In vitro translation of androgen receptor cRNA results in an activated androgen receptor protein

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Translation of androgen receptor (AR) cRNA in a reticulocyte lysate and subsequent analysis of the translation products by SDS/PAGE showed a protein with an apparent molecular mass of 108 kDa. Scatchard-plot analysis revealed a single binding component with high affinity for R1881 (K_d = 0.3 nM). All AR molecules synthesized specifically bound steroid. No evidence for AR phosphorylation during *in vitro* synthesis was found. When AR was labelled with [^{3}H]R1881 and analysed on sucrose-density gradients, a complex of approx. 6 S was observed. The complex was shifted to a higher sedimentation coefficient after incubation with a monoclonal AR antibody directed against an epitope in the DNA-binding domain. In the presence as well as the absence of hormone, AR molecules were able to bind to DNA–cellulose without an activation step. Gel retardation assays revealed that the AR forms complexes with a DNA element containing glucocorticoid-responsive element/androgen-responsive element sequences. Receptor–DNA interactions were stabilized by different polyclonal antibodies directed against either the N- or C-terminal part of the AR and were abolished by an antibody directed against the DNA-binding domain of the receptor. In conclusion, translation of AR cRNA *in vitro* yields an activated AR protein which binds steroid with high affinity. It is proposed that AR antibodies enhance AR–DNA binding by stabilizing AR dimers when bound to DNA.

INTRODUCTION

The androgen receptor (AR) can be isolated in the cytosol of target cell extracts as a large non-activated (i.e. non-DNA-binding) 8 S complex. Recently, it was shown that this complex contains the 90 kDa heat-shock protein (hsp90), the 70 kDa heat-shock protein (hsp70) and a 56–59 kDa protein [1]. This large multiprotein non-activated complex dissociates on hormone binding, thereby revealing the DNA-binding domain of the receptor [2]. The AR then binds to specific enhancer sequences referred to as hormone-responsive elements present in the 5′ flanking region of target genes and is supposed to regulate transcription via protein–DNA interactions and by interactions with other transcription factors [3].

The primary structure of the AR has been elucidated, and the domains responsible for ligand binding, DNA binding, nuclear localization and transcriptional modulation have been identified [4,5]. AR belongs to a superfamily of nuclear proteins including the receptors for the other classes of steroid hormones, thyroid hormone, vitamin D and retinoids [6–8].

It is important to understand the intracellular dynamics of steroid receptor proteins and the factors that control their steroid-binding capacity and DNA-binding activity. Expression of AR in a suitable host system could facilitate our ability to study the physical properties of the AR protein and the molecular basis of androgen action. A variety of systems has been used for the (over)expression of steroid receptors. The full-length progesterone and glucocorticoid receptors have been expressed in yeast, using *Saccharomyces cerevisiae* as a host system [9,10]. Segments of the glucocorticoid, progesterone and androgen receptor cDNAs were expressed in *Escherichia coli* and were found to exhibit similar biological activities when compared with the native receptors [11–14]. Attempts to produce full-length AR protein by expressing human AR in *E. coli*, yeast or insect cells have been problematical, resulting, in general, in the synthesis of insoluble proteins [13–16].

Rabbit reticulocyte lysates contain quite large amounts of several hsps such as hsp90 and hsp70 and are useful for the study of steroid–receptor complex formation with hsps as well as for the study of requirements for steroid binding and interaction with DNA [17–19].

In the present paper, the *in vitro* synthesis of the full-length human AR is described as well as the analysis of the steroid-binding properties, hydrodynamic characteristics and specific binding to an androgen-responsive element (ARE).

MATERIALS AND METHODS

Materials

[^{35}S]Methionine (specific radioactivity > 1000 Ci/mmol) and [γ-^{32}P]ATP were obtained from Amersham (Little Chalfont, Bucks., U.K.). The synthetic androgen 17β-hydroxy-17α-[^{3}H]methyl-4,9,11-oestratrien-3-one ([^{3}H]R1881; specific radioactivity approx. 87 Ci/mmol) and unlabelled R1881 were purchased from NEN–Dupont de Nemours ('s-Hertogenbosch, The Netherlands).

Recombinant RNasin and rabbit reticulocyte lysate were obtained from Promega Biotech (Madison, WI, U.S.A.). RNA transcription kit containing T7 and T3 RNA polymerase (EC 2.7.7.6) and pBluescript cloning vector were obtained from Stratagene (La Jolla, CA, U.S.A.).

The antisera sp060 (epitope amino acid residues 201–222), sp066 (epitope amino acid residues 899–917), sp197 (epitope amino acid residues 1–20) and sp063 (epitope amino acid residues

Abbreviations used: AR, androgen receptor; hsp, heat-shock protein; GRE, glucocorticoid-responsive element; ARE, androgen-responsive element; ERE, oestrogen-responsive element; R1881, 17β-hydroxy-17α-methyl-4,9,11-oestratrien-3-one; DTT, dithiothreitol.

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593–612) were prepared by previously published procedures [2,20,21]. The antisera sp197, sp060 and sp063 contain high-titre AR antibodies, as shown by immunoprecipitation and Western blotting [20–22]. The antiserum sp066 recognizes the 110–112 kDa AR on Western blots [20].

Mouse monoclonal antibody F 39.4.1 (designated F39) was prepared against the N-terminal domain of the androgen receptor [20]. Mouse monoclonal antibody F 52.24.4 (designated F52) was prepared against the C-terminal part of the DNA-binding domain of the androgen receptor [2].

Calf-thymus DNA–cellulose (4 mg of DNA/g of cellulose) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals and reagents were purchased from Merck (Darmstadt, Germany).

In vitro transcription and translation

The coding sequences of human AR cDNA were excised from the expression vector pSVAR0 [23] at the *Sal*I (EC 3.1.23.37) sites and inserted into the dual promotor (T7/T3) vector pBluescript II–KS (Stratagene) and was designated pAR0. For transcription the vector (1 μg) was linearized with *Xho*I (EC 3.1.23.42) (sense cRNA) or *Bam*HI (EC 3.1.23.6) (antisense cRNA) and transcribed *in vitro* with either T7 RNA polymerase (sense cRNA) or T3 RNA polymerase according to manufacturers' instructions.

Translation reactions (50 μl) in rabbit reticulocyte lysates were carried out as recommended by the supplier. Each reaction mixture included 35 μl of rabbit reticulocyte lysate, 40 units of RNasin, 10 μM $ZnCl_2$, 1 μl of 1 mM amino acid mixture without methionine, 45 μCi of [^{35}S]methionine and 1–2 μg of human AR RNA transcript. The samples were incubated for 1 h at 30 °C and thereafter stored at −20 °C or used directly. In order to measure the amount of [^{35}S]methionine incorporated, samples were subjected to SDS/PAGE (7% gels), sample lanes were cut in 2 mm slices and counted in liquid-scintillation cocktail for ^{35}S radioactivity.

Electrophoresis, electroblotting and autoradiography

These were carried out according to previously described procedures [24].

Metabolic labelling of lymph node carcinoma of the prostate (LNCaP) cells with [^{35}S]methionine

For labelling studies, 6×10^7 LNCaP cells [24] were incubated for 60 min at 37 °C with 20 μCi/ml [^{35}S]methionine (total 100 μCi) in methionine-free RPMI 1640 medium. Cell lysis and AR immunopurification were by published procedures [24].

Steroid binding

Translation reaction mixtures prepared with non-radioactive methionine were diluted four times with buffer A [40 mM Tris/HCl, pH 7.4, 1 mM EDTA, 10% (w/v) glycerol, 10 mM dithiothreitol (DTT), 10 mM Na_2MoO_4, 50 mM NaF) and incubated for 3 h at 4 °C with 0.1–10 nM [^{3}H]R1881 in the presence or absence of a 100-fold excess of unlabelled R1881. Thereafter, bound and unbound steroid were separated with dextran-coated charcoal.

Sucrose-density-gradient centrifugation

Sucrose density gradients [10–30% (w/v) sucrose] were prepared in buffer A with or without 0.4 M NaCl. Reticulocyte lysate containing translation products (50 μl) was mixed with 50 μl of buffer A and incubated with 10 nM [^{3}H]R1881 in the presence or absence of a 100-fold excess of unlabelled R1881 for 2 h at 4 °C. When appropriate, ascitic fluid (1 μl) with monoclonal antibody F39 or F52 or a non-specific antibody was added. Samples of 40 μl were loaded on to the gradients. The gradients were run for 20 h at 50000 rev./min in a SW-60 rotor (Beckman) at 4 °C in a Beckman L70 centrifuge. ^{14}C-labelled BSA (4.6 S) and [^{14}C]-aldolase (7.9 S) were used as internal markers. Fractions of the gradients were collected from the bottom and assayed for radioactivity.

When [^{35}S]methionine was used to label AR protein during *in vitro* synthesis, samples of the fractions were incubated for 1 h at 37 °C with 1 ml of 1 M NaOH/2% (v/v) H_2O_2. Proteins were then precipitated by the addition of 3 ml of 16% (w/v) trichloroacetic acid/2% (w/v) casamino acids, at 4 °C. After centrifugation (15 min at 3000 ***g***), pellets were washed with 0.5 ml of 5% trichloroacetic acid and thereafter solubilized in 1 M NaOH. After neutralization with conc. HCl, samples were analysed for ^{35}S radioactivity by liquid-scintillation counting.

DNA–cellulose chromatography

Reticulocyte lysate containing [^{35}S]methionine-labelled translation products or non-radioactive receptor was diluted five times with TEDG buffer [40 mM Tris/HCl, pH 7.4, 1 mM EDTA, 10 mM DTT and 10% (w/v) glycerol] and incubated for 2 h at 4 °C with buffer only or 10 nM [^{3}H]R1881 respectively.

A DNA–cellulose suspension (12.5%, w/v) of 750 μl was added to 300 μl of diluted lysate and incubated for 60–120 min at 4 °C with constant mixing. The mixture was poured into a small column and washed with TEDG buffer until no free radiolabelled steroid could be monitored. Bound ^{3}H-labelled steroid was eluted with TEDG buffer containing 1 M NaCl; fractions of 100 μl were collected. Alternatively, bound ^{3}H-labelled steroid was eluted with a gradient ranging from 50 to 300 mM NaCl in TEDG buffer. Conductivity was measured with a Philips PW-9505 conductivity meter equipped with a PW-9513 measuring cell (constant 1.62). Conductivity was compared with a standard curve of known NaCl concentration in the appropriate buffer. When [^{35}S]methionine-labelled lysates were used, fractions of the eluate were run on an SDS/7% polyacrylamide gel and blotted to nitrocellulose. After autoradiography, the blot was sliced and the slices were counted after solubilization in Filtercount cocktail (Packard Company).

Gel retardation assay

A glucocorticoid-responsive element (GRE) consensus oligonucleotide sequence 5′-TCGACTGTACAGGATGTTCTAGCTACT-3′ was obtained from Promega. Double-stranded oligonucleotide was labelled using T4 polynucleotide kinase with [γ-^{32}P]ATP to a specific radioactivity of 5×10^8 d.p.m./μg.

The binding reaction mixture (15 μl) contained typically 2 μl of reticulocyte lysate (1.5–2 fmol of AR protein) with 2 μg of poly(dI-dC)·poly(dI-dC) in buffer [10 mM Hepes, pH 7.9, 60 mM KCl, 1 mM DTT, 1 mM EDTA, 4% Ficoll] and 0.2 ng (12 fmol) of labelled GRE. After incubation for 30 min on ice, the protein-bound DNA complexes were separated from free probe on a 4% polyacrylamide gel (45:1) run in 0.25 × TBE (1 × TBE = 50 mM Tris base, 50 mM boric acid, 1 mM EDTA, pH 8.6) for 1.5–2 h at room temperature. Gels were fixed for

15 min in 10% acetic acid/10% methanol, dried and autoradiographed.

In experiments with AR antibodies, 0.1 μl portions of antisera were preincubated for 1 h at 4 °C with reticulocyte lysates before addition of reaction mixture.

Besides unlabelled GRE, two other oligonucleotides were used for competition experiments: one containing an ARE from the prostate-specific antigen promotor (position −170 to −156) sequence 5′-GATCCAGCTAGCACTTGCTGTTCTGCAAG-3′ according to Riegman et al. [25] and one containing an oestrogen-responsive element (ERE) [26] from the apo-very-low-density lipoprotein II promotor region (position −165 to −177), sequence 5′-GATCCTCAGGTCAGACTGACCTTCG-3′, kindly provided by Dr. G. AB, Department of Biochemistry, University of Groningen, The Netherlands.

Site-directed mutagenesis

The AR mutant in which Cys-602 and Cys-605 were mutated to Ser residues was constructed by site-directed mutagenesis using PCR DNA-amplification techniques on pSVAR0 [23]. The primer used to introduce the mutation was 5′-CCGAAGGA-AGAATTCTCCATCTTCTCGTC-3′. From the resulting mutant plasmid pSVAR65, a 700 bp *Bst*EII–*Asp*I fragment was excised and exchanged with pAR0. The mutant transcription vector was designated pAR65.

RESULTS

Characterization of AR synthesized in rabbit reticulocyte lysate

The rabbit reticulocyte lysate was programmed for protein synthesis with *in vitro* transcribed human AR cRNA in the presence of [^{35}S]methionine. Analysis of the translation products by SDS/PAGE showed a predominant band with an apparent molecular mass of 108 kDa (Figure 1). For comparison, the result of a labelling experiment with LNCaP cells is shown. The AR from LNCaP cells migrates as a closely spaced doublet of 110–112 kDa on SDS/PAGE as described previously [24]. This heterogeneity reflects differential AR phosphorylation [22,24]. From the results shown in Figure 1, it is clear that the AR in reticulocyte lysate is synthesized as a single 108 kDa protein, indicating that it is not phosphorylated during *in vitro* synthesis in the same way as during synthesis in LNCaP cells. Also, after incubation of reticulocyte lysates with [γ-^{32}P]ATP during AR synthesis, it was not possible to detect phosphorylated AR after immunopurification, whereas several other unknown phosphorylated proteins could be detected in the lysate (result not shown).

In order to analyse the steroid-binding properties of AR synthesized *in vitro*, the reticulocyte lysate was incubated at 4 °C for 3 h with increasing concentrations (0.1–10 nM) of [^{3}H]R1881 in the presence or absence of a 100-fold molar excess of unlabelled R1881. Linear transformation of saturation data revealed a uniform non-interacting population of binding sites for R1881 with a maximum binding capacity of 480 fmol/ml and a dissociation constant of 0.3 nM (Figure 2).

In parallel experiments, the amount of synthesized AR protein was determined by incorporation of [^{35}S]methionine. The amount was estimated from methionine pool size (5 μM according to the manufacturer), the amount of [^{35}S]methionine incorporated and the number of methionine residues in the receptor molecule. It is calculated that each of the intact (108 kDa) AR proteins binds 1.0 ± 0.1 ($n = 2$) molecules of R1881 at a saturating ligand concentration.

When the AR was labelled with [^{3}H]R1881 and analysed on sucrose density gradients, a single peak of specifically bound radioactivity (Figure 3a) was observed. The sedimentation coefficient of this complex was approx. 6 S. In the presence of high salt (0.4 M NaCl), the sedimentation coefficient of AR shifted to 4 S. When the AR was synthesized in the presence of [^{35}S]methionine, the sedimentation coefficient was also approx. 6 S, and shifted to 4 S in the presence of high salt (results not shown).

The monoclonal antibody F39 is directed against an epitope in the N-terminus of AR (amino acid residues 301–320) and was able to shift the 6 S AR complex to a sedimentation coefficient of about 10 S. Also, the monoclonal antibody F52 which is directed against an epitope in the DNA-binding domain (amino acid residues 593–612) was able to shift the 6 S complex to a higher sedimentation coefficient (8 S) (Figure 3b). This indicates that the DNA-binding domain is exposed in the 6 S AR complex. A

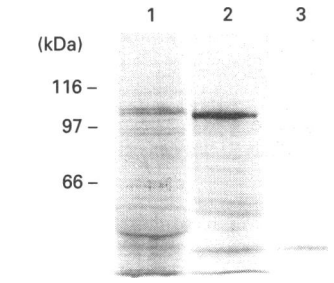

Figure 1 Synthesis of AR in rabbit reticulocyte lysate and in LNCaP cells

Transcription of pAR0 was carried out as described in the Materials and methods section. Translation was performed in the presence of [^{35}S]methionine, and total lysates were analysed by SDS/PAGE and autoradiography. For comparison, LNCaP cells preincubated for 60 min in methionine-free medium were incubated for 60 min with [^{35}S]methionine. Subsequently, AR was immunoprecipitated with F39 monoclonal antibody and analysed by SDS/PAGE. Lane 1, immunoprecipitate of LNCaP cells; lane 2, reticulocyte lysate (1 μl) with sense cRNA; lane 3, reticulocyte lysate (1 μl) with antisense cRNA. Molecular-mass markers (kDa) are indicated on the left.

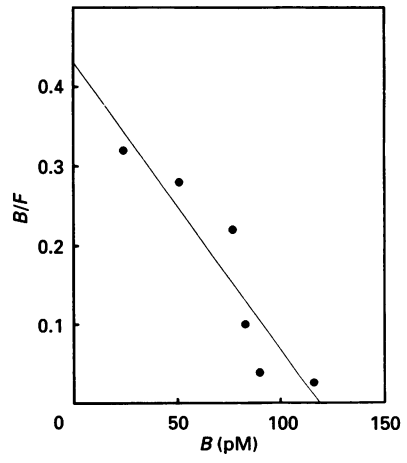

Figure 2 Scatchard plot of [^{3}H]R1881 binding to AR synthesized *in vitro*

Reticulocyte lysate was incubated with six concentrations of [^{3}H]R1881 ranging from 0.1 to 10 nM. Parallel tubes contained an additional 100-fold excess of non-radioactive R1881. Bound and free ligand were separated using a dextran-coated charcoal assay. The K_d (0.3 nM) was calculated from the slope of the line, and the number of binding sites was extrapolated from the intercept on the abscissa ($B_{max.}$ = 480 fmol/ml of translation mixture). The aspecific binding was less than 5% of total binding.

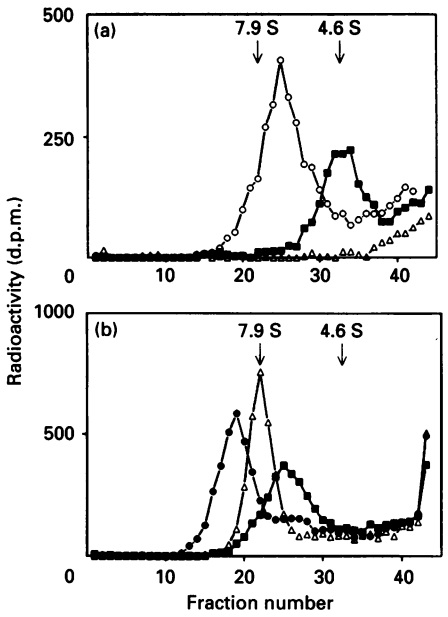

Figure 3 Sucrose-density-gradient profiles of human AR synthesized *in vitro*

AR was synthesized and incubated with [^{3}H]R1881 as described in the Materials and methods section. (**a**) The lysate (50 μl) was incubated for 2 h with [^{3}H]R1881 alone (○) or [^{3}H]R1881 and a 100-fold excess of unlabelled R1881 (△) and analysed by sucrose-density-gradient centrifugation without salt as described. Another sample (20 μl of lysate) was loaded on a 10–30% sucrose density gradient with 0.4 M NaCl (■). BSA (4.6 S) and aldolase (7.9 S) were used as sedimentation markers on a parallel gradient. (**b**) The lysate (50 μl) was incubated for 2 h with either 1 μl of ascitic fluid containing the androgen receptor antibody F39 (●) or F52 (△) or 1 μl of ascitic fluid containing a non-specific antibody (■). Samples (20 μl of lysate) were run on 10–30% sucrose density gradients without added salt as described in the Materials and methods section.

control sample of mouse ascites containing monoclonal antibodies against the Fos oncoprotein was not able to change the sedimentation coefficient of the 6 S AR complex (Figure 3b).

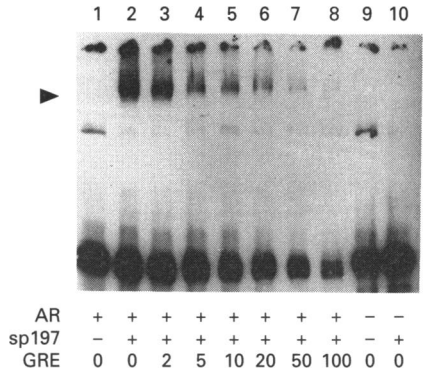

Figure 5 Binding of AR synthesized *in vitro* to a GRE

Lysates (2 μl) with AR protein (lanes 1–8) or without AR protein (lanes 9–10) were incubated with a ^{32}P-labelled GRE, and protein–DNA complexes were separated from free DNA by non-denaturing gel electrophoresis as described in the Materials and methods section. Lanes 1 and 9, preincubation of lysate with normal rabbit serum; lanes 2–8 and 10, preincubation with sp197 (0.1 μl) antiserum. In all lanes the same amount of ^{32}P-GRE was added in the absence (lanes 1, 2, 9, 10) or presence (lanes 3–8) of unlabelled GRE. The excess unlabelled GRE was 2-fold (lane 3), 5-fold (lane 4), 10-fold (lane 5), 20-fold (lane 6), 50-fold (lane 7) and 100-fold (lane 8). The AR–GRE complex is indicated by an arrow.

DNA-binding properties of human AR synthesized *in vitro*

Interaction of receptors with DNA–cellulose is generally used as a measure of activation to the DNA-binding state of steroid receptors. Human AR synthesized *in vitro* was tested for binding ability to DNA–cellulose after being labelled with [^{3}H]R1881 for 2 h at 4 °C. Figure 4(a) established that AR synthesized *in vitro* is retained on a DNA–cellulose column. The AR was also eluted from the DNA–cellulose with a linear salt gradient and was obtained in the fractions containing 120 mM NaCl ($n = 2$) (Figure 4b). Experiments with ^{35}S-labelled AR incubated with DNA–cellulose in the absence of R1881 gave similar results. So it appears that the presence of hormone is not essential for AR to be able to bind to DNA–cellulose.

In further experiments, we have tested the ability of the AR

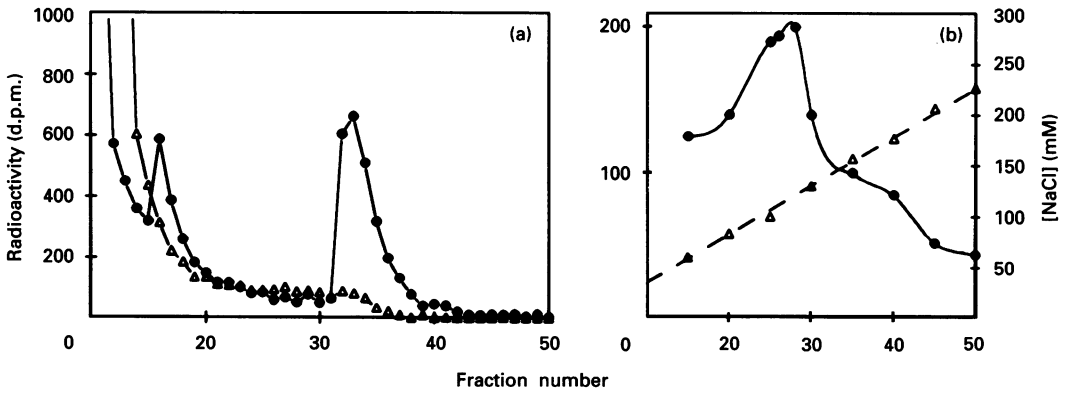

Figure 4 Elution from DNA–cellulose of AR synthesized *in vitro*

Reticulocyte lysates containing translational products of sense (●) or antisense (△) AR cRNA (**a**), or sense (●) AR cRNA (**b**) were diluted five times with TEDG buffer, pH 7.4, and incubated with [^{3}H]R1881 (10 nM for 2 h at 4 °C). A DNA–cellulose suspension was added and incubation was continued for 60 min at 4 °C with mixing. The suspension was poured in a column and washed with TEDG buffer until essentially no free [^{3}H]R1881 was detected. Bound AR was eluted with either TEDG buffer containing 1 M NaCl starting at fraction 30 (**a**) or a gradient ranging from 50 to 300 mM NaCl (△) in TEDG buffer (**b**).

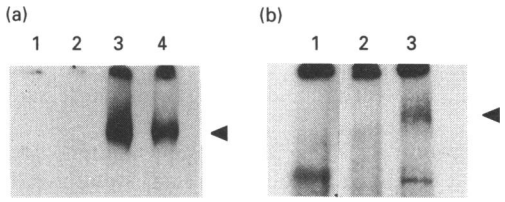

Figure 6 Binding of AR synthesized *in vitro* to ^{32}P-labelled GRE: influence of preincubation with ligand or salt

(**a**) Reticulocyte lysate was preincubated with 10 nM R1881 (lanes 2 and 4) or without R1881 (lanes 1 and 3) for 60 min at 4 °C. Thereafter lysates were incubated with sp197 antiserum (lanes 3 and 4) or with normal rabbit serum (lanes 1 and 2). Incubation with ^{32}P-labelled GRE and further analysis was as in Figure 5. (**b**) Reticulocyte lysates containing AR protein were incubated with NaCl (200 mM) for 60 min at 4 °C (lanes 2 and 3) or with buffer only (lane 1). Thereafter lysates were incubated with sp197 antiserum (lane 3) or normal rabbit serum (lanes 1 and 2) followed by incubation with ^{32}P-labelled GRE and further analysis as described. The AR–GRE complex is indicated by an arrow.

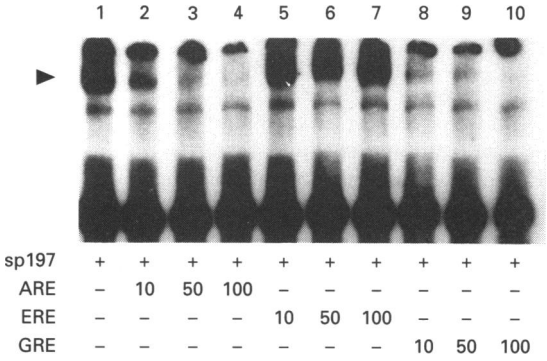

Figure 7 DNA-binding specificity of AR synthesized *in vitro*

Lysate containing AR (2 μl) was used in an incubation with ^{32}P-labelled GRE alone (lane 1) or in the presence of various competitors (lanes 2–10). In all lanes (1–10) the sp197 antiserum (0.1 μl) was also added. Protein–DNA complexes were separated from free DNA by non-denaturing electrophoresis as described in the Materials and methods section. Competitors used were: 10-fold ARE (lane 2), 50-fold ARE (lane 3), 100-fold ARE (lane 4), 10-fold ERE (lane 5), 50-fold ERE (lane 6), 100-fold ERE (lane 7), 10-fold GRE (lane 8), 50-fold GRE (lane 9) and 100-fold GRE (lane 10). The AR–GRE complex is indicated by an arrow. See the Materials and methods section for sequences of competitors.

synthesized *in vitro* to interact with specific DNA sequences. For these experiments, a consensus GRE was used, which has previously been shown to confer androgen-responsiveness to a reporter gene [4]. Reticulocyte lysates containing AR protein, but not lysates programmed with antisense AR cRNA, were able to interact with a 27-mer oligonucleotide containing the above-mentioned GRE, as shown by gel mobility-shift assays. However, the complexes observed were very faint. Incubation with an antiserum against AR, recognizing the first 20 amino acids (sp197), resulted in an apparent stabilization of the GRE–AR complexes (Figure 5). Addition of normal rabbit serum to reticulocyte lysates or antiserum against AR to a lysate not containing AR failed to show similar complexes. Competition with unlabelled GRE of the GRE–AR complex in the presence of antiserum sp197 was almost complete at a 20–50-fold excess of unlabelled GRE, confirming the specificity of the observed complexes (Figure 5).

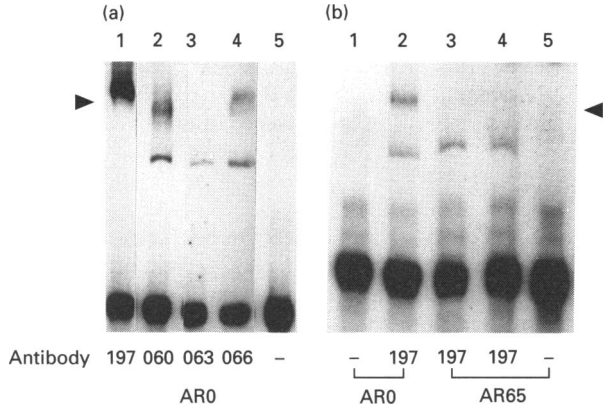

Figure 8 (a) Binding of AR synthesized *in vitro* to ^{32}P-labelled GRE in the presence of various AR antisera and (b) GRE binding of wild-type and a mutant AR

(**a**) Lysate containing AR (2 μl) was incubated with 0.1 μl of various AR antisera for 2 h at 4 °C and thereafter added to the incubation mixture containing ^{32}P-labelled GRE as described in the Materials and methods section. Antisera added were: sp197 (lane 1), sp060 (lane 2), sp063 (lane 3) and sp066 (lane 4) and no antiserum (lane 5). The antisera sp197 and sp060 are directed against the N-terminal domain, sp063 against the DNA-binding domain and sp066 against the steroid-binding domain of the AR. (**b**) The AR mutant pAR65 (C602→S, C605→S) was constructed as described and subsequently transcribed and translated *in vitro* in the same manner as pAR0. Lysates containing AR0 protein (lanes 1 and 2) and AR65 protein (lanes 3, 4 and 5) were preincubated with sp197 (lanes 2–4) antiserum or used directly without incubation with antiserum (lanes 1 and 5). Lysates were added to the incubation mixture containing ^{32}P-labelled GRE, and protein–DNA complexes were separated from free DNA by non-denaturing electrophoresis as described in the Materials and methods section. Lanes 1 and 2, 4 μl of lysate added; lane 3, 2 μl of lysate added; lanes 4 and 5, 4 μl of lysate added. The AR–GRE complex in lane 2 is indicated with an arrow.

After preincubation of the reticulocyte lysate with R1881, specific GRE–AR complexes could also only be observed in the presence of AR antiserum sp197 (Figure 6a). The reticulocyte lysates were pretreated with salt in order to convert the 6 S AR into the 4 S AR species (see Figure 4). In this case also, GRE–AR complexes were observed only in the presence of the sp197 antiserum (Figure 6b). The GRE–AR complex can compete with an ARE from the prostate-specific antigen promotor [25], which has been shown to confer androgen-responsiveness on a reporter gene (Figure 7). In contrast, the GRE–AR complex could not compete with an ERE from the apo-very-low-density lipoprotein II promotor region [26], confirming the specificity of the GRE–AR interaction.

In addition to the sp197 antiserum, several other AR antisera were able to stabilize the GRE–AR complex, although to a lesser extent (Figure 8a). Interestingly, after preincubation of the reticulocyte lysate with an antiserum directed against the second zinc finger of the DNA-binding domain (SP063 antiserum), no labelled GRE–AR complexes were observed. This confirmed that the DNA-binding domain of AR interacts with the labelled GRE.

The DNA-binding domain of steroid receptors contains two zinc ions which are tetrahedrally co-ordinated by cysteine residues. Mutagenesis experiments have shown that these cysteine residues are essential for DNA-binding capacity [27].

The cysteine residues at positions 602 and 605 in the DNA-binding domain of the AR protein were mutated to serine residues, an amino acid that is structurally closely related to cysteine but which has not been found to co-ordinate zinc ions in any zinc-containing protein [28]. The mutant obtained (designated pAR65) was transcribed and translated *in vitro*. Similar

amounts of AR0 and AR65 protein were tested in a gel mobility-shift assay with labelled GRE (Figure 8b). No AR65–GRE complexes were observed, showing that AR does indeed interact via the DNA-binding domain with the GRE, an interaction that is apparently stabilized by the presence of anti-AR antiserum.

DISCUSSION

The rabbit reticulocyte lysate appears to be a convenient system for the synthesis of AR protein. The AR protein produced has a high binding affinity for androgenic steroids (K_d 0.3 nM) which is in agreement with previously reported dissociation constants for human androgen receptors in various cellular systems [29–31]. The difference in apparent molecular mass between the AR synthesized *in vitro* (108 kDa) and the AR from LNCaP cells (110 kDa) is due to a difference in the length of the polyglutamine stretch in the N-terminus. Various lengths of the human AR polypeptide, deduced from cDNAs isolated in different laboratories, have been reported [13]. The vector pSVAR0 [23], which has been used for the construction of the pAR0 vector, contains 20 glutamine codons, whereas the AR cDNA isolated from the LNCaP cells contains 27 glutamine codons (J. Trapman and H. Sleddens, unpublished work). The doublet appearance of the AR from LNCaP cells (110–112 kDa) is caused by differential phosphorylation [24]. Alkaline phosphatase treatment of cytosols from LNCaP cells caused a gradual elimination of the 112 kDa isoform, with a concomitant increase in the 110 kDa isoform [24].

All AR molecules synthesized were able to bind steroid, and there was no evidence available for AR phosphorylation during *in vitro* synthesis. This suggests that AR phosphorylation is not essential for hormone binding, at least *in vitro*. This conclusion is corroborated by the finding that, in LNCaP cells, AR is not phosphorylated in the DNA- and steroid-binding domains [22]. In contrast, the *in vitro* synthesized oestrogen receptor binds oestradiol with high affinity, but low efficiency. Only a kinase purified from calf uterus was able to convert most of the non-hormone-binding oestrogen receptor into a steroid-binding state [18,32].

Other than AR, *in vitro* synthesized 1,25-dihydroxyvitamin D_3 receptor and glucocorticoid receptor were also able to bind steroid with high affinity without previous phosphorylation by an exogenous kinase [17,33]. For the *in vitro* synthesized human progesterone receptor also, it has been shown that phosphorylation is not essential for steroid binding [34].

Steroid hormone receptors can be isolated from the cytosol of target cell extracts as large non-activated 8 S complexes, containing hsp90, hsp70 and p56 [1–2].

In a recent study [2], the hormone-induced transformation of AR in human prostate carcinoma cells (LNCaP cell line) was described. On incubation of the cells with R1881 for 30 min at 37 °C, the sedimentation value of the cytosolic AR decreased from 8 S (non-DNA-binding form of AR) to an intermediate form of 6 S. The monoclonal antibody F52 (directed against the DNA-binding domain) specifically recognized the 6 S form of the AR from LNCaP cells, but not the non-DNA-binding 8 S AR complex [2]. It appears that the 6 S AR detected in reticulocyte lysate is similar to the 6 S AR detected in the cytosol of LNCaP cells after incubation with R1881 at 37 °C. The AR synthesized in reticulocyte lysate (6 S) can therefore be regarded as an activated protein. In previous studies, we have shown that the DNA-binding form of AR in calf uterus cytosol also has a sedimentation coefficient of 6 S [35], whereas the non-DNA-binding AR has a sedimentation coefficient of 8 S. In this respect it is interesting to note that AR from calf uterus cytosol and LNCaP cell nuclear extracts and AR synthesized *in vitro* (Figure 4b) are all eluted from DNA–cellulose at an NaCl concentration of 120–140 mM [35] (G. G. J. M. Kuiper, P. E. de Ruiter, J. Trapman, G. Jenster and A. O. Brinkmann, unpublished work).

The composition of the 6 S AR complex in reticulocyte lysate is at present unknown. It could represent a receptor dimer or a complex with other proteins such as hsp70. After immunoprecipitation of AR from reticulocyte lysate with F39 antibody and subsequent immunoblotting with anti-hsp90 and anti-hsp70 antibodies, no signal above background was detected. We concluded that the amount of AR protein present in reticulocyte lysate is not sufficient to produce a signal with anti-hsp antibodies on Western blots. In similar experiments, the 6 S AR complex from LNCaP cell cytosol was shown to contain hsp70 and essentially no hsp90 and p56 [2]. The anti-hsp70 antibody we used (N27 monoclonal antibody from Dr. W. J. Welch, University of California, San Francisco, CA, U.S.A.) does not recognize the native forms of the protein very well. Consequently, it was not possible to shift the 6 S complex on sucrose density gradients with N27 antibody, as we did with anti-AR antibodies.

Under similar conditions to those in the present paper, the glucocorticoid receptor was detected as a 9 S non-DNA-binding protein [19]. Only after incubation of the *in vitro* synthesized glucocorticoid receptor with the agonist dexamethasone and heat-treatment were receptors able to bind to DNA–cellulose. In the present investigation it is shown that AR is able to bind to DNA–cellulose, even in the absence of hormone and heat-treatment, indicating that it is present as an activated protein.

It is not known at present why the AR in reticulocyte lysate does not form a stable 8 S complex, despite the presence of molybdate. Molybdate has been shown to stabilize the 8 S AR complex in calf uterus cytosol, although its presence is not essential for the detection of this complex [35]. For the glucocorticoid receptor in reticulocyte lysates, it has been shown that hsp90 is necessary but not in itself sufficient for the formation of the non-activated (non-DNA-binding) receptor complex [36]. Other components might be needed for the formation of the 8 S AR complex which are either not present at all in the reticulocyte lysate or not in sufficient amounts.

The human AR synthesized *in vitro* displays specific binding to target DNA sequences as shown using the gel mobility-shift assay. The complexes formed are DNA-sequence-specific, as they form in the presence of high concentrations of poly(dI-dC)·poly(dI-dC) and can compete with low molar ratios of GRE/ARE sequences but not an unrelated ERE sequence. However, the affinity for these responsive elements is rather low. Only in the presence of specific AR antisera can stable complexes be detected. This is the case for the 4 S AR protein as well as for the 6 S AR complex (Figure 6). Such a stabilization has also been described in mobility-shift assays with antibodies directed against the progesterone receptor [37], and more recently also for complexes formed between a fragment of AR expressed in *E. coli* and ARE/GRE oligonucleotides [38]. Steroid hormone receptors can bind as symmetrical dimers to palindromic steroid responsive elements [27]. The stabilization of the AR–GRE complex in the presence of AR antisera could be explained by antibody-induced AR dimerization with the result that high-affinity DNA binding is achieved. For the *in vitro* synthesized mouse oestrogen receptor, it has been shown that there is a direct correlation between specific DNA binding and dimerization [39].

Another explanation, other than the absence of AR dimerization, for the low-affinity DNA binding might be that AR-associated proteins enhancing the interaction of AR protein and DNA are missing from the present system. There is strong

evidence available suggesting that receptors interact with other factors involved in transcriptional regulation and that such protein–protein contacts might increase the affinity of receptors for responsive elements [40–43]. The present system, involving AR synthesized in reticulocyte lysate, could serve as a valuable probe in measuring and isolating such putative factor(s) from nuclear extracts of target cells.

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