ANDROGEN RECEPTOR PHOSPHORYLATION

ANDROGEENRECEPTOR FOSFORYLERING

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CONTENTS

ABBREVIATIONS

Ala	alanine
AR	androgen receptor
ARE	androgen responsive element
ATP	adenosine 5'-triphosphate
BSA	bovine serum albumin
cAMP	adenosine 3':5'-cyclic-monophosphate
(c)DNA	(complementary) deoxyribonucleic acid
CHO	chinese hamster ovary cell line
COS	monkey kidney cell line
cpm	counts per minute
CV-1	monkey kidney cell line
DHT	5α-dihydrotestosterone (17β-hydroxy-5α-androstan-3-one)
DOC	sodium deoxycholate
dpm	disintegrations per minute
DTT	dithiothreitol
EDTA	ethylenediaminetetra acetic acid
GRE	glucocorticoid responsive element
HeLa	human cervix carcinoma cell line
HPLC	high performance liquid chromatography
HRE	hormone responsive element
hsp70	70 kDa heat-shock protein
hsp90	90 kDa heat-shock protein
kDa	kilo Dalton
LNCaP	lymph node carcinoma of the prostate
MEM	minimum essential medium
mibolerone	7α-17α-dimethyl-19-nortestosterone
MMTV-LTR	mouse mammary tumor virus long terminal repeat
(m)RNA	(messenger) ribonucleic acid
NMR	nuclear magnetic resonance
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulfonyl fluoride
R1881	17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one
RPMI	Roswell Park Memorial Institute
S	Svedberg unit
SDS	sodium dodecylsulphate
Ser	serine
T	testosterone (17β-hydroxy-4-androsten-3-one)
T47D	mammary tumor cell line
TCA	trichloroacetic acid
Thr	threonine
TFA	trifluoroacetic acid
TLC	thin layer chromatography
TLCK	N-tosyl-L-lysine-chloromethyl ketone
TPCK	N-tosyl-L-phenylalanine chloromethyl ketone

SCOPE OF THIS THESIS

Steroid hormone receptors are *trans*-acting gene regulatory proteins, involved in the accomplishment of steroid hormone-induced cellular responses. Upon binding of hormone, the receptor-hormone complex undergoes a conformational change, which is thought to precede binding of the complex to hormone responsive elements in the target cell genome. This results in the up- or down-regulation of the expression of target genes. Hence steroid hormone receptors can be viewed as ligand-activated transcription factors. The activities of transcription factors are often regulated through protein phosphorylation. Evidence has been provided that progesterone, glucocorticoid, estrogen, 1,25-dihydroxyvitamin D_3, and androgen receptors also exist as phosphoproteins in the cell.

The aim of the work presented in this thesis was to gain more insight in the possible functional role of androgen receptor phosphorylation.

To achieve this goal, studies were carried out to determine in detail the effect of ligand binding on androgen receptor phosphorylation, and attempts were made to identify individual receptor phosphorylation sites.

After an introduction on the role of the androgen receptor in the mechanism of action of androgens, and a detailed overview of steroid receptor phosphorylation (Chapters 1 and 2), investigations are described on androgen receptor protein heterogeneity, on the location of phosphorylation sites in the androgen receptor, and on the effect of ligand on androgen receptor phosphorylation (Chapters 3 to 6).

Finally, various possible functional roles of androgen receptor phosphorylation are considered (Chapter 7).

SUMMARY

Many physiological processes in organisms are regulated by a relatively small number of steroid hormones. Androgens are the so-called male sex steroid hormones which control growth, differentiation and functions of male reproductive and accessory sex tissues. Androgens are mainly produced in the testis and circulate in the blood. They diffuse in and out of all cells, but are retained with high affinity and specificity in target cells by an intranuclear binding protein, termed the androgen receptor. Once bound by androgens, the androgen receptor undergoes a conformational change allowing the receptor to bind with high affinity to DNA and to modulate transcription of certain genes. The androgen receptor appears to be a transcription factor, regulated by androgenic steroids. Phosphorylation is the predominant cellular mechanism for reversible modification of proteins, and the fact that many transcription factors are phosphoproteins suggests a regulatory role of phosphorylation. In this thesis, studies on phosphorylation of the androgen receptor in human prostate tumor cells (LNCaP) are described. In LNCaP cells, the androgen receptor protein is present as two isoforms with apparent molecular masses of 110 and 112 kDa during SDS-PAGE. The 112 kDa isoform reflects the phosphorylated receptor, whereas the 110 kDa isoform is the non-phosphorylated receptor. Both isoforms are able to bind androgens with high affinity and can subsequently be transformed to the DNA binding form. It appears to be unlikely that phosphorylation is involved in the regulation of steroid- or DNA binding affinity. Upon incubation of the prostate tumor cells with androgens, the phosphorylation degree of the androgen receptor was rapidly increased. Multiple phosphorylation sites on serine residues are located in the N-terminal *trans*-activation domain and not in the DNA- and ligand binding domains. Tryptic phosphopeptide maps of the androgen receptor show induction of

phosphorylation at a novel site(s) by hormone treatment. It is proposed that this extra phosphorylation in the N-terminal domain causes a conformational change, enabling protein-protein contacts of the *trans*-activation domain with other transcription factors or co-activators on a target gene promoter.

SAMENVATTING

De steroidhormonen testosteron en 5α-dihydrotestosteron (androgenen) zijn betrokken bij de ontwikkeling en het behoud van de mannelijke geslachts-kenmerken, inclusief de vorming van zaadcellen. Androgenen oefenen hun werking uit door binding aan een eiwit, de androgeenreceptor, dat aanwezig is in de kern van androgeen doelwitcellen. Binding van androgenen aan de receptor geeft aanleiding tot een aantal structurele veranderingen in het receptor eiwit, waardoor dit in staat is een interactie aan te gaan met bepaalde DNA sequenties in het promotor gebied van androgeen doelwitgenen. Dit resulteert uiteindelijk in stimulatie of remming van de transcriptie van gereguleerde genen. De androgeenreceptor kan dan ook opgevat worden als een hormoon-gereguleerde transcriptiefactor.

Fosforylering is een van de belangrijkste modificaties die eiwitten in de cel kunnen ondergaan, en het feit dat vele transcriptiefactoren fosfo-eiwitten zijn doet vermoeden dat fosforylering betrokken is bij de regulatie van de funktie van deze eiwitten. Ook de androgeenreceptor is een fosfo-eiwit. In dit proefschrift is de fosforylering van de androgeenreceptor in prostaat tumorcellen onderzocht. De androgeenreceptor is in deze cellen aanwezig als twee isovormen met molecuulmassa's van 110 en 112 kDa, zoals blijkt na SDS-PAGE. De 112 kDa isovorm ontstaat uit de 110 kDa isovorm door fosforylering. Beide isovormen zijn in staat om specifiek androgenen te binden, en ondergaan in de cel vervolgens een transformatie tot de DNA bindende vorm. Incubatie van prostaat tumorcellen met androgenen heeft een snelle toename van de fosforyleringsgraad van de androgeenreceptor tot gevolg. Bij nadere analyse blijkt dat de androgeen- receptor uitsluitend fosforylering ondergaat op serine residuen, en wel in het aminoterminale *trans*-activatie domein. Tevens blijkt dat hormoonbinding de fosforylering op één of meer nog niet gefosforyleerde serine

residue(n) tot gevolg heeft, terwijl de fosforyleringsgraad van andere plaatsen niet of nauwelijks verandert. Het is zeer goed mogelijk dat de hormoon-gestimuleerde fosforylering de *trans*-activatie capaciteit van de androgeenreceptor (mede)-bepaalt.

CHAPTER 1

THE ANDROGEN RECEPTOR

1.1 Introduction

Many physiological processes in organisms are regulated by a relatively small number of steroid hormones, all synthesized from cholesterol. Being small hydrophobic molecules, they are thought to be able to cross the plasma membrane of the cell by simple diffusion. In the cell, steroid hormones bind to specific intracellular receptor proteins, converting these proteins to functional transcription factors, which are then able to regulate in the nucleus the expression of specific genes (Jensen and DeSombre, 1973). The steroid hormones include gonadal hormones (estrogens, progestins and androgens) and adrenal cortical hormones (glucocorticoids and mineralocorticoids). This chapter is devoted to the mechanism of action of androgens and to the androgen receptor. Androgens (testosterone and 5α-dihydrotestosterone) control the growth, differentiation and function of reproductive and accessory sex tissues in the male, such as the prostate, epididymis, seminal vesicle, vas deferens, and penis. They also influence many other tissues such as skin, bone marrow, hair follicles, pituitary gland, hypothalamus, and behavioural centers in the brain. Testosterone (T) is, in combination with follicle-stimulating hormone (FSH), required for the initiation and maintenance of normal spermatogenesis (Marshall and Nieschlag, 1987; Roberts and Zirkin, 1991). Most prostate tumors are initially androgen dependent, and tumor growth can be arrested by removal of circulating androgens. However, at a later stage these prostate tumors usually become androgen independent and start growing again (Menon and Walsh, 1979).

1.2 Demonstration of the androgen receptor

Early attempts to demonstrate selective uptake and retention of androgens *in*

vivo, using ^{14}C-labeled steroids with low specific activity, did reveal some accumulation of androgens in target organs of the rat, such as the prostate and seminal vesicles, in comparison with other tissues (Harding and Samuels, 1962). Only when T labeled with tritium of high specific activity became available, a significant affinity of androgens for their target tissues could be demonstrated *in vivo*. In the period 1967-1969, the selective accumulation of ^{3}H-testosterone or metabolites thereof in the prostate and seminal vesicles of the guinea pig and rat, was described (Resko et al., 1967; Anderson and Liao, 1968; Bruchovsky and Wilson, 1968; Mainwaring, 1969). The ^{3}H-labeled steroid was found mainly in the nucleus, as was shown by subcellular fractionation and by dry-mount autoradiography (Anderson and Liao, 1968; Sar et al., 1970). An important advance in the understanding of the interaction of androgenic steroids with target tissues came with the discovery that the radioactivity which bound specifically in the prostate and in the seminal vesicles was not T but DHT (Anderson and Liao, 1968; Bruchovsky and Wilson, 1968). Reduction of T to DHT takes place in many target tissues but also in non-target tissues. However, some androgen responsive tissues in the human, such as muscle and the wolffian duct, show little capacity to reduce T to DHT, and in these organs T itself appears to interact directly with the receptor (Bardin and Catterall, 1981). The discovery that several sexual behavioural responses in the rat are stimulated by T but not by DHT, suggested that T acts directly on the androgen receptor in the brain. However, this situation is complicated by the fact that T, unlike DHT, is converted in the brain to estrogen which may produce the response (Sheridan, 1991, and references therein). DHT is responsible for virilization of the urogenital sinus and genital folds during embryogenesis, and for the development of most male secondary sex characteristics during puberty (Griffin and Wilson, 1992). DHT binds more tightly to the androgen receptor than T, and formation of DHT might therefore serve as an amplifying mechanism for

androgen action (Griffin and Wilson, 1992).

The DHT bound in nuclei of rat prostate exposed to T either *in vivo* or during tissue incubations, could be extracted with salt containing buffers as part of a complex sedimenting at 3S in sucrose density gradients (Liao and Fang, 1969). Cytosolic fractions of prostate contained a complex of 8-9S which dissociated to 3.5S by salt treatment (Liao and Fang, 1969, and references therein). These studies provided the first evidence for the existence of androgen receptor protein. The uptake of androgenic steroids into nuclei of minced prostatic tissue was found to be strongly temperature dependent, with essentially no uptake taking place at 0-2°C (Liao and Fang, 1969). Isolated nuclei from rat prostate or epididymis incubated at 37°C did not bind androgenic steroids. However, after the addition of cytosol to the incubation mixture the formation of an extractable 3S complex containing DHT was reported (Liao and Fang, 1969, and references therein). Further studies showed that cytosols from a variety of androgen target tissues promote the binding of ^{3}H-DHT to isolated chromatin (Mainwaring and Peterken, 1971). From these and many other studies it was concluded that androgen receptor complexes bound in target cell nuclei to chromatin, are derived from an initial complex formed in the cytoplasm, and that hormone binding is essential for the conversion of the receptor to a form that has high affinity for chromatin. This process, which was also described for the other steroid hormone receptors, was termed transformation (Gorski et al. 1968).

1.3 Characterization and purification of the androgen receptor

Upon homogenization in hypotonic buffers, the cytoplasmic androgen receptor from rat prostate has sedimentation coefficients between 7S and 12S (Liao et al., 1975). These large forms can be converted to the slower sedimenting 3-5S forms by incubation at 20-30°C or by raising the salt concentration to 0.4 M KCl

(Mulder et al., 1983; Tindall et al., 1984). Also from human prostate tissue, human genital skin fibroblasts, human hyperplastic prostate tissue, sheep seminal vesicle, and calf uterine tissue, androgen receptors with similar properties could be isolated (Foekens et al., 1981; Kovacs et al., 1983; Wilbert et al., 1983; Murthy et al., 1984; de Boer et al., 1986A). Steroid binding was specific for androgens, and the affinity decreased in the sequence R1881 (methyltrienolone, a synthetic steroid) > DHT > T >> progesterone > estradiol > cortisol (Bonne and Raynaud, 1975; Murthy et al., 1984). Androgen receptors have a high affinity for DHT and T (Kd ~0.5 nM), and a finite binding capacity. A protein fraction designated "8S androgen receptor promoting factor", that promoted the conversion of the 3-5S androgen receptor to the 8S androgen receptor form, was purified and found to inhibit the binding of the 3-5S androgen receptor to isolated nuclei and DNA cellulose in a concentration dependent manner (Colvard and Wilson, 1981; Colvard and Wilson, 1984). This study and others supported the hypothesis that the 8S androgen receptor is a complex of the 3-5S androgen receptor monomer with a non-steroid binding protein that renders the receptor incapable of binding to nuclei and DNA. Molybdate was found to stabilize the 8-10S androgen receptor forms *in vitro* (Rowley et al., 1984; de Boer et al., 1986B). The transition of the molybdate-stabilized 8-10S non-transformed receptor form into the transformed receptor (DNA binding form) is accompanied by a decrease in sedimentation value to 4S (Kovacs et al., 1983; de Boer et al., 1986A; Wilson et al., 1986; Colvard and Wilson, 1987). Transformation can be accomplished *in vitro* by treatment with salt, ammonium sulphate precipitation of cytosols, or incubation of cytosols at 20-30°C, and is associated with a strong increase in DNA cellulose binding capacity (Kovacs et al., 1983; de Boer et al., 1986B; Wilson et al., 1986). In-cell free binding experiments, transformed androgen receptors of rat ventral prostate cytosol bound with high affinity (Kd ~ 0.1 nM) to a saturable number of binding sites associated with the nuclear

matrix of the prostate. This binding was relatively high in prostate, but liver nuclear matrix, in contrast, contained only 15% of the sites found in the prostate nuclear matrix (Barrack, 1983; Buttyan et al., 1983; Colvard and Wilson, 1984).

Purification to homogeneity of the androgen receptor appeared to be a very difficult task, largely due to the relatively low expression level of androgen receptor protein in target tissues. In addition, the androgen receptor appeared to be very sensitive to proteolytic degradation. Two approaches have been used for the purification of the androgen receptor: differential chromatography with polyanion resins, and steroid affinity chromatography. In the first approach selective adsorption of the transformed androgen receptor by polyanions as DNA cellulose, phosphocellulose, heparin agarose, and ADP-sepharose is used after elimination of contaminating proteins, using the low affinity of non-transformed receptor for polyanions. Using chromatography on polyanion matrices a considerable purification of the androgen receptor from sheep seminal vesicle, calf uterus and rat prostate could be obtained (Mulder et al., 1979; Foekens et al., 1982; Mulder et al., 1985; van Loon et al., 1988). A complete purification of androgen receptor of rat ventral prostate was obtained by the introduction of a steroid affinity chromatography step after the polyanion chromatography steps (Chang et al., 1983; Davies and Thomas, 1984). The androgen receptor was purified approximately 120,000 fold and silver nitrate staining of a SDS-PAGE gel revealed a major polypeptide band migrating at 86,000 dalton (Chang et al. 1983).

Affinity labeling of the androgen receptor was used during various purification protocols to determine the molecular mass of the androgen receptor under denaturing (SDS-PAGE) conditions. Affinity labeling of steroid binding proteins can be performed either through electrophilic steroid affinity labels or through

photoactivation of highly conjugated synthetic steroid ligands (Katzenellenbogen et al., 1984; Gronemeyer and Govindan, 1986). Affinity labeling with the low affinity ligand [^{3}H]-dihydrotestosterone 17β-bromoacetate revealed a molecular mass of 86,000 dalton under denaturing conditions for the androgen receptor of rat prostate and of 118,000 dalton for the receptor in the R3327 Dunning prostate tumor (Chang et al., 1984; Rowley et al., 1984). Photoaffinity labeling of the androgen receptor with [^{3}H]-methyltrienolone (R1881) from calf uterine tissue, human genital skin fibroblasts and prostatic tumor cells (LNCaP cells), revealed molecular masses of 85,000-110,000 dalton under denaturing conditions (Brinkmann et al., 1985; Gyorki et al., 1986; Stamatiadis et al., 1987; Brinkmann et al., 1988; Gyorki et al., 1988). Limited proteolysis of androgen receptors by various proteases *in vitro*, and characterization of the resulting receptor fragments with regard to ligand- and DNA binding properties, revealed three different receptor domains, which are involved in steroid binding, DNA binding, and modulation of DNA binding (de Boer et al., 1987).

Because of the great difficulties in purifying the androgen receptor in sufficient amounts to serve as an immunogen, antibodies became only available with delay, in comparison with other steroid receptors. A surprise was the discovery of autoantibodies in the blood of patients with prostatic disease (Liao and Witte, 1985). The antibodies from serum samples were associated with a purified IgG fraction, and interacted with the 3.8S cytosolic androgen receptor complexes of rat ventral prostate to form 9 to 12S units. The antibodies interacted with the nuclear and cytosolic androgen receptor, either the DNA binding or the non-DNA binding form, but not with receptors for estrogen, progesterone or cortisol from several sources. By immortalizing blood lymphocytes of similar patients, cell lines were developed that produced monoclonal antibodies that reacted with human and rat androgen receptors. These antibodies identified the human

androgen receptor as an 118 kDa protein on Western blots (Young et al., 1988).

1.4 Structure of the androgen receptor

With the availability of purified progesterone-, estrogen-, and glucocorticoid receptors, and of specific antibodies that reacted with these proteins, it became possible to clone and express the cDNAs encoding these receptors. The amino acid sequence of these receptors was deduced from the nucleotide sequences of the respective cDNA clones. Expression cloning was carried out for the glucocorticoid receptor from human (Hollenberg et al., 1985; Govindan et al., 1985), rat (Miesfeld et al., 1986) and mouse (Danielsen et al., 1986), and for the human estrogen receptor (Green et al., 1986). In the same way the cDNAs for the progesterone receptor from rabbit (Loosfelt et al., 1986), chicken (Conneely et al., 1986; Gronemeyer et al., 1987) and human (Misrahi et al., 1987) were cloned. On the basis of the highly homologous sequences encoding the DNA binding domain of related receptors, it was assumed that the androgen receptor DNA binding domain would fall in the group that also contains the glucocorticoid, estrogen, and progesterone receptor. Using oligonucleotides corresponding to the most conserved region of the DNA binding domain, several investigators independently cloned the androgen receptor. First the human androgen receptor was cloned (Chang et al., 1988A; Chang et al., 1988B; Lubahn et al., 1988A; Lubahn et al., 1988B; Trapman et al., 1988; Faber et al., 1989; Tilley et al., 1989) and the rat androgen receptor (Chang et al., 1988B; Tan et al., 1988). Later the mouse androgen receptor was cloned (Gaspar et al., 1990; He et al., 1990; Faber et al., 1991A).

The open reading frame of the human androgen receptor cDNA encodes a

protein of 910-919 amino acid residues, with a calculated molecular mass of 99 kDa (Chang et al., 1988B; Lubahn et al 1988B; Brinkmann et al., 1989; Tilley et al., 1989). The N-terminal domain of the androgen receptor contains a variety of homopolymeric amino acid stretches, most notably glutamine and glycine stretches. For the human androgen receptor there is an intraspecies variation in the length of these stretches, resulting in variation in the length of the androgen receptor protein (910-919 amino acid residues) (Sleddens et al., 1992). The human androgen receptor gene has been localized on the X chromosome (Lubahn et al., 1988A; Trapman et al., 1988; Brown et al., 1989). The coding part of the gene is distributed over 8 exons. Based on the homology with other steroid receptors, it was concluded that the first exon encodes the whole N-terminal domain, exons 2 and 3 the DNA binding domain, while the information for the ligand binding domain is split over exons 4 to 8 (Kuiper et al., 1989; Lubahn et al., 1989). The promoters of the human (Tilley et al., 1990; Faber et al., 1991B; Mizokami et al., 1994), rat (Baarends et al., 1990) and mouse (Faber et al., 1991A) androgen receptors were characterized, and were found to be located approximately 1 kb upstream from the translation initiation codon in exon 1. There has only one androgen receptor gene been found, despite the fact that there are two ligands.

Using cDNA constructs encoding deletion mutants and their transient expression in COS cells, it was possible to identify individual domains in the androgen receptor molecule (Figure 1.1). Similar to all members of the steroid receptor superfamily, the human androgen receptor consists of a central DNA binding domain, a C-terminal ligand binding domain, and an N-terminal domain which is important for transcriptional activity (Jenster et al., 1991; Simental et al., 1991).

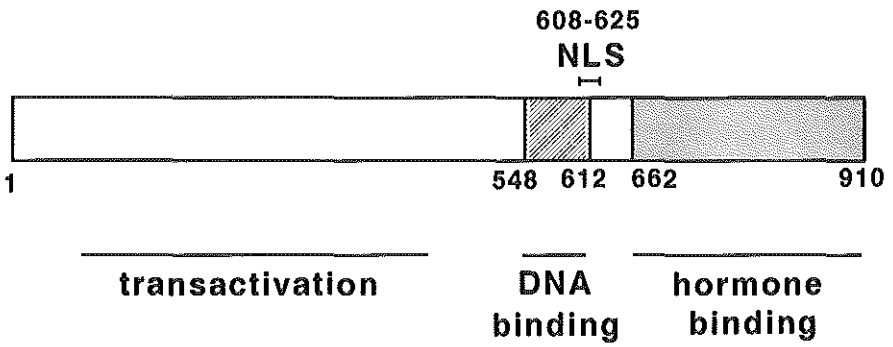

Figure 1.1. Structure of the human androgen receptor.
Indicated are the N-terminal *trans*-activation domain, the DNA binding domain, the nuclear localization signal (NLS), and the hormone binding domain. The DNA binding domain (amino acid residues 548 to 612) contains two zinc fingers and is 100% homologous to the DNA binding domain of the rat and mouse androgen receptors. The same accounts for the hormone binding domain (amino acid residues 662 to 910).

Apart from the glutamine and glycine amino acid repeats, another important characteristic of the androgen receptor N-terminal domain is the relatively high content of acidic amino acid residues (Faber et al., 1989; Faber et al., 1991A). Acidic regions in transcription factors are directly involved in activation of transcription (Ptashne, 1988; Mitchell and Tjian, 1989; Latchman, 1990). The N-terminal domain of the androgen receptor was indeed found to be essential for transcriptional activation by the androgen receptor, during transient expression experiments in COS and HeLa cells, with cotransfection of an androgen regulated reporter gene (Rundlett et al., 1990; Jenster et al., 1991; Simental et al., 1991; Palvimo et al., 1993). Deletion of a large part of this

domain (Δ51-211) inactivated the androgen receptor *trans*-activation capacity (Jenster et al., 1991). The importance of the N-terminal domain of the androgen receptor deduced from these transfection experiments, was confirmed *in vivo* in an individual with complete androgen insensitivity, who showed a large truncation of the N-terminal domain but no structural changes in the DNA- and hormone binding domains (Zoppi et al., 1993).

The ligand binding domain of the androgen receptor consists of approximately 250 amino acid residues, and binds the ligand in a hydrophobic pocket (Jenster et al., 1991). The integrity of the complete ligand binding domain is important for receptor activity. Deletions in the steroid binding domain as well as truncation of the last 12 C-terminal amino acid residues abolished hormone binding (Jenster et al., 1991). Point mutations in the androgen receptor ligand binding domain are a frequent cause of X-linked androgen insensitivity (Brinkmann and Trapman, 1992; Ris-Stalpers et al., 1994).

One of the motifs which have evolved for protein-DNA interactions are autonomously folding zinc binding domains, which require zinc for folding and for DNA binding activity (Klug and Rhodes, 1987). A certain type of so-called zinc fingers has been described in the steroid nuclear receptor family. This motif binds two zinc atoms to form a single folded domain of 66 amino acids, with four cysteine residues acting as ligands for each zinc atom (Schwabe and Rhodes, 1991). The crystal structures of the glucocorticoid- and estrogen receptors zinc fingers bound to their specific DNA target sequences have been determined (Luisi et al., 1991; Schwabe et al., 1993). The structures are very similar to those of the estrogen receptor and glucocorticoid receptor zinc finger domains as determined by NMR-spectroscopy, and show that the receptor binds to DNA as a dimer (Schwabe et al., 1990; Härd et al., 1990). Sequence specific DNA interactions occur through an α-helix, which lies in the major groove of the DNA. The most important amino acid residues in this regard are identical

for the glucocorticoid and androgen receptors, and the androgen receptor therefore can be expected to interact in a similar way with target DNA (Härd and Gustafsson 1993, and references therein).

The androgen receptor undergoes homodimer formation in association with DNA binding (Wong et al., 1993; De Vos et al., 1994; Kallio et al., 1994). The dimerization domain is located within the DNA binding domain and the hinge region of the receptor (Wong et al., 1993). Mutational analysis of the mouse estrogen receptor ligand binding domain has identified a subregion near the C-terminal end, involved in receptor dimerization (Fawell et al., 1990). Although this region is conserved in other receptors, the general importance of this region in steroid receptor dimerization remains to be confirmed for other receptors.

The cloning of the human androgen receptor cDNA, and the elucidation of the androgen receptor primary structure, have provided information to generate androgen receptor specific antibodies, using synthetic peptides as antigens in rabbits or mice (van Laar et al., 1989; Husmann et al., 1990; Shan et al., 1990; Zegers et al., 1991). In this way polyclonal and monoclonal antibodies were obtained against epitopes in the N-terminal domain, the DNA binding domain, and the ligand binding domain of the androgen receptor (van Laar et al., 1989; Zegers et al., 1991; Veldscholte et al., 1992B). The androgen receptor antibodies appeared to be a powerful research tool. For example, they were used to investigate nuclear import of the androgen receptor after transient expression of deletion and substitution mutants in various cell lines (Jenster et al., 1991; Simental et al., 1991; Kemppainen et al., 1992; Jenster et al., 1993; Zhou et al., 1994B). Expression of the wild type androgen receptor in different cell lines revealed a cell line-specific subcellular distribution of the unliganded receptor between the nuclear and cytoplasmic compartments (Jenster et al., 1993). However, upon addition of hormone the androgen receptor was located

exclusively in the nucleus, independent of the cell line used (Jenster et al., 1993). Mutagenesis studies revealed a bipartite nuclear targeting signal at amino acid residues 608-625 of the human androgen receptor (Figure 1.1), consisting of two clusters of basic amino acid residues (Jenster et al., 1993; Zhou et al., 1994B). Such a nuclear localization signal is present in many other nuclear proteins, including all steroid receptors (Dingwall and Laskey, 1991; Robbins et al., 1991). The human androgen receptor was also visualized immunohistochemically, using a mouse monoclonal antibody directed against a fragment of the N-terminal domain, in various human tissues and in prostate cancer tissue (Ruizeveld de Winter et al., 1991; van der Kwast et al., 1991). In all tissues investigated, nuclear localization of the androgen receptor was predominant if not exclusive, irrespective of the presence or absence of hormone.

1.5 Interaction of the androgen receptor with target genes

When it was recognized that hormone-induced transformation of steroid receptors caused them to interact with chromatin, many attempts were made to identify the acceptor sites to which the transformed receptors become attached (Jensen, 1979; Spelsberg et al., 1987; Rejman et al., 1991).

With regard to the chemical composition of the androgen receptor acceptor sites, non-histone proteins and DNA have been implicated as the major components (Mulder and Brinkmann, 1985, and references therein). On the basis of the relative binding affinities for androgen receptors, evidence was obtained for at least two classes of acceptor sites in rat ventral prostate chromatin (Davies and Thomas, 1984; Davies et al., 1985). One class has a high affinity and low capacity for the androgen receptor and is functionally associated with transcriptionally active chromatin, whereas the other class has a lower affinity but higher capacity for the androgen receptor (Davies et al., 1985). Studies

involving covalent cross-linking with formaldehyde of rat prostate androgen receptors bound to chromatin acceptor sites *in situ*, have supported the existence of two distinct classes of acceptor sites (Foekens et al., 1985; Rennie et al., 1987). These cross-linking experiments showed that only about 20% of the androgen receptor molecules are in a configuration allowing direct contact with acceptor DNA, whereas the remaining 80% is associated with acceptor proteins (Foekens et al., 1985). However, it is not certain whether these two fractions are indicative of acceptor sites associated with transcriptionally active and inactive regions of prostatic chromatin, respectively.

Studies with reconstituted systems using genomic DNA fragments have indicated preferential binding of purified androgen receptors to specific regions and nucleotide sequences of genes regulated by androgens. One such an androgen-regulated gene encodes prostatein, also known as prostatic binding protein (PBP) or prostatic secretion protein, which is the major secretory protein of rat ventral prostate (Forsgren et al., 1979; Peeters et al., 1982; Viskochil et al., 1983). This protein consists of three different subunits: C1, C2 and C3, arranged as two heterodimers (C1C3 and C2C3). The C3 subunit of rat prostatein can be encoded by two genes, but only one of these genes appears to be transcribed (Parker et al., 1983). It has been demonstrated that C3 mRNA is regulated acutely by androgens, and regulation appears to be at the levels of transcription and mRNA turnover (Peeters et al., 1980; Page and Parker, 1982). This regulation was thought to involve the interaction of the transformed androgen receptor with specific binding sites at or near the C3 gene transcription start site. Partially purified androgen receptor from rat prostate or prostatic adeno-carcinoma bound to two DNA fragments from within the C3 subunit gene as shown in DNA cellulose competition assays (Perry et al., 1985; Rushmere et al., 1987; van Dijck et al., 1989). The specific sequences bound by the androgen

receptor were not identified in these studies. However, both fragments, i.e. the 0.3 kb fragment 5'-upstream of the transcription initiation site and the 0.5 kb fragment of the first intron, contained 15 bp imperfect palindromic sequences resembling a GRE (glucocorticoid responsive element).

DNA sequences responsible for regulation by steroid hormones have been found in inducible genes by gene transfer experiments (Yamamoto, 1985, and references therein). In these experiments, a chimaeric gene is constructed consisting of a putative regulatory DNA fragment cloned in front of a reporter gene and subsequently introduced into a heterologous steroid hormone responsive cell line. In this way, a 15-mer consensus sequence for the glucocorticoid responsive element (GRE) and a 13-mer consensus sequence for the estrogen responsive element (ERE) were identified (Beato, 1989, and references therein). Subsequently, it was shown that the GRE is also able to mediate induction of a reporter gene by progesterone and testosterone (Strähle et al., 1987; Ham et al., 1988). Mutations within the GRE-like sequence in the 0.5 kb intron fragment of the C3 gene eliminated the response to androgens during transfer experiments in T47D cells, thus confirming that this sequence could function as an androgen responsive element (Claessens et al., 1989). The androgen responsive element in the first intron of the C3 gene was further investigated in transfection experiments, using CV1 and COS cells after co-transfection of an androgen receptor expression vector (Tan et al., 1992).

Additional examples of androgen regulated genes are the genes encoding human prostate specific antigen (Riegman et al., 1991A; Wolf et al., 1992), mouse sex limited protein (Adler et al., 1992), rat ornithine decarboxylase (Crozat et al., 1992), rat S-adenosylmethionine (Maric et al., 1992), rat probasin protein (Rennie et al., 1993), rat cystatin-related glycoproteins (De Vos et al., 1993), mouse vas deferens aldo-keto reductase (Fabre et al., 1994), human and rat prostate specific acid phosphatase (Virkkunen et al., 1994), and the human

glandular kallikrein-1 (hKLK2) protein (Riegman et al., 1991B; Murtha et al., 1993). Sequences, similar to the GRE were found in the promoter regions of all these genes, and from functional analysis a consensus ARE was defined as 5'GGA/TACAnnnTGTTCT, which is very similar to the consensus GRE 5'GGTACAnnnTGTTCT (Roche et al., 1992; Kallio et al., 1994). The recognition of a GRE-like sequence by the androgen receptor probably reflects the highly conserved P and D boxes in the DNA binding domains of the androgen- and glucocorticoid receptors. The P box recognizes a specific half site DNA sequence, while the D box facilitates binding of two receptors to the GRE by protein-protein interactions leading to receptor dimerization (Umesono and Evans, 1989; Freedman, 1992). Intact human and rat androgen receptors, or fragments thereof containing the DNA binding domain, have been produced in *E.Coli*, insect cells, COS cells or by *in vitro* translation, and were used for analysis of the androgen receptor DNA binding properties *in vitro* (Young et al., 1990; De Vos et al., 1991; Tan et al., 1992; Yan-Bo Xie et al., 1992; Kuiper et al., 1993; Palvimo et al., 1993; De Vos et al., 1994; Kallio et al., 1994; Krempelhuber et al., 1994). In all studies, sequence specific binding of androgen receptor to GRE/ARE containing oligonucleotides was shown, and evidence was provided that the receptor binds as a homodimer to DNA (Kallio et al., 1994). Segments flanking the DNA binding domain were reported to influence the stability of receptor-DNA complexes (Palvimo et al., 1993; Kallio et al., 1994), indicating that protein-protein contacts involving the N-terminal domain are stabilizing the dimeric receptors bound to DNA.

Although the presence of hormone was not needed for specific androgen receptor-DNA complex formation *in vitro*, the presence of androgens altered the mobility of receptor-DNA complexes during electrophoretic mobility shift analysis (Kaspar et al., 1993; Palvimo et al., 1993). This implies that binding of

ligand induces a conformational change in the androgen receptor, at least when bound to DNA. Nevertheless, there seems to be a discrepancy between the *in vitro* situation where hormone is not essential for receptor-DNA complex formation, and the *in vivo* situation where hormone is essential. The general model for steroid hormone action involves binding of the hormone to the receptor followed by a conformational change, called transformation, which leads to binding to chromatin (Jensen et al., 1968). This model was confirmed by genomic footprinting studies in which the hormone responsive element of the hepatic tyrosine aminotransferase gene was shown to be occupied only after hormone administration (Becker et al., 1986). One possible explanation for this apparent discrepancy is that *in vivo* the steroid free receptor is associated with hsp90, which prevents the receptor from binding to chromatin (Groyer et al., 1987; Denis et al., 1988; Pratt, 1990; Smith and Toft, 1993). Upon binding of the hormone, hsp90 dissociates, leaving the DNA binding domain of the receptor free to bind to chromatin. Also the androgen receptor is associated with hsp90, both in intact cells and after recovery of the unliganded receptor in hypotonic cell lysates. Furthermore, androgens promote the temperature dependent dissociation of androgen receptors from hsp90 (Veldscholte et al., 1992A; Veldscholte et al., 1992B).

The DNA binding domain of the androgen receptor has a high degree of amino acid sequence similarity with progesterone and glucocorticoid receptors, and as a consequence the receptors recognize very similar hormone responsive elements. However, the ligands of these different receptors elicit biological responses that are ligand specific, suggesting that accessory factors interacting with regions outside the DNA binding domain are important determinants for steroid- and receptor-specific actions. The mouse sex limited protein gene (Slp) is a duplicated complement C4 gene, whose expression in several tissues is

androgen dependent due to the influence of an inserted provirus (Stavenhagen and Robins, 1988; Adler et al., 1992). Within the 5' proviral long terminal repeat, which functions as a hormone-dependent enhancer, a GRE is present. However, the enhancer is activated by androgens and not by glucocorticoids or progestins (Adler et al., 1992). Potent androgen induction requires both the glucocorticoid responsive element and auxiliary elements also present within a 120 bp DNA fragment (Adler et al., 1992; Adler et al., 1993). Co-transfection assays with the glucocorticoid receptor revealed that the glucocorticoid receptor can bind to the responsive element, but is unable to *trans*-activate. The positive effect of the androgen receptor appears to require the N-terminal domain of the androgen receptor. Thus, the exclusive transcriptional response to androgens derives from interactions between cell specific non-receptor factors and the androgen receptor. These interactions are determined by the context of the receptor binding site, rather than by the sequence of the binding site itself.

It is generally accepted that steroid receptors are *trans*-acting regulatory factors with an enhancer function on steroid target genes (Beato, 1989). As outlined above, also for the androgen receptor clear evidence is available for the interaction with nucleotide sequences upstream of steroid regulated genes, and subsequent receptor-mediated stimulation of transcription initiation. This does not exclude, however, a possible participation of chromatin structure and alterations of chromatin structure in the mechanism of action of androgens. Upon castration of rats, ventral prostate chromatin regions containing acceptor sites and androgen responsive genes, are withdrawn into a more compact structure, inaccessible to micrococcal nuclease (Davies and Rushmere, 1988). This trend is reversed *in vivo* by administration of androgens (Davies et al., 1985). The androgen receptor is obviously able to alter chromatin structures into a transcriptionally more favourable form, and could act as a competence factor,

enabling separate factors to act as enhancers on certain genes. Detailed evidence has been presented for a role of the glucocorticoid receptor in removal of promoter repression due to nucleosome positioning on the MMTV promoter. In this mechanism of action, there is no direct interaction between the receptor and other transcription factors (Truss et al., 1992; Eriksson and Wränge, 1993).

1.6 Conclusions

Androgenic steroids circulate in the blood and diffuse in and out of cells, but are retained in target cells by binding with an intranuclear binding protein, termed androgen receptor. The androgen receptor binds its specific steroid with high affinity, having an equilibrium dissociation constant (Kd) in the range of 10^{-9}-10^{-10}M. The androgen receptor can be isolated from the cytosol of target cell extracts as a large non-transformed (i.e. non-DNA binding) 8S oligomeric complex, which contains hsp90. Once bound by androgens, the androgen receptor undergoes a conformational change termed "transformation", which involves dissociation of heat-shock proteins from the 8S complex. This transformation allows the androgen receptor to bind with high affinity to specific sites (acceptor sites) on the chromatin. The result of the binding to nuclear acceptor sites by the androgen receptor is the alteration of gene transcription (Figure 1.2). In which way the androgen receptor, once positioned on a hormone responsive element in the promoter of a target gene, precisely modulates transcriptional efficiency, is poorly understood. This probably requires functional interactions between receptor molecules as well as interactions with other essential transcription factors (Figure 1.2).

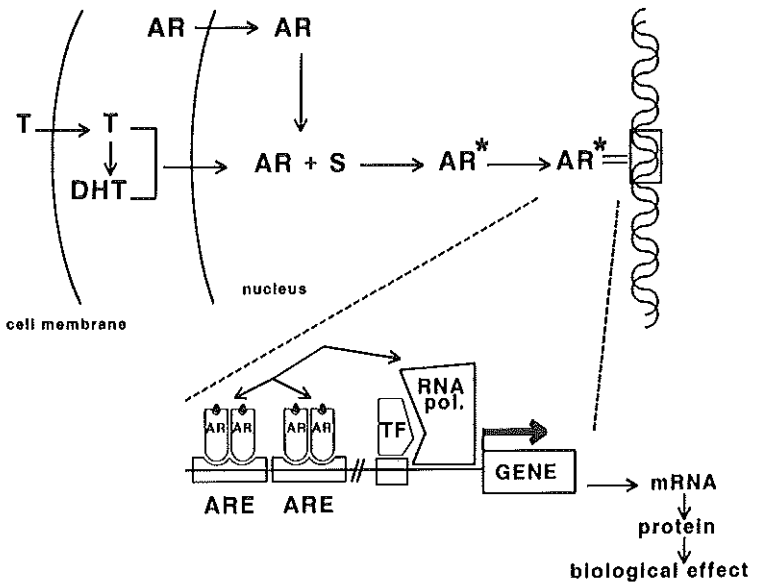

Figure 1.2. Schematic representation of the mechanism by which androgens regulate gene expression.
Testosterone (T) enters the cell and can be metabolized to 5α-dihydro-testosterone (DHT). T or DHT (=S) can bind to the androgen receptor (AR) which subsequently undergoes transformation. The transformed androgen receptor (AR*) binds to androgen responsive elements (AREs) in front of androgen regulated genes and interacts with other transcription factors (TF), and RNA polymerase.

References : see end of Chapter 2.

CHAPTER 2

STEROID HORMONE RECEPTOR PHOSPHORYLATION

2.1 Introduction

Protein phosphorylation is generally acknowledged as an important way of acute regulation of protein function in eukaryotic cells. Thousands of proteins are expressed in a typical eukaryotic cell, of which about a third is thought to be a phosphoprotein. Two hundred protein kinases and some hundred protein phosphatases have been identified, and it is estimated that 3% of all eukaryotic genes may code for protein kinases and phosphatases (Hubbard and Cohen, 1993). Protein phosphorylation is often concerned with switching of a cellular activity from one state to another. It is the major mechanism by which cells respond to extracellular signals, and it is responsible for the timing of events which must occur at defined stages during the cell cycle, such as DNA synthesis and mitosis.

Among the phosphoproteins, the nuclear proteins are a special group (Meek and Street, 1992). These include transcription factors as CREB, Jun, Fos, NFκb, Myc and Myb; tumor suppressor proteins as p53, and the retinoblastoma susceptibility gene protein product (pRB), and viral proteins as the SV40 large tumor antigen. Each of these proteins can be phosphorylated at multiple sites, most often by different kinases. In some cases phosphorylation at a certain site occurs in response to different signals. For transcription factors three main mechanisms of regulation by phosphorylation can be identified (Hunter and Karin, 1992). First, the DNA binding affinity of transcription factors can be modulated negatively or positively by phosphorylation. Second, phosphorylation can affect the interaction of *trans*-activation domains of transcription factors with components of the transcription initiation complex. Third, phosphorylation can influence the shuttling of transcription factors between the nuclear and cytoplasmic compartments, thereby regulating indirectly the activity of transcription factors in the nucleus. These mechanisms are by no means

mutually exclusive, and (de)phosphorylation at different sites by different kinases or phosphatases can result in differential regulation of a single transcription factor by several mechanisms (Jackson, 1992). Most members of the nuclear receptor superfamily, including the glucocorticoid, estrogen, progesterone, androgen, 1,25-dihydroxyvitamin D_3 and mineralocorticoid receptors are phosphoproteins. These receptors become rapidly extra phosphorylated upon binding of their respective ligands, suggesting that phosphorylation has an important role in the regulation of steroid receptor activity. The goal of this chapter is to describe the state of art of the rapidly advancing field of steroid receptor phosphorylation, and to indicate in which way phosphorylation might regulate steroid receptor activity.

2.2 Indirect evidence for a role of steroid hormone receptor phosphorylation

The first indications that steroid hormone receptor function might be regulated through phosphorylation processes came from studies, showing that cortisol binding by the glucocorticoid receptor in thymus cells varied with intracellular ATP levels (Munck and Brinck-Johnsen, 1968; Bell and Munck, 1973). Similarly, decrease of DHT binding in prostatic tissue homogenates after incubation of tissue slices with compounds causing loss of ATP (2,4-dinitro-phenol, cyanide, or azide) and subsequent increase of DHT binding by incubation of the homogenates with ATP, suggested that an ATP requiring process was involved in androgen receptor function (Liao and Fang, 1969; Liao et al., 1975; Rossini and Liao, 1982). These, and several other studies, led to a model of receptor cycling, in which an inert (non-ligand binding) form of the receptor is produced from the transformed receptor in the nucleus. Subsequently, an ATP-dependent process restores the ability to bind steroid and the receptor

is recycled to the nucleus after steroid binding (Rossini, 1984). Further indications for a possible role of phosphorylation in steroid hormone action came from studies involving phosphatases and phosphatase inhibitors. The loss in hormone binding capacity of glucocorticoid-, estrogen- and progesterone receptors that occurs on incubation of cytosolic extracts of target cells at 15-25°C, was prevented by phosphatase inhibitors such as molybdate and fluoride (Nielsen et al., 1977A; Auricchio and Migliaccio, 1980; Grody et al., 1980). Loss of hormone binding capacity also occurred on treatment of the glucocorticoid receptor with purified alkaline phosphatase, and on treatment of mouse uterus estrogen receptor with a purified nuclear phosphatase (Nielsen et al., 1977B; Auricchio et al., 1985). The binding ability of the mouse uterus estrogen receptor after phosphatase treatment could be restored by incubation with ATP and a kinase preparation purified from uterus (Auricchio et al., 1985). Collectively, these studies indicated that the steroid binding capacity of receptors was lost either by dephosphorylation of receptors or of associated proteins and could be restored by rephosphorylation.

More evidence that steroid hormone receptors are phosphoproteins came from studies in which partially purified receptor preparations were incubated with purified kinases. In experiments with cAMP-dependent protein kinase, it was shown that the progesterone receptor from chicken oviduct can undergo phosphorylation *in vitro* (Weigel et al., 1981; Singh et al., 1986). Also, the purified glucocorticoid receptor from rat liver could be phosphorylated *in vitro* by incubation with cAMP-dependent protein kinase (Singh and Moudgil, 1985). Purified estrogen receptor from calf uterus was shown to be phosphorylated *in vitro* by an purified calcium/calmodulin dependent protein kinase on tyrosine residues (Auricchio et al., 1985). Androgen receptor from rat prostate was suggested to be phosphorylated by a cAMP-independent protein kinase, although

in this study no direct evidence was provided that the phosphoprotein represented the androgen receptor (Goueli et al., 1984). Clearly, these studies, using partially purified receptor preparations, were limited by the difficulty of separating receptors from other proteins, including endogenous kinase and phosphatase activities.

In another series of experiments, target cells or target tissue slices were incubated with [^{32}P]-orthophosphate, and thereafter attempts were made to purify the respective steroid receptors. This purification involved "classical" methods as chromatography on DNA cellulose or phosphocellulose columns, and steroid affinity chromatography. In this way it was demonstrated that the glucocorticoid receptor (90-94 kDa) becomes phosphorylated in rat liver slices and in intact mouse L cells (Housley and Pratt, 1983; Grandics et al., 1984; Singh and Moudgil, 1985). Also the progesterone receptor was shown to be phosphorylated after incubation of chicken oviduct minces with [^{32}P]-orthophosphate and purification via affinity chromatography (Dougherty et al.,1982). In most of these studies molybdate-stabilized receptor forms have been used. Subsequent studies have shown that this form of receptor is associated with a non-hormone binding 90 kDa heat-shock protein (Joab et al., 1984). This protein was found to be phosphorylated in mouse fibroblasts (Housley et al., 1985). Therefore, a portion of the 90-94 kDa ^{32}P-labelled proteins observed in these reports could in fact belong to the 90 kDa non-hormone binding protein co-purifying with the glucocorticoid and progesterone receptors. Phosphorylation of the estrogen receptor (65 kDa) on tyrosine residues was observed after incubation of rat uterine tissue with [^{32}P]-orthophosphate and purification of the estrogen receptor with steroid affinity chromatography (Migliaccio et al., 1986). Considerable progress in the studies on steroid receptor phosphorylation occurred at the time when specific polyclonal and monoclonal antibodies suitable to purify receptors

from target cells or tissues became available.

2.3 Basal and hormone dependent phosphorylation of steroid receptors

When steroid target cells in culture or target tissue slices are incubated with [^{32}P]-orthophosphate, steroid hormone receptors can be shown to be phosphorylated after immunopurification and SDS-PAGE. The use of antibodies for the purification of receptors has the great advantage that rigorous washing steps (high salt and detergents) can be used, so that co-purification of contaminating phosphoproteins can be excluded. Furthermore, by immunopurification it is possible to compare the phosphorylation degree of steroid receptors in the absence or presence of ligand, which is impossible when "classical" purification strategies based on binding of tritiated ligands are employed. Steroid hormone receptors are already phosphoproteins in the absence of ligands, which will be designated basal phosphorylation. The progesterone receptor was shown to be phosphorylated in cytosolic extracts as well as in high salt nuclear extracts (transformed receptor) in rabbit uterine slices (Logeat et al., 1985). In later studies progesterone receptor phosphorylation was also described in human breast tumor cells (T47D cells), chicken oviduct slices, and in primary cultures of chicken oviduct cells (Garcia et al., 1986A; Denner et al., 1987; Wei et al., 1987; Sheridan et al., 1988; Sullivan et al., 1988A). Phosphorylation of the glucocorticoid receptor was described in WEHI-7 thymoma cells and mouse L-cells (Mendel et al., 1987; Tienrungroj et al., 1987). Estrogen receptor phosphorylation has been described in mouse uterus, calf uterus, and in the MCF7 human breast cancer cell line (Lahooti et al., 1990; Washburn et al., 1991; Denton et al., 1992), and human androgen receptor phosphorylation was found in human lymph node carcinoma of the prostate (LNCaP) cells (Van Laar et al., 1990). The effects of vitamin D are mediated by the receptor that binds

the active metabolite 1,25-dihydroxyvitamin D_3. Phosphorylation of this receptor was observed in organ culture studies of embryonic chicken duodenum (Brown and DeLuca, 1990). In contrast, no basal phosphorylation of the 1,25-dihydroxy-vitamin D_3 receptor was observed in mouse fibroblasts (Pike and Sleator, 1985). No data are available on phosphorylation of the mineralocorticoid receptor in target cells or tissue, only the mineralocorticoid receptor overexpressed in Sf9 insect cells was reported to be phosphorylated (Alnemri et al., 1991).

Steroid receptors become extra phosphorylated on incubation of target cells or tissues in culture with their respective ligands. This extra phosphorylation is a rapid process (within 30 min) and is associated with receptor transformation to the tight nuclear binding form in several systems. The rapid increase in receptor phosphorylation upon ligand binding is often used as an argument to stress the importance of steroid receptor phosphorylation (see Figure 2.1). Hormone stimulated extra phosphorylation has been reported for the glucocorticoid receptor in WEHI-7 thymoma cells, 3T3 fibroblasts and in hepatoma cells (Hoeck et al., 1989; Orti et al., 1989; Hoeck and Groner, 1990). The unliganded glucocorticoid receptor was found to exist as a heteromeric complex containing one steroid binding unit and two hsp90 monomers, and probably also other heat-shock proteins. Upon hormone binding at 37°C these heteromeric complexes dissociate, followed by receptor transformation to the DNA binding form. Kinetic studies in WEHI-7 cells revealed that the primary substrate for hormone stimulated extra phosphorylation was the transformed receptor (Orti et al., 1993). Also the non-transformed receptor became extra phosphorylated, albeit at a lower rate (Orti et al., 1989). This extra phosphorylation was induced by glucocorticoid agonists, but not by the antagonist RU486 (Hoeck et al., 1989), which suggested a functional role for glucocorticoid receptor phosphorylation.

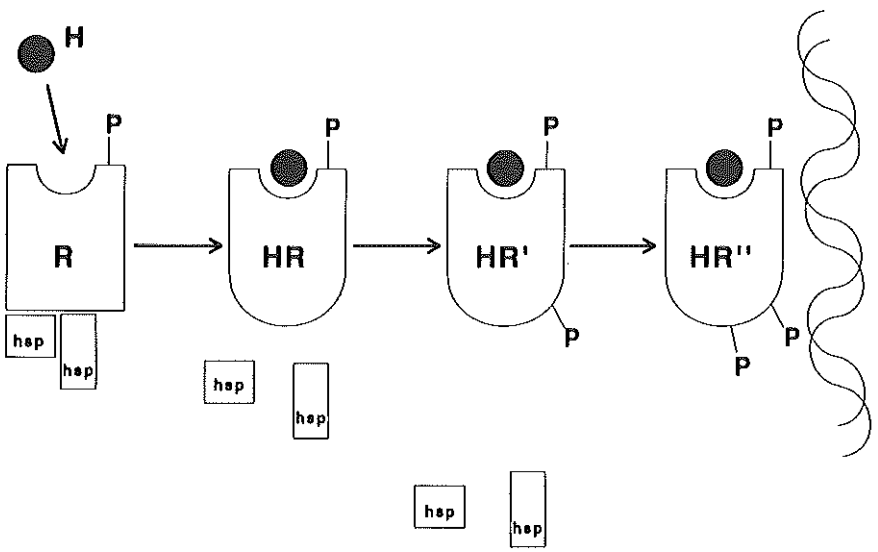

Figure 2.1. Basal and hormone dependent steroid hormone receptor phosphorylation.
The unliganded receptor (R) is already phosphorylated. Upon ligand binding the receptor (HR) undergoes a conformational change, and the heat-shock proteins dissociate. The transformed (DNA bound) steroid receptor (HR") and the non-transformed steroid receptor (HR') are extra phosphorylated.

Addition of progesterone to T47D cells or to chicken oviduct slices caused a rapid extra phosphorylation of the progesterone receptor (Wei et al., 1987; Sheridan et al., 1988; Sullivan et al., 1988B; Nakao et al., 1989). In contrast to the glucocorticoid receptor, RU486 did promote human progesterone receptor transformation and phosphorylation in T47D cells (Sheridan et al., 1989A), but

had no effect on the phosphorylation degree of the chicken oviduct progesterone receptor (Nakao et al., 1989). RU486 does not bind to chicken and hamster progesterone receptors, but does bind to the human progesterone receptor. A single amino acid species variation in the ligand binding domain was shown to be responsible for this differential antagonistic effect of RU486 (Groyer et al., 1985; Eliezer et al., 1987; Gronemeyer et al., 1992). A two-fold increase in androgen receptor phosphorylation in LNCaP cells was measured after incubation with R1881 (Van Laar et al., 1991). In transiently transfected COS-7 cells, it was found that the androgen receptor protein degraded rapidly ($t_{1/2}$= 1 h), except in the presence of androgen ($t_{1/2}$= 6 h), which accounted for an apparent 2-4 fold androgen-induced increase in androgen receptor phosphorylation (Kemppainen et al., 1992). Estrogen treatment of mouse uterine slices during metabolic labelling with [^{32}P]-orthophosphate caused extra phosphorylation of estrogen receptors (Washburn et al., 1991). In MCF-7 cells extra phosphorylation of the estrogen receptor after estradiol treatment was also found (Denton et al., 1992). Phosphorylation of the 1,25-dihydroxyvitamin D_3 receptor was strongly induced after incubation of mouse fibroblast and embryonal chicken duodenum organ cultures with 1,25-dihydroxyvitamin D_3 (Pike and Sleator, 1985; Brown and DeLuca, 1990). The stimulation factors reported ranged from 2 to 7, often for one type of steroid hormone receptor in comparable systems. This variation might be due to differences in experimental conditions.

Post-translational modifications, including phosphorylation, of proteins can be detected by resolving isoforms of different apparent molecular masses during SDS-PAGE. This is an alternative method to study steroid receptor phosphorylation, which has been used extensively. The photoaffinity-labelled progesterone receptor (using the synthetic progestin ^{3}H-R5020) gave a doublet

of the untransformed protein with molecular masses of 117 and 120 kDa (Horwitz et al., 1985). During the first 30 min of incubation with R5020, the progesterone receptor protein shifted entirely to the heavier form (120 kDa), most likely caused by a hormone-dependent covalent modification. In later studies it was shown that these isoforms of the progesterone receptor were caused by differential phosphorylation (Sheridan et al., 1989B). The mouse uterus estrogen receptor also appears as a doublet after SDS-PAGE, of which the higher molecular mass form is being induced by estrogens (Golding and Korach, 1988). Microheterogeneity after SDS-PAGE was detected also for the human glucocorticoid- and androgen receptors (Northrop et al., 1985; Van Laar et al., 1990). In rat osteosarcoma cells and mouse fibroblasts, treatment with 1,25-dihydroxyvitamin D_3 elicited a decrease in mobility of the 1,25-dihydroxy-vitamin D_3 receptor on SDS-PAGE (Pike and Sleator, 1985; Jurutka, 1993A).

2.4 Location of phosphorylation sites in steroid hormone receptor proteins

The majority of the glucocorticoid receptor phosphorylation sites could be located in the N-terminal *trans*-activation domain using chemical cleavage with hydroxylamine and cyanogenbromide or proteolytic digestion with trypsin and chymotrypsin (Dalman et al., 1988; Smith et al., 1989; Hoeck and Groner, 1990; Hutchison et al., 1993). There is some debate on additional phosphorylation of the DNA- or steroid binding domains of the glucocorticoid receptor (Hutchison et al., 1993). In rat hepatoma cells, in mouse L cells, and in Chinese hamster ovary cells, the glucocorticoid receptor becomes also phosphorylated in the DNA binding domain, but not in the steroid binding domain (Dalman et al., 1988; Hoeck and Groner, 1990; Hutchison et al., 1993). In contrast, in AtT-20 mouse pituitary tumor cells the glucocorticoid receptor was found to be non-phosphorylated in the DNA binding domain (Van der Weyden Benjamin et al.,

1990). Only in WEHI-7 mouse thymoma cells the glucocorticoid receptor was, apart from the N-terminal domain, also phosphorylated in the steroid binding domain, but not in the DNA binding domain (Smith et al., 1989). In a later study by the same laboratory it was found that a highly purified tryptic fragment containing the steroid binding domain is not phosphorylated, indicating that the earlier result might have been due to a contaminant (Bodwell et al., 1991). Progesterone receptors from chicken oviduct and from T47D cells are phosphorylated in the N-terminal domain (Sullivan et al., 1988B; Sheridan et al., 1989A). Also the rabbit progesterone receptor was shown to be predominantly phosphorylated in the N-terminal domain after transfection in COS cells (Chauchereau et al., 1991). The 1,25-dihydroxyvitamin D_3 receptor was found to be phosphorylated in a fragment spanning the hinge region and a large part of the ligand binding domain (Brown and DeLuca, 1991). In another study only phosphorylation in the hormone binding domain was reported (Jones et al., 1991). In conclusion, most steroid receptors that have been investigated are phosphorylated at least in the N-terminal *trans*-activation domain.

2.5 Identification of phosphorylation sites

Phosphoamino acid analysis has revealed that the glucocorticoid, progesterone-, and 1,25-dihydroxyvitamin D_3 receptors are phosphorylated on serine residues (Dalman et al., 1988; Sheridan et al., 1988; Smith et al., 1989; Hoeck and Groner, 1990; Brown and DeLuca, 1991). For the glucocorticoid receptor, minor phosphorylation on threonine residues was also reported (Hoeck and Groner, 1990). Results on phosphoamino acid analysis of estrogen receptors were confusing. While Auricchio and co-workers showed that the estrogen receptor was only phosphorylated on tyrosine residues, other investigators reported specific phosphorylation on serine residues (Auricchio, 1989; Washburn et al.,

1991; Denton et al., 1992; Lahooti et al., 1994; LeGoff et al., 1994). The reason for this discrepancy is at present unknown.

By phosphopeptide mapping and sequencing of tryptic peptides, seven phosphorylated sites have been identified in the mouse glucocorticoid receptor (Bodwell et al., 1991). These sites are identical in CHO cells in which the glucocorticoid receptor was overexpressed, and in mouse thymoma cells (WEHI-7). All sites are on serine residues, except one site on a threonine residue, and are located in the N-terminal domain (Ser 122, 150, 212, 220, 234, 315 and Thr 159). Three serine residues (212, 220 and 234) are in a region which is essential for transcriptional activity of the mouse glucocorticoid receptor, and are present in the core *trans*-activation domain of the human glucocorticoid receptor (Danielsen et al., 1987; Dahlman-Wright et al., 1994). Data on the influence of glucocorticoids on the phosphorylation state of these sites are not yet available. Four phosphorylated sites in the chicken progesterone receptor have been identified (Denner et al., 1990A; Poletti and Weigel, 1993). There are four Ser-Pro motifs in the chicken progesterone receptor, which are all phosphorylated. Two sites (Ser 211 and Ser 260) are substantially phosphorylated in the absence of ligand, with a small increase in the presence of ligand, while phosphorylation of two other sites (Ser 367 and Ser 530) was essentially ligand-inducible. Upon expression of chicken progesterone receptor in the yeast *Saccharomyces cerevisiae* the receptor was correctly phosphorylated on all four Ser-Pro motifs (Poletti et al., 1993). The effects of ligand on the phosphorylation degree of the four sites were consistent with the authentic chicken progesterone receptor, showing that the yeast system is applicable to the study of the role of phosphorylation in progesterone receptor function. The human 1,25-dihydroxy-vitamin D_3 receptor transiently expressed in COS-1 cells was phosphorylated on serine residues (Hilliard et al., 1994). In order to obtain sufficient quantities for

microsequencing of phosphopeptides, ^{32}P-labelled 1,25-dihydroxyvitamin D_3 receptor from COS cells was mixed with purified 1,25-dihydroxyvitamin D_3 receptor expressed in *Saccharomyces cerevisiae*. Sequencing of phosphopeptides obtained by digestion of receptor with various proteases, revealed phosphorylation at serine residue 208, within a consensus site for casein kinase II (Hilliard et al., 1994). Phosphorylation at this site was clearly stimulated (approx. 8 fold) by treatment of COS cells with 1,25-dihydroxyvitamin D_3.

Another approach for the identification of phosphorylation sites, is site-directed mutagenesis of consensus phosphorylation sites for various kinases, and subsequent transient expression of mutant proteins in COS cells or comparable systems. This strategy however is time-consuming and creates an additional problem, in that the abolition of a phosphorylation site by a point mutation might cause a conformational change. Furthermore, mutagenesis of a consensus phosphorylation site might promote phosphorylation at an alternative site, as has been shown for the human 1,25-dihydroxyvitamin D_3 receptor (Hilliard et al., 1994). Comparison of tryptic phosphopeptide maps of wild type and mutant human estrogen receptors expressed in COS cells, revealed phosphorylation at serine residues 104 and/or 106, and residue 118 (Ali et al., 1993; Le Goff et al., 1994). All three serine residues are part of a serine-proline motif, the preferred substrate of proline-directed protein kinases (Vulliet et al., 1989). By similar strategies the human 1,25-dihydroxyvitamin D_3 receptor has been shown to be phosphorylated at serine residue 51 (protein kinase C site) within the DNA binding domain and at serine residue 208 (casein kinase II site) within the ligand binding domain (Hsieh et al., 1991; Jurutka et al., 1993B).

2.6 Protein kinases involved in steroid hormone receptor phosphorylation

Several protein kinases have been reported to co-purify with the progesterone receptor. Co-purification of casein kinase-like protein kinases and magnesium dependent kinases with the rabbit and chicken progesterone receptors has been described. None of these kinases, however, showed hormone-stimulated phosphorylation of the progesterone receptor *in vitro*. The physiological significance of these findings is uncertain (Garcia et al., 1986B; Logeat et al., 1987). The chicken progesterone receptor is phosphorylated at all four Ser-Pro motifs *in vivo* (Denner et al., 1990A, Poletti and Weigel, 1993). The Ser/Thr-Pro motif is a portion of the consensus sequence for the cdc2 (cell division cycle) protein kinases and the mitogen-activated protein kinases, and these are therefore candidate kinases to phosphorylate the receptor (Vulliet et al., 1989; Mukhopadhyay et al., 1992). Chicken progesterone receptor is also phosphorylated by a DNA dependent protein kinase, during *in vitro* progesterone dependent transcription assays using HeLa cell nuclear extracts. This phosphorylation was strictly dependent on the presence of double-stranded DNA (Bagchi et al., 1992; Weigel et al., 1992). Further analysis of receptors isolated from chicken oviduct nuclear extracts will be necessary to determine whether this phosphorylation also occurs *in vivo*. The DNA dependent kinase that is involved also phosphorylates the transcription factor Sp1, and is a heterodimer composed of a catalytic subunit and a DNA binding subunit (Jackson et al., 1993). The latter probably directs the kinase to DNA and can be regarded as a "targeting subunit", making possible efficient phosphorylation of DNA-bound substrates (Hubbard and Cohen, 1993). The DNA dependency of progesterone receptor phosphorylation *in vitro* was confirmed during *in vivo* experiments (Takimoto et al., 1992). Analysis of mutant and wild type progesterone receptors expressed in COS-1 cells suggested that hormone-induced phosphorylation can

be subdivided into two stages. The first stage is DNA independent, for which a receptor mutant lacking DNA binding activity is a substrate; the second stage is DNA dependent, for which the same receptor mutant is not a substrate. The synthetic antiprogestin ZK98299 binds progesterone receptors but prevents their interaction with DNA. Compared with progestin agonists, this antagonist reduced phosphorylation of progesterone receptors in T47D cells to 60% (Takimoto et al., 1992). These results indicate that the hormone-induced phosphorylation of the progesterone receptor involves DNA binding independent as well as DNA binding dependent steps (Takimoto et al., 1992; Takimoto and Horwitz, 1993).

Cell-free phosphorylation of the calf uterine estrogen receptor has been found to occur on tyrosine residues (Auricchio, 1989). A nuclear phosphatase from calf uterus inactivated hormone binding, and the resulting dephosphorylated receptor form could be converted back to an estrogen binding form by a cytosolic calcium/calmodulin dependent protein tyrosine kinase activity (Aurrichio, 1989). This kinase activity has been purified to homogeneity (Castoria et al., 1993). The uterus protein tyrosine kinase is a 67 kDa protein on SDS-PAGE and binds calmodulin in a Ca^{2+} dependent manner. The kinase phosphorylates *in vitro* the phosphatase-treated calf uterus estrogen receptor on tyrosine, and thereby activates hormone binding. By site-directed mutagenesis of tyrosine residues, tyrosine residue 537 was identified as the phosphorylated residue. The mutant (Tyr 537 --> Phe) is neither phosphorylated nor activated with regard to estrogen binding by the kinase (Castoria et al., 1993). Remarkably, the kinase is dependent for its activity on the presence of purified calf uterus estrogen receptor. Whether the kinase becomes activated either by estrogen or by the estrogen receptor complex, or by an unknown compound in the purified receptor preparation, is presently unknown. Furthermore, it has not yet been shown that the estrogen receptor in calf uterus *in vivo* is also phosphorylated on tyrosine

residue 537, and that this phosphorylation is essential for hormone binding capacity. The purified porcine uterus estrogen receptor was shown to be non-phosphorylated in the ligand binding domain (Bökenkamp et al., 1994). In chicken oviduct cytosol, the estrogen receptor exists in three forms: two forms which bind estrogen with low and high affinity respectively, and one form which does not bind estrogens at all (Raymoure et al., 1986). Conversion of the non-binding form to the low affinity binding form *in vitro* requires Mg^{2+} and ATP (Raymoure et al., 1986). This conversion is mediated by a 40 kDa activation factor that is associated with protein kinase as well as protein phosphatase activity (Dayani et al., 1990; McNaught et al., 1990). The amino acid(s) that are phosphorylated or dephosphorylated by this activation factor are presently unknown. The human estrogen receptor is phosphorylated on three Ser-Pro motifs, which is the preferred motif in substrates of the proline directed protein kinases including cdc2 kinase and the mitogen activated kinases (Le Goff et al., 1994). For the glucocorticoid receptor, proline directed protein kinases are also predominant, since four out of seven identified phosphorylation sites are in proline directed protein kinases consensus sequences (Bodwell et al., 1991). Since the proline directed protein kinases also phosphorylate the progesterone receptor (Denner et al., 1990), it might be concluded that this protein kinase is the principal protein kinase involved in steroid hormone receptor phosphorylation.

2.7 Steroid hormone receptor phosphorylation and receptor function

There is increasing evidence that gene expression is regulated by phosphorylation of transcription factors (Hunter and Karin, 1992; Jackson, 1992). Since steroid receptors are ligand-dependent transcription factors and also exist as phosphoproteins, several studies have been performed to uncover the

physiological role(s) for receptor phosphorylation in steroid hormone action. The following receptor functions or activities linked to phosphorylation have been suggested: receptor association with heat shock proteins, activation of hormone binding capacity, nuclear import, subnuclear location, nucleocytoplasmic shuttling, modulation of binding to hormone responsive elements, receptor dimerization, and interactions with general transcription factors (Kuiper and Brinkmann, 1994).

The goal of this section is to describe the evidence available for the involvement of phosphorylation in some of these receptor functions.

2.7.1 Role of phosphorylation in estrogen receptor mediated *trans*-activation

In contrast to the results of Auricchio et al., there have been several reports showing estrogen receptor phosphorylation on serine residues (Auricchio, 1989; paragraph 2.5 and references therein). The liganded and unliganded estrogen receptor in MCF-7 breast carcinoma cells and calf uterus is phosphorylated on serine residues, and it was shown that treatment with acid phosphatase caused loss of affinity of the estrogen receptor for specific DNA sequences *in vitro* (Denton et al., 1992). Exposure of rat uterine cells in primary culture to estrogen, insulin-like growth factor I (IGF-I), or agents which alter intracellular cAMP levels, such as cholera toxin plus isobutylmethylxanthine (IBMX) and 8Br-cAMP, resulted in the upregulation of cellular levels of the progesterone receptor (Aronica and Katzenellenbogen, 1991). The progesterone receptor gene is a well known estrogen target gene. These effects were believed to be mediated through the estrogen receptor and phosphorylation pathways. It was indeed shown that in rat uterine cells transcription from a plasmid DNA containing two tandem estrogen responsive elements linked to a reporter gene,

could be stimulated by estrogen and also by 8Br-cAMP, cholera toxin plus IBMX, or IGF-I (Aronica and Katzenellenbogen, 1993). The level of estrogen receptor phosphorylation in uterine cells could be increased 3 to 5 fold upon exposure to one of the following: estrogen, cholera toxin plus IBMX, 8Br-cAMP or IGF-I (Aronica and Katzenellenbogen, 1993). However, the fact that an anti-estrogen (ICI 164384) evoked a similar increase in estrogen receptor phosphorylation, without a concomitant increase in estrogen receptor mediated transcription activation, indicated that an increase in overall phosphorylation does not necessarily result in increased transcriptional activity (Aronica and Katzenellenbogen, 1993). *Trans*-activation by the estrogen receptor, therefore, might depend upon phosphorylation or dephosphorylation of a specific site or sites, the modulation of which might not be detected in overall phosphorylation. The location of the estrogen receptor phosphorylation sites in the N-terminal transcription activation domain also suggested that phosphorylation might play a role in receptor *trans*-activation (Ali et al., 1993; Le Goff et al., 1994). Indeed, mutation of the three phosphorylated serine residues (residue 104, 106 and 118) caused a significant reduction (by 40%) in *trans*-activation activity in response to estrogens (Ali et al., 1993; Le Goff et al., 1994), but did not affect the DNA binding properties and nuclear import of the receptor. The sites phosphorylated in response to incubation with cholera toxin plus IBMX or 8Br-cAMP, remain to be identified (Le Goff et al., 1994). In the neuroblastoma cell line SK-ER3 it was also shown that IGF-I, in the absence of estrogen, could increase the activity of an estrogen responsive promoter (Ma et al., 1994). Phosphorylation of the estrogen receptor was not investigated in these cells. It is clear from these studies on estrogen receptors that site-specific phosphorylation affects transcriptional activation, although overall phosphorylation is not a parameter by which the differential transcriptional activity of estrogens versus anti-estrogens can be distinguished.

2.7.2 Role of phosphorylation in progesterone receptor mediated *trans*-activation

Studies on the phosphorylation of the progesterone receptor in T47D cells suggested that phosphorylation influences the ability of receptors to regulate gene transcription (Sheridan et al., 1988; Sheridan et al., 1989A). In the chicken progesterone receptor two hormone-induced phosphorylation sites (Ser 367 and Ser 530) have been identified (Denner et al., 1990A; Poletti and Weigel, 1993). Interestingly, each hormone dependent site is located in a separate area of the receptor, flanking the DNA binding domain and involved in *trans*-activation (Dobson et al., 1989). Substitution of serine residue 530 by an alanine residue in the chicken progesterone receptor resulted in a strong reduction in transcriptional activity, but only at low hormone concentrations (Bai et al., 1994). Effects of substitution of serine 367 alone or in combination with serine 530 were not studied. Mutation of serine residue 677 in the human progesterone receptor (homologous to serine residue 530 in the chicken receptor) reduced the *trans*-activation function by 30 to 50%, on a simple but not on a complex promoter in COS cells and in HeLa cells (Takimoto et al., 1991). It is important to note that extra phosphorylation of the progesterone receptor is not always correlated with increased transcriptional activity, because the antagonist RU486 provoked extra phosphorylation of the human progesterone receptor, while blocking *trans*-activation (Chaucherau et al., 1991). The site(s) phosphorylated in response to RU486 treatment have not been identified yet.

Analysis of human progesterone receptor phosphorylation in HeLa cell nuclear extracts during *in vitro* transcription assays, showed that phosphorylation increased in parallel with progesterone-induced enhancement of RNA synthesis from a promoter containing a progesterone responsive element (Bagchi et al., 1992). These data are correlative, because the presence of kinases in the

transcription extract made it impossible to study the *trans*-activating properties of the progesterone receptor when it had not undergone phosphorylation. However, it is interesting to note that progesterone receptor phosphorylation was at least in part dependent on the presence of progesterone (Bagchi et al., 1992). Treatment of CV-1 cells transiently expressing chicken progesterone receptor, with 8Br-cAMP or an inhibitor (okadaic acid) of protein phosphatases 1 and 2A, stimulated chicken progesterone receptor-mediated induction of transcription from a cotransfected reporter gene construct in the absence of progesterone (Denner et al., 1990B; Zhang et al., 1994). The ability to bypass hormonal activation of receptors with agents that modify cellular kinase or phosphatase activities, provided evidence that phosphorylation processes are involved in regulation of the transcriptional activity of progesterone receptors. In contrast, 8Br-cAMP or okadaic acid did not stimulate target gene expression in the absence of progesterone in a T47D-derived cloned cell line, containing a stable transfected MMTV reporter gene construct (Beck et al., 1992). Either compound augmented human progesterone receptor-mediated target gene transcription by 3 to 4 fold when added together with progesterone (Beck et al., 1992). The reason for the differential effects of treatment of cells with 8Br-cAMP and okadaic acid on progesterone receptor-mediated transcription of human- and chicken progesterone receptors, are unknown. Both modulators had no effect on the extent of human progesterone receptor phosphorylation in T47D cells (Beck et al., 1992), while possible effects of modulators on chicken progesterone receptor phosphorylation in CV-1 cells were not investigated (Denner et al., 1990B). The chicken and calf progesterone receptors are substrates *in vitro* for cAMP-dependent protein kinase (Hurd et al., 1989; Denner et al., 1990A; Nakao et al., 1992).

2.7.3 Role of phosphorylation in glucocorticoid receptor mediated *trans*-activation

The mouse glucocorticoid receptor is phosphorylated in the N-terminal domain at seven sites (serine residue 122, 150, 212, 220, 234, 315, and threonine residue 159), in WEHI-7 mouse thymoma cells and in CHO cells (Bodwell et al., 1991). Three serines (212, 220 and 234) are in a region which is essential for transcriptional activation, and are also present at homologous positions in the core *trans*-activation domain of the human glucocorticoid receptor (Danielsen et al., 1987; Dahlman-Wright et al., 1994). Substitution of serine residues by either alanine or aspartic acid residues did not influence receptor *trans*-activation. Only glucocorticoid receptors with five different serine phosphorylation sites substituted by alanine residues exhibited an approximately 20% decrease in *trans*-activation capacity (Mason and Housley, 1993). From these studies, it was concluded that phosphorylation of the glucocorticoid receptor at the identified sites is not a major determinant in glucocorticoid receptor transcriptional activity from the MMTV promotor in COS cells. The possibility that the large overexpression of the glucocorticoid receptor in COS cells compensates for reduced *trans*-activation capacity of the mutant could be excluded, since expression in E 8.2 cells (a GR negative variant of L fibroblasts) to a much lower level gave similar results (Mason and Housley, 1993).

In another study, the effects of okadaic acid, a protein phosphatase inhibitor, on *trans*-activation by the rat glucocorticoid receptor after transient expression in COS and CV-1 cells was investigated. In the absence of hormone, the glucocorticoid receptor was capable of enhancing transcription from the co-transfected MMTV-CAT reporter plasmid in response to okadaic acid treatment (Somers and DeFranco, 1992). Synergistic enhancement resulted from combined dexamethasone and okadaic acid treatment. By analysis of chromatin structure

it was shown previously that unliganded glucocorticoid receptors do not bind to glucocorticoid responsive elements *in vivo* (Becker et al., 1986). It is not known if *trans*-activation from glucocorticoid responsive elements by unliganded glucocorticoid receptors in okadaic acid treated COS and CV-1 cells is associated with the same alterations in chromatin structure as *in vivo*. The effects of okadaic acid on transcriptional enhancement did not correlate with major changes in glucocorticoid receptor phosphorylation, as was shown by two dimensional tryptic peptide mapping of ^{32}P-labelled receptors (Somers and DeFranco, 1992). Thus, changes in phosphorylation of unknown component(s) of the glucocorticoid receptor signal transduction pathway, and not the receptor itself, may influence its transcriptional enhancement activity. Expression vectors for the glucocorticoid receptor and a hormone-responsive reporter (MMTV) were stably introduced in T47D breast tumor cells (Moyer et al., 1993). In these cells the dexamethasone response could be enhanced 2 to 10 fold by activators of protein kinase A, protein kinase C, and inhibitors of phosphatases (okadaic acid). Okadaic acid alone had no effect in these cells, in contrast to the previous study employing transient transfection in COS or CV-1 cells (Somers and De Franco, 1992). Treatment of the T47D cells with protein kinase activators or phosphatase inhibitors had no effect on either cellular receptor content or dexamethasone binding. Also, treatment of cells with protein kinase activators or phosphatase inhibitors alone or in combination with dexamethasone did not change the phosphorylation pattern of the glucocorticoid receptor compared to the pattern before or after dexamethasone treatment, respectively (Moyer et al., 1993). So, again no link could be made between stimulation of glucocorticoid receptor-mediated transcription and phosphorylation of the glucocorticoid receptor.

2.7.4 Role of phosphorylation in subcellular location of the glucocorticoid receptor

In most studies it is shown that unoccupied glucocorticoid receptor proteins reside predominantly within the cytoplasmic compartment, and after ligand binding a rapid and efficient translocation of receptors to the nucleus occurs (Wikström et al., 1987). For rat hepatoma cells it was shown, however, that the glucocorticoid receptor is always in the nucleus (Brink et al., 1992). The nuclear glucocorticoid receptor is not indefinitely confined to the nuclear compartment after hormone treatment, but undergoes a recycling process involving the cytoplasm (Raaka and Samuels, 1983). When rat fibroblasts are treated with dexamethasone in the presence or absence of okadaic acid, the glucocorticoid receptor becomes extra phosphorylated and is translocated to the nucleus. When hormone is withdrawn the glucocorticoid receptor becomes cytoplasmic again. In cells continuously exposed to okadaic acid, however, the glucocorticoid receptor cannot re-enter the nucleus in the presence of dexamethasone and is trapped in the cytoplasmic compartment (DeFranco et al., 1991). It was postulated that okadaic acid blocks a dephosphorylation event that occurs either when the glucocorticoid receptor is exported from the nucleus or during the reassembly of a heteromeric complex with heat-shock proteins in the cytoplasm. These receptors, therefore, retain the extra phosphorylated state and are now trapped in the cytoplasmic compartment. Upon comparison of tryptic phospho-peptide maps of the glucocorticoid receptor, two unique phosphopeptides were detected after okadaic acid-treatment (DeFranco et al., 1991). Apparently, these phosphopeptides represent the site(s) which need to be dephosphorylated before the glucocorticoid receptor can recycle to the nuclear compartment. The effectiveness of glucocorticoid action can vary through the cell cycle. Glucocorticoids regulate epidermal growth factor (EGF) receptors in HeLa S3

cells in a reversible and cell cycle dependent manner. To be effective with regard to EGF receptor upregulation, glucocorticoids must be present during the late G1 and S phases, while they are ineffective during the G2/M and early G1 phases of the cell cycle (Fanger et al., 1986). Cells in the late G1 and S phases had consistently more nuclear glucocorticoid receptor compared to cells in the G2/M and early G1 phases. Site-specific alterations in glucocorticoid receptor phosphorylation were observed in mouse L cell fibroblasts during synchronization in the G2 phase, as compared to unsynchronized cells (Hsu et al., 1992). Together, these studies suggest that the subcellular distribution of the glucocorticoid receptor is influenced by its phosphorylation state.

2.7.5 Role of phosphorylation in DNA binding and *trans*-activation by the 1,25-dihydroxyvitamin D_3 receptor

Treatment of CV-1 cells expressing the 1,25-dihydroxyvitamin D_3 receptor with either 8Br-cAMP or okadaic acid and/or 1,25-dihydroxyvitamin D_3 resulted in *trans*-activation from a reporter gene in a receptor dependent fashion (Darwish et al., 1993). Under these conditions there was increased phosphorylation of the 1,25-dihydroxyvitamin D_3 receptor (Darwish et al., 1993). These results suggest that phosphorylation plays a central role in the *trans*-activation function of the 1,25-dihydroxyvitamin D_3 receptor. Also in COS-7 cells the 1,25-dihydroxy-vitamin D_3 receptor could be phosphorylated by protein kinase A (Jurutka et al., 1993C). The actual site(s) of 1,25-dihydroxyvitamin D_3 receptor phosphorylation by protein kinase A have not been identified. The human 1,25-dihydroxy-vitamin D_3 receptor is selectively phosphorylated by protein kinase C on serine at position 51 in CV-1 cells (Hsieh et al., 1991). This serine residue is located in a position between the two zinc fingers that constitute the DNA binding domain of the receptor. Substitution of this serine residue by a negatively

charged aspartic acid residue markedly reduced the binding of the receptor to a vitamin D-responsive element *in vitro*, and also reduced the 1,25-dihydroxyvitamin D_3 receptor-mediated transcription from a reporter gene in COS-7 cells (Hsieh et al., 1993). Substitution of serine 51 by alanine in the human 1,25-dihydroxyvitamin D_3 receptor reduced receptor phosphorylation in COS-7 cells, but preserved completely the DNA binding activity *in vitro* and also the *trans*-activation capacity. So, phosphorylation at serine residue 51 is not required for either DNA binding or *trans*-activation of the 1,25-dihydroxyvitamin D_3 receptor. It has been suggested that phosphorylation at serine residue 51 could silence the 1,25-dihydroxyvitamin D_3 receptor via a negative regulation of DNA binding when target cells are subject to protein kinase C activation events (Hsieh et al., 1993). In osteoblast-like cells the 1,25-dihydroxyvitamin D_3 stimulated synthesis of osteocalcin could be inhibited by several protein kinase C inhibitors (van Leeuwen et al., 1992), demonstrating a possible functional involvement of protein kinase C in the action of vitamin D on bone, although phosphorylation of the 1,25-dihydroxyvitamin D_3 receptor was not investigated.

2.8 Conclusions

Steroid hormone receptors are present in target cells as parts of oligomeric complexes with heat-shock proteins. The receptors are already phosphorylated in the unliganded form. After binding of their respective ligands, receptors become rapidly extra phosphorylated. Kinetic studies have revealed that this extra phosphorylation is preceded by dissociation of heat-shock proteins (notably hsp90) from the complex. Most of the phosphorylation sites identified so far, are located in the N-terminal domain. In the progesterone receptor protein one site is located in the hinge region, and in the 1,25-dihydroxyvitamin D_3 receptor one

site was reported to reside in the DNA binding domain. Serine is the main phosphorylated amino acid residue in steroid hormone receptors. Only the estrogen receptor is reported to be phosphorylated on tyrosine residues, although also phosphorylation on serine residues has been reported by various groups. Most of the sites that have been identified so far are only partially phosphorylated. This is in agreement with findings on steroid hormone receptor heterogeneity, showing that within a cell several subpopulations of receptors exist, each with different numbers or patterns of phosphorylated amino acids. Possibly differential phosphorylation of the same steroid hormone receptor molecule is involved in the regulation of different receptor functions. Most of the sites in progesterone-, glucocorticoid-, and estrogen receptors are homologous with consensus sequences for the proline-directed protein kinases. These kinases are involved in regulation of the cell cycle, suggesting a possible link between steroid hormone receptor phosphorylation and the cell cycle. The sensitivity of cells to steroid hormones could vary through the cell cycle, depending on receptor phosphorylation degree and pattern. Functions of receptors that are regulated by phosphorylation are only beginning to be investigated. Steroid hormone receptor phosphorylation might have a role in transcriptional regulation, regulation of steroid binding capacity, nucleocytoplasmic shuttling and DNA binding capacity.

Analysis of transcriptional promoters for RNA polymerase II has revealed that the precise selection of a start site and the efficiency of transcription are imparted by a variety of cis-acting elements that flank the transcription initiation site. The TATA box binds the general transcription factor TFIID-complex and directs the assembly of the general transcriptional apparatus on the promoter DNA. The efficiency of transcription initiation is affected by a group of sequence-specific DNA binding regulatory transcription factors, such as Sp1 and

CCAAT box binding proteins. Steroid hormone receptors can stimulate transcription by binding to enhancer elements at a relatively large distance from the transcriptional start site. By regulation of the activity of upstream regulatory factors, transcription is modulated in response to physiological stimuli such as hormones, growth factors, and vitamins. At present, it is not known in detail how phosphorylation influences transcriptional activation potential. Domains rich in acidic amino acid residues are often important for the activity of transcription factors, and phosphorylation might exert its effects by simply contributing extra negative charge (Ptashne, 1988). In Figure 2.2, a model for steroid hormone receptor- mediated transcription activation is depicted. The steroid hormone receptor molecule becomes extra phosphorylated in or close to a transcription activation domain after hormone binding, thereby activating transcription from a target gene promoter. Evidence for such a model is provided for the progesterone-, estrogen-, and 1,25-dihydroxyvitamin D_3 receptors (section 2.7).

Regulation of steroid hormone receptor activity via regulation of steroid hormone binding capacity would be an attractive model. However, only for the estrogen receptor some evidence for such a model has been found (see paragraph 2.6). The non- or under-phosphorylated estrogen receptor is unable to bind steroid. Only upon phosphorylation by a tyrosine protein kinase the receptor is able to bind hormone with high affinity. Phosphorylation may cause a conformational change in the ligand binding domain, enabling steroid binding. Alternatively, phosphorylation of the receptor may lead to dissociation or inactivation of a factor inhibiting steroid binding. The effect of ATP on the steroid binding capacity of the glucocorticoid receptor (paragraph 2.2) is probably due to an ATP dependent association of hsp90 to the receptor (Hu et al., 1994), and is not associated with phosphorylation of the receptor itself. Binding of hsp90 to the glucocorticoid receptor was shown to be essential for

the formation of a high affinity ligand binding receptor (Bresnick et al. 1989).

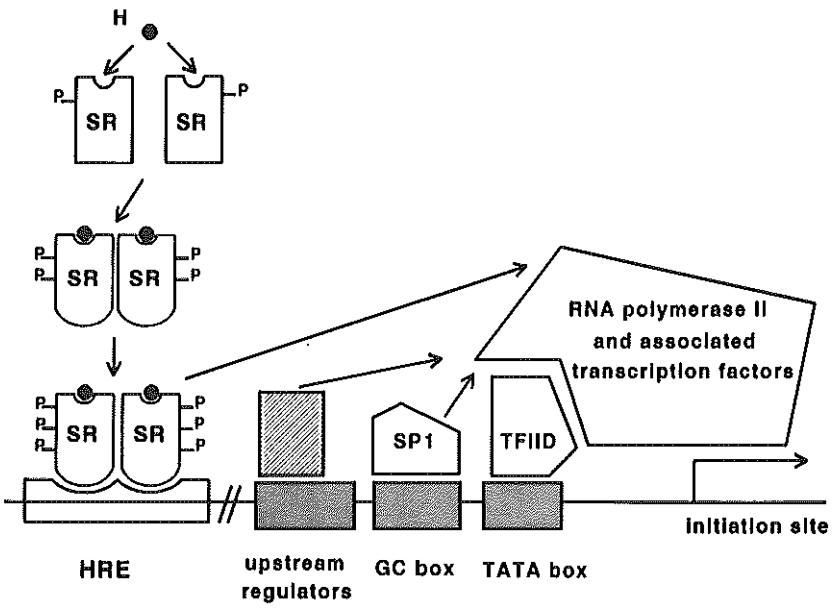

Figure 2.2. Schematic representation of steroid hormone receptor (SR) phosphorylation and transcription activation.

Depending on the cell type, steroid hormone receptors are located to a considerable extent in the cell nucleus already in the absence of hormone. Upon addition of hormone, receptors become extra phosphorylated and translocate rapidly to the cell nucleus. Steroid receptors probably can return to the cytoplasmic compartment. Only for the glucocorticoid receptor some evidence for a role of receptor phosphorylation in the regulation of receptor subcellular

location was provided (paragraph 2.7.4).

Phosphorylation of steroid hormone receptors in the DNA binding domain might influence their ability to bind to DNA. Evidence for such a model, however, is limited. Only for the 1,25-dihydroxyvitamin D_3 receptor some evidence is available for a negative effect of phosphorylation in the DNA binding domain on specific DNA binding (paragraph 2.7.5).

Future advances will depend on the identification, and elucidation of the regulation, of all kinases and phosphatases involved in steroid hormone receptor phosphorylation. Additional mutagenesis studies of phosphorylation sites might help to further elucidate and understand the consequences of the introduction of a negatively charged phosphate group for the activity of steroid hormone receptors.

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CHAPTER 3

SYNTHESIS AND POST-TRANSLATIONAL MODIFICATION OF THE ANDROGEN RECEPTOR IN LNCaP CELLS

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Summary

Androgen receptor synthesis and modification were studied in the human LNCaP cell line. Immunoblotting with a specific polyclonal antibody showed that the androgen receptor migrated as a closely spaced 110–112 kDa doublet on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels. Most of the receptor protein is present in the higher molecular mass form. Pulse labelling experiments with [^{35}S]methionine showed that the androgen receptor is synthesized as a single 110 kDa protein which is rapidly converted to a 112 kDa protein. Alkaline phosphatase treatment of cytosols from [^{35}S]methionine pulse labelled cells caused a gradual elimination of the 112 kDa isoform with a concomitant increase of the 110 kDa isoform. This indicates that the observed 110 to 112 kDa upshift of the newly synthesized androgen receptor reflects receptor phosphorylation. Both isoforms can bind hormone and can undergo a hormone dependent transformation to a tight nuclear binding form, indicating that the 110 to 112 kDa conversion is not an obligatory step for hormone binding or receptor transformation.

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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. The term 'receptor activation' describes the acquisition of the steroid binding state.

Introduction

Steroid hormone receptors are transacting gene regulating proteins, involved in the effectuation of steroid hormone induced cellular responses. Upon binding of hormone to its specific receptor, the receptor-hormone complex is known to undergo a conformational change called transformation, which is thought to precede binding of

the complex to hormone responsive elements in the target cell genome (Evans, 1988; Beato, 1989). Steroid hormone receptors are part of a dynamic system. The receptors are synthesized and degraded to maintain a steady state level in hormone untreated cells, and are up- or downregulated in hormone treated cells (Horwitz et al., 1982; McDonnell et al., 1987; Blok et al., 1989; Tilley et al., 1990). In general cellular hormone sensitivity might not only depend on changes in protein turnover rates, but also on covalent modifications that activate receptors to hormone binding states. The exact molecular details of the processes of hormone binding and receptor transformation are still unclear.

Ample evidence has been provided that steroid hormone receptors can exist as phosphoproteins in intact cells (Dougherty et al., 1984; Migliaccio et al., 1986; Sheridan et al., 1988; van Laar et al., 1990). Phosphorylation appears to be required for activation of estrogen receptors to a hormone binding state (Migliaccio et al., 1986). The receptors for progesterone, glucocorticoids and androgens are also phosphoproteins, but the function of this modification is presently unknown.

With the recent production of specific antibodies against the androgen receptor, tools were provided for more detailed analysis of the androgen receptor protein (van Laar et al., 1989; Quarmby et al., 1990). Using photoaffinity labelling and immunoblotting, it has been observed that the androgen receptor from LNCaP cells migrates as a closely spaced doublet of 110–112 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Furthermore, evidence has been obtained that the LNCaP cell androgen receptor is a phosphoprotein and that phosphorylation is stimulated by hormone (van Laar et al., 1990, 1991). The 110–112 kDa proteins might represent androgen receptor isoforms, possibly produced through various types of modification mechanisms, including phosphorylation. Phosphorylation and/or other covalent modifications of the androgen receptor protein might occur co-translationally, but it is also possible that a significant time lag could exist between synthesis and modification. In fact, apart from a co/post-translational ligand independent phosphorylation the androgen receptor does undergo ligand stimulated hyperphosphorylation (van Laar et al., 1991).

The present results obtained by [^{35}S]methionine incorporation and immunoprecipitation, show that the LNCaP cell androgen receptor is synthesized as a single 110 kDa protein which is rapidly converted into a form with a molecular mass of 112 kDa. It was also found that the conversion of the newly synthesized receptor reflects phosphorylation and is not of necessity for the acquisition of the hormone binding state and receptor transformation to the tight nuclear binding form.

Materials and methods

Materials

Radioactive methionine ([^{35}S]methionine, spec. act. > 800 Ci/mmol) was obtained from Amersham (Little Chalfont, U.K.). Culture media were obtained from Seromed (Berlin, F.R.G.). Fetal calf serum was obtained from Seralab (U.K). The synthetic androgen 17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one (R1881) was obtained from NEN (Boston, MA, U.S.A.). All other chemicals and reagents were purchased from either Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Merck (Darmstadt, F.R.G.). Phosphorylase *b* and β-galactosidase were obtained from Boehringer-Mannheim (Penzburg, F.R.G.), and labelled with [^{14}C]formaldehyde as described (Jentoft et al., 1979).

Cell culture

The LNCaP cell line (derived from a fast growing colony of a *L*ymph *N*ode *Ca*rcinoma of the *P*rostate) was a gift from Dr. Horoszewicz (Horoszewicz et al., 1983). The cells were cultured in plastic tissue culture flasks in RPMI 1640 culture medium, with added glutamine, streptomycin, penicillin and 7.5% (v/v) heat-inactivated (56°C) fetal calf serum, at 37°C in a humidified atmosphere of 5% CO_2 in air. Two to four days before an experiment, medium was replaced by the same medium, but containing 5% (v/v) heat inactivated charcoal treated fetal calf serum. LNCaP cells between the 65th and 72th passage in vitro were used.

Metabolic labelling with [^{35}S]methionine

For labelling studies, 10^7 LNCaP cells were pre-incubated for 1 h at 37°C with methionine free RPMI 1640 medium. Subsequently, [^{35}S]-methionine was added to a concentration of approximately 20 μCi/ml and the incubations were continued for different time periods at 37°C. For pulse-chase experiments, medium was removed after 30 min, the cells were washed once with phosphate buffered saline (PBS), and the incubations were continued in RPMI 1640 with 0.5 mM methionine. Incubations were stopped by removal of the medium immediately followed by a wash with PBS at 20°C.

Subsequently, the cells were lysed in buffer A (40 mM Tris-HCl pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM dithiothreitol, 10 mM Na_2MoO_4, 50 mM NaF), supplemented with 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.08% (w/v) SDS, 0.6 mM phenylmethylsulfonyl fluoride (PMSF) and 0.5 mM bacitracin at 4°C. The lysate was centrifuged (10 min, 2000 $\times g$) and androgen receptor was immunoprecipitated from the supernatant.

Preparation of cytosol and nuclear extract

Approximately 6×10^7 cells were collected in 5 ml of buffer A supplemented with 0.6 mM PMSF, 0.5 mM bacitracine and 0.2 mg/ml leupeptin. Cells were homogenized and the homogenate was centrifuged for 10 min at $800 \times g$. The cytosol was prepared by centrifugation of the supernatant at $105{,}000 \times g$ for 30 min at 4°C. The nuclear pellet was washed with buffer A containing 0.2% (v/v) Triton X-100, and then with buffer A without additions. Subsequently, the nuclear pellet was extracted with buffer A (pH 8.5) containing 0.4 M NaCl for 1 h at 4°C. The sample was centrifuged at $105{,}000 \times g$ for 30 min.

Immunoprecipitation

Immunoprecipitation was performed using mouse monoclonal antibody, raised against a synthetic peptide corresponding to the amino acid residues 301–320 of the human androgen receptor (van Laar et al., 1989). 1 μl of ascites, containing either monoclonal androgen receptor antibody or a non-specific mouse IgG, was mixed with 25 μl antimouse IgG agarose (packed gel) (Sigma) and 200 μl PBS containing 1% bovine serum albumin (BSA). The mixture was incubated for 2 h at 4°C using end-over-end rotation. Following centrifugation (20 s, $8000 \times g$), the supernatant was removed and the agarose beads were washed 3 times with buffer A.

The cell lysate, cytosol or nuclear extract was added to the agarose beads, and the mixture was incubated for 2 h at 4°C. After centrifugation for 20 s at $8000 \times g$ the pellets were washed 3 times with buffer A, containing 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate and 0.08% (w/v) SDS, 3 times with buffer A in the presence of 0.2% (v/v) Triton X-100 and 0.4 M NaCl and 3 times with buffer A only.

Electrophoresis, blotting and autoradiography

After washing of the immunoprecipitates, proteins were extracted with 50 μl sample buffer (40 mM Tris-HCl pH 6.8, 5% (v/v) glycerol, 2% (w/v) SDS, 10 mM dithiothreitol (DTT), 0.2% (w/v) bromophenol blue), boiled for 2 min and centrifuged ($5000 \times g$, 2 min). The supernatants were separated on 7% SDS-PAGE gels in the mini Protean II cell (Bio-Rad) according to the manufacturer's instructions. As marker proteins [^{14}C]β-galactosidase (116 kDa) and [^{14}C]phosphorylase *b* (97 kDa) were used. After electrophoresis the gels were positioned on nitrocellulose paper (Schleicher & Schüll (Dassel, F.R.G.), 0.45 μm) and placed in a Bio-Rad mini Trans-Blot cell filled with buffer (16.5 mM Tris, pH 8.3, 150 mM glycine and 20% v/v methanol). The transfer was performed for 60 min at 100 V. After blotting the paper was incubated with an androgen receptor specific polyclonal rabbit antiserum (SpO61) (van Laar et al., 1989), diluted 1:1000 in PBS/0.05% (v/v) Tween 20 (PBS-Tween) for 1 h at room temperature, washed 2 times for 10 min each with PBS-Tween, and incubated subsequently with alkaline phosphatase-conjugated goat antirabbit IgG (Sigma) diluted 1:1000 in PBS-Tween. After washing (2 times), the antibody complexes were visualized with a solution of 0.3% (w/v) 4-aminodiphenylamine diazonium sulphate (Sigma) and 0.1% (w/v) naphthol AS-MX phosphate (Sigma) in 0.2 M Tris-HCl, pH 9.1, 10 mM $MgCl_2$. Subsequently, the filter was air-dried and exposed to

Hyperfilm βmax (Amersham) at room temperature.

Receptor dephosphorylation

After labelling for 30 min with [^{35}S]methionine cells were suspended in TGD buffer (40 mM Tris-HCl, pH = 8.5, 10% v/v glycerol and 1 mM DTT) plus a mixture of protease inhibitors (see under Preparation of cytosol). Cells were homogenized and centrifuged (30 min, 30,000 × *g*) to obtain a cytosol. Cytosols (200 μl, protein conc. = 10 mg/ml) were incubated for 60 min at 37°C with no additions, with 100 or 200 units of calf alkaline phosphatase (Boehringer-Mannheim) or with alkaline phosphatase plus the phosphatase inhibitors 10 mM EDTA and 10 mM NaH_2PO_4. The samples were then placed on ice, diluted with buffer A (10 mM EDTA) and immunoprecipitated as described.

Results

Androgen receptor immunoblotting and pulse labelling

Immunoprecipitation with a monoclonal androgen receptor antibody and subsequent SDS-PAGE showed that the androgen receptor in LNCaP cells migrates as a closely spaced 110–112 kDa doublet (Fig. 1*A*). Most of the receptor protein was present in the higher molecular mass form according to the difference in intensity between the two bands. The androgen receptor isoforms were observed in the presence as well as in the absence of ligand, their relative amounts were constant and both proteins bound hormone as shown by photoaffinity labelling (see also van Laar et al., 1990).

In order to investigate the de novo synthesis of the two bands, LNCaP cells were incubated for various time periods with [^{35}S]methionine. In Fig. 1*B*, it is shown that, after incubation of hormone depleted LNCaP cells with radiolabelled methionine for 30 min, the amount of methionine incorporated into both isoforms was virtually the same. Upon prolonged incubation, however, a marked increase of the relative labelling intensity of the slower migrating isoform (112 kDa) was observed. The incorporation of [^{35}S]methionine in trichloroacetic acid (TCA) precipitable protein as well

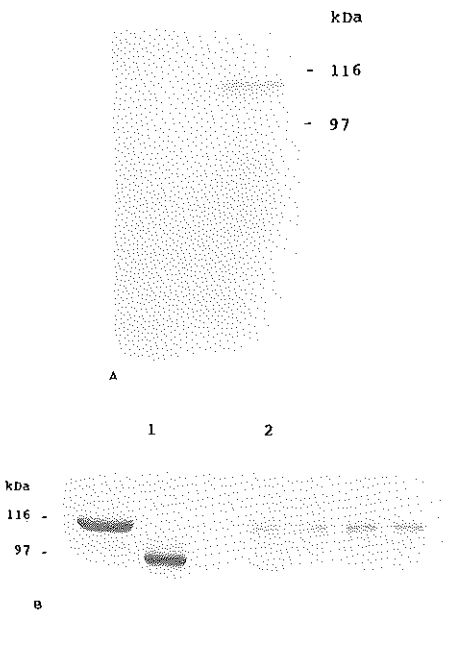

Fig. 1. *A:* Immunoblot of androgen receptor immunopurified from cell lysates of LNCaP cells. Androgen receptor was immunoprecipitated using a specific antibody (lane 2) or non-specific mouse IgG (lane 1) and subjected to SDS-PAGE and blotting. *B:* ^{35}S-Labelling of androgen receptor in LNCaP cells. LNCaP cells, pre-incubated for 60 min in methionine-free medium, were incubated for different time periods with [^{35}S]methionine. Subsequently, androgen receptor was immunoprecipitated from total cell lysates and analyzed by SDS-PAGE and blotting. Incubation with [^{35}S]methionine was for: 30 min (lane 1), 60 min (lane 2), 90 min (lane 3) and 120 min (lane 4).

as in androgen receptor protein increased linearly with time, reaching an optimum between 60 and 90 min after the start of the pulse labelling with [^{35}S]methionine (results not shown).

To investigate the apparent upshift in more detail, we performed a time study in which [^{35}S]methionine incorporation into the androgen receptor of hormone depleted LNCaP cells was investigated after 5, 10, 15, 20, 25 and 30 min of labelling. The result of this experiment is shown in Fig. 2. After 5 min, only the 110 kDa radioac-

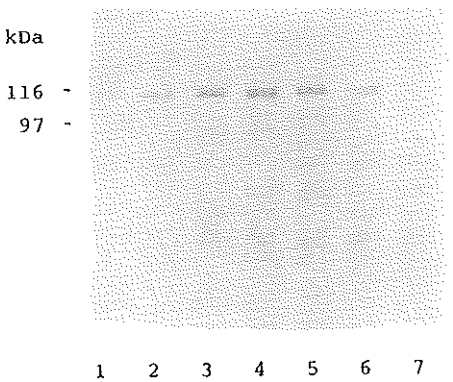

Fig. 2. Origin of the androgen receptor doublet. LNCaP cells, pre-incubated for 60 min in methionine-free medium, were incubated for different time periods with [^{35}S]methionine. Androgen receptor was immunoprecipitated using a specific antibody (lanes 1–6), or non-specific mouse IgG (lane 7) and analyzed by SDS-PAGE and blotting. Incubation with [^{35}S]methionine was for: 5 min (lane 1), 10 min (lane 2), 15 min (lane 3), 20 min (lane 4), 25 min (lane 5) and 30 min (lanes 6 and 7).

tively labelled androgen receptor protein could be detected; after longer incubation periods a gradual increase in the slower migrating androgen receptor protein (112 kDa) and no further increase in the 110 kDa protein was observed. The different kinetics of the de novo synthesis of the 110 kDa and 112 kDa androgen receptor proteins strongly suggest a time dependent conversion of the 110 kDa protein into the 112 kDa androgen receptor protein.

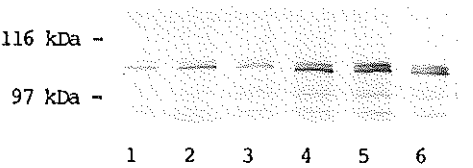

Fig. 3. Pulse labelling of androgen receptor in the presence of hormone. LNCaP cells, pre-incubated for 60 min in methionine-free medium, were incubated for different time periods with [^{35}S]methionine in the presence of 10 nM R1881. Androgen receptor was immunoprecipitated using a specific antibody and analyzed by SDS-PAGE and blotting. Incubation was for: 15 min (lane 1), 30 min (lane 2), 45 min (lane 3), 60 min (lane 4), 100 min (lane 5) and 130 min (lane 6).

Transformation of newly synthesized androgen receptor protein

In the next set of experiments, the possible influence of R1881 (methyltrienolone, a synthetic androgenic steroid) on the de novo synthesis and the conversion of the 110 kDa androgen receptor protein was investigated. To this end LNCaP cells were incubated with [^{35}S]methionine together with 10 nM R1881 for 15, 30, 45, 60, 100 and 130 min at 37°C. At each time point more radioactivity was found to be incorporated into the 110 kDa than in the 112 kDa androgen receptor protein (Fig. 3). From a comparison of the data presented in Figs. 1*B*, 2 and 3, it can be concluded that in

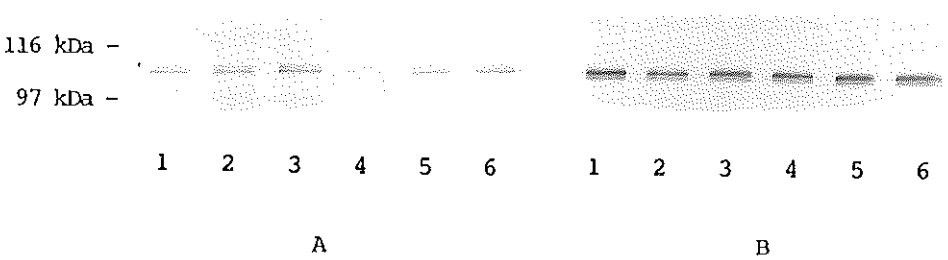

Fig. 4. Androgen receptor transformation. LNCaP cells, pre-incubated for 60 min in methionine-free medium, were incubated for different time periods with [^{35}S]methionine in the presence of 10 nM R1881. Thereafter cytosols and nuclear extracts were prepared and receptor was immunopurified as described. *A:* Autoradiogram. Lanes 1–3 cytosol, incubation was for: 10 min (lane 1), 20 min (lane 2), 30 min (lane 3). Lanes 4–6 nuclear extracts, incubation was for: 10 min (lane 4), 20 min (lane 5), 30 min (lane 6). *B:* Immunoblot. Lanes 1–3 cytosol and lanes 4–6 nuclear extracts. Incubation time periods as for *A*.

the presence of hormone the conversion may occur at a much slower rate than in the absence of hormone.

To address this point further, receptor transformation, as characterized by the acquisition of tight nuclear binding activity, was investigated as a function of time after addition of R1881. Cells were incubated with [^{35}S]methionine together with 10 nM R1881 for 10, 20 or 30 min at 37°C, and thereafter cytosols and nuclear extracts were prepared. In Fig. 4*A*, it is shown that at all time points the 110 kDa isoform was the dominant species. Upon prolonged incubation, a gradual increase of the 110 kDa protein in the nuclear extract was found, while in the cytosolic fraction the amounts are virtually constant. This indicates that the newly synthesized 110 kDa androgen receptor protein is able to bind hormone and that it can be transformed to a tight nuclear binding form immediately after synthesis. The transformed 110 kDa isoform is not further converted to the 112 kDa androgen receptor protein. From the immunoblot of Fig. 4*B* it is clear that the cytosol fraction cannot be fully depleted of receptor protein by the hormone.

Is the 112 kDa isoform also capable of undergoing transformation and is there a difference in transformation kinetics of both isoforms? In order to try to answer this question, hormone depleted LNCaP cells were incubated with [^{35}S]-methionine for 30 min. After this incubation, a chase with cold methionine was done either in the presence or absence of 10 nM R1881, for 10 and 20 min at 37°C. In Fig. 5*A* and *B* it is shown that only an extremely small amount of androgen receptor protein fractionated into the nuclear fraction obtained from cells which were incubated in the absence of hormone. During the incubation with R1881 for 10 or 20 min, both androgen receptor isoforms were able to undergo transformation to a tight nuclear binding form, showing similar transformation kinetics. A considerable amount of androgen receptor protein remained in the cytosolic fraction. This holds for the newly synthesized receptor as well as for the pre-existing receptor protein (see Figs. 4*B* and 5*B*). It is unlikely that the incomplete transformation is only caused by the short incubation time with R1881 because also after 1 h of incubation the cytosolic fraction was still not depleted (results not shown). Furthermore, it is well-known that the amount of receptor protein in nuclear extracts of LNCaP cells reaches a near optimum 15 min after addition of hormone and remains constant on longer incubations (van Laar et al., 1991).

In vitro androgen receptor dephosphorylation

It has been shown that the LNCaP cell androgen receptor is already phosphorylated in the absence of hormone and can undergo a rapid extra phosphorylation upon the addition of hormone (van Laar et al., 1991). The heterogeneity of the androgen receptor might therefore be linked to phosphorylation. To study this, LNCaP cells were incubated for 30 min with [^{35}S]methionine and subsequently a cytosolic fraction was prepared. Portions of the cytosolic fraction were incubated for 60 min at 37°C with calf alkaline phosphatase in the presence or absence of phosphatase inhibitors. Phosphatase treatment led to an almost complete elimination of the newly synthesized 112 kDa protein (Fig. 6*A*). Also pre-existing 112 kDa androgen receptor was gradually

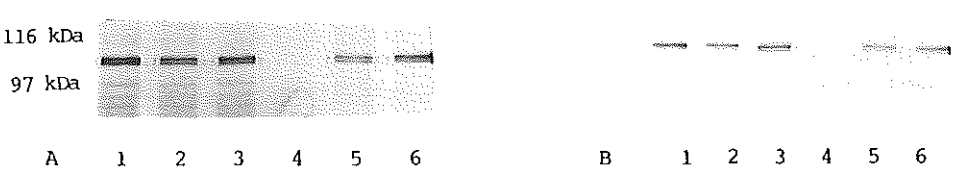

Fig. 5. Hormone responsiveness of androgen receptor. LNCaP cells, pre-incubated for 60 min in methionine-free medium, were incubated for 30 min with [^{35}S]methionine. Thereafter a chase with cold methionine was done either in the presence or absence of R1881, for 10 or 20 min at 37°C. Cytosols and nuclear extracts were prepared as described. *A:* Autoradiogram. Lanes 1–3 cytosol and lanes 4–6 nuclear extract. Incubation was for: 0 min (lanes 1 and 4), 10 min (lanes 2 and 5) or 20 min (lanes 3 and 6) in the presence of R1881. *B:* Immunoblot. Lanes 1–3 cytosol and lanes 4–6 nuclear extract. Incubation time periods as for *A*.

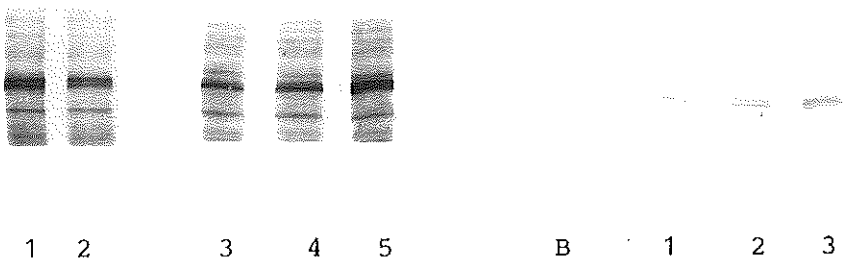

Fig. 6. Receptor dephosphorylation. Cytosols were prepared and incubated with alkaline phosphatase as described in Materials and Methods. *A:* Autoradiogram. Incubation in the absence of alkaline phosphatase (lane 1) and incubation with 100 units of alkaline phosphatase plus phosphatase inhibitors (lane 2) for 60 min at 37°C. Lane 3 incubation with 200 units phosphatase, lane 4 incubation with 100 units phosphatase and lane 5 incubation in the absence of phosphatase at 37°C for 60 min. *B:* Immunoblot. Immunoblot corresponding to *A* (lanes 3–5).

eliminated upon phosphatase treatment, with a concomitant increase of the 110 kD isoform, as seen in the immunoblot of Fig. 6*B*. Since the effect of alkaline phosphatase was blocked by the simultaneous addition of EDTA and NaH_2PO_4 (Fig. 6*A*), we conclude that the upshift in molecular weight from 110 kDa to 112 kDa is caused by receptor phosphorylation.

Discussion

The intracellular localization of steroid hormone receptors has been studied extensively (for review see Carson-Jurica et al., 1990). Steroid receptors are readily isolated from cellular cytosols in the absence of hormone, whereas after hormone treatment receptors are extractable only with salt from nuclei. With the production of specific antireceptor antibodies, the progesterone, estrogen and androgen receptors were found to be primarily nuclear in the absence of their respective ligands (King et al., 1984; Perrot-Applanat et al., 1985; Ruizeveld de Winter et al., 1990).

In the present report, it is shown that the androgen receptor is synthesized as a single protein of 110 kDa, and that a 112 kDa isoform is generated through a rapid post-translational modification. From dephosphorylation experiments with alkaline phosphatase it was concluded that the post-translational modification is in fact receptor phosphorylation. This phosphorylation occurred independent of hormone, and therefore the upshift in molecular weight reflects the hormone independent (= basal) phosphorylation of LNCaP cell androgen receptor. Recently, it was shown that administration of R1881 to LNCaP cells resulted in an increase of the amount of nuclear extractable androgen receptor and that the phosphate-to-protein ratio of androgen receptor extracted from nuclei 5, 15 and 30 min after R1881 administration to LNCAP cells was constant. This indicates that a hormone dependent phosphorylation step occurs before or during transformation to the tight nuclear binding form, but not after transformation (van Laar et al., 1991). The present results show that the 110 kDa androgen receptor protein can be transformed immediately after synthesis, upon the addition of hormone, and that the hormone dependent phosphorylation step is not reflected in a further increase in molecular mass. The 112 kDa isoform can undergo transformation upon addition of hormone as well.

The protein kinases which are involved in phosphorylation of the androgen receptor have not yet been identified. On the basis of our results, it can be suggested that the hormone independent phosphorylation occurs in the cytoplasm before translocation to the nucleus. It has been postulated for steroid receptors that the basal phosphorylation is necessary for the acquisition of hormone binding capacity (Migliaccio et al., 1986). It was shown that both isoforms of the androgen receptor can be photoaffinity labelled with [^{3}H]R1881 (van Laar et al., 1990). This would

imply that all the newly synthesized 110 kDa androgen receptor protein is phosphorylated on several sites. The phosphorylation at these sites may occur in a random fashion, whereby only one of the phosphorylations causes an apparent upshift in molecular mass. Recently it has been shown that the chicken oviduct progesterone receptor is differentially phosphorylated on three sites in the absence of hormone (Denner et al., 1990).

Another possible function for the hormone independent phosphorylation of androgen receptors might be that it serves to target the untransformed androgen receptor protein to the nucleus. In the absence of hormone androgen, receptors are only loosely bound to chromatin, and are easily extracted in the cytosolic fraction (Fig. 5*B*). Addition of ligand might result in the phosphorylation of a certain subset of androgen receptor proteins, and in the transformation to the tight nuclear binding form. Another subset of androgen receptor proteins is possibly not hyperphosphorylated upon addition of hormone, and is lost from the nucleus upon homogenization of cells. Surprisingly a rather large amount of androgen receptor protein seems to belong to this subset. In this respect, it would be interesting to compare the extent of phosphorylation of the transformed nuclear androgen receptor and the untransformed cytosolic androgen receptor after hormone stimulation of LNCaP cells.

After pulse labelling with [^{35}S]methionine of hormone depleted T47D human breast tumor cells, Sheridan et al. (1989) showed that the B form of the progesterone receptor is synthesized as a single B protein of 114 kDa. The mature B triplet is formed 6–10 h later by phosphorylation. Interestingly, phosphorylation of the newly synthesized androgen receptor in LNCaP cells was a fast process (10–30 min). The explanation for the difference in phosphorylation kinetics of the progesterone and androgen receptors in the two cell systems is not known.

Recently, the LNCaP cell androgen receptor cDNA was cloned and sequenced. One point mutation (G → A) was found near the C-terminus, which results in Thr-868 being converted to Ala in the LNCaP androgen receptor. The mutation was confirmed in several independent cDNA clones and by polymerase chain reaction of androgen receptor exon 8 in genomic LNCaP cell DNA (Harris et al., 1990; Veldscholte et al., 1990). Androgen receptor heterogeneity observed in LNCaP cells, however, is not associated with the described point mutation because the 110–112 kDa doublet is also observed after expression of wild type androgen receptor protein in COS cells (Jenster et al., manuscript in preparation).

The present investigation illustrates that androgen receptor heterogeneity in LNCaP cells is linked to receptor phosphorylation but not to the acquisition of hormone binding capacity or the hormone dependent transformation to a tight nuclear binding form.

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CHAPTER 4

LOCALIZATION AND HORMONAL STIMULATION OF PHOSPHORYLATION SITES IN THE LNCaP-CELL ANDROGEN RECEPTOR

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Localization and hormonal stimulation of phosphorylation sites in the LNCaP-cell androgen receptor

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Phosphorylation of the androgen receptor in human prostate tumour cells (LNCaP) is increased by addition of androgens to intact cells. Double-label studies, using [^{35}S]methionine incorporation into receptor protein, and [^{32}P]P_i to label metabolically receptor phosphorylation sites, have enabled us to determine the phosphate content, relative to receptor protein, of both non-transformed and transformed androgen receptors generated in intact LNCaP cells. No net change in the phosphorylation of the intact 110 kDa steroid-binding component of the androgen-receptor complex was found upon transformation to the tight nuclear binding form in the intact cell. Partial proteolysis of androgen receptor protein metabolically labelled with [^{32}P]P_i and photolabelled with [^{3}H]R1881 (methyltrienolone) revealed that phosphorylation occurs mainly in the N-terminal *trans*-activation domain, whereas no phosphorylation was detected in the steroid- and DNA-binding domains. The location of most (> 90%) of the hormonally regulated phosphorylation sites in the N-terminal *trans*-activation domain suggests a role of phosphorylation of the androgen receptor in transcription regulation.

INTRODUCTION

Steroid-hormone receptors are *trans*-acting gene-regulating proteins, involved in the accomplishment of steroid-hormone-induced cellular responses. Upon binding of hormone, the receptor–hormone complex undergoes a conformational change called transformation, which is thought to precede binding of the complex to hormone-responsive elements in the target-cell genome [1].

A putative role of receptor phosphorylation in steroid-hormone action has long been recognized. On the basis of effects of ATP on hormone binding and transformation of steroid receptors, it has been suggested that these two processes are influenced by phosphorylation/dephosphorylation events ([2], and references therein). Ample evidence has now been provided that the progesterone, glucocorticoid, oestrogen, androgen and vitamin D_3 receptors exist as phosphoproteins even in the absence of ligand [3–7]. Additional phosphorylation has been observed upon hormone binding [2]. It has been shown that basal phosphorylation is indispensable for the acquisition of ligand-binding activity of the oestradiol receptor [5]. Indirect evidence has been provided for a similar role of phosphorylation in ligand binding of several other steroid receptors [2]. Various functional roles for hormone-induced receptor phosphorylation have been proposed, e.g. dissociation of associated proteins (such as hsp 90), interaction with other transcription factors and specific binding to hormone-responsive elements [2]. All steroid receptors thus far described have multiple phosphorylation sites [8–14].

We have studied androgen-receptor heterogeneity and synthesis in the human LNCaP cell line (Lymph-Node Carcinoma of the Prostate cells) [7,15]. In these cells, the androgen receptor is a heterogeneous protein which is synthesized as a single 110 kDa protein, which becomes rapidly phosphorylated to a 112 kDa protein [15]. Metabolic labelling experiments using [^{32}P]P_i indicated that the androgen receptor is a phosphoprotein in hormone-depleted cells [7]. Immunoprecipitation of androgen receptors from total cell lysates showed an almost 2-fold increase in receptor phosphorylation within 30 min after hormone administration as compared with control cells [16].

It has also been shown that the amount of androgen-receptor protein in nuclear extracts of LNCaP cells reaches a near-optimum within 30 min after addition of hormone, whereas the cytosolic fraction is not completely depleted [15,16]. The fact that transformation to the tight nuclear binding form and hormone-induced hyperphosphorylation both reach an optimum within 30 min after addition of hormone to LNCaP cells suggests that there might be a link between both processes.

In the present study we have used monoclonal antibodies against the androgen receptor to purify cytosolic non-transformed and nuclear transformed complexes from ^{32}P-labelled and ^{35}S-labelled LNCaP cells exposed to androgens at 37 °C. Data obtained indicated that there is a similar degree of phosphorylation of the androgen receptor, before and after transformation to the tight nuclear binding form in the intact cell.

Using limited proteolysis it was established that most of the phosphorylation sites are localized in a *trans*-activation domain in the N-terminal part of the androgen-receptor protein.

MATERIALS AND METHODS

Materials

[^{35}S]Methionine (sp. radioactivity > 800 Ci/mmol) and [^{32}P]P_i (carrier free) was obtained from Amersham (Little Chalfont, Bucks., U.K.). Culture media were obtained from Seromed (Berlin, Germany). Fetal-calf serum was obtained from Serolab (U.K.). The synthetic androgen 17β-hydroxy-17α-[^{3}H]-methyl-4,9,11-oestratrien-3-one ([^{3}H]R1881; sp. radioactivity ~ 87 Ci/mmol) and unlabelled R1881 were purchased from NEN–Dupont (Dreieich, Germany).

The monoclonal antibody against the androgen receptor (F39.4.1, epitope amino acid residues 301–320) has been de-

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scribed previously [16–18]. The polyclonal antisera against the androgen receptor sp061 (epitope amino acid residues 301–320), sp066 (epitope amino acid residues 899–917) and sp197 (epitope amino acid residues 1–20) were prepared by previously published procedures [19]. The polyclonal antiserum (sp066) recognizes the 110–112 kDa androgen receptor on Western immunoblots, but is unable to interact with the native androgen receptor in solution complexed with radioactive ligand [19]. The polyclonal antisera sp197 (designed as described in [20]) and sp061 both contain high-titre antibodies against the androgen receptor, as was shown by immunoprecipitation and Western blotting [18].

All other chemicals and reagents were purchased from either Sigma Chemical Co. (St. Louis, MO, U.S.A.) or Merck (Darmstadt, Germany).

Cell culture

The LNCaP cell line was cultured as described previously [16].

Metabolic labelling with [^{35}S]methionine and/or [^{32}P]P_i

For phosphorylation studies, LNCaP cells were incubated for 4 h at 37 °C in a phosphate-free Krebs–Ringer buffer at pH 7.3 (118 mM NaCl, 4.75 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$) containing 0.2% (w/v) glucose and amino acids (minus methionine) according to the formulation of Eagle's Minimum Essential Medium, and 0.15 mCi/ml [^{32}P]P_i and 10 μCi/ml [^{35}S]methionine when appropriate.

Incubations were stopped by removal of the medium, immediately followed by a wash with PBS at 20 °C. Subsequently, the cells were lysed in buffer A [40 mM Tris/HCl, pH 7.4, 1 mM EDTA, 10% (v/v) glycerol, 10 mM dithiothreitol, 10 mM Na_2MoO_4, 50 mM NaF], supplemented with 1% (v/v) Triton X-100, 0.5% (w/v) sodium deoxycholate, 0.08% (w/v) SDS, 0.6 mM phenylmethanesulphonyl fluoride and 0.5 mM bacitracin at 4 °C. The lysate was centrifuged (30 min, 100000 *g*) and androgen receptor was immunoprecipitated from the supernatant.

Preparation of cytosol and nuclear extract

Approx. 6×10^7 cells were collected in 5 ml of buffer A supplemented with 0.6 mM phenylmethanesulphonyl fluoride, 0.5 mM bacitracin and 0.2 mg/ml leupeptin. Cells were homogenized, the homogenate was centrifuged for 10 min at 800 *g*, and the cytosol was prepared by centrifugation of the supernatant at 105000 *g* for 30 min at 4 °C. The nuclear pellet was washed with buffer A containing 0.2% Triton X-100, and then with buffer A without additions. Subsequently, the nuclear pellet was extracted with buffer A (pH 8.5) containing 0.4 M NaCl for 1 h at 4 °C. The nuclear extract was centrifuged at 105000 *g* for 30 min.

Immunoprecipitation, electrophoresis, blotting and autoradiography

These were done by previously published procedures [15,16].

Double labelling of LNCaP-cell androgen receptor with [^{3}H]R1881 and ^{32}P and cleavage with α-chymotrypsin

LNCaP cells were incubated with [^{32}P]P_i as described for 4 h. Then [^{3}H]R1881 was added to a final concentration of 10 nM and the incubation was continued for 30 min at 37 °C. After washing with ice-cold PBS, the cells were irradiated for 2 min at the surface of an u.v. trans-illuminator (wavelength 300 nm, Chromato-Vue-transilluminator; UV Products Inc., San Gabriel, CA, U.S.A.). A cell lysate was prepared, and 1 ml portions of it (corresponding to approx. 3×10^7 cells) were precipitated with the F39.4.1 monoclonal antibody against the androgen receptor, bound to goat anti-(mouse IgG)-agarose [15,16]. The pellets were transferred to a clean Eppendorf tube, and 100 μl of TEG buffer (40 mM Tris/HCl, pH 7.5, 1 mM EDTA and 10% glycerol) was added. Digestions were carried out with 0.5–1.0 μg of α-chymotrypsin (Merck; trace amounts of trypsin activity present) for 30 min at 4 °C, with constant mixing. At the end of the digestion, 25 μl of 5-fold-concentrated SDS/PAGE sample buffer was added, and the tubes were heated at 95 °C for 3 min. Samples were then centrifuged at 10000 *g* for 2 min and subjected to SDS/PAGE (11%-acrylamide gel). Lanes were cut into 2 mm slices, and the slices were incubated overnight in 1 ml of 0.1% SDS/TEG buffer. The radioactivity of 750 μl samples was determined with a Packard Tri-Carb 2500 TR liquid-scintillation counter, with a double-label setting for ^{3}H and ^{32}P.

Measurement of receptor-bound phosphate of transformed and non-transformed complexes after whole-cell incubation with R1881

Metabolic labelling of LNCaP cells with [^{32}P]P_i and [^{35}S]methionine was performed as described herein. Between 5 and 30 min before the end of the labelling period, R1881 was added to a final concentration of 10 nM and incubation was continued at 37 °C. Subsequently the cells were homogenized, and cytosolic fractions and nuclear extracts were made. After immunoprecipitation with the F39.4.1 monoclonal antibody, SDS/PAGE and blotting to nitrocellulose, the blots were incubated with the polyclonal antibody sp061 [16]. After colour development, each lane was cut into 2 mm slices and the slices were dissolved in 10 ml of Filtercount cocktail (Packard). Radioactivity was determined with a Packard Tri-Carb 2500 TR liquid-scintillation counter, with a double-label setting for ^{32}P and ^{35}S.

Labelling of LNCaP-cell androgen receptor with ^{32}P and partial proteolytic cleavage with α-chymotrypsin

LNCaP cells were incubated with [^{32}P]P_i as described herein. After labelling for 4 h, R1881 was added to a final concentration of 10 nM and the incubation was continued for 30 min at 37 °C. A total cell lysate was prepared and the androgen receptor was immunoprecipitated from 1 ml portions of the cell lysate (corresponding to about 3×10^7 cells) with the F39.4.1 monoclonal antibody against the androgen receptor bound to goat anti-(mouse IgG)-agarose [15,16]. Digestions were carried out with α-chymotrypsin (Merck) in the range 12.5–150 ng in 100 μl of TEG buffer (pH 7.4), for 30 min at 4 °C with constant mixing. At the end of digestion, SDS/PAGE sample buffer was added, and the tubes were heated at 95 °C for 3 min. Samples were then centrifuged at 10000 *g* for 2 min and subjected to SDS/PAGE (11%-acrylamide gel) and blotted on to nitrocellulose as described previously [15]. The filter was air-dried and exposed to Hyperfilm-MP (Amersham) with intensifying screens for 16–72 h at −80 °C. Thereafter blots were incubated with receptor-specific polyclonal antisera as described previously [15].

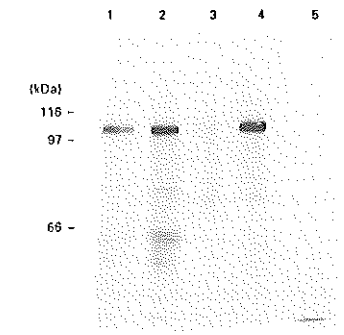

Figure 1 Hormone-dependent androgen-receptor phosphorylation and tight nuclear binding

LNCaP cells cultured for 4 h with [^{32}P]P_i were incubated in the absence (lanes 1 and 3) or presence (lanes 2 and 4) of 10 nM R1881. R1881 was added 30 min before the end of the incubation with [^{32}P]P_i. Receptors were immunoprecipitated from cytosol (lanes 1, 2 and 5) and nuclear extracts (lanes 3 and 4) with the F39.4.1 monoclonal antibody (lanes 1–4) or non-specific mouse IgG (lane 5). After SDS/PAGE on a 7% gel, the proteins were transferred to nitrocellulose. The blot was exposed to X-ray film for 18 h at −80 °C before development. Molecular mass markers (kDa) are indicated on the left.

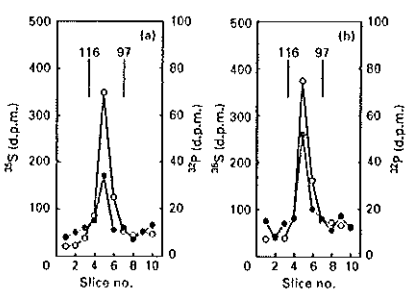

Figure 2 Hormone-dependent androgen-receptor phosphorylation

The figure shows the radioactivity in the nitrocellulose slices which contained the immunopurified receptor protein from cytosolic extracts of LNCaP cells after SDS/PAGE and immunoblotting. The cells were incubated for 4 h at 37 °C with [^{32}P]P_i and [^{35}S]methionine, (a) without or (b) with 10 nM R1881 for the last 30 min. The migration and mass (kDa) of molecular-mass markers are indicated at the top. ○, ^{35}S; ●, ^{32}P.

RESULTS

Receptor phosphorylation and tight nuclear binding

In the absence of hormone, androgen receptors are only loosely bound to chromatin, and are easily extracted in the cytosolic fraction. Upon addition of hormone, androgen receptors are transformed to a form which is tightly bound in the cell nucleus, and can only be recovered after extraction of nuclei with high salt (0.4 M NaCl). From the fact that transformation of the androgen receptor to the tight nuclear binding form and hormone-induced hyperphosphorylation both reach an optimum within 30 min after addition of hormone to LNCaP cells, it could be suggested that there is a link between both processes.

Table 1 Phosphorylation of androgen receptors in cytosolic (C) and nuclear (N) extracts

The experiment was performed as described in the legend to Figure 1. After colour development, 110 kDa receptor bands were excised and subjected to liquid-scintillation counting (see Figure 2). The amounts of each radioisotope (^{32}P and ^{35}S) associated with the androgen receptor were calculated after subtraction of the background under the peak. The ^{32}P/^{35}S ratios of two independent experiments are presented (* not determined).

Incubation time with R1881 (min)	^{32}P/^{35}S ratio			
	Expt 1		Expt 2	
	C	N	C	N
0 (no hormone)	0·11	–	0·05	–
5	0·12	–	*	–
15	0·14	0·15	0·07	0·07
30	0·19	0·19	0·11	0·11

In order to address this question, receptors were isolated by immunoprecipitation from both cytosol and nuclear salt extracts of ^{32}P-labelled LNCaP cells that were incubated at 37 °C for 30 min, in either the presence or the absence of hormone (R1881). Androgen receptor was subjected to SDS/PAGE, and blotted on to nitrocellulose. In Figure 1, an autoradiogram of such an experiment is shown. In the absence of hormone, almost all of the phosphorylated receptor is recovered in the cytosolic fraction (compare lanes 1 and 3 with lanes 2 and 4) and is therefore regarded as non-transformed. Upon addition of hormone, hyperphosphorylated androgen receptor is present in the nuclear extract (lane 4). However, not all androgen receptor becomes transformed to the tight nuclear binding form, because in the cytosolic fraction (lane 2) also hyperphosphorylated receptor is present.

The extent of receptor phosphorylation was determined in double-label experiments, using [^{35}S]methionine to measure receptor protein and [^{32}P]P_i to measure receptor phosphate. This approach enables the determination of the phosphate content, relative to the amount of receptor protein, of both the non-transformed androgen receptor and the androgen-receptor molecules that are transformed to the tight nuclear binding form in intact LNCaP cells exposed to R1881 at 37 °C. In Figures 2(a) and 2(b) the profiles of ^{35}S and ^{32}P radioactivity associated with the 110 kDa androgen-receptor protein are shown. In the presence as well as in the absence of hormone an equal amount of receptor protein was immunoprecipitated (^{35}S label). Incubation of the cells with hormone, however, increased the amount of radioactive phosphate associated with the receptor (Figure 2b). Data from two independent experiments in which cells were incubated for various time periods with R1881 are summarized in Table 1. The androgen receptor is already phosphorylated in the absence of hormone, and upon addition of hormone a 1.8-fold stimulation of phosphorylation for the cytosolic extract was observed. However, the ratio of ^{32}P to ^{35}S radioactivity specifically associated with the 110 kDa androgen-receptor protein did not change upon transformation to the tight nuclear binding form.

Localization of phosphorylation sites with regard to the functional domains of the androgen receptor

The androgen receptor from LNCaP cells after photolabelling *in situ* with [^{3}H]R1881 is a protein of 110 kDa [18,21]. Upon limited

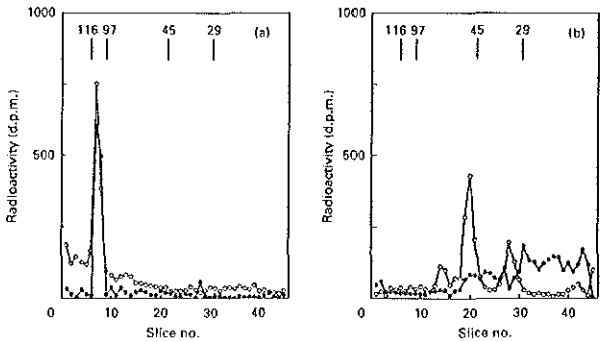

Figure 3 Chymotrypsin cleavage of ^{32}P- and ^{3}H-R1881-photolabelled androgen receptor

Androgen receptor from LNCaP cells was labelled with [^{32}P]P_i and ^{3}H-R1881. Portions of total cell lysate were immunoadsorbed to anti-(mouse IgG)–agarose with anti-androgen-receptor antibody. After the multiple wash procedure, the immunoadsorbed receptor was incubated for 30 min at 4 °C, in the absence (a) or the presence (b) of 1.0 μg of α-chymotrypsin. The digestion products were resolved by SDS/PAGE, the gel was sliced, and the radioactivity in the slices was counted for ^{32}P (●) and ^{3}H (○). The migration of molecular mass markers (kDa) is indicated at the top.

Table 2 Ratio of ^{32}P and ^{3}H-R1881 of the intact receptor and the generated chymotryptic fragments

The experiment was performed as described in the legend to Figure 3. The amounts of ^{3}H and ^{32}P radioactivity (d.p.m.) associated with each fragment was calculated from the data in Figure 3 after subtraction of background obtained.

Androgen receptor and fragments	^{32}P (d.p.m.)	^{3}H (d.p.m.)	Ratio ^{32}P/^{3}H
Intact	1067	875	1.2
~ 70 kDa fragment	18	123	0.14
~ 45 kDa fragment	85	695	0.12
~ 33 kDa fragment	15	230	0.06

proteolysis, a tryptic fragment of ~ 30 kDa containing the steroid-binding domain and a ~ 45 kDa chymotryptic fragment containing the DNA- and steroid-binding domain are generated [22]. We have now used photoaffinity labelling with [^{3}H]R1881 and metabolic labelling with [^{32}P]P_i of the LNCaP-cell androgen receptor to detect possible phosphorylation sites within the DNA- and/or ligand-binding domain. Double-labelled receptors were purified with the F39.4.1 monoclonal antibody against the androgen receptor, before proteolysis with α-chymotrypsin. Fragments were subsequently analysed by SDS/PAGE, followed by liquid-scintillation counting of gel slices. Figure 3(a) shows the result for the intact androgen receptor, and Figure 3(b) shows the result after partial digestion with α-chymotrypsin. Three ^{3}H-labelled fragments were identified: a ~ 33 kDa fragment containing the ligand-binding domain, a ~ 45 kDa fragment encompassing the DNA- and ligand-binding domain, and a fragment of ~ 70 kDa which, in addition to the ~ 45 kDa fragment, also contains part of the N-terminal domain of the receptor (see also Figure 5). All three fragments reacted on immunoblots with the sp066 antiserum, which is directed to the C-terminus of the androgen-receptor protein (epitope amino acid 899–917), indicating that the fragments contained at least the whole ligand-binding domain (result not shown). The degrees of phosphorylation of the various fragments generated by α-chymotrypsin and the intact receptor were determined and expressed as the ^{32}P/^{3}H ratio (Table 2). The ^{32}P/^{3}H ratio for the intact receptor protein was more than 10 times that for the various fragments generated containing the DNA- and steroid-binding domains.

It can be concluded that 90% or more of the phosphorylation sites are outside the DNA- and ligand-binding domain. In Figure 3(b) a large amount of ^{32}P is associated with fragments of < 29 kDa, probably proteolytic fragments of the N-terminal domain of the androgen receptor.

To analyse in more detail the proposed N-terminal domain phosphorylation of the LNCaP-cell androgen receptor, immunoprecipitated receptor was incubated with limited amounts (12.5–100 ng) of α-chymotrypsin. The partial digests of ^{32}P-labelled androgen receptor were analysed by SDS/PAGE, immunoblotting and autoradiography. The results of these experiments are shown in Figure 4. In this Figure, lane 10, containing the intact androgen receptor, displayed the presence of a single phosphorylated species of 110 kDa. Lanes 1–3 reflect the receptor preparations incubated with decreasing amounts of α-chymotrypsin. Strongly phosphorylated bands of 110 kDa, 85–90 kDa, ~ 50 kDa, ~ 40 kDa, ~ 25 kDa and a weakly phosphorylated band of ~ 15 kDa were observed.

In lanes 4–6 the corresponding immunoblot is shown after staining with the sp061 antiserum (epitope amino acid residues 301–320). All the phosphorylated bands from the autoradiogram (lanes 1–3) react with this antibody, except for the ~ 25 kDa band. Particularly interesting is the ~ 50 kDa fragment, which reacts with the sp061 antibody and is strongly phosphorylated, showing that indeed the N-terminal domain is heavily phosphorylated. This ~ 50 kDa fragment also reacts on blots with the sp197 antiserum, which is directed against an epitope at the N-terminus (amino acid residues 1–20), showing that this fragment encompasses the whole N-terminal domain of the receptor (see lanes 8 and 9). The ~ 50 kDa fragment does not react with the sp066 antiserum (epitope amino acid residues 899–917; result not shown). On the immunoblot an additional fragment of ~ 70 kDa is not phosphorylated (cf. lanes 3 and 6).

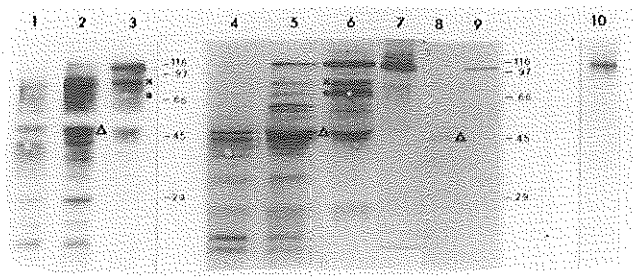

Figure 4 Proteolytic digestion of phosphorylated androgen receptors with α-chymotrypsin

Androgen receptor was labelled with [^{32}P]P_i, immunopurified from total cell lysates and digested with α-chymotrypsin. The digestion products were resolved by SDS/PAGE and the proteins were transferred to nitrocellulose. Lanes 1–3 show the autoradiogram of equal amounts of receptor preparations digested for 30 min at 4 °C with 100 ng (lane 1), 50 ng (lane 2) and 12.5 ng (lane 3) of α-chymotrypsin. Lanes 4–6 show the corresponding blot after staining with the sp061 antiserum. Lane 10 shows the autoradiogram of intact androgen receptor (no protease) and lane 7 the corresponding immunoblot. Lanes 8 and 9 show the immunoblot after staining with the sp197 antiserum, for the undigested androgen receptor (lane 9) and the receptor preparation digested with α-chymotrypsin (lane 8). Molecular-mass markers (kDa) are indicated. The symbols used indicate the fragments depicted in Figure 5.

This is most likely identical with the ~ 70 kDa fragment shown in Figure 3(b), which is essentially non-phosphorylated. In addition, a strongly phosphorylated fragment of 85–90 kDa was observed (cf. lanes 3 and 6). This fragment reacts on immunoblots with the sp061 antibody (lane 6) and also with the sp066 antiserum directed against an epitope at the C-terminus (amino acid residues 899–917; result not shown).

From these results, it is very likely that the main phosphorylation sites of the LNCaP-cell androgen receptor are located in the N-terminal domain, probably in the region of amino acid residues 1–300 (Figure 5).

Partial proteolysis of phosphorylated androgen receptor from control cells (not incubated in the presence of hormone) revealed a similar phosphopeptide pattern to that shown in Figure 4 for receptor in the presence of hormone. The only difference was the less strong labelling intensity of the intact receptor and the proteolytic fragments.

DISCUSSION

In the present study we have compared the degree of phosphorylation, in hormone-treated cells, of cytosolic receptors and 0.4 M NaCl-extracted receptors from nuclei. It was concluded that there is no net change in degree of phosphorylation between non-transformed receptor and receptor transformed to the tight nuclear binding form. This is analogous to results obtained for the glucocorticoid receptor in mouse L cells [23] and WEHI-7 cells [24].

Since our methods only measure the total amount of phosphate associated with the receptor, it is still possible that these phosphates are rearranged upon transformation. For the progesterone receptor in T47D cells, it has been shown that there are at least two different phosphopeptides in the transformed nuclear receptor, compared with the cytosolic non-transformed receptor [25]. Studies on phosphorylation of progesterone, glucocorticoid, 1,25-dihydroxyvitamin D_3 and oestrogen receptors in various systems provided evidence for hormone-dependent extra phosphorylation [6,12,14,26–29].

Also in the present study, using double labelling with [^{32}P]P_i and [^{35}S]methionine, we show that there is an almost 2-fold increase in degree of receptor phosphorylation on addition of hormone. This accounts for the cytosolic as well as for the nuclear-extractable androgen receptor. This hormone-induced extra phosphorylation is a rapid process, reaching an optimum within 30 min. The half-life of the androgen receptor in LNCaP cells is 2–3 h, in the presence as well as in the absence of ligand [16,30]. Only after longer incubations of LNCaP cells with androgens (24 h or longer) could a significant increase of androgen receptor protein be detected [31]. A possible stabilizing effect of androgens on receptor protein, mimicking receptor hyperphosphorylation, as recently shown for the androgen receptor in COS cells [32], can therefore be excluded.

Figure 5 presents a model for the location of phosphorylation sites with respect to the functional domains of the androgen receptor. It is possible that, when the receptor is cleaved by a protease, certain fragments become more sensitive to dephosphorylation, thereby giving the false impression that a certain fragment is not phosphorylated. However, digestions were carried out with immunoadsorbed receptor, which had been

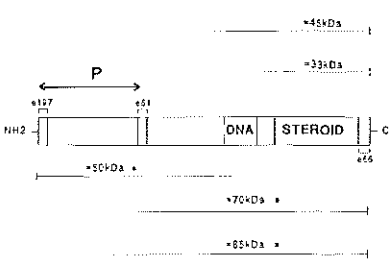

Figure 5 Model of phosphorylated region and functional domains of the human androgen receptor

Positions of the functional domains of the 110 kDa human androgen receptor (917 amino acid residues) are as described previously [17]. The epitopes (ε197, ε61 and ε66) for the anti-peptide antisera used are indicated. The continuous lines represent the various chymotryptic fragments generated in this study. The positions of these fragments are based on previous work [22] and on probing with the indicated anti-peptide antisera. Where boundaries are not exactly known, lines are dashed. Fragments indicated by symbols correspond to fragments indicated by the same symbols in Figure 4. The phosphorylated region (see the Discussion section) is indicated by an arrow and the letter 'P'.

thoroughly washed free of cellular constituents, and we regard this possibility as highly unlikely. The chymotryptic fragments of ~45 kDa (Figure 3) and ~50 kDa (Figure 4) react with polyclonal antibodies directed against the C- and N-terminus respectively. The possibility of missing phosphorylated sites in both termini can therefore be excluded. The predominant localization of phosphorylation sites in the N-terminal domain of the androgen receptor is consistent with reports on progesterone and glucocorticoid receptors [8–12]. Recently seven phosphorylated sites were identified in the N-terminus (between amino acids 120 and 320) of the mouse glucocorticoid receptor [13]. Also, after transfection of progesterone [33] and glucocorticoid [12] receptors into COS or CV-1 cells, it was shown that all phosphorylated sites were located in the N-terminal domain.

It is noteworthy that the region shown to be heavily phosphorylated (amino acid residues 1–300) has also been shown to be essential for transcriptional activation [17]. A deletion mutant lacking amino acids 51–211 (pAR7) showed a strongly decreased transcriptional activation capacity, which was only 5% of that of the wild-type androgen receptor. The wild-type androgen receptor expressed in COS-1 cells migrated as a 110–112 kDa doublet when analysed by SDS/PAGE. This doublet represents androgen-receptor isoforms produced by phosphorylation [15,17,30]. The deletion mutant pAR7, however, migrated as a single band with an apparent molecular mass of ~84 kDa after expression in COS cells [17]. It is tempting to speculate that in the deleted region one or more serine or threonine residues become phosphorylated in the wild-type androgen receptor, giving rise to the described doublet.

A possible mechanism by which phosphorylation might modulate transcription is by altering the transcriptional activation potential of a transcription factor. Evidence for this type of regulation has been provided by studies in which a correlation was found between the phosphorylation state of certain transcription factors and their ability to activate transcription, despite phosphorylation having no effect on the ability of these factors to bind to DNA [34]. Such a model could also be applied to members of the steroid-receptor superfamily. No definitive evidence has been provided so far that phosphorylation of steroid receptors directly influences DNA binding capacity [2]. Also, in the present study we have shown that there is no net change in the degree of receptor phosphorylation upon transformation in the intact cell.

The most definitive indication for a role of phosphorylation in regulating members of the receptor superfamily of transcription factors has been provided for the progesterone receptor and the v-*erb* A protein [35,36]. It was shown by site-directed mutagenesis that phosphorylation of a particular serine residue in the N-terminal part of v-*erb* A regulates biological activity, without affecting nuclear localization or DNA-binding capacity [35]. The progesterone receptor in the chicken oviduct is a phosphoprotein, and treatment with progesterone *in vivo* stimulates phosphorylation of the receptor [14]. In CV-1 cells transfected with the chicken progesterone receptor, it was shown that treatment of cells with kinase activators or phosphatase inhibitors mimicked progesterone-dependent receptor-mediated transcription in the absence of progesterone [36]. This suggested that phosphorylation of the progesterone receptor or other proteins in the transcription complex modulate progesterone-receptor-mediated transcription.

The N-terminal receptor region defined here to be predominantly phosphorylated in the LNCaP-cell androgen receptor contains a total of 12 potential phosphorylation sites for Ser-Pro-directed kinase, casein kinases and double-stranded-DNA-dependent kinase, on the basis of published consensus sequences [37]. Identification of the actual sites of phosphorylation should allow definitive determination of the role of phosphorylation in the mode of action of the androgen receptor.

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CHAPTER 5

PHOSPHOTRYPTIC PEPTIDE ANALYSIS OF THE HUMAN ANDROGEN RECEPTOR: DETECTION OF A HORMONE-INDUCED PHOSPHOPEPTIDE

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Phosphotryptic Peptide Analysis of the Human Androgen Receptor: Detection of a Hormone-Induced Phosphopeptide†

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ABSTRACT: Phosphorylation of the androgen receptor (AR) in human prostate tumor cells (LNCaP) is increased by androgens. The AR is expressed as two isoforms with apparent molecular masses of 110 and 112 kDa. Metabolic labeling experiments with [^{32}P]orthophosphate revealed that only the 112 kDa isoform is radioactively labeled. Phosphoamino acid analysis revealed only phosphorylation on serine residues. Phosphotryptic peptide analysis of human AR protein by two-dimensional peptide mapping and by reverse-phase HPLC showed phosphorylation at multiple sites. Comparison of phosphopeptide maps of AR protein from cells incubated in the absence or presence of the synthetic androgen R1881 indicated that the ligand-stimulated phosphorylation is probably due to induction of phosphorylation at a new site rather than increased phosphorylation at an existing site. This result suggests that hormone-dependent AR phosphorylation might play a role in the signal transduction pathway of androgens.

Steroid hormone receptors are transacting gene-regulatory proteins, involved in the accomplishment of steroid hormone-induced cellular responses. Upon binding of hormone, the receptor hormone complex undergoes a conformational change called transformation, which is thought to precede binding of the receptor to hormone-responsive elements in the target cell genome (Bagchi et al., 1992; Jensen, 1991).

Ample evidence has been provided that the progesterone, glucocorticoid, estrogen, androgen, and 1,25-dihydroxyvitamin D_3 receptors are phosphoproteins (Moudgil, 1990; Orti et al., 1992; Kuiper & Brinkmann, 1994). Additional phosphorylation has been observed upon hormone binding. All steroid receptors investigated thus far have multiple phosphorylation sites (Moudgil, 1990; Orti et al., 1992). Steroid hormone receptor phosphorylation has been directly implicated in activation of hormone binding capacity, nuclear–cytoplasmic shuttling of steroid receptors, modulation of binding to hormone response elements, and regulation of receptor transactivation function (Takimoto & Horwitz, 1993; Kuiper & Brinkmann, 1994).

Previously we have studied androgen receptor (AR)[1] synthesis and heterogeneity in the human LNCaP cell line (Van Laar et al., 1990; Kuiper et al., 1991). In these cells, the AR is synthesized as a protein with an apparent molecular mass of 110 kDa, which becomes rapidly phosphorylated, resulting in an upshift to 112 kDa on SDS–PAGE (Kuiper et al., 1991). In addition, metabolic labeling experiments using [^{32}P]P_i indicated that the AR is a phosphoprotein in hormone-depleted LNCaP cells, and also in COS cells after transfection with an AR expression vector (Van Laar et al., 1990; Kemppainen et al., 1992). Upon addition of hormone to LNCaP cells, a rapid 2-fold increase in AR phosphorylation degree was observed (Van Laar et al., 1991; Kuiper et al., 1993). Partial proteolysis of AR protein labeled with [^{32}P]P_i revealed that phosphorylation occurs mainly in the N-terminal transactivation domain (Kuiper et al., 1993). Also progesterone, glucocorticoid, and estrogen receptors are predominantly phosphorylated in the N-terminal transcription activation domain, suggesting that phosphorylation might play a role in regulation of receptor transactivation function (Orti et al., 1992; Kuiper & Brinkmann, 1994).

The N-terminal region of the AR, found to be predominantly phosphorylated, contains 12 potential phosphorylation sites for Ser/Thr-Pro-directed kinases, casein kinases, and double-stranded DNA-dependent kinase, on the basis of published consensus sequences (Kennelly & Krebs, 1991; Finnie et al., 1993; Kuiper et al., 1993). These kinases have been shown to phosphorylate various transcription factors and are supposed to be involved in the regulation of transcription factor activity (Hunter & Karin, 1992). No data are available on the number of AR phosphorylation sites, their regulation by hormone, and the kinases involved. In the present study, we have used monoclonal antibodies against the AR to purify receptor protein from total cell lysates of LNCaP cells, after metabolic labeling with [^{32}P]-P_i. The purified AR protein was digested with trypsin, to estimate the number of phosphorylation sites and the effect of hormone incubation on the various sites. The resulting phosphopeptides were resolved by 2D phosphopeptide mapping and by reverse-phase HPLC. It is described herein that the AR contains multiple phosphorylation sites and that a new phosphopeptide is detectable upon hormone treatment, indicating the induction of phosphorylation at a site which

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[1] Abbreviations: TFA, trifluoroacetic acid; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; DTT, dithiothreitol; HPLC, high-performance liquid chromatography; AR, androgen receptor; LNCaP, lymph node carcinoma of the prostate; MEM, minimum essential medium; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; TLCK, *N*-tosyl-L-lysine chloromethyl ketone; R1881, 17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one; DOC, sodium deoxycholate; PMSF, phenylmethanesulfonyl fluoride; BSA, bovine serum albumin; TCA, trichloroacetic acid.

is not phosphorylated in AR from cells incubated in the absence of hormone.

MATERIALS AND METHODS

Materials. [^{35}S]Methionine (> 1000 Ci/mmol) and [^{32}P]-orthophosphate (carrier free) were obtained from Amersham (Little Chalfont, Bucks, U.K.). Culture media were obtained from Gibco (Life Technologies, Breda, The Netherlands). The synthetic androgen 17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one (R1881) was purchased from NEN—Dupont ('s Hertogenbosch, The Netherlands). The monoclonal antibody against the AR (F39.4.1, epitope amino acid residues 301—320) and the polyclonal antiserum sp061 have been described previously (Van Laar et al., 1989, 1990). Trypsin (TPCK-treated) was from Sigma (St. Louis, MO), and bovine α-chymotrypsin was from Merck (Darmstadt, Germany). Sequencing-grade endoproteinase Glu-C (V8 protease) and TLCK (trypsin inhibitor) were obtained from Boehringer Mannheim (Germany). Thin-layer cellulose plates and all solvents for 2D phosphopeptide mapping were obtained from Merck. HPLC reagents were from Merck, and from J. T. Baker Chemical Co. (Phillipsburg, NY). All other chemicals and reagents were from commercial sources and high purity.

Cell Culture. The LNCaP cell line was cultured as described previously (Van Laar et al., 1991). LnCaP cells between the 66th and 74th passage in vitro were used for the present studies.

Metabolic Labeling. For phosphorylation studies, LNCaP cells were incubated for 12—16 h at 37 °C in phosphate-free MEM (Sigma) with 0.25 mCi/mL [^{32}P]orthophosphate, in the presence or absence of 10 nM R1881. Each 175 cm^2 flask contained about 7 × 10^7 cells in 10 mL of medium, and was kept in a humidified atmosphere of 5% CO_2 in air. For labeling with [^{35}S]methionine, 7 × 10^7 LNCaP cells were incubated for 60 min at 37 °C with 20 μCi/mL [^{35}S]-methionine in 5 mL of methionine-free RPMI 1640 medium. Incubations were stopped by removal of the medium, immediately followed by a wash with PBS at 20 °C.

Preparation of Cellular Lysates. Cells were lysed at 6—8 °C with 1.5 mL per flask of lysis buffer [40 mM Tris-HCl (pH 7.4), 5 mM EDTA, 10 mM sodium phosphate, 10 mM DTT, 10% v/v glycerol, 10 mM sodium molybdate, 50 mM NaF, 0.5 mM sodium orthovanadate, 1% Triton X-100, 0.5% w/v DOC, 0.08% SDS, 0.6 mM PMSF, and 0.5 mM bacitracin]. The lysate was cleared by centrifugation (30 min, 100000g) at 6 °C.

Immunoprecipitation and Gel Electrophoresis. Cell lysates were immunoprecipitated with anti-AR F39.4.1 monoclonal antibody linked to agarose beads (Van Laar et al., 1991). Typically, 1.5 mL portions of lysate were incubated with 25 μL (packed gel) of F39—agarose (~1 μL of F39 ascites) for 2 h at 4 °C with gentle shaking. As a control, lysates were immunoprecipitated with anti-PSA (prostate-specific antigen) monoclonal antibody linked to agarose beads. The agarose beads were then washed extensively with detergent and salt-containing buffers, as described previously (Kuiper et al., 1991). Proteins were extracted with sample buffer [40 mM Tris-HCl (pH 6.8), 5% (v/v) glycerol, 2% (w/v) SDS, and 10 mM DTT], boiled for 3 min, and centrifuged. The supernatants were separated on 7% SDS—PAGE gels in a mini Protean II cell (Bio-Rad) and subsequently blotted to nitrocellulose as described previously (Kuiper et al., 1991). The nitrocellulose membrane was dried for 30 min or longer, and incubations with polyclonal AR antiserum sp061 and secondary alkaline phosphatase-conjugated antibodies were done as described (Kuiper et al., 1991). The blot was air-dried and autoradiographed using Amersham Hyperfilm MP.

Generation of Phosphopeptides and Two-Dimensional Separation. Immunopurified receptors were separated on SDS—PAGE gels as described. The gels were washed with water and dried during 12 h. Dried gels were autoradiographed, and the autoradiogram was used as a guide to excise AR protein. Dried gel slices containing ^{32}P-labeled AR (equivalent to one flask; see Metabolic Labeling) were rehydrated in 0.75 mL of 50 mM NH_4HCO_3 (pH 8.0) and incubated for 16 h at 37 °C with 50 μg of TPCK-treated trypsin. Three more additions of 25 μg of trypsin each were made with 6—12 h intervals. The fractions were combined and lyophilized. For two-dimensional separation, tryptic phosphopeptides were separated first on thin-layer cellulose plates, by electrophoresis at 500 V for 45 min at room temperature in 1% w/v ammonium carbonate buffer (pH 9.1) in a chamber as described by Gracy et al. (1977). After drying, chromatography in the second dimension was done for 6—8 h at room temperature in butanol/isobutyric acid/pyridine/glacial acetic acid/water (2:65:5:3:29) (Scheidtmann et al., 1982). Plates were dried and subjected to autoradiography for 3—5 weeks at −70 °C.

HPLC Analysis. Immunopurified AR proteins from five to seven flasks of LNCaP cells (total 10—15 mCi of [^{32}P]-orthophosphate) were separated on SDS—PAGE gels and treated with trypsin as described. After lyophilization, the phosphopeptides were taken up in 30 μL of 0.1% TFA. Peptides were separated by reverse-phase HPLC using a 2 × 150 mm Waters Delta Pak C18 column (Waters Chromatography Division, Millipore Corp., Milford, MA). The HPLC equipment consisted of a Waters 625LC system with a 600E system controller and a Waters 486 UV detector. The sample (25 μL) was applied on the column in 0.1% TFA, and a linear gradient of 0—60% acetonitrile in 0.1% TFA was generated in 240 min. The flow rate was 0.18 mL/min, and fractions were collected every 1.5 min with an ISCO Retriever II fraction collector. Radioactivity of the fractions was determined by Cerenkov counting in a Packard 2500 TR counter (approximately 30% efficiency).

Phosphoamino Acid Analysis. Immunopurified AR protein was eluted from the F39 mAb—agarose beads with 0.75 mL of buffer [50 mM NH_4HCO_3 (pH 8.0)/0.1% SDS/10 mM DTT] for 3 min at 90 °C. After addition of 100 μg of BSA as carrier protein, precipitation occurred with 20% w/v TCA on ice. The precipitate was dissolved in 100 μL of 6 N ultrapure HCl (Pierce Chemical Co., Rockford, IL) and hydrolyzed for 1 h at 110 °C. After repeated lyophilization steps, the sample was resuspended in 10 μL of bidest containing 2 mg/mL phosphoamino acid standards each (Sigma) and spotted on a cellulose thin-layer plate. Two-dimensional electrophoretic separation of the partial hydrolysis products was done at pH 1.9 in formic acid/acetic acid/bidest (50:156:1794), and at pH 3.5 in acetic acid/pyridine/bidest (100:10:1890), for 90 min at 500 V in each dimension (van der Geer et al., 1993). Plates were autoradiographed for 1—2 weeks at −70 °C.

Stoichiometry of Phosphorylation. These experiments were performed as described for pp60$^{v\text{-}src}$ and phospholipase C-γ (Meisenhelder et al., 1989; Sefton et al., 1982; Sefton, 1991). In brief, (2–3) × 10^7 LNCaP cells were incubated for 24 h in 11 mL of a mixture of methionine-free and phosphate-free MEM/phosphate-free MEM (80:20), supplemented with $NaH_2PO_4{\cdot}H_2O$ to a final concentration of 0.95 mM. To this medium was added either approximately 100 μCi of [^{35}S]methionine or 4 mCi of [^{32}P]orthophosphate. The specific activity of the phosphate in the medium was calculated from the measured amount of ^{32}P in the medium at the end of the incubation and the known amount of phosphate. Cells were lysed at 6–8 °C, and the AR protein was immunoprecipitated as described and separated on a 7% SDS–PAGE gel. Following autoradiography, either the ^{35}S- or the ^{32}P-labeled AR bands were cut out from the dried gel and solubilized with Soluene (Packard) at 60 °C for 4 h before being counted in scintillation fluid. Total cellular protein was measured in a sample of the lysate by the Bradford dye method, using BSA as standard (Rubin & Warren, 1977). The radioactivity (^{35}S) of the cellular protein was measured in a fraction of the lysate, by TCA precipitation of protein onto a glass fiber filter which was counted in parallel with the ^{35}S-labeled gel bands.

RESULTS

Androgen Receptor Protein Heterogeneity and Phosphorylation. Pulse-labeling studies with [^{35}S]methionine showed that the AR is synthesized as a 100 kDa protein which is rapidly converted to a 112 kDa protein. Alkaline phosphatase treatment of cytosols either from LNCaP cells or from COS cells transiently expressing AR protein caused an elimination of the 112 kDa isoform with a concomitant increase of the 110 kDa isoform (Kuiper et al., 1991; G. Jenster et al., submitted for publication). These experiments indicated that the 112 kDa AR isoform is phosphorylated, but did not provide information on the phosphorylation status of the 110 kDa AR isoform. Therefore, LNCaP cells were metabolically labeled with [^{35}S]methionine for 1 h, and the AR protein was immunoextracted and separated on SDS–PAGE gels. The autoradiogram as well as the immunoblot showed an AR doublet of 110–112 kDa molecular mass, indicating that de novo synthesized as well as preexisting AR proteins exist as two isoforms in LNCaP cells (Figure 1, lanes 2 and 4). In a similar experiment, LNCaP cells were metabolically labeled with [^{32}P]orthophosphate for 16 h. The half-life of the AR protein in LNCaP cells is between 2 and 3 h, allowing the turnover of essentially all AR molecules during 16 h incubation (Van Laar et al., 1991). The autoradiogram (Figure 1, lane 6) showed only one radioactively labeled band, comigrating with the 112 kDa AR isoform. Also after incubation of cells with 10 nM of the synthetic androgen R1881, only the 112 kDa band was radioactively labeled (Figure 1, lane 7). From this experiment, we concluded that the 112 kDa AR isoform represents phosphorylated AR and that the 110 kDa AR isoform represents nonphosphorylated AR protein. From the immunoblots (Figure 1, lanes 1 and 2), it is not possible to calculate the ratio between both AR isoforms.

In an attempt to determine the extent to which AR protein is phosphorylated in LNCaP cells, we labeled cells for 24 h with either [^{35}S]methionine or [^{32}P]orthophosphate, and isolated AR protein by immunoprecipitation and SDS–PAGE. The number of moles of phosphate incorporated per mole of AR protein was calculated, with the assumptions that (1) after 24 h the specific activity of the phosphoamino acids in AR protein is the same as that of the phosphate in the labeling medium and that (2) the specific activity of the methionine in AR protein is the same as that in bulk cellular protein (see Materials and Methods). It was calculated that the AR protein contains 1.0 mol of phosphate per mole of protein (n = 3) when the LNCaP cells were not incubated with R1881 (see Table 1). Since only an aggregate value was measured, we cannot calculate exactly which fraction of the total population of AR molecules is phosphorylated. Since also after R1881 incubation of LNCaP cells the AR protein is detected as a 110–112 kDa doublet on immunoblots (Kuiper et al., 1991), we conclude that a significant fraction of AR molecules remains nonphosphorylated (110 kDa isoform) in the presence of hormone.

Phosphoamino Acid Analysis of AR Protein. In order to obtain some information on the type of kinases which phosphorylate human AR protein, we analyzed the phosphoamino acids of AR protein isolated from LNCaP cells incubated in the presence or absence of R1881. Conditions of acid hydrolysis (1 h) were optimal for the preservation of phosphoamino acids, especially phosphotyrosine (Duclos et al., 1991). After two-dimensional electrophoresis of the acid hydrolysate of immunopurified AR protein from cells incubated with 10 nM R1881, only phosphorylation on serine

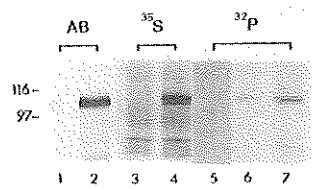

FIGURE 1: AR protein heterogeneity and phosphorylation. LNCaP cells were incubated for 16 h with [^{32}P]orthophosphate (lanes 5, 6, and 7), in the presence (lane 7) or absence (lanes 5 and 6) of 10 nM R1881. Another set of cells was incubated with [^{35}S]methionine for 1 h (lanes 1–4). Receptors immunoprecipitated from total cell lysates with the F39 monoclonal antibody (lanes 2, 4, 6, and 7) or nonspecific mouse IgG (lanes 1, 3, and 5). After SDS–PAGE on a 7% gel, the proteins were transferred to a nitrocellulose membrane and incubated with polyclonal AR antiserum sp061 and secondary alkaline phosphatase-conjugated antibodies (AB), as described (lanes 1 and 2). The gels prepared from the LNCaP cells incubated with [^{35}S]methionine (lanes 3 and 4) or [^{32}P]orthophosphate (lanes 5–7) were autoradiographed. Molecular mass markers (kDa) are indicated on the left.

Table 1: Stoichiometry[a] of AR Phosphorylation in LNCaP Cells

	flask				
	1	2	3	4	$X \pm SD^e$
^{32}P (cpm)	1180	1058	894		
^{32}P SA[a] (cpm/pmol)	660	659	614		
P in AR (pmol)	1.78	1.61	1.46		1.62 ± 0.16 (n = 3)
^{35}S (dpm)	323	481	666	431	
^{35}SSA[b] (dpm/μg)	2790	3022	2982	3216	
AR[c] (pmol)	1.18	1.62	2.28	1.37	1.61 ± 0.47 (n = 4)

[a] Specific activity per picomole of phosphate. [b] Specific activity per microgram of protein. [c] For the conversion of micrograms of AR protein to picomoles of AR protein, it was assumed that 10 pmol of AR protein equals 0.98 μg of AR protein. [d] Stoichiometry = (P in AR)/(AR) = (1.62 pmol)/(1.61 pmol) = 1.0 mol of P/mol of AR protein. [e] Mean ± standard deviation.

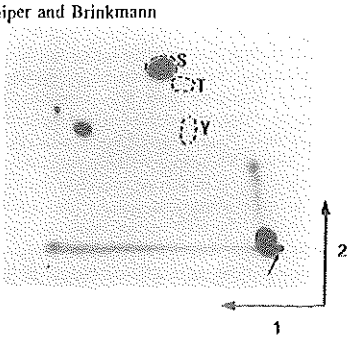

FIGURE 2: Phosphoamino acid analysis of AR protein. LNCaP cells were incubated for 16 h with [^{32}P]orthophosphate, and subsequently AR protein was immunopurified as described under Materials and Methods. The ^{32}P-labeled AR protein was subjected to partial acid hydrolysis (6 N HCl, 1 h at 110 °C). Partial hydrolysis products were mixed with unlabeled phosphoamino acid standards and resolved on thin-layer cellulose plates by electrophoresis at pH 1.9 (first dimension, 1) prior to electrophoresis at pH 3.5 (second dimension, 2). Unlabeled phosphoamino acids were visualized by ninhydrin staining and ^{32}P-labeled phosphoamino acids by autoradiography. The position of the phosphoserine (S), phosphothreonine (T), and phosphotyrosine (Y) standards is indicated. The origin is indicated by a small arrow.

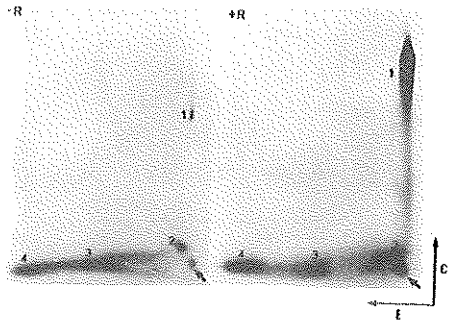

FIGURE 3: Two-dimensional phosphopeptide maps of trypsin-digested AR protein. AR protein was labeled in LNCaP cells with [^{32}P]orthophosphate during an incubation for 12 h, in the presence or absence of 10 nM R1881, and subsequently immunoprecipitated from total cell lysates. The proteins were resolved on a 7% SDS–PAGE gel (see Figure 1, lanes 6 and 7), and phosphoproteins were detected by autoradiography of the dried gel. The labeled AR protein was excised from the gel and digested with trypsin. Two-dimensional maps of the phosphopeptides were prepared on thin-layer cellulose plates through electrophoresis (E, anode on the left) and chromatography (C), and described under Materials and Methods. Shown are the autoradiograms (3 weeks exposure at −70 °C) of AR protein from cells incubated in the presence of 10 nM R1881 (+R) or without R1881 (−R). The origins are indicated by arrows.

residues was observed (see Figure 2). During one-dimensional electrophoresis, there was, apart from ^{32}P activity comigrating with the phosphoserine standard, also ^{32}P activity wholly or partially overlapping with the phosphotyrosine and phosphothreonine standards (result not shown). However, during 2D electrophoresis, this ^{32}P activity separated from the phosphotyrosine and phosphothreonine standards (see Figure 2), and probably results from nucleoside monophosphates, arising from RNA degradation during acid hydrolysis (Duclos et al., 1991). Also when AR protein was isolated from cells incubated without R1881, or after elution of intact AR protein from SDS–PAGE gels, only phosphorylation on serine residues was detected (not shown).

Peptide Analysis of the ^{32}P-Labeled Androgen Receptor. In previous studies, it was shown that the phosphorylation degree of the AR protein in LNCaP cells increases 2-fold upon incubation of cells with R1881 and that predominantly the N-terminal domain is phosphorylated (Van Laar et al., 1991; Kuiper et al., 1993). In order to obtain information on the number of AR phosphorylation sites and the effect of hormone treatment on individual sites, two-dimensional phosphopeptide maps were prepared. Immunopurified and SDS–PAGE gel-purified ^{32}P-labeled AR was digested with TPCK-treated trypsin (see Materials and Methods), and the phosphopeptides were separated on thin-layer cellulose plates by electrophoresis in the first dimension and by chromatography in the second dimension. In initial studies, various buffers for electrophoresis and chromatography were used, and the maps obtained were analyzed. When electrophoresis was performed at pH 3.5 or pH 1.9, all the phosphopeptides remained at or near the application site (results not shown). A possible explanation for this lack of mobility could be the fact that the N-terminal domain of the human AR is very hydrophilic and contains a large number of acidic amino acid residues, especially between amino acid residues 100 and 325 (Faber et al., 1989). When the pH of the electrophoresis buffer was raised to 9.1, most phosphopeptides moved toward the anode, indicating the relative enrichment of acidic amino acid residues in these peptides (see Figure 3). During chromatography in "isobutyric acid buffer", designed to resolve hydrophilic phosphopeptides (Scheidtmann et al., 1982), only one phosphopeptide moved in the second dimension, showing that most AR phosphopeptides are extremely hydrophilic (see Figure 3). In total, four phosphopeptides were identified, although it should be emphasized that the resolution of the hydrophilic peptides which do not move in the second dimension during chromatography is not optimal. In Figure 3, a comparison is shown between tryptic phosphopeptide maps of ^{32}P-labeled AR protein isolated from cells incubated either in the presence or in the absence of 10 nM R1881 (see also Figure 1, lanes 6 and 7). The location and number of phosphopeptides were the same in each case. A significant increase in the phosphorylation degree of the peptides which are moving in the second dimension during chromatography, however, was observed after hormone treatment of the cells. No significant changes in the phosphorylation degree of the other peptides were detected. The hormone-induced phosphopeptide spot is very broad, possibly due to phosphorylation of the same peptide at different positions, which affects its hydrophobicity to different degrees due to the context of the phosphorylation site (Boyle et al., 1991).

Although the two-dimensional thin-layer separation method was sensitive to quantitative changes in the phosphorylation degree of AR phosphopeptides, the resolution of most of the hydrophilic peptides was not optimal. Therefore, separation of tryptic phosphopeptides by reverse-phase HPLC was performed. The peptides were generated by tryptic digestion of AR protein as described (Materials and Methods) and applied to a C18 column in 0.1% trifluoroacetic acid. A total of 15 separate ^{32}P-labeled peaks above background (20 ± 2 cpm) were detected (see Figure 4). This was clearly

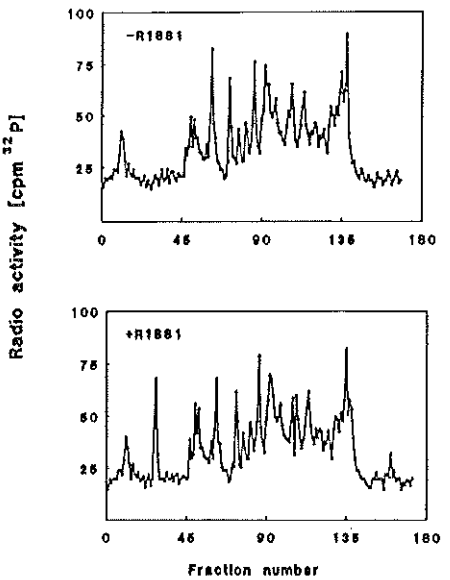

FIGURE 4: HPLC elution profiles of tryptic phosphopeptides of AR protein from LNCaP cells. AR protein was labeled in LNCaP cells with [^{32}P]orthophosphate during incubation for 12 h in the presence (Δ; lower graph) or absence (●; upper graph) of 10 nM R1881, and immunoprecipitated from total cell lysates. Following electrophoresis, the labeled AR protein was excised and digested with trypsin. The peptides were acidified and applied to a C18 column. The column was developed with a linear 0–60% acetonitrile gradient in 0.1% TFA, in 240 min. The flow rate was 0.18 mL/min, and fractions were collected every 1.5 min. Fractions were analyzed by Cerenkov counting.

more than the number of phosphopeptides detected by thin-layer separation (see Figure 3). Incubation of LNCaP cells with R1881 induced phosphorylation of a site in a peptide eluting in fractions 27–28 (retention time 40 min). Only minimal differences in the phosphorylation degree and elution positions of the other peptides were observed (Figure 4), indicating that the hormone-stimulated AR protein hyperphosphorylation is to a large extent due to phosphorylation of a new site.

DISCUSSION

Steroid hormone receptors, including the AR, are heterogeneous proteins that exist as multiple isoforms, migrating as doublets or triplets on SDS–PAGE gels. Upon ligand binding, the isoforms of progesterone, androgen, estradiol, and vitamin D receptors undergo an increase in apparent molecular mass, concomitantly with additional phosphorylation (Jurutka et al., 1993; Brown & DeLuca, 1990; Le Goff et al., 1994; Washburn et al., 1991; Beck et al., 1992; G. Jenster, personal communication). The reason for these mobility shifts is not clear, since the actual increase in molecular mass of the protein by phosphorylation is very small. A discrepancy exists between the theoretical calculated molecular mass of the AR protein (98 kDa) and the apparent molecular mass on SDS–PAGE gels (110–112 kDa). In the present study, it was shown that the nonphosphorylated AR protein has an apparent molecular mass of 110 kDa, indicating that this discrepancy is not due to phosphorylation. In another study, this discrepancy has been ascribed to some unknown features of the region between amino acid residues 51 and 211 of the human AR protein (Jenster et al., 1991). For the mouse glucocorticoid receptor, a similar discrepancy in molecular mass has been ascribed to the region between amino acid residues 108–324 (Hutchison et al., 1993). The two regions have in common a relatively high content of acidic amino acid residues, which might influence in both cases the association of SDS and thereby the mobility during SDS–PAGE. An alternative posttranslational modification, such as glycosylation, causing anomalous behavior on SDS–PAGE gels, cannot be excluded.

In the present study, it was calculated that the AR protein contains 1 mol of phosphate/mol of protein. The stoichiometry measurements may be an underestimation, if the specific activity of the phosphoamino acids in the AR protein is not equal to that of the phosphate in the labeling medium or if cellular phosphatases are active during cell lysis. It was attempted to minimize these factors by labeling the cells for 24 h and by adding phosphatase inhibitors to the lysis buffer. Phosphorylation after cell lysis was blocked by the addition of EDTA and nonradioactive phosphate to the lysis buffer. Given the rapid turnover of phosphate groups on proteins, this renders the labeling of the phosphoryl moieties in phosphoproteins to equilibrium much easier than the labeling of the polypeptide backbone. The average half-life of cellular proteins (40 h) is long relative to the length of time that many kinds of cells can tolerate radiolytic damage (Weber, 1972; Sefton, 1991). On the other hand, the specific activity ([^{35}S]methionine labeling) of a protein with a short half-life would be greater than that of bulk cellular protein if the labeling period was too short. The AR half-life is between 2 and 3 h (van Laar et al., 1991; Syms et al., 1985), so that after a labeling period of 24 h all AR protein molecules have been renewed.

In the present investigation, it is shown that the AR protein is exclusively phosphorylated on serine residues. Nuclear, but not cytosolic, AR protein from rat ventral prostate was shown previously to react with anti-phosphotyrosine antibodies, indicating that these receptors or associated protein(s) are possibly phosphorylated on tyrosine residues (Golsteyn et al., 1989; 1990). However, it should be noted that no intact 110 kDa AR protein was identified in these studies. Phosphoamino acid analysis has revealed that the glucocorticoid, progesterone, and 1,25-dihydroxyvitamin D_3 receptors are phosphorylated on serine residues (Dalman et al., 1988; Sheridan et al., 1988; Brown & DeLuca, 1991). Results on phosphoamino acid analysis of estrogen receptors are not conclusive. While Auricchio (1989) showed that estrogen receptors are only phosphorylated on tyrosine residues, other investigators reported phosphorylation only on serine residues (Washburn et al., 1991; Denton et al., 1992; LeGoff et al., 1994; Lahooti et al., 1994). Separation of tryptic phosphopeptides from human AR protein revealed that the AR is phosphorylated at multiple sites. When a comparison was made between cells incubated with R1881 and cells incubated without R1881, it was found that one additional phosphopeptide appeared after hormone treatment. No further significant changes (quantitatively and/or qualitatively) were detected in the two separation systems which were used.

It is difficult to give an accurate estimation on the number of AR protein phosphorylation sites. The N-terminal region shown to be heavily phosphorylated in the AR protein contains 33 serine residues and 12 consensus phosphorylation sites for casein kinases, Ser-Pro-directed kinases, and DNA-dependent kinase. Partial trypsin digestion products can be generated at adjacent arginine and/or lysine residues and also at arginine/lysine–aspartic acid/glutamic acid bonds (Boyle et al., 1991). Between amino acid residues 1 and 300 of the AR protein, six of these combinations known to cause partial digests are present, so that it is possible that a single phosphorylation site gives rise to several different phosphopeptides. Nevertheless, from the present results, it can be inferred that the number of AR phosphorylation sites is in the same order as those reported for the glucocorticoid and progesterone receptors. In the mouse glucocorticoid receptor, seven phosphorylation sites were identified, and in the chicken progesterone receptor, four sites were found (Poletti & Weigel, 1993; Bodwell et al., 1991; Poletti et al., 1993). Digestion of AR protein with α-chymotrypsin produced seven phosphopeptides after HPLC (result not shown). Digestions with V8 protease were not conclusive. The appearance of new phosphopeptides after hormone treatment was also shown by phosphopeptide mapping for the human and chicken progesterone receptor, the calf uterus estrogen receptor, and the rat glucocorticoid receptor (Denner et al., 1990; Sheridan et al., 1989; Nakao et al., 1992; Denton et al., 1992; DeFranco et al., 1991). In contrast, for the human estrogen receptor and the avian progesterone receptor, it was found that hormone-induced phosphorylation was not due to phosphorylation at a new site, but rather increased phosphorylation of existing sites (Sullivan et al., 1988; LeGoff et al., 1994).

Efforts will now be focused on the identification of the hormone-stimulated AR phosphorylation site(s), in order to be able to identify their significance in nuclear cytoplasmic AR shuttling, DNA binding of the AR protein, and transcriptional activation by the AR. Since the amount of AR protein which can be purified from LNCaP cells is rather limited, this will involve the use of an AR protein overexpression system in yeast cells or insect cells.

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CHAPTER 6

IN VITRO TRANSLATION OF ANDROGEN RECEPTOR cRNA RESULTS IN AN ACTIVATED ANDROGEN RECEPTOR PROTEIN

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

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

INTRODUCTION

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

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

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

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

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

MATERIALS AND METHODS

Materials

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

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

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

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

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

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

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

In vitro transcription and translation

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

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

Electrophoresis, electroblotting and autoradiography

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

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

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

Steroid binding

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

Sucrose-density-gradient centrifugation

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

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

DNA–cellulose chromatography

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

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

Gel retardation assay

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

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

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

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

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

Site-directed mutagenesis

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

RESULTS

Characterization of AR synthesized in rabbit reticulocyte lysate

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

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

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

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

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

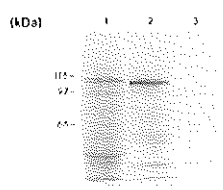

Figure 1 Synthesis of AR in rabbit reticulocyte lysate and in LNCaP cells

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

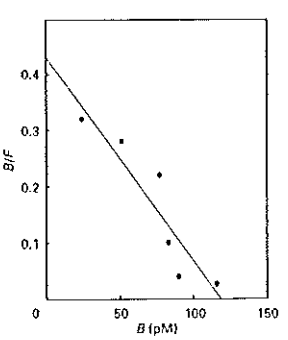

Figure 2 Scatchard plot of [^{3}H]R1881 binding to AR synthesized *in vitro*

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

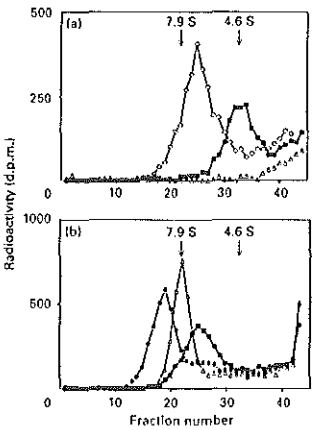

Figure 3 Sucrose-density-gradient profiles of human AR synthesized *in vitro*

AR was synthesized and incubated with [^{3}H]R1881 as described in the Materials and methods section. (**a**) The lysate (50 μl) was incubated for 2 h with [^{3}H]R1631 alone (○) or [^{3}H]R1881 and a 100-fold excess of unlabelled R1881 (△) and analysed by sucrose-density-gradient centrifugation without salt as described. Another sample (20 μl of lysate) was loaded on a 10–30% sucrose density gradient with 0.4 M NaCl (■). BSA (4.6 S) and aldolase (7.9 S) were used as sedimentation markers on a parallel gradient. (**b**) The lysate (50 μl) was incubated for 2 h with either 1 μl of ascitic fluid containing the androgen receptor antibody F39 (●) or F52 (△) or 1 μl of ascitic fluid containing a non-specific antibody (■). Samples (20 μl of lysate) were run on 10–30% sucrose density gradients without added salt as described in the Materials and methods section.

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

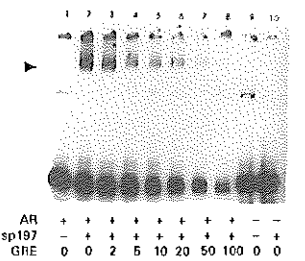

Figure 5 Binding of AR synthesized *in vitro* to a GRE

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

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

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

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

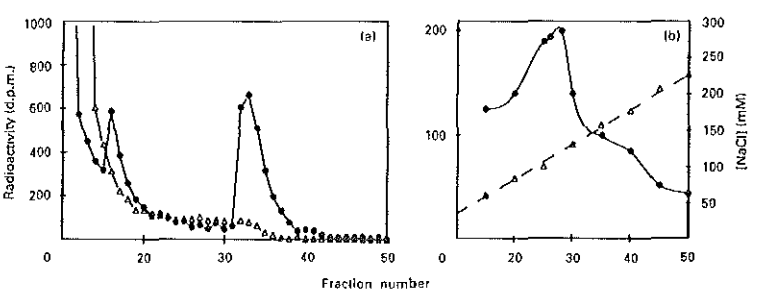

Figure 4 Elution from DNA–cellulose of AR synthesized *in vitro*

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

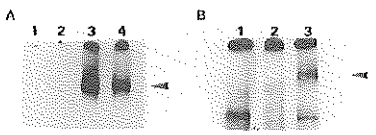

Figure 6 Binding of AR synthesized *in vitro* to ^{32}P-labelled GRE: influence of preincubation with ligand or salt

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

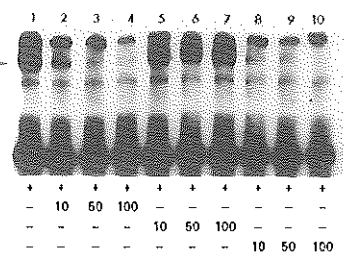

Figure 7 DNA-binding specificity of AR synthesized *in vitro*

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

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

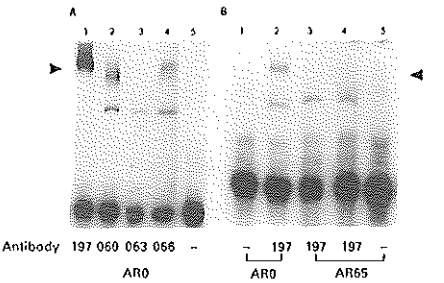

Figure 8 (a) Binding of AR synthesized *in vitro* to ^{32}P-labelled GRE in the presence of various AR antisera and (b) GRE binding of wild-type and a mutant AR

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

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

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

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

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

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

DISCUSSION

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

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

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

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

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

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

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

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

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

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

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

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CHAPTER 7

DISCUSSION

7.1 Androgen receptor protein in LNCaP cells

In many experiments described in this thesis, the LNCaP cell line, derived from a fast growing colony of a Lymph Node Carcinoma of the Prostate, was used as a model system to study androgen receptor phosphorylation. This cell line contains a high amount of androgen receptor molecules, and shows androgen-stimulated growth in culture (Horoszewicz et al., 1983; Schuurmans et al., 1988; van Steenbrugge et al., 1991). The growth of LNCaP cells was not only stimulated by androgens, but also by estrogens and progestins (Schuurmans et al., 1988). This was surprising, since the LNCaP cells do not contain estrogen and progesterone receptors (Berns et al., 1986; Schuurmans et al., 1988). Examination of the binding characteristics of the androgen receptor in LNCaP cells, in comparison with the binding characteristics of the wild type androgen receptor in COS cells, in normal human skin fibroblasts, and in PC-3 (prostate tumor) cells, revealed an abnormal high binding affinity for progesterone and estrogen of the LNCaP cell androgen receptor (Veldscholte et al.,1990A). A point mutation in the LNCaP cell androgen receptor gene was detected, which resulted in the substitution of Thr 868 by Ala (Veldscholte et al.,1990B). After transient expression of this mutated androgen receptor in COS or HeLa cells, it was shown that not only androgens, but also progestagens and estrogens, albeit it at high concentrations, could stimulate the expression of an androgen receptor responsive reporter gene (Veldscholte et al.,1990B; Ris-Stalpers et al.,1993). Nevertheless, the androgen receptor in LNCaP cells has a normal binding affinity for androgens (Kd 0.4 nM) (Veldscholte et al., 1990A). After transfection of GRE/ARE containing reporter constructs in LNCaP cells, the endogenous androgen receptor was able to mediate enhancement of transcription, upon incubation of cells with T or DHT (Denison et al.,1989; Warrian et al.,1994). Prostate specific antigen (PSA) mRNA expression could be

upregulated by androgens, as is the case in prostatic tissue (Riegman et al.,1991; Swinnen et al., 1994). No other properties of the androgen receptor in LNCaP cells, apart from the changed ligand binding specificity, are affected compared to the wild type androgen receptor, as far as is known. The high expression of androgen receptor protein and the androgen responsiveness make LNCaP cells a suitable model to study androgen receptor phosphorylation. Finally, the codon 868 mutation in the androgen receptor gene is not an unique property of LNCaP cells, nor an artefact of cell culture, since the same mutation was found in 6 out of 24 prostatic tissue specimens of patients with metastatic prostate cancer (Gaddipati et al.,1994). In another study, the codon 868 mutation was found in metastatic tissue from 1 out of 8 cases of endocrine therapy resistant prostate cancer (Suzuki et al., 1993). Possibly, the codon 868 mutant androgen receptor provides a selective growth advantage in the genesis of a subset of prostate cancers.

7.2 Heterogeneity of the androgen receptor

The androgen receptor isolated from LNCaP cells migrates as a closely spaced doublet of 110-112 kDa apparent molecular mass during SDS-PAGE (Chapter 3). The 112 kDa isoform reflects the phosphorylated androgen receptor, and the 110 kDa isoform the non-phosphorylated receptor (Chapters 5 and 6). Also other proteins, like for example the retinoblastoma protein (Buchkovich et al.,1989), pp60 c-*src* (Gould et al.,1985), and the *Dictyostelium* cAMP receptor (Hereld et al.,1994), show a decrease in their mobility on SDS-PAGE gels upon phosphorylation. These shifts are probably caused by variable binding of SDS, affecting the net charge of the protein-SDS complex (Blackshear,1984). The observed androgen receptor heterogeneity is not a characteristic of LNCaP cells

only, since the same androgen receptor doublet was also observed in genital skin fibroblasts, and in CHO cells stably transfected with human androgen receptor cDNA (Ris-Stalpers et al.,1994; unpublished results). The androgen receptor doublet is already present in LNCaP cells which have not been incubated with hormone, indicating that the doublet is caused by a hormone independent phosphorylation step (Chapters 3 and 5; Kuiper et al., 1992). This is confirmed by the fact that in genital skin fibroblasts from individuals with androgen insensitivity, expressing androgen receptors which are unable to bind hormone, the androgen receptor is also a doublet on SDS-PAGE (Ris-Stalpers et al.,1994). The wild type human androgen receptor and six different androgen receptor proteins specifically mutated to obtain different amino acid residues at position 868, were transiently expressed in COS cells. Again, in all cases the androgen receptor migrated as a doublet on SDS-PAGE, irrespective whether or not the mutants were able to bind hormone (Ris-Stalpers et al.,1993). In labeling experiments with [^{32}P]-orthophosphate of CHO cells expressing the human androgen receptor protein, only the 112 kDa isoform was labeled, as was shown for the androgen receptor from LNCaP cells (Chapter 5, and unpublished results).

In conclusion, these studies in different cell systems provide evidence for the existence of a hormone independent (basal) phosphorylation step and show that androgen receptor heterogeneity is caused by phosphorylation (Chapters 3 and 5). Furthermore, the fact that androgen receptor heterogeneity is observed in various cell types, even in cells not normally expressing androgen receptor protein, indicates that ubiquitous kinase(s) are involved.

7.2.1 Heterogeneity of the androgen receptor and ligand binding

Both isoforms of the androgen receptor are able to bind ligand, since both

isoforms are able to undergo hormone dependent transformation to the tight nuclear binding form in LNCaP cells (Chapter 3). Also, both isoforms can be labeled specifically with [^{3}H]-R1881 upon photolabeling of androgen receptors in LNCaP cells (van Laar et al.,1990). Since the 110 kDa isoform is not phosphorylated, this indicates that androgen receptor phosphorylation is not essential for the acquisition of ligand binding capacity in target cells. Previous studies showed that in rat prostate cells the androgen receptor protein is rapidly inactivated to a form that is unable to bind ligand upon treatment of cells with dinitrophenol (which results in uncoupling of mitochondrial respiration and ATP synthesis), and that this inactive form could be reactivated by an ATP dependent process (Rossini and Liao, 1982). However, there was no direct evidence provided that the ligand binding capacity is related to phosphorylation of the androgen receptor protein itself, although models were proposed in which androgen receptor phosphorylation was assumed to be essential for ligand binding (Rossini, 1984).

All the non-phosphorylated androgen receptor molecules synthesized in a cell-free system (reticulocyte lysate) were able to bind R1881 with similar affinity as receptors in target cells (Chapter 6). The fact that androgen receptors synthesized in reticulocyte lysate bind ligand, makes it also unlikely that phosphorylation of a putative target cell co-factor is essential for acquisition of ligand binding capacity.

7.2.2 Heterogeneity of the androgen receptor and DNA binding

Since both isoforms of the androgen receptor protein in LNCaP cells undergo transformation to the tight nuclear binding form, it is unlikely that phosphorylation is essential for DNA/chromatin binding by the receptor (Chapter 3). However, the possibility that both isoforms bind to different acceptor sites

in the nucleus cannot be excluded. The human androgen receptor synthesized *in vitro* displayed specific binding to ARE/GRE containing oligonucleotides in an electrophoretic mobility shift assay (Chapter 6). The affinity of the androgen receptor for these oligonucleotides was rather low. Only in the presence of specific androgen receptor antisera stable complexes could be detected (Chapter 6). The androgen receptor antibodies probably stimulate dimerization of the androgen receptor protein, thus enabling high affinity DNA binding. The low DNA binding affinity of the *in vitro* synthesized androgen receptor could in principle be due to the absence of phosphorylation or the presence in reticulocyte lysate of an inhibitor. In experiments using nuclear extracts of LNCaP cells in electrophoretic mobility shift assays, it was also impossible to detect specific complexes with GRE/ARE oligonucleotides. Only after the addition of specific androgen receptor antisera, stable complexes were detected (unpublished results). The androgen receptor in nuclear extracts is phosphorylated (Chapters 3 and 4), and lack of phosphorylation, is therefore, probably not the cause of the low DNA binding affinity. Moreover, Palvimo et al. (1993) showed specific binding of *in vitro* synthesized rat androgen receptor protein to the GRE/ARE from the tyrosine aminotransferase gene promoter in mobility shift assays. Complexes with DNA were already observed in the absence of androgen receptor antibodies. This is in contrast with the results described in Chapter 6. The reason for this discrepancy is at present unknown.

7.2.3 Role of basal androgen receptor phosphorylation

The role of the basal androgen receptor phosphorylation has not been elucidated. It is also not clear why only a part of the receptor population undergoes basal phosphorylation (Chapter 5). The basal phosphorylation is a rapid process (Chapter 3), and most likely occurs in the cytoplasm, before receptor

translocation to the nucleus. It is possible that the unliganded androgen receptor is only a substrate for kinase(s) during synthesis or during a short period after synthesis, when the receptor is probably not yet associated with heat-shock proteins. The detection of both isoforms could reflect the existence of phosphorylation and dephosphorylation events, causing constant interconversion of both isoforms. The possible existence of such a cycle, however, was not confirmed in chase experiments after labeling of androgen receptors in LNCaP cells with [^{35}S]-methionine. The rate of disappearance of the 112 kDa isoform was 2-3 h, while the rate of disappearance of the 110 kDa isoform was < 15 min. (Kuiper et al.,1992). This indicates only phosphorylation of androgen receptor protein (110 kDa to 112 kDa conversion), and no dephosphorylation. Or, alternatively, the protein half-life of the 110 kDa isoform is very short in comparison with that of the phosphorylated 112 kDa isoform. It should be noted that many proteins contain phosphorylated serine or threonine residues whose function does not appear to require reversible regulation. These phosphate groups turn over with the same half-life as the protein itself, and probably have a structural role. One could hypothesize that phosphorylation increases the half-life of androgen receptor protein in LNCaP cells. There is evidence that modulation of substrate proteins by covalent modifications, changes in hydrophobicity, and interactions with proteins or allosteric factors, may change the susceptibility of proteins to proteolysis and thereby functions to regulate protein degradation in the cell (Beynon, 1980; Holzer, 1980). It has been demonstrated that phosphorylation of serine residues reduces the sensitivity of polypeptides to proteolytic digestion (Holzer,1980). A possible influence of phosphorylation on androgen receptor stability can only be proven beyond doubt, after identification of all phosphorylation sites followed by measurement of the protein half-life of specific phosphorylation site mutants.

7.3 Hormone-stimulated androgen receptor phosphorylation

The androgen receptor in LNCaP cells is already phosphorylated in the absence of hormone, and undergoes a hormone-stimulated additional phosphorylation. After incubation with R1881, an almost 2-fold increase in the phosphorylation degree over non-stimulated control cells was reached (van Laar et al.,1991). Similar results, that is a basal level of phosphorylation which was increased by incubation with mibolerone, were obtained after overexpression of the androgen receptor in *Spodoptera frugiperda* (Sf9) cells, using the baculovirus system (Alarifi et al.,1994). In Chapters 4 and 5, the hormone-stimulated phosphorylation was investigated in more detail. The hormone-stimulated phosphorylation is a rapid process, indicating that it does not require *de novo* protein synthesis (Chapter 4). A 1.8 fold stimulation was observed for the non-transformed androgen receptor (Chapter 4). For the transformed androgen receptor the stimulation factor is not known, since there are no androgen receptors present in the high salt nuclear extract of non-hormone incubated cells. The mean phosphorylation degree of transformed and untransformed receptors is the same after incubation with hormone (Chapter 4). This indicates that there is no direct causal link between hormone-stimulated phosphorylation and transformation to the tight nuclear binding form.

Part of the androgen receptor transformation process consists of the dissociation of receptor associated heat-shock proteins. Upon incubation of LNCaP cells with R1881 at 37°C, most hsp90 is dissociated from the receptor within 3 min (Veldscholte et al., 1992). Thus the dissociation of heat-shock proteins is much faster than the increase in phosphorylation (Chapter 4), so that the substrate for the kinase(s) involved is the receptor free from heat-shock proteins. In fact, transformation as a whole is for most receptors a much faster process than the hormone-stimulated extra phosphorylation. Ultimately, about 40% of the total

amount of the androgen receptor in LNCaP cells becomes transformed, and within 10 min of incubation with R1881 about 70% of that maximum is already reached (Veldscholte et al.,1992). Phosphorylation of transformed receptors continues to increase after 10 min incubation with R1881, indicating that at least a part of the extra phosphorylation of the transformed receptors occurs after chromatin binding (Chapter 4). Consequently, there could exist a difference in the phosphorylation pattern between transformed and untransformed receptors, despite the fact that both receptor types are phosphorylated to the same degree. In COS cells, hormone treatment induces an extra receptor isoform with an apparent molecular mass of 114 kDa, resulting in a 110-112-114 kDa triplet on SDS-PAGE (Jenster et al., 1994). Mutations in the DNA binding domain caused a reduction in the amount of the 114 kDa isoform upon treatment of COS cells with R1881, indicating that part of the extra phosphorylation is influenced by the ability of the receptor to bind to DNA (Jenster et al., 1994). After incubation with R1881, a substantial amount of the 110 kDa non-phosphorylated isoform is still present (Chapter 5). This accounts for the transformed as well as for the untransformed receptors (Chapter 3). Whether there is a functional difference between both isoforms (phosphorylated versus non-phosphorylated) of the transformed receptor is at present unknown. Also, after prolonged incubation of LNCaP cells with R1881 (12-16 h), still both isoforms were detected (Chapter 5). In another study, involving incubation of LNCaP cells with mibolerone for up to 49 h, both isoforms were detected on immunoblots (Krongrad et al.,1991). For some proteins, multisite and hierarchal protein phosphorylation has been described (Roach, 1991). Whether such a hierarchy also exists for the androgen receptor, in a sense that only the basal phosphorylated receptor protein is a substrate for the hormone-stimulated extra phosphorylation, is presently unknown.

7.4 Identification and functional role of androgen receptor phosphorylation sites

By using limited proteolysis of purified ^{32}P-labeled androgen receptors it was established that most, if not all, phosphorylation sites are located in the N-terminal domain. This accounts for the basal and the hormone-stimulated phosphorylation sites (Chapter 4). Only phosphorylation on serine residues was detected (Chapter 5). Many, but not all consensus phosphorylation sites for various kinases, known to phosphorylate nuclear proteins on serine residues, are located in the N-terminal domain of the androgen receptor protein (see Table 7.1). From a comparison of many phosphoproteins it is known that multiple phosphorylation sites tend not to be randomly distributed, but are usually concentrated in relatively short segments of protein molecules. Often, these phosphorylated regions are located at the extreme amino- or carboxyl termini of proteins (Roach, 1991). The N-terminal domain phosphorylation of the androgen receptor in LNCaP cells was confirmed in experiments involving the expression of androgen receptor mutants in COS cells (Jenster et al., 1994; Zhou et al., 1994). These studies provided evidence for phosphorylation at serine residues 80 and 93, besides phosphorylation at other unknown sites in COS cells. Serine residues 80 and 93 are part of a Ser-Pro-X-X motif, which is recognized by the Ser-Pro directed kinase (Vulliet et al.,1989). The Ser-Pro-X-X and Thr-Pro-X-X motifs are found much more frequently in gene regulatory proteins (such as *homeotic* gene proteins, *segmentation* gene proteins, steroid hormone receptor proteins, and certain oncogene proteins) than in DNA binding proteins that are not directly involved in gene regulation (such as the core histones) and also more frequently than in general proteins (Suzuki et al.,1989). Within the steroid/thyroid hormone receptor superfamily these motifs are not located in the zinc fingers and only seldom in the ligand binding region, but preferably in the

Table 7.1 POTENTIAL SERINE PHOSPHORYLATION SITES IN THE HUMAN ANDROGEN RECEPTOR

Protein kinase	Consensus recognition motif	Potential sites in AR
Casein kinase II	Ser-X-X-Glu/Asp	29 118 217 255* 290 299 641* 653 694
cAMP-dependent protein kinase	Arg-X-X-Ser	16* 212*
DNA-dependent protein kinase	Ser-Gln in a non-basic region	114
Ser-Pro directed kinase	Ser-Pro-X	80 93 255* 307 506 641*
Protein kinase C	Lys/Arg-X-X-Ser	16* 212* 241

* denotes serine residues which are part of the recognition motif of more than one kinase.

There are 17 potential phosphorylation sites for these kinases, of which 14 are located in the N-terminal domain (exon 1, amino acid residues 1-528).

Protein kinase consensus specificity motifs were obtained from Pearson and Kemp (1991) and Finnie et al., (1993).

The amino acid residue numbering is based on an AR cDNA coding for 910 amino acid residues, with 20 glutamine residues and 16 glycine residues in exon 1 (Faber et al., 1989).

hypervariable N-terminal domain or in the hinge region connecting the zinc fingers and the ligand binding domain. The strong evolutionary conservation in the otherwise variable N-terminus emphasizes the importance of these Ser-Pro motifs. Serine residue 114 of the human androgen receptor is within a consensus sequence for DNA dependent protein kinase (Table 7.1). DNA dependent protein kinase is a DNA-binding protein, and is active *in vitro* only when bound to the same DNA molecule as the substrate (Jackson et al., 1993). It has been suggested that restricting phosphorylation to DNA-bound substrates might be a mechanism to activate transcription factors only when they are ready to engage in transcription activation (Jackson et al., 1993). Whether the DNA bound androgen receptor protein is phosphorylated by DNA dependent protein kinase is unknown. Nine potential phosphorylation sites for casein kinase II can be found in the androgen receptor protein (Table 7.1). Casein kinase II is a ubiquitous cyclic nucleotide-independent protein kinase, and it has been reported to be activated by insulin, insulin-like growth factor, and epidermal growth factor (Pinna, 1990). Of the steroid hormone receptors only the estrogen receptor is known to be phosphorylated *in vivo* by casein kinase II (Arnold et al., 1994).

The N-terminal region of the androgen receptor, shown to be the predominant phosphorylated part of the androgen receptor, is essential for transcription activation (Jenster et al.,1991). There are some examples in which phosphorylation has been implicated in regulating the activity of transcription factors. One of the best examples of this type of regulation is provided by the transcription factor CREB (cAMP responsive element binding protein), which mediates transcription in response to activation of the cAMP-dependent protein kinase (PK-A) signal transduction pathway. PK-A appears to stimulate the *trans*-activation capacity of CREB by phosphorylating CREB in a domain required for transcriptional activity (Gonzalez and Montminy, 1989). In addition,

phosphorylation in or around the *trans*-activation domains of the human serum response factor protein, the CREMτ protein (cAMP responsive element modulator), and human c-*myc*, directly influence the *trans*-activation potential of these transcription factors (de Groot et al., 1993; Henriksson et al.,1993; Liu et al., 1993). The observation that negatively charged amino acid residues are important for the activity of many transcriptional activation domains, suggests that phosphorylation simply operates by adding extra negative charge (Ptashne, 1988). Indeed, the N-terminal *trans*-activation domain of the human androgen receptor is very hydrophilic and rich in acidic amino acid residues, especially between residues 100 and 325 (Faber et al.,1989), and phosphorylation in this area could add extra negative charge.

By tryptic phosphopeptide analysis it was shown that the androgen receptor contains multiple phosphorylation sites, and that R1881 treatment strongly stimulates the phosphorylation of one peptide, with only minor effects on other phosphopeptides (Chapter 5). Perhaps, this extra hormone-induced phosphorylation causes a major conformational change in the N-terminal domain, thus enabling protein-protein contacts between the N-terminal *trans*-activation domain and other transcription factors or coactivators on a target gene promoter, causing transcription induction. Evidence for such a model has been provided for the CREB protein, which after phosphorylation at serine residue 133 by protein kinase A undergoes a major conformational change, enabling interaction with CBP (CREB binding protein) which itself interacts with TFIIB and the RNA polymerase II holoenzyme (Arias et al., 1994; Kwok et al., 1994; Nordheim, 1994). Identification of all androgen receptor phosphorylation sites and subsequent site-directed mutagenesis is obviously essential. Unfortunately, microsequencing of several phosphopeptide peaks from the elution profile of the reverse phase separation analysis (Chapter 5, Figure 4), revealed that the samples were heterogenous, and therefore, sequences could not be determined

(not shown). Since the amount of androgen receptor protein which can be purified from LNCaP cells is rather limited, a more rigorous purification of androgen receptor phosphopeptides is only possible after overexpression of androgen receptor protein in yeast or insect cells. Androgen receptor protein from these cells, purified to near homogeneity, could then be mixed with a small amount of [^{32}P]-labeled androgen receptor protein from LNCaP cells, followed by digestion with trypsin. Sequential cleavage of purified phosphopeptides with other protease(s), and re-purification by reverse phase HPLC could then provide material suitable for microsequencing and identification of phosphorylation sites. Functional studies on the role of phosphorylation of specific sites in androgen receptor action on the regulation of target genes, could then be undertaken.

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CURRICULUM VITAE

De schrijver van dit proefschrift werd geboren op 23 mei 1961 te Maastricht. Na het behalen van het HAVO diploma aan het R.K. Veldeke College te Maastricht volgde hij de HBO-B opleiding tot analist aan het Van 't Hoff Instituut te Rotterdam. Vanaf 1 mei 1984 was hij werkzaam als analist binnen het Instituut Endocrinologie & Voortplanting van de Erasmus Universiteit te Rotterdam. Vanaf september 1985 volgde hij tevens de deeltijd studie Biologie aan de Rijksuniversiteit te Utrecht. Het doctoraal examen met als hoofdvak Moleculaire Biologie werd afgelegd in september 1991. Vanaf 1 september 1991 tot 1 september 1994 was hij als wetenschappelijk onderzoeker werkzaam binnen het Instituut Endocrinologie & Voortplanting van de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit te Rotterdam, alwaar het in dit proefschrift beschreven onderzoek werd verricht. Sinds 1 januari 1995 is hij werkzaam binnen het Center for Biotechnology (CBT), Karolinska Institute, te Huddinge, Zweden, op basis van een NWO stipendium.

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