The putative androgen receptor-A form results from *in vitro* proteolysis

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ABSTRACT

Activation domains in the 114 kDa androgen receptor (AR) NH₂- and carboxyl-terminal regions are thought to contribute to different extents to AR-mediated transactivation. We investigated using anti-peptide antibodies whether smaller AR forms that migrate like the previously described 87 kDa AR-A occur *in vivo* resulting in constitutive or increased gene activation. Immunoblots of prostate cancer and fibroblast cell culture extracts revealed 114 and 84 kDa AR forms. Antibody mapping indicated the 84 kDa AR lacked the ligand-binding domain and comigrated with the constitutively active AR fragment AR1-660. AR expressed in COS cells was 114 and 92 kDa. Migration of the 92 kDa AR was slightly slower than that of a 90 kDa expressed fragment that was designed to initiate at

the second methionine (residue 189) and lacked the NH₂-terminal FxxLF interaction sequence. The 92 kDa AR did not result from alternative initiation since it was observed when the second methionine was changed to alanine. Optimization of extraction conditions indicated that both 84 and 92 kDa forms resulted from *in vitro* proteolytic cleavage and that cleavage by caspase-3 could account for the 92 kDa form. The results suggest that AR forms with gel mobility similar to that of the previously described 87 kDa AR-A result from *in vitro* proteolytic cleavage of NH₂- or carboxyl-terminal regions during cell extraction and storage and that smaller forms with increased transcriptional activity do not occur *in vivo*.

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INTRODUCTION

Steroid hormone receptors occur naturally in multiple forms. These include A and B forms of the progesterone receptor (PR) (Horwitz & Alexander 1983, Krett *et al.* 1988, Kastner *et al.* 1990b), estrogen receptor α and β (Kuiper *et al.* 1996, Mosselman *et al.* 1996), glucocorticoid receptor (GR) α and β (Hollenberg *et al.* 1985, Oakley *et al.* 1996), and most recently, GR-A and -B (Yudt & Cidlowski 2001). Estrogen receptor and GR α and β are products of separate genes (Kuiper *et al.* 1996, Oakley *et al.* 1996) whereas PR-A and -B result from alternative promoter usage of the same gene (Kastner *et al.* 1990b) and GR-A and -B derive from alternative initiation at the first and second

methionines (Yudt & Cidlowski 2001). The shorter 769 amino acid PR-A lacks 164 NH₂-terminal amino acid residues whereas full-length PR-B is 933 amino acid residues in length (Horwitz & Alexander 1983, Krett *et al.* 1988, Kastner *et al.* 1990b). The two PR forms can be present at nearly equimolar amounts (Horwitz & Alexander 1983, Feil *et al.* 1988) and are hormonally and developmentally regulated (Spelsberg & Halberg 1980, Kato *et al.* 1993, Mangal *et al.* 1997). Differences occur in their subcellular localization (Lim *et al.* 1999), activation by cAMP (Sartorius *et al.* 1994) and extent of phosphorylation (Clemm *et al.* 2000).

A and B forms were also reported for the androgen receptor (AR). An 87 kDa AR-A lacked part of the NH₂-terminal region analogous to PR-A

and comigrated with an expressed fragment that initiated at the second methionine at residue 189 (Wilson & McPhaul 1994). A survey of tissues indicated different ratios of the two AR forms, but in contrast to PR, the amounts of AR-A were only ~10% of the amount of intact AR (Wilson & McPhaul 1996, Gao & McPhaul 1998). It was postulated that, like PR, AR-A occurs *in vivo* and has functional properties distinct from full-length AR (Wilson & McPhaul 1994, Gao & McPhaul 1998). Previous studies relied in part on AR expression where the sequence flanking the second methionine was altered to increase initiation at this position (Gao & McPhaul 1998). Multiple forms of the AR were also reported in several fish species including rainbow trout (Takeo & Yamashita 1999) and the Japanese eel (Ikeuchi *et al.* 1999). AR β in rainbow trout fails to bind androgen whereas eel AR β is functionally active. The lack of an additional AR sequence forthcoming from the human genome database combined with the phenotype of the syndrome of androgen insensitivity (Quigley *et al.* 1995) strongly supports the presence of only one active AR gene in humans.

Whether alternative forms of AR are expressed that lack either the NH₂- or carboxyl-terminal region is critical to understanding AR activity *in vivo*. For example, deletion of the carboxyl-terminal domain could result in a constitutively active receptor that increases androgen-regulated gene activation independently of ligand. Similarly, partial deletion of the NH₂-terminal region as suggested for the previously reported AR-A would delete the ²³FQNLF²⁷ sequence, which upon AR binding of high-affinity androgens, interacts with activation function 2 (AF2) in the ligand-binding domain (He *et al.* 1999). Interaction of ²³FQNLF²⁷ with AF2 in the NH₂- and carboxyl-terminal interaction may limit AR activation by p160 coactivators (He *et al.* 2000, 2001). Loss of this NH₂-terminal region in AR-A could potentially expose AF2 to enhanced activation and increased AR-mediated gene activation.

Our earlier investigations into multiple forms of AR indicated that they result from proteolytic degradation during isolation (Wilson & French 1979). However, these studies were performed prior to the cloning of AR complementary DNA (Chang *et al.* 1988, Lubahn *et al.* 1988) and before the availability of anti-peptide antibodies directed against specific sequences in the AR (Tan *et al.* 1988, Quarman *et al.* 1990, Prins *et al.* 1991, Zegers *et al.* 1991). We therefore reevaluated the origin of the 87 kDa AR-A reported in various tissues and cell lines to determine whether these forms occur *in vivo* or are a result of proteolytic degradation.

In agreement with previous reports (Wilson & McPhaul 1994, 1996, Gao & McPhaul 1998), AR forms that migrate like AR-A were detected in cell and tissue extracts, particularly in frozen extracts prepared in the absence of dihydrotestosterone (DHT). By the analysis of several AR mutants and the use of anti-peptide antibodies specific to different regions of the AR and extraction conditions that minimize proteolysis, the data indicate that the previously described 87 kDa AR-A and forms with similar migration by SDS gel electrophoresis result from *in vitro* proteolysis of the NH₂- or carboxyl-terminal region. The results further indicate that the presence of AR-A could be accounted for by *in vitro* cleavage at a predicted caspase-3 cleavage site in the NH₂-terminal domain.

MATERIALS AND METHODS

Materials

LNCaP, PC3 and COS-1 cell lines were purchased from the American Type Culture Collection (Rockville, MD, USA) and LNCaP-C4-2 cells from UroCor, Inc. (Oklahoma City, OK, USA). Control human foreskin fibroblast cultures (HFF-1) were previously described (Van Wyk *et al.* 1985, De Bellis *et al.* 1994). Fetal bovine serum was purchased from Hyclone Laboratories, Inc. (Logan, UT, USA), and DHT, antibiotics and cell culture media reagents were from Sigma Corp. (St Louis, MO, USA) or Gibco-BRL (Life Technologies, Grand Island, NY, USA).

Tumor transplantation

Male athymic nude mice 4–5 weeks of age were obtained from Harlan Sprague Dawley, Inc. (Indianapolis, IN, USA). CWR22 tumors were transplanted s.c. as dissociated cells in Matrigel (Wainstein *et al.* 1994, Nagabhushan *et al.* 1996) into mice containing testosterone pellets s.c. (12.5 mg for sustained release of ~10 µg testosterone/day; Innovative Research of America, Sarasota, FL, USA) to normalize mice to serum testosterone levels at 4 ng/ml. Intact mice bearing androgen-stimulated tumors and recurrent CWR22 tumors resected 150 days after castration were exposed to methoxyflurane and killed by cervical dislocation. Tumors were removed immediately and frozen in liquid nitrogen. Animal procedures were approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Cell culture, plasmids and transfections

LNCaP cells were maintained in RPMI medium containing 10% fetal bovine serum (Pietrzkowski *et al.* 1993). The LNCaP-C4-2 cell line derived from LNCaP cells after prolonged culture in the absence of androgen (Horoszewicz *et al.* 1983, Thalmann *et al.* 1994, Wu *et al.* 1994) was grown in T medium (DMEM/F12 with 5% fetal bovine serum, 5 µg/ml insulin, 13.65 pg/ml triiodothyronine, 5 µg/ml apotransferrin, 0.244 µg/ml d-biotin, and 25 µg/ml adenine). The CWR-R1 cell line developed from a recurrent CWR22 human prostate cancer xenograft (Gregory *et al.* 2001b) was maintained in prostate growth medium (Presnell *et al.* 1998) comprised of Richter's Improved MEM (Irvine Scientific, Santa Ana, CA, USA) supplemented with 10 mM nicotinamide, 20 ng/ml epidermal growth factor, 5 µg/ml transferrin, 5 µg/ml insulin, 5 ng/ml selenium, 100 units/ml penicillin, 100 µg/ml streptomycin, 0.25 µg/ml fungizone and 2% fetal bovine serum. Monkey kidney COS-1 cells were maintained in DMEM with high glucose and 10% bovine calf serum, 20 mM Hepes, pH 7.2, L-glutamine and antibiotics. COS cells (1.2×10^6 cells/10 cm dish) were transfected with wild-type and mutant pCMVhAR expression vector DNA (10 µg) using DEAE dextran (Yarborough *et al.* 1990). Human prostate cancer PC3 cells were maintained in DMEM/F-12 containing 10% fetal bovine serum, 15 mM Hepes, pH 7.2, L-glutamine and antibiotics. PC3 cells were transfected with pCMVhAR (0.25 µg) using Effectine Transfection Reagent (Qiagen, Valencia, CA, USA) according to manufacturer's instructions. HFF-1 human foreskin fibroblasts were maintained in DMEM with high glucose containing 10% fetal bovine serum, L-glutamine, non-essential amino acids and antibiotics. HFF-1 cells derived from a primary culture of normal human foreskin were passed at least 14 times. Cell lines were passed every 4 days using trypsin/EDTA (Gibco-BRL).

Human AR expression vector pCMVhAR was modified to delete the first 188 residues. AR189–919a deletion mutant was prepared by digesting pCMVhAR with BglII and AflII, followed by blunt ending and ligation. This construct deletes the first methionine and leaves the endogenous sequence that flanks the second methionine at residue 189 for initiation. A second mutant AR189–919b was created by inserting 5' flanking sequence of the first methionine prior to the second methionine. This was done using an oligonucleotide primer similar to that previously described (Gao & McPhaul 1998) that contains a 5' BglII site followed by 28 base pairs of 5' flanking non-coding sequence, ATG

codon 189 and subsequent human AR sequence. pCMVhAR was digested with BglII and BstEII and ligated with the BglII/BstEII PCR amplified mutant insert. No significant differences in expression levels were noted for the two AR189–919 plasmids, suggesting that the sequence flanking the second methionine is competent for initiation in the absence of sequence coding for the first methionine. To create pCMVhAR-M189A, methionine 189 was changed to alanine in full-length AR using PCR mutagenesis within an AflII/BstEII fragment which was ligated into pCMVhAR digested with the same enzymes. pCMVhAR156–919 was created by PCR mutagenesis by amplifying a BglII/BstEII fragment with an ATG nucleotide sequence placed prior to the sequence coding for residue 156, followed by ligation into pCMVhAR digested with the same enzymes. pCMVhAR1–660 that expresses the AR NH₂-terminal and DNA-binding regions and part of the hinge region was previously described (Simental *et al.* 1991). All regions amplified by PCR using Vent-polymerase (BioRad, Hercules, CA, USA) were verified by DNA sequencing.

Immunoblot analysis

Tissue and cell lysates were prepared in RIPA buffer containing protease inhibitors (1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecyl sulfate, 0.15 M NaCl, 5 mM EDTA, 50 mM Tris-HCl pH 7.4 containing 0.5 mM phenylmethylsulfonyl fluoride, 10 µM pepstatin, 80 mg/ml leupeptin, 4 µM aprotinin, 0.2 mM sodium vanadate and 5 mM benzamidine) with and without 1 µM DHT as indicated in the figure legends. Tissue was homogenized 30 s on ice and incubated 30 min on ice followed by two centrifugations at 10 000 *g* for 20 min each. Cell lines (CWR-R1, LNCaP, LNCaP-C4-2, PC3, HFF-1 and COS) were harvested from 10 cm dishes by scraping in PBS and centrifugation at 4 °C for 10 min to pellet the cells. RIPA with protease inhibitors with or without 1 µM DHT was added to cell pellets and lysates were prepared by passing the cell suspensions through 22-gauge needles five times and trituration. Lysates were incubated on ice for 30 min followed by centrifugation at 10 000 *g* for 20 min. Supernatants were used with or without freezing and storage at –20 °C as indicated prior to separation on polyacrylamide gels containing SDS. Proteins were electroblotted to nitrocellulose membranes.

AR32 rabbit polyclonal antibody was raised against a peptide with sequence of human AR amino acid residues 9–28 (Quarmby *et al.* 1990). PG21 is a rabbit anti-peptide antibody raised

against a peptide with sequence of AR NH₂-terminal residues 1–21 (Prins *et al.* 1991) (Research Diagnostics, Inc., Flanders, NJ, USA). AR52 rabbit polyclonal antibody was raised against a peptide with a sequence of human AR residues 544–558, which is NH₂-terminal to the DNA-binding domain (Tan *et al.* 1988). AR32 and AR52 IgG fractions were prepared and used for immunoblotting at 1 µg/ml. Mouse monoclonal antibody F39·4·1 (F39) was raised against a peptide with sequence derived from human AR NH₂-terminal residues 302–321 (Zegers *et al.* 1991) (Biogenex, San Ramon, CA, USA) and used for immunoblots at 1:10 000 dilution. Rabbit polyclonal antibody C19 was raised against a peptide with sequence of human AR carboxyl-terminal residues 901–919 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and used for immunoblotting at 0·2 µg/ml. Secondary antibody (goat-anti-mouse IgG or goat anti-rabbit IgG conjugated to horseradish peroxidase (Amersham Corp., Arlington Heights, IL, USA)) was used for detection by enhanced chemiluminescence (Pierce, Rockford, IL, USA).

RESULTS

AR in cell lines and tissues

Immunoblot analysis revealed a predominant 114 kDa full-length AR in extracts of prostate cancer cell lines LNCaP, LNCaP-C4-2 and CWR-R1 (Fig. 1, lanes 1–3). Full-length AR was also evident in extracts of the CWR22 human prostate cancer xenograft propagated in nude mice (Wainstein *et al.* 1994, Nagabhushan *et al.* 1996, Tan *et al.* 1997) from both androgen-dependent tumors and recurrent tumors that arose after prolonged androgen deprivation by castration (Fig. 1, lanes 5–6). In addition, an 84 kDa immunoreactive band was detected in the CWR-R1 cell line and CWR22 recurrent tumor extracts. There were only small amounts of the 84 kDa form in LNCaP and LNCaP-C4-2 cell lines and the androgen-dependent CWR22 tumor extracts (Fig. 1). Cultures of normal human foreskin fibroblasts showed the predominant 114 kDa AR with minor amounts of the smaller form (Fig. 1, lane 4). In contrast, immunoblot analysis of human AR transiently expressed in PC3 prostate cancer cells resulted in only the 114 kDa band detected when DHT was included in the incubation media (Fig. 2A, lane 3). The increase in transiently expressed AR in PC3 in the presence of DHT most likely resulted from AR stabilization by androgen as we showed previously that AR is unstable in the absence of androgen (Kemppainen *et al.* 1992). AR

FIGURE 1. Immunoblot of endogenous AR from cell lines and CWR22 prostate tumor xenografts. Protein lysates (10 µg/lane) from LNCaP, LNCaP-C4-2, CWR-R1 and human foreskin fibroblast (HFF) cell lines and androgen-dependent (CWR22) and recurrent CWR22 tumors were analyzed on immunoblots using AR monoclonal antibody F39 (epitope 302–321). Lysates were prepared in RIPA buffer without DHT and analyzed after storage at –20 °C. Migration positions of the major AR bands at 114 (full-length) and 84 kDa (arrows) and molecular mass markers are indicated. The data shown are representative of five separate immunoblots.

expression in COS cells in the presence of DHT revealed the 114 kDa full-length AR and an additional 92 kDa immunoreactive band (Fig. 2B, lane 1). No AR bands were detected in COS (not shown) or PC3 cells (Fig. 2A, lane 1, pCMV5) when transfected with the parent vector that lacked AR coding sequence as previously reported (Kaighn *et al.* 1979, Tilley *et al.* 1990). Since the electrophoretic migration of both smaller forms was similar to that of the previously described 87 kDa AR-A (Wilson & McPhaul 1994, 1996, Gao & McPhaul 1998), we investigated their origin and domain composition.

Anti-peptide antibodies that react with specific epitopes within the AR sequence were used on immunoblots to detect the presence of the different AR domains. These included NH₂-terminal antibodies PG21 (epitope at amino acid residues 1–21) (Prins *et al.* 1991), AR32 (epitope 9–28) (Quarmby *et al.* 1990), F39 (epitope 302–321) (Zegers *et al.* 1991) and AR52 (epitope 544–558) (Tan *et al.* 1988), as well as carboxyl-terminal antibody C19 (epitope 901–919) (Fig. 3). We also addressed whether the smaller AR forms observed above resulted from initiation at the second methionine at residue 189 in a manner analogous to that of PR-A (Kastner *et al.* 1990b) and as previously suggested for AR-A (Wilson & McPhaul 1994). Two different AR189–919 mutants (a and b) were created so that

FIGURE 2. Immunoblots of AR and AR mutants transiently expressed in PC3 and COS cells. (A) Human prostate cancer PC3 cells were transfected with empty parent vector pCMV5 (p5) or with wild-type human AR expression vector pCMVhAR (AR). PC3 cells were incubated in the absence (lane 2) and presence (lane 3) of 10 nM DHT for 24 h prior to harvest. Protein lysates were prepared in RIPA buffer containing 1 μ M DHT and 10 μ g protein samples were analyzed on immunoblots using AR polyclonal antibody C19 (epitope 901–919). (B) COS cells were transfected with the following AR expression vectors: wild-type pCMVhAR (AR, lane 1), pCMVhAR189–919a in which amino acid residues 1–171 were deleted to retain the flanking sequence of methionine 189 (lane 2), pCMVhAR189–919b where 28 nucleotides of sequence derived from the 5' flanking sequence of the first methionine were inserted before the second methionine at residues 189 (lane 3), and pCMVhARM189A, in which methionine 189 was changed to alanine in full-length pCMVhAR (lane 4). Transfected COS cells were incubated with 500 nM DHT 24 h prior to harvest. Lysates were prepared in RIPA buffer containing 1 μ M DHT and 10 μ g samples were analyzed on immunoblots using AR polyclonal antibody C19 (epitope 901–919). Migration positions of the major AR bands (arrows on right) and molecular mass markers (left) are indicated. The data are representative of two to four separate experiments.

the first NH₂-terminal 188 amino acid residues were not expressed (Fig. 3, AR189–919). The coding sequence in pCMVhAR for residues 1–171 was deleted by restriction enzyme digestion (BglII/AflII) in AR189–919a so that the wild-type sequence flanking methionine 189 was retained. For AR189–919b we used PCR mutagenesis to insert

the 28 nucleotide sequence 5' of the first methionine codon in front of the codon for the second methionine at residue 189. Expression of both recombinant AR189–919a and b mutants resulted in 90 kDa bands that were indistinguishable or slightly smaller than the fragment observed when full-length AR was expressed in COS cells (Fig. 2B, lanes 1–3). The results indicate that under these expression conditions, the naturally occurring flanking sequence of the second methionine is sufficient for robust initiation of a 90 kDa truncated AR in a construct in which the coding sequence of the first methionine and subsequent amino acids was deleted.

Data from the AR189–919 mutants raised the possibility that the smaller AR bands observed in cell and tissue extracts and in transient expression studies resulted from initiation at methionine 189. To address this, a full-length AR expression construct was created in which the second methionine at residue 189 was replaced by alanine. Surprisingly, expression of AR-M189A yielded a similar proportion of full-length 114 kDa AR and the 92 kDa immunoreactive band (Fig. 2B, lane 4). The results indicate that the smaller form did not result from initiation at the second methionine.

Analysis of AR fragments using AR-specific anti-peptide antibodies

Peptide-specific AR antibodies were used to probe immunoblots to establish whether the smaller AR forms detected in cell and tumor extracts resulted from the absence of the NH₂- or carboxyl-terminal region. The positions of the peptides used in raising the antibodies are shown relative to the AR sequence (Fig. 3). The 84 kDa AR band present in CWR-R1 cell extracts was detected using AR NH₂-terminal antibodies AR32 and PG21, but was not detected with AR carboxyl-terminal antibody C19 (Fig. 4). The 84 kDa band from the CWR-R1 cell extracts was also detected with NH₂-terminal domain antibodies F39 (see Fig. 1) and AR52 (not shown). Consistent with these results, the 84 kDa band comigrated with AR deletion mutant AR1–660 (Fig. 4, last lane) that comprises the AR NH₂-terminal, DNA-binding domain and hinge regions and lacks the carboxyl-terminal ligand-binding domain (see Fig. 3). Thus the 84 kDa form observed in the prostate cancer cell lines and tumors lacks the carboxyl-terminal ligand-binding domain. Based on previous transient transfection studies using AR1–660 (Simental *et al.* 1991), the presence of this 84 kDa fragment *in vivo* would result in constitutive AR transcriptional activity.

In contrast, the 92 kDa band evident after expression of AR in COS cells was not detected

FIGURE 3. Schematic diagram of wild-type and mutant human AR coding regions, the position of antibody epitopes and apparent molecular masses. Regions from which peptides were designed and used to produce anti-peptide AR antibodies PG21, AR32, F39, AR52 and C19 are indicated above the diagram. Diagrams of the coding regions are shown for full-length wild-type AR amino acid residues 1–919 (WT), AR1–660 comprising the NH₂-terminal, DNA-binding domain and hinge region amino acid residues 1–660, AR156–919 in which there is deletion of 155 residues of the NH₂-terminal sequence, representing a caspase-3 cleavage site, and AR189–919 designed to initiate at the second methionine at residue 189. AR189–919a and AR189–919b were designed as described in Fig. 2 legend and in Materials and Methods. Positions of the first (*Met 1*) and second (*Met 189*) methionines are indicated as well as the amino acid residue boundaries of the NH₂-terminal (gray), DNA-binding domain (white), hinge region (black), steroid-binding domain (hatched) and apparent molecular masses (kDa) determined by SDS gel electrophoresis.

by AR NH₂-terminal antibody AR32 but was recognized by antibodies F39, AR52 and C19 (Fig. 5A). An additional minor band was detected by the F39 antibody forming a doublet of the lower band (Fig. 5A, lane 2) and likely resulted from the greater sensitivity of the F39 antibody. The results

are consistent with observations above (see Fig. 2B) that the 92 kDa AR fragment is similar to the expressed mutant AR189–919 that was designed to initiate at the second methionine. The 92 kDa fragment therefore lacks slightly fewer than 190 amino acid residues from the AR NH₂-terminus

FIGURE 4. Immunoblots of endogenous AR from the CWR-R1 human prostate cancer cell line and the AR1–660 deletion mutant expressed in COS cells. CWR-R1 cells were cultured in the absence and presence of 10 nM DHT as indicated for 48 h prior to harvest. COS cells were transfected as described in Materials and Methods with pCMVhAR1–660, which codes for the NH₂-terminal, DNA-binding domain and hinge regions (see Fig. 3). Immunoblots of 10 µg protein extracts prepared in the presence of 1 µM DHT were analyzed using anti-peptide antibodies AR32 (epitope 9–28), PG21 (epitope 1–21) and C19 (epitope 901–919) as indicated. AR32 antibody was used for AR1–660. Positions of the major AR bands (arrows on right), molecular mass markers (left) and the antibodies used for detection are indicated. The data are representative of three separate experiments.

FIGURE 5. Immunoblots of wild-type AR and deletion mutant AR156–919 transiently expressed in COS cells. Protein samples were analyzed from cells incubated in the presence of 500 nM DHT 24 h prior to harvest in RIPA buffer containing 1 μ M DHT. (A) COS cell extracts (10 μ g protein) after expression of full-length pCMVhAR were analyzed on immunoblots using AR antibodies AR32 (epitope 9–28), F39 (epitope 302–321), AR52 (epitope 544–558) and C19 (epitope 901–919) as indicated. (B) Full-length pCMVhAR (AR) and deletion fragment pCMVhAR156–919 were transiently expressed in COS cells and cell extracts (10 μ g protein) were analyzed on immunoblots using antibody C19 (epitope 901–919). Positions of the major AR bands (arrows on right) and molecular mass markers (left) are indicated. The data are representative of two or three separate experiments.

and, as shown above, does not result from initiation at methionine 189. The results indicate that, depending on the cell type, AR forms that migrate like the previously described AR-A (Wilson & McPhaul 1994, 1996, Gao & McPhaul 1998) lack either the NH₂- or carboxyl-terminal region.

In vitro proteolytic degradation

To investigate whether the smaller AR forms result from *in vitro* proteolytic degradation during tissue or cell extraction or sample storage, extraction conditions were modified and frozen extracts compared with freshly prepared samples. When CWR-R1 cells were extracted in the same RIPA buffer so as to maintain 4 °C in the presence of 1 μ M DHT and extracts immediately analyzed on immunoblots, only the single full-length 114 kDa AR band was observed (Fig. 6, lane 1). Similar analysis of LNCaP and LNCaP-C4-2 cells also revealed the full-length AR and almost undetectable bands for the smaller forms (Fig. 6, lanes 3 and 5). If the same extracts were stored frozen overnight

FIGURE 6. Immunoblot of endogenous AR from CWR-R1, LNCaP and LNCaP-C4-2 cell lines. CWR-R1 (lane 1), LNCaP (lane 3) and LNCaP-C4-2 cells (lane 5) were extracted in ice-cold PBS on ice in RIPA buffer containing 1 μ M DHT and samples were analyzed immediately on gels. In lane 2, a parallel sample of CWR-R1 extract was prepared as in lane 1 but was stored frozen overnight at –20 °C prior to analysis (lane 2, 1 $\times$). Lysates of LNCaP cells (lane 4) and LNCaP-C4-2 cells (lane 6) were stored frozen for 3 weeks at –20 °C during which time they were thawed and frozen five times (5 $\times$) prior to analysis. The immunoblot was probed with F39 monoclonal antibody (epitope 901–919). Positions of the major AR bands (arrows on right) and molecular mass markers (left) are indicated. The data shown are representative of two or three separate experiments.

and reanalyzed, the 84 kDa form was prominent with a minor 92 kDa band (Fig. 6, lane 2). After repeated freezing and thawing and storage, shown for LNCaP and LNCaP-C4-2 extracts (Fig. 6, lanes 4 and 6), the 84 kDa fragment was predominant with a minor 92 kDa band, whereas the full-length 114 kDa AR was not detected. Similar results were obtained after storage of the CWR22 cell line extracts (data not shown). DHT in the extraction media increased the recovery of full-length 114 kDa AR and reduced the amount of the smaller forms (data not shown); however, the presence of a cocktail of standard protease inhibitors which was present in all of the studies (see Materials and Methods) was not sufficient to block the *in vitro* breakdown of AR when samples were stored and later analyzed. Similar extraction and immunoblot studies of AR expression in COS cells indicated that the 92 kDa band results from proteolytic cleavage during extraction.

An *in vitro* caspase-3 cleavage site was reported at human AR aspartic acid residue 146 (Ellerby *et al.* 1999). Because of the polymorphic length of the NH₂-terminal glutamine repeat (Choong & Wilson 1998), numbering of AR amino acid residues can vary. Based on the AR residue numbering of Lubahn *et al.* (1988) which is used by the AR mutation database (Gottlieb *et al.* 1998), and considering the consensus sequences for caspase cleavage (Earnshaw *et al.* 1999), caspase-3 cleavage would occur between human AR aspartic acid

residue 155 and serine 156. To determine whether caspase cleavage could account for the 92 kDa fragment, we compared the migration of AR deletion mutant AR156–919 (see Fig. 3) with AR fragments observed in the COS cell extracts. Migration of the AR156–919 fragment, which simulates the AR fragment resulting from a proposed caspase cleavage site, was at 92 kDa (Fig. 5B) and thus it comigrated with the 92 kDa fragments described above and migrated slightly faster than the AR fragments that initiate at the second methionine. The results suggest that during preparation and analysis, smaller forms of AR evident in cell and tissue extracts result from proteolytic cleavage of the NH₂- or carboxyl-terminal domain, resulting in fragments with electrophoretic migration similar to that of the previously described AR-A.

DISCUSSION

We investigated whether AR occurs in physiologically relevant smaller forms as described previously for PR (Horwitz & Alexander 1983, Krett *et al.* 1988, Kastner *et al.* 1990b). The PR gene was shown to initiate at the first or second methionine, depending on promoter usage (Kastner *et al.* 1990b). A short 87 kDa form designated AR-A was also reported to be physiologically significant, but in contrast to PR, the smaller form of AR was detected in quantities only about 10% of those of the full-length 114 kDa AR (Wilson & McPhaul 1994, 1996, Gao & McPhaul 1998). We observed the 114 kDa full-length AR and an 84 kDa AR fragment in extracts from LNCaP and CWR22 human prostate cancer cell lines, from xenograft tumor samples and from normal human foreskin fibroblast cultures. Peptide-specific antibodies indicated that the smaller 84 kDa AR fragment lacked the carboxyl-terminal ligand-binding domain. Full-length AR transiently expressed in COS cells revealed the 114 kDa AR and a 92 kDa form that migrated similarly to AR mutants that were created to initiate at the second methionine. However, when the second methionine was changed to alanine by PCR mutagenesis in the full-length AR expression vector, the 92 kDa form was again observed, indicating that the smaller form did not result from initiation at the second methionine. Peptide-specific AR antibodies provided evidence that the 92 kDa form lacked part of the NH₂-terminal region as suggested previously for AR-A (Wilson & McPhaul 1994, 1996, Gao & McPhaul 1998) and could be accounted for by *in vitro* cleavage at a caspase-3 site. Both the 84 and 92 kDa forms were eliminated by the use of extraction and

storage conditions that minimized *in vitro* proteolytic degradation, indicating that the putative A form of AR most likely results from *in vitro* proteolytic cleavage of the carboxyl- or NH₂-terminal regions during extraction. While it cannot be definitively ruled out that very small amounts of AR-A form through alternative pathways, the data suggest that differences in intensity of AR-A previously reported in different tissues (Gao & McPhaul 1998) reflect tissue- and cell-specific differences in protease activity during extraction and storage of protein extracts rather than differences in expression of multiple AR forms. Absence of the smaller forms after AR expression in prostate cancer PC3 cells suggests that PC3 cells have less protease activity during cell extraction.

The consensus Kozak initiation sequence for optimal translation initiation is A/GNNATGGNN (Kozak 1999), where N can be any nucleotide. The first translation initiation sequence for human, mouse and rat AR (A⁻³GGATGG⁺⁴AA/G) conforms to this consensus sequence, whereas sequence flanking the second methionine does not (A⁻³CCATGC⁺⁴AA) (Table 1). According to a leaky scanning theory proposed by Kozak (1999), translation does not initiate from a second weaker start site of a single transcript when the first start site conforms to the consensus sequence. Initiation at a second methionine can occur, however, when the sequence flanking the second methionine more closely adheres to the consensus sequence than does the first initiation start site, as recently reported for GR (Yudt & Cidlowski 2001). In support of the model, mutagenesis to create a consensus translation initiation sequence for the first ATG in GR abolished expression from the second ATG site (Yudt & Cidlowski 2001). The leaky scanning mechanism is also supported by the inability of chicken and human PR-A to be translated from a single PR-B transcript (Kastner *et al.* 1990a). The first translation initiation site in human and chicken PR-B conforms to the Kozak consensus translation initiation sequence better than the second start site sequence (Table 1). Thus, although transient expression studies presented here indicated that the second AR methionine 189 could initiate translation when the coding sequence for the first methionine was deleted, the leaky scanning hypothesis predicts that the presence of a consensus translation initiation sequence at the first methionine in AR inhibits initiation at the second methionine. The results of the present study support the concept that smaller AR forms do not result from initiation at the second methionine.

While our studies cannot rule out the possibility of a low abundance alternative splice variant for AR,

TABLE 1. Nucleotide sequences for the consensus Kozak translation initiation sequence (Kozak 1999), and for the first and second possible translation initiation start sites for the human AR (Lubahn *et al.* 1988), mouse AR (Charest *et al.* 1991), rat AR (Tan *et al.* 1988), human PR (Kastner *et al.* 1990a,b), chicken PR (Conneely *et al.* 1987, Gronemeyer *et al.* 1987) and human GR (Hollenberg *et al.* 1985)

	Species	1st start site			2nd start site		
		A/GNN	ATG	GNN	A/GNN	ATG	GNN
AR	Human	AGG	ATG	GAA	ACC	ATG	CAA
	Mouse	AGG	ATG	GAG	ACC	ATG	CAA
	Rat	AGG	ATG	GAG	ACC	ATG	CAA
PR	Human	GTC	ATG	ACT	CTC	ATG	AGC
	Chicken	AGC	ATG	ACC	CCG	ATG	AGC
GR	Human	CTG	ATG	GAC	GTC	ATG	GAC

the data are consistent with AR being highly susceptible to proteolytic cleavage. Further analysis of the transcriptional activity of the smaller AR forms was therefore not pursued, although the results of cotransfection assays indicated that the NH₂-terminally truncated mutant AR189–919 lacked dominant negative activity in contrast to the dominant negative effect of PR-A on PR-B activity (Tung *et al.* 1993, Vegeto *et al.* 1993). A constitutively active AR-A form that lacks the carboxyl-terminal ligand-binding domain in CWR22 and LNCaP prostate cancer cell lines therefore cannot explain the increased expression of androgen-regulated genes observed in recurrent tumor models (Gregory *et al.* 1998), which could result from p160 coactivator overexpression (Gregory *et al.* 2001a). The data similarly argue against an NH₂-terminally deleted form as previously postulated for AR-A, which could be accounted for by *in vitro* caspase-3 cleavage. Because cleavage by caspase-3 removes the NH₂-terminal ²³FQNLF²⁷ sequence that interacts with AF2 in the ligand-binding domain (He *et al.* 2000), it was important to establish whether this occurs *in vivo* or *in vitro*. *In vivo* cleavage could allow AF2 in the ligand-binding domain to more efficiently recruit p160 coactivators (He *et al.* 2000).

AR differs from other steroid receptors in having a single copy gene on the X chromosome, and in males, a single allele. AR apparently also appears to differ from other steroid receptors by occurring only in its full-length form when other receptors have multiple genes or forms. Retention of the entire NH₂-terminal domain is required for the androgen-induced AR NH₂- and carboxyl-terminal interaction (Langley *et al.* 1995, 1998). The androgen insensitivity syndrome highlights the singular importance of the AR gene in male sexual development (Quigley *et al.* 1995) and the essential

role of an ever increasing number of amino acid residues in the DNA- and steroid-binding domains. Substitution mutations of more than 200 amino acids cause different degrees of androgen resistance (Gottlieb *et al.* 1998). The absence, with few exceptions (Choong *et al.* 1996, Ghadessy *et al.* 1999), of single missense mutations in the NH₂-terminal domain associated with androgen insensitivity is striking, considering the critical role of this region in AR transcriptional activity. The studies presented here support the requirement for full-length AR expression *in vivo*.

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