Ku Is a Novel Transcriptional Recycling Coactivator of the Androgen Receptor in Prostate Cancer Cells*

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The androgen receptor (AR) dynamically assembles and disassembles multicomponent receptor complexes in order to respond rapidly and reversibly to fluctuations in androgen levels. We are interested in identifying the basal factors that compose the AR aporeceptor and holoreceptor complexes and impact the transcriptional process. Using tandem mass spectroscopy analysis, we identified the trimeric DNA-dependent protein kinase (DNA-PK) complex as the major AR-interacting proteins. AR directly interacts with both Ku70 and Ku80 in vivo and in vitro, as shown by co-immunoprecipitation, glutathione S-transferase pull-down, and Sf9 cell/baculovirus expression. The interaction was localized to the androgen receptor ligand binding domain and is independent of DNA interactions. Ku interacts with AR in the cytoplasm and nucleus regardless of the presence or absence of androgen. Ku acts as a coactivator of AR activity in a luciferase reporter assay employing both Ku-defective cells and Ku small interfering RNA knockdown in a prostate cancer cell line. DNA-PK catalytic subunit (DNA-PKcs) also acts as a coactivator of androgen receptor activity in a luciferase reporter assay employing DNA-PKcs defective cells. AR nuclear translocation is not affected in Ku defective cells, implying Ku functionality may be mainly nuclear. Chromatin immunoprecipitation experiments demonstrated that both Ku70 and Ku80 interact with the prostate-specific antigen promoter in an androgen-dependant manner. Finally, in vitro transcription assays demonstrated Ku involvement in transcriptional recycling with androgen dependent promoters.

Androgens, testosterone, and dihydrotestosterone (DHT)1 play a role in a multitude of physiological and developmental responses. Theses responses are mediated by the androgen receptor (AR), a 110-kDa member of the nuclear receptor superfamily. The structure of the AR is consistent with members of this family, consisting of an amino-terminal activation function (AF-1), the DNA binding domain, the hinge region, a carboxy-terminal ligand binding domain (LBD), and a second AF (AF-2) in the LBD. The AR is involved in growth, differentiation, and the progression of prostate cancer (for review, see Ref. 1).

For the AR to rapidly respond to fluctuations in hormone levels, it functions as a member of a multicomponent complex (2). Simplistically, the aporeceptor complex, primarily residing in the cytoplasm, translocates into the nucleus upon hormone binding. The AR-hormone complex binds the androgen response element (ARE), forming the holoreceptor complex. Both the aporeceptor and holoreceptor complexes act to facilitate, stabilize, and enhance the AR activity. One such complex consists of molecular chaperones that are involved in the stabilization of AR as well as its delivery to the nucleus. It is likely other complexes exist of which we have very little knowledge.

A broad family of proteins, which interact with the AR, have previously been identified called coregulators. Coregulators are generally categorized into two classes; coactivators, which enhance the transcriptional activity of AR, and corepressors, which reduce AR transcriptional activity. Several AR coactivators have been identified, including cAMP response element-binding protein-binding protein (3–5), SRC-1 (5), TRAP-Mediator complex (6), TIF2 (7), SWI/SNF (8), complex AR70 (9), and filamin (10). However, these coactivators have not been identified as members of either the aporeceptor nor holoreceptor complexes.

In an attempt to identify novel members of the AR aporeceptor and holoreceptor complexes, we used tandem mass spectroscopy to study proteins associated with AR. As expected, we identified multiple members of the heat shock protein family which have previously been shown to stabilize the unligated receptor in the cytoplasm, and to regulate ligand affinity (11, 12). Additionally, we identified all members of the DNA-dependent protein kinase (DNA-PK) heterotrimeric complex. DNA-PK is composed of the heterodimeric Ku and the DNA-PK catalytic subunit (DNA-PKcs) (13, 14). Ku, the regulatory subunit, consists of proteins with approximate molecular masses of 70 and 80 kDa (Ku70 and Ku80, respectively). DNA-PKcs is a nuclear serine/threonine protein kinase, a member of the phosphatidylinositol 3-kinase family (15), with an approximate molecular mass of 470 kDa.

Ku was originally characterized by Mimori et al. (16) as an autoantigen recognized by the sera of patients with polymyositis-scleroderma overlap syndrome and now is often found in patients with other autoimmune diseases. Ku plays a role in a multitude of nuclear processes; that is, DNA repair, telomere maintenance, V(D)J recombination, and transcriptional regu-
loration (for review, see Ref. 17). Ku is probably best known for its role in nonhomologous DNA-end-joining (NHEJ) repair, a process responsible for repairing a majority of DNA double strand breaks. The importance of Ku is demonstrated by the fact that cells deficient in either Ku are more sensitive to ionizing radiation (18–20).

The critical function of Ku in DNA repair not withstanding, recently there has been an evolving role for Ku in transcription. Initial reports identified Ku as a transcription factor that directly bound to sequence-specific promoter elements (21–23). Other reports emerged that Ku was associated with RNA polymerase II sites (24), and Ku is also directly associated with the RNA polymerase II complex (25). The entire DNA-PK complex is also involved in transcriptional regulation. DNA-PK has been shown to interact with and/or phosphorylate a number of transcription factors including epidermal growth factor receptor (26), c-Myc (27), c-Jun (28), glucocorticoid receptor (22), and Ku is also directly associated with RNA polymerase II sites (24), and Ku is also directly associated with transcription related proteins more efficiently (37).

Recently there has been an evolving role for Ku in transcription. Ku by itself and as part of the DNA-PK complex appears to be integrally important for transcriptional control. Additionally, Ku has previously been identified as being involved in secondary initiation events (35, 36). Cells deficient for Ku showed greatly reduced transcription, which has been attributed to defective reinitiation. Reinitiation is enhanced by the recycling of promoter and other transcription related proteins more efficiently (37).

Here we identify Ku as a transcriptional recycling coactivator of AR function. We show that 1) AR interacts directly with both Ku70 and Ku80 and indirectly with DNA-PKcs, 2) the interaction of Ku70 and Ku80 with AR is via the AR LBD, 3) both Ku and DNA-PKcs enhance AR activity in transactivation assays, 4) Ku is recruited to the prostate-specific antigen (PSA) promoter in an androgen-dependent manner, and 5) Ku enhances the transcriptional activity of AR through recycling of the transcriptional factors. These results define Ku as a recycle coactivator of AR.

EXPERIMENTAL PROCEDURES

Reagents and Antibodies—All cell culture reagents were from Invitrogen unless otherwise noted. FBS and charcoal dextran-treated FBS were from Omega Scientific. Antibodies to the androgen receptor were from Upstate and Neomarkers. Antibodies to Ku were from Neo-markers, antibodies to DNA-PKcs were from Upstate, and antibodies to tubulin were from Santa Cruz Biotechnology. siRNA oligos were from Dharmacon.

Expression Constructs and Protein Expression—All DNA-PK plasmids were generous gifts of Dr. David J. Chen (University of Texas Southwest Medical Center). AR fragments were constructed by PCR and ligated into pcDNA3.1. Insect cell viruses were generous gifts of Hong-Wu Southwestern Medical Center. AR fragments were constructed by PCR and ligated into pcDNA3.1. GST-ARC fragment was expressed, partially purified on phenol red-free RPMI1640 supplemented with 10% charcoal-dextran-stripped fetal bovine serum (FBS) and charcoal-dextran-treated FBS were from Omega Scientific. Antibodies to the androgen receptor were from Upstate and Neomarkers. Antibodies to Ku were from Neo-markers, antibodies to DNA-PKcs were from Upstate, and antibodies to tubulin were from Santa Cruz Biotechnology. siRNA oligos were from Dharmacon.

Cell Culture, Transfection, and Lysis—LNCaP cells were grown in RPMI1640 media supplemented with 10% FBS, for androgen deprivation LNCaP cells were grown in phenol red-free RPMI1640 supplemented with 10% charcoal-dextran-stripped fetal bovine serum. PC-3(AR) cells were grown in phenol red-free RPMI1640 supplemented with 10% charcoal-dextran-stripped fetal bovine serum. Androgen stimulation for all cell lines was done in phenol red-free RPMI supplemented with 10% charcoal-dextran-stripped fetal bovine serum and 10 nM dihydrotestosterone (DHT). XR-V15B-vector (XRV15B) and XR-V15B-Ku86 (XRV15-Ku) cells were grown in Ham’s F-12 media supplemented with 10% FBS, CHO V3 and CHO AA8 cells were grown in minimum Eagle’s medium supplemented with 10% FBS. Transient transfection was done using Effectene (Qiagen) following the manufacturer’s recommendation. Sf9 cells were grown in SF900-II SFM. Cells were lysed with ice-cold lysis buffer (1% Nonidet P-40, 50 mM Tris, pH 7.4, 10% glycerol, 50 mM KCl, 50 mM β-mercaptoethanol, 50 mM NaF, 5 mM EDTA, 1 mM Na3VO4, Complete protease inhibitor (Roche Applied Science)) unless otherwise indicated. For nuclear/cytosolic fractionation the NE-PER (Pierce) kit was used according to manufacturer's instructions.

RESULTS

DNA-PK Complex Interacts with the Carboxyl-terminal Region of the Androgen Receptor—A great deal of attention has been paid to identifying AR cofactors that modulate its activity in an androgen-dependent manner. Our laboratory has been interested in identifying basal factors that associate with AR in an androgen-independent manner. To facilitate identification of potential members of the multicomponent AR complex, the AR was divided into both amino-terminal (amino acids 1–478) and carboxy-terminal (ARC, amino acids 479–919) fragments to facilitate bacterial expression of the GST-tagged constructs. The GST-ARC fragment was expressed, partially purified on GST resin, and incubated with nuclear extracts of LNCaP cells (Figs. 1, A and B). LNCaP cells were either deprived of androgens (C) or stimulated with 10 nM DHT (D) to allow for any androgen-stimulated post-translational modifications for 2 h before lysis as described under “Experimental Procedures.” Lysates were treated with DNase I, and complete digestion was confirmed by EtBr staining of extracts (data not shown). Interacting proteins were separated by SDS-PAGE.

Multiple bands in the region of 28–400 kDa were observed. Only the predominant bands were isolated for further identifi-
with AR carboxyl terminus. Masses are identified on the proteins. A GST-ARC fragment was incubated with unstimulated (C) or DHT-stimulated (D) LNCaP nuclear extracts as indicated. Bound proteins were identified by SDS-PAGE and Sypro-Ruby stain. Molecular masses are identified on the left, and confirmed proteins are on the right. DBD, DNA binding domain. Pol II, RNA polymerase II; Topo I, topoisomerase I. B, schematic diagram illustrating the regions of AR used in the manuscript. Numbers indicate amino acids. AR functional domains are identified. D+LBD, DNA binding domain and the LBD.

fication by microsequence analysis. These proteins were isolated from the SDS-PAGE, and their identities were determined by proteolytic peptide sequencing. Three of the proteins analyzed belong to the DNA-PK complex; the 470-kDa DNA-PK catalytic subunit, the 70-kDa DNA-PK regulatory subunit Ku70, and the 80-kDa regulatory subunit Ku80. Other proteins identified were 100-kDa poly(ADP-ribose) polymerase, 90-kDa topoisomerase I, and 220-kDa RNA polymerase II polypeptide A, which have also been shown to be associated with hormone receptors (30). The stoichiometry of ARC and DNA-PK interaction is very high, indicating a higher affinity binding. The presence of multiple peptide fragments of each of the components of the DNA complex in tandem mass spectroscopy analysis gave us confidence in the assignment of these molecules as AR-interacting proteins. These interactions appear to be ligand-independent as the association is detected in both DHT-treated and untreated cell extracts. The in vitro interactions of DNA-PK complex with AR were confirmed by in vivo large scale immunoprecipitation of endogenous AR from LNCaP nuclear extracts followed by tandem mass spectroscopy. Table I shows the major co-immunoprecipitated proteins identified by tandem mass spectroscopy. In the molecular range of 70–120 kDa, the major interacting complexes of the androgen receptor are the heat shock protein complex and the DNA-PK complex. Ku70 and Ku80 Directly Interact with AR in Vitro—The DNA-PK interaction was selected as the main focus due to the higher affinity of the complex, the increasing importance of DNA-PK as a nuclear hormone receptor transcriptional regulator (29, 30), and the involvement of Ku in transcriptional recycling (35, 36). To confirm the direct interaction of DNA-PK subunits with AR, GST pull-down assays were employed. GST-ARC was expressed in Escherichia coli and partially purified on GST resin. Ku70, Ku80, and DNA-PKcs were expressed and [35S]methionine-labeled in the rabbit reticulocyte lysate system. Due to the large size of DNA-PKcs (~470 kDa), which rendered the expression in E. coli impossible, DNA-PKcs were expressed in three separate overlapping constructs, the amino-terminal region of DNA-PKcs (N-DNAPK), middle region of DNA-PKcs (M-DNAPK), and carboxyl-terminal region of DNA-PKcs (C-DNAPK) (Fig. 2A) GST-ARC interacted with both Ku70 and Ku80; however, the Ku70 interaction appears to be stronger (Fig. 2B). There appears to be no direct interaction between GST-ARC and any of the fragments of DNA-PKcs. We interpret this to mean that DNA-PKcs association with AR is via Ku70 and Ku80. Importantly, DNase treatment had no effect on the interaction of Ku70 and Ku80 with AR, suggesting the association is not due to contaminating DNA. No interaction was observed when GST resin alone was employed. Once a direct interaction between AR and Ku70 and Ku80 was confirmed, fragments of AR encompassing the DNA binding domain (DBD), ligand binding domain (LBD) and a larger fragment encompassing both the DNA binding domain and the LBD (D+LBD) were constructed (Fig. 1B). The AR fragments along with full-length AR and green fluorescent protein were expressed and [35S]methionine-labeled in rabbit reticulocyte lysate. GST-Ku70 and GST-Ku80 were expressed in E. coli and partially purified on GST resin. Both the AR LBD and D+LBD fragments interacted with both Ku70 and Ku80 (Fig. 2B). The full-length AR had a weaker but noteworthy interaction with Ku70 and Ku80. Inclusion of 10 nM DHT did not enhance interaction with full-length AR. No interaction with GST resin alone was observed. The finding that the AR LBD but not the DNA binding domain is the interacting domain further strengthened the fact that the interaction is not DNA dependent.

Ku70 and Ku80 Directly Interact with AR in Vivo—Co-immunoprecipitation of endogenous AR and Ku70 from LNCaP cells again confirms the AR-DNA-PK interaction (Fig. 3A). LNCaP cells were deprived of androgen for 3 days then either left unstimulated or stimulated with 10 nM DHT for 2 h as described under “Experimental Procedures.” Cells were subsequently lysed, fractionated into cytosolic and nuclear fractions, and immunoprecipitated with anti-AR or anti-Ku70 antibodies. Immunoprecipitates were separated by SDS-PAGE and subjected to immunoblot with anti-DNA-PKcs, anti-AR, anti-Ku80, or anti-Ku70 antibodies. AR interacted strongly with Ku70 in the cytosol in an androgen-independent manner. AR interaction with Ku70 in the nucleus appeared to be enhanced by DHT, likely due to the increase of AR in the nucleus after DHT stimulation. AR interaction with DNA-PKcs appears to be predominately nuclear. This interaction has been confirmed by the colocalization of AR and DNA-PK in LNCaP cells using immunocytochemistry (data not shown).

Further confirmation of the interactions was obtained in Si9 insect cells. Si9 cells were infected individually and in combination with baculovirus carrying FLAG-AR, His-Ku70, and Ku80 for 36–48 h. Cells were lysed and immunoprecipitated with anti-FLAG resin. Distinct interactions were observed between Ku70 and AR and between Ku80 and AR as well as between the Ku homodimer and AR (Fig. 3B). The formation of the AR-Ku complex in Si9 cells is further confirmed by Sypro-Ruby staining of FLAG-immunoprecipitated AR from baculovirus-infected Si9 cells (Fig. 3C).

Ku Homodimer as Well as DNA-PKcs Are Co-activators of Androgen Receptor Activity—To examine the influence of Ku on AR activity, full-length human AR together with PSA-Luc plasmids were transfected into XR-V15B, a cell line lacking Ku80 and Ku70, or its transfected variant, XR-V15B-Ku, which expresses both Ku70 and Ku80. The Renilla luciferase construct served as an internal transfection efficiency control. After transfection cells were treated with 10 nM DHT, and the luciferase levels were quantified. Ku-containing cells showed a substantial increase in AR activity for the 6.0-kilobase PSA reporter construct (Fig. 4A). Ku also enhanced AR activity for the 4 × ARE and mouse mammary tumor virus reporter constructs employed (data not shown). This suggests that Ku enhances AR-mediated transactivation.

Because AR plays a major role in prostate cancer, we sought
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**Table 1**

| Protein                                      | Accession number | Molecular mass | Score |
|----------------------------------------------|------------------|----------------|-------|
| Heat shock protein                           | P08187           | 70.1           | 30.0  |
| Heat shock 70-kDa protein                    | P09874           | 112.9          | 53.0  |
| Androgen receptor                            | P10275           | 98.9           | 62.6  |
| 78-kDa glucose-regulated protein precursor   | P11142           | 70.8           | 84.0  |
| Heat shock protein hsp90                      | P11421           | 72.1           | 73.0  |
| 94-kDa glucose-regulated protein             | P11387           | 90.7           | 30.0  |
| DNA topoisomerase 1                          | P14625           | 92.4           | 30.0  |
| Poly(ADP-ribose) polymerase 1                | P07900           | 84.6           | 20.0  |
| Heat shock protein                            | P12956           | 69.7           | 35.8  |
| Ku70                                         | P10275           | 70.1           | 30.0  |

**Fig. 2. AR interacts with Ku through its ligand binding domain in vitro.** A, the panel shows the interaction of in vitro translated, [35S]methionine-labeled Ku70, Ku80, three fragments of DNA-PKcs identified in C, or green fluorescent protein-negative control with ARC. Labeled proteins were incubated with affinity-purified GST-ARC, GST-ARC with DNase I treatment, or GST resin. Full-length in vitro translated proteins are identified by arrows on the left. N-DNAPK, amino-terminal region of DNA-PKcs; M-DNAPK, middle region of DNA-PKcs; C-DNAPK, carboxyl-terminal region of DNA-PKcs. B, the panel shows the in vitro translated, [35S]methionine-labeled fragments of AR identified in Fig. 1B and green fluorescent protein (GFP)-negative control, which interact with Ku70 and Ku80. Labeled fragments were incubated with affinity-purified GST-Ku70, GST-Ku80, or GST resin. Full-length in vitro translated proteins are identified by arrows on the left. C, schematic diagram illustrating the regions of DNA-PKcs used in the GST pull-down experiments. Numbers indicate amino acids. DNA-PKcs functional domains are identified. FL, full-length.

...to examine the effect of Ku knock-down on AR activity in the prostate cancer cell line. We chose PC-3(AR), a prostate cancer cell line expressing AR, because of its demonstrated androgen response and the relatively high transfection efficiency. To knock down Ku levels, a siRNA duplex against Ku80 was employed. Treatment of the prostate cancer cell line PC-3(AR) with Ku80 siRNA (39) resulted in a reduction of Ku80 expression versus a scrambled control (Fig. 4B). As previously reported, Ku70 expression was also reduced in the Ku80 knockdown cells (39) because the expression of Ku70 is tightly regulated by Ku80. If Ku is involved in AR-mediated transactivation, then endogenous AR activity should diminish subsequent to Ku siRNA transfection. Treatment with two concentrations of siRNA duplex both resulted in reductions in AR activity as measured by a 6.0-kilobase PSA promoter luciferase reporter versus the control (Fig. 4C). These data are all consistent with Ku being a co-activator of AR activity.

Having demonstrated the role of Ku in AR transactivation, we asked whether the associated DNA-PKcs is also important in this process. To examine the influence of DNA-PKcs on AR activity, full-length human AR construct was transiently transfected into CHO V3 (DNA-PKcs mutant) or the parental CHO AA8 (wild type) cells along with AR luciferase reporter constructs. We tested two reporter constructs, 4XARE-luciferase and mouse mammary tumor virus luciferase, suitable for CHO cells. "DNAPK-" cells showed considerable enhancement of AR activity over "DNAPK-" cells for both reporters (Fig. 4D), suggesting DNA-PKcs is also AR transactivator.

*Ku Is Recruited to the PSA Promoter after DHT Stimulation of LNCaP Cells*—If Ku is involved in the AR transcriptional process, one would expect that Ku would associate with the ARE in a manner similar to AR. To determine the association of Ku proteins with the PSA promoter after DHT treatment of LNCaP cells, we performed chromatin immunoprecipitation experiments. Cells were cross-linked with formaldehyde, and after sonication the chromatin to a length of about 500 base pairs, DNA was immunoprecipitated with antibodies that recognize...
Cells were deprived of or stimulated with 10 nM DHT. The previously been shown to be involved in transcriptional recy-
ering three repeats of the androgen response regions of the rat probasin gene (40) immediately upstream of a 360-nucleotide 
portion of the PSA promoter region showed

DNA ends. Nuclear extracts from XRV15B (Ku

Fig. 5. Ku interacts with the PSA promoter and is involved in transcrip-
tion recycling. A, map of the PSA gene identifying the location of primers used in the chromatin immunoprecipita-
tion (IP) assay. B, LNCaP cells were deprived or stimulated with 10 nM DHT. Cells were formaldehyde-cross-linked and sonicated to shear DNA into ~500-bp fragments. Lysates were immunoprecipitated with AR, Ku70, and Ku80 as indicated above. Cross-linking was reversed and protein-
digested, and DNA was amplified using primers identified previously. Antibodies used for immunoprecipitation are indicated above the panels, and primers used for amplification are indicated on the left.

versus secondary initiation was distinguished by the addition of heparin in identified lanes (41). Heparin prevents the formation of new initiation complexes without disrupting actively transcribing complexes. Reactions were allowed to proceed an additional 45 min. Reactions were separated by 6% denaturing polyacrylamide gel electrophoresis (Fig. 6B). The ratio of the band in the absence of heparin to that in the presence indicates the efficiency reinitiation in this system. As shown in Fig. 6B, Ku enhanced AR-mediated transcription of the reporter constructs. The efficiency of reinitiation was much higher when Ku was present. To further confirm these results, XRV15B and

Fig. 6. Ku enhances AR transcription by recycling. In vitro transcription assay to determine the involvement of Ku in transcriptional recycling. The androgen-dependent construct p(ARR3)LovTATA was transcribed in lysates with or without Ku as identified. A, the panel shows the dependence of p(ARR3)LovTATA transcription in a Ku

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Ku Is Involved in Transcriptional Recycling of AR—Ku has previously been shown to be involved in transcriptional recycling (35, 36). The G-less cassette p(ARR3)LovTATA, containing three repeats of the androgen response regions of the rat probasin gene (40) immediately upstream of a 360-nucleotide segment lacking guanosine, was used to determine the effects of Ku in AR recycling. The G-less cassette allows for in vitro transcription to be terminated at the first G residue without linearizing the plasmid. Employing a circular, supercoiled plasmid is essential to more accurately replicating in vivo transcription and to eliminating the possibility of Ku binding to DNA ends. Nuclear extracts from XRV15B (Ku

DNA-PK is a co-activator of AR activity. A, dual luciferase reporter assay representing the fold stimulation in AR activity after DHT stimulation. XRV15B lacking Ku and XRV15B-Ku (expressing Ku) cells were transiently transfected with AR, firefly luciferase reporter with a 6.0-kilobase PSA promoter, and Renilla luciferase control. Cells were deprived of or stimulated with 10 nM DHT. The bar graph represents the -fold stimulation in AR activity after the DHT stimulation. B, Western blot analysis of Ku proteins and tubulin expressed in PC-3(AR) cells transfected with control or Ku80 siRNA. Two different siRNA concentrations used are indicated above. Tubulin was used as a loading control. C, PC-3(AR) cells were transiently transfected with Ku80 siRNA, firefly luciferase reporter with a 6.0-kilobase PSA promoter, and Renilla luciferase control. Cells were deprived of or stimulated with 10 nM DHT. The bar graph represents the -fold stimulation in AR activity after the DHT stimulation. D, CHO V3 (lacking active DNA-PKcs) and CHO AA8 (expressing active DNA-PKcs) cells were transiently transfected with AR, firefly luciferase reporter with 4XARE, or mouse mammary tumor virus (MMTV) promoter and Renilla luciferase control. Cells were deprived of or stimulated with 10 nM DHT. The bar graph represents the -fold stimulation in AR activity after the DHT stimulation.
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XRV15B-Ku cells were transiently transfected with AR (Ku/AR and Ku+/AR, respectively). Nuclear lysates were prepared as before, except with no addition of exogenous AR to the transcription reactions. Lysates were added to transcription reactions, and as previously observed the presence of Ku enhanced the ability of AR to perform multiple rounds of transcription (Fig. 6C). The increased transcription could be attributed to the ∼2-fold greater reinitiation of the Ku-containing extracts. Average -fold increase over heparin-treated cells is noted beneath the lanes. Fold increase is the average of at least three independent experiments. As previously reported (35) Ku− cell lines showed decrease transcription relative to Ku− in heparin-treated lanes. The greater difference for heparin treatment in the Ku− cells is indicative of the involvement of Ku in transcriptional recycling.

**DISCUSSION**

We undertook a targeted discovery of potential factors that comprise the aporeceptor and holoreceptor complexes of the androgen receptor. We began by attempting to identify proteins that interact with the carboxyl termini of the androgen receptor. Mass spectroscopy analysis identified trimeric DNA-PK complex as the major bands interacting with the ARC. DNA-PK consists of the Ku70/Ku80 heterodimer and the catalytic subunit DNA-PKcs. Recently, Ku itself and as part of the DNA-PK complex has emerged as a part of the transcriptional machinery (21, 23, 35, 36). Specifically DNA-PK has been identified as a co-activator of several steroidial transcription factors (22, 29, 30).

We first confirmed the identified interactions by multiple methods. GST pull-down assays and SiF9 cell protein interaction studies indicated that both Ku70 and Ku80 directly and independently interacted with AR; however, no interaction was observed with DNA-PKcs. This could indicate that Ku functions as a docking protein for DNA-PKcs as has been shown for a variety DNA-PKcs substrates (42). Although it has been previously shown that Ku subunits do not independently interact with DNA (43, 44), we felt it necessary, due to the fact both the Ku heterodimer and AR are DNA binding proteins, to DNase-treat interactions to exclude the possibility that the interaction was bridged by DNA. As expected the interactions were not impaired by DNase treatment. Subsequently the interaction was localized to the LBD of AR. It has previously been shown that LXXLL and FXLXXL motifs are responsible for protein interactions with the AR LBD (45–47). Ku70 contains a LXXLL motif, and Ku80 contains the FXLXX motif. However, mutation of the canonical Ku70 LXXLL motif and Ku80 FXLXXL motif does not affect their binding to AR, indicating that they are not important for AR binding (data not shown). This is not totally unexpected because both Ku70 and Ku80 bind AR in the cytoplasm in the absence of ligand, when the hydrophobic groove in the LBD is occupied by the FXLXX of the AR amino-terminal domain.

**After confirmation of the direct interaction,** we examined the role of Ku in AR activity. If Ku is a member of the AR macromolecular complex, Ku might regulate AR transcriptional activity. Luciferase reporter assays indicated that Ku was a positive regulator of AR activity for multiple promoters. Although the cell line employed in these experiments is only mutant for Ku80, Ku70 levels were also greatly reduced or eliminated (19), and expression of exogenous Ku80 rescued Ku70 expression, making it impossible to determine whether the presence of one subunit is sufficient for Ku co-activator activity. These results were further confirmed in the more relevant prostate cell line PC-3(AR), stably expressing exogenous human AR, employing Ku80 siRNA adapted from Belenkov et al. (39). As before, knock down of Ku80 also reduced Ku70 levels, thus complicating interpretation of the data. Even in Si9 cells Ku70 expression was heavily dependent upon Ku80 expression.

We next examined several potential mechanism of Ku coactivator activity. Co-immunoprecipitation assays indicated that Ku interacted with AR both in the cytoplasm and in the nucleus. Both Ku70 and Ku80 contain nuclear localization signals and are actively transported into the nucleus (24, 48, 49). Potentially Ku was involved in nuclear localization of AR after ligand binding. Green fluorescent protein-tagged AR localization was observed both in Ku mutant and Ku rescued cells. Lack of Ku did not appreciably affect AR localization either in the presence or absence of DHT (data not shown), indicating Ku is not involved in nuclear localization of AR. Next we examined the role of the other member of the DNA-PK complex, DNA-PKcs in AR activity. A cell line mutant for DNA-PKcs and the parental cell line were employed in the same AR activity assay used for Ku. DNA-PKcs showed similar co-activator activity to Ku. This indicated that DNA-PKcs may be involved in the coactivator activity of AR. It has been shown previously that Ku can bridge interactions between a protein and DNA-PKcs, allowing DNA-PKcs to phosphorylate the target protein (42). AR contains multiple consensus (S/T)Q DNA-PK phosphorylation motifs clustered mainly in or near the LBD and has also previously been shown that DNA-PK phosphorylates AR (50). Although it is likely that one of the functions of Ku is to bridge the AR- DNA-PKcs interaction, Ku has several other potential co-activator functions. Ku has been identified as a member of the RNA polymerase II complex (24, 25), which also was identified by liquid chromatograph-tandem mass spectroscopy as an ARC-interacting protein. This interaction itself may account for some of the co-activator function. Additionally, Ku has been implicated in the transcriptional reinitiation process (35, 36), which may account for co-activator function.

In an effort to further understand the role of Ku plays in enhancing AR transcription, chromatin immunoprecipitation assays were performed. If Ku were involved in recycling AR, one would expect that Ku would colocalize with AR at an ARE in an androgen-dependent manner to release AR from the ARE after one round of transcription. Both Ku70 and Ku80 were found associated with the PSA promoter in an androgen-dependent manner. This association within the promoter would support the role of Ku in reinitiation. If the role of Ku was limited to DNA-PKcs recruitment, one might expect rapid disassociation after DNA-PKcs phosphorylation. The definitive test of AR recycling is the actual examination of Ku recycling abilities. Ku had previously been implicated in the transcriptional recycling employing several non-steroidal promoters (35, 36). Utilizing an androgen receptor-dependent promoter system, we demonstrated the role of Ku in reinitiation. A Ku-deficient cell line underwent secondary rounds of transcription initiation at a much lower rate than the Ku-rescued cell lines. This was independent of the source of AR. Additionally, XRV15B-Ku86 cells showed a much higher level of transcription.

In addition to the DNA-PK subunits, several other interesting proteins were also found associated to ARC including poly-(ADP-ribose) polymerase and DNA topoisomerase I that have not yet been studied. These factors commonly appear in interactions with other transcription factors (30). Both poly(ADP-ribose) polymerase (51, 52) and topoisomerase I (53, 54) are involved in “preparing” a gene for transcription. It appears the AR can recruit a “pre-initiation” complex of several of the factors required for transcriptional initiation. All these factors, Ku, DNA-PKcs, topoisomerase I, and poly(ADP-ribose) polymerase, have been implicated in cancer. However, by Western blot Ku levels are not significantly altered in several prostate...
cancer cell lines (CWR22R, DU145, LNCaP, PC-3) versus normal prostate epithelial cells (data not shown). Additionally there was no consistent microarray data indicating a substantial change in Ku or DNA-PKcs message levels from normal to various levels of cancerous prostate cells (55, 56). However, this does not imply that DNA-PK does not play a role in prostate cancer progression. It has recently been shown that although there are no significant differences in gene expression for members of the non-homologous end-joining pathway between malignant and non-malignant prostate cells, malignant cells were defective for DNA repair (57). This has many interesting implications for the role of DNA damage in AR-dependent transcription and the role of the AR-DNA-PK interaction in DNA repair.

The continuous recycling hypothesis of steroid receptor regulation (2, 58) is an attractive mechanism for explaining the rapid and modulated response of AR to androgens. Ku meets all identified criteria for being a recycling factor. 1) Ku interacts with the androgen receptor ligand binding domain, 2) Ku is an ATPase, and 3) Ku is involved in transcriptional recycling. The model consists of three phase: signaling, assembly, and disassembly. The recycling factors are proposed to be involved in both the disassembly and signaling phases. Therefore, one would expect that AR and Ku would interact in the cytoplasm and the nucleus both in the presence and absence of androgen, which is exactly what was found. Finally, Ku is a coactivator of AR activity as would be expected for a recycling factor.

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