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Structure and function of the androgen receptor

A. O. Brinkmann[1], P. Klaasen[2], G. G. J. M. Kuiper[1], J. A. G. M. van der Korput[2], J. Bolt[1], W. de Boer[1], A. Smit[1], P. W. Faber[2], H. C. J. van Rooij[2], A. Geurts van Kessel[3], M. M. Voorhorst[1], E. Mulder[1], and J. Trapman[2]

Departments of [1]Biochemistry II, [2]Pathology and [3]Cell Biology and Genetics, Erasmus University, Rotterdam, The Netherlands

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Summary. The androgen receptor in several species (human, rat, calf) is a monomeric protein with a molecular mass of 100–110 kDa. The steroid binding domain is confined to a region of 30 kDa, while the DNA-binding domain has the size of approx. 10 kDa. A 40 kDa fragment containing both the DNA and steroid binding domain displayed a higher DNA binding activity than did the intact 100 kDa molecule. cDNA encoding the major part of the human androgen receptor was isolated. The cDNA contains an open reading frame of 2,277 bp but still lacks part of the 5'-coding sequence. Homology with the progesterone and glucocorticoid receptor was about 80% in the DNA binding domain and 50% in the steroid binding domain. The present data provide evidence that the androgen receptor belongs to the superfamily of ligand responsive transcriptional regulators and consists of three distinct domains each with a specialized function.

Key words: Androgen – Receptor – cDNA – Structure – Human

Introduction

Steroid hormones are a class of intercellular messengers that mediate physiological responses in target cells by interacting with intracellular high affinity receptors. Upon activation by the ligand the steroid receptor complex modulates transcription of target genes via direct interaction with specific hormone responsive elements [21]. Receptors for glucocorticoids, estrogens, progestins and very recently also for androgens have been cloned and found to be members of a family of DNA-binding proteins that also include the thyroid hormone receptors, the vitamin D receptor and the retinoic acid receptor [1, 6, 10, 11, 14, 17, 19, 20, 22]. The human androgen receptor, however, still remains the least well characterized steroid hormone receptor. The considerably lower tissue concentration and the extreme susceptibility to proteolytic breakdown have hampered progress in androgen receptor characterization. The exact size of the androgen receptor is still not known. The purpose of the present investigation was to study the domain structure of the androgen receptor. Furthermore, data are presented on the molecular cloning, structural analysis and expression of cDNA's encoding the human androgen receptor.

Materials and methods

Materials

[1, 2, 4, 5, 6, 7-^{3}H]5α-dihydrotestosterone ([^{3}H]17β-hydroxy-17α-methyl, 4, 9, 11-estratrien-3-one, [^{3}H]-R1881; Spec.Act 86 Ci/mmol) were purchased from the Radiochemical Centre (Amersham, UK) and from Nen-Dupont (Boston, MA) respectively. Non-radioactive steroids were obtained from Steraloids, Inc. (Wilton, NH). α Chymotrypsin (Merck, Darmstadt, FRG) and trypsin (Worthington Biochemical Corp. Freehold, NY) were dissolved in 40 mM Tris-HCl, 1 mM EDTA, 10% glycerol pH 7.4 (TEG-buffer) diluted to 1 mg/ml and then stored at −20 °C.

Preparation and labeling of cytosolic receptors

Cytosols were prepared in 2–4 volumes of TEG-buffer containing 10 mM dithiothreitol (TEDG-buffer), 10 mM sodium molybdate and 0.6 mM phenylmethylsulfonyl fluoride as described previously [8].

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Equilibration occurred at 4°C for 1–2 h with 5–10 nM [^{3}H]-R1881 or [^{3}H]DHT. Either in the presence or absence of a 100-fold molar excess of non-radioactive steroid. The incubations with [^{3}H]-R1881 were always performed in the presence of a 500 fold molar excess of non-radioactive triamcinolone actonide in order to prevent any ^{3}H-R1881 binding to progesterone receptors [23].

Scatchard plot analysis and competition studies

Scatchard plot analysis of androgen binding in COS-cells was performed after total cell uptake of increasing concentrations of [^{3}H]-R1881 [0.4–10 nM]. Separation of bound from unbound steroid was achieved with an oil-micro-assay method [15]. Sucrose gradient centrifugation of nuclear extracts was performed as described previously [3].

Preparation of proteolytic fragments

Chymotrypsin and trypsin in TEG-buffer were added to the AR preparations to a final concentration of 0.8–1.6 µg/mg and 15 µg/mg protein respectively and incubated at 0°C for 60 min. Prostate cytosol was mixed with AR preparations in a ratio of 1:1 and incubated at 0°C for 60 min.

DNA-cellulose binding assay

Analysis of AR-binding to DNA-cellulose was conducted by a previously described method [2]. Bound AR was eluted with linear gradients of NaCl (0–0.5 M) or $MgCl_2$ (0–0.04 M) in TEDG-buffer.

AR-photoaffinity labeling

[^{3}H]-R1881-AR complexes obtained from DNA-cellulose by $MgCl_2$ elution or from intact LNCaP-cells were irradiated with u.v. light as described previously [2, 4]. Analysis of covalently labeled AR complexes was carried out by SDS-polyacrylamide gel electrophoresis as published elsewhere [2, 4].

cDNA library screening

λgt10 cDNA libraries prepared from mRNA of human breast cancer were kindly provided by Dr. E. Milgrom (Bicetre, France). An oligonucleotide corresponding to the most homologous part of the human progesterone, estrogen and glucocorticoid receptor DNA-binding domain (3′-GGACGCTTTCGACGTTTCGGAA-GAAGTTTTCTTGTTACCTTCCTGT-5′) was synthesized, ^{32}P-end labeled and used for screening of one of the cDNA libraries. Duplicate nitrocellulose filters (Milipore, Molsheim, France) were hybridized overnight at 42°C in 6×SSC, 10xDenhardt solution containing 0.1% SDS and 100 µg/ml salmon sperm DNA. Filters were washed in 3×SSC, 0.1% SDS (2×20 min) at 42°C and 1×SSC (1×20 min) at room temperature. After drying, filters were exposed for 16 to 64 h to a Kodak X-AR5 film at −70°C using intensifyer screens. Positive plaques were isolated by two cycles of purification.

Characterization of cDNA

EcoRI restriction fragments of positive clones were isolated, subcloned into pUC9, and a restriction map was prepared. For sequencing by the dideoxy chain termination method fragments were subcloned into M13mp18/19.

Northern blot analysis

Total cellular RNA was isolated by the guanidinium thiocyanate method. PolyA^+ RNA was prepared by oligo-dT cellulose chromatography. RNA was denatured by glyoxal treatment, separated by electrophoresis on a 1% agarose gel and transferred to a nylon membrane (GeneScreen; NEN, Boston). Filters were hybridized with cDNA probes under stringent conditions (42°C in 50% formamide). After washing, filters were exposed to X-ray film. Southern blot analysis and chromosome mapping were performed as described previously [22].

Transfections

The 1.4 kb Asp718-EcoRI fragment of the cDNA clone was inserted into the SmaI-EcoRI site of the eukaryotic expression vector pSV328A. The pSV-AR construct was transiently expressed in COS cells using the DEAE-dextran method and 2 µg/ml of the expression vector. Cells were maintained for 3 days in Dulbecco's modification of Eagle's minimal essential medium supplemented with 5% fetal calf serum and antibiotics.

Results

Molecular size

A recently developed photoaffinity labeling technique for androgen receptors [2] was applied to determine molecular masses of androgen receptors from different tissues under denaturing conditions. For this purpose human prostate cancer cells (LNCaP-cells) were incubated with ^{3}H-R1881 (methyltrienolone, a synthetic non-metabolizable androgen) and were irradiated in situ [4]. Prostatic and epididymal androgen receptors from 1 day castrated rats and androgen receptors from calf uteri were partially purified by DNA-cellulose chromotography before covalent linkage with ^{3}H-R1881. Analysis on polyacrylamide gels showed that the androgen receptor from the three different species studied was a monomeric protein with a molecular mass of 100–110 kDa (Fig. 1). In the rat prostate cytosol, however, a 50 kDa androgen receptor was observed. Endogenous proteases present in the cytosol were probably the cause of the smaller receptor forms found in this tissue [8].

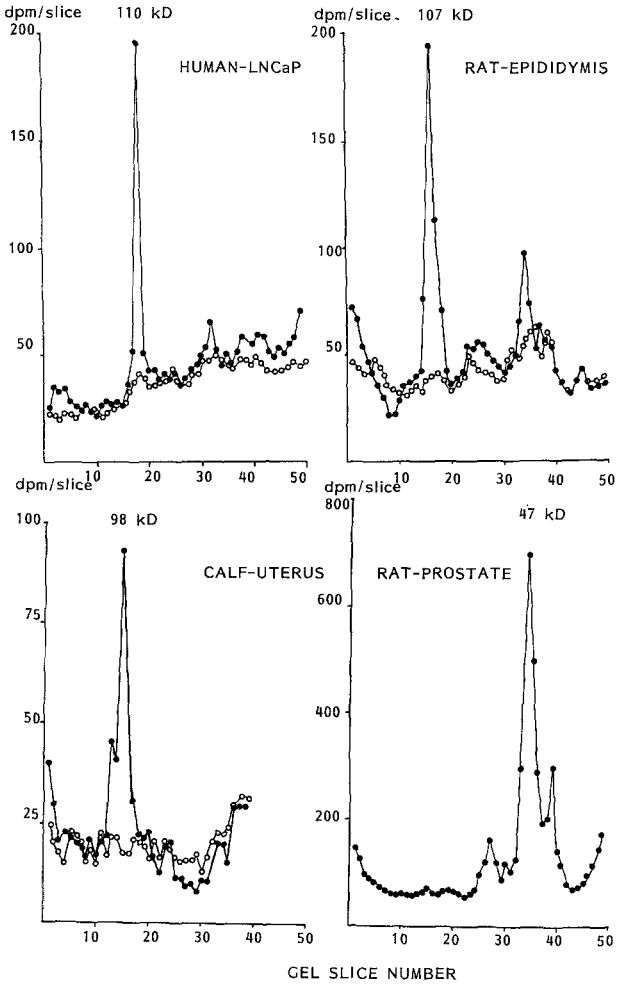

Fig. 1. SDS-PAGE profiles on 7.5–19% acrylamide gels of [^{3}H]-R1881 photolabeled androgen receptors from human LNCaP cells, rat prostate, rat epididymis and calf uterus. Photoaffinity labeling occurred after incubation with 10 nM [^{3}H]-R1881 in the presence (○) or absence (●) of 1 μM unlabeled R1881

Table 1. Effect of proteolytic enzymes on the DNA binding and molecular size of the androgen receptor

Treatment	DNA-binding AR eluted at mM $MgCl_2$	Molecular size
Control	9	100 kD
Chymotrypsin	14	40 kD
Trypsin	n.d.	30
Prostate cytosol	15	50 kD

n.d. = not detectable

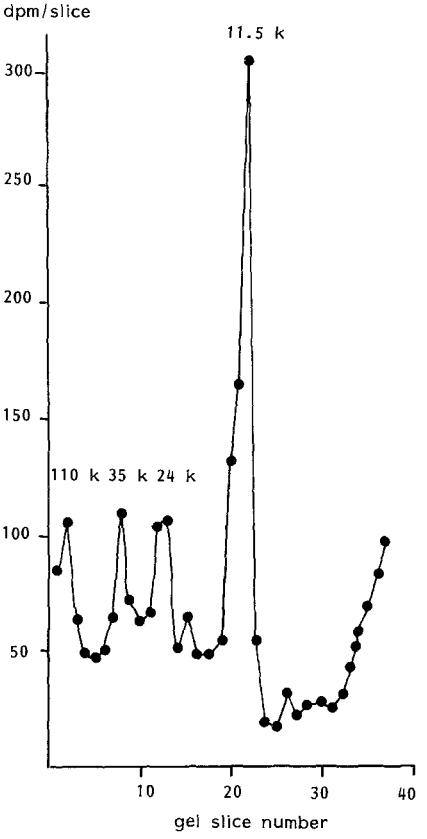

Fig. 2. SDS-PAGE profile on 8–26% acrylamide gel of [^{3}H]-R1881 photolabeled androgen receptors. 110 kDa photolabeled androgen receptor was eluted from a preparative SDS-PAGE gel and subsequently treated with chymotrypsin

Domain structure

Limited digestion by chymotrypsin, trypsin or proteases present in rat prostate cytosol was employed as a tool for producing androgen receptor fragments [8]. Upon treatment of the DNA binding form of the calf uterine androgen receptor with chymotrypsin and rat prostate cytosol a 40–50 kDa fragment was generated which retained both the steroid and DNA binding sites (Table 1). The affinity of this fragment for DNA was increased as is illustrated by the higher concentration of $MgCl_2$ needed for complete elution of the bound receptor. Trypsin cleaved the native androgen receptor at a site distinct from the chymotrypsin site, since a much smaller fragment (30 kDa) was generated, which had lost its DNA binding capacity but retained steroid-binding properties (Table 1). Experiments with higher proteolytic enzyme concentrations resulted in a complete loss of binding activity. Proteolytic digestion of the 100 kDa photoaffinity labeled androgen receptor from calf uterus yielded a 11.5 kDa fragment, while under similar conditions for the human androgen receptor a fragment was generated of 15 kDa (Fig. 2) [4].

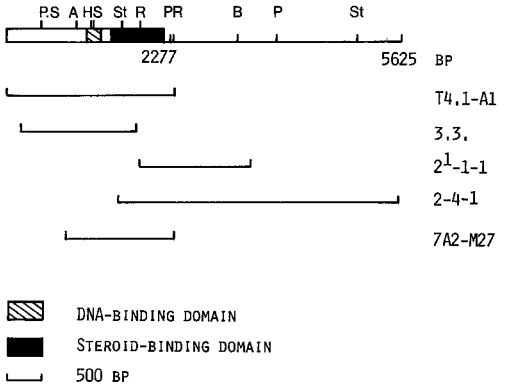

Fig. 3. Restriction map of human androgen receptor cDNA-clones. A = Asp718; B = BamH1; H = HindIII; P = PstI; R = EcoR1, S = SacI; St = StuI

Molecular cloning of androgen receptor cDNA

A T47D (human mammary cell line) cDNA library was screened under low stringency conditions with an oligonucleotide probe (see Materials and methods) corresponding with part of the DNA binding domain of the human progesterone and glucocorticoid receptor. One positive cDNA clone (1,500 bp) with a restriction map different from other steroid receptors was isolated. Using a panel of human hamster somatic hybrid cell lines the corresponding gene was found to be localized on the X-chromosome [22].

Subsequently fragments of the isolated clone were used for isolation of additional overlaping cDNA's. In total 5 cDNA clones were isolated spanning over 5 kb (Fig. 3). Sequencing revealed an open reading frame of about 2.3 kb (Fig. 3). The amino acid sequence was deduced from the nucleotide sequence of the isolated cDNA's and was compared to that of the human progesterone receptor (PR) [16] and human glucocorticoid receptor (GR) [11]. Two major functional regions were identified. These comprise the DNA binding region and the hormone binding domain and are contained within a 350 amino acid stretch extending from the C-terminal end.

The most conserved region is the DNA binding domain. This domain consists of a stretch of 75 amino acids characterized by a high content of cysteines and basic amino acid residues. The homology between the isolated cDNA and hPR and hGR is 83% and 80% respectively (Fig. 4). The arrangement of the conserved cysteines has formed the basis for the suggested Zn^{2+}-dependent "finger" structure in analogy with that proposed for the TFIIIA transcription factor [16]. There is no direct evidence that a similar structure exists in steroid hormone receptors, although in several other DNA binding proteins the cysteine motif has been observed.

The second most conserved region was contained within the hormone binding domain and was localized at the C-terminal end. This domain consisted of stretches of hydrophobic amino acids and showed a 53% to 50% homology with the same domain of hPR and hGR.

In order to examine the hormone binding properties of polypeptides encoded by the receptor cDNA, appropriate fragments were cloned in the eukaryotic expression vector pSV328A and transiently expressed in androgen receptor negative COS cells. 48 h after transfection the cells were incubated with ^{3}H-R1881 and a high salt nuclear extract was prepared. The extracts were subjected to sucrose gradient centrifugation. The ^{3}H-R1881 complex sedimented at 3–4S and the radio-

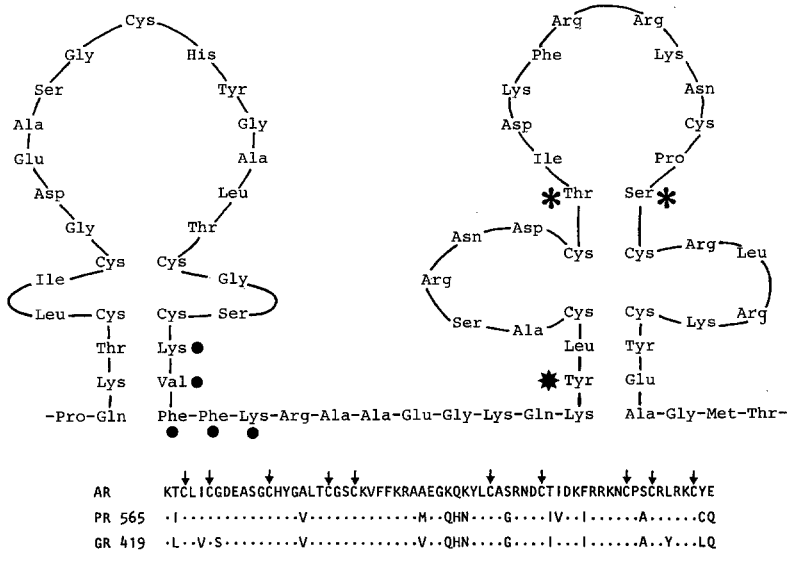

Fig. 4. Deduced aminoacid sequence of the DNA-binding domain of the human androgen receptor (AR) and comparison with the human progesterone (PR) and glucocorticoid (GR) receptors. A possible nuclear transfer signal (●) and possible phosphorilation sites [★ and ∗] are indicated

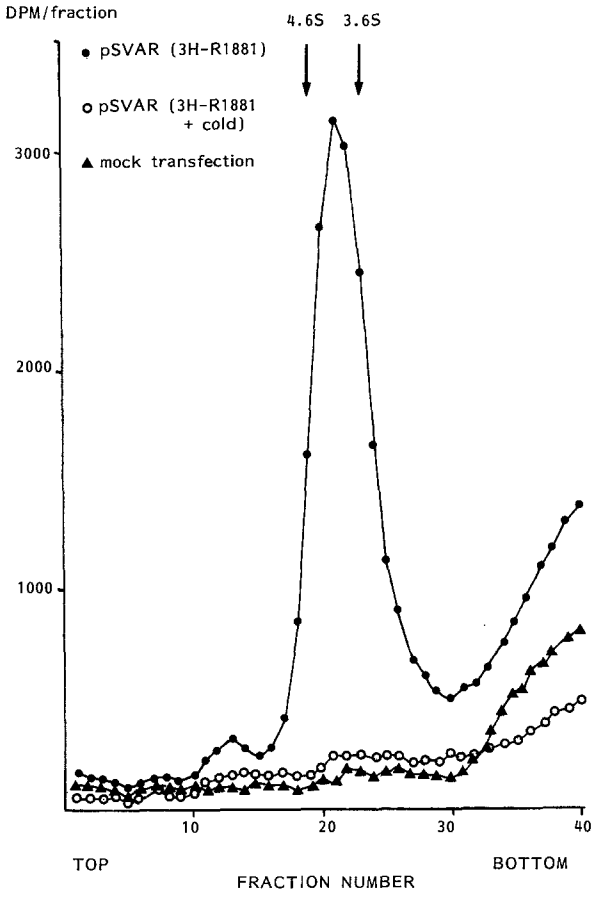

Fig. 5. Sucrose density gradient centrifugation of specific [^{3}H]-R1881 binding in nuclear extracts from COS cells either after transfection with pSVAR (human androgen receptor cDNA construct) (● and ○) or after mock transfection (▲)

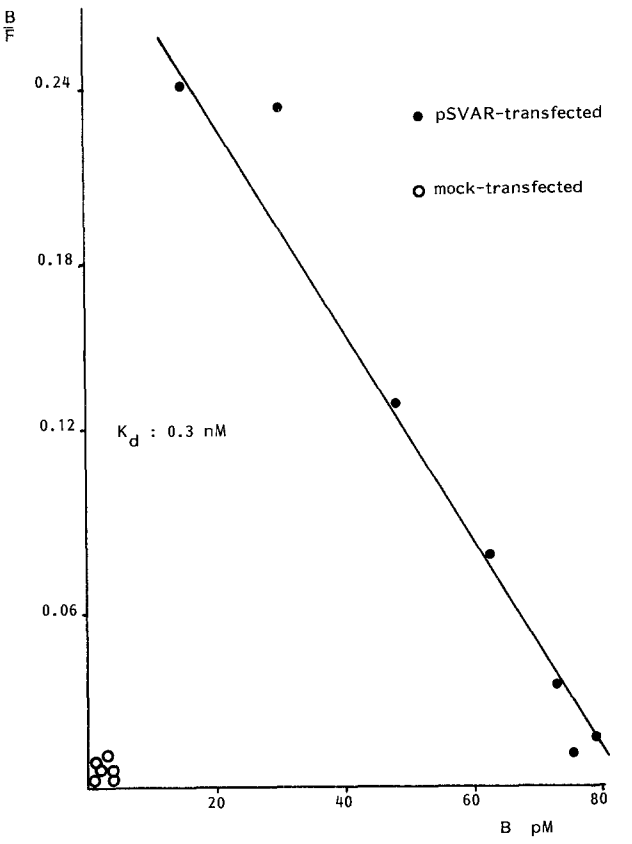

Fig. 6. Scatchard plot analysis of ^{3}H-R1881 binding by COS cells either transfected with pSVAR [●] or after mock transfection (○)

Table 2. Relative binding affinities (RBAs) for the steroid binding protein present in the cytosol fraction of COS cells transfected with pSV-AR. The RBA for DHT was arbitrarily chosen at 100

Dihydrotestosterone (DHT)	100
R1881 (methyltrienolone)	85
Testosterone	15
Progesterone	6
R5020 (Promegestone)	3
Estradiol	4
Triamcinolone acetonide	<1

active peak was completely suppressed by a 100-fold molar excess of non-radioactive ligand (Fig. 5). The ligand specificity of the protein was further examined in a competition binding assay by incubating cytosolic extracts with ^{3}H-5α-dihydrotestosterone in the presence of variable amounts of non-radioactive ligands. From the binding data a relative binding affinity was calculated. The results in Table 2 illustrate that the protein product of the cDNA isolated displays selectivity for androgens, particularly 5α-dihydrotestosterone, R1881 and testosterone. In this respect the binding specificity is identical to that of wild type androgen receptors [3]. Finally, Scatchard plot analysis was performed after total cell uptake of [^{3}H]-R1881 (Fig. 6). A Kd of 0.3 nM could be calculated for a limited number of binding sites.

The expression of mRNA corresponding to the isolated cDNA was investigated in the LNCaP (prostate carcinoma) cell line which contains high levels of androgen receptor [4]. Three major mRNA species with a size of 11, 8.5 and 4.7 kb respectively were detected (Fig. 7).

Discussion

The present investigation demonstrated that androgen receptors from different species (e.g. human, rat, calf)

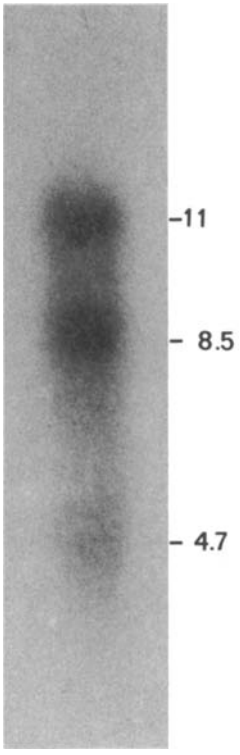

Fig. 7. Northern blot analysis of mRNA isolated from LNCaP-cells (Lymphe Node Carcinoma of the Prostate) hybridized with a 267-pb cDNA fragment. Sizes of hybridizing bands are in kb

and from different tissues (e.g. prostate carcinoma cells, epididymis, uterus) were monomeric proteins and had a comparable molecular mass of 100–110 kDa. The molecular mass of the androgen receptor was in the same range as had been reported for the glucocorticoid and progesterone receptors [11, 17]. The smaller form found in the rat prostate cytosol can be completely ascribed to an active protease present in this tissue (Table 1) [8]. The increase in DNA-binding activity after treatment by chymotrypsin was interesting and indicated that a DNA-binding modulating domain was cleaved from the native androgen receptor. A similar DNA-binding modulating domain has been reported for the glucocorticoid receptor [5]. This modulating domain could play a role in decreasing non-specific DNA-binding and improve the discriminating ability of the receptor between specific and nonspecific DNA binding sites as has been suggested for the glucocorticoid receptor [7]. Furthermore the finding that at least two other domains outside the DNA and steroid binding domains were involved in transcriptional activity strengthened the idea that the DNA-binding region was not exclusively involved in interaction with genomic DNA [9].

The detailed analysis of the human androgen receptor cDNA's confirms the proposed domain structure based on protein analysis studies. The difference in the molecular masses between the photoaffinity labeled human androgen receptor (110 kDa) and the polypeptide of 759 amino acids deduced from the cloned cDNA (81.5 kDa) indicated that part of the 5'-coding sequence is lacking.

Structural analysis revealed that the second DNA-binding finger deviated considerably in amino acid composition as compared with the hGR and hPR. A histidine residue present in all known steroid receptors is absent in the androgen receptor. The tyrosine residue present in this region is a potential phosphorylation site for a tyrosine kinase [18]. Interestingly two other potential phosphorylation sites are present (Serine and Threonine) in this domain [12]. The presence of these potential phosphorylation sites does not necessarily mean that the human androgen receptor is phosphorylated in this position but phosphorylation of the androgen receptor in the DNA-binding region is an attractive possibility. The phosphorylation process could play an important role in the interaction with genomic acceptor sites as has been suggested for the progesterone receptor [13].

With respect to the steroid binding domain a considerable homology was found with the hPR and hGR. The receptor binding specificity of the high affinity ligand methyltrienolone [R1881] for both the AR and PR is understandable in the light of the observed homology between both receptors. The 30 kDa receptor fragment generated after trypsin treatment of the native AR probably represents the meroreceptor form of the AR: the smallest polypeptide containing the complete steroid binding domain. The actual interaction of the ligand with aminoacid residues within the steroid binding domain is confined to a much smaller region of 12–15 kDa as was established after extensive chymotrypsin treatment of the ^{3}H-R1881 covalently labeled AR:

The molecular cloning of the cDNA coding for the human androgen receptor suggests several possibilities for androgen receptor research which were not possible in the past because of the extreme difficulties in obtaining sufficient quantities of the protein in the pure form. Particularly important is the opportuniy to generate antibodies against the androgen receptor, which can be used for a further detailed domain structure analysis. Another aspect which can be studied is androgen receptor structure and expression in several forms of androgen resistance and in prostate cancer.

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Dr. A. O. Brinkmann
Department of Biochemistry II
Erasmus University Rotterdam
P.O. Box 1738
3000 DR Rotterdam
The Netherlands