MECHANISMS OF ACTION OF ANDROGEN RECEPTOR AGONISTS AND ANTAGONISTS

WERKINGSMECHANISMEN VAN ANDROGEENRECEPTOR AGONISTEN EN ANTAGONISTEN

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Contents:

Abbreviations

ADP	adenosine diphosphate
(h)AR	(human) androgen receptor
ARE	androgen response element
ATP	adenosine triphosphate
ATPase	enzyme for ATP to ADP conversion
BiP	binding protein (grp78)
BPH	benign prostate hyperplasia
cAMP	adenosine cyclic-3':5'-monophosphate
CAT	chloramphenicol acetyltransferase
cDNA	complementary deoxyribonucleic acid
CHO	chinese hamster ovary cell line
COS	monkey kidney cell line
COUP	chicken ovalbumin upstream promoter
COUP-TF	COUP transcription factor
CPA	cyproterone acetate (antiandrogen)
CV-1	monkey kidney cell line
(k)Da	(kilo) Dalton, molecular weight
DHT	5α-dihydrotestosterone
DMSO	dimethylsulfoxide
DMP	dimethyl pimelimidate
DTT	dithiothreitol
E_2	estradiol
EcR	ecdysone receptor
EcRE	ecdysone response element
EDTA	ethylenediaminetetra-acetic acid
eIF-2α	eukaryotic initiation factor 2α
ELISA	enzyme-linked immunosorbent assay
(h)ER	(human) estrogen receptor
ERE	estrogen response element
ERR1	estrogen receptor related 1
ERR2	estrogen receptor related 2
FK506	immunosuppressant drug
FKBP	FK506-binding protein
FSH	follicle-stimulating hormone
GnRH	gonadotropin-releasing hormone
GnRH-R	GnRH receptor
(h)GR	(human) glucocorticoid receptor
GRE	glucocorticoid response element
grp78	glucose-regulated protein of 78 kDa (BiP)
grp90	glucose-regulated protein of 90 kDa
H-2RIIBP	H-2 region II-binding protein
HeLa	human cervix carcinoma cell line
HF	hydroxyflutamide (antiandrogen)
HRE	hormone response element
HRI	heme-regulated protein kinase
hsc70	70-kDa heat-shock cognate protein (hsp73)

hsp	heat-shock protein
hsp56	56-kDa heat-shock protein
hsp56-60	56-60-kDa heat-shock protein
hsp70	70-kDa heat-shock protein (hsp72 and hsp73)
hsp72	72-kDa heat-shock protein
hsp73	73-kDa heat-shock protein (hsc70)
hsp90	90-kDa heat-shock protein
ICI 176 334	casodex, a trade mark of ICI Pharmaceuticals (antiandrogen)
LH	luteinizing hormone
LH-R	LH receptor
LNCaP	human lymph node carcinoma of the prostate (cell line)
MAb	monoclonal antibody
MHCI-RII	murine major histocompatibility class I regulatory region II
MMTV-LTR	mouse mammary tumor virus long terminal repeat
MPA	medroxyprogesterone acetate (antiandrogen)
MR	mineralocorticoid receptor
MRE	mineralocorticoid response element
mRNA	messenger ribonucleic acid
NHIK	human cervix carcinoma cell line
NLS	nuclear localization signal
PAGE	polyacrylamide gel electrophoresis
PAP	prostatic acid phosphatase
pAR0	plasmid construct encoding wild-type AR
pARL	plasmid construct encoding LNCaP mutant AR
PC3	human prostate cancer cell line
PC-EW	human prostate cancer cell line
PCR	polymerase chain reaction
PMSF	phenylmethanesulfonyl fluoride
$pp60^{src}$	60 kDa transforming kinase of Rous sarcoma virus
(h/c)PR	(human/chicken) progesterone receptor
PRE	progesterone response element
PSA	prostate-specific antigen
R1881	methyltrienolone (synthetic androgen)
R5020	promegestone (synthetic progestin)
RAR	retinoic acid receptor
RARE	retinoic acid response element
RBA	relative binding affinity
RU486	mifepristone (antiglucocorticoid, antiprogestin)
RXR	retinoic X receptor
RXRE	retinoic X response element
S	Svedberg unit, sedimentation coefficient.
SDS	sodium dodecyl sulfate
SHBG	sex hormone-binding globulin
SHR	steroid hormone receptor
SV40	Simian Virus 40
T	testosterone
TAA	triamcinolone acetonide (synthetic glucocorticoid)
TAF	transcription activation function
TAU	transcription activation unit

TR	thyroid hormone receptor
TRE	thyroid hormone response element
VDR	vitamin D_3 receptor
VDRE	vitamin D_3 response element

Chapter 1

Introduction and Scope of the Thesis

1.1 Introduction

1.1.1 Androgens

The androgen receptor (AR) is a ligand-dependent transcription factor. The ligand binds to the receptor with high specificity and affinity, and induces a change in the conformation of the receptor that activates receptor functions in the cell nucleus. For the androgen receptor, the ligand can be either testosterone (T) or its metabolite 5α-dihydrotestosterone (DHT). The steroid hormone testosterone is mainly produced and secreted by the Leydig cells in the testis, and can be converted both intratesticulary and peripherally to DHT by the enzyme 5α-reductase. DHT is the more potent androgen, and has a higher affinity than testosterone for the receptor. The hormones are transported by the blood, mainly bound to proteins such as albumin and sex hormone-binding globulin (SHBG) (Westphal, 1978), and have several functions throughout the body. The androgens can probably reach the receptor, that is mainly located in the nucleus of the target cell, by diffusion across the plasma and nuclear membranes.

The changed conformation of the receptor molecule following hormone binding results in the capacity of the protein to bind as a dimer to regulatory elements on DNA, the so-called hormone response elements (HREs). A HRE which can bind the AR is called an androgen response element (ARE). This interaction of the receptor with an ARE in the promoter of a gene induces or represses transcription of that androgen-responsive gene. Transcription regulation is believed to result from interaction of other transcription factors with the receptor (Beato, 1989).

In prenatal life, androgens play an important role in the development of the male genital tract. Postnatally, androgens play an important role in the full development and functional maintenance of male internal sex organs (e.g., testis, epididymis, prostate) and the production of factors by these organs. Testosterone is required, in combination with follicle-stimulating hormone (FSH), for normal spermatogenesis (Marshall & Nieschlag, 1987; Matsumoto et al., 1986). Androgens also play a crucial role in the development of external sex organs and the secondary sex traits in boys, such as the changes in hair growth, musculature, and vocal cords which occur at puberty.

1.1.2 Antiandrogens

Antiandrogens are used clinically for treatment of diseases which have an androgen dependent etiology, and/or show an undesirable response to circulating androgens. Antiandrogens are used for inhibition of androgen action. This effect of these compounds is a consequence of their potential to compete with androgens for AR occupancy, without eliciting androgen activity themselves. In addition to their role in the clinic, these receptor antagonists can be used for research purposes, to study the molecular mechanism of action of the AR. Because different antiandrogens potentially affect different aspects of receptor function, it is of interest to study the mechanism of action of many antiandrogens in order to acquire as much information as possible on receptor function and malfunction.

1.1.3 Aim of the Investigations

One aim of the investigations presented in this thesis was to explain the unexpected androgenic effects of some antiandrogens, progestins and estradiol on the prostate cancer cell line LNCaP (Lymph Node Carcinoma of the Prostate). A second goal was to study the interaction of the AR with other cellular proteins, in particular several heat-shock proteins, after binding of androgens or antiandrogens to the receptor. A more detailed scope of the thesis will be presented in the last paragraph of this chapter. As an introduction to that section, more detailed information on receptor function needs to be provided. Therefore, in the next paragraphs, the structure of the AR and its mechanism of action will be discussed. Then, the association of the AR with heat-shock proteins and the possible roles of these associations will be considered. In addition, some general characteristics and functions of heat-shock proteins will be reviewed. In the subsequent part, the clinical significance of antiandrogens is addressed. In the last section of this chapter, the scope of this thesis is described.

1.2 The Mechanism of Action of Androgens

1.2.1 A Family of Regulators With Similar Structure

The AR is a member of a super-family of ligand-responsive transcription factors (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988; Faber et al., 1989). The members show a high level of molecular identity. The arrangement of the different domains in the receptors, is essentially the same for all members. This arrangement involves a centrally located DNA-binding domain, a COOH-terminally located ligand-binding domain linked to the former by a region called the hinge region, and a highly variable N-terminal region (Figure 1). Both the DNA-binding domains and the ligand-binding domains share a high degree of sequence identity among the different members of this family (Table I).

By using recombinant DNA technology, the domains of different receptors can be artificially interchanged, resulting in new functional receptors with characteristics combined from the receptors used (Green & Chambon, 1987; Webster et al., 1988). The steroid binding domain even functions, with respect to repression of transcription, in combination with the DNA-binding domain of the non-related transcription factor GAL4. This repression can be relieved by the addition of hormone (Webster et al., 1988). The ligand-binding domain of the steroid receptors not only binds the ligand, but also contains some conserved sub-domains with other functions, including trans-activation and receptor dimerization (see next paragraphs).

The DNA-binding domain is about 70 amino acid residues in size and has a high content of basic amino acid residues and contains two zinc fingers or zinc twists (see Figure 1). These zinc fingers both bind one zinc ion, which is centrally located in between tetrahedrally located cysteine residues. The most N-terminally located finger is responsible for DNA binding and determines response element specificity (Green et al., 1988); three amino acid residues in the so called P-box are responsible for the specificity of binding (Umesono & Evans, 1989). The other zinc finger is, in addition to the ligand-binding domain, involved in dimerization of two receptor molecules (Green & Chambon, 1989). For the glucocorticoid receptor (GR) and estrogen receptor (ER) the structure of the DNA-binding regions in solution were determined by nuclear magnetic resonance

techniques (Härd et al., 1990; Schwabe et al., 1990). Both these studies, and the crystallographic analysis of the interaction of the GR with DNA (Luisi et al., 1991), are in agreement with a role of the first zinc finger in the interaction of the receptor with DNA and of the second zinc finger in protein-protein interactions in the receptor dimer.

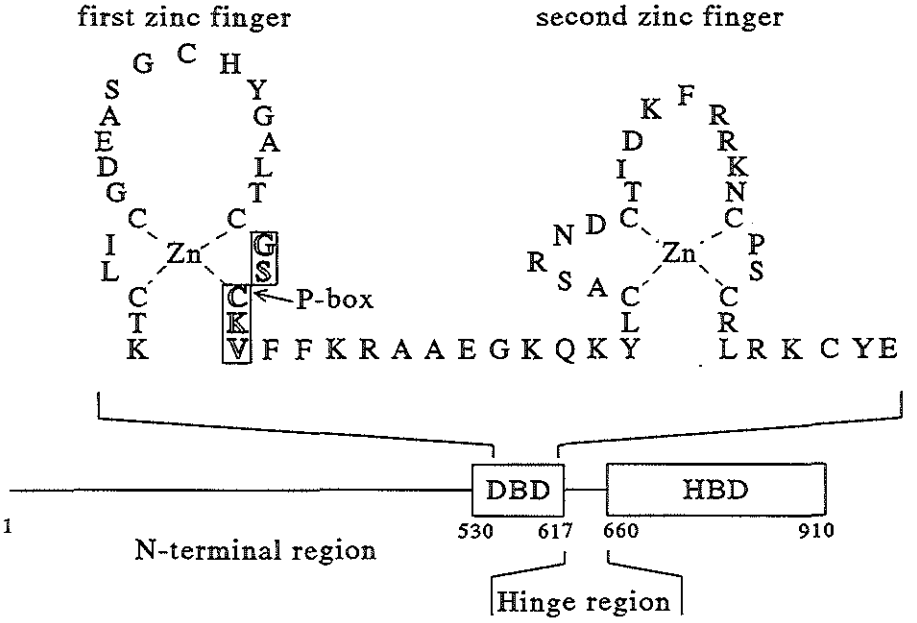

Figure 1. The structure of the androgen receptor and part of the DNA-binding domain. Numbers refer to amino acid numbers. Zn: zinc; DBD: DNA-binding domain; HBD: hormone-binding domain.(Faber et al., 1989).

The ligand-binding domain and the zinc finger region are connected by a region called the hinge region, containing a nuclear localization signal similar to that of SV40 large T antigen (SV40 NLS), responsible for nuclear translocation of the receptor.

The N-terminal region is highly variable and contains a second trans-activation function in addition to the one in the ligand-binding domain. For most members of the steroid receptor family, this region also is the most immunogenic part of the molecule (Beato, 1989; Carson-Jurica, 1990).

The ligands to which the different receptors respond include both hormones and other substances. The receptor super-family consists of responders to steroid hormones,

but also includes the thyroid hormone receptor (TR). Other members of the super-family are e.g., the retinoic acid receptor (RAR), vitamin D_3 receptor (VDR), and in *Drosophila* the ecdysone receptor (EcR). Of several receptors, different genes and different transcripts derived from alternative promoter usage or alternative splicing have been described, which encode different protein variants (see Table II). Compilated from: Carson-Jurica et al., 1990; Fuller et al., 1991; Koele et al., 1991; Laudet et al., 1992.

Table I

Percentage identity between DNA-binding domains and between hormone-binding domains of members of the steroid receptor family.

	hAR	hPR	hMR	hGR	hER	hERR1	hERR2
hAR		81	78	78	59	53	53
hPR	56		90	90	56	54	54
hMR	52	57		93	57	59	59
hGR	51	54	57		57	57	57
hER	22	27	24	27		69	72
hERR1	23	24	23	25	32		63
hERR2	22	25	21	25	33	90	

The percentages identity between the DNA-binding regions (upper right half of the table) and the hormone-binding domains (lower left half of the table) are depicted. Two classes of receptors, based on type of response element which are recognized, are shown (see below). The homology of the DNA-binding domain (shaded) and the hormone-binding domain (boxed) is generally higher between members of one class than between members of different classes. For abbreviations, see Table II. Modified from Koele et al., 1991.

The receptors are important in the prenatal development or organization of either the organism as a whole (e.g., RAR) or of specialized organs (e.g., AR). In addition, postnatally, most of them regulate gene transcription in specialized tissues in response to hormones or other ligands.

Some members of the family were found by screening for new receptors on the basis of a high level of identity of the nucleotide sequence encoding the DNA-binding domains. These members are believed to have a homologous mode of action as compared to the other members of the family, but because the respective ligands have not yet been found, these members are called orphan receptors (e.g., estrogen receptor related 1 and 2, ERR1 and ERR2, and the Chicken Ovalbumin Upstream Promoter Transcription Factor, COUP-TF).

Table II
The steroid/thyroid hormone receptor superfamily.

Receptor/Gene locus	Abbreviation (s) = splice variant (p) = different promoter usage	Ligand
MAMMALIAN RECEPTORS:		
Receptors with known ligand:		
glucocorticoid	GR	glucocorticoids (cortisol)
mineralocorticoid	MR	corticosteroids (aldosterone)
progesterone	PR A(p)	progesterone
	PR B(p)	"
androgen	AR	(dihydro)testosterone
estrogen	ER	estradiol
thyroid	TRα-1/c-erbAα1(s)	T_3
	TRα-2/c-erbAα2(s)	not T_3
	TRα-3/c-erbAα3(s)	not T_3
	TRß-1(s)	T_3
	TRß-2(s)	T_3
	v-erbA (viral)	not T_3
vitamin D_3	VDR	vitamin D_3
retinoic acid	RARα	retinoic acid
	RARß	"
	RARγ	"
novel retinoic acid	RXRα	retinoic acid (9-cis stereoisomer)
	RXRß	"
mouse peroxisome proliferator-activated receptor	mPPAR	peroxisome proliferators
Orphan receptors:		
human estrogen receptor related	hERR1	
	hERR2	
reverse erbA/erbA-related 1	REV-erbα/ear1	not T_3
murine H-2 region II Binding Protein/ erbA related 2	H-2RIIB/ear2	
Chicken Ovalbumin Up-stream Promoter TF/erbA related 3	COUP-TF/ear3	
hepatocyte nuclear factor 4	HNF-4 (LF-A1?)	a retinoid?
apolipoprotein regulatory protein 1	ARP-1	
DROSOPHILA GENES:		
ecdysone receptor	EcR/DHR3?	20-hydroxyecdysone
seven-up	svp1	
	svp2	
tailless	tll	
ultraspiracle/s15 chorion gene promoter sequence (Cs15-CF1)	usp/XR2C/CF1	
fushi tarazu	FTZ-F1	
early puff gene prod. E75	E75 A(p)	
	E75 B(p)	
knirps	kni	
knirps-related	knrl	
embryonic gonad	egon	

1.2.2 The Subfamilies

It is believed that the different members of the super-family are derived from one ancestral receptor gene. Duplications and mutations of this gene could have resulted in the variety of receptors that exists today. In some instances, the DNA-binding domain and the ligand-binding domain belonging to one member, may have evoluted independently. These members are believed to originate from swapping events between domains of different origin (Laudet et al., 1992). The super-family can therefore be divided into different subfamilies by comparing the extent of homology of the amino acid sequence of either the DNA-binding domains or the most conserved part of the ligand-binding domains. The N-terminal domain is so poorly conserved that it is not possible to make groups on the basis of these sequences. The steroid hormone receptors belong to one of three subfamilies, with a high degree of homology between both ligand-binding domains and the DNA-binding domains. Relatively early in the evolution process, the GR, PR, MR, and AR have separated from the other members of this subfamily (Laudet et al., 1992). This group also forms a separate class of receptors when the type of recognized HREs is concerned (see Table I and below).

1.2.3 Classes of HREs

In addition to classification of the receptors on a structural basis, the members of the superfamily can also be classified according to similarity between the HREs in the promoter regions of the responsive genes which are recognized by the receptors. The members of the superfamily bind as dimers to their respective HREs, and both participants of the dimer interact with DNA (Luisi et al., 1991). Therefore, most HREs consist of two halfsites with a consensus sequence specific for the type of HRE. These halfsites are either organized as direct repeats (halfsite sequences on same strand), or as inverted repeats, also referred to as palindromic sequences (halfsite sequences on opposite strands). In addition to the consensus sequence, also the spacing of the halfsites is of importance for receptor specificity (for a review see De Luca, 1991, Glass et al., 1991). In relation to the HRE specificity, the classes can be distinguished by the P-box sequences in the first zinc finger (See Figure 1 and Table III). This division is distinct from the division in sub-families. Four major classes of nuclear receptors have been described on basis of HRE binding specificity (Forman & Samuels, 1990), and three additional classes have been suggested (Laudet et al., 1992). The steroid receptor subfamily contains two of the classes as a whole: the GR/PR/AR/MR class, and the ER/ERR1/ERR2 class. The members of the first class recognize the glucocorticoid response element (GRE) with the palindromic consensus sequence AGTACA nnn TGTTCT (see Table III). No response elements for the PR, AR, and MR (PRE, ARE, and MRE, respectively) have been found that do not fit the GRE consensus sequence. The P-box sequence in this class is GSCKV. The ER, which together with the ER-related orphan receptors ERR1 and ERR2 forms the second class, recognizes the consensus sequence N_AGGTCA nnn TGACCN_T. N_A and N_T are not necessary for ERE function, but are most commonly an A and a T, respectively. In Table III, a small selection of family members with their consensus HREs and P-box sequences are depicted.

Table III
Consensus sequences of response elements for members of the steroid/thyroid hormone receptor super family.

Class	HRE	Consensus	Repeat type	Receptor	P-box
1	GRE/PRE/ ARE/MRE	AGAACA NNN TGTTCT G G C C TTT A	I.R.	GR,PR,AR,MR	GSCKV
2	ERE	NGGTCA NNN TGACCN T	I.R.	ER	EGCKA
3	COUP	AGGTCA $N^{1/6}$ AGGTCA GT G	D.R.	COUP-TF	EGCKS
4	TRE/RARE	AGGTCA $N^{0/1}$ TGACCT A G G	I.R.	TR, RAR	EGCKG
4	VDRE	TGGTGA N TCACCG	I.R.	VD_3R	EGCKG
4	EcRE	AGGTCA N TGACCT G T A C	I.R.	EcR	EGCKG
4	MHCI-RII	AGGTCAGGGG	N.R.	H-2RIIBP	EGCKG
4	Cs15-CF1	AGGTCACGT	N.R.	CF1	EGCKG

One class of response elements (1) can be distinguished from three other classes (2, 3, and 4) by one invariant nucleotide in each half site of the response elements (doubly underlined) and by two invariant amino acids (doubly underlined) in the P-box amino acid sequence in the first zinc finger. The members of this GR-like class (1), to which also the AR belongs, contain the P-box sequence GSCKV, and their response elements consist of common inverted repeats separated by three spacer nucleotides (N). The other three classes contain the P-box sequence EGCKA, EGCKS, and EGCKG, respectively. Their response elements consist either of inverted repeats (I.R.), direct repeats (D.R.), or no repeat (N.R.). The underlined nucleotides in the VDRE are exceptionally different from the other response elements. The repeats are separated by 0 to 6 spacer nucleotides, depending on the type of element ($N^{x/y}$ indicates x or y nucleotides). MHCI-RII: murine major histocompatibility class I regulatory region II, which binds the murine H-2 region II binding protein (H-2RIIBP); COUP: chicken ovalbumin upstream promoter element; EcRE: ecdysone response element (EcRE); Cs15-CF1; Drosophila s15 chorion gene promoter sequence. The other abbreviations are described in the text. Compilated from: Carson-Jurica et al. (1990); Forman & Samuels (1990); Fuller (1991); Martinez et al. (1991); Segraves 1991).

In Class 4 HREs, in addition to the inverted repeats depicted in Table III, several direct repeats have been described, in which only the spacing (1, 3, 4 or 5 nucleotides) determines whether it functions as a response element for either the retinoic X receptor

(RXRE), vitamin D_3 receptor (VDRE), thyroid hormone receptor (TRE), or retinoic acid receptor (RARE), respectively. For reviews, see De Luca (1991) and Glass et al. (1991). In addition, several examples of heterodimerization have been described between members of RXR, RAR, TR, and VDR (Forman et al., 1989; Glass et al., 1989; Yu et al., 1991). The type of heterodimers may be dependent on the structure of the response element (e.g., inverted repeat, direct repeat, no repeat) and the exact composition of the halfsites. For the steroid receptors, no heterodimerization has been described.

1.2.4 Receptor Specific Regulation

The release of hormones in the blood results in their presence throughout the organism. Only the target organs should respond to the message, and the specificity of the message is the result of hormone interaction with specific receptors. Therefore it is of utmost importance that the ligand shows specificity to its receptor. Moreover, only the ligand, and no other compounds, should bind to, and transform the receptor to an active form. In general, there is little or no cross-responsiveness between the different steroid receptors and their respective ligands. In contrast to this, the AR binds to, and activates transcription through GRE-like elements. These elements are also recognized by the GR, MR, and the PR. Despite the extensive search for AR specific response elements, no elements have been found which do not fit the consensus GRE. It is therefore likely that the GR, MR, PR, and AR make use of the same elements. But how can receptor specificity then be achieved?

First of all, not all receptors are present and/or active in all cells. Cell- and tissue-specific expression of the receptors might be important for the specificity of the hormonal response (Sträle et al., 1989). However, because more than one type of receptor is often expressed in one cell (the GR is expressed in nearly all cells), this cannot be the only mechanism. The relative levels of expression of the different receptors might also be important for hormone specific actions. In addition, rapid metabolism of one of the steroids to an inactive form could effectively silence one of the hormonal signals (Funder et al., 1988).

Secondly, there are several possible response elements which have a sequence similar to, but not completely identical to the consensus sequence. These different response elements might have different affinities for the different receptors.

Thirdly, receptor- and promoter-specific transcription factors could play a role. This is illustrated by the following example. The mouse mammary tumour virus long terminal repeat (MMTV-LTR), which contains several GRE consensus binding sites, can be mutated, resulting in different effects on either progesterone stimulation or glucocorticoid stimulation. In particular, a binding site for nuclear factor I is required for glucocorticoid action, but not for progesterone action (Gowland & Buetti, 1989).

1.2.5 Subcellular Localization of Steroid Receptors

The localization of the steroid receptors in the non-occupied state was for a very long time an issue of many discussions, especially because after cell rupture receptors were found in the cytosol. The conclusion of the most recent data in the literature is, that all receptors are divided over both the cytoplasm and the nucleus, and that in this respect mainly quantitative differences exist between the different receptors. In the absence of

ligand, the AR, PR and ER are predominantly located in the nucleus (King & Greene, 1984; Perrot-Applanat et al., 1985; Husmann et al., 1990). The GR was initially found mainly in the cytoplasm in the absence of hormones, and translocated to the nucleus after ligand binding (Picard & Yamamoto, 1987; Wickström et al., 1987). The localization of the unoccupied GR in the cytoplasm has also been stated to be the result of diffusion of the receptor from the nucleus during tissue preparation and fixation of the cells for immunocytochemistry (Gasc et al., 1989; Brink et al., 1992). In this case, the difference between the GR on the one hand, and the AR, ER, and PR on the other hand, could be a difference in affinity of the unoccupied receptors for the nuclear compartment. This difference would be revealed after tissue preparation for immunocytochemistry. This is different from the situation after cell rupture described above, which results in leakage of all unoccupied steroid receptors from the nucleus. Guiochon-Mantel et al. (1991) showed nucleo-cytoplasmic shuttling of the PR and the ER. The receptors were actively transported into the nucleus and then diffused back into the cytoplasm. It was stated that the residency of the receptors in either the nuclear or the cytoplasmic compartment might reflect a dynamic situation. The difference between the GR and the sex steroid receptors, in this respect, would then be mainly quantitative (Guiochon-Mantel et al., 1991).

1.2.6 How Are the Receptors Transported to the Nucleus?

Small molecules can reach the nucleus by passive diffusion through the nuclear pores. Molecules larger than 20—40 kDa are probably actively transported across the nuclear envelope, through the nuclear pore complex (Feldherr et al., 1983, 1984). Proteins are directed by a nuclear targeting sequence. Many different nuclear targeting sequences have been described, and one of the most well-characterized is that of SV40 large T antigen, PKKKRKV (Kalderon et al., 1984). It might be that this sequence is an exceptionally efficient variant of the COOH-terminal part of a more general bipartite nuclear localization signal (NLS, Dingwall & Laskey, 1991). The bipartite NLS consists of two basic amino acid residues, a spacer region of any ten amino acid residues, and a basic cluster in which three out of the next five amino acid residues must be basic (Robbins et al., 1991). This bipartite sequence motif is conserved throughout the steroid receptor family, including the AR, and is located at the boundary of the exon encoding the second zinc finger and the exon encoding the hinge region and the first part of the ligand-binding domain (Dingwall & Laskey, 1991). The sequence shown to be necessary for the hormone-dependent nuclear translocation of the GR is the more COOH-terminally located element of this motif (called the SV40 large T antigen-like motif). A second motif may be present in the steroid-binding domain (Picard & Yamamoto, 1987).

For the PR, not the hormone-dependent, but the hormone-independent nuclear localization was described as being dependent upon this SV40 large T antigen-like NLS (Guiochon-Mantel et al., 1989). A second, hormone-dependent translocation signal in the PR is present in the DNA-binding domain (Guiochon-Mantel et al., 1988).

For the AR, the situation is the reverse of that for the PR: deletion of the DNA-binding domain and part of the hinge region, including the SV40-like signal, results in a block of nuclear translocation in the absence of hormone. Addition of hormone, however, results in translocation of the mutant receptor to the nucleus, indicating that also the AR contains two nuclear localization signals, of which one is ligand dependent (Jenster et al., 1991).

Short peptides homologous to the SV40-like NLS from RAR, GR, ER, and AR, chemically coupled to bovine serum albumin and introduced into cells by viral cointernalization, were able to direct the conjugate to the nucleus (Hamy et al., 1992). This indicates that the SV40-like signal is not only necessary for either hormone-dependent or hormone-independent translocation of the steroid hormone receptors, but is also sufficient for nuclear translocation of other peptides.

It is not known how the NLS sequences are involved in directing the proteins into the nucleus. Do these sequences interact directly with the nuclear pore complex, or do they interact with soluble proteins which in their turn interact with the nuclear pore complex? Proteins have been described, which bind with high affinity to synthetic peptides containing the nuclear localization signal. These proteins were present in cytosol, nuclei and a nuclear envelope fraction, suggesting that they play a role in nuclear translocation (Adam et al., 1989). Furthermore, proteins without their functional targeting sequences can still be transported into the nucleus as part of a complex with a protein that has a nuclear targeting sequence (Dingwall & Laskey, 1991). This was also shown for the PR; a receptor mutant which was not able to translocate to the nucleus in either a hormone-dependent or a hormone-independent way, could be translocated when a receptor mutant which was non-defective in hormone-dependent translocation was co-transfected and hormone was added (Guiochon-Mantel et al., 1989). It can be conceived that this co-translocation by dimerization is a special form of a more general phenomenon. It might be that also the hormone-independent receptor translocation depends upon co-transportation with other proteins. In this respect, it is of interest to note, that hsp70, with which the receptor can be associated, also contains a nuclear localization signal and is also found in the nucleus (Koskinen et al., 1991).

1.2.7 Association of Steroid Hormone Receptors With Heat-Shock Proteins: Fact or Artifact?

As was described above, the AR is located predominantly in the nucleus, both in the presence and absence of hormones. Rupture of cells in the absence of hormones results in high quantities of receptor protein in the cytosolic fraction. After addition of hormone to cells, the receptor becomes more tightly bound to the nucleus and is recovered in the nuclear pellet, but can still be extracted with high concentrations of salt.

The steroid hormone receptor recovered from the cytosolic fraction, sediments as a large heteromeric complex with a sedimentation coefficient of 8—10 S, that has an apparent molecular weight of about 300,000. This form of receptor does not bind to DNA. Addition of hormone at elevated temperatures (from room temperature to 37°C), *in vivo* (cultured cells) and also *in vitro* (cell-free systems), results in both dissociation of the complex to a form with a sedimentation coefficient of 4—5 S, and the ability of the receptor to bind to DNA. This process is called transformation. Dissociation of the heteromeric complex also occurs in the presence of high concentrations of salt, high levels of ATP, or after dilution, even when no ligand is bound. The unliganded, but 'transformed' PR and GR also bind specifically to DNA (Bailly et al., 1986; Willmann & Beato, 1986). The 8—10 S complex is stabilized by the group 6A transition metal oxyanions molybdate, vanadate, and tungstate. It is also stabilized by aluminium fluoride, and hydrogen peroxide. Stabilization of the complex always coincides with the inability of the receptor to bind to DNA (for reviews see Grody et al., 1982; Pratt et al., 1989).

The definition of 'cytosol' is a biochemical one. It is the supernatant of broken cells

after ultra-speed centrifugation. Although the cytosol is therefore not the same as the cytoplasm of cells, the fractionation protocol suggests that nuclear components are not present and that soluble components of the cytoplasm are present. The presence of hormone receptors in the cytosol fraction, even when most of the receptors can be localized in the nucleus of intact cells by immunohistochemical methods, therefore suggests that leakage from the nucleus during cell fractionation is responsible for this phenomenon. The discrepancy, however, has caused considerable debate about the relevance of some biochemical studies on cytosolic steroid receptors. Therefore, when the first reports appeared, showing that the unoccupied GR, PR, ER, and AR were associated with a 90 kDa heat-shock protein (hsp90; Joab et al., 1984; Sullivan et al., 1985), the biological relevance of this association was questioned and the association was thought to be an artifact caused by the rupture of the cells.

Because the biological relevance of the isolated cytosolic complexes was debated, several groups have tried to obtain evidence that the association of the receptors with hsp90 occurs within living cells. It was shown that, *in vivo*, newly synthesized GRs predominantly associate with newly synthesized hsp90 (Howard & Distelhorst, 1988). In addition, it was possible to cross-link the GR to hsp90 in intact cells in the presence of DMSO (Rexin et al., 1988). Renoir et al. (1990a) stabilized the PR complex with a combination of tungstate and the receptor antagonist RU486, and could extract complexes containing both hsp90 and receptor molecules from nuclei. The latter two types of experiments, however, are subject to debate, because one can argue about the intactness of cells in the presence of DMSO and cross-linkers, or in the presence of tungstate and RU486. However, there is other evidence for a role of hsp90 in normal receptor function. The GR is not able to bind hormone, or binds hormone with a relatively low affinity, in the absence of hsp90 (Bresnick et al., 1989; Nemoto et al., 1990). Reconstitution of the association between the GR and hsp90 in a rabbit reticulocyte system, results in recovery of the steroid binding activity (Scherrer et al., 1990). Furthermore, in a yeast strain with an inducible hsp90 homologue it was shown that the artificially expressed GR could stimulate transcription from a glucocorticoid responsive reporter gene, only when hsp90 was expressed in sufficient amounts to bind the receptor (Picard et al., 1990). This indicates that hsp90 represses receptor-DNA binding, but that interaction of the receptor with hsp90 also facilitates the subsequent ligand-induced activity of the receptor (Picard et al., 1990).

In addition to hsp90, other proteins, including a 70-kDa and a 56- to 60-kDa (heat-shock) protein have been shown to be associated with steroid hormone receptors. More detailed information on hsp90 and the other receptor-associated proteins will be given in Paragraph 1.3. At this point in the Introduction, it is important to emphasize that the AR in intact cells, in the absence of hormones, is bound to other proteins, and that binding of hormone results in a derepression of DNA binding and subsequently leads to transcription activation.

1.2.8 Is Dissociation of the Receptor—Heat-Shock Protein Complex Sufficient for DNA Binding and Transcription Regulation?

The fact that steroid hormone receptors, when not bound to hsp90, can bind specifically to DNA even in the absence of hormone, has often been used as an argument for the idea that, in the heteromeric complex, the DNA-binding domain of the receptor is masked. Dissociation of hsp90 from the receptor would expose the DNA-

binding domain and result in DNA-binding capacity. It is clear that dissociation of the heteromeric complex is needed, but there are several reports indicating that it is not sufficient. Also after dissociation of hsp90 from the receptor, hormone is required for binding of the receptor to hormone response elements. In addition, the subsequent transcription activation might require ligand-receptor interaction. In studies on DNA-binding, the PR and GR sometimes require hormone for DNA binding, but sometimes not. Hormone was found to be required and sufficient (Bagchi et al., 1990a, 1991; Reik et al., 1991), required but not sufficient (Edwards et al., 1991), or even not necessary for DNA-binding of steroid receptors (Schauer et al., 1989; Bagchi et al., 1990b; Brown & Sharp, 1990; Curtis & Korach, 1990; Fawell et al., 1990; Kalff et al., 1990; Klein-Hitpass et al., 1990; Tsai et al., 1990). In these experiments, the purity of receptor preparations and cell type specific differences might play an important role.

In *in vitro* transcription assays, the effect of hormone on transcription activation has been measured. When hormone was not required for DNA binding, also transcription activation was seen without hormone (Bagchi et al., 1990b; Kalff et al., 1990; Klein-Hitpass et al., 1990). In two studies, the PR antagonist RU486 was used to study the effect on transcription. In one of these studies, where no hormone was required for DNA-binding, it could block *in vitro* transcription (Kalff et al., 1990), whereas in another study it stimulated receptor-DNA binding, and also transcription (Bagchi et al., 1990a).

In all studies described above, disruption of hsp-binding was necessary, although not always sufficient, for both DNA-binding and transcription activation. Whether the hormone has more tasks than dissociating the receptor complex, therefore, remains uncertain from these *in vitro* experiments. When the ER was expressed in yeast, hormone was required for both DNA binding in intact yeast cells, and for transcription activation (McDonnell et al., 1991). However, when the expression of ER was very high, no hormone was needed for binding to DNA, but still was required for transcription activation. The results obtained with the yeast cells suggest a two-step model in which the ligand first causes dissociation of the receptor from an inhibitory complex, resulting in a DNA-binding form. The ligand then converts the receptor into a transcriptionally competent form (McDonnell et al., 1991).

1.2.9 Dimerization and DNA Binding

Steroid receptors bind to regulatory DNA sequences as dimers, and transformation of receptors to a DNA-binding form therefore includes the transformation to a dimerization competent form. It is not very clear, however, whether dimerization takes place before binding of the receptors to DNA, or is a consequence of binding to DNA. There are reports of dimerization either on DNA (Kumar & Chambon, 1989; Tsai et al., 1988), or in solution (Cairns et al., 1991; DeMarzo et al., 1991; Rodriguez et al., 1990; Wrange et al., 1989). It has been shown for the GR that the binding of a receptor molecule to the second half-site in the HRE is facilitated by the occupancy of the first half-site (Dahlman-Wright et al., 1990). Changing the distance between the half-sites within the HRE impairs both binding of a second receptor molecule, and thus dimerization, and receptor enhanced transcription activation (Dahlman-Wright et al., 1990; Chalepakis et al., 1990). Impairment of ER dimerization, either by mutagenesis of the ER or by binding of certain antiestrogens to the ER, results in inhibited binding of the receptor to DNA (Fawell et al., 1990a,b). This inability of anti-estrogens to promote dimerization can be overcome by binding of an antibody, which forms a complex with

two receptor molecules and brings the two receptor molecules in close proximity. Thus by mimicking a receptor dimer, restoration of DNA-binding is observed (Fawell et al., 1990a). In addition to the role of the second zinc-finger in dimerization (Green & Chambon, 1989; Härd et al., 1990; Schwabe et al., 1990; Luisi et al., 1991: Dahlman-Wright et al., 1991), a region in the steroid-binding domain, conserved among the steroid receptor family, has been implicated to play a role in dimerization of the ER (Fawell et al., 1990a; Lees et al., 1990).

In conclusion, dimerization of steroid hormone receptors is a prerequisite for stable binding of the receptors to DNA. Dimerization might occur in solution, but in the presence of HREs, either this process is enhanced, or preexisting dimers are stabilized by binding of the dimer to the HRE. These interactions may be described by the following reactions, resulting in an equilibrium situation:

$$\begin{array}{ccc} R + R + HRE & \rightleftarrows & RR + HRE \\ \uparrow\downarrow & & \uparrow\downarrow \\ R + R\text{-}HRE & \rightleftarrows & RR\text{-}HRE \end{array}$$

In this scheme, R is the receptor, RR is a receptor dimer, HRE is the hormone response element, and R-HRE and RR-HRE are HREs bound to one or two (dimer) receptors, respectively.

1.2.10 Transcription Activation

There are several possible mechanisms by which binding of a receptor-dimer to a HRE could enhance transcription of the gene in question. In one model, the binding of the receptor-dimer induces a disruption of the chromatin structure which allows the transcription machinery to do its work. The receptor-dimer either stays bound to the HRE (Pham et al., 1991; Reik et al., 1991), or it leaves the site and makes it accessible for other factors via a 'hit-and-run' mechanism (Rigaud, et al., 1991). The PR was shown to enhance the formation of a stable preinitiation complex at the target gene promoter (Klein-Hitpass et al., 1990). Alterations in the chromatin structure induced by the ER were associated with the establishment of active transcription complexes (Pham et al., 1991). This was also dependent upon domains of the ER which are important for transcription activation. The commonly accepted idea is, that the transcription activation domains contact transcription factors, which in their turn, directly or indirectly, activate the transcription machinery. According to definition, a transcription-activation domain is a region of the receptor that, when combined with a DNA-binding region, can increase the frequency of transcription initiation (Ptashne, 1988; Ptashne & Gann, 1990).

Several subregions of steroid hormone receptors have been assigned to be important for transcription activation. Some have been very well defined, and have been shown to function as transcription regulators also in combination with only a DNA-binding domain. These regions were called transcription activating functions (TAFs). Other regions important for transcription regulation are less well defined. Both the N-terminal domains and the steroid-binding domains of the GR (Giguère et al., 1986; Godowski et al., 1987), PR (Meyer et al., 1990), AR (Jenster et al., 1991; Simental et al., 1991), and ER (Tora et al., 1989a; Webster et al., 1988) contain a more or less well defined region that is important for transcription activation. It might be that for some receptors, there are more than two regions important for transcription activation. For the GR

(Hollenberg et al., 1987; Schena et al., 1989) and ER (Nardulli et al., 1991) the DNA-binding domains also have a transcription-activation function.

The recognition that steroid hormone receptors contain two, or even more regions involved in transcription activation, is very important. It is a crucial point for comparing the results obtained by different groups studying transcription activation by steroid hormone receptors, especially concerning the effects of steroid receptor antagonists. A significant feature is the fact that two TAFs of one receptor have different characteristics. The N-terminally located TAF (TAF-1) can be constitutively active, whereas the C-terminally located TAF (TAF-2) is only active after binding of ligand. Furthermore, TAF-1-mediated transcription activation is dependent on cell type and promoter context (Berry et al., 1990; Meyer et al., 1990; Tora et al., 1989a). Hence, transcription activation can be observed only in the proper system with respect to cell type and promoter type. The existence of a constitutively active TAF also can explain the partial agonistic effects of some antagonists (see Paragraph 1.2.12).

1.2.11 Phosphorylation

Probably all steroid hormone receptors are phosphorylated. The function of this phosphorylation is unknown, but effects of ligands on the level of phosphorylation suggest that a functional role does exist (for a review see Orti et al., 1992). Both ligand-binding capacity of the GR and hormone-independent PR-mediated transcription activation have been suggested to depend on receptor phosphorylation status of these receptors (Munck et al., 1972; Bell & Munck, 1973; Nielsen et al., 1977; Denner et al., 1990).

The AR is phosphorylated both in an androgen independent and in an androgen dependent way (Van Laar et al., 1990, 1991; Kuiper et al., 1991; Kemppainen et al., 1992). The amino acid residues that are phosphorylated are not known. Furthermore, the relation between the different transformation steps and the phosphorylation status of the receptor have not been determined yet. It can be envisaged that e.g., after dissociation of the receptor-hsp complex a phosphorylation site is exposed. Phosphorylation may be necessary for subsequent steps in transcription activation. Alternatively, hormone-induced phosphorylation may be a prerequisite for dissociation of the receptor-hsp complex. Another possibility is that dissociation of the receptor-hsp complex and phosphorylation are two independent results of steroid binding. Possibly, steroid receptor antagonists have some indirect effect on receptor phosphorylation.

1.2.12 Mechanisms of Antagonist Action

Many molecular aspects of steroid hormone receptor function are potential targets for antagonist action. Antagonists compete with agonists for binding to the receptor, but do not result in full transformation of the receptor to a transcriptionally active form. Processes which have been suggested to be blocked by antagonists include translocation of steroid receptors to the nucleus (Lindemeyer et al., 1990; Segnitz & Gehring, 1990), dissociation of receptor-hsp complexes (Distelhorst & Howard, 1990; Lefebvre et al., 1988; Moudgil & Hurd, 1987; Segnitz & Gehring, 1990; Renoir et al., 1990a), dimerization (Fawell et al., 1990; Klein-Hitpass et al., 1991), DNA-binding (Berry et al., 1990), and interaction with transcription factors (Guiochon-Mantel et al., 1988; El-Ashry et al., 1989; Berry et al., 1990; Meyer et al., 1990; Sabbah et al., 1990; Klein-Hitpass et

al., 1991; Pham et al., 1991) (see Figure 2).

Hormone-independent, N-terminally located TAFs (e.g., TAF-1 of ER and PR) are only activated by binding of the receptor to DNA. Hence, antagonists which block the interaction of the hormone-dependent TAF of steroid hormone receptors with transcription factors, but allow binding of the receptor to DNA, have potentially agonistic properties. Because the function of hormone-independent TAFs is also cell type- and promoter-specific, such compounds can be both agonist and antagonist for one receptor, depending on the context. This might explain the existence of partial agonists (Berry et al., 1990; Green, 1990; Meyer et al., 1990; Reese & Katzenellenbogen, 1991). A more elaborate discussion on partial antagonists can be found in Chapter 6.

Several investigators have described characteristics of receptors bound to either agonists or antagonists, such as different conformations (Moudgil et al., 1988; Meyer et al., 1990) or different electrophoretic mobility (Sabbah et al., 1991). These characteristics may be responsible for, or contribute to, antagonist-induced inhibition of receptor function, but do not necessarily involve alternative modes of action of antagonists, because they possibly are caused by one of the mechanisms described above. It is therefore important to make a clear distinction between the target of action, in terms of which transformation step is blocked, and the molecular basis which causes the block.

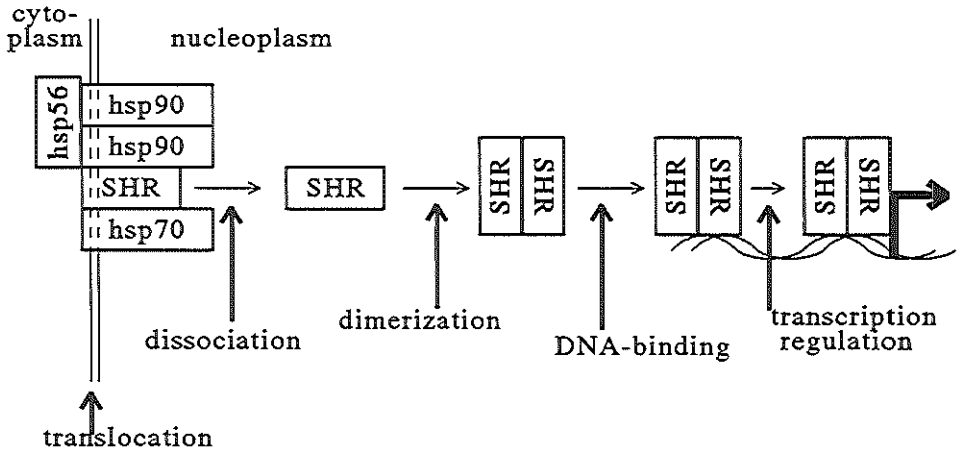

Figure 2: Possible steps in the action of steroid hormone receptors (SHR) which might be blocked by steroid receptor antagonists (↑).

1.3 Heat-Shock Proteins

In the previous section it was discussed that heat-shock proteins play an important role in several aspects of steroid hormone receptor action. To understand this role, knowledge about the composition of the heteromeric complex of receptor and associating proteins, and the sites of interactions between the components is needed. In the following paragraphs, it is discussed that heat-shock proteins are very common among all life forms and are important for several cellular house-keeping functions. The term 'heat-shock' is misleading; heat-shock proteins are also present in cells that have not been exposed to

a heat-shock, and their role in steroid hormone receptor function are only a small fraction of their tasks.

1.3.1 Heat-Shock Proteins Are Induced By Stress

The first report on heat-shock response was on the induction of a new puffing pattern in *Drosophila* (Ritossa, 1962). The puffing pattern was related to the synthesis of new mRNAs. Later it was reported that cells of all known organisms respond to heat treatment or other stress conditions with enhanced synthesis of a variety of proteins. These proteins were first called 'heat-shock' proteins, but a more suitable name would be 'stress' proteins. It is thought that increased levels of these proteins protect cells against the otherwise damaging influence of stress conditions. Prove for this protective role came from reports that deletion of some of the genes coding for heat-shock proteins in bacterial and yeast cells had lethal effects only when the cells were exposed to increased temperatures (Saito & Uchida, 1977; Itakawa & Ryu, 1979; Craigh & Jacobson, 1984). There is a high degree of sequence similarity for several classes of heat-shock proteins between organisms as diverse as bacteria, plants and higher eukaryotes (Craigh, 1985; Lindquist, 1986). The size of the proteins ranges from as small as 8 kDa to as large as 110 kDa. The idea is that heat-shock proteins play a role in normal cell functioning, but are even more important under stress conditions. Therefore, stress either enhances the expression of heat-shock proteins which are already present, or it induces the expression of new heat-shock proteins which are closely related, but are better equipped to function under stress conditions. There are two members of the 70 kDa heat-shock protein (hsp70) family, for example, which are highly related but distinct gene products (Craigh, 1985; Lindquist, 1986). One of these, hsp73, is constitutively expressed under non-stress conditions. The other, hsp72, is expressed at very high levels after stress, but is not expressed under non-stress conditions with the exception of primate cells (Welch et al., 1983; Welch, 1990).

1.3.2 What Is the Role of Heat-Shock Proteins and Their Constitutively Expressed Counterparts Under Non-Stress Conditions? The Molecular Chaperone Concept.

Newly synthesized proteins have to adopt a functional conformation. The conventional idea is that a native protein can self-assemble into a conformation of lower free energy. In this concept, oligomerization can be a function of a protein, so that the requirements for this process are intrinsically present in the structure of the monomeric proteins. In cases where the probability for incorrect inter- and intra-molecular interactions is high, which may result in non-functional structures, chaperone proteins assist in both finding the optimal conformation and the assembly of oligomeric structures. Molecular chaperones are currently defined as a family of unrelated classes of proteins that mediate the correct self-assembly of other proteins, but are not themselves components of the final functional structures (Ellis & Hemmingsen, 1989). Their function is to inhibit the processes which lead to incorrect protein folding or the assembly of non-functional complexes. Processes which might need chaperoning are protein synthesis (to prevent unfavourable interactions in partly synthesized proteins during synthesis), protein transport across membranes (to prevent translocation-incompetent conformations and to refold the proteins after translocation), functional changes in subunit-subunit interactions,

organelle biogenesis (assembly of protein complexes consisting of subunits derived from both intra-organellic and cytoplasmic source), and stress responses (prevention of aggregation of denatured proteins, stimulation of renaturation of denatured proteins, or assistance in the degradation of non-functional proteins); reviewed by Ellis & Van der Vies (1991). It is thought that heat-shock proteins are chaperones, and so the stress response would be an amplification of the basic chaperone function (Beckmann et al., 1990; Pelham, 1990).

1.3.3 The hsp90 Family

Hsp90 is a phosphoprotein which is abundantly present (1—2% of the total cellular protein content) in all prokaryotic and eukaryotic cells examined thus far. Up to now there are no indications for enzymatic actions of hsp90 on the proteins with which it interacts, but the heat-shock protein has an intrinsic kinase activity, which can result in autophosphorylation on serine residues (Csermely & Kahn, 1991). Hsp90 is present in both cytoplasm and nucleus of rabbit uterus cells (Gasc et al., 1990). It was the first heat-shock protein which was recognized to be associated with steroid hormone receptors (Joab et al, 1984; Sullivan et al., 1985). There are two genes in mammals which encode hsp90. Hsp90α and hsp90ß are highly homologous (70% conserved amino acid sequence; Rebbe et al., 1987; Hickey et al., 1989). A third protein which is related to the cytoplasmic/nuclear hsp90 proteins is GRP90 (glucose-regulated protein), which is associated with the endoplasmic reticulum. It is retained in the membrane of the endoplasmic reticulum, due to its 4 amino acid extension at the COOH-terminus (Munro & Pelham, 1987).

Hsp90α and ß are present in cell lysates as complexes, with a wide variety in size, indicative for interactions with several other proteins (Welch, 1990). One of these complexes is particularly interesting because, in addition to hsp90, it also contains hsp70 and hsp56 (Sanchez et al., 1990a), which are also associated with steroid receptors.

Hsp90 transiently interacts with the oncogene product $pp60^{src}$ in the cytoplasm, inhibiting its kinase activity, until it is deposited at the inner side of the plasma membrane where it regains its kinase activity (Brugge et al., 1981; Brugge, 1986). Similar associations with hsp90 have been reported for several other transforming kinases (Ziemiecki, 1986; Ziemiecki et al., 1986) and for casein kinase II (Dougherty et al. 1987).

Moreover, hsp90 was found to associate with the microfilamental and microtubular network (Koyashu et al., 1986; Nishida et al., 1986; Sanchez et al., 1988). Heat shock induces an increase in nuclear hsp90 (Collier & Schlesinger, 1986). These latter observations may indicate that the cytoskeleton is involved in the transport of hsp90 towards the nucleus, to the perinuclear area. It has been suggested that the non-transformed GR is bound to actin filaments through hsp90 (Miyata & Yahara, 1991). Similarly, hsp90 may be associated with both tubulin and the nontransformed GR (Pratt et al., 1989). In addition, the GR was co-localized with cytoplasmic microtubules (Akner et al., 1991). It can be envisaged that both the cytoskeleton and hsp90 play a role in transport of steroid receptors towards the nucleus during the transformation process.

Another protein which interacts with hsp90 is the heme-regulated protein kinase (HRI) which phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF-2α)(Rose et al., 1989). Activation of the kinase by hemin depletion results in dissociation of the HRI-hsp90 complex (Matts & Hurst, 1989). The activation of the kinase is dependent on the phosphorylation state of hsp90. The ability to increase HRI activity

upon dissociation of the HRI-hsp90 complex is lost when hsp90 is dephosphorylated (Szyszka et al., 1989). Rephosphorylation of hsp90 by casein kinase II restores its activity. This indicates that hsp90 binding to HRI is needed for HRI activation. Some aspects of this model are reminiscent of the steroid receptor-hsp90 association model; hormone-induced activity of the GR in yeast is only possible when a transient association of the receptor with the yeast homologue of hsp90 takes place (Picard et al., 1990).

1.3.4 The hsp70 Family

Members of the hsp70 family are present in both prokaryotes and eukaryotes, and reside in different compartments of eukaryotic cells. The members of the hsp70 family have in common that they interact with incompletely processed and matured proteins (Beckmann et al., 1990; Chirico et al, 1988; Zimmerman et al., 1988; Kang et al., 1990). It has also been proposed that hsp70 proteins can bind to mature proteins when these are unfolded under stress conditions. This association would enhance the refolding of the damaged proteins (Pelham, 1986). In mammals there are two cytoplasmic family members derived from two different genes: a stress inducible form, hsp72, and a constitutively expressed form, hsp73. The latter is also referred to as hsc70 (Craigh, 1985; Lindquist, 1986). Both cytosolic forms translocate to the nucleus upon heat-shock, possibly to bind denatured pre-ribosomes (Welch & Suhan, 1986).

The role of hsp70 proteins is not restricted to stress related events. Most of the functions of molecular chaperones in general are also performed by one or more members of the hsp70 family. Newly synthesized proteins might become associated with hsc70 to ensure a proper folding (Beckmann et al., 1990). Hsc70 also binds to clathrin and accelerates the removal of the clathrin triskelion subunits from clathrin-coated vesicles (Chappell et al., 1986; Ungewickel et al., 1985). In addition, hsc70 is needed for efficient translocation of bacteriophage M13 protein into vesicles of the rough endoplasmic reticulum, suggesting a role in transmembrane transport (Chirico et al., 1988; Zimmerman et al., 1988). In yeast, a hsp70 member in the mitochondrial matrix is required for protein translocation and the correct folding of proteins imported into the matrix (Kang et al., 1990). The endoplasmic reticulum of eukaryotic cells contains a hsp70 member, termed either grp78 (glucose-regulated protein, i.e., upregulated by glucose starvation) or BiP (Binding Protein), which plays a role in the assembly of multimeric protein complexes (Lee, 1987; Haas & Wabl, 1983).

All hsp70s examined bind ATP and ADP (Welch & Feramisco, 1985; Flaherty et al., 1990). The affinity of hsp70 for unfolded proteins is high when it is bound to ADP. Binding of an unfolded protein results in folding of the protein and displacement of the ADP by ATP. The release of hsp70-bound proteins requires the hydrolysis of ATP to ADP. The affinity of the resulting ADP-bound hsp70 is high for unfolded proteins, so that the cycle can be repeated. The net result of this 'three-state cycle' (Palleros et al., 1991) is the proper folding of partially unfolded proteins and the hydrolysis of ATP. How the hsp70 class of proteins discriminates unfolded proteins from folded proteins, is subject of extensive research.

Folding of the nascent steroid receptor protein, complex formation with other proteins, nuclear translocation, and dimerization, are all candidates for hsp70-assisted processes. The fact that antibodies against hsp70 can inhibit reassociation of the PR to hsp90 and even destabilizes preexisting hsp90-hsp70-PR complexes (Smith et al., 1990b; Smith et al., 1992), indicates that hsp70 plays a role in complex-formation. The

association of steroid receptors with hsp70 can be disrupted by ATP, but it is not certain whether this plays a role in the receptor transformation process (Smith et al., 1992). The ATP dependent dissociation of the receptors and hsp70, however, indicates that their interaction might be similar to the type of interaction between hsp70 and denatured proteins described above.

1.3.5 Hsp56/p56—60

Until recently, only very little was known about p56 or p59. They were described as steroid hormone receptor-associated proteins, which could be detected by the antibody EC1 (Tai et al., 1986; Renoir et al., 1990b; Sanchez et al., 1990a). P56 is found in human cells, and its synthesis is enhanced in human IM-9 cells upon incubation of the cells at 43°C for 4 h or by chemical stress, and p56 therefore was called a heat-shock protein (hsp56; Sanchez, 1990).

The rabbit 59 kDa protein has been cloned and sequenced (Lebeau et al., 1992). Its N-terminal sequence was identical for 15 out of 19 amino acid residues with that of human hsp56. The protein does not bear significant sequence similarity with any other known heat-shock protein. Its sequence showed putative phosphorylation sites, a putative ATP binding site, and a possible calmodulin binding site, and a 96 amino acid stretch with 55% homology to peptidyl-prolyl isomerase (Lebeau et al., 1992).

Other investigators isolated a 60-kDa protein from human Jurkat cells and calf thymus using a FK506 affinity matrix (Yem et al., 1992). FK506 is an immunosuppressant drug that inhibits T-cell proliferation by binding to regulatory proteins. The isolated human and calf proteins had N-terminal sequences which were nearly identical. Moreover, the N-terminal sequence of the human protein was identical to that of hsp56 (Yem et al., 1992). Therefore, these FK506-binding proteins (FKBPs) might be p56(hsp56) and the calf homologue of this protein, respectively. Part of the calf thymus protein showed homology to a region near the COOH-terminus of two other FK506 binding proteins, FKBP-12 and FKBP-13 (Yem et al., 1992). The immunosuppressant binding proteins FKBP-12, FKBP-13, and cyclophilin (which binds the immunosuppressant cyclosporin A) all possess peptidyl prolyl cis-trans isomerase (or rotamase) activity that catalyses the cis-trans isomerization of proline peptide bonds and accelerates rate limiting steps in the folding of proline-containing proteins (Chang et al., 1991). The homology of the rabbit 59 kDa and calf 60 kDa proteins with these proteins might indicate that hsp56/p56—60 is related to, and possibly has similar activity as peptidyl-prolyl isomerase. Whether this activity plays a role in the steroid hormone complexes remains to be elucidated. It was speculated that p59 could regulate the function of hsp90 (Lebeau et al., 1992). This protein was found mainly in the nucleus (Gasc et al., 1990).

1.3.6 How Many Hsp90 Molecules Are Associated with One Receptor Molecule?

Several groups have attempted to calculate the stoichiometry of hsp90-receptor interaction. Most investigators found a molecular ratio hsp90:receptor of 2:1. Mendel & Orti (1988) calculated that one GR molecule was associated with two hsp90 molecules. The calculation was made by measuring the relative amounts of labelled methionine incorporated into the two immunoadsorbed proteins. The same conclusion was reached

using a combination of immunoaffinity chromatography and high-performance anion-exchange liquid chromatography (Denis et al., 1987). This stoichiometry correlates with the one found by Rexin et al. (1988), who, by examining (partially) cross-linked complexes, calculated that one heteromeric GR-complex consists of one steroid-binding subunit, two 90 kDa subunits, and a ≈50 kDa subunit. Furthermore, hsp90 is in a dimeric form when it is released from the PR (Radanyi et al., 1989). Bresnick et al. (1990) used three different techniques, including a more rapid method of immune isolation and a more gentle washing procedure, which resulted in ratios of two to ten hsp90 molecules to one GR molecule. They concluded that the 9S complex which is generally found in the cytosol must be a 'core unit' containing two hsp90 and one GR, which is derived from a larger structure.

1.3.7 Other Receptor-Associated Proteins

As described in Paragraph 1.3.5, the second heat-shock protein which was discovered to be associated with steroid hormone receptors was a protein of 56—60 kDa, the molecular mass depending on the species. It was first detected as a 59 kDa subunit in rabbit PR-, GR-, AR-, and ER-complexes (Tai et al., 1986). The human homologue, p56, was also found to exist in cytosol in a higher order complex containing both hsp90 and hsp70, but no steroid receptor (Sanchez et al. 1990a). Furthermore, it was shown that the rabbit homologue, p59, is bound to hsp90 but not to the hormone-binding subunit of the steroid-receptor complexes (Renoir et al., 1990b). These findings suggest that p56—60 does not interact with the receptor itself, but might play a role in regulating or modifying the function of hsp90, as suggested in Paragraph 1.3.5. Moreover, p56 was shown to be a heat-shock protein (Sanchez, 1990). In view of the chaperoning function of heat-shock proteins (see above), it might be that hsp56 plays a role in modulation of hsp90 function.

As mentioned in Paragraph 1.2.7, in addition to hsp90 and hsp56, hsp70 is also associated with steroid receptors. It was first discovered in immunoprecipitation studies with the avian PR from quail oviduct. Precipitation of the receptor resulted in co-precipitation of 90, 70, 54, 50 and 23 kDa proteins (Kost et al., 1989; Smith et al., 1990a). The 90 kDa protein is hsp90. The 54 kDa protein (Smith et al., 1990a) might be the avian homologue of human hsp56, but the antibody reacting with human hsp56 or the rabbit p59 did not cross-react. The smaller proteins have not been further characterized up till now. The 70 kDa protein was identified as hsp70. It binds directly to the receptor, and this association can be disrupted by addition of ATP at 23°C (Kost et al., 1989). Mouse GR, over-expressed in chinese hamster ovary cells (CHO cells), also is associated with hsp70 (Sanchez et al., 1990b). However, the endogenous untransformed mouse GR in L cell (fibroblast) cytosol is not associated with hsp70. In the CHO cells the receptor was present in the nucleus, while in L cells it was present in the cytoplasm. It was therefore speculated that the association of the receptor with hsp70 reflects its presence in an inactive 'docking' complex in the nucleus (Sanchez et al., 1990b; see below). Hormone-induced transformation of both quail PR and mouse GR, and even high concentrations of salt, do not result in hsp70 dissociation from the receptors (Kost et al., 1989; Smith et al., 1990a; Sanchez et al., 1990b). ATP-mediated dissociation of hsp70 from human PR does neither enhance, nor impair the ability of PR to bind to specific sites on DNA, indicating that hsp70 does not play a role in the DNA binding process (Oñate et al., 1991). Hsp70 does play a role, however, in the reconstitution process of PR-complexes in rabbit reticulocyte lysate. Restoration of hsp90 binding to chicken

oviduct PR requires rabbit reticulocyte lysate that contains both hsp90 and hsp70, absence of progesterone and oviduct cytosol, presence of ATP and Mg^{2+}, and an elevated temperature (30°C). Addition of antibodies to hsp70 inhibits hsp90-PR association and destabilizes complexes already present (Smith et al., 1990b; Smith et al., 1992). Whether hsp70 also plays a role in the assembly of receptor complexes *in vivo* is not known.

In conclusion, untransformed steroid hormone receptors are associated with at least three heat-shock proteins: hsp90, hsp70, and hsp56 (p56—60). A proportion of these hsp's are also present in cells as complexes which are not associated with members of the steroid hormone receptor family, indicating that these complexes serve a more general role. Little is known about the 54-, 50-, and 23-kDa receptor-associated proteins in quail oviduct, although the 54-kDa protein might be the avian homologue of human hsp56.

1.3.8 What Parts of the Receptor Molecule Interact With Hsp90 or Hsp70?

Because steroid receptors consist of several functional domains, which, to a certain degree can function independently from each other, it is of interest to know which parts (domains) of the receptors interact with the receptor-associated proteins. This knowledge might give clues about the function of such associations. Several groups have tried to locate the domains of steroid receptors that interact with hsp90. Before it had been described that also hsp70 was associated with steroid hormone receptors, the investigations focused either on the detection of the hsp90 protein, or on the size of the receptor complexes as a whole. A decrease in complex size was interpreted as a loss of receptor-hsp90 association, but now we know that a decrease in size of the receptor complexes might also result from the dissociation of hsp70 and other proteins. Most studies were performed for GRs. The first reports indicated that the steroid-binding domain binds hsp90 (Denis et al., 1988a; Pratt et al., 1988). Originally, it was speculated that a 20 amino acid sequence, conserved among steroid receptors (a.a. 583—602 of the mouse GR), was the site of interaction with hsp90 (Danielsen et al., 1986). Later it was proposed that a second site was important for molybdate mediated stabilization of the complex (Housley et al., 1990; Dalman et al., 1991a). In this two-site model, region 574—632 is important for molybdate independent association of hsp90, and region 632—659 is important for the association which is stabilized by molybdate. Howard et al. (1990) claimed for the rat GR that only receptor sequences between amino acid residues 568 and 616 (corresponding to mouse GR 556—604) were necessary for hsp90 complex formation. Recent studies do not support the notion that only a small region of the receptor is involved in interaction with hsp90. Cadepond et al. (1991) divided the ligand-binding domain of the human GR in three subregions. Each of the subregions could be deleted without loss of 8S complex formation. Moreover, each of the subregions was sufficient for at least some 8S complex formation.

For the PR, the situation is quite similar to the one described for the GR. Only deletion of the entire steroid-binding domain eliminated binding of both hsp90 and hsp70, and three separate regions were able to partially restore hsp90 and hsp70 binding to this mutant protein. Binding of both hsp90 and hsp70 was not abolished when one of these, or other, regions of the steroid-binding domain were deleted (Schowalter et al., 1991). A similar conclusion was reached for hsp90 binding to PR by Carson-Jurica et al. (1989). Also for the ER no limited subregion of the ligand-binding domain was absolutely required for hsp90 binding. However, the C-terminal part of the DNA-binding domain (hER: amino acid residues 251—271, containing the nuclear localization domain)

was necessary, but not sufficient, for complex formation (Chambraud et al., 1990).

In some of the investigations described above, the mutant receptors which lacked binding of hsp90 were also used for transcription activation studies. It is interesting to note that in the studies with GR (Cadepond et al., 1991), PR (Carson-Jurica et al., 1989), and ER (Chambraud et al., 1990), there was a strong correlation between the binding of hsp90 and the repression of receptor-mediated transcription activation; all constitutively active mutants had lost their association with hsp90. This indicates that hsp90 binding might play a physiological role in maintaining the receptor in a nonfunctional state.

For the AR, no studies have been published on the region(s) of this receptor responsible for binding to hsp90 and hsp70. Some unpublished results from our laboratory (Jenster, G. & Thelen, M.) indicate that also for the AR the steroid-binding domain is involved, and that no small regions can be detected which are either sufficient or necessary for binding to hsp's.

1.3.9 Is There a Role For Hsp's in Nuclear Translocation of Steroid Hormone Receptors?

As described above, unoccupied steroid hormone receptors probably reside in both cytoplasm and nucleus. The unoccupied nuclear receptors are loosely associated with the nucleus, since disruption of the cells results in loss of nuclear association, and only hormone-induced transformation results in tight nuclear binding. Some members of the steroid/thyroid hormone receptor superfamily, however, are tightly associated with the nucleus also in the absence of ligand. There is a correlation between the type of nuclear binding in the absence of ligand - tight or loose - and the association of the unoccupied receptor with heat-shock proteins. Receptors which are tightly bound to the nucleus in the absence of ligand, e.g., TR (Samuels et al., 1988) and RAR (Nervi et al., 1989), appear not to associate with hsp90 (Dalman et al., 1990, 1991b). The viral homologue of the TR, v-*erb* A, is found partly as a tight nuclear binding form and partly as a cytosolic form. The latter is, in contrast to the former, also associated with hsp90 (Privalsky, 1991). In addition, GR mutants which are constitutively active are always recovered as small 4S complexes, indicating that hsp90 is absent. It was suggested that the class of receptors which associates with hsp90 forms 'docking' complexes with the heat-shock proteins in either the nucleus (e.g., AR, PR, ER) or the cytoplasm (GR) (Dalman et al, 1991b). The ligand would be the trigger to release the receptor from the docking site and to induce DNA binding (tight nuclear binding). The presence or absence of hsp70 in the GR complexes also correlates with the presence of the receptor either in the nucleus or in the cytoplasm, respectively (Pratt, 1990). These examples indicate that heat-shock proteins play a role in the subcellular localization of steroid hormone receptors. The absence of binding of the receptor to a proposed 'docking' complex correlates with tight nuclear binding (e.g., TR, RAR), and the inclusion of hsp70 in the complex correlates with a (non-tight) nuclear localization. As was discussed above, several isoforms of hsp70 are known to be important for transport of proteins across membranes. It can be envisaged that also for translocation of steroid receptors to the nucleus, heat-shock proteins play a role.

1.4 The Clinical Significance of Antiandrogens

Antiandrogens have been developed, and new ones are still being designed, for pharmacological use. Their capacity to block androgen action makes them useful in the treatment of diseases which respond undesirably to circulating androgens. The greatest impact of these compounds probably has been on treatment of prostate abnormalities, although the effects are mostly palliative (Schröder, 1991). However, there are some other disorders which can be treated successfully with antiandrogens. Moreover, there are some cancers which are potentially androgen responsive, but have not been tested for response to antiandrogens.

1.4.1 The Role of Androgens in Prostate Abnormalities

In addition to the functional maintenance of the prostate, androgens also play an important role in the growth of prostate cancer and benign prostatic hyperplasia (BPH). Several methods have been developed to suppress the amount or activity of androgens in the target tissue. Some of these treatments make use of antiandrogens which negatively regulate the activity of the AR. Antiandrogens are used either alone or in combination therapy, for the treatment of both prostatic carcinomas and BPH.

Adenocarcinoma of the prostate is characterized by malignant growth and is a major cause of cancer deaths among men (Cunha et al., 1987; Carter et al., 1990; Carter & Coffey, 1990). Prostatectomy is the first step in the treatment of prostate cancer, and when the tumor is detected at an early stage, the patient is likely to be cured. However, at time of diagnosis, in most of the patients metastasis already has occurred, and tumors develop in peripheral tissues (Schröder, 1988; Prostate Cancer Working Group, 1991). The idea that growth of prostate carcinomas might be dependent on androgens was first introduced by Huggins and Hodges (1941). They showed that surgical castration or chemical castration with estrogens resulted in a marked improvement for the patients. Also other groups showed beneficial effects of castration, and an improvement of 5-year survival of castrated versus noncastrated patients with advanced metastatic prostate cancer (Nesbit & Baum, 1950; Emmett et al., 1960). However, the major problem is that, eventually, nearly all tumors become independent of androgens for growth, and therefore become unresponsive to androgen depletion. It is not known whether androgen independent tumors originate from formation of androgen independent cells in a population of androgen dependent cells, or whether androgen independent cells are selected from an originally heterogeneous tumor. The process which renders the prostate cells independent of androgens for growth is subject of extensive research throughout the world (Peeling & Griffiths, 1986; Prostate Cancer Working Group, 1991).

BPH is characterized by a nonmalignant nodular enlargement of prostatic tissue, resulting in an obstruction of the urethra. It is estimated that half of all men over the age of 65 have some prostatic enlargement, and at least one-third of these men have clinical symptoms of BPH (e.g., obstruction of the urethra; Hieble & Caine, 1986; Shida, 1986). For the treatment of BPH, in addition to surgery, several hormonal therapies have been described. Medical castration with gonadotropin-releasing hormone (GnRH) analogues, the use of antiandrogens, and 5α-reductase inhibitors all reduce prostate size and decrease urinary obstruction to some extent (Caine et al., 1975; Geller et al., 1979; Gabrilove et al., 1987; Schweikert & Tunn, 1987; Stone, 1989). These therapies vary in effectiveness and some have undesirable side effects (Sciarra et al., 1990). The therapies

employed for treatment of BPH are similar to the treatment of prostate cancer. The difference is, however, that the latter disease is life threatening. The next paragraphs will give some information on prostate cancer therapies and their mechanisms.

1.4.2 Suppression of Androgen Levels

Surgical or chemical castration is the most direct way to reduce the serum level of androgens, and therefore is often employed to reduce the growth of prostate cancer metastases.

By surgical castration (orchiectomy) the main source of androgen production is removed. The testes normally produce 95% of circulating androgens; the other 5% are produced by the adrenals. Weak androgens such as androstenedione and dehydroepiandrosterone and its sulfate are produced in large quantities by the adrenals, and converted to T and DHT in the prostate (Harper et al., 1974) (see Figure 3).

Chemical castration can be accomplished by giving the patients compounds that inhibit the production of androgens. Treatment with estrogens, e.g., diethylstilbestrol, blocks luteinizing hormone (LH) release from the pituitary gland and thereby the production of testicular (but not of adrenal) androgens (see Figure 3). There are, however, several disadvantages of these treatments, including impotence, gynecomastia, cardiovascular and cerebrovascular problems, which make this treatment unfavourable (Veterans Administration Cooperative Urological Research Group, 1967).

Treatment with GnRH agonists results in down-regulation of GnRH-receptors in the pituitary gland, and thereby in a reduction of LH, resulting in a reduction of circulating T to castrate levels (Labrie et al., 1980; Tolis et al., 1982; Warner et al., 1983; Waxman et al., 1983; Labrie et al., 1987) (see Figure 3). A disadvantage of this treatment is an initial increase in LH-release, and hence increased testicular T production, resulting in increased bone pain (and possibly growth stimulation of the cancer cells). The effects of the initial increase in plasma T levels can be counteracted by simultaneous administration of antiandrogens. The mean time to progression of prostate tumors following standard dose estrogen therapy has been shown by the Leuprolide Study Group (1984) to be similar to the mean time to progression following GnRH agonist treatment and surgical castration.

In addition to interference with the normal hormonal regulation of testicular androgen production, androgen synthesis can also be inhibited at the enzymatic level. One type of inhibitor acts at the last step towards the production of the most active androgen, DHT. It blocks the action of the enzyme 5α-reductase, which converts both testicular and adrenal androgens to DHT (see Figure 3). One such an inhibitor, MK-906 (finasteride) has no known side effects, but tissue T levels rise and the therapeutic effect on advanced prostate cancer might be limited (Geller, 1991a). Other inhibitors of androgen production, such as ketoconazole, act at an earlier step in the production of androgens. Not only testicular, but also adrenal androgen production is inhibited (see Figure 3). However, since also corticosteroid secretion is inhibited, these treatments require additional treatment with corticosteroids. In addition, the effects of ketoconazole on plasma T levels are only transient, and the effective dose has several negative side effects (Geller, 1991a).

1.4.3 Suppression of Androgen Action: Antiandrogens

The advantage of the use of antiandrogens over castration is that also adrenal androgens cannot affect tumor growth. After chemical or surgical castration the weak adrenal androgens androstenedione and dehydroepiandrosterone sulfate can be converted to DHT in peripheral tissues or within the prostate itself (Harper et al., 1974). It was therefore suggested to block androgen action completely, by combining surgical or chemical castration with treatment with antiandrogens (Labrie et al., 1982). This treatment, however, is controversial because it is still not very clear to what extent adrenal androgens play a role in the growth of prostate cancers. The contribution of adrenal androgens to the total amount of circulating androgens is very low; these low concentrations cannot prevent tumor regression in studies with animal models (Van Weerden et al., 1990).

The antiandrogens can be divided into two functional groups: the steroidal antiandrogens (e.g., cyproterone acetate, megestrol acetate, medroxyprogesterone acetate, chlormadinone acetate), and the non-steroidal antiandrogens e.g., flutamide or its active metabolite hydroxyflutamide, nilutamide (anandron), and casodex. The steroidal antiandrogens block androgen action, but in addition have progestational and glucocorticoid activities and are therefore called non-pure antiandrogens. The progestational activity results in a down-regulation of GnRH, and consequently of LH, T, and DHT (see Figure 3). The non-steroidal antiandrogens, however, stimulate the hypothalamus-pituitary-gonadal axis, and consequently lead to increased T and DHT levels (Mowszowicz et al., 1974; Neumann & Töpert, 1986; Raynaud & Ojasoo, 1986), and are therefore also referred to as pure antiandrogens (see Figure 3).

Antiandrogens have been used in monotherapy, and also in combination with surgical or chemical castration. In monotherapies using cyproterone acetate or megestrol acetate, plasma T levels escape to normal values within half a year. Another side effect is impotence. In combination with small doses of estrogen, T levels stay at castrate levels for sustained periods (Geller, 1991b; Venner et al., 1988). The main advantage of the pure antiandrogen flutamide is that it has no negative effect on potency and libido, but disadvantages include an increase in T levels, gynecomastia and diarrhea (Geller, 1991a).

In general, treatment of patients with prostate cancer with antiandrogens has no significant effect on survival rates, but time to progression increases, and consequently a reduction of pain and better functional status of the patient are the most important effects (Schröder, 1991).

1.4.4 The Use of Antiandrogens in Other Clinical Therapies

There are several androgen-dependent diseases which are candidates for antiandrogen therapy. Disorders caused by hyperandrogenism, including female hirsutism (male pattern of hair growth in women) and virilism, acne, seborrhea, precocious puberty and hypersexuality can be treated with antiandrogens. The rationale for antiandrogen treatment is, that the effects of abnormal high androgen levels are blocked by the competing compounds. Pure, as well as non-pure antiandrogens have been used successfully, e.g., in the treatment of hirsutism (Cusan et al., 1990; Sciarra et al., 1990) and polycystic ovary syndrome (Cusan & Dupont, 1989).

In addition, bladder carcinomas, pancreatic carcinomas, laryngeal carcinomas, and hepatocellular carcinomas, all contain ARs and are therefore likely candidates for antiandrogen therapy (Noronha & Rao, 1986; Lipton et al., 1990; Toral et al., 1990; Nagasue et al., 1991).

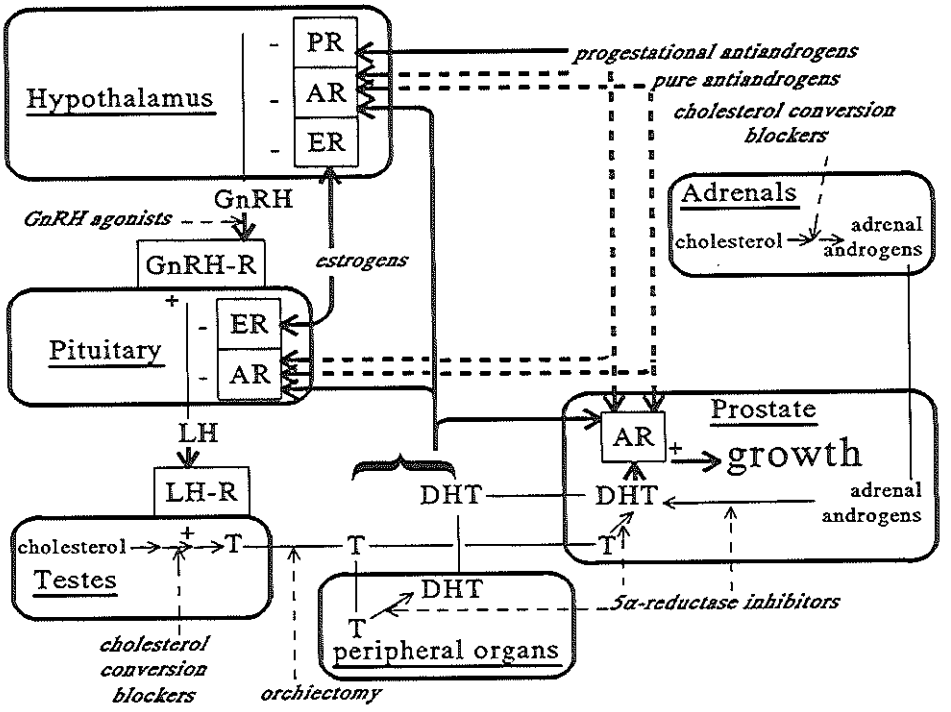

Figure 3: Regulation of androgen production and androgen action. Hypothalamic GnRH stimulates the production of LH by the pituitary gland. LH stimulates the conversion of cholesterol to T via a number of intermediary steroids in the testes. T can be converted to DHT in the peripheral organs, including the prostate. T (after conversion to DHT) and DHT stimulate the growth of the prostate, but also inhibit hypothalamic GnRH secretion and pituitary LH secretion through the androgen receptor. This negative feedback-loop is important for sustaining physiological androgen levels. In addition, the adrenals produce androgens which in the prostate can be converted to the most active androgen DHT. Stimulating actions of the hormones are depicted by thick arrows, and inhibiting actions by thick dashed arrows. The receptors (boxes) can have stimulating (+) or inhibiting (-) effects on a process. Treatments that have negative effects on androgen production or action are depicted in italics. Stimulating effects of these treatments are depicted by thick arrows, negative effects are depicted by dashed arrows. Hormones: T: testosterone; DHT: 5α-dihydrotestosterone; LH: luteinizing hormone; GnRH: gonadotropin-releasing hormone; Receptors: ER, PR, AR: the intracellularly located estrogen-, progesterone-, and androgen-receptor, respectively. GnRH-R and LH-R: the GnRH- and LH-receptor, respectively, located in the membrane of the target cells.

1.5 SCOPE OF THIS THESIS

1.5.1 LNCaP Cells As a Model System for Prostate Cancer

There is one human prostate cell line that is androgen responsive in growth and that can be cultured *in vitro*: the Lymph Node Carcinoma of the Prostate cell line 'LNCaP' (Horoszewicz et al., 1980, 1983). From this cell line, there are several sublines which are either unresponsive or responsive to, or dependent on androgens for growth (for a review on the origin of LNCaP and its sublines, see Van Steenbrugge et al., 1991). LNCaP cells are used as a source for large quantities of AR for biochemical studies, to study androgen responsive genes and processes, and as a model to study androgen and growth factor effects on prostate cells. However, several reports indicated that the response of LNCaP cells to non-androgenic steroids (progestins, estrogens, and antiandrogens) is non-classical, and suggested abnormal AR mediated responses (Schuurmans et al., 1988, 1990; Wilding et al., 1989). LNCaP cells do not contain any steroid hormone receptors other than the AR (Berns et al., 1986; Schuurmans et al., 1988). Moreover, the affinity of the cytosolic AR in LNCaP cells for estrogens and progestins was found to be unexpectedly high (Schuurmans et al., 1988). This suggested that growth stimulation of LNCaP cells by estrogens, progestins, and antiandrogens was mediated by an aberrant AR in these cells.

1.5.2 The Scope of This Thesis

The aim of the studies described in the next chapters was, first, to prove that the AR in LNCaP cells is abnormal with respect to ligand binding characteristics, and to find an explanation for this defect. Second, it was investigated whether this aberration could account for the growth stimulating effects of antiandrogens on this cell line. Third, the effects of both androgens and antiandrogens were investigated at the biochemical level, with much emphasis on receptor interactions with other (heat-shock) proteins.

Chapter 2 describes investigations to study the binding affinities of several steroidal and non-steroidal ligands for the AR in LNCaP cells. These binding affinities were compared with the binding affinities for the AR from other sources, including cells expressing wild type AR (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988; Faber et al., 1989). From studies with nuclear preparations, devoid of cytoplasmic contaminations, it was concluded that the binding affinity of the AR in LNCaP cells was abnormal.

The third chapter describes that the AR gene in LNCaP cells contains a mutation. The expression of the mutant receptor in LNCaP cells was confirmed by cDNA sequence analysis. In transfection studies, the binding specificity of the mutant receptor was compared with the binding specificity of the wild type receptor expressed in the same cell type. Also the ability of both the mutant and wild type receptor to activate transcription from an AR responsive construct in response to androgens, antiandrogens, progestins and estrogens was investigated.

One antiandrogen, ICI 176 334 ("casodex", a trade mark of ICI Pharmaceuticals), was found which could not stimulate growth of LNCaP cells, but inhibited the effect mediated by androgens (Chapter 4). It was investigated whether there is a difference between antiandrogens such as hydroxyflutamide, which induce growth of LNCaP cells,

on the one hand, and casodex, which does not induce growth of LNCaP cells, on the other hand. The ability of these compounds to provoke a dissociation of the AR—heat-shock protein-complex was studied. In addition, it was investigated whether the three heat-shock proteins hsp90, hsp70, and hsp56 could be detected in the heteromeric complexes.

In Chapter 5 of this thesis, the effects of incubation of LNCaP cells with androgens on the AR—heat-shock protein-complex is described. Both changes in complex-size and composition, and changes in affinity of the receptor for the nucleus were analyzed. In addition, the development of an antibody against part of the DNA-binding domain of the AR is described. This antibody was used to examine whether its epitope was exposed on the surface of untransformed and transformed ARs. It was also tested whether this antibody could be used to specifically precipitate wholly or partially transformed receptors.

Finally, in Chapter 6, the results from the former chapters are discussed in a broader context. The effects of the mutation in the AR of LNCaP cells on results obtained with estrogens, progestins and antiandrogens are discussed. The possible role of the different heat-shock proteins in receptor transformation is considered. Suggestions are made for future investigations.

Chapter 2

Unusual Specificity of the Androgen Receptor in the Human Prostate Tumor Cell Line LNCaP: High Affinity for Progestagenic and Estrogenic Steroids

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Summary

LNCaP tumor cells, derived from a metastatic lesion of a human prostatic carcinoma, are androgen-sensitive in cell culture. Although increase in growth rate is observed with low doses of progestagens or estradiol, these cells contain exclusively androgen receptors. In the present study the binding affinity of different ligands for both non-DNA- and DNA-binding (transformed) forms of the androgen receptor were analyzed. The cytosolic (non-transformed) form of the receptor displayed an abnormal high affinity for progestagens and estradiol when compared with the cytosolic androgen receptor from other sources. Subsequently the non-transformed form of the androgen receptor obtained from LNCaP cell nuclei was studied. A high binding affinity was found not only for dihydrotestosterone, but also for progesterone and the synthetic progestagen R5020 (relative binding affinity 42% and 10% of dihydrotestosterone). The binding characteristics of the transformed androgen receptor were examined in intact cells at 37°C. LNCaP cells were compared in this respect with COS cells containing the cloned human androgen receptor, normal human skin fibroblasts, and PC3 (prostate) and NHIK (cervix) human tumor cell lines. The affinity of the transformed androgen receptors for the progestin R5020 in LNCaP cells was significantly higher than in the other cell systems, although the differences were less pronounced than for the non-transformed receptor form. In conclusion: the LNCaP tumor cells contain an androgen receptor with an abnormal binding site. This might be due to a mutation and/or a post-transcriptional effect.

Introduction

The actions of steroid hormones on their target cells are mediated by specific receptor proteins. The hormone binds to the receptor and the receptor is transformed to a DNA-binding form with a high affinity for the hormone-responsive enhancer elements of the hormone-responsive genes. Binding of the transformed receptor to these enhancer elements is an essential step in transcriptional activation. The specificity of hormonal action is accomplished both by the specific recognition of the enhancer element by the DNA binding part of the receptor and by the specificity of the hormone—receptor interaction, determined by the steroid-binding part of the receptor (Grody et al., 1982; Parker, 1983).

LNCaP tumor cells derived from a metastatic lesion of a human prostatic carcinoma contain androgen receptors and respond to androgens with growth in cell culture (Horoszewicz et al., 1983; Berns et al., 1986). In addition, increase in growth rate is observed in the presence of low doses of estrogens (Horoszewicz et al., 1983; Schulz et al., 1985) and progestins (Schuurmans et al., 1988), but these cells do not contain progestin (Schuurmans et al., 1988) or estrogen receptors (Berns et al., 1986), as has been shown in our laboratory previously with specific antibodies against these receptor proteins. Additional proof for the absence of progesterone receptors was found in studies with the synthetic ligand R1881, which has equal affinity for both androgen and progestin receptors (Asselin et al., 1976). Specific antibodies against the androgen receptor complexed with all R1881 bound to receptors, thereby demonstrating the absence of R1881 binding to progesterone receptors (Van Laar et al., 1989a).

Preliminary experiments indicated that the cytosolic androgen receptor had a high affinity for both progestins and estrogens (Schuurmans et al., 1988). Cytosolic receptor preparations, however, contain only the non-DNA binding form of the

receptor and are often exposed to proteolytic breakdown or are contaminated with other steroid-binding proteins as sex-hormone-binding globulin or lower affinity binders. Therefore, we studied the steroid binding specificity of the unoccupied androgen receptor in LNCaP cells in intact isolated nuclei at 4°C. Because the receptor has to go through a multi-step transformation process, before hormone-sensitive genes are activated, also the steroid-binding affinity of the nuclear transformed receptor was analyzed in intact cells incubated with steroids at the physiological temperature. The affinity of the androgen receptor for the progestin R5020 in these cells was compared with the affinity of the androgen receptor in three other human cell types and with androgen receptors obtained from COS cells transfected with androgen receptor cDNA (Trapman et al., 1988).

We demonstrate that the androgen receptor of LNCaP prostate tumor cells has an unusual high affinity for several steroids and especially for progestins.

Experimental

Materials

[^{3}H]R1881 (^{3}H-labeled 17ß-Hydroxy-17α-methyl-estra-4,9,11-trien-3-one), s.a., 87 Ci/mmol, and unlabeled R1881 and R5020 (17α,21-dimethyl-19-norpregna-4,9-dione-3,-20-dione) were purchased from New England Nuclear (Boston, U.S.A.). [1,2,6,7-^{3}H]Progesterone, s.a., 84 Ci/mmol, was obtained from Amersham (U.K.). Triamcinolone acetonide (9α-fluoro-11ß-16α,17,21-tetrahydroxy-1,4-pregnadiene-3,20-dione 16,17-acetonide) was from Sigma (St. Louis, U.S.A.). All other steroids were purchased from Steraloids (Wilton, U.S.A.).

Buffers. Buffer A: 40 mM Tris-HCl, 1 mM EDTA, 10% (w/v) glycerol, 10 mM dithiotreitol (DTT), 0.6 mM phenylmethylsulfonyl fluoride (PMSF) (pH 7.4); buffer B: buffer A supplemented with 10 mM molybdate; buffer C: buffer B supplemented with 0.5 mM bacitracin and 0.25 mM leupeptin; buffer D: buffer A supplemented with 1 mM leupeptin; buffer E: buffer D adjusted to pH 8.5, with additionally 0.5 M NaCl; buffer F: buffer A supplemented with 0.5 mM bacitracin; buffer G: buffer F adjusted to pH 8.5, with additionally 0.5 M NaCl; buffer H: buffer A containing 0.4 M KCl. Homogenization buffer I: prepared in essence according to Gorski et al. (1986): 10 mM Hepes, 25 mM NaCl, 0.15 mM spermine, 0.5 mM spermidine, 1 mM EDTA, 2 M sucrose, 0.6 mM PMSF, 10 mM dithiotreitol, 10% (w/v) glycerol (pH 7.6); buffer J: 0.5 M sucrose, further similar to buffer I.

Cells and Tissues

The LNCaP cell line (derived from a fast-growing colony of a lymph node carcinoma of the prostate (Horoszewicz et al., 1983)) was a gift from Dr. Horoszewicz (Buffalo, U.S.A.). The human prostatic tumor cell line PC3 (Kaighn et al., 1979) was kindly provided by Dr. Van Steenbrugge, Erasmus University Rotterdam. Both cell types were cultured in RPMI 1640 medium (GIBCO) with added penicillin and streptomycin, supplemented with 7.5% (v/v) heat-inactivated fetal calf serum (GIBCO) at 37°C in a humidified atmosphere of 5% CO_2 in air. The NHIK cell line (obtained from a human cervix carcinoma, Mulder et al., 1978) and genital skin (preputium) fibroblasts (fibroblasts were kindly provided by Dr. Degenhart, Erasmus University Rotterdam) were cultured in Eagle's minimal essential medium (GIBCO)

supplemented with 10% (v/v) heat inactivated fetal calf serum (GIBCO) and non-essential amino acids (GIBCO). COS-1 cells were grown in Dulbecco's modified Eagle's medium (GIBCO) supplemented with 5% fetal calf serum. Further additions/conditions were as described for LNCaP cells and PC3 cells. Media were changed every 3 or 4 days and cells were passaged once a week by plating out trypsinized cell suspensions. Experiments were done with passages 65—72 (LNCaP), 13—16 (NHIK), 14—16 (fibroblasts) and 45—47 (PC3).

The PC-EW human prostate tumor (Hoehn et al., 1984), grown in a nude mouse, was kindly provided by Dr. Van Steenbrugge. The mouse was castrated 4 days before death. The tumor was kept on ice and used immediately for the competition assay.

Rat prostates were dissected from adult Wistar rats (sub-strain RP), castrated 24 hours before killing.

Methodology

Cytosol Preparations. LNCaP cells were cultured for 2 days in 5% dextran—charcoal stripped (stripped) serum. After trypsinization and addition of soybean trypsin inhibitor the cells were pelleted and homogenized in ice cold buffer B with 15 strokes of a Potter-Elvehjem homogenizer at 900 rpm. The cytosol was prepared by centrifugation of the homogenate at 105 000 × *g* for 1 h at 2°C.

Rat prostates from adult Wistar rats (sub-strain RP), castrated 24 hours before killing, were homogenized at 0°C with a Thurrax homogenizer in buffer B, three times 4 s, then centrifuged for 10 min 16 300 × *g*. The cytosol was prepared by centrifugation of the supernatant at 105 000 × *g* for 1 h at 2°C.

The PC-EW tumor was homogenized in ice cold buffer C by three bursts of 10 s with a Thurrax homogenizer, then centrifuged for 10 min at 16 300 × *g*. The cytosol was prepared by centrifugation of the homogenate at 105 000 × *g* for 1 h at 2°C. The supernatant was pre-incubated for 30 min with 500 nM triamcinolone acetonide (a synthetic glucocorticoid with high affinity for the progesterone receptor) to occupy possible progesterone receptors (Asselin et al., 1979; Zava et al., 1979). This preparation was used for a competition assay.

Isolation of LNCaP Nuclei Prior to Incubation With Steroids. Nuclei were isolated from LNCaP cells cultured for 2 or 3 days in medium without fetal calf serum or 5% stripped serum. Cells washed in phosphate-buffered saline were harvested by scraping in ice-cold homogenization buffer I. The cells were then homogenized with five strokes in a glass/Teflon homogenizer at 1100 rpm. Then half the volume of buffer J was added and the suspension was centrifuged for 30 min, 105 000 × *g*, at 2°C. The pellet was rehomogenized in buffer I and half the volume of buffer J was added. The suspension was layered on a buffer I cushion and centrifuged for 30 min 105 000 × *g* at 2°C. Pelleted nuclei were resuspended in buffer D and used for competition assays.

Scatchard Analysis. LNCaP cell cytosol was incubated with increasing concentrations (0.5—10 nM) of [^{3}H]R1881 or [^{3}H]progesterone at 4°C for 18 h. In parallel incubations 1 μM of unlabeled R1881 or progesterone was included to assess nonspecific binding. Bound and free steroids were separated using a dextran-coated charcoal assay and Scatchard analysis of the binding data was performed (Mulder et al., 1978).

Competition Assay. The resuspended nuclei were incubated for 18 h at 4 °C with 5 nM [^{3}H]R1881 in the presence of unlabeled steroids (R1881, dihydrotestosterone, progesterone and R5020 (a synthetic, non-metabolizable progestin)), ranging from 0 to 100-fold the concentration of the label. Nuclei were extracted in buffer E for one h

at 4°C and centrifuged for 30 min at 14 900 × *g*. Separation of bound and unbound steroid was achieved by incubating the extract for 5 min with 1 vol. of 20 mM pyridoxal phosphate in 10 mM borate buffer (pH 8.1) and precipitating proteins for 10 min with 10 vol. of protamine sulfate (0.5 mg/ml) (Mulder et al., 1981). After centrifugation (15 min at 4000 × *g*) pellets were washed, solubilized in soluene (15 min at 60 °C) and radioactivity was estimated in 10 ml of the following mixture: Instagel (Packard) with 0.1% butylated hydroxy toluene (w/v) and 1% acetic acid (v/v).

Competition studies with cytosols were essentially performed as described above for nuclear suspensions. For LNCaP cell cytosol and rat prostate cytosol the dextran-coated charcoal assay was used for separation of bound and unbound steroid.

Affinity Labeling of the Androgen Receptor. LNCaP cells which had been kept on medium containing 5% stripped serum for 3 days were incubated with 10 nM [^{3}H]R1881 with or without 100 nM unlabeled R5020 in serum free medium for 1 h at 37°C. In situ photolabeling of the receptor was then performed as described by Van Laar et al. (1989b). In brief: after two washes with ice cold phosphate-buffered saline, the culture flasks were put on a 300 nM ultraviolet-transilluminator (UVP, U.S.A.) and the cells were irradiated for 2 min. Then nuclei were isolated and extracted as described above. The amount of DNA in the pellet was measured to correct for the amount of cells in each incubation.

SDS-PAGE of the Affinity-Labeled Receptor. The androgen receptor was precipitated from the extract with 10% trichloroacetic acid overnight at 4°C, then extensively washed with 10% trichloroacetic acid (3 ×) and subsequently with ethyl acetate (3 ×). The precipitate was dissolved in SDS sample buffer by boiling for 2 min and SDS-polyacrylamide gel electrophoresis using 8% gels was done according to Laemmli (1970). The slab gel was then cut in 2 mm slices. The slices were dissolved in soluene (Packard) for 4 h at 45°C and radioactivity was estimated as described above. Parallel lanes were run with high molecular weight markers (Sigma, 29 000—200 000).

Transfection of COS-1 Cells. The androgen receptor expression plasmid pAR0 was constructed by ligating a 3037 bp *Bgl*II-*Pst*I cDNA fragment, containing the complete androgen receptor protein coding region (Trapman et al., 1988; Faber et al., 1989), in the eukaryotic expression vector pBR328A+ (Van Heuvel et al., 1986) using standard procedures.

COS-1 cells were grown in Dulbecco's modified Eagle's medium supplemented with 5% stripped fetal calf serum and antibiotics. Approx. 40% confluent cell cultures in 10 cm petri dishes were transfected with 10 μg pAR0 and 10 μg pTZ carrier plasmid using the calcium phosphate precipitation method (Chen & Okayama, 1987). 48 h after transfection, cells were used for the steroid binding assay.

Competition Studies With Intact Cells. Competition studies were performed with LNCaP cells, PC3 cells, NHIK cells, fibroblasts, and with COS-cells containing the transfected cDNA of the human androgen receptor. The cells were kept on 5% stripped serum containing medium for 1—3 days and washed two times with phosphate-buffered saline prior to the incubations with the steroids. The cells were incubated for 1 h at 37°C with 10 nM tritiated R1881 with or without 100-fold unlabeled R1881 to assess nonspecific binding. R5020 was used as a competitor at 10- or 100-fold the molar concentration of the labeled R1881. After two washes with ice-cold phosphate-buffered saline the cells were harvested by scraping in buffer F and centrifugated at 800 × *g*. Cell pellets were homogenized by 6 strokes with a

glass/Teflon homogenizer, followed by 10 min centrifugation (800 × *g*), 5 min incubation in buffer F containing 0.2 % (v/v) Triton X-100, 10 min centrifugation (800 × *g*), resuspension in buffer F and a final centrifugation step. Pelleted nuclei were extracted in buffer G for 1 h at 4°C. Part of the extract was used for protamine sulfate precipitation of the receptor as described under competition assay, part of it was analyzed on a 10—30% sucrose gradient in buffer H (De Boer et al., 1986). [^{14}C]-labeled bovine serum albumin (4.6 S) and [^{14}C]ovalbumin (3.6S) were used as sedimentation markers. After 20 h 400 000 × *g* centrifugation at 2°C the gradients were collected in fractions and assayed for radioactivity.

After extraction of the nuclei, the pellets were dissolved in 1M NaOH and used for counting the amount of non-extractable [^{3}H]R1881-bound receptors by scintillation counting.

DNA Measurements. DNA content of the extracted nuclei was measured according to Hinegardner (1976).

Results

Androgen Receptor in Cytosol Fractions

In preliminary experiments we determined the binding characteristics of androgen receptors in the cytosol fraction obtained from LNCaP cells for dihydrotestosterone, for the synthetic ligand R1881 and for progesterone. The binding affinity for dihydrotestosterone and for R1881 (K_d: 0.4 nM) was higher than the affinity for progesterone (K_d: 3.9 nM), but the number of binding sites was about equal for all three ligands. R1881 binds to both androgen and progesterone receptors with equal affinity (Asselin et al., 1979), but immunological data showed that progesterone receptors are absent in LNCaP cells (Schuurmans et al., 1988). The non-metabolizable ligand R1881 was therefore preferred as androgen receptor ligand in subsequent studies.

The steroid binding specificity of the androgen receptor in cytosol obtained from LNCaP cells was compared with the specificity of receptors from two other sources: rat prostate and the transplantable human prostate tumor PC-EW (Table I). In LNCaP cells the affinities of the receptor for both progesterone and R5020 (a synthetic, non-metabolizable progestin), are much higher then in both PC-EW tumor cells and in rat prostate. In addition, the receptor obtained from the LNCaP cells showed considerable affinity for estradiol.

The synthetic glucocorticoid triamcinolone acetonide has been used in assays for estimation of androgen receptors in the presence of progesterone receptors (Asselin et al., 1979; Zava et al., 1979). It binds to progesterone receptors but not to androgen receptors. The very low affinity of triamcinolone acetonide for the receptor in LNCaP cytosol (Table I) provides additional evidence that no progesterone receptors are present.

Quality of Androgen Receptor Preparations Obtained From the Cell Nucleus

The aim of these studies was to isolate a pure preparation of intact nuclei containing the receptor in ligand-free form and not degraded by proteolytic enzymes (i.e., present in the native 99 kDa form).

TABLE I

Relative binding affinities of different steroids for the androgen receptor in cytosol fractions of rat prostate, PC-EW cells, and LNCaP cells

Cytosols obtained from rat prostate, PC-EW tumor, and LNCaP cells were used for competition assays as described in the Experimental section. The relative binding affinity (RBA) is expressed in % as the ratio of the amounts of non-labeled R1881 and competing steroid which are needed for 50 % inhibition of binding of tritiated R1881. The RBA for R1881 was set at 100% (n.d., not determined).

Competitor	RBA value		
	rat prostate	PC-EW cells	LNCaP cells
R1881	100	100	100
Dihydrotestosterone	54	83	88
Testosterone	12	n.d.	25
R5020	n.d.	0.3	8.4
Progesterone	0.1	0.3	17
Estradiol	0.2	n.d.	2.4
Triamcinolone acetonide	<0.1	n.d.	<0.1

The nuclear preparation obtained by sedimentation through a heavy sucrose cushion was free of cytoplasmic contaminants, cellular debris and intact cells, as monitored by phase contrast microscopy. The activity of the cytoplasmic marker enzyme lactate dehydrogenase in these preparations was low (less than 0.2% of that of intact cells), indicating that no intact cells were present and cytoplasmic contaminations are minimal.

The molecular size and intactness of the nuclear receptor was estimated by SDS-polyacrylamide electrophoresis after photoaffinity labeling of the receptor with R1881 (Figure 1). One major peak is seen in these preparations, approximately at the position of 110 kDa, in agreement with previous studies (Van Laar et al., 1989b) which also showed this position on SDS gels for the native 99 kDa form (Trapman et al., 1988) of the receptor. Proteolytic breakdown of the receptor is therefore minimal.

Figure 1 also shows that labeling of receptors in the presence of 10-fold excess of the synthetic progestin R5020 results in a decrease of covalently labeled receptors (77% of control). This decrease in binding of tritiated R1881 to the androgen receptor, in the presence of R5020 illustrates the high affinity of the androgen receptor for R5020.

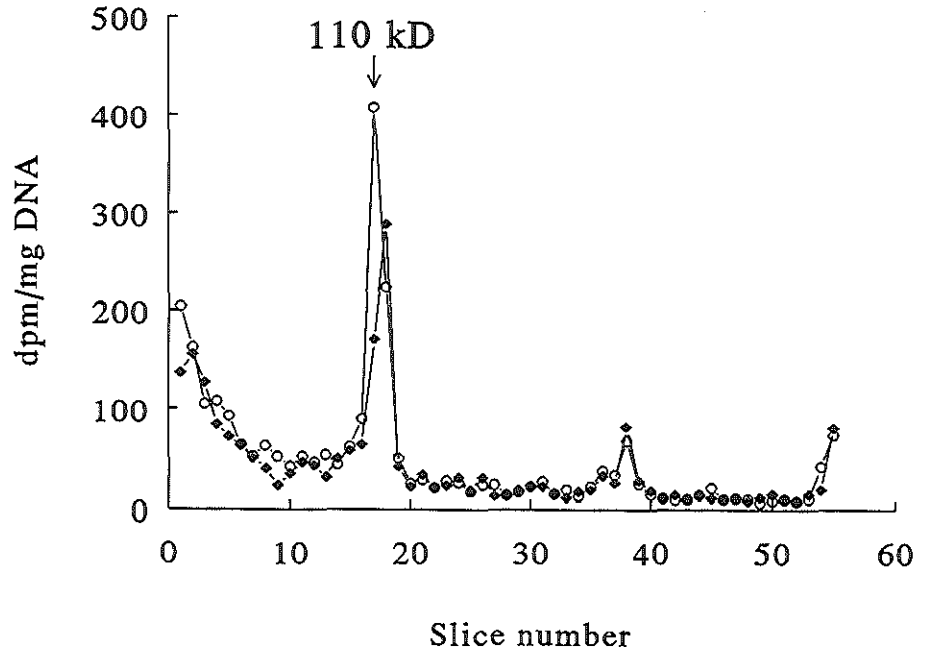

Figure 1. SDS-polyacrylamide gel electrophoresis profiles of [^{3}H]R1881 affinity labeled nuclear androgen receptor of LNCaP cells labeled at 37°C in the absence (○) and presence (◆) of 10-fold excess of R5020.

The isolation procedure of nuclei in buffers containing spermine and spermidine and a high concentration of sucrose, had proven to be useful for the isolation of rat liver nuclei, containing tissue specific transcription factors (Gorski et al., 1986). Using this strategy, we found 3000 binding sites for R1881 per nucleus; when the cells were kept free from ligand prior to the isolation of the nuclei. The amount of binding sites for R1881 typically increased to 20 000 sites per nucleus when the cells were preincubated with ligand.

Binding Specificity of Receptors in the Isolated Nucleus

LNCaP cells were grown on steroid-depleted medium and nuclei which contained unoccupied receptors were isolated. The results of competition studies for nuclei incubated at 4°C with R1881, dihydrotestosterone, progesterone and R5020 are shown in Figure 2. At a relatively small excess of the competing steroids, the binding of labeled R1881 is considerably decreased. The nuclear receptor clearly has a high affinity not only for R1881 and dihydrotestosterone but also for progesterone and R5020 (The relative binding affinities for dihydrotestosterone, progesterone and R5020 are respectively 135%, 57%, and 13% of the affinity for R1881).

Excess non-radioactive dihydrotestosterone reduced the amount of labeled R1881 bound to the receptors to very low values (Figure 2B), indicating that all R1881 is bound to androgen receptors. The glucocorticoid triamcinolone acetonide (not shown) did not compete for the nuclear binding sites, in agreement with the results obtained with cytosolic receptor preparations described above.

The Steroid Binding Specificity of the Transformed Androgen Receptor in Different Cell Types

In the next series of experiments, the binding specificity of the transformed (DNA-binding form) androgen receptor was estimated in intact cells which were incubated for 1 h at 37°C with different steroids. Two different non-metabolizable ligands were used for comparison of the binding specificity: the progestin R5020 and the androgen receptor ligand R1881. For this study receptors in LNCaP cells were compared with receptors in the human tumor PC3 and NHIK cells, in normal human fibroblasts, and in COS cells in which the human androgen receptor cDNA was expressed. These cells do not contain progesterone receptors (Mulder et al., 1978; Kaighn et al., 1979; Brown et al., 1981).

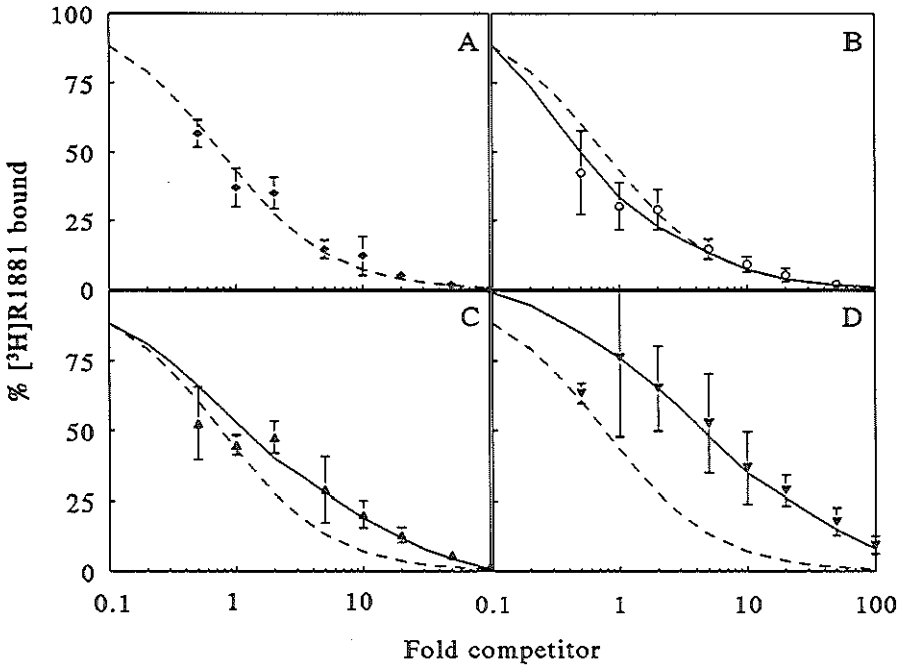

Figure 2. Competitive binding curves of different steroids for the non-occupied androgen receptor in intact isolated nuclei from LNCaP cells at 4°C. The given values are means ± S.D. of three separate experiments. The curve for R1881 (dashed line) is shown in all four panels. Panel A: R1881 (◆); B: dihydrotestosterone (○); C: progesterone (▲); D: R5020 (▼).

Figure 3 shows the amounts of [^{3}H]R1881 in nuclear extracts obtained from the different cells, incubated in the presence of competing R5020. In the presence of a 100-fold molar excess of unlabeled R5020, the labeling of the receptors was significantly lower for LNCaP cells than for the other cells: $0.01 > p > 0.002$

(Student's *t*-test), when compared with NHIK cells, and $p < 0.001$ when compared with the other cells. We also observed some variation in receptor labeling for the other cells. Only between the fibroblasts and the COS-cells this was just significant ($0.05 > p > 0.02$).

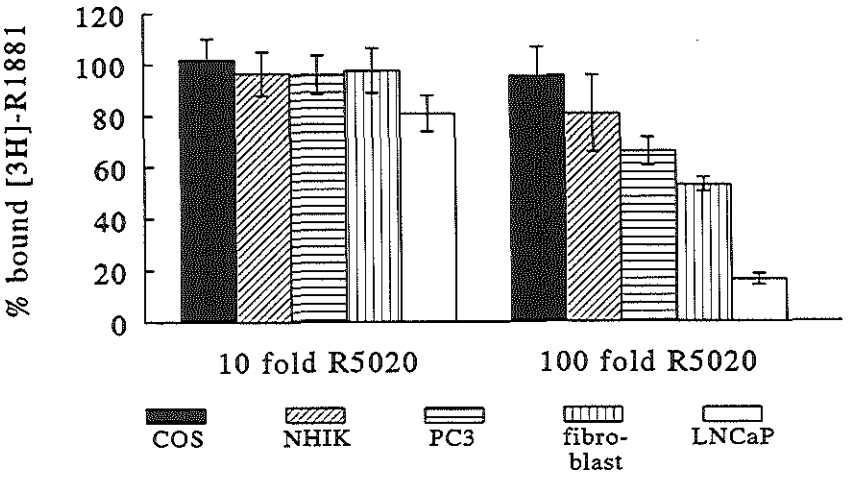

Figure 3. Competition of the progestin R5020 for androgen receptor binding sites in different cell types. Intact tumor cells (LNCaP, NHIK, and PC3), normal human skin fibroblasts, and COS-cells transfected with the human androgen receptor were incubated at 37°C with [^{3}H]R1881 and a 10- or 100-fold molar excess of unlabeled R5020. Receptors were extracted from the nuclei as described in the Experimental section. The amount of specific [^{3}H]R1881 binding/mg DNA in the absence of competitor (R5020) was set at 100%. The values are means ± S.E. of three (NHIK-, PC3, COS-cells, and fibroblasts) or four (LNCaP cells) separate experiments.

In Figure 4 the results of sucrose density gradient centrifugation of the labeled nuclear extracts for LNCaP and NHIK cells are shown. The amount of label recovered from the peak fractions of the gradients, was identical to the amount of label found after protamine sulfate precipitation of the nuclear extracts. This indicates that all receptors present in the extract are precipitated in the protamine sulfate assay.

The amount of label extracted from the nuclei varied between 41% and 73% for the different cells. For the residual, not extracted receptors, the results of the competition studies were similar to those presented in Figure 3. The low amount of [^{3}H]R1881 extracted from LNCaP cell nuclei, in the presence of R5020, as observed above, is therefore not due to a R5020-dependent change in extraction efficiency of the binding sites in the nuclear fraction. In fact, the sum of extractable and non-extractable values gave results similar to those presented in Figure 3.

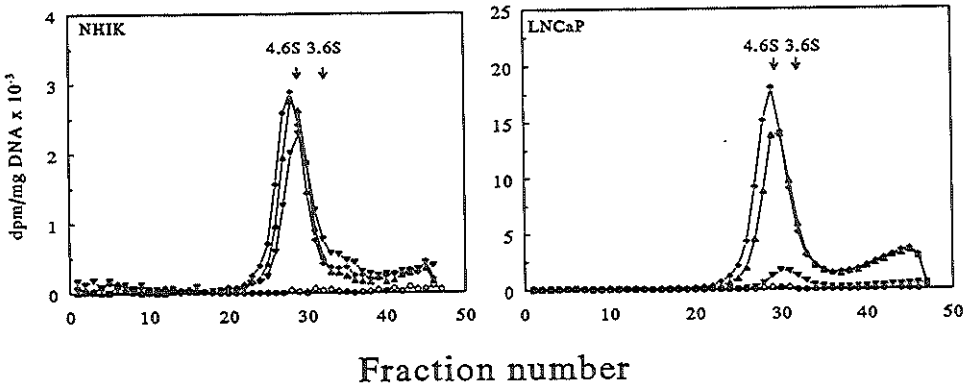

Figure 4. Sucrose density gradient centrifugation of androgen receptors extracted from nuclei of LNCaP and NHIK cells. Nuclei were isolated and extracted from cells incubated with 10 nM [^{3}H]R1881 alone (◆), or in the presence of 100-fold unlabeled R1881 (○), 10-fold R5020 (▲) or 100-fold R5020 (▼).

Discussion

The results in the present study show that the androgen receptor of the prostatic cell line LNCaP has a broad specificity. In addition to androgens, especially progestins are bound with high affinity. The results also show that the progestin binding capacity of the LNCaP cells was not due to the presence of progesterone receptors. This observation is in agreement with immunological data from previous studies which showed that progesterone receptors are absent (Schuurmans et al., 1988) and that only androgen receptors are present in LNCaP cells (Van Laar et al., 1989a).

The binding affinity of the androgen receptor in LNCaP cells was studied with two synthetic non-metabolizable ligands: R1881, a steroid with equal affinity for androgen receptors and progesterone receptors (Asselin et al., 1979) and generally used in androgen receptor binding studies (Robel et al., 1985), and R5020, a progestin with a very low affinity for androgen receptors (Ojasoo & Raynaud, 1978). Receptors were labeled at 4°C to study the untransformed (non-DNA-binding) form of the receptor and at 37°C to study the transformed (DNA-binding) form of the receptor.

The results obtained with the cytosolic fractions of LNCaP cells, rat prostate and PC-EW tumor cells strongly indicate that the binding specificity of the androgen receptor in LNCaP cells is abnormal. In further studies the receptors were isolated from purified nuclei to exclude an unusual binding specificity either due to contaminations of the cytosol with low-affinity binding proteins or to formation of proteolytic fragments of the receptor. Low-affinity binders for estrogens and other steroids have previously been determined in several tissues (Panko et al., 1981). Rapid breakdown of androgen receptors has been shown for receptors in prostate tissue (Mulder & Brinkmann, 1985) and an effect in steroid binding cannot be

excluded. The androgen receptors which were obtained from the purified nuclei were intact. The photoaffinity-labeled receptor migrated as an 110 kDa protein on SDS-PAGE as was found for the native 99 kDa receptor (Trapman et al., 1988; Van Laar et al., 1989b).

The non-transformed (non-DNA binding) receptor in the purified nuclear fraction showed affinities for progesterone and the progestin R5020 (42% and 10% respectively of dihydrotestosterone) which are extremely high for an androgen receptor compared to observations in other studies, see Table II.

TABLE II

Relative binding affinities of different steroids for the androgen receptor in different sources: Literature data

Relative binding affinities (RBA) of some steroids for the androgen receptor in different cell types, calculated as described in the legend of Table I (n.d., not determined). Data were obtained from:
1: Asselin et al. (1979), hypertrophic human prostate cytosol, incubated at 0—4°C.
2: Asselin et al. (1976), rat ventral prostate cytosol, incubated at 0—4°C.
3: Bergink et al. (1983), human breast cancer cells MCF-7 cytosol, incubated at 4°C.
4: Bergink et al. (1983), MCF-7 cells, whole cell assay, incubated at 37°C.
5: Brown et al. (1981), human genital skin fibroblasts, whole cell assay, incubated at 37°C.

Competitor	RBA value				
Data from:	1	2	3	4	5
R1881	100	100	100	100	100
Dihydrotestosterone	42	61	100	89	54
Testosterone	9	36	33	34	n.d.
Progesterone	2	<1	5	0.5	0.6
R5020	<1	<1	n.d.	n.d.	n.d.
Estradiol	<1	<1	n.d.	n.d.	0.8
Triamcinolone acetonide	<1	n.d.	n.d.	n.d.	0.3

The binding characteristics of the transformed, DNA-binding form of the androgen receptor were examined in intact cells at 37°C. LNCaP cells were compared in this respect with COS cells containing the cloned human androgen receptor, normal human skin fibroblasts, and PC3 (prostate) and NHIK (cervix) human tumor cell lines. The affinity of the transformed androgen receptors for the progestin R5020

in LNCaP cells was significantly higher than in the other cell systems, although the differences were less pronounced than for the non-transformed receptor form. This difference in affinity between transformed and non-transformed receptors might be due to a modulation of affinity of the receptor for steroids during the transformation process. Weichman and Notides (1980) showed differences in ligand—receptor dissociation rates for the estrogen receptor in the transformed and untransformed form, and observed that the ratios of the dissociation rates for different steroids were not identical for these receptor forms. This might result in different affinity constants and relative binding affinities for a series of steroids depending on whether or not the assay conditions allow transformation of the receptor. Effects of assay conditions on steroid binding affinities, have been observed by Raynaud et al. (1980) who showed differences in relative binding affinity of several steroids for different receptors depending on incubation time or temperature of the assay. A temperature dependent change in affinity for estradiol was also shown for the human estrogen receptor containing an artefactual point mutation in the hormone-binding domain (Tora et al., 1989b).

In the present study we used a mild procedure for isolation of nuclei (Gorski et al., 1986) to prevent unoccupied androgen receptors from leaking out of the nucleus during isolation. The nuclei isolated from steroid-depleted cells, however, contained only 15% of the number of receptors found in the nuclei after incubation of the cells with androgens. Two explanations for this result are possible: either the unoccupied receptors do not reside in the nucleus, or the isolation procedure does not prevent leakage of androgen receptors out of the nucleus. Histochemical studies with specific antibodies against the androgen receptor are needed to obtain a definite answer about the localization of the unoccupied receptor. For estrogen and progestin receptors a predominant nuclear localization was observed (Gasc et al., 1984; King & Greene, 1984), but unoccupied glucocorticoid receptors are also present in the cytoplasmic compartment (Robertson et al., 1987; Wikström et al., 1987).

Studies of cell systems containing androgen receptors with altered steroid specificity are scarce. Brown et al. (1982) studied a mutant androgen receptor in human fibroblasts of certain patients with the androgen insensitivity syndrome and observed increased binding of progestins. However, in contrast to our studies with LNCaP cells, also a decrease in affinity for androgens was found. Recently it was reported that the androgen receptor in these cells contained a mutation in the steroid-binding domain, which resulted in replacement of valine in the normal sequence with a methionine in the mutated androgen receptor gene (Lubahn et al., 1989a). The changed binding pattern of the LNCaP-cell androgen receptor could be due to a mutation in the steroid-binding domain, although it might also be envisaged that post-transcriptional processing of the receptor is changed in the tumor cell (e.g., by phosphorylation).

If an abnormal binding pattern of steroids to a receptor is found, ligands that normally do not bind and transform the receptor, might lead to enhanced transcription of specific genes, but only when all subsequent steps towards gene activation are effectuated in a comparable way as by the natural ligand. The growth effects on LNCaP cells of progestins described by Schuurmans et al. (1988) indicate that some progestins indeed have the capacity to transform the receptor and subsequently induce growth stimulatory effects. In addition it has been recently shown that LNCaP cells behave aberrantly with respect to the response to antiandrogens. Both Wilding et al. (1989) and studies in our laboratory (unpublished observations) showed

increase in growth rate and excretion of prostate specific acid phosphatase with different antiandrogens (cyproterone acetate and flutamide derivatives). It is tempting to speculate that there is a relationship between the abnormal steroid binding specificity of the androgen receptors and the androgenic actions of progestins and antiandrogens in LNCaP cells.

Acknowledgments

We wish to thank Dr. Van Steenbrugge for providing us with the PC-EW prostate tumor and the PC3 cell line, and Dr. Degenhart for providing us with the human preputium skin fibroblasts.

Chapter 3

A Mutation in the Ligand-Binding Domain of the Androgen Receptor of Human LNCaP Cells Affects Steroid Binding Characteristics and Response to Antiandrogens

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Abstract

LNCaP prostate tumor cells contain an abnormal androgen receptor system. Progestins, estradiol, and antiandrogens can compete with androgens for binding to the androgen receptor and can stimulate both cell growth and excretion of prostate specific acid phosphatase. We have discovered in the LNCaP androgen receptor a single point mutation changing the sense of codon 868 (Thr to Ala) in the ligand-binding domain. Expression vectors containing the normal or mutated androgen receptor sequence were transfected into COS or HeLa cells. Androgens, progestins, estrogens, and antiandrogens bind the mutated androgen receptor protein and activate the expression of an androgen-regulated reporter gene construct (GRE-tk-CAT). The mutation therefore influences both binding and the induction of gene expression by different steroids and antisteroids.

Introduction

Interaction of androgens with their target cells is a process which involves an integrated sequence of molecular events. The hormone binds to a receptor and the receptor is transformed to a DNA-binding form that interacts with the hormone responsive genes. Binding of the transformed receptor to the hormone response elements of these genes is an essential step in transcriptional activation. Steroid hormone receptors consist of three domains: an N-terminal part, a DNA-binding domain, and a steroid-binding domain at the C-terminus. The specificity of hormonal action is accomplished both by the specific recognition of the hormone response element by the DNA-binding part of the receptor and by the specificity of the hormone—receptor interaction, determined by the ligand-binding part of the receptor (Beato, 1989).
LNCaP tumor cells derived from a metastatic lesion of a human prostatic carcinoma contain androgen receptors and respond to androgens with growth in cell culture. In addition, increase in growth rate is observed in the presence of low doses of estrogens and progestins, but these cells do not contain estrogen or progesterone receptors as has been shown previously with specific antibodies against these receptor proteins (Horoszewicz et al., 1983; Schuurmans et al., 1988). Contrary to expectation, antiandrogens exert striking stimulatory effects on the proliferation of LNCaP cells (Wilding et al., 1989; Schuurmans et al., 1990). The androgen receptors in these cells contain an abnormal binding site with significantly increased binding affinity for progestagenic and estrogenic steroids (Schuurmans et al., 1988; Veldscholte et al., 1990b).

In this paper we report that the abnormal binding characteristics are due to a point mutation in the ligand-binding domain of the androgen receptor and demonstrate that both the abnormal binding characteristics and the induction of gene expression by different steroids and antisteroids is entirely due to this mutation.

Materials and Methods

Materials

[^{3}H]R1881, s.a., 87 Ci/mmol, unlabeled R1881, and R5020 were purchased from NEN (Boston, US). Triamcinolone acetonide (TAA) was obtained from Sigma (St. Louis, US). Anandron (RU 23908) was a gift from Roussel Uclaf (Paris, France). Cyproterone

acetate was a gift from Schering (Berlin, FRG), Tamoxifen (ICI 46,474) was obtained from ICI (Cheshire, U.K.). All other steroids were purchased from Steraloids (Wilton, US). [^{14}C]chloramphenicol was obtained from Amersham (Little Chalfont, UK). Butyryl-CoA was obtained from Sigma (St. Louis, US).

Cell Culture

The LNCaP prostate tumor cell line was a gift from Dr. Horoszewicz (Buffalo, NY). These cells were cultured as described previously (Veldscholte et al., 1990b). COS cells and HeLa cells were cultured in Eagle's minimal essential medium (GIBCO) supplemented with 5% (v/v) heat inactivated fetal calf serum (Sera Lab), antibiotics, and non-essential amino acids (GIBCO). Media were changed every 3 or 4 days and cells were passaged once a week by plating out trypsinized cell suspensions. Before transfection (COS cells and HeLa cells) or Western blot analysis (LNCaP cells), cells were cultured in medium with 5% dextran-charcoal treated serum.

Methodology

RNA Preparation. Total cellular RNA was isolated by the guanidinium isothiocyanate method (Chirgwin et al., 1977). cDNA was synthesized using 4 μg of total RNA, 100 ng of oligodeoxynucleotide primer (E8: 5'-AAGGCACTGCAGAGGAGTA-3'), 10 units of avian myeloblastosis virus reverse transcriptase (Promega), and 10 units of RNase inhibitor (RNasin; Promega). Synthesis was done according to the standard protocol (Promega).

DNA Amplification and Sequencing. Amplification by the polymerase chain reaction (PCR, Saiki et al., 1988) took place in 100 μl reaction mixtures containing 1 μg of genomic DNA or 2% of the cDNA-synthesis reaction mixture. PCR mixtures contained 50 mM KCl, 10 mM Tris-HCl (pH 8.3), 1.5 mM $MgCl_2$, 0.2 μmol of each dNTP, 17 μg of bovine serum albumin, 2 units of Thermus aquaticus (Taq) DNA polymerase (Amersham), and 600 ng of each oligonucleotide. Amplification was performed during 24 cycles; each cycle included denaturation for 1 minute at 92°C, primer annealing for 2 minutes at 60°C and primer extension for 1—5 minutes at 70°C. Amplified fragments were made blunt ended and inserted into the *Sma*I site of M13mp18 (Messing, 1983) prior to sequencing by the dideoxy chain termination method (Sanger et al., 1977).

Construction of the expression vectors. A human androgen receptor—cDNA expression vector (pAR0) was constructed using the SV40 early promoter and the rabbit ß-globin poly-A signal (Brinkmann et al., 1989). The pARL expression vector was generated by exchanging the 500 bp *Eco*RI fragment of pAR0 with the mutant 500 bp *Eco*RI fragment which was obtained from amplified LNCaP cDNA.

Transfection. Transfection of COS and HeLa cells was done by the calcium phosphate precipitation method (Chen & Okayama, 1987). For binding studies 5 dishes with each $1.2x10^6$ COS cells were transfected with either 10 μg pAR0 or 10 μg pARL and 10 μg pTZ (Pharmacia) carrier plasmid per dish. For immunoblotting studies $1.2x10^6$ COS cells were transfected with either 10 μg pAR0 or 10 μg pARL and 10 μg pTZ carrier plasmid. For transcription regulating studies $5x10^5$ HeLa cells were transfected with either 5 μg pAR0 or 5 μg pARL and 2.5 μg pG29G-tk-CAT reporter gene (Schüle et al., 1988). The pG29G-tk-CAT construct was kindly provided by Dr. Renkawitz. Carrier DNA (pTZ) was added to a total of 10 μg per dish.

Western Blot Analysis. Androgen receptor was immunoprecipitated from LNCaP and COS cells with a monoclonal antibody against the androgen receptor, subjected to SDS-PAGE electrophoresis, blotted and stained for the presence of receptor as described

previously (Van Laar et al., 1989a; Zegers et al., 1991).

Hormone-binding assay. COS cells transfected with either pAR0 or pARL were collected by scraping in buffer, homogenized and a cytosol fraction was prepared as described previously (Veldscholte et al., 1990b). The cytosol was incubated overnight at 4°C with 5 nM [^{3}H]R1881 in the presence of unlabeled steroids ranging from 0 to 1000-fold the concentration of the label. Separation of bound and unbound steroid was achieved by protamine sulfate precipitation (Veldscholte et al., 1990b).

CAT assays. One day before harvesting the cells, hormones were added to the cells in concentrations ranging from 10^{-12} to 10^{-7} M. The CAT assay was essentially performed as described (Seed & Sheen, 1988), using the method of xylene extraction of butyrylated chloramphenicol. The CAT activity per mg of extracted protein was calculated. Background CAT activity (no steroid added) was set at 0%. For each steroid tested, the amount of CAT activity/mg protein after extraction of background activity, was expressed as percentage of the highest level of CAT activity/mg protein that was found for cells incubated with R1881. Background activity was about 5% of the highest levels of CAT-activity (at 10^{-9} to 10^{-7} M R1881).

Results and Discussion

Exons 2 to 8 coding for the DNA-binding domain and steroid-binding domain of the androgen receptor were amplified from genomic DNA isolated from LNCaP cells, using the polymerase chain reaction (PCR, Saiki et al., 1988). Each exon was amplified individually using exon flanking sequences as oligonucleotide primers (Kuiper et al., 1989). In case of exon 8 the 3' primer was deduced from the 3' untranslated sequence of the mRNA. Sequences of the fragments were found to be identical to the previously published wild-type structure with only one exception: an A to G mutation was found in exon 8. This results in an amino acid change (Thr to Ala) in the steroid-binding domain at position 868 (Figure 1). LNCaP cells contain two X chromosomes (Horoszewicz et al., 1983). Five independent clones derived from genomic DNA all contained the mutated sequence (in 2 separate PCR amplifications). Therefore, it is most likely that LNCaP

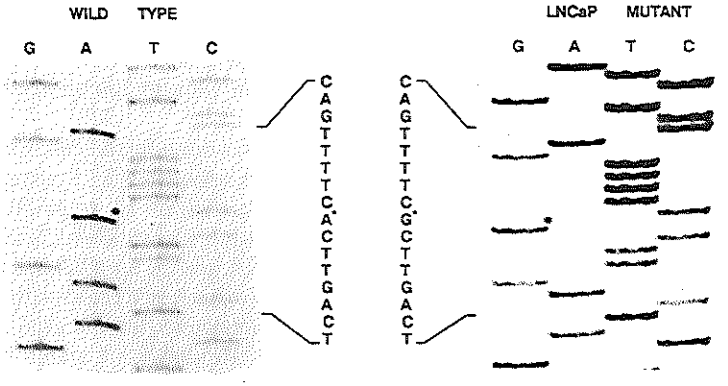

Figure 1. Sequence comparison of part of exon 8 of the wild-type and LNCaP androgen receptor. The asterisks indicate the nucleotide in codon 868 which is an A in the wild-type sequence and is substituted by a G in LNCaP sequence.

cells are homozygous for the mutated allele. Sequencing of cDNA obtained from mRNA isolated from LNCaP cells confirmed that the mutant receptor is expressed in these cells. (Recently the same mutation was reported by S.E. Harris, 1990).

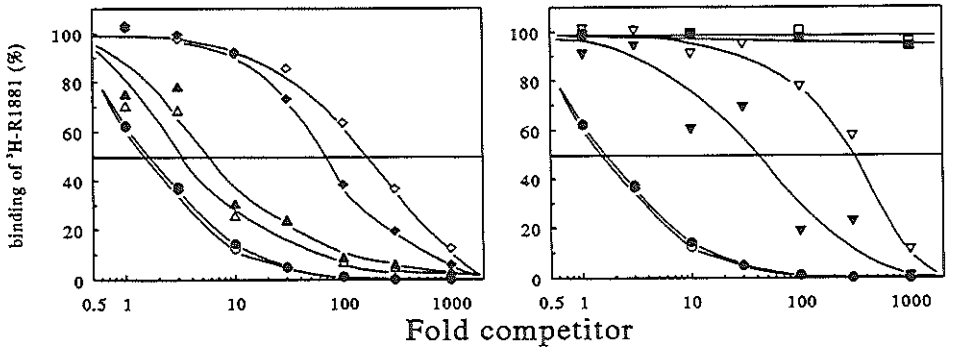

Figure 2. Competitive binding curves of different steroids for the cytosolic androgen receptor in COS cells transfected with either pAR0 (open symbols) or pARL (closed symbols). The left panel shows: R1881-pAR0 (○); R1881-pARL (●); DHT-pAR0 (△); DHT-pARL (▲); estradiol-pAR0 (◇); estradiol-pARL (◆). The right panel shows: R1881-pAR0 (○); R1881-pARL (●);R5020-pAR0 (▽); R5020-pARL (▼); TAA-pAR0 (□); TAA-pARL (■).

Expression vectors containing either the wild-type sequence (pAR0) or the mutated sequence (pARL) were transiently expressed in COS cells. Competition experiments performed on the cytosols of these cells, showed that the two receptors had similar affinities for androgenic compounds (dihydrotestosterone, R1881), but showed striking differences in a series of non-androgenic compounds (Figure 2 and Table I). Especially progestins (progesterone, R5020) and estradiol were bound with high affinity. This result indicates that the mutation is responsible for the high affinity of the androgen receptor for these compounds in LNCaP cells. The mutant receptor and wild-type receptor, both expressed in COS cells, and the receptor from LNCaP cells were immunoprecipitated with a monoclonal antibody against the androgen receptor. The apparent size of the receptor was 110 kDa on SDS-PAGE (Figure 3), the same as previously found for the androgen receptor in LNCaP cells (Van Laar et al., 1989b). This indicates that no major alterations (leading to changed apparent size) of the receptor occur due to the mutation. In addition, some bands at lower molecular weight positions were stained, probably due to partial degradation of the receptor in the COS cells.

Several other mutations of androgen receptors (related to androgen insensitivity syndromes) have been reported, however, these mutations generally lead to decreased or absence of androgen binding affinity for normal sized androgen receptors or absence of binding in the case of mutations leading to receptors of shorter size (Lubahn et al., 1989b; Ris-Stalpers et al., 1990).

To investigate whether the mutation described above was not only responsible for the altered binding characteristics of the receptor, but also for the stimulatory effects of non-androgenic compounds on the growth rate of LNCaP cells, HeLa cells were cotransfected

TABLE I

Relative binding affinities of different compounds for the androgen receptor in cytosol fractions of COS cells transfected with either pAR0 or with pARL, of PC-EW cells (a human prostate tumor cell line), and of LNCaP cells

Competition assays were performed as described in the method section. The relative binding affinity (RBA) is expressed in % as the ratio of the amounts of non-labeled R1881 and competing compound which are needed for 50% inhibition of binding of tritiated R1881. The RBA for R1881 was set at 100% (n.d., not determined). For comparison, data for PC-EW cells and LNCaP cells are included (from Veldscholte et al., 1990b).

Competitor	RBA value			
	COS cells			
	pAR0	pARL	PC-EW cells	LNCaP cells
R1881	100	100	100	100
Dihydrotestosterone	33.3	29	83	88
R5020	0.5	5	0.3	8.4
Progesterone	0.4	4	0.3	17
Estradiol	1	6	n.d.	2.4
Cyproterone acetate	1.4	2.6	n.d.	4.3
Anandron	0.1	0.4	n.d.	n.d.
Triamcinolone acetonide	<0.1	<0.1	n.d.	<0.1

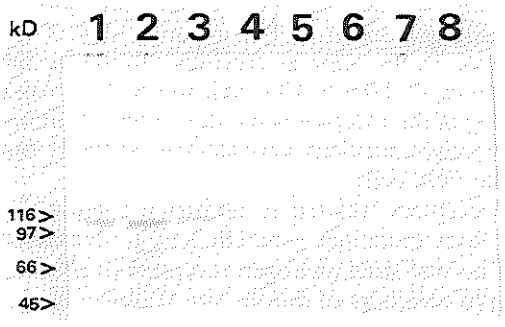

Figure 3. Immunoblot of androgen receptor immunopurified from LNCaP cells (lanes 1 and 5), from COS cells transfected with either pARL (lanes 2 and 6), or pAR0 (lanes 3 and 7), and COS cells which were not transfected (lanes 4 and 8). Androgen receptors were immunopurified using a specific monoclonal antibody (lanes 1, 2, 3, and 4) or with a nonspecific antibody (lanes 5, 6, 7, and 8). After SDS-PAGE the proteins were blotted and analyzed with an polyclonal antiserum against the androgen receptor.

with pAR0 or pARL and an androgen responsive reporter gene construct. It has been shown that the glucocorticoid response element (GRE) can also act as androgen response element (see for a review Beato, 1989). Therefore, the GRE- driven vector pG29G-tk-CAT was used for these studies. Androgens (R1881 and DHT) but also progestins (progesterone and R5020), estradiol, and even antiandrogens (cyproterone acetate and anandron) could induce CAT activity in the cells transfected with pARL, whereas only androgens induced CAT activity in the cells containing the pAR0 construct at low ligand concentrations (Figure 4). The Hela cells we used contain an endogenous glucocorticoid receptor. CAT activity was therefore induced by triamcinolone acetonide both in cells with pAR0 and pARL constructs. Tamoxifen, an anti-estrogen, had no effect on CAT induction.

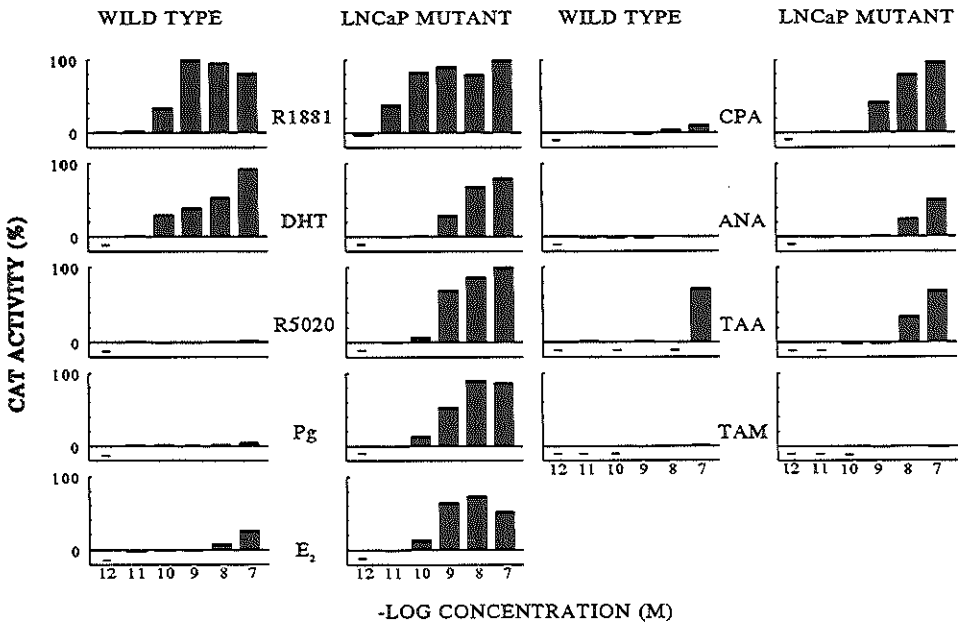

Figure 4. Induction of CAT activity in HeLa cells after cotransfection with either the wild-type androgen receptor or the LNCaP mutant receptor and a GRE-tk-CAT construct. R1881: methyltrienolone; DHT: dihydrotestosterone; R5020: promegestone; Pg: progesterone; E_2: estradiol; CPA: cyproterone acetate; ANA: anandron; TAA: triamcinolone acetonide; TAM: tamoxifen. -: not determined.

In conclusion: A single mutation in an essential part of the ligand-binding domain of the androgen receptor leads to a decrease in steroid binding specificity and, interestingly, completely reverses the effect of commonly used antiandrogens (Neumann & Töpert, 1986; Raynaud & Ojasoo, 1986). This mutation provides a tool for further studies on the molecular mechanism of steroid hormone action and antiandrogen blockade of receptor activation and transcription stimulation.

Acknowledgement

We thank Dr. Renkawitz for providing the pG29G-tk-CAT plasmid construct.

Chapter 4

Antiandrogens and the Mutated Androgen Receptor of LNCaP Cells: Differential Effects on Binding Affinity, Heat-Shock Protein Interaction, and Transcription Activation

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Abstract

Previous studies from this laboratory have described that LNCaP prostate tumor cells contain an androgen receptor (AR) with a point mutation in the steroid-binding domain (codon 868, Thr to Ala). This defect leads to a change in specificity of the AR. Estrogens, progestins, and some antiandrogens (e.g., cyproterone acetate, hydroxyflutamide, nilutamide) stimulate LNCaP cell growth rate through the AR. The present studies indicate that not all antiandrogens showed agonistic effects with the mutated receptor. The growth rate of LNCaP cells did not increase with the antiandrogen ICI 176 334, nor could this compound increase transcription activation of the reporter gene construct via the mutant receptor in a cotransfection system [HeLa cell cotransfection system with an androgen-regulated reporter gene construct (pG29G-tk-CAT) and the mutant receptor as trans-vector]. Interaction of the AR of LNCaP cells with heat-shock proteins was studied by isolation of the receptor with a specific monoclonal antibody and characterization of associated proteins. Hsp90, hsp70, and hsp56 were found to coprecipitate with the AR. Incubation of the cells at 37 °C with androgen (R1881, 10 nM) or the antiandrogen hydroxyflutamide, prior to receptor isolation, resulted in dissociation of the AR—heat-shock protein complex. This dissociation is paralleled by the transformation to a tight nuclear binding form of the AR. In contrast, ICI 176 334 could not induce a release of heat-shock proteins and did not increase nuclear binding, but inhibited the transformation process induced by R1881. From these results, we propose a mechanism of action of antiandrogens in LNCaP cells in which these compounds affect different steps in the processes of receptor transformation and transcription activation. In LNCaP cells, ICI 176 334 shows decreased affinity for the AR and affects steps before DNA binding occurs. In contrast other antiandrogens including hydroxyflutamide show increased affinity for the mutant AR, transform the receptor to the DNA-binding state, and permit interaction of the receptor with the transcription machinery.

Introduction

Effects of steroids in target cells are mediated by their respective receptors. After binding of the hormone, these ligand dependent transcription factors are transformed to a DNA-binding form with high affinity for hormone response elements (HREs)[1] of target genes. Subsequently, the transcription of these genes is modulated by binding of the transformed steroid—receptor complex and interaction with other transcription factors (Beato, 1989). All steroid hormone receptors appear to be composed of several functional domains, including a large C-terminal ligand-binding domain and a central basic region responsible for DNA binding. In addition, domains involved in the transactivating function of the receptor have been identified in both the N- and the C-terminal part of progesterone, glucocorticoid, and estrogen receptors (Carson-Jurica et al., 1990). The primary structure of the androgen receptor has been determined, but the

[1] Abbreviations: AR, androgen receptor; LNCaP, lymph node carcinoma of the prostate; GRE, glucocorticoid responsive element; CAT, chloramphenicol acetyltransferase; hsp90, 90 kDa heat-shock protein; hsp70, 70 kDa heat-shock protein; hsp56, 56 kDa heat-shock protein; TAF, transcription activation function; HRE, hormone responsive element; LH, luteinizing hormone; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulphate; PAGE, polyacrylamide gel electrophoresis; RBA, relative binding affinity.

exact location of domains involved in transcription activation has not yet been described (Chang et al., 1988; Trapman et al., 1988; Lubahn et al., 1988; Faber et al., 1989).

In the absence of hormones, steroid receptors are thought to exist in a non-DNA-binding (nontransformed)[2] state, associated with several other proteins. The 90-kDa heat-shock protein (hsp90) was shown to be associated with the androgen, progesterone, glucocorticoid, and estrogen receptors (Joab et al., 1984; Sullivan et al., 1985). Another component of the receptor complex is a protein of 56—59 kDa. The antibody EC1, developed by Nakao et al. (1985), reacts specifically with a 59-kDa protein present in rabbit progesterone—, glucocorticoid—, androgen—, and estrogen—receptor complexes. Recently, it was shown that this protein also is a heat-shock protein (Sanchez, 1990). In addition to the 90- and 56—59-kDa proteins, the 70-kDa heat-shock protein (hsp70) has been found in the nontransformed progesterone— and glucocorticoid—receptor complexes (Kost et al., 1989; Smith et al., 1990a; Sanchez et al., 1990a). Thus far, association of hsp70 with other steroid receptors has not been shown.

The large multiprotein—receptor complex is considered to dissociate upon hormone binding, thereby revealing the DNA-binding domain of the receptor. The receptor then dimerizes, binds to the response element of the regulated gene, and interacts with other participants in the transcription machinery (Carson-Jurica et al., 1990).

The precise mechanisms of the effects of steroid receptor antagonists at the receptor level are not known. Several mechanisms have been proposed, ranging from induction of an abnormal conformation (Moudgil et al., 1989), impaired translocation of the receptor to the nucleus (Lindemyer et al., 1990; Segnitz & Gehring, 1990), impaired dissociation of the heteromeric receptor complex (Moudgil & Hurd, 1987; Segnitz & Gehring, 1990), impaired receptor dimerization and binding of the receptor to DNA (Berry et al., 1990; Fawell et al., 1990a; Klein-Hitpass et al., 1991) to impaired interaction of the DNA-bound receptor with transcription factors (Guiochon-Mantel et al., 1988; Berry et al., 1990; Sabbah et al., 1991; Klein-Hitpass et al., 1991). Antiandrogens act by inhibition of the binding of androgens to the receptor, but their precise molecular mechanisms of action are at present not known. With respect to their physiological effect, antiandrogens can be divided into two groups. The nonpure (steroidal) antiandrogens (e.g., cyproterone acetate, megestrol acetate, medroxyprogesterone acetate, chlormadione acetate) block androgen action, but in addition have progestational and glucocorticoid activities. The pure (nonsteroidal) antiandrogens (e.g., flutamide, nilutamide) block the action of androgens and have a stimulating effect on the hypothalamus—pituitary—gonadal axis and consequently lead to increased LH and testosterone levels (Mowszowicz et al., 1974; Neumann & Töpert, 1986; Raynaud & Ojassoo, 1986). ICI 176 334 is a pure but peripherally-selective antiandrogen in rats and dogs (Furr et al., 1987; Chandolia et al., 1991), but in a clinical study in men it caused a small but significant elevation of serum LH and testosterone, suggesting that it does affect androgen receptors at the hypothalamic level in men (Mahler & Denis, 1990).

In the present study, we have used the LNCaP cell line, derived from a human lymph node carcinoma of the prostate, for investigations on the mechanism of action of antiandrogens. The LNCaP cell line is the only available human cell line that shows both hormone dependency and continuous growth in vitro (Horoszewicz, 1983). Although the

[2]It should be noted that the term "transformation" will be used to describe the process whereby the steroid-bound receptor is converted from a non-DNA-binding state to a tight nuclear binding form.

cells do not contain steroid receptors other than the androgen receptor, growth can also be stimulated by progesterone, estradiol, and the antiandrogens cyproterone acetate and nilutamide (Schuurmans et al., 1988, 1990; Wilding et al., 1989). The LNCaP cell line contains an androgen receptor with a mutation in the ligand-binding domain: amino acid 868, Thr replaced by Ala (Veldscholte et al., 1990a). In transfected cells, the mutant receptor was found to enhance transcription from an androgen-regulated reporter gene construct (GRE-tk-CAT), not only in the presence of androgens and different other steroids but also in the presence of some antiandrogens (Veldscholte et al., 1990a). In the present study, it is shown that not all antiandrogens have similar, stimulatory effects through the mutant receptor of LNCaP tumor cells. The antiandrogens used in this study have different effects on dissociation of the receptor—heat-shock protein complex, on tight nuclear binding of the receptor, and on transactivation of an androgen receptor regulated gene.

Experimental Procedures

Materials

[^{3}H]R1881, (87 Ci/mmol), unlabeled R1881 (methyltrienolone), and R5020 (promegestone) were purchased from NEN (Boston, MA); triamcinolone acetonide and butyryl-CoA were from Sigma (St. Louis, MO). Nilutamide ("Anandron", RU 23908) was a gift from Roussel Uclaf (Paris, France), cyproterone acetate from Schering (Berlin, FRG), hydroxyflutamide from Schering, USA (Bloomfield, NJ), and ICI 176 334 (trademark "Casodex") from ICI Pharmaceuticals (Macclesfield, Cheshire, U.K.). ICI 176 334 was freshly dissolved before each experiment. All other steroids were purchased from Steraloids (Wilton, NH). [^{14}C]Chloramphenicol (50—60 mCi/mmol) was obtained from Amersham (U.K.).

The glucocorticoid/progesterone/androgen responsive CAT construct pG29GtkCAT (Schüle et al., 1988) was generously provided by Dr. R. Renkawitz. The mouse monoclonals AC88, N27, and KN382/EC1 were generously provided by Dr. D. O. Toft, Dr. W. J. Welch, and Dr. L. E. Faber, respectively.

Cell Culture

LNCaP prostate tumor cells, obtained from Dr. Horoszewicz, were cultured in RPMI 1640 as described previously (Veldscholte et al., 1990b). COS-1 cells and HeLa cells were cultured in Eagle's minimal essential medium (GIBCO, Breda, The Netherlands) supplemented with 5% heat inactivated fetal calf serum (Sera Lab, Uden, The Netherlands), antibiotics, and nonessential amino acids (GIBCO) (medium A). Before transfection, cells were cultured in medium A with 5% (v/v) dextran—charcoal-treated serum (medium B).

Growth Studies. LNCaP cells (passage 20) were plated in 24-multi-well dishes (Falcon, Oxnard, CA) at a density of 2×10^4 cells/cm^2, in RPMI 1640 medium supplemented with 5% (v/v) dextran—charcoal-treated serum (medium C). After 2 days, medium was changed, and cells were kept on experimental medium (medium C with R1881, hydroxyflutamide, and ICI 176 334, at the indicated concentrations) with one medium change after 3 days. At day 6, cells were washed twice with phosphate-buffered saline, pH 7.5 (buffer I), and dissolved in 1 M NaOH for determination of DNA content (Hinegardner, 1976).

Incubation of LNCaP Cells and Subcellular Fractionation. LNCaP cells at confluency were kept on medium C for 2—4 days and washed twice with buffer I. Half of the number of flasks (175 cm^2) were put on ice, ice-cold serum-free RPMI 1640 medium with either R1881, hydroxyflutamide, or ICI 176 334 at the indicated concentrations was added, and the cells were incubated for 30 min at 4 °C. The cells in the other half of the flasks were incubated at 37° C for 30 min with the same experimental media. Subsequently, the cells were washed with ice-cold buffer I and scraped in ice cold buffer II [10 mM sodium phosphate, 1.5 mM EDTA, 12 mM 1α-thioglycerol, 10 mM DTT, 10 mM sodium molybdate, 0.6 mM PMSF, 0.25 mM leupeptin, 0.5 mM bacitracin, and 10% (v/v) glycerol, pH 7.4]. The cells were then homogenized with a glass/Teflon homogenizer and centrifuged at 800*g* for 5 min. The supernatant was then centrifuged for 30 min, 105000*g* at 2° C. The supernatant (cytosol) was used for immunopurification of the receptor complexes and Western blot analysis. The crude nuclear (800*g*) pellet was resuspended in buffer II with 0.2% (v/v) Triton X-100. After 5 min, the nuclei were pelleted and washed with buffer II. Nuclear extracts were made by incubating the nuclei with an extraction buffer [0.5 mL/flask; 40 mM Tris-HCl, 1.5 mM EDTA, 10 mM DTT, 0.6 mM PMSF, 0.25 mM leupeptin, 0.5 mM bacitracin, 0.5 M NaCl, and 10% (v/v) glycerol, pH 8.5] for 1 h at 4°C. After centrifugation for 30 min, 105000*g* at 2° C, the supernatant was used for receptor immunoprecipitation and Western blot analysis. Experiments were performed in triplicate.

Immunoaffinity Purification of the Receptor Complexes and Western Blot Analysis. The monoclonal antibody F39.4.1 directed against amino acids 301—320 in the N-terminal domain of the androgen receptor (Zegers et al., 1991) was chemically cross-linked directly to protein A—Sepharose by the method of Schneider et al. (1982). Ascitic fluid (400 μL) was used to prepare 1 mL of affinity matrix. In each experiment, 15 μL of matrix was used for immunoprecipitation of the receptor from cytosol (1.4 mg of cytosolic protein) or nuclear extract (0.6 mg of nuclear protein). The immunoprecipitation was performed at 4° C by incubating the affinity resin with the cytosols or extracts for 2 h under rotation. The resin was then washed 3 times with buffer I (buffer I with 10 mM sodium molybdate after binding of cytosolic receptors). Before the last washing step, the resin was transferred to a new vial. Thereafter, the pellet was boiled for 2—3 min in SDS—sample buffer, and SDS—PAGE was carried out according to Laemmli (1970) using 7% polyacrylamide gels on a Mini Protean II system (Bio-Rad). After electrophoresis, the slab gel was subjected to Western blotting essentially as described previously (Van Laar et al., 1989a). The Mini Protean II system was used for the transfer of the protein onto nitrocellulose (Schleicher & Schuell), for 1 h at 100 V.

The monoclonal antibodies F39.4.1, specific for the androgen receptor, AC88, specific for hsp90 (Riehl et al., 1985), N27, specific for hsp70 (Vass et al., 1988), and KN382/EC1, specific for hsp56—59 (Nakao et al., 1985), were used as primary antibodies for protein detection. F39.4.1, AC88, and KN382/EC1 were used at a concentration of 10 μg/mL. N27 was used at a dilution of 1:1000. Alkaline phosphatase conjugated goat anti-mouse IgG (Sigma) was used as secondary antibody to detect the proteins on the blot.

Construction of the Expression Vectors and Transfections. Construction of expression vectors (pAR0 for wild type, pARL for LNCaP mutant androgen receptor) was described previously (Veldscholte et al., 1990a). Transfection of COS-1 and HeLa cells was done by the calcium phosphate precipitation method (Chen & Okayama, 1987). For binding studies, 12 dishes (75 cm^2, Nunclon) each with 1.2×10^6 COS cells were transfected with either 10 μg of pAR0 or 10 μg of pARL and 10 μg of pTZ (Pharmacia) carrier plasmid

per dish. For transcription regulation studies, 5×10^5 HeLa cells/dish (30 cm^2, Nunclon) were transfected with either 1 μg of pAR0 or 1 μg of pARL (the optimal amount for this assay, unpublished results) and 1 μg of pG29GtkCAT reporter gene (Schüle et al., 1988). Carrier DNA (pTZ) was added to a total of 10 μg per dish. After 1 day, cells were washed, and experimental media (medium B with hormones at the indicated concentrations) were added. Two days after the transfection, cells were harvested for the CAT assay.

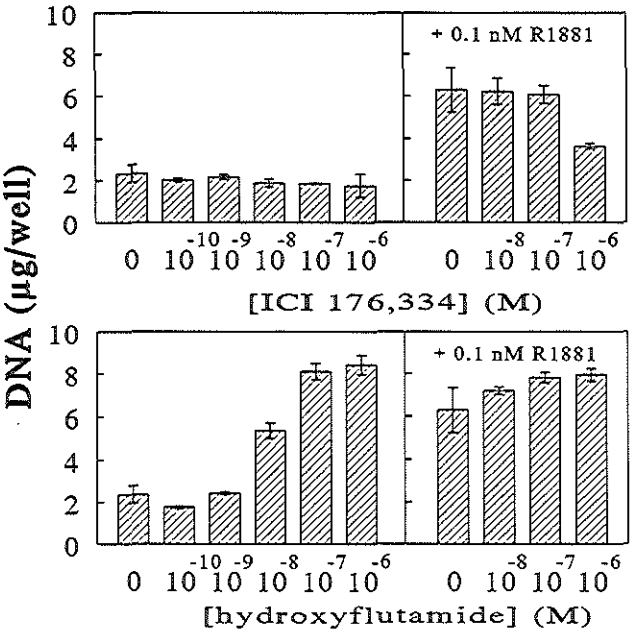

Figure 1: Effects of ICI 176 334 and hydroxyflutamide on growth of LNCaP cells. Various concentrations of ICI 176 334 (upper panels) and hydroxyflutamide (lower panels) were added alone (left panels) or in combination with 0.1 nM R1881 (right panels) with one medium change after 3 days, as described under Experimental Procedures. DNA content was determined after 6-days culture. Means and standard deviations of four measurements are shown.

Hormone-Binding Assay. COS cells transfected with either pAR0 or pARL were collected by scraping in buffer and homogenized, and a cytosol fraction was prepared as described previously (Veldscholte et al., 1990b). The cytosol was incubated overnight at 4° C with 5 nM [^{3}H]R1881 in the presence of unlabeled steroids ranging from 0 to 1000-fold the concentration of the label. Separation of bound and unbound steroid was achieved by protamine sulfate precipitation (Veldscholte et al., 1990b). Relative binding affinity (RBA, expressed in percent) represents the ratio of the amount of nonlabeled R1881 and competing compound which are needed for 50% inhibition of the binding of tritiated R1881.

CAT Assays. The CAT (chloramphenicol acetyltransferase) assay was essentially performed as described by Seed and Sheen (1988), using the method of xylene extraction

of butyrylated chloramphenicol. The CAT activity per dish was calculated; background CAT activity (vehicle only; 0.2% ethanol) was set at 0%. For each steroid (or combination of steroids) tested, the amount of CAT activity after subtraction of background activity was expressed as a percentage of the highest level of CAT activity that was found for cells incubated with R1881. Background activity was less then 5% of the highest levels of CAT activity (at 10^{-9}—10^{-8} M R1881). Experiments were performed in triplicate.

Results

LNCaP Growth Studies. The synthetic androgen R1881 increases the growth rate of LNCaP cells in charcoal-stripped medium at concentrations of 10^{-11} M and higher, with maximal stimulation at 10^{-10} M (Schuurmans et al., 1988). In the present experiments, 10^{-10} M R1881 gave a 2.7-fold increase in DNA content versus control cultures (Figure 1, compare first bar in the left panels with the first bar in the right panels). ICI 176 334 did not have any effect on the growth rate from 10^{-10} up to 10^{-6} M (Figure 1). However, ICI 176 334 partly inhibited the effect of 10^{-10} M R1881 on the cell growth at 10^{-6} M (upper right panel of Figure 1). In contrast, hydroxyflutamide induced cell growth at concentrations ranging from 10^{-8} to 10^{-6} M. In cells submaximally stimulated with R1881, this antiandrogen further increased the growth rate (not shown).

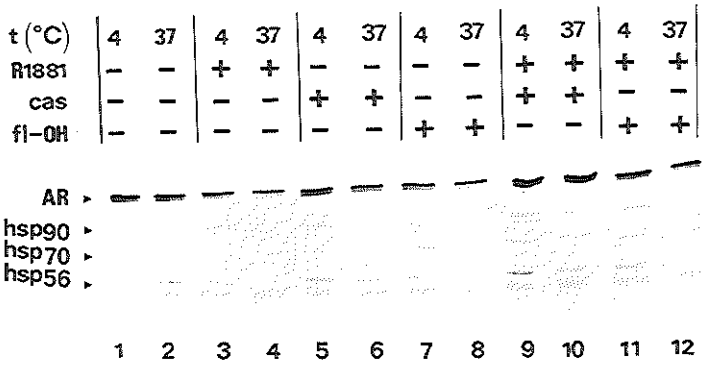

Figure 2: Heat-shock protein interaction with the androgen receptor isolated from LNCaP cell cytosol. The cells were incubated for 30 min at 4° C (lanes 1, 3, 5, 7, 9, and 11) or at 37° C (lanes 2, 4, 6, 8, 10, and 12). The androgen receptor was immunopurified from the cytosol with the monoclonal antibody F39.4.1 and after SDS electrophoresis visualized on a Western blot with the same antibody. Equal amounts of cytosolic protein (1.4 mg) were used for the immunopurification procedure. Hsp90, hsp70 and hsp56 were stained with the specific antibodies AC88, N27, and KN382/EC1, respectively. In all lanes, staining of IgG is visible. AR, androgen receptor; cas, ICI 176,334; fl-OH, hydroxyflutamide. Compounds tested: vehicle only (lanes 1 and 2); 10^{-8} M R1881 (lanes 3 and 4); 5×10^{-5} M ICI 176 334 (lanes 5 and 6); 5×10^{-5} M hydroxyflutamide (lanes 7 and 8); 10^{-8} M R1881 + 5×10^{-5} M ICI 176 334 (lanes 9 and 10); and 10^{-8} M R1881 + 5×10^{-5} M hydroxyflutamide (lanes 11 and 12).

Binding of Heat-Shock Proteins to Androgen Receptors. To investigate the effects of androgens and antiandrogens on the interaction of the androgen receptor with heat-shock proteins, the LNCaP cells were incubated either at 4° C (control) or at the physiological temperature of 37° C with androgens or antiandrogens. The receptor complexes were isolated from the cytosol using an antibody specific for the human androgen receptor and subjected to electrophoresis. Subsequently, Western blots were incubated with antibodies specific for the androgen receptor and the heat-shock proteins hsp90, hsp70, and hsp56-59, respectively.

When only vehicle (ethanol) was added to the cells, incubation at 37° C did not induce changes in the interactions of the AR with the three different heat-shock proteins (Figure 2, compare lanes 1 and 2). Incubation of the cells with the androgen receptor agonist R1881 at 37° C resulted in a loss of hsp90 and hsp56 from the receptor complex and in a decrease in the amount of hsp70 bound (Figure 2, compare lanes 3 and 4). Incubation of the cells with hydroxyflutamide at 37° C, both in the absence and in the presence of R1881, resulted in dissociation of the receptor complex (Figure 2, compare lane 7 with lane 8, and lane 11 with lane 12). In contrast, ICI 176 334 did not affect receptor complex dissociation (Figure 2, compare lanes 5 and 6) and antagonized the effect of the androgen R1881 (Figure 2, compare lanes 9 and 10).

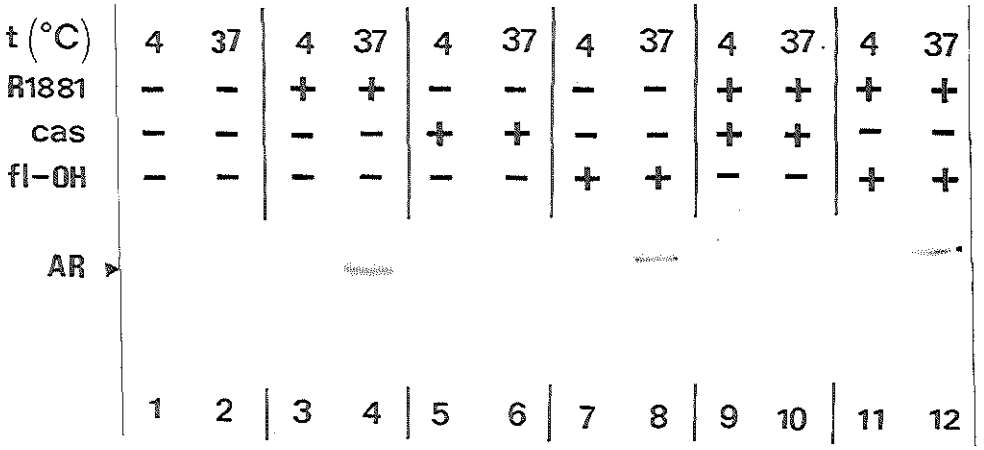

Figure 3: Retention of the androgen receptor in the nucleus of LNCaP cells. The cells were incubated for 30 min at 4° C (lanes 1, 3, 5, 7, 9, and 11) or at 37° C (lanes 2, 4, 6, 8, 10, and 12). The androgen receptor was immunopurified from nuclear extracts with the monoclonal antibody F39.4.1 and after SDS electrophoresis visualized on a Western blot with the same antibody. AR, androgen receptor; cas, ICI 176 334; fl-OH, hydroxyflutamide. Equal amounts of nuclear protein (0.6 mg) were used for the immunopurification procedure. Compounds tested: vehicle only (lanes 1 and 2); 10^{-8} M R1881 (lanes 3 and 4); 5×10^{-5} M ICI 176 334 (lanes 5 and 6); 5×10^{-5} M hydroxyflutamide (lanes 7 and 8); 10^{-8} M R1881 + 5×10^{-5} M ICI 176 334 (lanes 9 and 10); and 10^{-8} M R1881 + 5×10^{-5} M hydroxyflutamide (lanes 11 and 12).

Nuclear Retention of the Androgen Receptor. The presence of receptors in nuclear extracts is indicative for the transformation process of the steroid—receptor complex to a tight nuclear binding form (Beato, 1989). To investigate the effects of androgens and antiandrogens on the binding of the androgen receptor in the nucleus, LNCaP cells were incubated either at 4° C, or at 37° C with androgens or antiandrogens. Androgen receptors were isolated from nuclear extracts of these cells and subjected to electrophoresis and Western blotting and staining with a specific antibody for the androgen receptor. A small amount of receptor was found in the nuclear extracts after incubation of the cells at 4° C in the absence of hormones and in the presence of R1881, ICI 176 334, or hydroxyflutamide, respectively (Figure 3, lanes 1, 3, 5, and 7). The amount of tight nuclear-bound receptor increased when the cells were incubated at 37° C only in the presence of R1881 (lane 4) and hydroxyflutamide (lane 8), but not in the presence of ICI 176 334 (lane 6) or in the absence of hormones (lane 2). Furthermore, ICI 176 334 inhibits tight nuclear binding of the receptor induced by R1881 (Figure 3, lanes 9 and 10).

Binding Affinities. To compare the binding affinities of different compounds under identical conditions, expression vectors containing either the wild type sequence (normal androgen receptor; pAR0) or the mutant sequence (LNCaP cell androgen receptor; pARL; see Figure 4) were transiently expressed in COS cells. Competition experiments performed on the cytosols of these cells showed that the two receptors had similar relative binding affinities (RBA's) for androgenic compounds (dihydrotestosterone and R1881) but showed striking differences for some nonandrogenic compounds (increased RBA of progestins and estradiol for the mutant receptor, Veldscholte et al., 1990a). For a series of antiandrogens, a slight increased RBA was observed for the mutant receptor: cyproterone acetate, 2.6 vs 1.4; nilutamide, 0.4 vs 0.1; hydroxyflutamide, 2.4 vs 0.4. The RBA for ICI 176 334 is negatively influenced by the mutation (RBA 0.1 vs 0.3, mutant vs wild type AR).

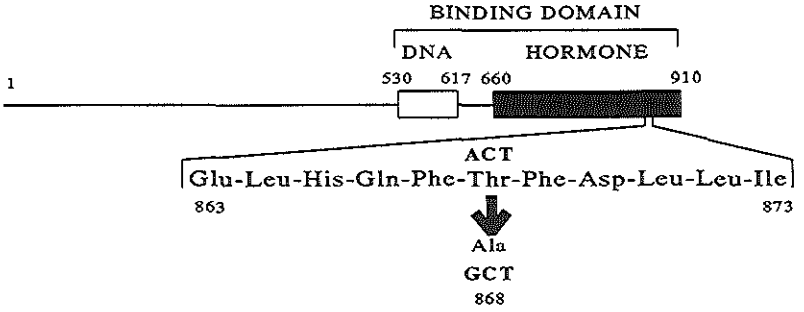

Figure 4: Androgen receptor of LNCaP cells. In codon 868, A is replaced by G, which results in the substitution of an alanine for a threonine residue. The numbers indicate the amino acid residue numbers at the domain boundaries [From Veldscholte et al., (1990a)].

CAT Induction. Cotransfection of the expression vector for either the wild-type or the mutant androgen receptor with an androgen-regulated reporter gene was performed to study differences in effect on transcription of the antiandrogens. It has been shown that

the glucocorticoid response element (GRE) can also act as androgen response element (Beato, 1989). Therefore, the GRE-driven vector pG29GtkCAT (Schüle et al., 1988) was used as reporter gene in the cotransfection experiments. The androgen R1881 stimulated CAT activity, when added to cells containing either the wild-type or the mutant androgen receptor (Figure 5). When ICI 176 334 was tested in this cotransfection system, this compound did not stimulate CAT activity in cells containing either the normal or the mutant receptor. However, hydroxyflutamide stimulated CAT activity of cells expressing the mutant receptor. The ability of the two antiandrogens to antagonize the CAT induction by R1881 was also tested (Figure 6). In the presence of the wild-type receptor, both hydroxyflutamide and ICI 176 334 antagonized the effect of R1881. The antagonistic effect of hydroxyflutamide was observed at concentrations of 1000-fold or higher the concentration of R1881, and ICI 176 334 mediated antagonism was observed at 10 000-fold or higher the concentration of R1881. When the mutant receptor was expressed, hydroxyflutamide had only limited effects on R1881-mediated CAT induction, but ICI 176 334 showed an antagonistic effect, as in the case of the wild-type receptor.

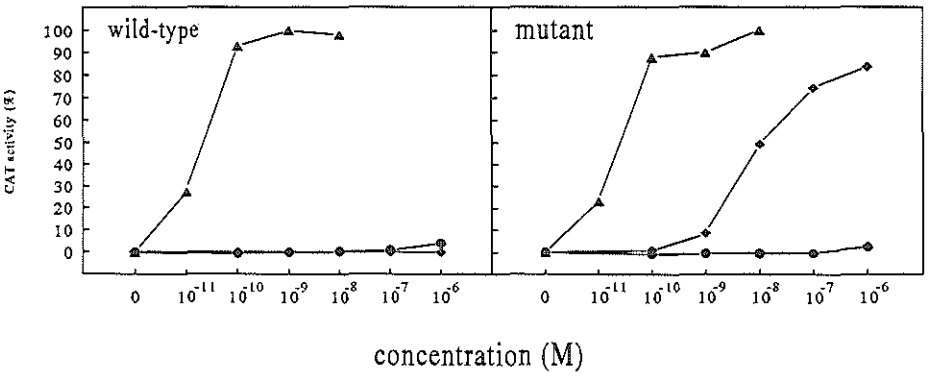

Figure 5: Induction of CAT activity in transfected HeLa cells. The cells were cotransfected with the expression vector either encoding the wild-type androgen receptor (left panel) or coding for the LNCaP mutant receptor (right panel) and a GRE-tk-CAT construct. CAT activity was determined after incubation of the cells with R1881 (▲), hydroxyflutamide (◆), or ICI 176 334 (●) as described under Experimental Procedures.

Discussion

The androgen receptor, like other members of the steroid hormone receptor family, is thought to be present in its untransformed state as a heteromolecular complex, containing several proteins, including heat-shock proteins. Heat-shock proteins are predominantly cytoplasmic, while most steroid receptors (with the exception of the glucocorticoid receptor) are primarily nuclear (Carson-Jurica et al., 1990). In one model explaining the action of steroid hormones, the ligand induces a dissociation of the heteromeric complex (step 1), thereby revealing the DNA-binding domain which can interact with the hormone response element (Pratt et al., 1989; Renoir et al., 1990a). Receptor dimerization (step 2) has been shown to play a role in receptor binding to the glucocorticoid and estradiol response elements (Chalepakis et al., 1990; Fawell et al.,

1990a). For progesterone receptors, the ability to form stable dimers in the absence of DNA was found to correlate with the release of 90 kDa heat-shock protein (DeMarzo et al., 1991). Interaction of the receptor with transcription factors (step 3) is the final step leading to transcription regulation of target genes. Antihormones may exert their effect at one or more steps in this scheme, and as a consequence differ in their mechanism with respect to inhibition of steroid-induced transcription.

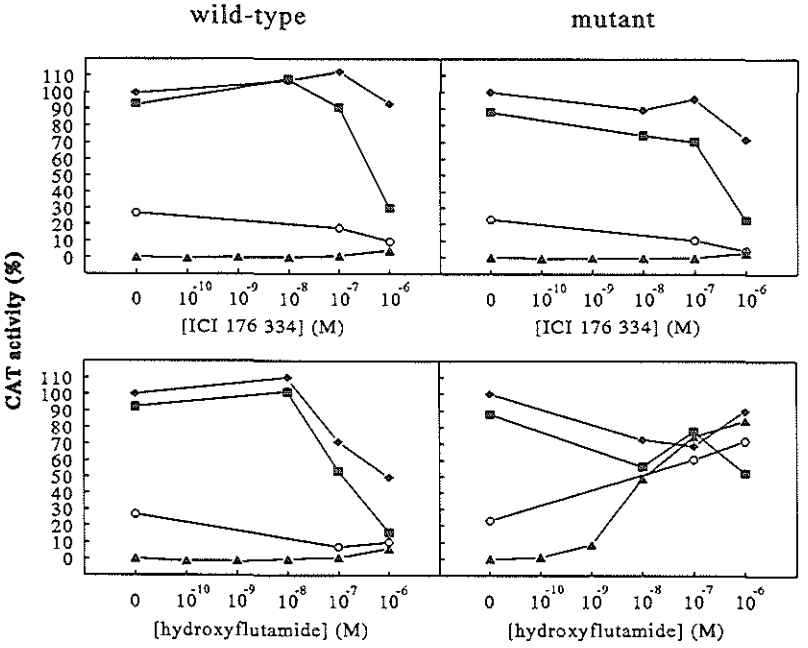

Figure 6: Effects of R1881, ICI 176 334, and hydroxyflutamide on the induction of CAT activity in transfected HeLa cells. The cells were cotransfected with the expression vector either encoding the wild-type androgen receptor (left panels) or encoding the LNCaP mutant receptor (right panels), and a GRE-tk-CAT construct. CAT activity was determined after incubation of the cells with ICI 176 334 or hydroxyflutamide alone (▲) or in combination with R1881 at a concentration of 0.01 nM (○), 0.1 nM (■), or 1 nM (◆) as described under Experimental Procedures.

In the present study, we have used the androgen sensitive LNCaP prostate tumor cell line to study the mechanism of action of some antiandrogens. The androgen receptor in the LNCaP cells contains a mutation in the steroid-binding domain (Thr to Ala at position 868; Veldscholte et al., 1990a). Two structurally related, nonsteroidal antiandrogens (called "pure" antiandrogens because their mechanism of action is thought to interfere only in androgen action) showed opposite effects on growth of the tumor cells. While ICI 176 334 inhibited LNCaP tumor growth, hydroxyflutamide behaved as an agonist and stimulated LNCaP cell proliferation. In previous studies, the agonistic properties of some antiandrogens on growth of LNCaP cells have been described (Wilding et al., 1989; Schuurmans et al.; 1990). Also the secretion of prostatic acid

phosphatase by LNCaP cells is increased not only by androgens but also by estradiol and the antiandrogens cyproterone acetate and nilutamide (Schuurmans et al., 1990). Cyproterone acetate gives a down regulation of the androgen receptor mRNA, indicative of an agonistic effect (Quarmby et al., 1990). To prove that the deviant effects of antiandrogens on LNCaP cells are solely due to altered ligand-binding characteristics and to an altered transcription activation mechanism of the mutant androgen receptor, HeLa cells were transfected with a reporter gene and androgen receptor expressing plasmid constructs differing only with respect to the bases coding for the mutated amino acid residue. Similar results were obtained as in the growth studies: the antiandrogen ICI 176 334 retained inhibitory characteristics for both the mutant and wild-type androgen receptor, whereas hydroxyflutamide behaved as an inhibitor of normal androgen receptor function but as a stimulator of the mutant receptor. For the estrogen and glucocorticoid receptors, partial agonistic properties of some antagonists have been shown. It was theorized that, depending on cell type and promoter context, the N-terminally located TAF-1 (transcription activation function of the receptor) was activated by those compounds (Meyer et al., 1990; Klein-Hitpass et al., 1991). We did not observe any agonistic action of antiandrogens for the wild type receptor transfected into Hela cells together with a GRE-tk-CAT containing reporter gene construct.

To compare the binding characteristics of antiandrogens for wild-type and mutant receptors, these receptors were overexpressed in COS cells, and competition analysis was performed. The binding affinity of the antiandrogens for the AR was only a few percent of the affinity of androgens. For ICI 176 334, the binding affinity was decreased for the mutant receptor as compared with the wild-type receptor, whereas the affinity of hydroxyflutamide was increased for the mutant receptor. The lower affinity of ICI 176 334 for the mutant receptor might be related to a faster dissociation rate. A strict relationship between the dissociation rate of antiandrogens and antagonistic activity, however, has not been found (Wakeling et al., 1981). It is therefore unlikely that the differences in affinity alone could explain agonistic or antagonistic properties of the antiandrogens for the receptor in LNCaP cells.

Our in vitro studies with LNCaP cell cytosol showed that the androgen receptor in its transcriptionally inactive state (i.e., the steroid—receptor complex before transformation to the DNA-binding state) is present as a heteromolecular complex with different heat-shock proteins. Isolation of the androgen receptor with a monoclonal antibody against the receptor resulted in coprecipitation of hsp90, hsp70, and hsp56. Association of the androgen receptor with hsp90 and hsp56 was shown before (Joab et al., 1984; Sullivan et al., 1985; Nakao et al., 1985, Tai et al., 1986). The association with hsp70 was shown for both the progesterone receptor and the glucocorticoid receptor (Kost et al., 1989; Sanchez et al., 1990a) but, thus far, not for the androgen receptor. Incubation of intact LNCaP cells with the synthetic androgen R1881 at 37 °C resulted in dissociation of heat-shock proteins from the receptor complex. In line with the agonistic properties of hydroxyflutamide in LNCaP cells, this compound also induced release of heat-shock proteins at 37 °C. ICI 176 334 exerted a stabilizing effect on the heteromeric androgen—receptor complex. Furthermore, its antagonistic properties in LNCaP cells were displayed by the inhibitory effects on androgen-induced heat-shock protein release. Similarly, stabilizing effects on a multiprotein heteromeric complex by glucocorticoid and progesterone receptor antagonists have been proposed as a mechanism of antagonism (Lefebvre, 1988; Segnitz & Gehring, 1990; Distelhorst & Howard, 1990; Renoir et al., 1990a). Stabilization of the complex is thought to prevent the receptor from dimerizing and binding to regulatory sequences of responsive genes.

We therefore also measured the effect of ICI 176 334 and hydroxyflutamide on binding of the androgen receptor to the nucleus in LNCaP cells. The loss of association of the receptor with heat-shock proteins is accompanied by an increase in the amount of tight nuclear-bound receptor, an indication that a transformed, DNA-binding form of the receptor is obtained. R1881 and hydroxyflutamide, both agonistic in LNCaP cells, increase the amount of tight nuclear-bound receptor in these cells, whereas the antagonist ICI 176 334 does not stimulate tight nuclear binding of the receptor but rather inhibits the effect mediated by R1881. Our data indicate that in LNCaP cells ICI 176 334 acts as an antagonist by inhibiting both dissociation of the heteromeric complex of the AR with heat-shock proteins and the subsequent high-affinity binding of the receptor to the nucleus.

The progesterone and estrogen antagonists have been tentatively divided into two classes depending on their level of action (Klein-Hitpass et al., 1991; Green, 1990). The so-called "type I" or "pure" antagonists interfere with the binding of the receptor to DNA (Berry et al., 1990; Fawell et al., 1990a; Klein-Hitpass et al., 1991). Impaired receptor dimerization and subsequent binding to DNA in vitro were shown for ICI 164 384, an estrogen receptor antagonist (Fawell et al., 1990a), although recently a stimulatory effect on receptor—DNA binding was also observed for this compound (Sabbah et al., 1991; Pham et al., 1991). The other class of antihormones (type II; including, e.g., the progesterone/glucocorticoid receptor antagonist RU486) does induce DNA binding of the receptor but blocks the transcription activation function TAF-2, a region located in the C-terminal steroid-binding domain of the receptor (Meyer et al., 1990). According to this scheme, the antiandrogen ICI 176 334 would be classified for LNCaP cells as a type I antagonist, interfering with the transformation of the androgen receptor complex to the DNA-binding state (the receptor form that interacts with the hormone response element). It is tempting to speculate that in LNCaP cells, due to the mutation, antagonists do not impair functioning of a TAF-2-like transcription activation function in the androgen receptor.

In conclusion, we propose from our results a mechanism of action of antiandrogens in LNCaP cells in which these compounds affect different steps in the processes of receptor transformation and transcription activation. In LNCaP cells ICI 176 334 shows decreased affinity for the AR and affects steps before DNA binding occurs. In contrast, other antiandrogens including hydroxyflutamide show increased affinity for the mutant AR, transform the receptor to the DNA-binding state, and permit interaction of the AR with the transcription machinery.

Acknowledgments

We thank Dr. R. Renkawitz for his kind gift of the pG29GtkCAT plasmid construct and Dr. D. O. Toft, Dr. W. J. Welch, and Dr. L. E. Faber for generously providing the monoclonal antibodies AC88, N27, and KN382/EC1, respectively.

Chapter 5

Hormone-Induced Dissociation of the Androgen Receptor—Heat-Shock Protein Complex: Use of a New Monoclonal Antibody To Distinguish Transformed from Nontransformed Receptors

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Abstract

The hormone-induced transformation process of the androgen receptor in the androgen-responsive human prostatic carcinoma cell line LNCaP was studied. Immunoprecipitation of the nontransformed cytosolic receptor (8 S on sucrose gradients) with a specific monoclonal antibody (F39.4.1) resulted in coprecipitation of three heat-shock proteins (hsp90, hsp70, and hsp56). Upon incubation of the cells with the synthetic androgen R1881, the sedimentation value of the receptor complex decreased to an intermediate form of 6S, and an almost complete loss of coprecipitating heat-shock proteins was observed. After a 2-h incubation, the receptor was recovered in considerable part from the nuclear fraction (extraction with high salt; 4.6S form). By use of the bifunctional cross-linker dimethyl pimelimidate, dissociation of the 8S complex, but not of the 6S complex, was blocked. A newly developed monoclonal antibody (F52.24.4), directed against the C-terminal part of the DNA-binding domain of the androgen receptor, specifically recognized both the 4.6S and the 6S forms of the receptor but did not react with the nontransformed 8S form. It is concluded that the unoccupied androgen receptor is associated with several heat-shock proteins and that transformation of the receptor to the tight nuclear-binding form is a multistep process that involves the dissociation of heat-shock proteins from the receptor.

Introduction

Steroid hormone receptors act as ligand-dependent transcription factors in the process of steroid-induced effects on target cells. Upon steroid binding, the receptor is converted from a non-DNA-binding state to a tight nuclear-binding form. It is believed that binding of the receptor to DNA takes place in specialized regions, called the hormone-response elements, mostly present in front of the regulated genes. This ligand-induced, specific interaction between the receptor and a target gene, results in interaction of other transcription factors with the gene and ultimately in modulation of transcription (Beato, 1989). All steroid hormone receptors appear to be composed of several functional domains, including a large C-terminal ligand-binding domain and a central basic region involved in DNA binding. For the progesterone, glucocorticoid, and estrogen receptors, domains involved in transcription activation have been identified in both the N- and the C-terminal part of the receptor (Carson-Jurica et al., 1990). The primary structure of the androgen receptor was determined some years ago (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988; Faber et al., 1989) but only recently, transcription activation functions have been ascribed to the N-terminal domain and were suggested for the steroid-binding region (Jenster et al., 1991; Simental et al., 1991).

The process of steroid hormone receptor transformation[1] to a tight nuclear-binding form has been studied extensively (Grody et al., 1982; Joab et al., 1984; Sullivan et al., 1985; Bailly et al., 1986; Mendel et al., 1986; Tai et al., 1986; Aràny et al., 1988; Denis et al., 1988b; Howard & Distelhorst, 1988; Kost et al., 1989) [for recent reviews see Pratt (1987) and Pratt et al. (1989)]. Most investigations have focused on *in vitro* transformation of receptors and have shown that, after cell rupture, the untransformed

[1]It should be noted that the term "transformation" is used herein to describe the process whereby the steroid-bound receptor is converted from a non-DNA binding state to a tight nuclear-binding form.

(non-DNA-binding) receptor is associated with several other proteins. It is now generally accepted that a 90-kDa heat-shock protein (hsp90)[2] is associated with androgen, progesterone, glucocorticoid, and estrogen receptors (Joab et al., 1984; Sullivan et al., 1985). Another component of the receptor complex is a protein of 54—60 kDa, with small variations in size for different species. It was shown that this protein is also a heat-shock protein and was therefore called hsp56 (Sanchez, 1990). The antibody EC1, developed by Nakao et al. (1985), reacts specifically with a 59-kDa protein present in rabbit progesterone, glucocorticoid, androgen, and estrogen receptor complexes (Tai et al., 1986). Recently, Yem et al. (1992) identified a 60-kDa protein of which the N-terminal sequence was identical to that of hsp56 and showed immunosuppressant binding properties. Also recently, the cDNA of a similar protein (p59) from rabbit liver was cloned (Lebeau et al., 1992). The sequence in the N-terminal part showed a considerable homology to peptidyl-prolyl isomerase. It was speculated that these 56—60-kDa immunosuppressant-binding proteins play a role in intracellular trafficking of heterooligomeric forms of steroid hormone receptors (Lebeau et al., 1992). A third member of the group of heat-shock proteins associated with steroid receptors is a 70-kDa heat-shock protein, hsp70, shown to be present in nontransformed progesterone, glucocorticoid, and androgen receptor complexes (Kost et al., 1989; Smith et al., 1990a; Sanchez et al., 1990b; Veldscholte et al., 1992).

Transformation of the non-DNA-binding receptor complex (8—9 S on sucrose density gradients), either by warming in the presence of hormone or by high salt treatment (0.4—0.5 M), leads to a decrease in size (4—5 S) and induces the ability of this smaller receptor form to bind to nuclei and DNA or other polyanions [reviewed by Pratt (1987) and Pratt et al. (1989)]. It has been shown for the glucocorticoid and progesterone receptors that hsp90 and hsp56 dissociate from the complex during this process (Mendel et al., 1986; Denis et al., 1988b; Kost et al., 1989; Sanchez et al., 1990b; Smith et al., 1990a). The *in vitro* studies suggest that the receptor complex dissociates to a 4—5 S form, thereby revealing the DNA-binding domain, resulting in binding of the receptor to the hormone response element. Additional arguments in favor of this unmasking hypothesis are that nonliganded glucocorticoid and progesterone receptors also have a high affinity for DNA if they are free of associating proteins (Bailly et al., 1986; Willmann & Beato, 1986) and that nonliganded thyroid receptors do not bind hsp90 and readily associate with DNA (Dalman et al., 1990). Furthermore, it was found that glucocorticoid receptor mutants which were constitutively active, when transfected into COS cells, were recovered in the 4S form, whereas the steroid-inducible forms were recovered as 9S complexes (Pratt et al., 1988). *In vivo* studies indicated that hormone-induced dissociation of hsp90 from the glucocorticoid and progesterone receptors indeed does occur (Howard & Distelhorst, 1988; Smith et al., 1990a). In the latter study, however, it was shown that *in vivo* treatment with hormone does not result in complete dissociation of the receptor complexes. The association of hsp70 with the progesterone receptor seems not to be lost, even after hormone injection *in vivo* (Smith et al., 1990a). Hsp70 is not involved in stabilization of the receptor complex to DNA (Oñate et al.,

[2]Abbreviations: hAR, human androgen receptor; hGR, human glucocorticoid receptor; hPR, human progesterone receptor; cPR, chicken progesterone receptor; hER, human estrogen receptor; LNCaP, lymph node carcinoma of the prostate; hsp90, 90 kDa heat-shock protein; hsp70, 70 kDa heat-shock protein; hsp56, 56 kDa heat-shock protein; DMP, dimethyl pimelimidate; DTT, dithiothreitol; PMSF, phenylmethylsulfonyl fluoride; MAb, monoclonal antibody; ELISA, enzyme-linked immunosorbent assay.

1991). It has been shown that hsp70 functions as a protein chaperone and assists in unfolding and renaturation of proteins (Palleros et al, 1991; Smith et al., 1992), but as for the other heat-shock proteins, its function in steroid receptor transformation is not understood.

In the present study, the composition of the androgen receptor protein complex was investigated during the process of hormone-induced receptor transformation *in vivo*, in intact cells. The composition of the protein complex was probed with antibodies recognizing the heat-shock proteins hsp90, hsp70, and hsp56, respectively, and with a newly developed antibody that specifically reacts with the DNA-binding domain of the androgen receptor. We found that, prior to tight nuclear binding, the receptor complex undergoes large rearrangements resulting in sequential loss of the different heat-shock proteins, leading to disclosure of the antigenic epitope in the DNA-binding domain of the androgen receptor.

Materials and Methods

Materials. [^{3}H]R1881 (87 Ci/mmol), unlabeled R1881 (methyltrienolone), [^{3}H]R5020 (72.4 Ci/mmol), and unlabeled R5020 (promegestone) were purchased from NEN (Boston, MA); [^{3}H]Oestradiol (94 Ci/mmol) and [^{3}H]dexamethasone (94 Ci/mmol) were obtained from Amersham (Cardiff, U.K.). Dimethyl pimelimidate (DMP) was obtained from Sigma (St. Louis, MO). AMPPD alkaline phosphatase substrate, Sapphire Chemiluminescence Amplifier, I-Block reagent, and Nitro-Block reagent were obtained from Trophix, Inc. (Bedford, MA). All other reagents were of analytical grade. Mouse monoclonal antibody F39.4.1 (Sanbio, Uden, The Netherlands) was prepared against the N-terminal domain of the androgen receptor (Zegers et al., 1991). The mouse monoclonals AC88 (recognizing hsp90), N27 (recognizing hsp70), and KN382/EC1 (recognizing hsp56) were generously provided by Dr. D. O. Toft (Mayo Clinic, Rochester, MN), Dr. W. J. Welch (School of Medicine, San Francisco, CA), and Dr. L. E. Faber (Medical College of Ohio, Toledo, OH), respectively.

Buffer Solutions. Buffer I, phosphate-buffered saline, pH 7.5; buffer II, 10 mM sodium phosphate, 1.5 mM EDTA, 12 mM α-thioglycerol, 10 mM Na_2MoO_4, 0.6 mM PMSF, 0.25 mM leupeptin, 0.5 mM bacitracin, and 10% (v/v) glycerol, pH 7.4; buffer III, buffer II supplemented with 0.2% (v/v) Triton X-100, but without leupeptin; buffer IV, 40 mM Tris-HCl, 1.5 mM EDTA, 10 mM DTT, 0.6 mM PMSF, 0.25 mM leupeptin, 0.5 mM bacitracin, 0.5 M NaCl, and 10% (v/v) glycerol, pH 8.5; buffer V, 10 mM sodium phosphate, 1.5 mM EDTA, 12 mM α-thioglycerol, and 10% (v/v) glycerol, pH 7.4; buffer VI, buffer I, containing 0.1% (v/v) Tween 20; buffer VII, 0.05 M Na_2CO_3, 1 mM $MgCl_2$, pH 9.5.

Cell Culture. LNCaP prostate tumor cells (Horoszewicz et al., 1983), obtained from Dr. Horoszewicz, were cultured in RPMI 1640 as described previously (Veldscholte et al., 1990b). Culture of cell lines NHIK, MCF-7, and T47D was described previously (Veldscholte et al., 1990b; Berns et al., 1984; Van Laar et al., 1989a).

Development of Monoclonal Antibody F52.24.4. MAb F52.24.4 was developed essentially as described for MAb F39.4.1, which recognizes amino acids 301—320 (Zegers et al., 1991). Briefly, synthetic peptides homologous to amino acid sequence 593—612 (Thr-Ile-Asp-Lys-Phe-Arg-Arg-Lys-Asn-Cys-Pro-Ser-Cys-Arg-Leu-Arg-Lys-Cys-Tyr-Glu) in the DNA-binding region of the human androgen receptor were synthesized on RapidAmide resin beads and coupled to keyhole limpet hemocyanin for immunization

of mice. Sera were tested in a direct ELISA for anti-peptide response, and in an immunoprecipitation assay for androgen receptor specificity. From specific serum antibody producing mice, spleen cells were fused with SP2/0 cells. Antibody-producing clones were first identified in a primary selection in anti-peptide ELISA, and then MAbs were selected for the ability to immunoprecipitate androgen receptors prepared from LNCaP cell nuclear extract. Balb/c mice were injected intraperitoneally with 0.5 mL of pristane (2,6,10,14-tetramethylpentadecane, 96%, Ega-chemie, Steinheim, F.R.G.). Seven days later, the mice were injected with 10^6 monoclonal hybridoma cells in 0.25 mL of buffer I. Ascitic fluid was collected under anaesthesia.

Receptor specificity of subclone F52.24.4 was tested with a double immunoprecipitation assay of [^{3}H]R1881-labeled hAR, [^{3}H]oestradiol-labeled hER, [^{3}H]R5020-labeled hPR, and [^{3}H]dexamethasone-labeled hGR preparations from nuclear extracts obtained from LNCaP, MCF-7, T47D, and NHIK cells, respectively (Zegers et al., 1991). F39.4.1 and F52.24.4 ascites (0.5 μL) were incubated at 4 °C for 2 h with goat anti-mouse agarose in buffer I, and after extensive washing of the resin with buffer I, nuclear extracts containing comparable amounts of labeled receptors (1.2×10^4 dpm) were added. After incubation for 2 h at 4 °C, the resin was washed and the amount of precipitated receptor was estimated by scintillation counting. The amount of labeled receptors present in the nuclear extracts was measured in a protamine sulfate assay as described by Veldscholte et al. (1990b).

Receptor Transformation. LNCaP cells (passage 65—72) at confluency were kept on RPMI 1640 medium with 5% dextran—charcoal-stripped fetal calf serum for 2—8 days and washed twice with buffer I, then serum-free RPMI 1640 medium with 10 nM [^{3}H]R1881 was added. For sucrose gradient experiments, the control cells (containing nontransformed receptors) were incubated with 10 nM [^{3}H]R1881 on ice for 2 h to label the receptors. For all transformation studies, the cells were transferred to a water bath of 37 °C for 1—3 min and then transferred to the incubator (37 °C) in the case of longer incubation times. Receptor transformation was stopped by putting the flasks on ice. In the indicated experiments, labeling of the receptors with tritiated R1881 was stopped by adding a 100-fold excess of unlabeled R1881. The cells were washed with ice-cold buffer I and scraped in ice-cold buffer II. The cells were then homogenized with a glass—Teflon homogenizer and centrifuged at 800*g* for 5 min. The supernatant was then centrifuged for 30 min at 105000*g*, at 2 °C. The high-speed supernatant (cytosol) was used for cross-linking studies, sucrose density gradient analysis, and Western immunoblot analysis. The crude nuclear pellet (800*g* pellet) was resuspended in buffer III. After 5 min the nuclei were pelleted and washed with buffer III without Triton X-100. Nuclei were extracted by incubation in buffer IV for 1 h at 4 °C. After centrifugation for 30 min at 105000*g*, at 2 °C, the supernatant was used for sucrose gradient analysis, receptor immunoprecipitation, and Western immunoblot analysis. The amount of labeled receptors present in the nuclear extracts was measured in a protamine sulfate assay as described by Veldscholte et al. (1990a).

Cross-Linking of the Receptor Complexes. Protein—protein cross-linking was performed in cytosol made in buffer II supplemented with 10 mM DTT, by the method of Arányi et al. (1988). In brief, the pH was adjusted to pH 9.0 with $^1/_{10}$ volume of a 2.2 M triethanolamine buffer. Then $^1/_5$ volume of a 0.1 M dimethyl pimelimidate (DMP), freshly dissolved in a 0.2 M triethanolamine buffer was added. Cross-linking was performed for 30 min at 10 °C. The reaction was stopped by adding $^1/_{100}$ volume of a 5 M hydroxylamide solution.

Interaction of Monoclonal Antibodies F39.4.1 and F52.24.4 with Receptor. Cytosols were prepared from LNCaP cells grown on RPMI 1640 with 5% dextran—charcoal-treated serum. The cells were harvested by trypsinization, and the reaction was stopped with an excess of trypsin inhibitor. After two washings with buffer I, the cells were homogenized in ice-cold buffer II with 15 strokes of a glass—glass homogenizer and spun at 105000*g* for 30 min, at 2 °C. The supernatant was incubated with 10 nM [^{3}H]R1881 for 2 h, and unbound label was removed by dextran—charcoal adsorption (Mulder et al., 1978). Then, 100 μL of cytosol was incubated for 2 h with 2 μL of ascitic fluid either with monoclonal antibodies F39.4.1 or F52.24.4 or with a nonspecific antibody, in the presence or absence of 0.5 M NaCl. Interaction of the antibodies with the receptor complex was assayed by sucrose density gradient centrifugation.

Sucrose Density Gradient Centrifugation. The samples were treated with dextran—coated charcoal to remove the unbound label and then were applied on sucrose gradients (10—30% sucrose) prepared in buffer V. In the cross-linking experiments, and in the experiments where interaction of the antibodies with the salt-dissociated receptor was investigated, 0.5 M NaCl was included in the gradient. The gradients were run for 20 h at 250000*g*, at 2 °C. ^{14}C-Labeled bovine serum albumin (4.6 S) and alkaline phosphatase (6.2 S) were used as internal sedimentation markers. Fractions of the gradients were collected from the bottom and assayed for radioactivity and alkaline phosphatase activity.

Immunoaffinity Purification of the Receptor and Western Immunoblot Analysis. The MAb F39 [against amino acids 301—320; Zegers et al. (1991)] and the MAb F52 (against amino acids 593—612) were chemically cross-linked directly to protein A—Sepharose by the method of Schneider et al. (1982). Ascitic fluid (400 μL) was used to prepare 1 mL of affinity matrix. This affinity matrix was used for immunoprecipitation of the receptor. In each experiment, either 15 (F39) or 25 μL (F52) of matrix was used for immunoprecipitation of the receptor from either cytosol (1.2 mg of cytosolic protein) or nuclear extract (3.6 mg of nuclear protein). The immunoprecipitation and Western immunoblot analysis was performed as described previously (Veldscholte et al., 1992). In most experiments, bound antibodies were detected by chemiluminescence as described below.

Chemiluminescence Detection of Proteins. Chemiluminescence detection was performed essentially as described by the manufacturer (Trophics, Bedford, MA). After transfer of proteins to the nitrocellulose membrane, the membrane was dried for 30 min or longer, then washed for 5 min in buffer VI, and subsequently incubated in 0.2% (w/v) I-Block reagent in buffer VI. The membrane was then washed in buffer VI for 5 min, and incubations with primary and secondary alkaline phosphatase conjugated antibodies were performed as described previously (Veldscholte et al., 1992), except that buffer VI (0.1% Tween) instead of buffer I with 0.05% Tween was used. The membrane was then washed 2 × 5 min in buffer VII, incubated for 5 min in Nitro-Block reagent (0.5 mg/mL in buffer VII), washed 2 × 5 min in buffer VII, and subsequently incubated for 2 h in a AMPPD alkaline phosphatase substrate solution (0.24 mM AMPPD and 1 mg/mL Sapphire amplifier in buffer VII) for formation of the chemiluminescent product. The immunoblots were wrapped in catering foil and placed in contact with X-ray film (Hyperfilm MP, Amersham, Cardiff, U.K.) for 1—45 min, depending on the intensity of chemiluminescence. Films were developed according to standard procedures.

Results

Generation of the Monoclonal F52. The procedure to obtain antibodies recognizing the epitope in the DNA-binding domain of the androgen receptor (amino acids 593—612) resulted in a clone producing antibodies of the IgG1 isotype. In an immunoprecipitation assay, ascitic fluid of clone F52.24.4 (in short, F52) was incubated with nuclear extracts containing equal amounts of either androgen receptor, glucocorticoid receptor, estrogen receptor, or progesterone receptor, labeled with the respective, receptor-specific, tritiated ligands. The antibody—receptor complexes were precipitated with goat anti-mouse agarose, and the amount of precipitated receptor was estimated and expressed as a percentage of the total amount of receptors in the reaction mixture (Table I). The percentages of receptors precipitated with MAb F52 were compared with the percentages obtained with MAb F39.4.1 (in short, F39), which recognizes amino acids 301—320. MAb F52 precipitated about 40% of the added labeled androgen receptor from LNCaP cell nuclear extracts (Table I). Such a relatively low percentage was also found for MAb F39, directed against the N-terminal domain of the receptor. This might be caused by dissociation of the ligand from the receptor during the precipitation procedure. Equivalent results have also been described for a set of polyclonal antisera against the androgen receptor (Van Laar et al., 1989a). In addition to binding of the antibody to the androgen receptor, F52 could also precipitate considerable amounts of other steroid receptors (Table I). This cross-reactivity of the MAb is probably due to the high level of homology between the different receptors in this region. Of the 20 amino acid residues in the peptide used for the immunization of the mice, 14 are conserved in the human progesterone receptor and in the human glucocorticoid receptor and 16 are conserved in the human estrogen receptor (Misrahi et al., 1987; Hollenberg et al., 1985; Green et al., 1986). In contrast, the antibody F39, developed against an amino acid sequence in the N-terminal region (Zegers et al., 1991), is highly specific, as shown by the low amounts of glucocorticoid, estrogen, and progesterone receptors precipitated with this latter antibody (Table I). In our studies with

Table I:

Percentage of Steroid Hormone Receptors Precipitated by Two Monoclonal Antibodies[a]

	Labeled receptor precipitated (%)			
MAb	hAR	hGR	hER	hPR
F52.24.4	38	53	6.5	3.6
F39.4.1	39	0	2.2	0.1

[a]Nuclear extracts of LNCaP, NHIK, MCF7, and T47D cells were incubated for 2 h at 4 °C with goat anti-mouse agarose. The amount of precipitated radioactivity was expressed as the percentage of the total amount (12 000 dpm) added.

LNCaP cells, the low receptor specificity of MAb F52 does not influence the interpretation of the results, because these cells only contain androgen receptors and no other receptors of the same family.

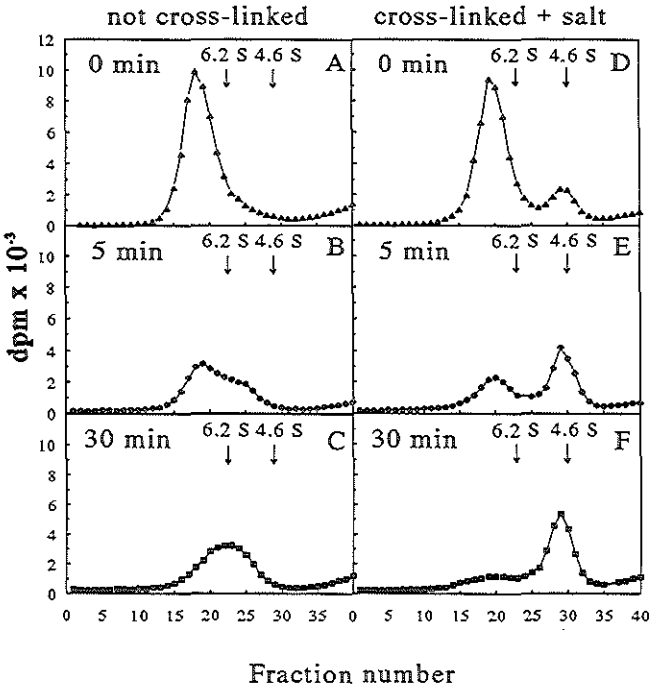

Figure 1: Sucrose density gradient profiles of androgen receptor from LNCaP cells. Cells were incubated for 2 h at 0 °C in the presence of 10 nM [³H]R1881 and subsequently incubated for 0 (A and D), 5 (B and E), or 30 min (C and F) at 37 °C. Cytosol was prepared and half of it was run on a 10—30% sucrose gradient without additional salt, as described under Materials and Methods (A—C). The other half was treated with the cross-linker DMP and run on a 10—30% sucrose gradient containing 0.5 M NaCl (D—F). Alkaline phosphatase (6.2 S) and bovine serum albumin (4.6 S) were used as internal sedimentation markers.

Transformation of the Androgen Receptor in Intact Cells Resulting in a Decreased Size of the Receptor Complex and Changed Protein Interactions. To describe the transformation of the AR in terms of changes in the configuration of the heterogeneous receptor protein complex, the receptor was analyzed on sucrose density gradients after various time periods of hormone-induced transformation. The nontransformed androgen receptor in LNCaP cells was recovered as one peak, sedimenting approximately as an 8S complex (Figure 1, panel A). After incubation of the cells with the tritiated synthetic androgen R1881 for 5 min at 37 °C, in addition to the 8S receptor, a second receptor form with a lower sedimentation value appeared (Figure 1, panel B). Incubation of the cells with

tritiated R1881 for 30 min at 37 °C led to a decrease in sedimentation value of the receptor to approximately 6S (Figure 1, panel C). Concurrently, the total amount of labeled receptor in the cytosol fraction was decreased and an increasing amount of receptor was found in the nuclear fraction. During a period up to 2 h, the amount of labeled receptor recovered from the nuclear fraction steadily increased (Figure 6; discussed below).

The association of proteins with the receptor was further investigated with a bifunctional cross-linker dimethyl pimelimidate (DMP). This cross-linker covalently links lysine residues at a spatial distance of approximately 9 Å from each other, provided that these residues are accessible for the reagent. Cross-linking for 30 min at 10 °C with DMP was found to be optimal for stabilization of the 8S form of the receptor complex and prevented it from dissociating in the presence of 0.5 M NaCl (Figure 1, panel D). The small amount of receptor present in the 4.6S region at 0-min incubation indicates either that not all receptor molecules are initially present in the 8S form or that the cross-linking efficiency is below 100%. The 6S receptor complex which is formed on incubation with hormone (Figure 1, panels B and C) was not prevented from dissociating in high salt after reaction with the cross-linking reagent, and a smaller, approximately 4.6S form of the receptor was obtained (Figure 1, panels E and F). We conclude that the cross-linker does not couple the proteins contained in the 6S receptor complex.

These results of the gradient centrifugation studies indicate that the transformation of the receptor to the tight nuclear binding form is a multistep process with regard to changes in size and conformation of the proteins. First the receptor complex changes in sedimentation value from 8 S to approximately 6 S, and then it gains high affinity for the nucleus and is no longer recovered in the cytosol fraction.

Exposure of a Specific Epitope on the Surface of the Receptor Complex during the Transformation Process. To demonstrate that rearrangements of proteins on the surface of the receptor complex have occurred during the transformation process, we used the monoclonal antibody described above (MAb F52) that is directed against an epitope in the DNA-binding region of the receptor. This antibody caused a shift of the 4.6S androgen receptor to higher sedimentation values (Figure 2A) but did not provoke a shift of the 8S complex on sucrose gradients (Figure 2B). In contrast, MAb F39, recognizing an epitope in the N-terminal domain of the receptor, caused shifts of both the 4.6S and 8S forms of the receptor to complexes with higher sedimentation values (Figure 2). These results show that the epitope for MAb F52 is exposed in the 4.6S receptor but not in the nontransformed 8S receptor complex. Mab F39 shifted the receptor complex over a greater distance than did F52 (Figure 2A). Because the sedimentation behavior of proteins is affected by the shape of the proteins, it can be envisaged that the conformation of the protein complexes is different when an antibody is bound either to the central DNA-binding domain or to the more distal N-terminal domain of the receptor.

Next we examined whether the epitope for F52 (in the DNA-binding region of the receptor) was also exposed in the intermediate 6S receptor complex. Therefore, LNCaP cells were first incubated with tritiated R1881, and cytosol fractions obtained from these cells were probed for interaction with MAb F52. When analyzed on sucrose density gradients, in the absence of antibodies, a receptor peak of approximately 8 S was found when cells were incubated in the cold and a 6S receptor peak was shown after the cells were warmed for 30 min at 37 °C (Figure 3). The 8S receptor complex did not interact with MAb F52 and remained in the same position (Figure 3A). The 6S receptor complex

was shifted toward higher sedimentation values in the presence of MAb F52 (Figure 3B). These results show that the 6S, but not the larger 8S, receptor complex exposes the epitope for the MAb F52.

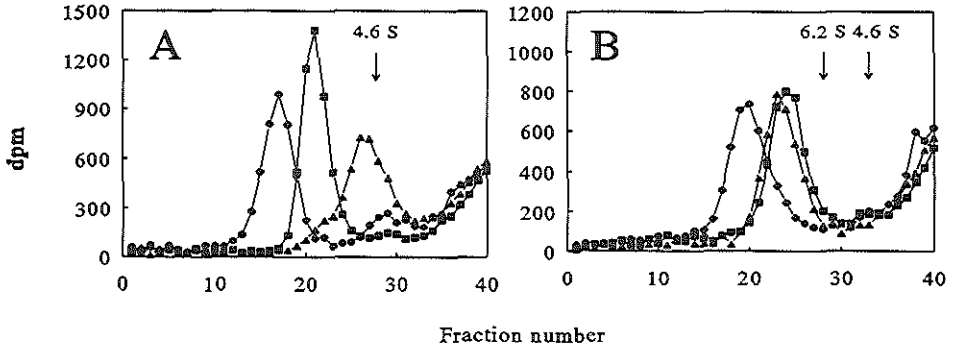

Figure 2: Sucrose density gradient profiles of androgen receptor from LNCaP cells. Cytosol was labeled with [^{3}H]R1881 for 2 h, and excess label was removed as decribed under Materials and Methods. The cytosol (100 µL) was then incubated for 2 h either with 2 µL of ascitic fluid of the androgen receptor antibody F39 (●) or F52 (■) or with 2 µL of ascitic fluid of a nonspecific antibody (▲). Incubation of the cytosol with antibodies and running of the 10—30% sucrose gradients was performed either in the presence (A) or in the absence (B) of 0.5 M NaCl. Bovine serum albumin and alkaline phosphatase were used as internal 4.6S and 6.2S sedimentation markers, respectively.

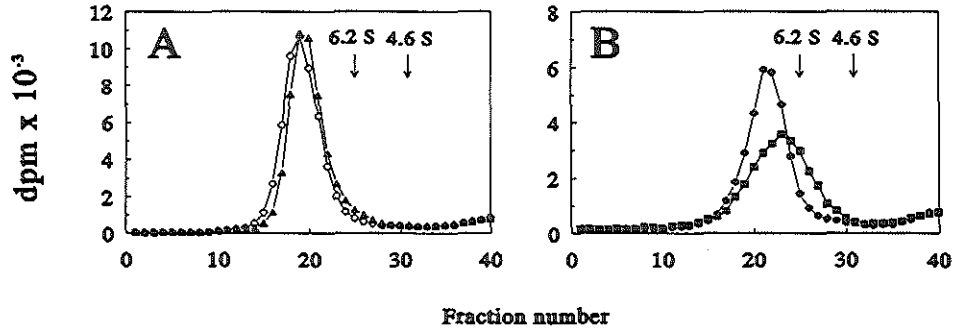

Figure 3: Sucrose density gradient profiles of androgen receptor from LNCaP cells. Cells were incubated in the presence of 10 nM [^{3}H]R1881, either for 2 h at 4 °C (untransformed receptor) or for 30 min at 37 °C (transformed receptor). Cytosols were prepared and cleared from unbound steroid by dextran-coated charcoal and then incubated for 2 h at 4 °C in the presence or absence of 2.5 µL of ascites F52. The samples were run on 10—30% sucrose gradients without additional NaCl, as described under Materials and Methods. Alkaline phosphatase (6.2 S) and bovine serum albumin (4.6 S) were used as internal sedimentation markers: (A) untransformed receptor, incubated either with (○) or without (▲) antibody; (B) transformed receptor, incubated either with (●) or without (■) antibody.

Loss of Receptor-Associated Heat-Shock Proteins during the Transformation process. To investigate whether the hormone-induced transition of the 8S to the 6S form is the result of dissociation of associated proteins from the larger complex, these receptor complexes were immunopurified and screened for coprecipitating proteins that are known to be present in other steroid hormone receptor complexes. We used buffers containing 10 mM molybdate, a condition known to stabilize the 8S complex during isolation.

When the androgen receptor was immunoprecipitated with MAb F39 from the cytosol obtained from LNCaP cells incubated at 4 °C, the heat-shock proteins hsp90, hsp70, and hsp56 were coprecipitated. Incubation of the cells with R1881 at 37 °C led to a fast decrease in the amount of coprecipitating hsp90 and hsp56 (Figure 4, lanes 1 and 3—6). Coprecipitated hsp56 was already absent after 3 min of incubation, and most hsp90 had dissociated within 10 min. The amount of coprecipitated hsp70 also decreased, but this was a somewhat slower process. The observed loss of coprecipitated proteins is also observed after incubation of the cells at 4 °C, although at a much lower rate (Figure 4; compare lanes 1 and 2).

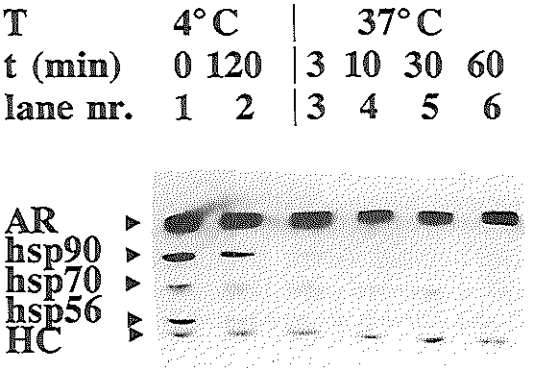

Figure 4: Immune purification with monoclonal F39 of androgen receptor complexes from cytosols of LNCaP cells incubated for various time periods with R1881. Receptor complexes were purified using F39—protein A—Sepharose and subjected to electrophoresis. Chemiluminescence exposures of Western immunoblots were prepared as described under Materials and Methods. MAbs F39, AC88, N27, and EC1 were used to identify the AR, hsp90, hsp70, and hsp56, respectively. HC, antibody heavy chain. Cells incubated without hormone, with 10 nM R1881 for 2 h at 4 °C, and with 10 nM R1881 at 37 °C for 3, 10, 30, and 60 min are represented by lanes 1—6, respectively.

Coinciding with a decrease in the amount of receptor in the cytosol fraction, an increase in the amount of immunoprecipitable nuclear receptor was found (Figure 5). The Western immunoblot only shows immunodetectable androgen receptor. We did not detect coimmunoprecipitated heat-shock proteins after extraction of nuclei with 0.5 M NaCl. This high-salt condition increases the dissociation rate of heteromeric complexes but is required to release the receptors that are tightly bound in the nucleus to DNA. At

lower ionic strength, most AR remained in the nuclear pellet. Not only the amount of immunodetectable receptors in the nuclear fraction increased on incubation of the cells at 37 °C, but in addition, the amount of tightly nuclear bound receptor labeled with [^{3}H]R1881 increased after long incubation times with the radioactive androgen (Figure 6). This indicates that the receptor becomes tightly bound to the nucleus after it has bound ligand. After 2 h of incubation, the amount of androgen receptor present in both cytosol and nuclear extract was estimated by protamine sulfate precipitation. The results showed that about 40% of the total amount of labeled receptor was at that time present in the nuclear extract.

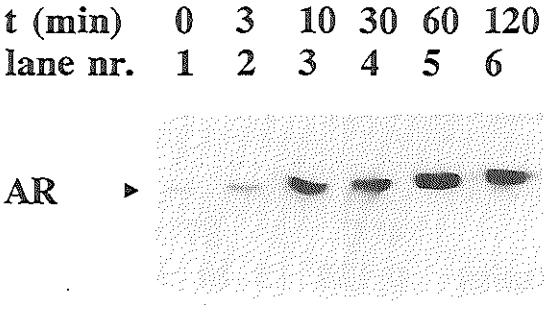

Figure 5: Androgen receptor isolated from LNCaP cell nuclear extracts of cells incubated for various time periods with R1881. Receptor molecules were purified using F39—protein A—Sepharose and subjected to electrophoresis. Chemiluminescence exposures of Western immunoblots were prepared as described under Materials and Methods. Cells incubated at 37 °C for 0, 3, 10, 30, 60, and 120 min are represented by lanes 1—6, respectively.

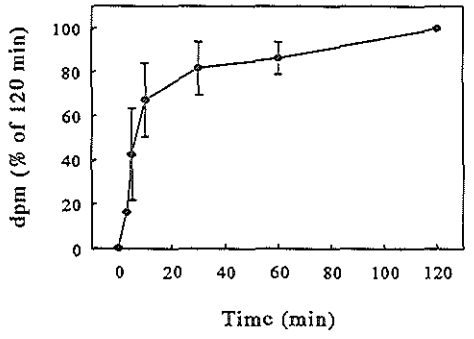

Figure 6: Labeling of androgen receptor extracted from nuclei of LNCaP cells incubated for various time periods with [^{3}H]R1881 at 37 °C. At the end of the labeling period, 1 μM of unlabeled R1881 was added to stop the specific labeling. The amount of label is expressed as the percentage of the amount found after 120 min of incubation. Preparation of nuclear extracts and the protamine sulfate assay for measurement of the amount of labeled receptor are described under Materials and Methods.

Lack of Coimmunoprecipitation of Hsp90 and Hsp56 with the 6S Receptor Complex. The antibody F52 forms a complex with the intermediate-sized 6S receptor protein

complex, but not with the large 8S receptor complex (sucrose gradients studies shown above; see Figure 2). Antibody F52 can therefore be used to isolate the 6S complex from mixtures of both 6S and 8S receptor complexes. LNCaP cells were kept at 4 °C either with or without androgen, and subsequently, receptor complexes were immunoprecipitated with MAb F52. Then, in addition to the receptor band, a hsp70 band and a faint band of hsp90 were visible on the Western immunoblot (Figure 7, lanes 1 and 2). Hsp90 was absent when the last wash step of the resin was extended for several hours, which is indicative of a low binding affinity of hsp90 in the complex precipitated with F52 antibody (not shown). Hsp56 was not visible on the blots.

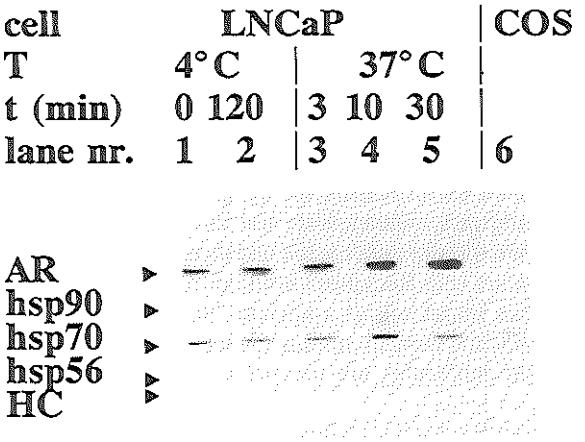

Figure 7: Immune precipitation with monoclonal F52 of androgen receptor complexes from cytosols of LNCaP cells incubated for various time periods with R1881. Receptor complexes were purified using F52—protein A—Sepharose and subjected to electrophoresis. Chemiluminiscence exposures of Western immunoblots were prepared as described under Materials and Methods. MAbs F39, AC88, N27, and EC1 were used to identify AR, hsp90, hsp70, and hsp56, respectively. HC, antibody heavy chain. Cells incubated without hormone, with 10 nM R1881 for 2 h at 4 °C, and with 10 nM R1881 at 37 °C for 5, 10, and 30 min are represented by lanes 1—5, respectively. The sample in lane 6 is an immunoprecipitate from COS-1 cells (control).

The amount of androgen receptor present on the blots increased when the cells were incubated with R1881 at 37 °C (Figure 7, lanes 3—5), indicating that more F52-precipitable receptor complexes are formed upon prolonged exposure of the cells to the receptor ligand. Hsp70 was present in all precipitates, but the amount of this heat-shock protein recovered varied considerably between different experiments. When the immunoprecipitation with MAb F52 was performed on a cytosol from a control cell line without androgen receptors (COS-1 cells), a small amount of hsp70 was detected on the blot (Figure 7, lane 6). This result shows that hsp70 also binds nonspecifically to MAb F52. With the antibody F39, which precipitates both large (8 S) and intermediate (6 S) receptor complexes, we observed a lower association of the receptor complex with hsp70 after prolonged incubation with androgen than found with F52 (compare Figures 4 and 7). This difference is an extra indication that hsp70 binds nonspecifically to the F52 resin.

These results suggest that the 6S complex contains mainly androgen receptor, probably in the form of a dimer (the monomer sediments at 4.6 S). However, we cannot exclude the presence of small amounts of hsp70 or other proteins that are not detected by the antibodies used in this study.

Discussion

In the absence of hormones, steroid receptors are present in the cells as heteromeric complexes with several different proteins and have a low affinity for DNA. Ligand-induced release of regulatory proteins is thought to be an important step required for activation of the DNA-binding function of the receptor. In this process, called transformation, the heteromeric complex dissociates, thereby unmasking the DNA-binding domain of the receptor (Carson-Jurica et al., 1990; Pratt, 1990). In the present study, we analyzed the composition of the androgen receptor complex during ligand-induced receptor transformation *in vivo*, in intact LNCaP prostate tumor cells. In this process, an untransformed 8S form from the cytoplasmic cell fraction is converted to a 4.6S nuclear form. In addition, intermediate size complexes (6 S) were observed in the cytosolic cell fraction. We did not address the question of the localization of the different receptor forms in the intact cell. Receptors isolated from cytosolic fractions may actually have been located in the intact cell at a nuclear "docking place" (Pratt, 1990). Recent evidence from immunohistochemical studies (Jenster et al., 1991) suggests that in fact most androgen receptor molecules are located in the nucleus or are associated with perinuclear structures.

A new monoclonal antibody (F52) against the DNA-binding domain of the androgen receptor was generated. This antibody was used to show that androgen receptor complex intermediates are formed in the transformation process and that the epitope for this antibody in the DNA-binding region becomes exposed during this process. It was demonstrated that the epitope for this antibody is exposed not only in the 4.6S receptor but also in the 6S intermediate form of the receptor. Other antibodies against synthetic peptides have been described which also specifically or preferably recognize monomeric receptor forms (Wilson et al., 1988; Smith et al., 1988; Urda et al., 1989). In Figure 8, two peptide sequences derived from the progesterone receptor are shown, which overlap the homologous sequence in the androgen receptor to which the F52 antibody was raised [p266, p269; Smith et al. (1988) and Wilson et al. (1988)]. The antiserum AP64 (Urda et al., 1989) contains antibodies raised against the human glucocorticoid receptor sequence Cys_{500}—Lys_{517}, overlapping the carboxy-terminal end of the DNA-binding domain and the amino terminus of the hinge region (Figure 8). Antisera developed against a region of the human estradiol receptor homologous to the region in the DNA-binding domain of the other steroid receptors, however, did bind the nontransformed estradiol receptor on sucrose gradients [Traish et al. (1989); sequence p2 and p3 in Figure 8]. This might be due to differences in tertiary structure of the regions flanking the peptide sequence in the less homologous estradiol receptor. However, specific recognition of the transformed receptor only was also observed for antibodies raised against the chicken progesterone receptor sequence Leu_{523}—Pro_{536} (Weigel et al., 1989). This sequence is located in the hinge region, indicating that the antigenic sites outside the DNA-binding domain also become exposed after transformation of the receptor.

Using the bifunctional cross-linker dimethyl pimelimidate for cross-linking of the androgen receptor complex, we have shown formation of a covalently linked complex

sedimenting at 8S that did not dissociate on sucrose gradients in the presence of salt. In studies with glucocorticoid receptors it has been shown that, in the untransformed complex, the cross-linker dimethyl suberimidate could cross-link the receptor to two hsp90 molecules and one 50 kDa unknown protein (Rexin et al., 1988). The 6S, intermediate size, androgen receptor complex was not stabilized by DMP, indicating a change in structure of the complex which prevents receptor cross-linking to other proteins. This suggests that the receptor can be covalently linked only to one of the fast dissociating proteins (e.g., hsp90 or hsp56) that are absent in the 6S form of the receptor complex. Alternatively, in the intermediate form of the receptor complex, the distances between the reactive amino acid residues (lysines) have changed in a way that makes coupling of the receptor with the other proteins impossible. In cross-linking reactions with bis(imidates), the distance between the reacting residues is very important for optimal coupling of the proteins (Aránayi et al., 1988).

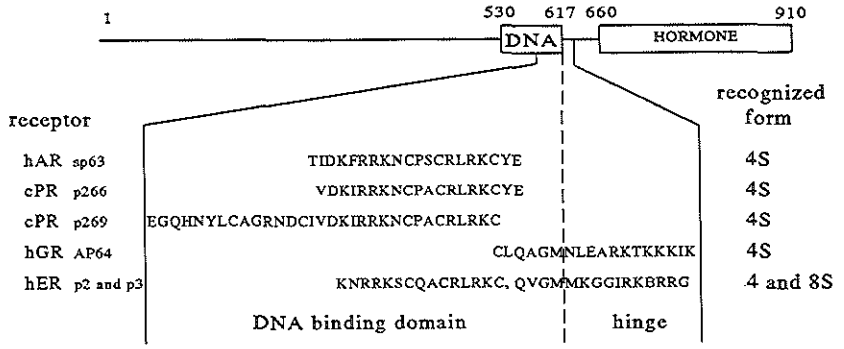

Figure 8: Schematic presentation of the peptides used for raising antibodies against steroid receptors. The peptide sp63 was used in the development of MAb F52. The peptides p266 and p269 are derived from the chicken glucocorticoid and progesterone receptor sequences in the homologues' region (Smith et al., 1988; Wilson et al., 1988). The antiserum AP64 (Urda et al., 1989) contains antibodies raised against the hGR sequence Cys_{500}—Lys_{517}, overlapping only the three amino acid residues that are homologues to the three carboxy-terminal amino acid residues of sp63. The peptides p2 and p3 are derived from human estrogen receptor sequences Lys_{213}—Cys_{245}, totally overlapping the homologues' sequence of peptide sp63, and Glu_{247}—Gly_{261}, homologues to part of AP64, respectively. DNA, DNA-binding domain; Hormone, hormone-binding domain; hinge, hinge region. The numbers indicate the amino acid residue numbers at the domain boundaries of the androgen receptor (Trapman et al., 1988; Faber et al., 1989).

In the present study, two different monoclonal antibodies against the androgen receptor were used for the characterization of heat-shock proteins interacting with the receptor. The antibody F39, recognizing an epitope in the N-terminal region, precipitates all different forms of the receptor and was used to study initial steps in heat-shock protein release. Before incubation of the LNCaP cells with hormone, the heat-shock proteins hsp90, hsp70, and hsp56 were precipitated together with the receptor by antibody F39. Other proteins might also be present in the complexes, as has been

described for the progesterone receptor (Smith et al., 1990a), but the repertoire of antibodies against heat-shock proteins used in this study does not permit their detection. The dissociation rate of hsp70 from the androgen receptor complex, during incubation of the cells with hormone, is lower than that observed for hsp90 and hsp56. However, after 1 h of incubation of the cells with hormone, the associated hsp70 level was reduced considerably. Investigations of Sanchez et al. (1990a) suggested that hsp56 exists in cytosol in a higher order complex containing hsp70 and hsp90. Furthermore, p59 (the rabbit homologue of hsp56) is bound to hsp90 and not to the hormone-binding subunit of steroid receptor complexes (Renoir et al., 1990b, Lebeau et al., 1992). This implies that hsp56 dissociates from the receptor complex either together with or in advance of hsp90. For the progesterone and glucocorticoid receptor, a similar steroid-induced dissociation process of the multiprotein receptor complex was found. As in our study of the androgen receptor, also for the progesterone and glucocorticoid receptor, hsp70 remained partly bound to the receptor. In contrast to our observations, however, in the latter studies, hsp70 also remained bound to the receptor complex in the presence of high concentrations of salt (Kost et al., 1989; Sanchez et al., 1990b; Smith et al., 1990a; Smith et al., 1990b). ATP is probably required for the *in vitro* dissociation of hsp70 from the progesterone receptor (Smith et al., 1992).

Immunoprecipitation of the intermediate 6S form of the androgen receptor with the newly developed F52 antibody resulted in coprecipitation of hsp70, but not of hsp56. Only limited amounts of hsp90 were coprecipitated, and after extended washing of the precipitate, this heat-shock protein was no longer present. This indicates that the carboxy-terminal part of the DNA-binding domain is exposed to the F52 antibody, after removal of hsp56 and most of hsp90. In contrast, hsp70 was coprecipitated and could not be removed by washing of the antibody—receptor complex. However, as described under Results, the experiments indicate that a considerable part of the hsp70 is nonspecifically bound. This nonspecifically bound (not receptor associated) hsp70 may have prevented the detection of a small amount of specifically bound (receptor associated) hsp70.

In summary, it appears that the first step of androgen receptor transformation of the 8S androgen receptor complex results in loss of association of hsp90 and hsp56, leaving a smaller 6S receptor complex. This intermediate receptor complex, in contrast to the 8S complex, cannot be stabilized by cross-linking with DMP, indicative of the changes in association of the receptor with the associating proteins. Furthermore, F52, a monoclonal antibody raised against part of the DNA-binding domain of the androgen receptor, binds to the 6S as well as to the 4.6S form, but not to the 8S receptor form, on sucrose gradients. This demonstrates that the C-terminal part of the DNA-binding domain is exposed in the 6S, hormonally transformed receptor. It cannot be excluded that the 6S receptor form detected on sucrose gradients consists of heteromeric complexes with hsp70, or other as yet undefined proteins. The possibility that receptor-bound protein factors might play a role in transcription activation should not be excluded (Lewin, 1990). Alternatively, the 6S complex may predominantly consist of homodimeric receptor complexes. This is not unlikely, since formation of homodimers preceding receptor binding to DNA has been shown for human and chick progesterone receptors (Demarzo et al., 1991; Rodrigues et al., 1990). Further analysis of the 6S intermediate forms of the androgen receptor will be the next step in the study of the hormone-induced receptor transformation process in intact cells.

Acknowledgments

We thank Drs. D. O. Toft, W. J. Welch, and L. E. Faber, for generously providing the monoclonal antibodies AC88, N27, and KN382/EC1, respectively. We also are grateful to Dr. W. J. A. Boersma for the constructive remarks on the manuscript.

Chapter 6

General Discussion

6.1 The LNCaP Cell as a Model System to Study Androgen Responsiveness: the Impact of the Mutated AR in LNCaP Cells on Research Results

Since the establishment of the prostate tumor cell line LNCaP (Horoszewicz et al., 1980, 1983), this cell line has been used as an *in vitro* model for androgen responsive growth (proliferation) of epithelial prostate cancer cells (for a review on the origin of LNCaP and its sublines, see Van Steenbrugge et al., 1991). The subline used in our studies is the FGC (Fast Growing Colony) subline, which is androgen sensitive, but not androgen dependent, for growth. Other sublines have been described, with properties that range from totally dependent on, to unresponsive to, androgens (Van Steenbrugge et al., 1991). Growth of prostate cancer cells initially is highly dependent upon androgen action, and it is of utmost importance that the androgen response system in a model cell system is mechanistically similar to that in normal prostate or prostate tumor epithelium cells. There are several indications, however, that for LNCaP cells this may not be the case. Despite the absence of ER and PR in LNCaP cells (Berns et al., 1986; Schuurmans et al., 1988), growth of these cells is not only enhanced by androgens, but also by estradiol and progesterone (Horoszewicz et al., 1983; Schulz et al., 1985; Schuurmans et al., 1988). Furhermore, antiandrogens showed stimulatory rather then inhibiting effects (Wilding et al., 1989; Schuurmans et al., 1990). Indeed, it has been described that the affinity of the cytosolic AR in LNCaP cells for several non-androgenic compounds is relatively high (Schuurmans et al., 1988). In Chapter 2, detailed studies on the steroid binding specificity of both cytosolic and nuclear AR are presented. By using very pure nuclear preparations, steroid binding to contaminating cytoplasmic components was circumvented. Moreover, the use of the nonmetabolizable AR ligand R1881 (Bonne & Raynaud, 1975) and PR ligand R5020 (Raynaud et al., 1980) made it possible to compare the binding affinities of the AR in several cell types with potentially different steroid metabolizing capacities. The results showed that the steroid binding specificity of both cytosolic and nuclear forms of the AR were abnormal. In Chapter 3, it is shown that a mutation in the steroid-binding domain of the AR of LNCaP cells, resulting in the replacement of a threonine residue at amino acid position 868 by an alanine residue, is responsible for the changed steroid binding specificity. Moreover, in transfection experiments, the mutant receptor, but not the wild-type receptor, enhanced transcription of an androgen responsive reporter gene in response to progestins, estrogens, and some antiandrogens. The existence of the mutation and the aberrant response of this receptor to non-androgens was confirmed by Harris et al. (1991) and Young et al. (1991).

The original karyotype of LNCaP cells (nearly tetraploid) is well preserved among the sublines (König et al., 1989). Therefore, also the duplication of the X chromosome, which carries the AR gene (Trapman et al., 1988), may have occurred in the parental subline. Only the mutated form of the AR was found after genomic sequencing (Chapter 3), and one of the early passages (passage 20) also contains the mutant receptor (C. Ris-Stalpers, personal communication). Therefore, it is likely that all sublines are homozygous for the mutation.

An increasing number of researchers use LNCaP cells in their studies. In most cases, the cells are used either as a model for androgen action, or as a model for prostate cancer cells. In Figure 1, the number of publications per year that contain data on research with LNCaP cells is depicted. It is clear that each year an increasing number

of LNCaP cell-related articles are published.

The presence of a mutated AR in LNCaP cells highly influences research results concerning AR mediated effects in these cells. Several effects of non-androgenic compounds, e.g., down-regulation of AR mRNA by CPA (Quarmby et al., 1990) and AR-hyper-phosphorylation by estradiol and progestins (Van Laar et al., 1991), can be explained by the mutation in the AR.

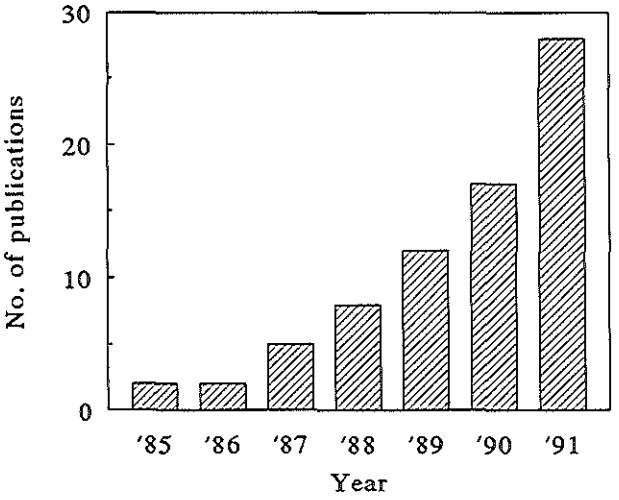

Figure 1. Number of papers describing research with LNCaP cells. A search for 'LNCaP' was done on a cd-rom system covering the 1200 journals with the highest impact (MedLine), in all fields (including abstracts), and the references found were checked for hits not related to the LNCaP cell line.

6.2 The Agonistic Effects of Antiandrogens Have Not Always Been Recognized

6.2.1 Growth rate of LNCaP cells. The stimulatory effect of androgens, estrogens, progestins, and antiandrogens on growth of LNCaP cells show a biphasic dose-response relationship when the cells are cultured in medium with dextran—charcoal-stripped serum (Schuurmans et al., 1988, 1990; Wilding et al., 1989; Olea et al., 1990; Simard et al., 1991; De Launoit et al., 1991; Harris et al., 1991). Up to a certain optimal concentration, which is different for each ligand, the growth rate increases with increasing concentrations. At concentrations higher than this optimum, growth rate is stimulated to a much lesser extent (see Figure 2). The molecular mechanism of this biphasic effect is not understood. However, for the purpose of this paragraph it is important to know that the AR is involved and that sub-maximal stimulation is not the result of a non-specific effect of high concentrations of ligand. This is concluded from the observation that casodex, the only antiandrogen tested which shows antagonistic effects in LNCaP cells, is able to antagonize the effect of a supra-optimal concentration of androgens. Casodex competes with androgens for receptor occupation and reduces the actual amount of AR occupied with androgen.

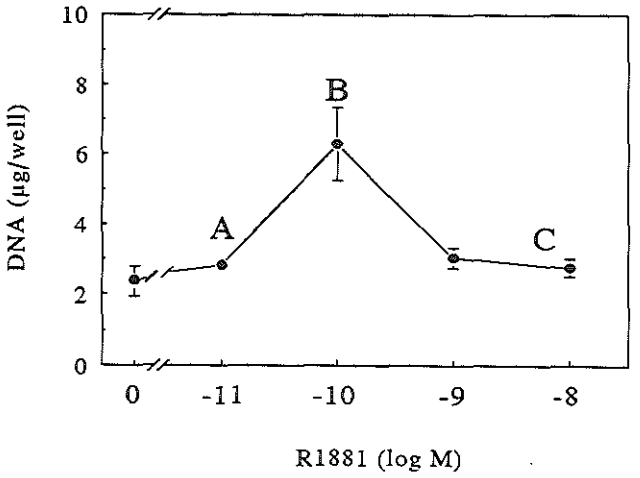

Figure 2. Effects of the synthetic androgen R1881 on growth of LNCaP cells during a 6 day culture period. Medium was changed after 3 days. Means and standard deviations of four measurements are shown. The results are from the same experiment as described in Figure 1 of Chapter 4.

In some studies, the agonistic effects of some antiandrogens on LNCaP cell growth were either not recognized, or were explained by effects not mediated by the androgen receptor (Olea et al., 1990; Wolf et al., 1991), and the report by Olea et al. (1990) will be discussed in more detail. In this study, in which antagonistic actions of several antiandrogens on LNCaP cell growth were claimed, these antiandrogens in fact inhibited the growth stimulating effect of DHT (Olea et al., 1990). One explanation could be that this apparent antagonistic effect of antiandrogens is due to the use of very high concentrations of DHT (Figure 2, range B to C). Therefore, the addition of the antiandrogens may have resulted in an increase in the total amount of agonist present, and consequently may have led to a shift in the biphasic dose-response curve to a point with lower growth stimulating potency (comparable with a shift from B to C in Figure 2). A similar effect could be expected from any combination of compounds with agonistic properties for LNCaP cells. Indeed, DHT inhibited the growth induced by antiandrogens (Olea et al., 1990).

Another explanation for the apparent antagonistic effect of antiandrogens on LNCaP cells could be that when two types of ligands are applied together, the receptor is activated less efficiently. This might be concluded from the observation that androgens as well as antiandrogens stimulate growth rate of LNCaP cells independent of each other, whereas their combined action resulted in lower proliferation rates (Olea et al., 1990). This unexpected effect might be the result of a failure to form dimers with two different ligands (AR(ligand-1)—AR(ligand-2) dimers). For the PR, such an effect has been shown: receptors bound to the agonist R5020 did not dimerize with receptors bound to the antagonist RU486 (Meyer et al., 1990).

Sonnenschein et al. (1989) and Olea et al. (1990) theorized that the stimulating effects of androgens, and also of estrogens, progestins, and antiandrogens, on LNCaP cell proliferation, are mediated by binding of these compounds to, and inhibition of the action of, serum factors with proliferation inhibitory activity. In Chapter 3 of this thesis it is shown, however, that androgens, estrogens, progestins, and antiandrogens can activate transcription in HeLa cells through the mutant 'LNCaP AR'. The transcription activation correlated very well with the effects of these compounds on growth rate of LNCaP cells. The simplest explanation, therefore, is that stimulation of proliferation of LNCaP cells by these non-androgenic compounds is mediated through the AR in these cells: it seems that the serum factors postulated by Sonnenschein et al. (1989) and Olea et al. (1990) may not play a significant role in the aberrant responses of LNCaP cells to non-androgens.

6.2.2 Other androgen receptor dependent effects in LNCaP cells. In addition to effects on growth, there are other processes in LNCaP cells which depend on AR action. The induction of epidermal growth factor receptor, the production of apolipoprotein D, and the secretion of the prostate marker prostatic acid phosphatase (PAP), are not only regulated by androgens, but also by progestins, estrogens, and antiandrogens. The dose-response curves of these effects are also biphasic (Schuurmans et al., 1988; Simard et al., 1991; Henttu & Vihko, 1992).

For another prostate tumor marker, prostate specific antigen (PSA), no biphasic dose-response curve was found. Its mRNA levels in LNCaP cells and the levels of secretion are both elevated in a monophasic mode by androgens and non-androgens (Young et al., 1991; Henttu & Vihko, 1992; Henttu et al., 1992). In agreement with this, HF stimulated PSA mRNA levels (Henttu & Vihko, 1992). In contrast, this compound could partially decrease DHT-induced PSA mRNA levels in another study (Young et al., 1991).

In LNCaP cells, androgens also decrease AR mRNA levels, while increasing AR protein levels. AR mRNA levels were decreased by testosterone, R1881, and CPA (Quarmby et al., 1990), the synthetic androgen mibolerone (Krongrad et al., 1991), estradiol, progesterone, and R1881 (Henttu et al., 1992).

6.2.3 In conclusion: In LNCaP cells, several androgen dependent effects are elicited also by estrogens, progestins, and some antiandrogens. In some cases, antagonistic effects of antiandrogens were claimed, but these effects may in fact represent agonistic effects. Misinterpretations can occur when a compound is tested at only one concentration, especially when a biphasic pattern of stimulation or repression is concerned. It is therefore not sufficient to test the antagonistic activity of a compound on LNCaP cells at only one ratio of concentrations of agonist and antagonist.

6.3 By What Mechanism Did the Antiandrogens Become Agonists?

6.3.1 The role of dissociation rate. It has been suggested that a fast dissociation of a steroid receptor ligand would explain the antagonistic properties of this compound (Raynaud et al., 1980). For a number of HF-derivatives, however, no strict correlation was found between dissociation rate and biological potency (Wakeling et al., 1981). This indicates that, even if dissociation of a compound plays a role in the antagonistic action of some compounds, it is not the only existing mechanism of antagonism.

In Chapters 3 and 4, the wild-type receptor and the LNCaP mutant receptor were compared with respect to the binding affinities for several compounds. The relative binding affinities (RBA; relative to a known high-affinity compound) of CPA, HF, and anandron for the mutant receptor were higher then the RBAs for the wild-type receptor expressed in the same cell type, whereas the RBA of casodex for the mutant receptor was lower. RBA values are determined both by association and dissociation rates of ligand-receptor complexes. However, the differences in RBA were so small, that it seems unlikely that the dissociation rates of CPA, HF, and anandron for the mutant receptor were sufficiently changed to play a role in the change from antagonistic to agonistic properties.

6.3.2 Also theoretically, dissociation rate alone cannot explain antagonistic activity. Also on theoretical grounds one can predict that a fast ligand dissociation *per se* does not lead to an antagonistic action. Transformation might proceed during a reversible dissociation process. However, it cannot be excluded that the unoccupied receptor is partially transformed, and left in an irreversible, non ligand binding state, incapable of transcription activation.

	L		L		L	
	+		+		+	
	AR		AR'		AR"	AR"' ▸ AR""
	↑↓		↑↓		↑	↑
	AR	⇄	*AR'*	⇄	*AR"*	→ *AR"'* ▸ *AR""*
step no.			1		2	3

L=ligand
AR=androgen receptor
AR=ligand-bound AR
→ irreversible step (hormone dependent)
⇄ reversible step (hormone dependent)
▸ irreversible hormone-independent step
AR' to AR"": several forms of partially transformed receptors
AR"" or AR""=transcriptionally active receptor

Figure 3: Hypothetical receptor-ligand association/dissociation scheme. Every transformation step can lead to a change in association or dissociation rate (affinity) of a ligand for the receptor. This change may be different for each ligand, and therefore the most likely point of dissociation may be different for each ligand. In this model, ligands which predominantly dissociate between steps 1 and 2 of receptor transformation leave the receptor in a form which is able to re-bind new ligand, and be transformed towards a transcriptionally active form. However, a compound which predominantly dissociates between steps 2 and 3, is an antagonist because it leaves the receptor in an irreversibly non-active state. A compound which dissociates after step 3 leaves the receptor in a transcriptionally inactive state, but the receptor can be converted to a transcriptionally active state, independent of ligand, and thus this ligand is an agonist.

Figure 3 shows a hypothetical model for receptor transformation. This model is supported e.g., by results for the ER (Weichman & Notides, 1980), for which it was shown that transformation results in a changed ligand dissociation rate. If the antagonistic action of a certain compound is elicited by its fast dissociation from the receptor, than at least one irreversible step is required, because otherwise a high concentration of the compound would drive the receptor towards transcription activation, in which case this compound would be an agonist. It is not the dissociation *per se* which blocks receptor action, but the condition in which the receptor is left unliganded.

6.4 Antagonists May Act at Different Steps in the Transformation Cascade

There are several steps in the cascade of receptor transformation which may be blocked by antagonists (Figure 4). These blockades can be the result of either a high rate of dissociation of the antagonist and a subsequent irreversible process (as described in the previous paragraph), or an aberrant interaction of an antagonist with the receptor, without dissociation of that compound from the receptor. In addition, more than one of the steps shown in Figure 4 may be involved in the inhibitory actions of antagonists.

6.4.1 Which steps in the cascade of AR transformation are blocked by antiandrogens? Theoretically, the antiandrogen-mediated blockade of receptor function can be at different transformation steps for each compound (Figure 4). However, because the antiandrogens CPA, HF, and anandron have become agonists for the mutant receptor in LNCaP cells, it is most likely that these antagonists block androgen action in the wild-type receptor through a similar mechanism. There are three possible explanations for the agonistic actions of CPA, HF, and anandron on the mutant receptor (hypothesis A to C in Figure 4).

1) In the first hypothesis, a blockade of receptor-hsp dissociation by these compounds, but not the one induced by casodex, is overthrown by the mutation. A comparison of the wild-type receptor and the mutant receptor with respect to antiandrogen-mediated receptor—hsp-complex dissociation will be necessary to test this theory experimentally.

2) Since in LNCaP cells casodex blocks the dissociation of the receptor—hsp-complex and acts as antagonist for both the wild-type and mutant receptor (Chapter 4), it is conceivable that the mutation has not altered receptor-hsp interactions. Therefore, CPA, HF, and anandron possibly block a receptor transformation step which succeeds hsp-complex dissociation, and this block might be eliminated by the mutation. The LNCaP mutation at amino acid position 868 is located in the so-called heptad repeat region. It contains a heptad repeat of hydrophobic amino acid residues, which is highly conserved among the steroid/thyroid hormone receptor superfamily and has been suggested to be involved in dimerization of the ER (Fawell et al. 1990b). It seems possible, therefore, that the dimerization step is blocked by CPA, HF, and anandron, but that this blocking effect is lost by the mutation (hypothesis B in Figure 4).

3) A third possibility (hypothesis C in Figure 4) is that antiandrogens still allow receptor dimerization and consequently can transform the wild-type receptor to a DNA-binding state, but that the ligand-receptor complex subsequently inhibits transcription (see Figure 5). There is some circumstantial evidence which supports this possibility. It has been shown that CPA can induce AR-mediated transcription of a reporter gene construct in CV-1 cells, and thus may act as partial agonist for the wild-type AR (Kemppainen et al., 1992). Using the same androgen responsive reporter gene construct

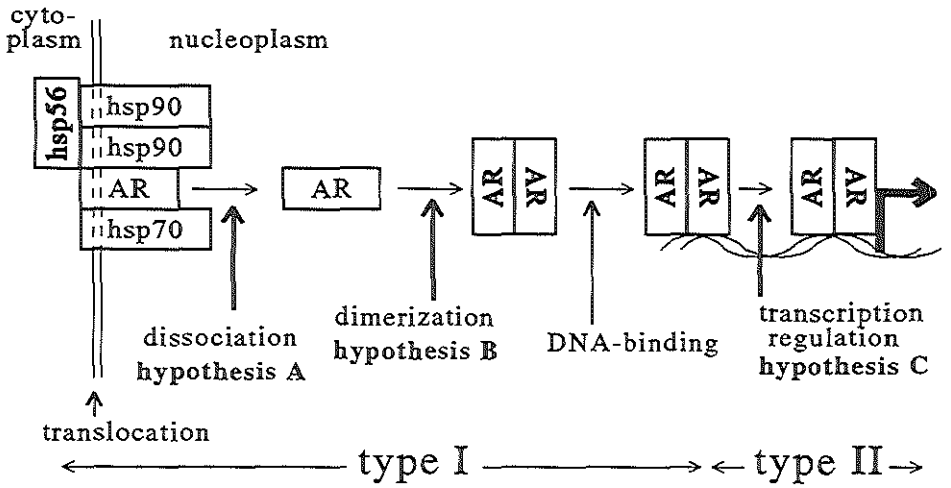

Figure 4. Several steps in the cascade of receptor transformation which are possibly blocked by antagonists. In hypothesis A, CPA, HF, and anandron block the dissociation step of the wild-type receptor. In hypothesis B, these antiandrogens block the dimerization step, and in hypothesis C, the transcription activation step is the target. A distinction was made between type I antagonists which block receptor action before DNA-binding occurs and type II antagonists which block receptor action following DNA binding. When hypothesis A or B is correct, the antiandrogens described above can be designated type I antagonist. When hypothesis C is correct, these antiandrogens can be designated type II antagonists.

(pG29GtkCAT; Schüle et al., 1988), we did not observe agonistic effects of CPA, HF, and anandron in HeLa cells transfected with the wild-type receptor (Chapters 3 and 4). The agonistic effects of CPA in CV-1 cells reported by Kemppainen et al. (1992), might involve the action of a hormone-independent transcription activation function (like TAF-1 from ER and PR; see Chapter 1) which might function in CV-1 cells but not in HeLa cells (see Figure 5). Binding of the AR to the hormone response element is sufficient for transcription activation through this hormone-independent TAF. The other TAF (like TAF-2 from ER and PR; see Chapter 1) is activated only by binding of an agonist to the wild-type receptor, and is thus ligand dependent. When CPA indeed can direct the wild-type AR to a DNA-binding form, as can be concluded from the CV-1 results, then the simplest explanation for agonistic action on the mutant AR in LNCaP cells and in transfected HeLa cells is, that the ligand-dependent TAF can be activated by CPA as a consequence of the mutation (hypothesis C in Figure 4). An additional argument in favour of this hypothesis is that for the ER, TAF-2 has been described as a small conserved stretch of amino acid residues, important for ligand-mediated transcription activation (Danielian et al., 1992). The homologous stretch in the AR, amino acid residues 884—891, is very close to the mutation at position 868 in the AR in LNCaP cells. For the AR, a COOH-terminally located TAF was indeed suggested (Simental et al., 1991). It can be envisaged that the mutation has changed the conformation in this region, in a way that allows antiandrogen-mediated transcription activation through the COOH-terminal TAF (see Figure 5).

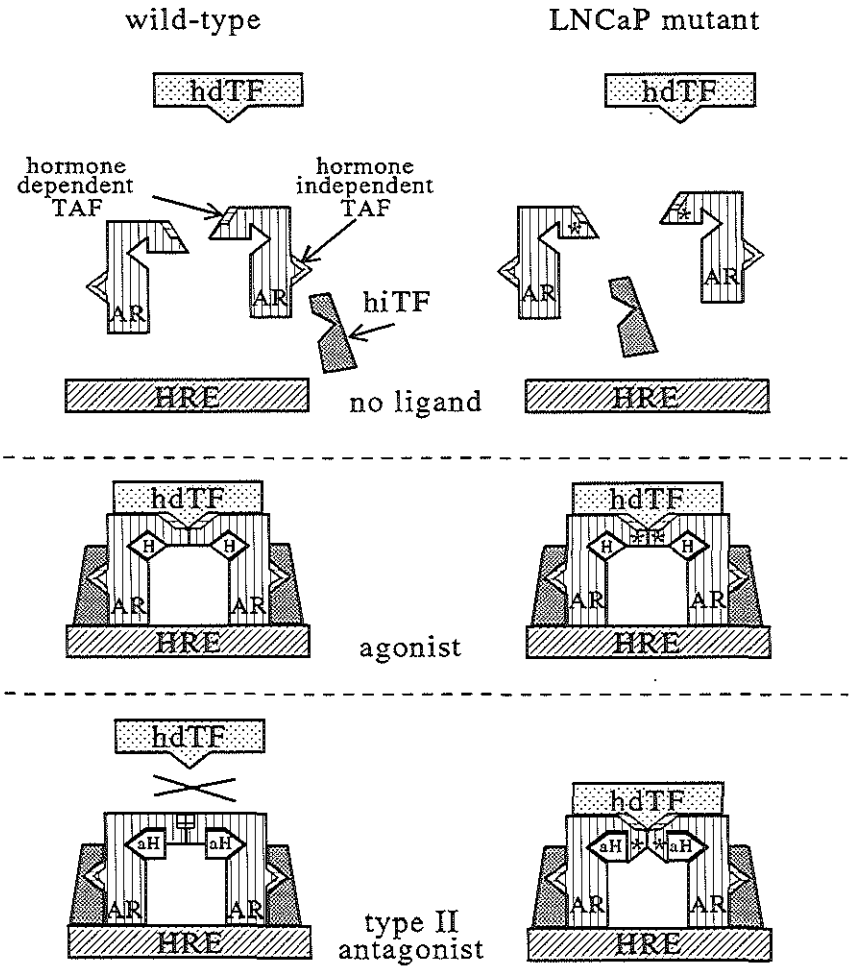

Figure 5. The putative effects of the mutation in the AR of LNCaP cells on the actions of agonists and type II antagonists. For clarity, hsp's have not been included in the figure. In both wild-type and mutant receptor, hormones (H) induce a change in the receptor molecule (AR) that allows dimerization and binding of the dimer to the HRE. In addition, the ligand induces a change in conformation which allows the hormone-dependent TAF to interact with a transcription factor (hdTF), resulting in transcription activation. In the wild-type receptor, type II antagonists (aH) induce dimerization and binding of the dimer to the HRE, but the interaction with hdTF is inhibited. Whether or not transcription occurs, is now dependent upon the presence of a second type of transcription factor (hiTF) (and possibly promoter context) which can interact with a hormone-independent TAF. In this model, this hiTF might not be present in HeLa cells and LNCaP cells. In the mutant receptor, the mutation (*) resulted in a changed response to type II antagonists. Despite the occupancy of the receptor by the antagonist, the hormone-dependent TAF is able to interact with a hdTF transcription factor, resulting in transcription activation.

For some partial ER- and PR-antagonists the cell and gene specific agonistic effects have also been explained by the action of ligand-independent TAFs (Berry et al., 1990; Green, 1990; Klein-Hitpass et al., 1991). Antagonists which block the binding of the receptor to the HRE were called type I antagonists. The antagonists which allow binding of the receptor to the HRE, but fail to activate the hormone-dependent TAF, were called type II antagonists (Klein-Hitpass et al., 1991). The reversed typification was used by others (Reese & Katzenellenbogen, 1991; Meyer et al., 1992). When the terminology of Klein-Hitpass is used for AR antagonists, then - at least in LNCaP cells - casodex is a type I antagonist. CPA, HF, and anandron are type I antagonists for the wild-type AR if either hypothesis A or hypothesis B is correct, but are type II antagonists if hypothesis C is correct (Figure 4).

6.5 Transformation of the AR Results in Loss of Association with Hsp90, Hsp70, and Hsp56

6.5.1 At first, transformation results in a decrease in complex size, and then the receptor gains high affinity for the nucleus. To examine the role of receptor-associated proteins (including heat-shock proteins) during hormone-regulated transformation of the receptor, experiments were undertaken to study whether this process could be separated into several distinct steps. In Chapter 5 it is described that short-term incubation of LNCaP cells with hormone results in a concomitant decrease in size of the cytosolic AR-complex from 8 to 6 S on sucrose gradients. Longer incubations resulted in decreasing amounts of receptor recovered from the cytosol, but an increased amount that was tightly bound to the nucleus. The latter form (salt extractable) sediments in the 4.6 S region in sucrose gradients. The salt extraction procedure, however, also disrupts the association of the AR with other proteins and therefore prevents their detection. Experimental protocols designed to cross-link the receptor to the proteins which are associated with the receptor in intact cells, prevented the extraction of the receptor (unpublished observations). Therefore, the association of the tightly nuclear bound receptor with hsp's could not be demonstrated.

6.5.2 The decrease in size of the receptor complex is the result of a loss of associating hsp's (Figure 6). Examination of proteins that were co-precipitated with the AR during precipitation with AR-specific antibodies indicated that the decrease in sedimentation value of the cytosol receptor induced by ligand binding was the result of dissociation of three different heat-shock proteins from the receptor. This dissociation process might be a two step process (Chapter 5), in which first hsp90 and hsp56 dissociate, and then in a second step binding of hsp70 is lost (Figure 6A). However, it was not possible to prove that AR-hsp70 intermediates exist.

The 6S form of the receptor could be isolated with a newly developed antibody (F52), directed against the COOH-terminal half of the DNA-binding region of the receptor. F52 binds specifically to the 6S- but not the 8S- complexes. This indicates that the COOH-terminal half of the DNA-binding domain is exposed in the 6S receptor complex. Incubation of LNCaP cells with hormone initially caused an increase in the amount of cytosolic receptor precipitated by the F52 antibody. In addition, the amount of co-precipitating hsp70 increased (Chapter 5).

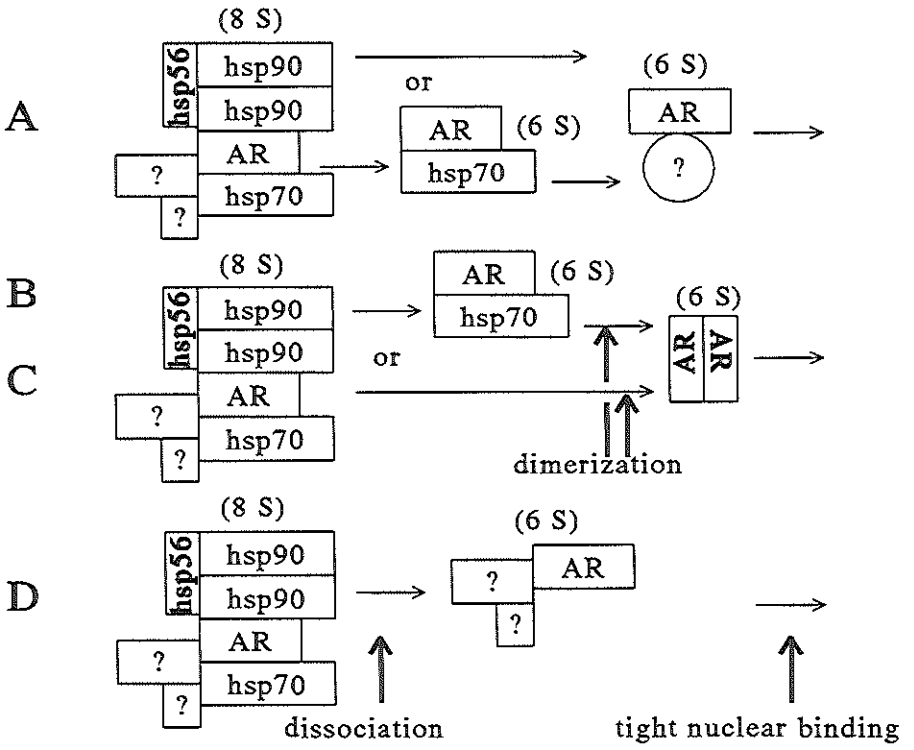

Figure 6. Effects of transformation of the AR on the composition of the AR-complex. The untransformed cytosolic 8S AR-complex consists in addition to the receptor molecule of hsp56, hsp70, two hsp90 molecules, and likely some other proteins (?). Binding of hormone to the AR results in dissociation of the AR-complex. An intermediate 6S complex is detected in the cytosol after short term incubations. The 6S complex may consist either of AR-hsp70 complexes and possibly some other AR-complexes (A), AR-hsp70 complexes and AR homodimers (B), or solely of AR homodimers (C). In addition, the 6S complex may be comprised of an AR either bound to proteins already present in the initial 8S complex or bound to other proteins (D). The next step in transformation results in tight nuclear binding of the AR, leading to decreased amounts of receptor in the cytosol, and increased amounts of salt extractable receptor in the nucleus.

Hormone-induced transformation of both quail PR and mouse GR, and even high concentrations of salt, did not result in complete hsp70 dissociation from these receptors (Kost et al., 1989; Smith et al., 1990a; Sanchez et al., 1990b). This contrasts with the results for the AR (Chapters 4 and 5). It might be that the difference between dissociation of the AR-hsp70 complexes on the one hand, and the PR- and GR-hsp70 complexes on the other hand, are a reflection of subtle differences in the kinetics of receptor transformation or affinity of hsp70 for the AR.

Some processes which are more speculative, are described in the next two paragraphs.

6.5.3 Either dissociation of the AR complex directly results in formation of AR homodimers, or these dimers are formed subsequently to the release of hsp70 from intermediate hsp70-AR complexes (Figure 6B and 6C). The 6S receptor peak might solely consists of homodimers. Alternatively, hsp70-AR complexes and AR homodimers may coexist in the 6S region of the gradient (Figure 6B and 6C). Homodimerization is important for binding of steroid hormone receptors to their HREs (see Chapter 1). To date there is no precedence for stable AR homodimers in solution. The fact that the 6S receptor was recovered from the cytosol, implies that it was probably not bound to DNA, and therefore argues against the presence of homodimers in the 6S peak. In contrast to ER dimers (Fawell et al., 1990b), GR- and PR- homodimers were not stable during gradient centrifugation or gel electrophoresis, unless they were stabilized by chemical crosslinking or the addition of DNA (Wrange et al., 1989; Rodriguez et al., 1990). In many respects the AR is more similar to the PR and GR than to the ER, and therefore the existence of AR dimers in solution which are stable enough to withstand sucrose gradient centrifugation, without stabilization by crosslinking or the addition of DNA, is less likely.

6.5.4 The 6S intermediate might be a multimer comprised of the AR and (an) unknown protein(s) (Figure 6D). Another possibility is that the 6S complex consists of one receptor molecule, bound to one or more proteins which were not detectable with the antibodies used in the present study. Similar to the avian PR, the untransformed AR might be associated with proteins other than the three types of hsp's (see Chapter 1). Therefore, the 6S AR intermediate might be a complex of the receptor with one or more of these proteins, or with proteins which are not present in the 8S complex, but rather associate with the AR during the transformation process (Figure 6D).

6.6 What is the Function of the Association of Hsp's with the AR?

The AR complexes which were analyzed, were present in the cytosol fraction after cell rupture, but most likely the receptor has leaked out from the nucleus during cell fractionation (see Chapter 1). It is not known whether the association with the heat-shock proteins occurred before or after leakage of the AR from the nucleus. In the latter case, the associations would mainly reflect homogenization-induced interactions of the receptor with heat-shock proteins. There are, however, several indications that the association of steroid receptors with at least hsp90 occurs intracellularly (Howard & Distelhorst, 1988; Rexin et al., 1988: see Chapter 1) and thus is no artifact. Renoir et al. (1990a) treated cells with a combination of tungstate and the receptor antagonist RU486 to stabilize the PR-hsp complex, and could extract complexes containing both hsp90 and receptor molecules from nuclei. Moreover, hsp90 plays a role for the GR in acquiring hormone binding capacity (Bresnick et al., 1989; Nemoto et al., 1990; Scherrer et al., 1990) and in the capacity to stimulate transcription in yeast cells (Picard et al., 1990; see Chapter 1). Therefore, the association of the cytosolic AR with hsp90 most likely is the result of its association within the nucleus.

In addition, hsp90 binding might also play a role in the cytoplasm of the intact cell. Several observations for the GR (Pratt et al., 1989; Akner et al., 1991; Miyata & Yahara, 1991) suggest that hsp90 functions as an attachment site of steroid receptors to the cytoskeleton, and might play a role in transport of the receptor in the cytoplasm (see Chapter 1).

For hsp70, no role has been found for functioning of steroid hormone receptors *in vivo*, although several reports on association of receptors with hsp70 suggest that hsp70 may play a role in steroid receptor action. Moreover, for the PR it was shown that hsp70 was required for re-association with hsp90 in a reticulocyte lysate (Smith et al., 1992). This indicates that at least *in vitro*, hsp70 can direct the receptor to associate with other proteins. This is reminiscent of the function of the hsp70 family member BiP, which in intact cells also plays a role in the assembly of multimeric protein complexes (Haas & Wabl, 1983; Lee, 1987). In addition, several hsp70 family members are important for translocation of proteins across membranes. Since the receptor has to pass across the nuclear membrane, it can be envisaged that hsp70 plays a role in this process.

The possible interactions of steroid receptors with the hsp's, result in the following model (Figure 7): After translation, the receptor associates with hsp70,which may assist in proper folding of the receptor. Then, hsp70 directs binding of the receptor to hsp90, which either is already attached to, or will subsequently attach to actin filaments. The hsp90-hsp70-receptor complex is transported towards the nuclear pore complex. Here hsp70 plays an important role in the translocation of the receptor-hsp complex. The complex either first dissociates into the different components, or it is translocated as a whole. At the nuclear side of the membrane, the receptor-hsp complex is attached to the

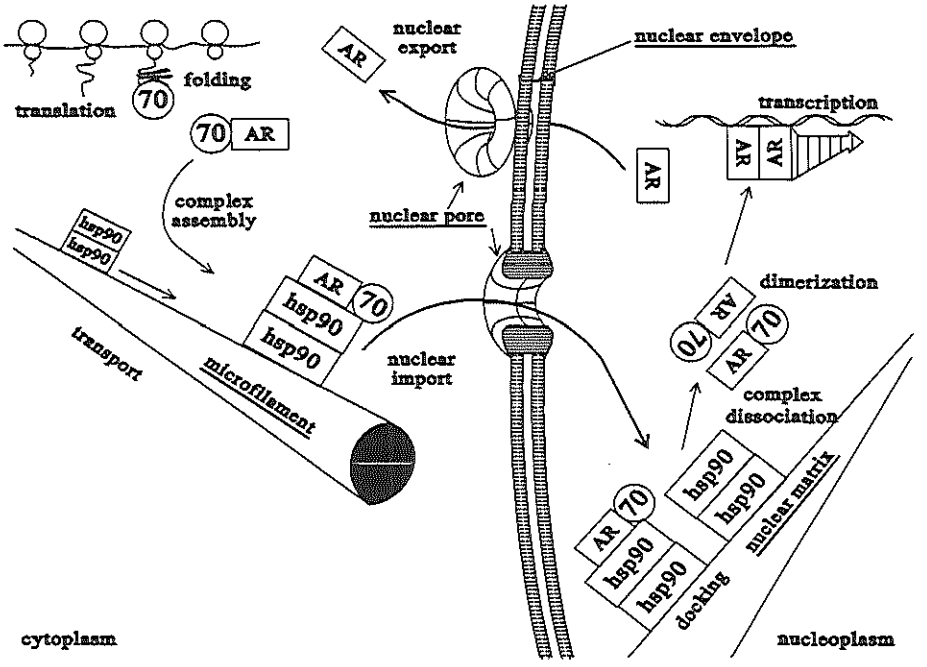

Figure 7. Theoretical model for chaperoning of steroid hormone receptors by heat-shock proteins. For a description of processes see text.

nuclear matrix on which it is transported to a 'docking' site (see Chapter 1). When the nuclear receptor binds hormone, association of the receptor with hsp90 is lost and the remaining hsp70-receptor complex is released. Then hsp70 directs receptor dimerization. The receptor dimers will bind androgen response elements and transcription will be initiated. The other receptor-associated protein(s) can have various roles. The 59 kDa rabbit homologue of hsp56 is bound to hsp90 and not to the receptor directly (Renoir et al., 1990b), and might regulate some function of hsp90 (Callebaut et al., 1992). For simplicity, hsp56 and possible other proteins are not depicted in this model.

6.7 Future Investigations

6.7.1 Receptor-hsp complex dissociation and receptor dimerization. In this thesis, the focus is mainly on what we now think is the first step in hormone-induced receptor transformation: the dissociation of the receptor-hsp complex. It will be interesting to investigate through what mechanism this step is induced. How does the ligand provoke dissociation of the complex? Does ligand binding directly induce a change in receptor conformation which leads to the release of the hsp's, or alternatively, does it make the receptor susceptible to phosphorylation which then results in the release of the adhering proteins? Furthermore, it would enhance our knowledge greatly if we knew the precise role of the receptor-associated proteins in nuclear translocation, dimerization, and phosphorylation. The existence of hsp90-hsp70-hsp56 complexes in the absence of steroid receptors or other proteins indicates that there is a more general role for this association than modulating receptor activity. To learn about the function of this hsp-complex in receptor action, could also improve our knowledge about other cellular processes.

One of the questions which remain unsolved in this thesis is whether the 6S receptor form represents AR homodimers. Although the existence of such a dimer in solution was questioned in Paragraph 6.5.3, it neither can be excluded. Therefore, it would be of interest to examine whether the 6S receptor described here, represents a dimer that is able to bind DNA. Gel retardation experiments could be applied to study both receptor dimerization and receptor DNA interactions.

6.7.2 TAFs and transcription factors. Steroid receptor antagonists block one or more steps in receptor action. Therefore, they are useful tools to study particular steps of receptor transformation and transcription regulation. Not only large changes in receptor interactions, but also small changes in receptor conformation or phosphorylation may be studied. Subtle conformational changes of TAF regions of receptors might occur during receptor activation, and antagonists might have specific effects on these changes. These changes could possibly be detected by e.g., a changed mobility of receptor fragments during electrophoresis, changes in susceptibility to proteolytic enzymes of the receptor, or by NMR studies.

Since the functioning of different TAFs is often cell and promoter specific, it would be of interest to study the transcription factors which specifically interact with these TAFs. Cells which are incompetent to activate transcription through a certain TAF, might become competent after transfection with a cDNA encoding the interacting transcription factor(s). One method to clone such a transcription factor could therefore proceed as follows: First, cells are selected that do not mediate transcription via a hormone-independent TAF (e.g., HeLa cells, Paragraph 6.4.1). These cells are stably transfected with a GRE-tk-neomycin-resistance gene construct through selection in

neomycin containing medium. The endogenous GR is used to induce expression of the neomycin resistance gene during the selection procedure. The clones containing this construct could subsequently be stably transfected with the wild-type AR. Now, selection could be performed by adding androgens (acting through the GRE) and neomycin. In neomycin containing medium, the arising clones cannot survive in the presence of those antiandrogens that transform the receptor to the DNA-binding state but block the hormone-dependent TAF (type II antiandrogens). In Hela cells, these antiandrogens do not show partial agonistic activity mediated by the hormone-independent TAF, due to lack of the necessary transcription factors. Therefore, introduction of cDNAs, encoding these transcription factors, should enable the transfected HeLa cells described above, to be resqued by type II antiandrogens in medium containing neomycin. The cDNA library, containing the cDNA encoding this transcription factor, can be made from cells in which the hormone-independent TAF of the AR does function, e.g., CV-1 cells (Paragraph 6.4.1).

6.7.3 Practical implications. The use of the mutated AR of LNCaP cells in the studies described in this thesis, provided a useful tool to obtain more insight into the mechanisms of inhibition of androgen action by antiandrogens. The various antiandrogens showed differences in their mechanisms of action, and therefore it can be envisaged that in the treatment of androgen-dependent disorders, these compounds also show differences in effectiveness and side effects. Cell and promoter context specific regulation of gene transcription, as discussed in the previous sections, might play an important role in the mechanisms underlying the effects of antiandrogens in a complex organism. More knowledge about the relation between the structure of antagonists and their modes of action may be very helpful in the design of new steroid hormone receptor antagonists.

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Summary

Androgens are very important in the development and maintenance of male sex organs, including the prostate. In addition to this biologically functional role, androgens stimulate the growth of benign prostate hyperplasia and prostate cancer. Hormonal treatment of these diseases involves either lowering of the concentration of the active androgens at the site of action or the administration of antiandrogens. Antiandrogens act by competing with androgens for binding to the androgen receptor (AR), thereby inhibiting its function. The mechanisms by which androgens elicit AR action and by which antiandrogens block this process, are subject of this thesis.

The hormonal actions of androgens are specific, because only the AR is activated to enhance transcription of specific genes. Moreover, only androgens but not the other steroid hormones are involved in this process. The LNCaP cell line, derived from a human lymph node carcinoma of the prostate, is an exception to this rule. Both progestins and estrogens elicit AR-mediated effects, including cell growth. Moreover, instead of blocking the action of androgens, some antiandrogens also evoke the AR-controlled growth induction. It was found that the AR in LNCaP cells has an increased affinity for non-androgenic compounds, including progesterone, estradiol, and the antiandrogen cyproterone acetate (Chapter 2).

The changed steroid binding specificity is caused by a threonine to alanine mutation at position 868 in the steroid-binding domain of the AR in these cells (Chapter 3). Introduction of either the wild-type receptor or the mutant receptor, together with an androgen responsive gene construct encoding the enzyme chloramphenicol acetyltransferase (CAT) into HeLa cells, made it possible to compare the actions of the LNCaP-derived mutant receptor with those of the wild-type receptor. Transfection of the mutant receptor, but not the wild-type receptor, resulted in estrogen-, progestin-, and antiandrogen-mediated CAT induction in HeLa cells. Therefore, the aberrant responses of LNCaP cells to non-androgens could be fully explained by the mutation in the AR in these cells.

In contrast to the antiandrogens cyproterone acetate, anandron, and hydroxyflutamide, the antiandrogen casodex (ICI 176 334) did not stimulate the proliferation rate of LNCaP cells, nor did it enhance transcription from the androgen responsive CAT-gene in HeLa cells transfected with the mutant AR (Chapter 4). This suggested that the mechanism of action of casodex might be different from the mechanism(s) of action of the other antiandrogens. In steroid free medium, the AR of LNCaP cells is not tightly bound to nuclear components and after rupture of the cells, recovered in the cytosol fraction, in association with other proteins. Binding of hormone to these cytosolic receptor complexes induces dissociation of the other proteins. Therefore, LNCaP cells were incubated with either androgens or antiandrogens, and subsequently the AR-complexes were purified from the cytosol with a specific antibody, and studied by Western blotting. When cells were incubated in the absence of hormones, heat-shock proteins (hsp90, hsp70, and hsp56) were coprecipitated together with the receptor. Incubation of the cells with either the synthetic androgen R1881 or with the antiandrogen hydroxyflutamide resulted in loss of hsp's coprecipitating with the AR. Incubation of the cells with casodex, however, did not result in a loss of receptor-associated hsp's, and even blocked the effect of R1881. The results suggest that R1881

and the antiandrogen hydroxyflutamide, in agreement with their agonistic effects on the AR in LNCaP cells, induce receptor-hsp complex dissociation. Casodex blocks this process and therefore acts as an antagonist of androgen action.

In Chapter 5, the androgen-induced dissociation of the AR-hsp complex was studied in more detail. Short term incubations of LNCaP cells with R1881 resulted in a decrease in size of the complex from 8 S to 6 S on a sucrose density gradient. A concomitant loss of receptor-associated hsp's was observed on Western blots. The dissociation was slowest for hsp70. Longer incubation times of the cells with R1881 resulted in a decreased amount of receptor recovered in the cytosol fraction, and an increased amount of nuclear-bound receptor extractable with 0.5 M salt. The 6S receptor form is still larger than the monomeric (4.6S) AR. To investigate the composition of the 6S form, an AR-specific monoclonal antibody (F52), directed against the DNA-binding domain of the receptor, was used. This antibody could bind to the 6S receptor complex, but not to the 8S receptor complex. Sucrose density gradient shift experiments and immunoprecipitation studies with F52 showed that in the 6S complex, the epitope for this antibody in the DNA-binding domain was exposed. Precipitation of 6S complex with this antibody resulted in isolation of AR molecules and hsp70 molecules, but not of hsp90 and hsp56 molecules. It was not clear whether the 6S form of the receptor consisted either of hsp70-receptor complexes, of homodimers of the receptor, or of combinations with still unrecognized proteins.

In Chapter 6, the results from the former chapters are incorporated into a model. In the model, the unoccupied AR predominantly resides in the nucleus of a target cell. The receptor is associated with hsp90, hsp70, hsp56, and possibly some other proteins. Rupture of the cells results in leakage of the receptor-hsp complex out of the nucleus, which is then found in the cytosol fraction. The cytosolic form of the receptor sediments as an 8S complex in sucrose density gradients, and contains in addition to the receptor molecule, hsp90, hsp70, and hsp56. Incubation of cells with androgens results in loss of association between the receptor and hsp90 and hsp56, and in a gradual loss of association with hsp70. Consequently, the size of the receptor complexes found in the cytosol decreases to 6S. Also, more and more receptor molecules are transformed to a tightly-bound nuclear form which can only be extracted with 0.5 M salt.

Some steroid receptor antagonists (type I) prevent the binding of the receptor to hormone response elements on the DNA. In LNCaP cells, casodex does this by blocking the dissociation between the receptor molecule and the hsp's. Other type I steroid receptor antagonists do provoke a dissociation of the receptor-hsp complex but block a step of receptor transformation which succeeds complex dissociation, such as receptor dimerization and binding of the dimer to DNA. Type II antagonists do induce binding of the receptor dimer to DNA but block the interaction of the receptor with transcription factors. In LNCaP cells the mutation in the AR eliminates this blockade for some antagonists.

The use of the mutated AR of LNCaP cells provided a useful tool to obtain more insight into the mechanisms of inhibition of androgen action by antiandrogens. Obviously, further studies, with both mutant and wild-type receptors, are needed to provide the basic knowledge necessary for the development of new strategies to regulate or inhibit androgen action.

Samenvatting

Androgenen zijn steroïdhormonen, belangrijk voor ontwikkeling en behoud van functies van de mannelijke geslachtsorganen, inclusief de prostaat. Naast deze biologisch functionele rol, stimuleren androgenen ook de groei van prostaatkanker en benigne prostaat hyperplasie, een goedaardige vergroting van de prostaat die o.a. een obstructie van de urinebuis kan veroorzaken. Verder zijn er verschillende aandoeningen en ziekten die worden veroorzaakt door hoge concentraties androgenen in de circulatie. Deze aandoeningen kunnen o.a. worden behandeld door in te grijpen in de productie van androgenen. Dit kan worden bereikt door operatief de androgeenproducerende testikels te verwijderen (castratie), of door toediening van stoffen die tot gevolg hebben dat de androgeenproductie in de testikels geremd wordt (chemische castratie). Ook is het mogelijk de enzymen te remmen die de zwakke bijnierandrogenen zoals androsteendion en dehydroepiandrosteron omzetten naar het krachtige androgeen dihydrotestosteron. Naast deze behandelingsmethoden zijn er andere ontwikkeld die gebruik maken van de toediening van antiandrogenen. Antiandrogen oefenen hun werking uit doordat ze competeren met androgenen voor binding aan de androgeenreceptor die de werking van de androgenen mogelijk maakt. Het mechanisme waardoor androgenen hun receptor in de doelwitcellen aanzetten tot activiteit en de wijze waarop antiandrogenen deze werking blokkeren, zijn de onderwerpen van dit proefschrift. De achtergrond van dit onderzoek is beschreven in Hoofdstuk 1.

De hormonale werking van androgenen ontleent zijn specificiteit aan het feit dat alleen androgeenreceptoren, maar geen andere receptoren, worden geactiveerd door androgenen. Deze activatie bestaat hieruit dat de receptoren binden aan specifieke gebieden op het DNA van androgeen-gereguleerde genen (hormoon respons elementen), en daar de productie van boodschapper RNA stimuleren. Dit boodschapper RNA codeert voor eiwitten die op hun beurt bepaalde effecten teweeg kunnen brengen zoals bijvoorbeeld celgroei. Bovendien is het zo dat alleen androgenen, maar niet de andere, structureel gelijkende steroïdhormonen, de androgeenreceptor kunnen activeren. De LNCaP cellijn, ontstaan uit een lymfeklier uitzaaiing van prostaatkanker, is een uitzondering op deze regel: ook andere steroïdhormonen kunnen LNCaP cellen via de androgeenreceptor stimuleren. De steroïdhormonen progesteron en oestradiol kunnen cultures van deze cellen in het laboratorium sneller doen groeien, terwijl LNCaP cellen voor deze hormonen geen receptoren bezitten. Bovendien is de activatie van de androgeenreceptor in deze cellen gestoord, zoals blijkt uit het feit dat sommige antiandrogenen, in plaats van de werking van androgenen te onderdrukken, zelf de androgeenreceptor activeren en celgroei stimuleren. Na meting van de bindingsaffiniteit van verschillende hormonen voor de androgeenreceptor in LNCaP cellen, bleek dat deze was verhoogd voor progesteron, oestradiol en het antiandrogeen cyproteron acetaat (Hoofdstuk 2).

Deze verandering in steroïd bindingsspecificiteit wordt veroorzaakt door een verandering in het DNA dat de genetische informatie voor het androgeenreceptor-eiwit bevat (een mutatie in het androgeenreceptor-gen). Door deze mutatie wordt één van de 910 aminozuren waaruit de androgeenreceptor is opgebouwd, fout gecodeerd. Op positie 868 wordt nu het aminozuur alanine in plaats van het aminozuur threonine ingebouwd.

Deze fout zit in het gedeelte van de receptor dat androgenen moet binden. Hierdoor komt het dat nu ook andere hormonen goed binden (Hoofdstuk 3).

Het is mogelijk stukken DNA die coderen voor de androgeenreceptor te introduceren in andere cellen die geen receptor bezitten (transfectie-techniek). Deze cellen maken dan androgeenreceptormoleculen volgens het geïntroduceerde DNA. Zo kunnen ze ook worden aangezet tot het maken van de gemuteerde receptor. De normale (wild-type) receptor en de gemuteerde receptor kunnen in dit transfectie-systeem worden vergeleken. Samen met het DNA coderend voor de androgeenreceptor, kan een stuk DNA geïntroduceerd worden dat codeert voor een gemakkelijk te meten eiwit (chlooramphenicol acetyltransferase, CAT). De productie van dit zgn. reporter eiwit wordt gereguleerd door de androgeenreceptor, omdat het DNA dat voor CAT codeert wordt gekoppeld aan een hormoon respons element. Op deze wijze kan de mate van activiteit van de wild-type androgeenreceptor worden vergeleken met die van de gemuteerde receptor, door de hoeveelheid geproduceerd CAT te meten. Zo werd aangetoond dat progesteron, oestradiol en sommige antiandrogenen, de gemuteerde receptor wél, maar de wild-type receptor niet activeren. Hiermee kon verklaard worden dat deze hormonen de groei van LNCaP cellen kunnen induceren. De mutatie in de receptor is tevens een mogelijke verklaring voor het feit dat in de patient de tumor doorgroeide tijdens oestrogeen-therapie. In tegenstelling tot de antiandrogenen cyproteron acetaat, anandron en hydroxyflutamide, stimuleerde het antiandrogeen casodex (ICI 176 334) niet de groei van LNCaP cellen. Bovendien was deze laatste stof niet in staat het reporter eiwit te induceren (Hoofdstuk 4). Daarom wordt verondersteld dat casodex werkt via een mechanisme dat verschilt van die van de andere antiandrogenen.

Wanneer LNCaP cellen worden gekweekt in steroïd-vrij medium is de androgeenreceptor niet stevig gebonden aan kerncomponenten; na het breken van de cellen, wordt de receptor gevonden in het zgn. cytosol. In het cytosol is de receptor geassocieerd met andere eiwitten (receptor-complex). Binding van hormoon aan de receptor resulteert in een dissociatie van het receptorcomplex. De vraag was of dit ook gebeurt na binding van antiandrogenen. Hiertoe werden LNCaP cellen met androgenen of antiandrogenen geïncubeerd, en vervolgens werd de androgeenreceptor geïsoleerd met antilichamen die zeer specifiek aan de androgeenreceptor binden. Tijdens deze procedure worden eiwitten die geassocieerd zijn met de receptor meegeïsoleerd. Met behulp van eiwitelectroforese en andere antilichamen werden deze eiwitten geïdentificeerd. Wanneer LNCaP cellen niet met androgenen of antiandrogenen werden geïncubeerd, werden tegelijk met de androgeenreceptor drie verschillende zgn. heat-shock eiwitten (hsp90, hsp70 en hsp56) uit de cytosolfractie van de cellen geïsoleerd. Zowel incubatie van de cellen met het synthetisch androgeen R1881 als met het antiandrogeen hydroxyflutamide, resulteerde in een afname van de hoeveelheid heat-shock eiwitten die meegeïsoleerd werden met de receptor. Casodex had dit effect niet en kon zelfs het effect van R1881 opheffen. Deze resultaten wijzen erop dat R1881 en hydroxyflutamide, in overeenstemming met hun receptor-activerende eigenschappen, een dissociatie bewerkstelligen van de androgeenreceptor en de geassocieerde heat-shock eiwitten. Casodex blokkeert dit proces en heeft daarom een antagonistische werking op de androgeenreceptor.

In Hoofdstuk 5 wordt beschreven hoe de androgeen-geïnduceerde dissociatie van het androgeenreceptor—heat-shock eiwit complex meer gedetailleerd werd onderzocht. Incubaties van korte duur, van LNCaP cellen met R1881 resulteerden in een geleidelijke afname in complexgrootte van 8S naar 6S op een sucrose dichtheidsgradiënt (de S-

waarde is groter naarmate het complex groter is). Bestudering van het 6S receptor-complex leerde dat er ook een verlies van heat-shock eiwit associatie met de receptor had plaatsgevonden. Hsp90 en hsp56 bleken het snelst hun associatie met de receptor te verliezen, hsp70 iets langzamer. Na langere incubaties van de cellen met R1881 werd steeds minder receptoreiwit in het cytosol gevonden. Dit kwam doordat de receptor na dissociatie van de heat-shock eiwitten een hoge affiniteit kreeg voor de celkern: na het breken van de cellen kwam de receptor dus niet meer in het cytosol terecht. De kern-gebonden receptoren konden alleen met een oplossing met hoge zoutconcentratie uit de kernen worden geëxtraheerd.

De 6S receptor is groter dan de monomere, ongebonden (4.6S), receptor. Om de gedeeltelijk gedissocieerde, 6S receptor-complexen uit het cytosol te kunnen bestuderen werd gebruik gemaakt van een speciaal monoclonaal antilichaam. Dit antilichaam (F52) bindt aan een gedeelte van de androgeenreceptor dat niet geëxposeerd is in de 8S receptor- maar wel in de 6S receptor-complexen. Hiermee konden specifiek de 6S receptor-complexen geïsoleerd worden. Isolatie m.b.v. F52 van receptorcomplexen uit LNCaP cellen die geïncubeerd waren met hormonen, resulteerde in isolatie van de androgeenreceptor en hsp70, maar niet van hsp90 en hsp56. Dit duidt er op dat de 6S receptorcomplexen die specifiek met F52 geïsoleerd worden bestaan uit receptormoleculen, gebonden aan hsp70. Het 6S complex zou inderdaad kunnen bestaan uit een receptor-hsp70 hetero-dimeer. Het is echter ook mogelijk dat het bestaat uit twee receptormoleculen (homo-dimeer). Een laatste mogelijkheid is dat de 6S receptor bestaat uit receptor-complexen met eiwitten die niet gedetecteerd konden worden met de hier gebruikte antilichamen.

In Hoofdstuk 6 worden de resultaten van de vorige hoofdstukken verwerkt in een model dat het werkingsmechanisme van de androgeenreceptor beschrijft na binding van androgenen of antiandrogenen. Volgens dit model bevindt de onbezette androgeenreceptor zich vooral in de kern van de doelwitcel. Daar is de receptor geassocieerd met hsp90, hsp70 en hsp56, en mogelijk nog andere eiwitten. Wanneer de cel wordt gebroken, lekt het receptor-complex uit de kern en komt zodoende in het cytosol terecht. Deze cytosolische receptor heeft een sedimentatiewaarde van 8 S op een sucrose dichtheidsgradiënt en is nog steeds gebonden aan de drie typen heat-shock eiwitten. Wanneer de cellen, voordat ze gebroken worden, worden geïncubeerd met androgenen, dissocieert het 8S complex. De associatie met de heat-shock eiwitten gaat verloren (eerst hsp90 en hsp56, dan hsp70). Als gevolg hiervan, wordt de receptor zo stevig aan het DNA in de kern gebonden, dat deze niet meer uit de kern weglekt wanneer de cel gebroken wordt.

Sommige antiandrogenen (type I) blokkeren de binding van de receptor aan de hormoon respons elementen op het DNA. In LNCaP cellen veroorzaakt casodex dit blijkbaar door de dissociatie van de receptor en de heat-shock eiwitten te verhinderen. Andere antiandrogenen induceren wel deze dissociatie maar blokkeren bij de wild-type receptor één van de stappen die daarop volgen; receptor dimerisatie, binding van de receptor dimeer aan DNA (hormoon respons elementen) of de interactie van de receptor met transcriptiefactoren. Deze laatste interactie regelt de gentranscriptie (synthese van boodschapper RNA) waardoor androgeeneffecten in een cel kunnen worden bewerkstelligd. De mutatie in de androgeenreceptor van LNCaP cellen is er de oorzaak van dat sommige antiandrogenen niet meer in staat zijn de werking van de receptor te blokkeren, maar daarentegen de gemuteerde receptor activeren.

Het gebruik van de gemuteerde androgeenreceptor in LNCaP cellen is een goed bruikbaaar gereedschap om meer inzicht te krijgen in de mechanismen van remming van

androgeen effecten door antiandrogenen. Het is duidelijk dat verder onderzoek, zowel met normale als gemuteerde receptoren, nodig is om deze kennis verder te vergroten. Deze kennis zal dan kunnen bijdragen aan de ontwikkeling van nieuwe strategieën om androgeen effecten te reguleren of te onderdrukken.

List of Publications

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Curriculum Vitae

Jos Veldscholte werd geboren op 18 oktober 1963 te Weerselo. Na het VWO op het "Lyceum de Grundel" te Hengelo (ov), begon hij in 1982 met de studie Biologie aan de R.U. te Utrecht. Tijdens deze studie deed hij onderzoek aan de ontwikkeling van de zeeslak *Patella vulgata*, bij de vakgroep Experimentele Dierkunde (Prof. dr. J.A. Van den Biggelaar). Verder werd door hem onderzoek verricht aan de opname van ijzer en transferrine door Sertolicellen van de rat en germinale cellen van de muis bij de vakgroep Celbiologie van de Geneeskunde faculteit (Prof. dr. J.A. Van der Donk). Het Doctoraalexamen werd afgelegd op 29 februari 1988. Van 1 maart 1988 tot 1 maart 1992 was hij werkzaam als assistent in opleiding op de afdeling Biochemie II, welke later opging in de vakgroep Endocrinologie & Voortplanting van de Erasmus Universiteit Rotterdam. Hier werd het onderzoek, zoals beschreven in dit proefschrift verricht onder begeleiding van Dr. E. Mulder. Thans is hij werkzaam bij voorgenoemde vakgroep als tijdelijk wetenschappelijk onderzoeker op een project van de Nederlandse Kankerbestrijding (NKB), betreffende onderzoek aan cellulaire en moleculaire veranderingen welke samengaan met verlies van androgeen-afhankelijke groei van LNCaP cellen.