residues.

The importance of the NH$_2$-terminal domain for AR functional activity is attributed largely to activation function-1 (AF1) between amino acid residues 142 and 337 (6). This region was further resolved into two noncontiguous activation segments, AF1a and AF1b (7). The functional importance of the AR NH$_2$-terminal region is supported by the extensive post-translational modification that occurs in the region, including phosphorylation (8–10), sumoylation (11), and ubiquitylation (12, 13). In addition, a conserved region between AR residues 225 and 259 in the region of AF1 was predicted to form an α-helix and to contribute to AR transactivation and was shown to be a binding site for the general transcription factor TFIIF (14). Earlier evidence also suggested that the AR NH$_2$-terminal region contains functional domains involved in transcriptional repression (15). In contrast, activation function-2 (AF2) in the AR ligand-binding domain, although highly conserved, has little inherent transcriptional activity when assayed in mammalian cells (16).

In this study, we identify sequence 234AKELCKAVS-VSMGL247 in the human AR NH$_2$-terminal domain, which is highly conserved in all AR sequences characterized thus far through multiple fish species. Based on the premise that strict amino acid sequence conservation indicates a site of critical function, we investigated the functional importance of human AR residues 234–247 using the yeast two-hybrid system. The COOH terminus of the Hsp70-interacting protein (CHIP) was identified as a binding partner and was found to contribute to AR down-regulation.

MATERIALS AND METHODS

Plasmids—The yeast vector pGBTs was kindly provided by Yue Xiong (University of North Carolina, Chapel Hill). pCMV-hAR-L237A was kindly provided by Yue Xiong (University of North Carolina, Chapel Hill). pCMV-hAR-L237A

*This work was supported by United States Public Health Service Grant HD16910 from NICHD, National Institutes of Health; by Cooperative Agreement U54-HD35041 as part of the Specialized Cooperative Centers Program in Reproductive Health; and by Fogarty International Center Grant RO3TW001234 from the National Institutes of Health (to Frank S. Frenck). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ Present address: Dept. of Molecular and Cellular Biology, Baylor College of Medicine, Houston, TX 77030.

§ To whom correspondence should be addressed: Labs. for Reproductive Biology, CB 7500, Medical Biomolecular Research Bldg., Rm. 3340, 103 Mason Farm Rd., University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-966-5168; Fax: 919-966-2203; E-mail: emw@med.unc.edu.

This paper is available on line at http://www.jbc.org

© 2004 by The American Society for Biochemistry and Molecular Biology, Inc.

Printed in U.S.A.

This is an Open Access article under the CC BY license.
**Identification of an AR NH2-terminal Conserved Motif—**

Comparison of AR NH2-terminal sequences from human to lower vertebrates shows that much of the region is not conserved (Fig. 1A). This is despite the important functional role of the AR NH2-terminal domain in transactivation (6). Among the

**K239VM/V241A/V243A, pCMV-hAR-A234T, and pCMV-hAR-E236G** (17) were expressed using a double PCR strategy by cloning the fragment into the pAII and BstEI sites of pCMV-hAR. The yeast bait vector pGBDT8-AR(220–270) contained the conserved sequence 234KELCKAVS-VSMGL24T and was constructed by PCR amplifying the corresponding region of human AR and inserting the fragment into the EcoRI and BamHI sites in the pGBK7 vector and was confirmed by DNA sequencing. 

**Washed beads were boiled in SDS-containing buffer. Input lanes were further tested for activity using a two-hybrid interaction filter assay performed following the instructions of the manufacturer. Colonies grown on synthetic medium lacking Leu, Trp, and His were verified for the absence of random errors.**

**A two-hybrid screen of a human testis cDNA library was performed by sequential transformation of bait and library vectors. The yeast strain HFC was transformed with the bait vector pCMV5-CHIP and pCMV5-CHIP4TPR, expressing CHIP with a deletion of the tetratricopeptide repeat (TPR) domain at residues 27–147 (see Fig. 3A), were created by digesting pCDNA3 CHIP and pCDNA3-CHIP4TPR with EcoRI and XbaI and ligating the inserts to the EcoRI and NotI sites of pCMV5-Chip.**

**Immunoblot Analysis—** Monkey kidney COS cells were cultured in Dulbecco’s modified Eagle’s medium containing 10% bovine calf serum (HyClone Laboratories, Logan, UT), 20 μg/ml HEPES (pH 7.2), and penicillin and streptomycin. Cells were plated at 2×105 cells/well on 12-well tissue culture plates and transfected with the indicated concentrations of Effectene as described above. Samples (10 μg of protein/lane) were separated on 10 or 16% acrylamide gels containing SDS. After electrophoresis, gel proteins were electroblotted onto nitrocellulose membranes. The blots were incubated with 1 μg/ml rabbit antibody AR32, raised against an AR NH2-terminal peptide, or with a 1:500 dilution of mouse anti-CHIP monoclonal antibody (Sigma) and incubated for 1 h at 0°C. After centrifugation at 12,000 rpm for 15 min at 4°C, the supernatant protein concentration was determined using the Bio-Rad assay. Transfected cells were fixed with freshly prepared 3% paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked with 0.5% bovine serum albumin as described (20), and incubated for 1 h with primary antibodies: mouse anti-FLAG monoclonal antibody 1D4 (BD Biosciences, San Diego, CA), rabbit anti-CHIP polyclonal antibody (Sigma) and rabbit anti-AR polyclonal antibody 3510 (1:250 dilution; Abcam, Cambridge, MA). Immunoreactive bands were visualized using chemiluminescence (SuperSignal Western Dura extended duration substrate, Pierce).

**Identification of an AR NH2-terminal Conserved Motif—**

Comparison of AR NH2-terminal sequences from human to lower vertebrates shows that much of the region is not conserved (Fig. 1A). This is despite the important functional role of the AR NH2-terminal domain in transactivation (6). Among the
poorly conserved regions are the previously reported AR trans-
activation subdomains AF1a (rat AR residues 154–167 and
human AR residues 173–186) and AF1b (rat AR residues 295–
359 and human AR residues 297–361), which are critical for
transcriptional activity (7). Lack of sequence conservation in
these regions is surprising and suggests that the NH2-terminal

FIG. 1. Alignment of the AR NH2-terminal amino acid sequence from different species. A, AR sequences were obtained from the GenBank™/EBI Data Bank with the following accession numbers: human AR, M20132 and J03180; Norway rat AR, J06454; Xenopus laevis African clawed frog AR, U67129; goldfish AR, AY004868; Japanese eel AR-α, AB023960; Japanese eel AR-β, AB025361; rainbow trout AR-α, AB012095; rainbow trout AR-β, AB012096; Nile tilapia AR-β, AB045212; red sea bream AR, AB017158; Haplochromis burtoni cichlid fish AR, AF121257 and AY082342; and Nile tilapia AR-α, AB045211. Amino acid residues conserved by amino acid, charge, or structure are shown in red, and partially conserved residues are shown in blue. The AR FXXLF motif is at residues 23–27, and the NH2-terminal conserved motif is at residues 234–247. Amino acid positions are based on the human AR sequence (22). The region shown is human AR NH2-terminal residues 1–543.

B, shown is an amino acid sequence alignment of the AR NH2-terminal conserved motif and flanking sequence, the AR consensus sequence, and the corresponding homologous sequence in GR. Sequences shown were extracted from the sites listed above and include human GR (X03225 and M10901) and X. laevis African clawed frog GR (X72211). Conserved residues are shown in orange-red, and partially conserved residues are shown in maroon. The human AR phosphorylation site Ser256 (10) and AR mutations from TRAMP prostate cancer tumors (A234T and E236G) are indicated. Shown are amino acid sequences corresponding to human AR residues 226–257 with numbering as previously reported (22) and human GR residues 67–98 (72).
domain evolved with its interacting partners. In contrast, several short regions of the AR NH$_2$-terminal region show a high degree of sequence conservation. The previously described FXXLF motif at human AR NH$_2$-terminal residues 234–247 (18, 21) is conserved as (F/Y/Q/N/S/I)LV/F from lower vertebrates through primates, although some species of fish seem to lack the NH$_2$-terminal 5′-coding region of the AR gene that includes the FXXLF motif (Fig. 1A). Absence of the extreme NH$_2$-terminal region could reflect incomplete sequence analysis or that certain species of fish have yet to evolve the AR FXXLF motif. An evolving FXXLF motif during vertebrate expansion is conceivable since the WXXLF motif at human AR residues 433–437, which contributes to the FXXLF motif-mediated AR NH$_2$- and COOH-terminal interaction (18), is conserved from primates to Xenopus, but not in fish and other lower vertebrates (Fig. 1A).

Another AR NH$_2$-terminal region that shows striking sequence conservation across species is positioned at human AR residues 234–247 (Fig. 1A) with the consensus sequence $^{238}$A/K/R/V/E(L/C/S)KAVSVS/MgL$^{247}$ (Fig. 1B), numbered according to the human AR sequence of Lubahn et al. (22) and the AR mutation data base.$^2$ Sequence conservation of hydrophobic residues in the region was noted previously (14). Also notable is Ser$^{256}$-Pro$^{257}$ since Ser$^{256}$ was shown previously to be phosphorylated in human AR (10). Ser$^{256}$ and Pro$^{257}$ are not conserved; but remarkably, the negatively charge residues Asp and Glu most often replace Ser$^{256}$ in lower species (Fig. 1, A and B), suggesting that a negative charge at this position is important for AR function.

Sequence conservation of AR NH$_2$-terminal residues 234–247 suggests a conserved motif for the AR gene family. A search of the protein sequence data base supports this, with one exception. GR from human and Xenopus, but not GR from fish, contains sequence $^{77}$DLSKAVSLMG$^{88}$ at human GR residues 77–88, which is homologous to 12 of the 14 residues in the AR NH$_2$-terminal conserved motif (Fig. 1B). The closer evolutionary distance between AR and the progesterone receptor compared with AR and GR (4) and the absence of the conserved motif in the progesterone receptor suggest that AR and GR acquired the sequence independently for a common function by convergent evolution.

Analysis of the predicted order and disorder composition of the AR NH$_2$-terminal domain using the PONDR Protein Disorder Predictor$^3$ indicates that the FXXLF motif and the AR NH$_2$-terminal conserved motif form short structurally ordered regions as indicated by PONDR scores of <0.1. Each motif is predicted to form an amphipathic $\alpha$-helix, indicating the presence of short structured segments in an NH$_2$-terminal region that is otherwise predicted to be largely unstructured.

**Intrinsic Activity of the AR NH$_2$-terminal Conserved Motif**—
We tested for the presence of transcriptional activity inherent to the AR NH$_2$-terminal conserved motif by expressing Gal4 DNA-binding domain-AR fusion proteins in mammalian cells (Fig. 2A). The NH$_2$-terminal conserved motif residues 234–247 lacked activity, but a low level of activity was detectable when the sequence was extended in Gal-AR-(229–254). A larger fragment, Gal-AR-(220–270), elicited 20% of the activity of the AR NH$_2$-terminal domain in Gal-AR-(1–503). When mutations at four residues (L237A/K239M/V241A/V243A) were introduced into the NH$_2$-terminal conserved motif in Gal-AR-(220–270) (Gal-AR-(220–270m4)) (Fig. 2A), the transcriptional response was reduced to background levels. Expression levels of the Gal-AR fusion proteins were similar as determined by immunoblotting (Fig. 2C, left panel).

The results suggest that although the NH$_2$-terminal conserved motif itself lacks intrinsic transcriptional activity, a larger region that includes flanking sequence of the conserved motif induces a transcriptional response that depends on residues within the AR NH$_2$-terminal conserved motif. The flanking region may therefore be necessary for proper folding or presentation of the NH$_2$-terminal conserved motif.

**Contribution of the Core Conserved Motif to AR Transcriptional Activity**—
Activity of the AR NH$_2$-terminal conserved motif in mammalian cells was investigated further by testing several mutants of full-length AR. Deletion of region 220–270 (AR220–270) caused a $\sim$50% decrease in AR transcriptional activity using the MMTV-Luc and PSA-Enb-Luc reporter vectors (Fig. 2B), confirming the importance of the region for AR activity. The decrease in transcriptional activity by AR220–270 and additional mutants was independent of AR expression levels (Fig. 2C, right panel). Deleting only the NH$_2$-terminal conserved motif (AR224–247) caused less of a decrease in activity, and the decrease was similar to that seen with ARm4. Mutating the flanking Ser$^{256}$ (Fig. 1B) to alanine to inhibit phosphorylation at this previously reported AR phosphorylation site (10) or to aspartic acid to mimic the negative charge of a phosphate group had no effect on the AR transcriptional response. The results suggest that the AR NH$_2$-terminal conserved motif and closely flanking sequence contribute to AR transactivation.

**AR NH$_2$-terminal Conserved Motif Interaction with CHIP**—
The high sequence conservation of the AR NH$_2$-terminal motif in AR gene evolution, its predicted structure, and evidence for a role in AR transcriptional activation led us to perform a yeast two-hybrid screen using a 51-amino acid residue fusion peptide as bait to probe a human testis cDNA library. Yeast strain HF7c was initially transformed with pGBT8-AR-(220–270), coding for the Gal4 DNA-binding domain and human AR residues 220–270. In agreement with the transcriptional response seen with Gal-AR-(220–270) in mammalian cells, the addition of 0.8 mM 3-amino-1,2,4-triazole was required to reduce intrinsic activity of pGBT8-AR-(220–270). Screening 16 clones of an amplified library revealed 138 positive clones that grew on synthetic medium lacking Leu, Trp, and His. Of these, 48 clones were positive in a $\beta$-galactosidase filter assay performed in yeast. Sequence analysis revealed five identical clones coding for CHIP. The clones encoded for CHIP residues 20–303, representing full-length CHIP lacking the first 19 NH$_2$-terminal amino acids. That the interaction was specific was suggested by a parallel screen of the same library using a similarly sized AR fragment from a different region of the AR NH$_2$-terminal domain. In this case, none of the positive clones that grew on synthetic medium lacking Leu, Trp, and His encoded for CHIP.$^4$

**CHIP** is a U-box-containing ubiquitin-protein isopeptidase lidade (E3) (23–26) that interacts with heat shock proteins Hsp70 and Hsp90 through its NH$_2$-terminal TPR domain (Fig. 3A) and has been reported to cause ubiquitylation of AR (13). To further investigate the role of the AR NH$_2$-terminal conserved motif in the AR and CHIP interaction, we tested several AR mutants in a mammalian two-hybrid assay using a Gal-CHIP-(20–303) fusion protein. Deletion of AR residues 220–270 (AR220–270) strongly reduced the interaction with CHIP (Fig. 3B), supporting the role of the NH$_2$-terminal conserved motif region in the AR interaction with CHIP. A small portion of this reduction was attributed to the slight decrease in background AR activity that resulted from the deletion of residues 220–270, in agreement with the results of Fig. 2 (A and B) showing that residues 220–270 harbor a transcriptional activation function. The background AR activity evident upon co-

---

$^2$ Available at www2.mcgill.ca/androgendb/data.htm.

$^3$ Available at www.pondr.com/PONDR/pondr.cgi.

$^4$ S. Bai, B. He, and E. M. Wilson, unpublished data.
**Fig. 2. Transcriptional activity of the AR NH₂-terminal conserved motif.** A, the transcriptional activity of the AR NH₂-terminal conserved motif and flanking sequence was determined by expressing Gal4 DNA-binding domain fusion proteins with no insert (GAL4-DBD) or with AR fragments in Gal-AR-(1–503), Gal-AR-(234–247), Gal-AR-(229–254), Gal-AR-(220–270), and Gal-AR-(220–270m4). Activity was determined in HepG2 cells using 50 ng of pGal vector and 0.1 μg of 5xGal4-Luc3 reporter vector. Cells were transfected using Effectene as described under "Materials and Methods." The transcriptional response is indicated in optical light units and error and are representative of three independent experiments. B, shown is the effect of the NH₂-terminal conserved motif region on AR transactivation. HepG2 cells were transiently transfected as described under "Materials and Methods" with 0.25 μg of MMTV-Luc or 0.25 μg of PSA-Enh-Luc together with 50 ng of pCMV-hAR with wild-type sequence (WT), with deletion of the conserved region (ARA220–270 and ARA234–247), with the four-residue mutation in the NH₂-terminal conserved motif (ARm4 (m4)), and with mutations S256A (SA) and S256D (SD) at an AR phosphorylation site. C, shown are immunoblots of AR mutants. Wild-type and mutant Gal-AR (10 μg/10-cm dish) and pCMV5-AR plasmids (2 μg/dish) were transiently expressed in COS cells (1.8 × 10⁶/dish) using DEAE-dextran in the absence of added androgen. For the Gal-AR vectors, cells were incubated in serum-containing medium with 1 μM MG132 proteasome inhibitor for 1 h at 37 °C prior to harvest. Protein extracts (10 μg/lane) were analyzed using anti-Gal4 DNA-binding domain antibody (left panel) for the Gal-AR fusion proteins tested for transcriptional activity in A or antibody AR32 (right panel) for the AR mutants tested for transcriptional activity in B.
**FIG. 3. Interaction of CHIP with the AR NH₂-terminal conserved motif.**

**A.** Schematic diagram of full-length CHIP (303 amino acids) containing the NH₂-terminal TPR domain at residues 27–147, which interacts with Hsp70 and Hsp90, and the COOH-terminal U-box domain at residues 231–288, which imparts E3 (ubiquitin-protein isopeptide ligase (Ub ligase)) activity (23, 24). Lys³⁰ is required for the interaction between the TPR domain and Hsp70 (C. Patterson, unpublished data). The results from GST affinity binding studies shown in **C** indicate that the TPR and U-box domains of CHIP are required for interaction with AR (shown by the bracket).

**B.** Interaction of CHIP with full-length AR in a mammalian two-hybrid assay. The assay was performed in HeLa cells using full-length wild-type AR, ARΔ220–270, ARΔ234–247, and ARm4 and cotransfected with the parent pGAL0 empty plasmid (GAL 0) or Gal-CHIP-(20–303) (GAL CHIP). HeLa cells were transfected using 10 ng of AR, 50 ng of Gal expression plasmid, and 0.1 µg of 5xGAL4-Luc3 in 12-well plates using Effectene as described. Optical light units reflect luciferase activity and error and are representative of three independent experiments.

**C.** In vitro interaction between CHIP and the AR NH₂-terminal conserved motif. ³⁵S-Labeled full-length wild-type CHIP (WT; lanes 1, 5, and 9), CHIPΔTPR (lacking the TPR domain; lanes 2, 6, and 10), CHIPΔU (lacking the U-box domain; lanes 3, 7, and 11), and CHIP-K30A (lanes 4, 8, and 12) were incubated with GST (GST0; lacking AR sequence; lanes 1–4), GST-AR(220–270) (containing...
expression of the pGal0 empty vector results from a cryptic androgen response element in the 5xGal4-Luc3 reporter (19). Deleting the NH2-terminal conserved motif in AR234–247 or introducing the multiple mutations L237A/K239M/V241A/V243A into AR (ARm4) did not decrease background AR transcriptional activity and reduced (but did not eliminate) the AR and CHIP interaction.

The results support a role for the NH2-terminal conserved motif in the AR and CHIP interaction, but also suggest that additional direct or indirect interactions occur between AR and CHIP. One possible interaction site is the AR ligand-binding domain, which was shown to bind the Hsp70/Hsp90 heterocomplex (27, 28), and Hsp70/Hsp90 interacts with CHIP (23). A recent study demonstrating that CHIP functions as a dimer provides a basis for this type of interaction (29).

**GST in Vitro Adsorption Assay**—To confirm a direct interaction between the AR NH2-terminal conserved motif and CHIP, GST affinity matrix binding assays were performed. Wild-type GST-AR-(220–270) and GST-AR-(220–270m4) were expressed in *E. coli*, purified, and incubated with 35S-labeled in vitro translated full-length CHIP and several CHIP mutants. A strong interaction was evident between GST-AR-(220–270) and 35S-labeled CHIP that was not detected using the pGST0 empty vector control (Fig. 3C, lanes 1 and 5). The AR interaction with CHIP was eliminated by deleting the CHIP TPR domain (lane 6), by deleting the CHIP U-box domain (lane 7), or by introducing a K30A mutation into the CHIP TPR domain (lane 8). Lys30 is highly conserved in the TPR domain (30) and by introducing a K30A mutation into the CHIP TPR domain (lane 8) was shown to be required for the interaction between the CHIP TPR domain and Hsp70 or Hsp90.5

Two AR mutations identified in the transgenic adenocarcinoma of mouse prostate (TRAMP) model for prostate cancer, E236G and A234T (17), also reduced the AR and CHIP interaction by 43 and 16%, respectively (Fig. 3D). In contrast, no decrease in the AR and CHIP interaction was seen using GST-AR-(220–270m4), in which four mutations (L237A/K239M/V241A/V243A) were introduced into the NH2-terminal conserved motif (Fig. 3C).

The data indicate a direct interaction between AR and CHIP mediated by the short AR NH2-terminal fragment that contains the conserved motif. A reduced AR and CHIP interaction by AR mutations derived from the TRAMP model suggests that the AR NH2-terminal conserved motif functions to limit AR activity and that mutations in this region confer increased AR transcriptional activity and that mutations in this region that confer increased AR transcriptional activity and reduced (but did not eliminate) the AR and CHIP interaction.

The results support a role for the NH2-terminal conserved motif in the AR and CHIP interaction, but also suggest that additional direct or indirect interactions occur between AR and CHIP. One possible interaction site is the AR ligand-binding domain, which was shown to bind the Hsp70/Hsp90 heterocomplex (27, 28), and Hsp70/Hsp90 interacts with CHIP (23). A recent study demonstrating that CHIP functions as a dimer provides a basis for this type of interaction (29).

**CHIP-induced Inhibition of AR Transcriptional Activity and AR steady-state levels**—A HepG2 cells (0.2 × 10⁶/well of a 12-well plate) were transiently transfected as described under “Materials and Methods” with 0.25 μg of MMTV-Luc/well using Effectene in the presence of (per well) 50 ng of pCMV-hAR (AR), 50 ng of pCMV-hGR (GR), 10 ng of pCMV-AR-(1–660), or 10 ng pCMV-AR-(1–660)Δ234–247, together with further addition (−), the pCMV5 empty vector (p5), pCMV5-CHIP (C), or pCMV5-CHIPΔTPR (ΔTPR). Cells were incubated for 24 h in the absence of hormone, with 0.1 nM R1881 for AR, or with 10 nM dexamethasone for GR. B, shown is an immunoblot of AR coexpressed with CHIP and the CHIP mutant lacking the TPR domain. COS cells were transiently transfected using Effectene as described under “Materials and Methods” with 1 μg of pCMV-hAR in the presence of 1 μg of pCMV5 empty vector (lanes 1 and 2), 1 μg of pCMV5-CHIP (lanes 2 and 3), and pCMV5-CHIPΔTPR (lanes 5 and 6). Cells were incubated 24 h after transfection in the absence and presence of 50 μM DHT as indicated, harvested, and analyzed by immunoblotting using antibody AR32 as described under “Materials and Methods.” Immunoblots of parallel experiments using mouse anti-β-actin monoclonal antibody (1:2500 dilution; Abcam, Inc.) confirmed similar protein loading between the lanes (data not shown). The data are representative of three independent experiments.

or with coexpression of the CHIP mutant lacking the TPR domain (CHIPΔTPR) (Fig. 4A, A7TP). Surprisingly, deletion of the conserved sequence in AR234–247 or AR220–270 did not eliminate the inhibitory effect of CHIP on AR transactivation (data not shown). Furthermore, the constitutive transcriptional activities of AR truncation mutant AR-(1–660), which

---

5 C. Patterson, unpublished data.
lacks the ligand-binding domain, and AR-(1–660)Δ234–247, which lacks, in addition, the NH₂-terminal conserved motif, were both strongly inhibited by CHIP (Fig. 4A). That this inhibitory effect of CHIP was mediated by sequences outside of the NH₂-terminal conserved motif was also supported by our observations that CHIP inhibited, to a similar extent, transactivation by the progesterone receptor, a receptor that lacks the conserved motif (data not shown). When the activity of a pCMV-Luc constitutive reporter was tested in the presence of CHIP expression, there was no significant decrease in transcriptional activity above that observed with the empty vector control or with pCMV5-CHIPΔTPR (data not shown), suggesting that the inhibitory effect of CHIP is not due to inhibition of general transcription mechanisms.

The results indicate that CHIP strongly represses AR and GR transcriptional activity independent of the NH₂-terminal conserved motif and independent of the ligand-binding domain. This is despite the interaction mediated by the AR NH₂-terminal conserved motif and CHIP. The requirement for the TPR domain of CHIP for transcriptional repression of AR, AR-(1–660), and GR suggests that the heat shock protein heterocomplex is involved since the CHIP TPR domain binds heat shock proteins Hsp70 and Hsp90 (31). CHIP may modulate AR and GR transcriptional activity through mechanisms independent of the NH₂-terminal conserved motif.

The influence of CHIP on AR steady-state levels was also tested. The low level of AR detected in the absence of androgen was further reduced by coexpression of CHIP (Fig. 4B, lanes 1 and 3). CHIP also decreased the stabilizing influence of androgen on AR levels (lanes 2 and 4). In contrast, deletion of the CHIP TPR region eliminated the reduction in AR levels induced by coexpression of CHIP. The results indicate that the TPR region of CHIP is required for the inhibitory effects of CHIP on AR steady-state levels and AR transcriptional activity.

Colocalization of CHIP and AR—The subcellular localization of CHIP and AR was determined in the absence and presence of DHT by immunocytochemistry. In the absence of DHT, transiently expressed AR (green) and FLAG-tagged CHIP (red) localized in the cytoplasm, with no evidence of nuclear staining (Fig. 5A). This is in agreement with a previous report that CHIP is a cytoplasmic protein (23). The partial colocalization of CHIP and AR in the cytoplasm supports their interaction in the absence of hormone. In the presence of DHT, AR was nuclear, whereas CHIP remained primarily in the cytoplasm (Fig. 5B).

The data suggest that CHIP regulates AR degradation prior to androgen binding and that androgen-induced nuclear targeting sequesters AR from the inhibitory effects of CHIP.

DISCUSSION

The NH₂-terminal regions of steroid receptors lack strict structural requirements like those of the DNA- and ligand-binding domains. To address structure-function relationships of the AR NH₂-terminal domain, we searched for evolutionarily conserved sequences that predict a critical role in AR function. Our approach was based in part on studies of the AR NH₂-terminal FXXLF motif, which is one of few highly conserved regions in the AR NH₂-terminal domain (32). The FXXLF motif has a dominant role in the androgen-dependent interdomain NH₂- and COOH-terminal interaction required for AR function, whereas the less well conserved AR NH₂-terminal WXXLF motif contributes only weakly to the AR NH₂- and COOH-terminal interaction (18, 32). Considering its essential role in transcriptional activation, it is surprising that more of the AR NH₂-terminal domain is not conserved (6). In particular, the two major activation regions of AF1 lack significant sequence conservation across species. This suggests that the NH₂-terminal activation domain has evolved in parallel with its interacting partners, with one interacting partner being the AR COOH-terminal ligand-binding domain. In contrast to the NH₂-terminal region, the COOH-terminal region including AF2 is structurally conserved. AF2 interacts with the LXXLL motifs of p160 coactivators (33–36) and, for AR, interacts in addition with the FXXLF motif that is present in the AR NH₂-terminal region (16, 18) and in some AR coregulators (19, 37, 38).

In this study, we have identified an extraordinarily conserved 14-amino acid NH₂-terminal sequence at human AR amino acid residues 234–247 that is common to all published sequences of AR, including the lower vertebrate fish species. The conserved motif lacks intrinsic transcriptional activity per se, but contributes to the transcriptional response of a larger region between residues 220 and 270. The AR NH₂-terminal conserved motif lies within a previously described α-helical region (2) and is NH₂-terminal to a recently reported hydrophobic motif identified among cellular and viral transcription factors that act as binding sites for transcription factor TFIIF (14), a general transcription factor reported to interact with AR (39, 40). To study the function of the AR NH₂-terminal conserved motif, we performed a yeast two-hybrid screen and

![Figure 5](image-url)
identified CHIP as the interacting partner for the conserved AR NH$_2$-terminal conserved motif. CHIP is highly conserved across species and interacts with the NH$_2$-terminal motif that is conserved throughout the entire AR gene family. Coexpression studies have shown that CHIP limits AR function by promoting AR degradation most likely through the previously described role of CHIP in ubiquitylation (13).

CHIP, named for its interaction with Hsp70, was originally identified from a screen for TPR domains. The TPR, first described role of CHIP in ubiquitylation (13).

The addition of DHT translocated AR to the nucleus, but CHIP reduced degradation (13). CHIP may act as a molecular link between the protein folding chaperone functions and proteasome-mediated protein degradation processes of the Hsp70/Hsp90 heterocomplex (52). CHIP also directly modulates the stress response by activating heat shock factor-1 (53) and influences protein trafficking (54).

A direct interaction between CHIP and the AR NH$_2$-terminal conserved motif was confirmed in mammalian two-hybrid and GST affinity matrix binding assays, supporting the initial identification of CHIP in a yeast two-hybrid screen as a bona fide AR-interacting protein. A BLAST search of the protein data base showed that besides AR, GR from Xenopus and higher species is the only other protein that contains a homologous sequence. However, in the yeast two-hybrid interaction assay, CHIP did not interact with a GR fragment containing the partial sequence (data not shown), even though CHIP increased GR transcriptional activity to an extent similar to the decrease in AR activity. It is conceivable that CHIP also associates with AR and GR indirectly via the ligand-binding domain through an association with Hsp70 and Hsp90. The requirement of the TPR region of CHIP for inhibition of AR and GR transcriptional activity suggests a mechanism involving CHIP interaction with the heat shock protein complex. The AR-CHIP-Hsp complex may be further stabilized by the NH$_2$-terminal conserved motif. On the other hand, CHIP also decreased the transcriptional activity of an AR deletion mutant lacking the ligand-binding domain, and deletion of the conserved sequence did not eliminate the inhibitory effect. The lack of a requirement for the AR NH$_2$-terminal conserved motif and the ligand-binding domain for the inhibitory effect of CHIP on AR transactivation suggests that other factors are involved.

Several spontaneous somatic AR mutations were reported in the TRAMP model of prostate cancer during tumor progression, two of which (E236G and A234T) are within the AR NH$_2$-terminal conserved motif. The data suggest that AR and CHIP interact in the cytoplasmic and nuclear compartments, leading to increased degradation by the proteasome (24, 26, 48). CHIP was shown to promote ubiquitylation and degradation through the proteasome (31). AR (13), and other proteins (26, 49), including ErbB2/Neu (50, 51). However, it was previously suggested that the predominant effect of CHIP on AR is inhibition of protein folding and reduced degradation (13). CHIP may act as a molecular link between the protein folding chaperone functions and proteasome-mediated protein degradation processes of the Hsp70/Hsp90 heterocomplex (52). CHIP also directly modulates the stress response by activating heat shock factor-1 (53) and influences protein trafficking (54).

A requirement for Hsp90 in androgen binding to AR was reported previously (27). Geldanamycin, an inhibitor of the Hsp90 chaperone function (58), inhibits androgen binding, increases AR degradation, and inhibits AR functional activity (27, 59). CHIP was reported to decrease AR synthesis, to inhibit folding of AR by actions not involving its E3 activity, and to reduce AR degradation (13). The studies reported here support that CHIP increases AR degradation in the absence and presence of androgen. This is consistent with a previous study showing that CHIP promotes ubiquitylation of AR, but conflicts with observations that CHIP does not increase AR degradation (13). A region at human AR residues 11–172, which is NH$_2$-terminal to the conserved AR motif, was also reported to interact with a different ubiquitin ligase, a RING domain-containing protein called AR-NIP (AR NH$_2$-terminus-interacting protein) (12).

Several spontaneous somatic AR mutations were reported in the TRAMP model of prostate cancer during tumor progression, two of which (E236G and A234T) are within the AR NH$_2$-terminal conserved motif. TRAMP tumors were derived in transgenic mice in response to overexpression of the SV40 large T antigen under the control of the minimal −426 to +28 regulatory sequence of the prostate-specific rat probasin promoter (60). The interaction between AR and CHIP was reduced as a result of these AR mutations. This is in support of the AR NH$_2$-terminal conserved motif functioning to limit AR activity. Interference with the AR and CHIP interac-
tion would be expected to increase AR activity, which could lead to increased prostate cancer growth. In agreement with this, recent studies have shown that the E236G mutation, which we have found inhibited the AR and CHIP interaction to the greatest extent, is associated with an increased incidence of tumor formation when the AR mutant is overexpressed. Furthermore, it was reported that the E236G mutant increases AR transactivation in the presence of AR coactivators (17). The results support the inhibitory role of the AR NH\textsubscript{2}-terminal conserved motif mediated by CHIP. With the recent report of increased AR levels as a common feature of hormone refractory recurrent prostate cancer (61), down-regulation of AR mutations in CHIP may contribute to increased AR steady-state levels during prostate cancer progression.

However, increased degradation of AR cannot necessarily be construed as a decrease in AR functional activity. In many instances, ligand-activated transcription factors are rapidly degraded. Among the steroid receptors, AR (55) and the vitamin D receptor (62) are exceptions. AR is distinguished by its rapid degradation in the absence of ligand (55) and androgen-induced stabilization (56). Part of the mechanism for androgen-induced AR stabilization involves the androgen-dependent NH\textsubscript{2}- and COOH-terminal interaction (57, 63) mediated by the androgen-dependent interaction with AR and CHIP stabilization involves the androgen-dependent NH\textsubscript{2}- and COOH-terminal interaction (57, 63) mediated by the FXXLF and WXXLF motifs mentioned above (18, 21). It is plausible that through the interaction of CHIP and the AR NH\textsubscript{2}-terminal conserved motif, CHIP helps to maintain low levels of AR in normal cells. We have shown that CHIP is mainly in the cytoplasm, and CHIP has been reported to be highly expressed in non-proliferating cells (23). In contrast to the low level of AR in non-proliferating cells, AR levels in prostate cancer are elevated (61), and AR has increased stability, which results in a greater sensitivity to low levels of circulating androgen (64) or to androgens present in prostate cancer tissue (65). It is possible that nuclear localization of AR that is detected in prostate cancer cells in the absence of androgen (66) contributes to increased AR levels by sequestering AR in the nucleus away from the degrading influence of CHIP in the cytoplasm. Alterations in CHIP levels, mutations in CHIP, or, as shown here, mutations in AR could decrease AR degradation, resulting in increased AR-dependent proliferation of recurrent prostate cancer cells.

Sequence homology comparisons in the AR gene family show that Ser\textsuperscript{256} in human and rat AR replaces the negatively charged residue aspartic or glutamic acid in AR from lower species. Phosphorylation at Ser\textsuperscript{256} was reported for human AR (10) and effectively reintroduces the constitutive negative charge present in lower species. The proximity of Ser\textsuperscript{256} to the NH\textsubscript{2}-terminal conserved motif suggests that a negative charge at this position modulates protein interactions at the NH\textsubscript{2}-terminal conserved motif and that phosphorylation at this site is a regulatory switch. Although the interaction between CHIP and AR was not significantly influenced by the S256A and S256D mutations in mammalian two-hybrid assays, the transition from the constitutive negative charge of lower species to the conditional charge regulated by phosphorylation may influence other interacting partners at this site. Higher vertebrates gain the ability to regulate the negative charge by phosphorylation. This is also suggested by the human AR phosphorylation site Ser\textsuperscript{256} (8, 10), which is Asp in lower species. Similarly, the human estrogen receptor-\(\alpha\) phosphorylation site Ser\textsuperscript{118} (67, 68) is Asp in the lamprey estrogen receptor (4). Increased regulation introduced by phosphorylation likely helps to modulate protein interactions to increase the overall level of homeostatic control.

NH\textsubscript{2}-terminal domains of steroid receptors are largely unstructured, and amino acid sequence is poorly conserved; yet computational programs that predict protein structure can identify short – 20-amino acid residue segments with ordered secondary structure, some of which are conserved across species. The POND\textsuperscript{R} Protein Disorder Predictor indicates that the FXXLF and WXXLF motifs are structured, and similarly, an ordered amphipathic \(\alpha\)-helical structure is predicted for the AR NH\textsubscript{2}-terminal conserved motif described in this work. We designed a yeast two-hybrid screening approach based on sequence conservation and structure predictions that short ordered regions, especially amphipathic \(\alpha\)-helices, are surfaces for protein-protein interactions. In support of this, the highly conserved AR FXXLF motif and flanking sequence mediate the androgen-dependent interaction with AF2 in the ligand-binding domain (18, 21) and the interaction of coregulatory proteins (19, 38). The predicted amphipathic \(\alpha\)-helices of the p160 coactivator LXXLL motifs were also confirmed by structural studies (69, 70). Thus, although transcriptional activation domains of regulatory proteins are largely unstructured and not conserved, short segments within these regions are predicted to form amphipathic \(\alpha\)-helices whose structure is likely stabilized by binding coregulatory proteins (2, 71). We previously proposed a reversal of the acidic transcriptional activation domain-coactivator interaction model, implicating charge interactions that precede nonpolar contacts of hydrophobic residues in protein-protein interactions mediated by amphipathic \(\alpha\)-helices (21). In the present study, we used comparative sequence alignment and structure predictions to identify a 14-amino acid AR NH\textsubscript{2}-terminal conserved motif conserved in all AR sequences throughout the lower vertebrates. We have demonstrated the usefulness of yeast two-hybrid library screening using short – 50-amino acid fusion peptides containing conserved sequence and predicted structure in the identification of interacting partners. The results demonstrate a novel strategy to identify interacting partners and to probe the functional significance of short conserved sequences present in steroid receptor NH\textsubscript{2}-terminal domains.

Acknowledgments—We are grateful for the excellent technical assistance of K. Michelle Cobb and De-Ying Yang. We thank Frank S. French, who kindly reviewed the manuscript.
