PHOSPHORYLATION OF THE ANDROGEN RECEPTOR IN A HUMAN PROSTATE TUMOR CELL LINE

FOSFORYLERING VAN DE ANDROGEEN RECEPTOR IN EEN HUMANE PROSTAAT TUMOR CELLIJN

PROEFSCHRIFT

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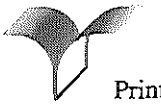

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CONTENTS

CHAPTER 1

GENERAL INTRODUCTION

1.1 Action of androgens

Testosterone and dihydrotestosterone, the principal androgenic hormones, possess masculinizing activities. During fetal life, testosterone mediates the virilization of the Wolffian ducts into the epididymides, vasa deferens, and seminal vesicles. The development of the urogenital sinus into prostate, urethra, and external genitalia, as well as the growth and maintenance of the male reproductive tract tissues, require the conversion of testosterone into dihydrotestosterone by the enzyme 5α-reductase (Griffin and Wilson, 1989). The different roles for testosterone and dihydrotestosterone in male sexual differentiation have been substantiated by studies in males with 5α-reductase deficiency. The wolffian duct structures in these individuals are developed normally, but the external genitalia are masculinized incompletely (Imperato-McGinley et al., 1974). It is not certain whether spermatogenesis requires the conversion of testosterone into dihydrotestosterone. Spermatogenesis is profoundly impaired or absent in males with 5α-reductase deficiency, but the spermatogenic arrest might either be due to a deficiency of dihydrotestosterone formation in the testis or to a testicular maldescent in these persons (Griffin and Wilson, 1989).

Androgens belong to the group of steroid hormones. Testosterone, which is the major circulating androgen, is synthesized from cholesterol in the Leydig cells of the testis and secreted into the blood. The production of testosterone is stimulated by luteinizing hormone (LH), which is produced by the pituitary gland. The release of LH is under control of the hypothalamus through luteinizing hormone-releasing hormone, and inhibited by testosterone via a negative feedback mechanism (Schwartz and McCormack, 1972).

Approximately 98% of circulating testosterone is bound to plasma proteins such as albumin and sex hormone-binding globulin (Westphal, 1978). Free testosterone is thought to enter androgen target cells via diffusion across the cell membrane, and is transported through the cytoplasm to the nucleus by means of a still not identified mechanism. The conversion of testosterone into dihydrotestosterone possibly occurs in the nuclear membrane (Houston et al., 1985).

Testosterone and dihydrotestosterone exert their androgenic effects by interacting with the same nuclear receptor protein (Liao et al., 1973; Grino et al., 1990). The androgen receptor is present most prominent in the male sexual organs but is also found

in a variety of other tissues in males and females (Liao, 1975; Takeda et al., 1990). When occupied by the appropriate ligand, the androgen receptor becomes a transacting factor, that regulates the transcription of specific genes (Paragraph 1.2).

The final proof for the indispensable role of the androgen receptor in androgen action has come from studies in individuals with the X-linked syndrome of androgen insensitivity. This syndrome is caused by mutations that lead to decreased androgen receptor levels or to qualitative defects in the receptor protein (Paragraph 1.4), or even to the absence of the receptor gene (Trifiro et al., 1991). Defects in the androgen receptor appear to be relatively common in comparison with defects in other steroid hormone receptors, which might be due to the fact that defects in the androgen receptor are not lethal. Furthermore, mutations in this X-encoded gene can be inherited through the mother and affect males, which are hemizygous for the X chromosome. The phenotypic expression in humans ranges from partial androgen insensitivity, in which 46,XY individuals are phenotypic males but infertile, to the complete syndrome, in which the phenotype of 46,XY individuals is female, but with undescended testis (Griffin and Wilson, 1989).

In agreement with the important role of androgens in the development and function of the normal prostate, androgens are thought to be involved in the growth of prostate tumors. Carcinoma of the prostate is, second to lung carcinoma, the most common cancer in men. Many forms of prostate cancer are initially androgen-dependent (Blackard et al., 1973; Menon and Walsh, 1979), and in most patients prostate tumor growth can be arrested by removal of the major source of circulating androgens, by castration in the early stages of the disease. However, this response is often transient. In a later stage, prostatic tumors, subjected to androgen ablation therapy, generally become androgen-independent. The etiology of prostate cancer and the mechanism underlying the transition from androgen-dependent to androgen-independent tumor growth are largely unknown. Recently it has been suggested that complete or partial down-regulation of the androgen receptor is involved in progression from an androgen-sensitive to an androgen-insensitive state (Quarmby et al., 1990; Tilley et al., 1990a). Alternatively, qualitative defects in the androgen receptor, as observed in the human LNCaP (Lymph Node Carcinoma of the Prostate) cell line (Veldscholte et al., 1990), or defects at the level of transcription control (Darbre and King, 1987) may lead to altered steroid

sensitivity of tumor cells. Elucidation of such defects will require a detailed knowledge of androgen receptor structure and function.

1.2 Role of steroid receptors in steroid hormone action

Steroid hormone receptors, which include androgen, progesterone, estrogen, glucocorticoid, and mineralocorticoid receptors, act as intracellular transducers of the hormonal signal. Androgen, progesterone, and estrogen receptors are present in the nuclei of target cells, irrespective of the presence of the corresponding hormone (Welshons et al., 1984; Perrot-Applanat et al., 1985; Husmann et al., 1990). In contrast, the unliganded glucocorticoid receptor is localized mainly in the cytoplasm and does undergo a ligand-induced translocation from the cytoplasm into the nucleus (Picard and Yamamoto, 1987; Wikström et al., 1987). It has been suggested that differences in subcellular localization between the unliganded glucocorticoid receptor and other steroid receptors reflect kinetic differences in receptor cycling into and out of the nucleus. For the glucocorticoid receptor, the rate-limiting steps might reside in the cytoplasmic part of the cycle (Pratt et al., 1989).

There are indications that newly synthesized steroid receptors must undergo an activation process in order to acquire hormone-binding activity. Phosphorylation might have an important role in steroid receptor activation (Paragraph 1.5), but a 90 kDa heat shock protein may be involved in the activation process as well (see below).

Ligand-binding results in alteration of properties of the receptor protein, termed "transformation" (Jensen et al., 1968). Transformation is characterized by exposition of the DNA-binding domain of the receptor molecule, which is masked in the unliganded receptor. The transformed hormone-receptor complex is able to interact specifically with defined DNA sequences, the hormone responsive elements. These *cis*-acting, enhancer-like, regulatory sequences are located in flanking regions of target genes and are responsible for hormonal regulation of the target genes (Renkawitz et al., 1984; Karin et al., 1984). Steroid receptors bind to hormone responsive elements as dimers (Kumar and Chambon, 1988; Tsai et al., 1988) and form a transcription initiation complex with other transcription factors at the target gene promoter (Klein-Hitpass et al., 1990). This

ultimately leads to modulation of gene transcription, and subsequently in modification of cell function (Evans, 1988; Beato, 1989) (Figure 1.1).

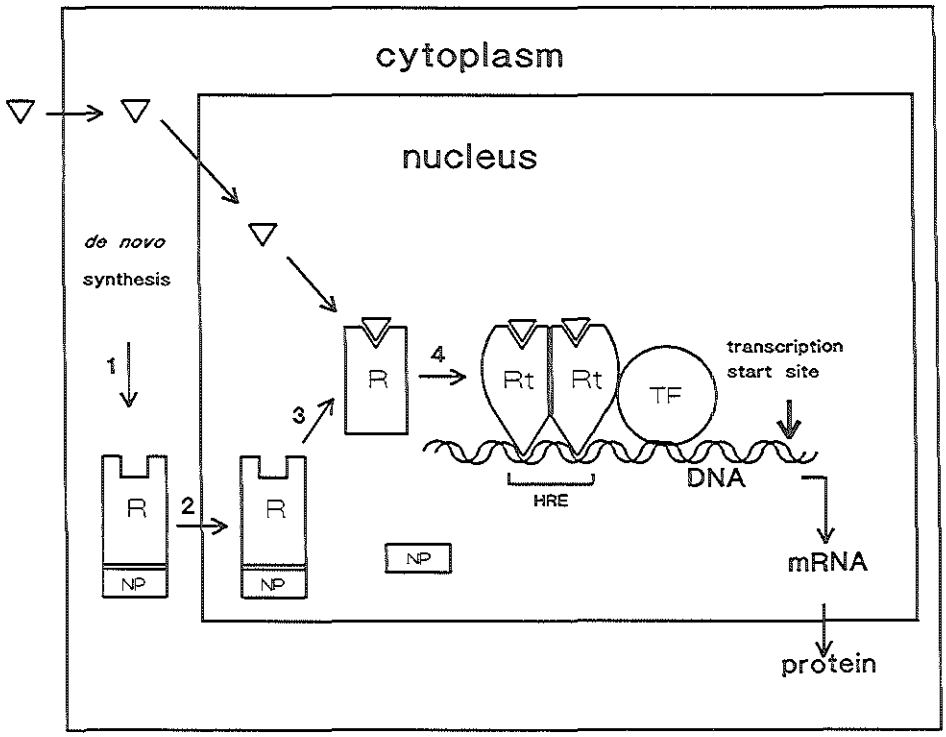

Figure 1.1. Schematic diagram of events involved in the acquisition of transcription regulatory activity of steroid receptors. (1), association of newly synthesized receptors with non-receptor proteins; (2), nuclear translocation; (3), receptor activation; (4), receptor transformation.
▽, steroid; NP, different non-receptor proteins; R, steroid receptor; Rt, transformed receptor; HRE, hormone responsive elements; TF, different transcription factors.

The cascade of events that finally results in acquisition of transcription regulating activity of the receptor proteins is only partially understood. As will be discussed in Paragraph 1.5, phosphorylation of steroid hormone receptors may be part of the cascade.

Non-receptor proteins most likely also have a role in the regulation of steroid receptor activity. In this respect, the function of a 90 kDa heat shock protein (hsp90) has been studied extensively. This protein is thought to be associated with unliganded steroid receptors in intact cells (Denis and Gustafsson, 1989). It has been proposed that hsp90 establishes or stabilizes the conformation of the hormone-binding domain of the receptor protein (Picard et al., 1990), and inactivates other functional domains of the unliganded steroid receptor (Dalman et al., 1990; Picard et al., 1990). Hsp90 might also actively unfold the receptor protein upon hormone binding. This function of hsp90 would match the function of a 70 kDa heat shock protein, which prevents the folding of newly synthesized proteins to facilitate their intracellular transport across membranes (Chirico et al., 1988; Deshaies et al., 1988). The observation that hsp90 and the unoccupied glucocorticoid receptor colocalize with microtubuli in intact cells led to the suggestion that hsp90 might be involved in transferring steroid receptors across the nuclear membrane via an association with microtubuli (Pratt et al., 1989).

It is still not clear what happens to the receptor proteins after they have exerted their biological effects. It has been proposed that chromatin-bound, biologically active receptors are recycled to their unliganded forms (Rossini, 1984; Orti et al., 1989a). The recycling may involve reassociation with non-receptor proteins and alterations in receptor phosphorylation. However, the relatively short half-lives of androgen and estradiol receptors (2-4 h), may argue against a need for receptor recycling (Nardulli and Katzenellenbogen, 1986; van Laar et al., 1991).

1.3 Structure of steroid receptors

Although steroid hormones and vitamin D are neither structurally nor biosynthetically related to thyroid hormones and retinoid acid, the specific receptor proteins of all these ligands belong to the same superfamily of functionally and structurally related proteins (Figure 1.2) that most likely descend from a common ancestral gene. The members of the superfamily are ligand-responsive, nuclear transcription factors. At the protein structural level, all these receptors are organized in discrete functional domains (Evans, 1988). The central domain is very conserved and is directly involved in DNA-binding. Screening for genes containing regions homologous to the DNA-binding domains of the

receptor proteins resulted in the identification of more members of the family with known ligands, and also in the finding of unknown genes. These family members are called "orphan receptors" since their ligands are unknown (Evans, 1988).

The DNA-binding domain is rich in basic amino acids and conserved cysteine residues. The positioning of the cysteine residues suggests that they form two "finger"

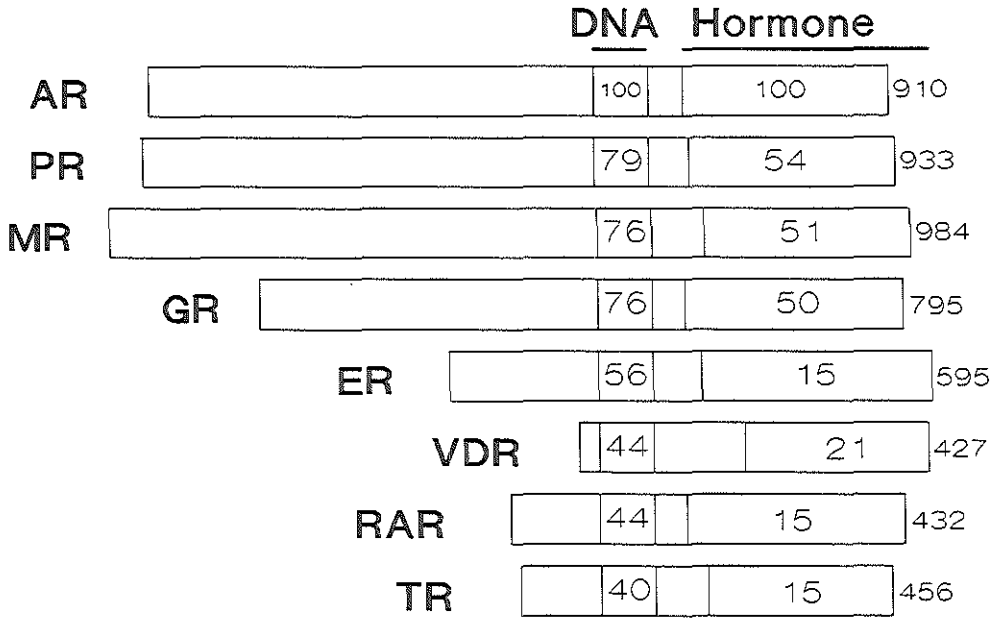

Figure 1.2. Schematic amino acid comparison between the hormone- and DNA-binding domains of the androgen receptor and those of various other members of the superfamily of ligand-responsive transcription factors. Amino acid sequences have been aligned according to the functional domain structure of the receptor proteins. The percentage amino acid identity of each receptor with the androgen receptor is indicated inside the DNA-binding domain ("DNA"), and hormone-binding domain ("Hormone"). The numbers outside the boxes refer to the total number of amino acid residues of each receptor protein.
AR, androgen receptor; PR, progesterone receptor; MR, mineralocorticoid receptor; GR, glucocorticoid receptor; ER, estrogen receptor; VDR, vitamin D receptor; RAR, retinoic acid receptor; TR thyroid hormone receptor.

motifs, which are likely to be stabilized by zinc, analogous to the DNA-binding elements in Xenopus transcription factor TFIIIA (Miller et al., 1985). The first zinc finger binds the hormone responsive elements (Green et al., 1988). It has been shown that only three

amino acids within this finger motif are responsible for the specific recognition of the hormone responsive elements. Since these amino acids are identical in androgen, progesterone, glucocorticoid, and mineralocorticoid receptors, these receptor proteins can act via the same hormone responsive elements (Ham et al., 1988; Mader et al., 1989). It has been suggested that the second zinc finger is involved in protein-protein interactions within the receptor dimer or in the interaction with other proteins of the transcription machinery (Härd et al., 1990). The ligand-binding domain is located at the C-terminal half of the molecule, relatively well conserved, and rich in hydrophobic amino acids. In contrast, the N-terminal domain is hypervariable in size and amino acid composition. This domain has been shown to be the most immunogenic domain of steroid receptors (Okret et al., 1984; Vu Hai et al., 1989), and is indispensable for transcription regulation (Hollenberg et al., 1987; Kumar et al, 1987). It has been shown that the hinge region, which separates the DNA-binding and steroid-binding domains, exhibits a transactivation function as well (Hollenberg and Evans, 1988; Dobson et al., 1989).

Amino acid sequences, involved in other aspects of receptor function, are less well characterized. Two sequences, that mediate hormone-dependent nuclear localization have been detected within the rat glucocorticoid receptor. One of the nuclear transfer signals contains a short region of homology with the nuclear translocation signal of SV40-large T antigen and has been localized immediately adjacent to the C-terminal zinc finger of the glucocorticoid receptor. A second nuclear transfer signal may be located in the carboxy-terminal half, overlapping the steroid-binding domain (Picard and Yamamoto, 1987). The amino acid sequence that appears to be responsible for the interaction with hsp90 resides in the same region of the receptor molecule (Howard et al., 1990). The estradiol receptor exhibits a weak constitutive dimerization function within the DNA-binding domain and a strong dimerization function within the ligand-binding domain. The latter was shown to be hormone-dependent (Kumar and Chambon, 1988; Fawell et al., 1990).

1.4 Structure of the androgen receptor

The androgen receptor gene has been localized on the X chromosome (Migeon et al., 1981; Brown et al., 1989). The protein-encoding part of the gene is distributed over 8

exons. The first exon encodes the N-terminal domain. The DNA-binding domain is encoded by two exons, each coding for one putative zinc-finger (Paragraph 1.3). One exon encodes the hinge region and the 5'-end of the steroid-binding domain, while 4 exons code for the remaining part of the steroid-binding domain and the untranslated 3'-region (Kuiper et al., 1989, Lubahn et al., 1989). The promoters of the human- (Tilley et al., 1990b) and rat androgen receptor genes (Baarends et al., 1990) have been identified and were shown to be located at approximately 1.1 kilobases upstream of the translation start codon of the androgen receptor protein.

The open reading frame of the human androgen receptor cDNA encodes a protein of 910 - 919 amino acids with a calculated molecular mass of approximately 99 kDa (Chang et al., 1988a,b; Lubahn et al., 1988a,b; Trapman et al., 1988; Faber et al., 1989; Liao et al., 1989). The rat and mouse androgen receptors are somewhat smaller and are composed of 902 (Chang et al., 1988a,b; Tan et al., 1988) and 899 residues (He et al., 1990), respectively.

There is considerable sequence homology between androgen receptors and glucocorticoid, progesterone, and mineralocorticoid receptors, within the steroid-binding and DNA-binding domains (Figure 1.2) (Hollenberg et al., 1985; Miesfeld et al., 1986; Arriza et al., 1987; Misrahi et al., 1987; Trapman et al., 1988). The N-terminal region of the androgen receptor shows the presence of several homopolymeric amino acid stretches. The human androgen receptor contains a stretch of 16-27 glycine residues, a stretch of 17-25 glutamine residues, as well as several smaller stretches of homopolymeric amino acid sequences (Chang et al., 1988b; Lubahn et al., 1988b; Faber et al., 1989; Young et al., 1990). Amino acid repeats are also present within the N-terminal domains of the chicken progesterone receptor (Conneely et al., 1987), rat, mouse, and human estrogen receptors (Greene et al., 1986; Koike et al., 1987; White et al., 1987), rat glucocorticoid receptor (Miesfeld et al., 1986), and in several other proteins (Davies et al., 1982; Grima et al., 1985; Poole et al., 1985; Wharton et al., 1985).

Another characteristic of the androgen receptor N-terminal domain is the relatively high content of acidic amino acids, resulting in a net negative charge of this domain at physiological pH. In two yeast factors, highly acidic regions are directly involved in transactivation of transcription (Ma and Ptashne, 1987; Hope et al., 1988), but it remains to be shown whether the acidic region of the human androgen receptor has a similar function.

Naturally occurring mutations in the androgen receptor gene, causing complete, or partial insensitivity to androgens, offer the opportunity to identify functionally important amino acid sequences in the receptor protein. Several point mutations or small deletions within the androgen receptor steroid-binding domain have been correlated with impaired hormone binding activity (Lubahn et al., 1989; Govindan, 1990; Ris-Stalpers et al., 1990; Sai et al., 1990). A point mutation within the steroid-binding domain of the androgen receptor from the human LNCaP (Lymph Node Carcinoma of the Prostate) cell line, changing a threonine residue into an alanine residue, has been shown to be responsible for an unusual broad binding specificity of this receptor protein towards steroid hormones (Veldscholte et al., 1990). The analysis of androgen receptors from persons with (partial) androgen insensitivity but with normal androgen-binding characteristics might reveal amino acid sequences involved in other androgen receptor characteristics, such as DNA-binding or transcription activation.

1.5 Phosphorylation of steroid receptors

Phosphorylation of receptors plays a key role in the mechanism of action of growth factors (e.g. EGF, insulin, IGF-I, and PDGF). The action of growth factors is initiated by the binding to specific transmembrane receptor proteins, which are composed of a large extracellular ligand-binding domain, a hydrophobic transmembrane region and a cytoplasmic domain that possesses tyrosine kinase activity. Ligand-binding results in autophosphorylation of the receptors on tyrosine residues. This, in turn, facilitates the phosphorylation of cellular substrates by the tyrosine kinase activity of the receptor. The substrates identified thus far include proto-oncogene products, or factors that regulate the activity of proto-oncogene products, and components of second messenger pathways, such as phospholipase C. Phosphorylation of phospholipase C ultimately results in release of Ca^{2+} from intracellular compartments and generation of diacylglycerol, the activator of protein kinase C. It is assumed that the internal stimulus for cell growth is provided by phosphorylation of cellular substrates on tyrosine by receptor and non-receptor tyrosine kinases, on serine/threonine by protein kinase C and by other serine/threonine kinases, together with alterations in the ionic content of the cell (Ullrich and Schlessinger, 1990).

In contrast to the plasma membrane receptors, described above, steroid receptors do not possess intrinsic protein kinase activity. The fact, however, that progesterone, glucocorticoid, and estradiol receptors exist as phosphoproteins in intact cells may imply a role for receptor phosphorylation in steroid hormone action as well (Housley and Pratt, 1983; Dougherty et al., 1984; Migliaccio et al., 1986; Sheridan et al., 1988). In addition, the vitamin D, and thyroid hormone receptors are phosphorylated (Goldberg et al., 1988; Brown and DeLuca, 1990). It has been shown that phosphorylation is indispensable for the acquisition of ligand-binding activity (receptor activation) of the estradiol receptor (Migliaccio et al., 1989), and indirect evidence has been provided for a similar role of phosphorylation in ligand-binding of various other steroid receptors (Nielsen et al., 1977; Rossini and Liao, 1982; Puri et al., 1984; Golsteyn et al., 1989; Dayani et al., 1990). Interestingly, a very different steroid-binding protein, the pregnenolone-binding protein, also has been shown to require phosphorylation in order to bind pregnenolone (Driscoll et al., 1990).

Following hormone administration to target cells, steroid receptor proteins and the vitamin D receptor undergo additional phosphorylation (Auricchio et al., 1987; Sheridan et al., 1988; Sullivan et al., 1988; Hoeck et al., 1989; Orti et al., 1989b; Brown and DeLuca, 1990). The increase of receptor phosphorylation by agonists lends further support to the idea that phosphorylation is involved in steroid receptor function.

A small population of nuclear, ligand-bound glucocorticoid receptors have been shown to undergo a partial dephosphorylation (Orti et al., 1989b). Dephosphorylation was associated with a very tight binding of the receptor proteins in the nucleus. The physiological role of the dephosphorylated receptors is still not known. Phosphorylation/ dephosphorylation might also provide a mechanism to turn on and off receptor functions at a high rate (Orti et al., 1989a).

1.6 Phosphorylation of the androgen receptor

Loss of androgen-binding activity in rat ventral prostate homogenates has been observed when the cells were ATP-depleted before homogenization. The binding activity could be regained by an energy-dependent process (Rossini and Liao, 1982). In addition, in cell-free systems, inhibition of endogenous phosphatase activity was correlated with increased

hormone-binding activity of the androgen receptor (Golsteyn et al., 1989). These results, which have also been obtained in similar experiments with other steroid hormone receptors (Paragraph 1.5), suggested a role for androgen receptor phosphorylation in the acquisition of hormone binding activity. The target for phosphorylation, however, could not be identified by this type of experiments; the putative phosphorylation might either involve the receptor protein itself, or some unidentified cofactor that may be required for steroid binding.

Using anti-phosphotyrosine antibodies, androgen-binding activity could be precipitated from nuclear extracts of androgen-treated rat ventral prostate cells, suggesting that the transformed androgen receptor is phosphorylated on tyrosine (Golsteyn et al., 1990). The precipitated protein had a molecular mass of 35 kDa, and was suggested to represent a receptor fragment. However, direct evidence has not been provided for this supposition, and it cannot be excluded that the 35 kDa protein is not related to the androgen receptor. In addition, the putative androgen receptor fragment might have been coprecipitated with a receptor-associated phosphotyrosine protein.

In 1984, Goueli et al. reported phosphorylation of purified androgen receptor from rat ventral prostate by a cAMP-dependent protein kinase. In this study, no direct evidence was provided that the purified phosphoprotein represented the androgen receptor. The phosphoprotein migrated on SDS-PAGE with an apparent molecular mass of 87 kDa, which deviates from the apparent molecular mass of approximately 110 kDa reported for the rat, calf, and human androgen receptor (Brinkmann et al., 1985; Brinkmann et al., 1989; van Laar et al., 1989). It cannot be excluded that the purified protein represents the receptor-associated 90 kDa heat shock protein (Paragraph 1.2) since this protein is known to be highly phosphorylated (Dougherty et al., 1984; Sanchez et al., 1985).

1.7 Scope of this thesis

The aim of the study described in this thesis was to determine whether the androgen receptor is a phosphoprotein, resembling in this respect the other members of the steroid/vitamin D/thyroid hormone receptor family. Furthermore, it was investigated whether the androgen receptor undergoes a hormone-dependent phosphorylation.

For all studies described in this thesis, the human LNCaP (Lymp Node Carcinoma of the Prostate) cell line was used. The androgen receptor in these cells was characterized with respect to molecular mass and ligand-binding properties (Chapter 3). The characterization of androgen receptor-specific polyclonal antibodies, which are useful tools for the phosphorylation studies, is described in Chapter 4. Studies on androgen receptor phosphorylation are presented in the Chapters 5 and 6. In addition to polyclonal antibodies, an androgen receptor-specific monoclonal antibody has been used for these studies. The preparation of the monoclonal antibody has been described briefly in Chapter 5. A detailed description of the generation and characterization of this antibody has been presented elsewhere (Zegers et al., 1991).

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CHAPTER 2

NEW TECHNIQUES FOR BIOCHEMICAL ANALYSIS OF THE ANDROGEN RECEPTOR

2.1 *In situ* photoaffinity labeling

In most studies described in this thesis, 17β-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one ([^{3}H]R1881) has been used. R1881 is a highly conjugated synthetic androgen that can be attached covalently to the androgen receptor by photoactivation (Brinkmann et al., 1985). This steroid has an even higher affinity for the androgen receptor than dihydrotestosterone (Bonne and Raynaud, 1975). R1881 has also been used for studies that did not require the covalent attachment of androgen to the androgen receptor. The reason for this was that, unlike testosterone and dihydrotestosterone, R1881 cannot be metabolized (Bonne and Raynaud, 1976). Hence, cells can be exposed for prolonged times to constant levels of R1881, which offers the opportunity for time-course studies on the response of the androgen receptor to R1881 (Chapters 5 and 6).

Up to a few years ago, only a limited number of analytical techniques were available for biochemical analysis of the androgen receptor. These techniques, including sucrose density gradient centrifugation and gel filtration, were based on the noncovalent binding of tritiated ligands to the receptor protein. The usefulness of these methods for detailed structural and functional analysis is limited, the more so because the androgen receptor protein is very unstable under cell-free conditions. Moreover, the receptor concentration in androgen target tissues is exceedingly low, viz. approximately 0.001% of total cellular protein.

With the development of a photoaffinity labeling procedure, it became possible to analyse the androgen receptor under protein denaturing conditions, e.g. on SDS-polyacrylamide gels (Brinkmann et al., 1985). However, this technique requires partial receptor purification prior to photoactivation. In Chapter 3, the development and application has been described of an *in situ* photoaffinity labeling procedure. This technique can be used to study the androgen receptor in intact cells under different hormonal conditions (Chapters 5 and 6). Since the covalent labeling of androgen receptors occurs in the intact cells, rather than in partially purified receptor preparations, this technique largely avoids *in vitro* incubations of cell-free preparations, which can involve proteolytic degradation.

2.2 Androgen receptor-specific antibodies

Major difficulties experienced in attempts to purify the androgen receptor protein in substantial amounts for immunization have hampered the development of specific antibodies. Following the elucidation of the nucleotide and amino acid sequence of the androgen receptor, however, androgen receptor-specific polyclonal and monoclonal antibodies could be generated without the necessity of receptor purification (Chapters 4 and 5; Zegers et al., 1991).

The antibodies appeared to be effective agents for immunoblotting and for isolation of androgen receptor from cell lysates and subcellular fractions. These techniques were used to isolate specifically [^{32}P]orthophosphate-labeled androgen receptor and to study the kinetics of androgen receptor phosphorylation (Chapter 6). Using these techniques, the unliganded androgen receptor can also be studied, which was not possible up to now.

Because it has been shown that the N-terminal domain is the most immunogenic part of steroid hormone receptors (Westphal et al., 1982; Lorenzo et al., 1988), regions within this domain were initially used to develop androgen receptor-specific antibodies. However, detailed functional and structural analysis of the androgen receptor, e.g. identification of amino acid sequences involved in the association with heat shock proteins, nuclear translocation, dimerization, and association with other transcription factors, might also require antibodies directed against various other regions of the androgen receptor. Antibodies that recognize different regions of the receptor molecule may help to identify the exact sites of phosphorylation.

2.3 Determination of the relative phosphate content of the androgen receptor

Determination of the relative phosphate content of androgen receptors under different hormonal conditions required the normalization of incorporated [^{32}P]orthophosphate in relation to the receptor protein concentration (Chapter 6). To this end, a procedure has been developed to estimate the relative receptor protein concentration and phosphate incorporation simultaneously. This method has been described in detail in the paragraph "Measurement of receptor-specific phosphate levels" of the "Experimental Procedures"

section of Chapter 6. Briefly, immunopurified androgen receptor, labeled metabolically with [^{32}P]orthophosphate, was analysed on SDS-polyacrylamide gels and transferred to a nitrocellulose filter. The filter was incubated successively with an androgen receptor-specific polyclonal antiserum, with [^{125}I]-protein A, and with alkaline phosphatase-conjugated goat-anti-rabbit IgG. Each lane was cut in 2 mm slices, and the slices were dissolved and counted for both ^{32}P and ^{125}I radioactivities. The amount of each isotope, associated with the androgen receptor, was determined after subtraction of the background.

In the experiment presented in Figure 2.1, the suitability of the procedure, using protein A to determine the relative androgen receptor levels, has been tested. In this experiment, different amounts of immunoprecipitated, unlabeled androgen receptor were subjected to SDS-PAGE and Western blot analysis, using ^{125}I-protein A. The amounts of androgen receptor that were used (approximately 225-1125 fmol androgen receptor, based on ligand-binding studies), represented the receptor levels in the experiments for which the technique has been developed. It is shown that the amount of ^{125}I-radioactivity,

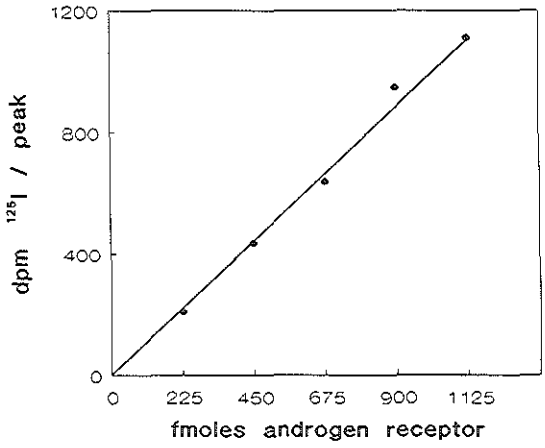

Figure 2.1. Relationship between the amount of ^{125}I-radioactivity, associated with the androgen receptor on the Western blot, and the amount of androgen receptor initially applied to the SDS-gel. The androgen receptor was isolated from LNCaP cell lysates, using an andogen receptor-specific monoclonal antibody, and solubilized in SDS-sample buffer as described in the "Experimental Procedures" section of Chapter 6. Different volumes of the receptor solution, containing 225-1125 fmoles androgen receptor, were subjected to SDS-PAGE and Western blot analysis, using a specific androgen receptor antiserum and ^{125}I-protein A, respectively. The amount of ^{125}I radioactivity associated with the androgen receptor protein bands was determined as described above.

associated with the androgen receptor on the Western blot, was directly proportional to the amount of receptor protein initially applied to the SDS-polyacrylamide gel. Hence, this procedure can be used to determine the relative receptor levels in different samples within one experiment. It is not possible, however, to quantify the absolute amount of receptor protein on the Western blot, because neither the specific activity of ^{125}I-protein A, nor the affinity of ^{125}I-protein A for the rabbit polyclonal antiserum and the affinity of the polyclonal antiserum for the androgen receptor, are known.

Figure 2.2 illustrates the typical patterns of ^{32}P and ^{125}I radioactivities, obtained when ^{32}P-labeled androgen receptor was subjected to SDS-PAGE and Western blot analysis using ^{125}I-protein A. The ^{32}P and ^{125}I radioactivities colocalize on the Western blot at the position of the 110 kDa androgen receptor (Chapter 3). In similar experiments, described in Chapter 6, the total amount of each isotope, associated with the androgen receptor, has been obtained by adding up the amount of radioactivity in the individual slices (slice numbers 9-11 in this particular experiment), after subtraction of the background.

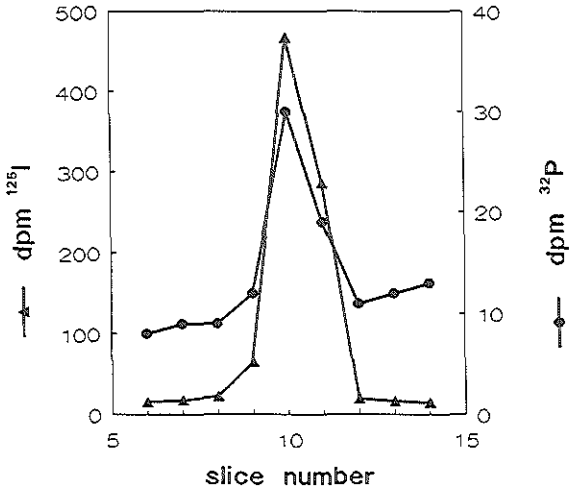

Figure 2.2. Profiles of ^{32}P and ^{125}I radioactivities, associated with ^{32}P-labeled androgen receptor after SDS-PAGE and Western blot analysis, using ^{125}I-protein A. LNCaP cells were cultured in the presence of [^{32}P]orthophosphate. The androgen receptor was isolated from total cell lysates with an androgen receptor-specific antibody and subjected to SDS-PAGE and Western blot analysis (Chapter 6). The nitrocellulose filter was cut into 2mm slices, and radioactivity was counted in the individual slices.

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CHAPTER 3

THE HUMAN ANDROGEN RECEPTOR IS A 110 kDa PROTEIN

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Key words: Androgen; Receptor; (Human); (In situ photolabeling); (Molecular weight); (LNCaP cells)

Summary

The androgen receptor in human prostate carcinoma cells (LNCaP) has been studied after in situ photolabeling with [^{3}H]R1881. Sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis of whole cell extracts revealed the presence of two specifically labeled proteins of 110 kDa and 43 kDa. Both photolabeled proteins were stable in cell homogenates and generated different chymotryptic maps, suggesting that the two proteins were different. From ligand binding specificity studies could be concluded that the 110 kDa protein represents the androgen receptor. The 43 kDa protein showed binding specificity only for R1881. Both photolabeled proteins were recovered from LNCaP nuclei, but the 43 kDa protein showed a relatively higher affinity for nuclei than the 110 kDa protein. The function of this protein is unknown. It is concluded that the human androgen receptor is a protein with a molecular mass of 110 kDa.

Introduction

Steroid hormone action in target tissues is mediated by specific intracellular receptor proteins. Upon ligand binding the receptor protein interacts in a specific way with hormone-regulatory elements which ultimately results in the regulation of transcription of specific genes (Kandala et al., 1985; Zhang et al., 1985; Montpetit et al., 1986; Salzman et al., 1987).

The androgen receptor remains the least well-characterized steroid receptor due to its considerable lower tissue concentration, its extreme lability and susceptibility to proteolytic breakdown. Recently, cloning and sequence analysis of cDNAs which partially encode the human androgen receptor were reported (Chang et al., 1988; Lubahn et al., 1988; Trapman et al., 1988). The exact size of the human androgen receptor, however, is still not known. The purpose of the present investigation was to determine the molecular mass of the human androgen receptor with the in situ photoaffinity labeling procedure (Horwitz and Alexander, 1983). This technique avoids in vitro incubation and isolation artefacts as much as possible. The previously established human LNCaP (lymph node carcinoma of the prostate) cell line was used for this study because this cell line contains androgen receptors and is responsive to androgens with respect to growth and specific protein synthesis (Horoszewicz et al., 1983; Berns et al., 1986). We provide evidence that the human androgen receptor is a protein with a molecular mass of 110 kDa when analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Fur-

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thermore, the LNCaP cell line contains a 43 kDa R1881 binding protein that is present in nuclei. This protein is most likely not a proteolytic degradation product of the androgen receptor. The function of this protein is unknown.

Materials and methods

Materials

[^{3}H]17β-Hydroxy-17α-methyl-estra-4,9,11-trien-3-one ([^{3}H]R1881, spec. act. 87 Ci/mmol) and radioinert R1881 were purchased from NEN-Dupont, Dreieich, F.R.G. Nonradioactive triamcinolone acetonide (9α-fluoro-11β,16α,17,21-tetrahydroxy-1,4-pregnadiene-3,20-dione 16,17-acetonide) was obtained from Sigma (St. Louis, MO, U.S.A.). All other steroids were purchased from Steraloids (Wilton, NH, U.S.A.). α-Chymotrypsin was obtained from Sigma. 4-Azaheptamethyldiamine was purchased from Merck-Schuchardt.

Cell culture

The LNCaP cell line (derived from a fast growing colony of the lymph node carcinoma of the prostate) was a gift from Dr. Horoszewicz (Buffalo, NY, U.S.A.). The cells were cultured in plastic Nunc flasks at 37 °C in RPMI-1640 medium, with added glutamine, streptomycin, penicillin and 7.5% (v/v) heat-inactivated fetal calf serum in a humidified atmosphere of 5% CO_2 in air. Four to seven days before the photolabeling experiments 7.5% (v/v) heat-inactivated fetal calf serum was replaced by 5% heat-inactivated charcoal-stripped fetal calf serum in the same medium. LNCaP cells between the 67th and 71th passage in vitro were used for the studies in this report.

In situ photolabeling

In situ photolabeling was performed according to the procedure described originally by Horwitz and Alexander (1983). LNCaP cells were incubated for 1 h at 37 °C with 10 nM [^{3}H]R1881 in serum-free medium in the presence or absence of unlabeled steroids. After incubation, the cell surface was washed twice with ice-cold phosphate-buffered saline. The flask was put on the surface of a 300 nm UV transilluminator (UVP, San Gabriel, CA, U.S.A.) and the cells were irradiated for 2 min. From the irradiated cells either a homogenate or a nuclear extract was prepared.

Preparation of lysates of in situ photolabeled cells

Immediately after in situ irradiation cells were solubilized in lysis buffer (40 mM Tris, pH 7.4, 1 mM EDTA, 4% glycerol, 10 mM dithiothreitol (DTT), 4% SDS) in the presence or absence of protease inhibitors (0.6 mM phenylmethylsulfonyl fluoride (PMSF), 1 mM leupeptin and 0.5 mM bacitracin), boiled for 3 min and centrifuged at 8000 $\times$ g for 10 min. The supernatant was analyzed by SDS-PAGE.

Preparation of nuclear extracts

After irradiation cells were harvested with a rubber policeman and homogenized in TEDGP buffer, pH 7.4 (40 mM Tris, 1 mM EDTA, 10% glycerol (w/v), 1 mM DTT, 0.6 mM PMSF, 1 mM leupeptin and 0.5 mM bacitracin) at 0 °C by using a glass homogenizer. The homogenate was centrifuged at 800 $\times$ g for 10 min at 4 °C. The pellet was washed once with TEDGP buffer containing 0.2% Triton X-100 and twice with TEDGP buffer alone. The nuclear pellet was resuspended in TEDGP buffer, pH 8.5, with 0.5 M NaCl and incubated for 1 h at 4 °C. The sample was centrifuged at 100 000 $\times$ g for 30 min. Proteins were precipitated from the supernatant with 10% trichloroacetic acid (TCA), solubilized in lysis buffer and boiled for 2 min. This preparation was analyzed by SDS-PAGE.

SDS-PAGE

SDS-PAGE of photolabeled samples was carried out according to Laemmli (1970) using 8% or 9–26% polyacrylamide gels. Following electrophoresis the slab gel was cut in 2 mm slices and radioactivity was eluted with a mixture of 8% Triton X-100 and 8% 4-azaheptamethyldiamine in water for 18 h at room temperature. Radioactivity was counted in picofluor. High molecular weight markers of Sigma (29 000–200 000 Da) in the presence or absence of low molecular weight markers of Pharmacia (2560–17 200 Da), run in parallel lanes, were used as references for molecular weight estimation.

Sonication of photolabeled LNCaP cells

After in situ photolabeling LNCaP cells were sonicated in a mixture of 40 mM Tris, pH 7.4, 1 mM EDTA and 4% glycerol for 5 s at high speed.

Chymotryptic digestion

The 110 kDa and 43 kDa photolabeled proteins were eluted from gel slices after SDS-PAGE in 40 mM Tris, pH 7.4, 1 mM EDTA, 10% glycerol and 0.1% SDS for 18 h at 20 °C. The eluate was incubated at 37 °C for 30 min in the presence or absence of 35 μg/ml α-chymotrypsin. The samples were analyzed by SDS-PAGE on 9–26% polyacrylamide gradient gels.

Results

Binding studies were performed with [^{3}H]R1881 because this ligand showed a high affinity for androgen receptors and its highly conjugated nature makes it extremely suitable as photoaffinity label for androgen receptors. LNCaP cells were incubated with 10 nM [^{3}H]R1881 and in situ irradiated. Whole cells were solubilized with a 4% SDS buffer and the extract was analyzed by SDS-PAGE. The profile of radioactivity is shown in Fig. 1. Two radioactive proteins were detected with molecular masses of 43 kDa and 110 kDa. A 100-fold molar excess of nonradioactive R1881 could suppress both radioactive protein peaks completely, indicating that they are specifically labeled. Table 1 shows the steroid specificity of both [^{3}H]R1881 binding proteins in LNCaP cells. The presence of a 100-fold molar excess of testosterone or dihydrotestosterone suppressed the covalent labeling of the 110 kDa protein with [^{3}H]R1881 completely. The 110 kDa protein also showed some affinity for the synthetic progestagen R5020. A 10-fold and a 100-fold molar excess of unlabeled R5020 reduced covalent labeling of the 110 kDa protein with [^{3}H]R1881 to 67% and 11% respectively. In contrast, testosterone, dihydrotestosterone and triamcinolone acetonide did not compete with [^{3}H]R1881 for binding to the 43 kDa protein, while a 100-fold molar excess of R5020 competed only to a limited extent (Table 1). This indicates that the 43 kDa and the 110 kDa proteins have different ligand specificities.

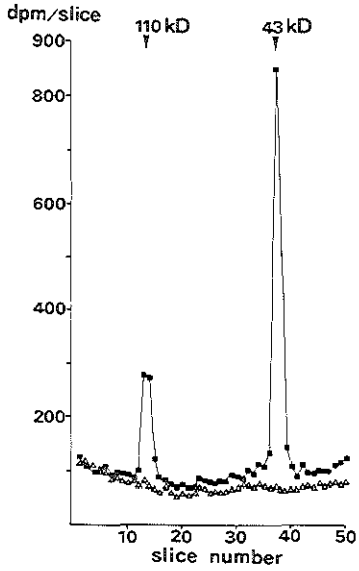

Fig. 1. SDS-PAGE pattern of photolabeled proteins of LNCaP cells after incubation with 10 nM [^{3}H]R1881 alone (■), or with 10 nM [^{3}H]R1881 in the presence of a 100-fold molar excess of unlabeled R1881 (△).

TABLE 1

STEROID BINDING SPECIFICITY OF THE 110 kDa AND 43 kDA PROTEINS IN LNCaP CELLS

LNCaP cells were in situ photolabeled with 10 nM [^{3}H]R1881 in the presence or absence of an excess of unlabeled steroids and separated on SDS-PAGE. The areas under the protein peaks were used to quantify the amount of covalently labeled protein. The amount of covalently bound [^{3}H]R1881 in the absence of unlabeled steroids was arbitrarily set at 100%.

Competing ligand	Molar excess	% Photolabeling	
		110 kDa	43 kDa
–	–	100	100
R1881	10	7	11
R1881	100	0	0
Testosterone	10	17	109
Testosterone	100	0	94
Dihydrotestosterone	10	25	104
Dihydrotestosterone	100	3	107
R5020	10	67	93
R5020	100	11	54
Triamcinolone acetonide	500	102	109

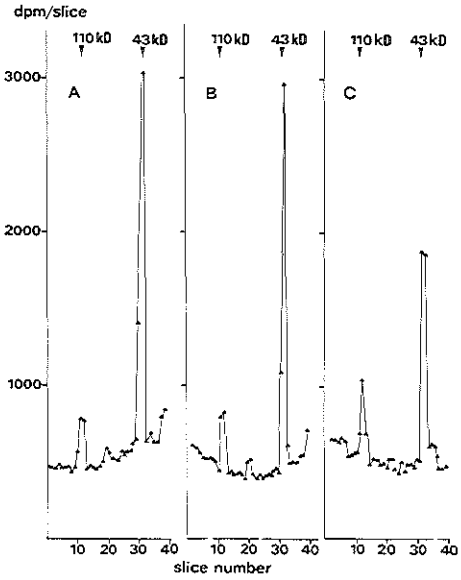

Fig. 2. Stability of in situ photolabeled AR in LNCaP cell sonicates. LNCaP cells were incubated with [^{3}H]R1881, in situ irradiated and sonicated. The sonicate was incubated at 20 °C. After 0 (*A*), 30 (*B*) and 60 (*C*) min samples were removed, adjusted to 4% (w/v) SDS and 10 mM DTT, boiled for 3 min and analyzed on SDS-PAGE.

The ratio between the amounts of the 43 kDa and 110 kDa photolabeled proteins was 3.7 ± 0.8 ($n = 8$). The presence or absence of several protease inhibitors (0.6 mM PMSF, 0.5 mM bacitracin, 1 mM leupeptin) in the lysis buffer had no effect on this ratio. The question whether the 43 kDa protein was a degradation product of the 110 kDa protein was studied next. LNCaP cells were in situ photolabeled and subsequently the cells were sonicated. In Fig. 2 the stability of both covalently labeled proteins in the sonicate is shown. Incubation of the sonicate for 1 h at 20 °C fails to degrade the 110 kDa photolabeled protein to the 43 kDa protein or to any other fragment. This indicates that the 43 kDa protein is not the result of in vitro degradation of the 110 kDa protein by endogenous proteases.

More evidence that the 43 kDa protein is not related to the 110 kDa protein was obtained after limited proteolysis with chymotrypsin. Fig. 3*A* shows the [^{3}H]R1881-labeled chymotryptic fragments generated from photolabeled 43 kDa protein. Digestion with 35 μg enzyme/ml resulted in the generation of fragments with molecular masses of 28, 25, 11 and 7 kDa. Limited chymotryptic digestion of the 110 kDa androgen receptor resulted in one single photolabeled fragment of 15 kDa (Fig. 3*B*), without any intermediate fragments.

The 43 kDa protein is, as the 110 kDa protein, present in nuclei. In Fig. 4*A* a SDS-PAGE pattern is shown of photolabeled proteins present in a 0.5 M NaCl extract of nuclei, prepared of in situ photolabeled LNCaP cells. In Fig. 4*B* the same nuclear pellet was solubilized in a 4% SDS buffer after the NaCl extraction. It can be concluded that most of the 110 kDa protein was extracted from nuclei with 0.5 M NaCl. The 43 kDa protein could only be released after the more drastic solubiliza-

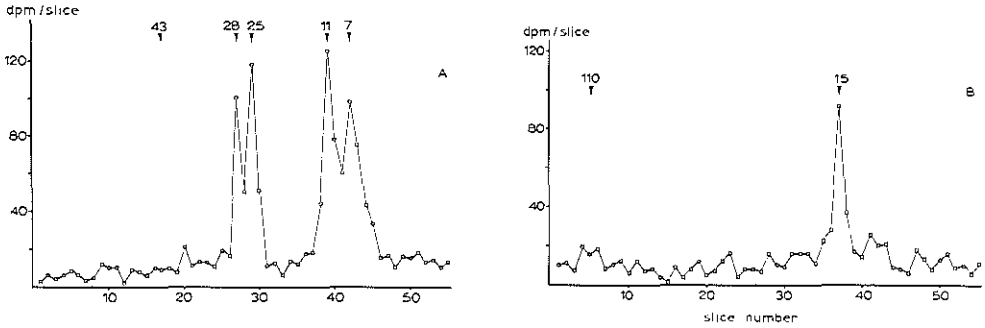

Fig. 3. SDS-PAGE patterns on 9–26% gels of chymotryptic digests of photolabeled 43 kDa protein (*A*) and 110 kDa androgen receptor (*B*).

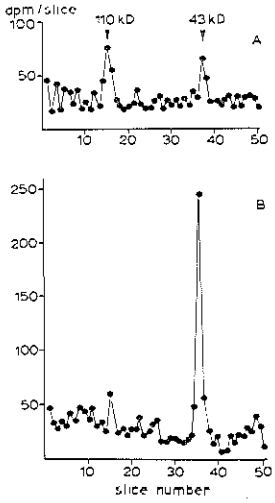

Fig. 4. SDS-PAGE patterns of photolabeled proteins, isolated from nuclei prepared from in situ photolabeled LNCaP cells. Nuclei were either extracted with 0.5 M NaCl (*A*) or with 4% SDS after NaCl extraction (*B*).

tion with SDS, suggesting that this protein is more tightly bound to nuclei.

Discussion

The exact size of the human androgen receptor is still not known due to its low tissue concentration, its extreme lability and susceptibility for proteolytic breakdown. Different molecular weights have been reported for covalently labeled androgen receptors on SDS-PAGE, ranging from 40 to 90 kDa (Gyorki et al., 1986; Brinkmann et al., 1987; Stamitiadis et al., 1987; Kodama et al., 1988; Kovacs and Truney, 1988). We estimated the molecular mass of the human androgen receptor in the LNCaP cell line with the in situ photolabeling procedure. With this technique in vitro incubation and isolation artefacts can be avoided as much as possible. Two proteins with molecular weights of 110 kDa and 43 kDa were specifically labeled. The fact that covalent labeling of the 110 kDa protein with [^{3}H]R1881 was suppressed completely in the presence of a 100-fold molar excess of unlabeled testosterone or dihydrotestosterone indicates that the 110 kDa protein is the androgen receptor. This molecular mass is in agreement with the molecular mass of the androgen receptor in calf uterus and rat epididymis (Brinkmann et al., 1985, 1989). The molecular mass of the human androgen receptor is in this respect in the same range as has been reported for the human glucocorticoid (Hollenberg et al., 1985) and progesterone receptor (Misrahi et al., 1987).

Endogenous proteases in LNCaP cells could be responsible for breakdown of the 110 kDa protein to a 43 kDa proteolytic fragment. However, the fact that both photolabeled proteins were stable for at least 1 h in LNCaP cell sonicates at 20 °C suggests that the 43 kDa protein is not generated during the homogenization procedure from the 110 kDa protein. Furthermore, it is not likely that the 43 kDa protein is generated from the 110 kDa protein during the photoactivation reaction, since irradiation of the cells for up to 7 min did not result in different ratios between both photolabeled proteins (result not shown). More evidence that the 110 kDa and 43 kDa proteins are not related was obtained after limited proteolytic digestion with chymotrypsin, which resulted in the generation of different photolabeled chymotryptic fragments for both photolabeled proteins. Furthermore, the proteins differ in their steroid specificity.

The 43 kDa protein binds specifically to R1881, but has no affinity for testosterone, dihydrotestosterone and triamcinolone acetonide. Furthermore, this protein has less affinity for R5020 than the 110 kDa androgen receptor, suggesting that the 43 kDa protein is not related to or derived from an androgen, glucocorticoid or progesterone receptor.

The relative binding activity of both the androgen R1881 and the progestagen R5020 for the human androgen receptor is understandable in the light of the observed homology between the steroid binding domains of the human androgen receptor and the human progesterone receptor (Trapman et al., 1988). Since R5020 has, like R1881, a highly conjugated structure, it is also possible that R5020 interferes with the photolabeling process of the 110 kDa androgen receptor and the 43 kDa protein. It can therefore not be excluded that the observed competition between R1881 and R5020 for the 43 kDa protein is in-

fluenced by the covalent attachment of nonradioactive R5020.

The 43 kDa protein is, like steroid hormone receptors, a nuclear protein with a relatively high affinity for nuclei. An increase in DNA-binding activity has also been found for the calf uterus androgen receptor (de Boer et al., 1987) and for the glucocorticoid receptor (Carlstedt-Duke et al., 1982) after removal of the DNA-binding modulating domain from the receptor. It cannot be excluded that this protein is related to a steroid hormone receptor which lacks the DNA-binding modulating domain.

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CHARACTERIZATION OF POLYCLONAL ANTIBODIES AGAINST THE N-TERMINAL DOMAIN OF THE HUMAN ANDROGEN RECEPTOR

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Characterization of polyclonal antibodies against the N-terminal domain of the human androgen receptor

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Summary

Antibodies against the N-terminal domain of the human androgen receptor (hAR) were prepared by two different approaches. Firstly, rabbits were immunized with a β-galactosidase-hAR (amino acids (aa) 174–353) fusion protein. Secondly, two synthetic peptides corresponding to potentially antigenic sites located within this fragment (aa 201–222 and 301–320) were used as immunogens. The obtained antisera contained high titer anti-hAR antibodies as was established with several independent methods (e.g. sucrose gradient centrifugation, immunoprecipitation, Western blotting). The two anti-peptide antisera specifically stained nuclei of glandular epithelial cells in frozen sections of human prostate tissue. Progesterone, estradiol and glucocorticoid receptors were not immunoprecipitated with these antisera.

The specific hAR antibodies provide new tools for the characterization of this steroid receptor as well as for diagnostic purposes in pathology of the human prostate and androgen resistance.

Introduction

Like all steroid hormone receptors, the androgen receptor (AR) is a ligand-responsive transcriptional regulator consisting of a putative DNA-binding domain, a steroid-binding domain at the C-terminal end and a large N-terminally located domain that might be involved in the regulation of gene transcription (Weinberger et al., 1986; Petrovich et al., 1987).

Antibodies against purified preparations of the oestrogen (ER), progesterone (PR) and glucocorticoid receptors (GR) have been used as tools in elucidating the function of these regulatory proteins (Okret et al., 1984; Logeat et al., 1985; Westphal et al., 1984). In addition, monoclonal antibodies directed against PR and ER are used for the quantification of these receptors in breast tumors, which is an important criterium for endocrine therapy. Similarly, antibodies against the human androgen receptor (hAR) might be useful for studies concerning the functional properties, quantification and localization of this protein. The antibodies could also be of high clinical importance for the characterization of defective receptor forms in individuals with the (partial) androgen

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insensitivity syndrome as well as for the quantification of AR in prostate cancer specimens.

Until recently antibodies against the hAR were not available because the low concentration of hAR in target tissues as well as the lability and susceptibility to proteolytic breakdown have hampered large scale purification of the receptor protein. However, autoimmune antibodies (Liao et al., 1985) as well as antibodies produced by immortalized human lymphocytes have been reported (Young et al., 1988). With the recent cloning of cDNA encoding the hAR and the subsequent elucidation of the hAR primary structure (Chang et al., 1988a; Lubahn et al., 1988a; Trapman et al., 1988), tools were provided for the preparation of large amounts of hAR protein fragments that can be used as antigen in the production of antibodies.

In this paper we report the preparation and characterization of a polyclonal anti-hAR antiserum, raised in rabbits against a fusion protein product containing a part of the hAR N-terminal domain. In addition we describe the properties of antibodies against synthetic peptides corresponding to possible predicted antigenic areas in the N-terminal domain of the hAR. The antibodies are shown to be valuable tools in various immunobiochemical techniques: precipitation, blotting and in situ enzyme-immunohistochemistry.

Materials and methods

[17α-*methyl*-^{3}H]Methyltrienolone ([^{3}H]R1881), spec. act. 87 Ci/mmol and [17α-*methyl*-^{3}H]promegestone ([^{3}H]R5020), spec. act. 72.4 Ci/mmol were purchased from NEN-Dupont, Dreieich, F.R.G. [2,4,6,7-^{3}H]Oestradiol, spec. act. 94 Ci/mmol and [1,2,4,6,7-^{3}H]dexamethasone, spec. act. 94 Ci/mmol, were obtained from Amersham, Cardiff, U.K.

Preparation of hAR fusion protein and generation of antibodies

The 542 bp *Pvu*II-*Pvu*II hAR cDNA fragment (derived from clone T4.1. A1 and corresponding to amino acids (aa) 174–353, see Faber et al. (1989), Figs. 1 and 2) was ligated in the *Sma*I site in the polylinker of the bacterial expression vector pEX2 (Stanley and Luzio, 1984) using standard recombinant DNA technology (Maniatis et al., 1982). The orientation of the integrated fragment was established by digestion of the plasmid (pEX-β-gal-hAR) with *Pst*I. β-Galactosidase-hAR fusion protein was produced in *Escherichia coli* POP 2136 during a 2 h induction period at 42° C. Protein was isolated from the bacterial pellet by extraction in a 60 mM Tris buffer (pH 6.8) containing 3% sodium dodecyl sulphate (SDS), 6 M urea, 5% β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF). After centrifugation (10 min, 12000 × g) the supernatant was dialyzed overnight at 4° C against phosphate-buffered saline (PBS) containing 1 mM PMSF. The protein extract was once again centrifuged and subsequently used for SDS-polyacrylamide gel electrophoresis (PAGE) analysis or immunization. A New Zealand white rabbit was immunized subcutaneously 2 times at 4-week intervals with approximately 250 μg of protein. The first injection was in complete Freund's adjuvant, the second in incomplete adjuvant and subsequent boosters in PBS. Two weeks after each immunization serum was analyzed for reactivity with the fusion protein by Western blotting.

Selection of putative antigenic sequences

For the selection of putative antigenic sequences predictive algorithms were used: hydrophilicity (Hopp and Woods, 1981; Kyte and Doolittle, 1982), α-helix and β-turns (Chou and Fassman, 1978; Garnier et al., 1978), surface probability (Emini, 1985), flexibility (Karplus and Schulz, 1985), culminating in an antigenicity index according to Jameson and Wolf (1988). Two peptides, SpO60 (amino acids 201–222) and SpO61 (amino acids 301–320), were synthesized.

Synthesis of peptides and immunization protocol

Solid-phase synthesis on Rapid-Amide resin beads was performed essentially according to Merrifield (1963) using Fmoc-protected amino acids (Dupont, Wilmington, U.S.A.) in the procedure as described for the RaMPS System (Dupont, Medical Products, Biotechnology Systems, U.S.A.). High-performance liquid chromatography (HPLC)-grade reagents were used throughout the peptide production procedure. For deprotection piperidine was used. Elongation was controlled at

each step (Kaiser et al., 1970), in case of a proline coupling elongation was checked according to a modification (Pritchard and Aufret, 1986) of the method originally described by Kaiser et al. (1980). Final deprotection and cleavage were performed using a mixture of trifluoroacetic acid, phenol and ethanedithiol followed by precipitation and filtration from diethyl ether. The peptides contained cysteins with a tertiary butyl protection group which was removed using mercuric (II) acetate. The peptides were purified as described elsewhere (Van Denderen et al., 1989). Briefly, the peptides were reduced using β-mercaptoethanol and subsequently purified over G-15 Sephadex (Pharmacia, Uppsala, Sweden) in 5% acetic acid. Fractions showing a single peak in reversed-phase chromatography in a gradient of acetonitrile with 0.1% trifluoroacetic acid, monitored at 214 nm, were pooled and freeze dried. The amino acid composition was confirmed using amino acid analysis of the peptides. The selected AR sequences were prolonged with a cystein to allow coupling to a carrier protein, keyhole limpet hemocyanin with maleimidobenzoyl-*N*-hydroxysuccinimide ester as described elsewhere (Boersma et al., 1988). Control conjugates of the peptides with bovine serum albumin (BSA), based on coupling of the peptides with a carbodiimide (Boersma et al., 1988), were used in enzyme-linked immunosorbent assay (ELISA) for analyses of the anti-peptide responses. Two rabbits (Flemisch Giant random bred, MBL-TNO, Rijswijk, The Netherlands) were immunized with each peptide conjugate; 3 times at 4-week intervals with 250 μg of the conjugate with Freund's complete adjuvant the first time and with incomplete Freund's adjuvant for the other immunizations. Two weeks after each immunization serum was analyzed for reactivity with the peptides and conjugates in an ELISA (Boersma et al., 1988). Pre-immune sera served as controls.

Cell culture

The cell lines LNCaP, NHIK, MCF-7 and T47D were cultured as described previously (Mulder et al., 1978; Keydar et al., 1979; Berns et al., 1984; Van Laar et al., 1989).

Preparation of nuclear extract

LNCaP, MCF-7 and NHIK cells were incubated with 10 nM [^{3}H]R1881, [^{3}H]oestradiol and [^{3}H]dexamethasone respectively for 1 h at 37 °C. T47D cells were incubated with 40 nM [^{3}H]R5020 for 1 h at 37 °C. After incubation, the cells were collected in TEDGP buffer, pH 7.4 (40 mM Tris, 1 mM EDTA, 10% glycerol (w/v), 10 mM dithiothreitol, 0.6 mM PMSF, 0.5 mM bacitracin) at 0 °C and homogenized by using a glass/Teflon homogenizer. The homogenate was centrifuged at 800 $\times$ *g* for 10 min at 4 °C. The pellet was washed with TEDGP buffer pH 7.4 containing 0.2% Triton X-100 and with TEDGP buffer pH 7.4 without additions.

The nuclear pellet was resuspended in TEDGP buffer, pH 8.5, in the presence of 0.5 M NaCl and 0.25 mM leupeptin and extracted for 1 h at 0 °C. The sample was centrifuged at 100 000 $\times$ *g* for 30 min.

Measurement of steroid binding

Protamine sulphate (Organon, Oss, The Netherlands) solution (500 μl of 0.5 mg/ml) was mixed with 50 μl nuclear extract and precipitated for 5–10 min on ice in glass tubes which were precoated with 0.1% BSA for 30 min at 30 °C. The precipitate was centrifuged for 15 min at 2000 $\times$ *g* and washed 4 times with 0.5 ml ice-cold TEDGP pH 7.4 buffer. The precipitate was dissolved in 0.5 ml Soluene-350 (Packard, Downers Grove, IL, U.S.A.) for 15 min at 60 °C. Precipitated radioactivity was counted in 5 ml Instagel (Packard). Nonspecific binding (less than 10%) was determined in nuclear extracts of cells which were incubated with radiolabeled ligand in the presence of a 100-fold molar excess of unlabeled ligand.

Sucrose gradient centrifugation

150 μl of [^{3}H]R1881-labeled nuclear extract (0.15 M NaCl), which was incubated for 6 h at 4 °C with anti- or pre-immune serum was layered on 10–30% sucrose gradients in the presence of 0.3 M NaCl and centrifuged for 20 h at 370 000 $\times$ *g* at 4 °C (De Boer et al., 1986).

Double immunoprecipitation

20 μl of [^{3}H]R1881-labeled LNCaP nuclear extract, diluted until the NaCl concentration was 0.15 M, was incubated with 20 μl antiserum or pre-immune serum in various dilutions for 18 h at

4°C. 50 µl goat anti-rabbit serum and 50 µl 1% normal rabbit serum were added and the incubation was continued for 2 h at 4°C. 1 ml of polyethylene glycol (molecular weight (MW) 6000–7000) was then added and the mixture was incubated for an additional 2 h at 4°C before centrifugation at 2400 × *g* for 30 min. The precipitate was washed once with 1 ml PBS containing 1% BSA, centrifuged for 10 min at 2400 × *g* and counted for radioactivity in 10 ml Instagel (Packard).

Photoaffinity labeling of nuclear extracts

Photoaffinity labeling of the androgen receptor with [^{3}H]R1881 in nuclear extracts was performed via irradiation of the sample with an Osram HBO 100 W/W-2 high pressure mercury lamp as described previously (Brinkmann et al., 1985).

Methanol precipitation

One volume of photolabeled nuclear extract from LNCaP cells was added to 5 volumes of methanol, precipitated for 18 h at −80°C and centrifuged at 10 000 × *g* for 30 min. The precipitate was prepared for SDS-PAGE.

SDS-PAGE

SDS-PAGE was carried out according to Laemmli (1970) using 8% polyacrylamide gels. After electrophoresis, the slab gel was either subjected to Western blotting or counted for radioactivity as described (Van Laar et al., 1989).

Western blotting

The slab gels were positioned on nitrocellulose paper (Schleicher and Schuell, 0.45 µm) and placed in a Bio-Rad (Richmond, CA, U.S.A.) Trans-Blot cell, filled with 16.5 mM Tris/150 mM glycine/20% methanol (pH 8.3). The transfer was performed at 4°C using 65 V for 17 h. The paper was incubated with the antiserum diluted 1:50 (Tp4) or 1:200 (SpO60 and SpO61) in PBS/0.05% Tween-20 (PBS-Tween) for 1 h at room temperature, washed 3 times for 20 min each with PBS-Tween, and incubated for another hour at room temperature with alkaline phosphatase-conjugated goat anti-rabbit IgG (Sigma, St. Louis, MO, U.S.A.), diluted 1:2000 in PBS-Tween. After washing of the nitrocellulose paper, the antibody complexes were stained with a solution of 0.3% (w/v) 4-aminodiphenylamine diazonium sulphate (Sigma), 0.1% (w/v) naphthol phosphate (disodium salt) (Sigma) and 10 mM $MgCl_2$ in 0.2 M Tris-HCl, pH 9.1.

Immunohistochemistry

6 µm-thick cryostat sections of freshly frozen prostatic tissues with nodular glandular hyperplasia were fixed in 4% buffered formalin and dehydrated in chilled methanol and acetone. Incubation with the oligopeptide-specific rabbit antisera (SpO60 and SpO61) diluted 1:500 in PBS containing 0.5% BSA and 0.1% sodium azide (pH 7.8) was done overnight at 4°C. After rinsing in PBS (pH 7.4) an indirect conjugated peroxidase method was applied using a swine anti-rabbit immunoglobulin conjugate (Dakopatts, Glostrup, Denmark) as second step reagent. Reactivity was visualized using hydrogen peroxide and diaminobenzidine (Sigma) as substrate. For distinction of nuclei counterstaining with Mayer's haematoxylin was sometimes applied. Pre-immune sera were used as negative controls. Freshly frozen lymphoma tissue processed similarly as the prostatic tissue served as a specificity control.

Results

Generation of the antibodies

A *Pvu*II-*Pvu*II cDNA restriction fragment (Tp4) encoding part of the hAR N-terminal domain (aa 174–353, see Faber et al., 1989) was ligated to the *lacZ* gene and expressed in *E. coli* (Fig. 1). The lysate, containing large amounts of the fusion protein (about 25%), was used for immunization of rabbits. Antisera which were purified over an affinity column prepared from lysates

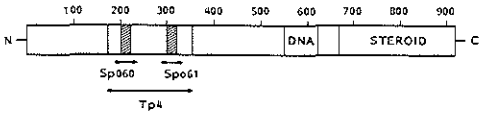

Fig. 1. Schematic diagram of the hAR, illustrating the location of the functional domains and the location of the peptides (SpO60 and SpO61) and the *Pvu*II-*Pvu*II fragment (Tp4) that were used for immunizations. Numbers indicate the amino acid residues.

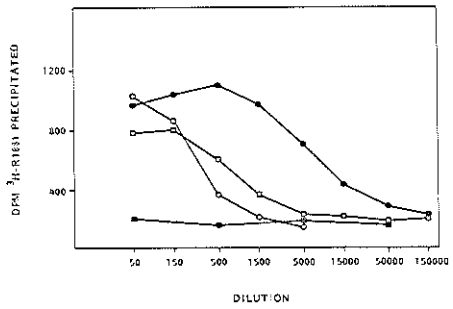

Fig. 2. Immunoprecipitation of [^{3}H]R1881-labeled hAR in nuclear extracts with the antisera Tp4 (○), SpO60 (□), SpO61 (●) or SpO61 pre-immune serum (■). A constant amount of nuclear extract containing 2480 dpm [^{3}H]R1881-labeled hAR was incubated with serial dilutions of the sera. Goat anti-rabbit antiserum was used as a second antibody.

of β-galactosidase producing bacteria were reactive with the β-gal-hAR fusion protein in a Western blot (not shown), indicating that antibodies against the hAR part of the fusion protein were indeed generated. With prediction programme analyzing immunogenicity (Chou and Fassman, 1978; Garnier et al., 1978; Hopp and Woods, 1981; Kyte and Doolittle, 1982), two possible antigenic areas were selected within the same hAR fragment which was used for the preparation of the fusion protein. Two peptides, SpO60 (aa 201–222) and SpO61 (aa 301–320, see Fig. 1 and Faber et al., 1989) were used to raise anti-hAR antibodies in rabbits. In an ELISA assay the anti-peptide antisera were reactive with the material used for immunization. In addition, the antisera recognized the β-gal-hAR (aa 174–353) fusion protein in a Western blot (not shown).

Double immunoprecipitation

The interaction of the three antisera with the native hAR was determined by double immunoprecipitation. A constant amount of [^{3}H]-R1881-labeled nuclear extract, prepared from

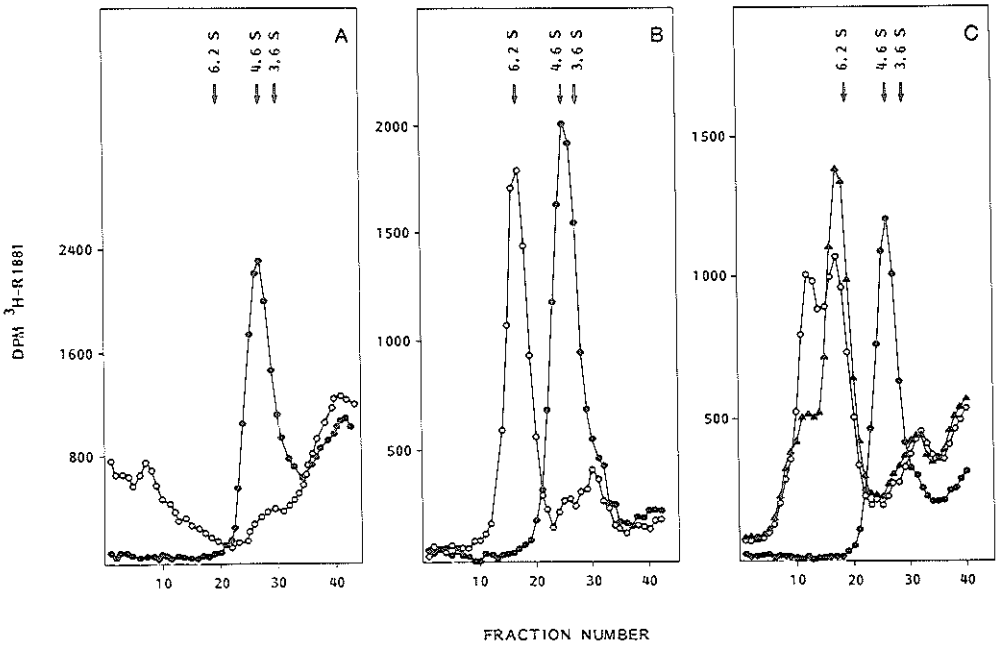

Fig. 3. Sedimentation profiles in 10–30% sucrose gradients of 137 μl [^{3}H]R1881-labeled hAR in LNCaP nuclear extracts (0.15 M NaCl in TEDGP buffer) incubated with (*A*) 13 μl Tp4 (○) or 13 μl pre-immune serum (●); (*B*) 13 μl SpO60 antiserum (○) or pre-immune serum (●); (*C*) 4 μl (○) or 0.8 μl (▲) SpO61 antiserum or 4 μl pre-immune serum (●).

LNCaP cells, was incubated with serial dilutions of the antisera. Immune complexes were precipitated with a second antibody. As shown in Fig. 2, use of serial dilutions of the antisera proportionally decreased the amount of precipitated [^{3}H]-R1881-AR complexes, indicating the presence of specific antibodies against the hAR.

The titres of anti-hAR antibodies in both anti-peptide sera (SpO60 and SpO61) were higher than in the serum raised against the fusion protein (Tp4). Approximately 50% of the [^{3}H]R1881 binding activity could be precipitated with a 1 : 300 dilution of the Tp4 antiserum. Dilutions of 1 : 7000 and 1 : 900 of the antisera SpO61 and SpO60 respectively were able to precipitate 50% of [^{3}H]R1881 binding activity.

Sucrose gradient analysis

In Fig. 3 sucrose density sedimentation analysis is shown of [^{3}H]R1881-labeled hAR in nuclear extracts of LNCaP cells in the presence of the antisera. Incubation of labeled nuclear extracts with each of the antisera resulted in faster sedimentation rates of the 4.6S AR, indicating the presence of specific antibodies in the three antisera that recognize the native hAR. No shifts were observed with pre-immune sera. In the presence of the SpO60 antiserum a 6.7S peak was formed (Fig. 3*B*). In contrast, incubation with the SpO61 antiserum resulted in the generation of two labeled protein peaks of 6.7S and 8.1S respectively (Fig. 3*C*). The ratio between both labeled protein peaks was dependent on the antiserum concentration. The 6.7S complex shifted completely to the 8.1S form in the presence of more antiserum. This suggests that the SpO61 antiserum is directed against two different epitopes on the receptor molecule, whereas one epitope is recognized by the SpO60 antiserum. The Tp4 antiserum shifted the steroid receptor complex to the bottom of the tube (Fig. 3*A*), indicating the formation of large immune complexes.

Specificity of the antibodies

In order to exclude any crossreactivity of the three different antisera with other steroid receptors, we tested receptor specificity with a double-immunoprecipitation assay. For this purpose nuclear extracts containing hPR, hER, hGR and hAR were prepared from T47D, MCF-7, NHIK and LNCaP cells respectively. The results of the immunoprecipitation studies with the three different antisera and the particular receptor preparation are shown in Table 1. Only with the AR preparation a significant radioactive immunoprecipitate was obtained, indicating that none of the other steroid hormone receptors was recognized by the antisera.

TABLE 1

STEROID RECEPTOR SPECIFICITY OF THE ANTISERA

Radiolabeled hAR (2480 dpm), GR (1050 dpm), ER (840 dpm) and PR (2000 dpm) in nuclear extracts of LNCaP, NHIK, MCF-7 and T47D cells, respectively, were incubated with anti- or pre-immune serum in a 1:50 dilution. Goat anti-rabbit antiserum was used as a second antibody. Data are expressed as dpm ^{3}H precipitated.

	hAR	hGR	hER	hPR
Tp4	1018	40	94	45
SpO60	787	43	88	47
SpO61	967	38	94	38
Pre-immune	204	37	100	30

Immunoprecipitation of covalently labeled hAR

To verify that the antisera recognize the intact hAR, receptors that had been covalently labeled with [^{3}H]R1881 were precipitated from LNCaP nuclear extracts with a double-immunoprecipitation assay. The immune precipitate was analyzed on SDS-PAGE. Fig. 4*A* shows the presence of one labeled protein at a position of 110 kDa after methanol precipitation of proteins from photo-labeled LNCaP nuclear extracts. This protein peak represents the intact hAR (Van Laar et al., 1989). The SpO61 and Tp4 antisera precipitated a radioactive labeled protein with exactly the same molecular mass as was precipitated with methanol (Fig. 4*B* and *C*). Similar results were obtained with the SpO60 antiserum.

Western blotting

To examine the interaction of the antisera with the denatured hAR, photolabeled LNCaP cell nuclear extract was fractionated by SDS-PAGE. After transfer to nitrocellulose membranes, blots were incubated with each of the sera. Fig. 5 shows

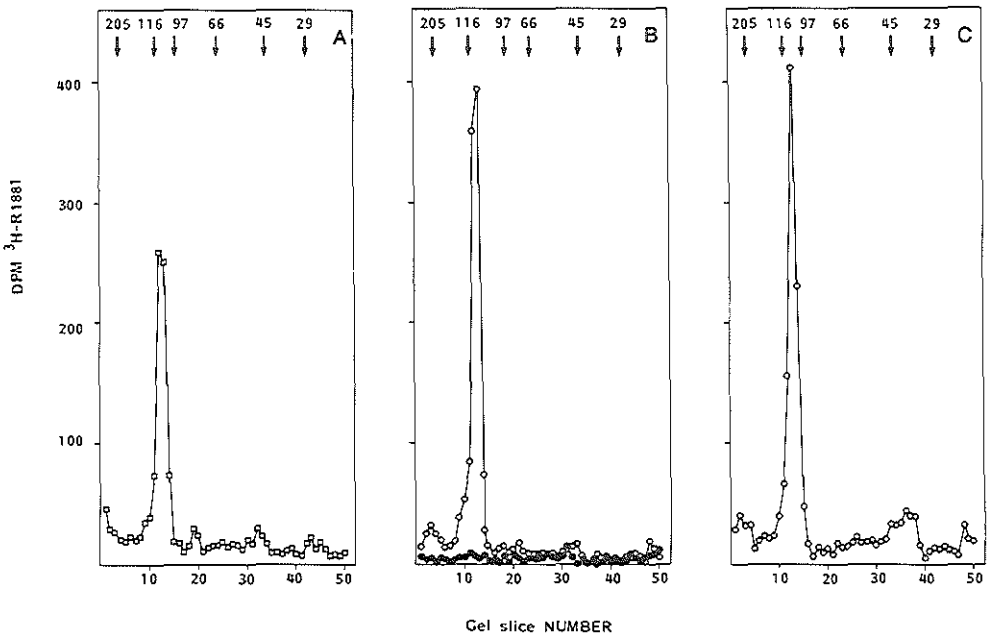

Fig. 4. SDS-PAGE patterns of covalently labeled hAR, precipitated from 800 μl LNCaP nuclear extracts (in 0.15 M NaCl in TEDGP buffer) with (*A*) methanol; (*B*) 4 μl SpO61 antiserum (○) or pre-immune serum (●); (*C*) 16 μl Tp4 antiserum. Immune complexes were isolated with a second antibody.

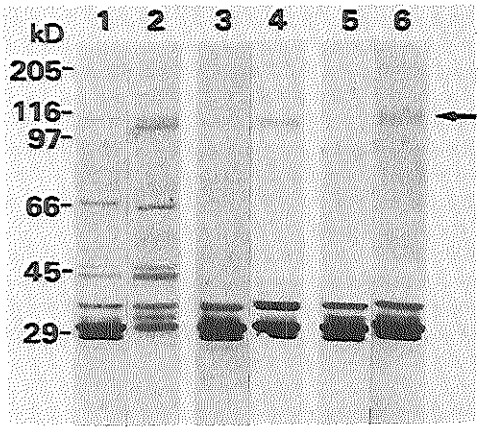

Fig. 5. Immunoblotting of hAR. After SDS-PAGE of nuclear extracts protein was transferred to nitrocellulose and blots were incubated with Tp4, 1:50 (lane 2), SpO60, 1:2000 (lane 4) and SpO61, 1:2000 (lane 6) or the corresponding pre-immune sera in the same dilutions (lanes 1, 3, and 5 respectively).

that a protein with an apparent molecular mass of 110 kDa was identified by the antisera (lanes 2, 4, and 6). A similar protein band was not detectable with the pre-immune sera (lanes 1, 3, and 5). Radioactivity comigrates with the specifically stained protein band.

Immunohistochemistry

Immunostaining with both antiserum SpO60 and SpO61 resulted in a selective nuclear staining of the secretory epithelial cells lining the prostatic glands, whereas no reactivity with the basal cell layer was observed. Reactivity with SpO61 was more intense as compared to the SpO60 antiserum. A rather weak staining reaction with varying proportions of nuclei of stromal cells with antiserum SpO61 was consistently present. Neither antisera yielded reactivity with lymphoma tissues (Fig. 6). In addition, pre-immune sera were negative for nuclear staining of prostate tissue.

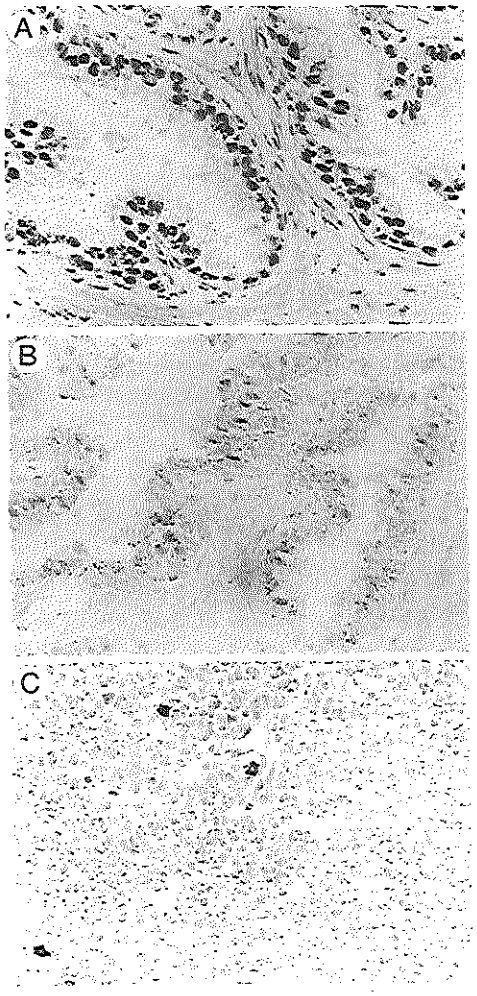

Fig. 6. *A*: Nuclear staining of the secretory epithelial cells of prostatic glands after immunohistochemistry with anti-AR antiserum SpO61. *B*: Prostatic tissue incubated with the pre-immune serum. *C*: Lymphoma tissue incubated with anti-AR antibody SpO61. Magnification 375×, no nuclear counterstaining.

Discussion

This paper describes two different approaches for the preparation of anti-hAR antisera. Both approaches resulted in the generation of high-titre antisera that recognize the native hAR as well as the hAR protein in Western blots. In addition, the peptide-antisera recognize the hAR in nuclei of epithelial cells of benign hyperplastic prostate tissue. The antibodies generated were highly specific for the hAR because crossreactivity with hGR, hPR and hER was absent. In addition, it appeared that AR from other species (e.g. calf) was also recognized (unpublished results). The species specificity, however, has to be investigated further.

In the first approach a bacterially expressed fusion protein product (Tp4), containing a fragment of 180 amino acid residues of the hAR was used for immunization. For two reasons a fragment localized in the N-terminal domain of the receptor protein had been chosen. Firstly, it was known that antibodies against purified preparations of the GR (Carlstedt-Duke et al., 1982; Westphal et al., 1982) and PR (Lorenzo et al., 1988) were predominantly directed against sites in the N-terminal domain, suggesting that this domain is the most immunogenic part of steroid hormone receptors. Secondly, since the steroid- and DNA-binding domains of the hAR share a high homology with similar domains in the hPR, hGR and mineralocorticoid receptor (Chang et al., 1988b; Lubahn et al., 1988b; Trapman et al., 1988) antibodies generated against regions in these domains might crossreact with other steroid hormone receptors.

In the second approach two synthetic peptides (SpO60 and SpO61), corresponding to potentially antigenic regions in the receptor molecule, were selected as antigen. Both amino acid stretches are present within the hAR fragment of the fusion protein product. A similar approach has also been successfully used for the generation of antibodies against a peptide homologous to a hAR fragment flanking the N-terminal zinc finger motif (Lubahn et al., 1988b).

Sucrose gradient analysis of [^{3}H]R1881-labeled hAR in the presence of the Tp4 antiserum resulted in the formation of large immune complexes sedimenting to the bottom of the tube. It is likely that a mixed population of antibody molecules had been generated, recognizing different epitopes on the hAR molecule. This could be expected because a relatively large fragment was used for immuniza-

tion. This result is different from the discrete shifts induced by the anti-peptide sera which are indicative for single-epitope recognition on the labeled hAR. In the presence of the SpO60 antiserum the sedimentation coefficient of the 4.6S [^{3}H]R1881-labeled hAR was shifted to 6.7S suggesting the formation of 1 : 1 complexes of hAR and antibodies. In the presence of the SpO61 antiserum antigen-antibody complexes with sedimentation coefficients of 6.7S and 8.1S were formed. Furthermore, the 6.7S form was converted to 8.1S with increasing antiserum concentrations. This sedimentation profile might be explained by the fact that the antiserum recognizes probably two different epitopes on the receptor molecule.

Both the pre-immune sera and antisera recognize protein bands in Western blots of LNCaP nuclear extracts. One protein band at a position of 110 kDa was stained exclusively with the hAR antisera. There is strong evidence that this band represents the hAR. Firstly, the hAR migrates as a 110 kDa protein on SDS-PAGE (Van Laar et al., 1989). Secondly, although the antisera were raised against different epitopes on the receptor molecule, they all recognized a protein at a similar position in the gel. Thirdly, [^{3}H]R1881 radioactivity comigrated with the stained protein band.

For the characterization of the antisera we exclusively used ligand-bound nuclear hAR. Whether the untransformed hAR is also recognized by the antisera remains to be investigated. Since transformation of steroid receptors might imply dissociation of the receptor from a macromolecular complex including a 90 kDa heat shock protein (Schuh et al., 1985; Sanchez et al., 1987) and/or a conformational change in the receptor molecule, it cannot be excluded that the epitopes, recognized by the antisera are masked in the untransformed receptor form.

The peptide antisera recognize specifically antigens in nuclei of the secretory epithelial cells lining the prostatic glands in frozen sections of prostatic tissue. Some nuclei of stromal cells showed a weak reactivity. A similar AR localization has been observed in rat and human prostatic tissue with different antisera (Lubahn et al., 1988b; Tan et al., 1988) and with radiolabeled R1881 as a ligand (Peters and Barrack, 1987).

The hAR-directed antibodies provide important tools which can be used for studying many aspects of receptor structure and function. It might be possible now to detect the AR without the use of radioactive ligands. Furthermore, AR mutants which lack completely or partially the steroid-binding domain can be detected now. The development of detection methods for aberrant hARs is highly relevant for studies concerning the androgen insensitivity syndrome and for investigations on androgen independency of prostate cancer. Other potential applications of specific AR antibodies are their use for receptor determination with enzyme-linked or radioimmunoassays, and for purification purposes on immunoadsorbent columns.

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CHAPTER 5

ANDROGEN RECEPTOR HETEROGENEITY AND PHOSPHORYLATION IN HUMAN LNCaP CELLS

J.H. van Laar, J. Bolt-de Vries, N.D. Zegers, J. Trapman
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ANDROGEN RECEPTOR HETEROGENEITY AND PHOSPHORYLATION IN HUMAN LNCaP CELLS

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Androgen receptor heterogeneity and phosphorylation were studied in the human LNCaP cell line. Fluorography after photoafffinity labeling as well as immunoblotting with a specific polyclonal antibody revealed that the human androgen receptor migrated as a closely spaced 110 kD doublet on SDS-polyacrylamide gels. A time-dependent change in the ratio between the two isoforms was not observed after R1881 treatment of intact cells. In nuclear extracts of LNCaP cells that were incubated with [^{32}P]orthophosphate in the presence of 10 nM R1881, a 110 kD phosphorylated protein was demonstrated after immunopurification using a monoclonal antibody against the human androgen receptor. Only a very small amount of this phosphoprotein was detected in the nuclear fraction from cells not treated with R1881. These results indicate that the human androgen receptor in LNCaP cells can be phosphorylated. © 1990 Academic Press, Inc.

Steroid hormone receptors are intracellular proteins which are involved in transcriptional regulation of specific genes in target tissues. The first step in steroid hormone action is ligand binding. This results in transformation of the steroid-receptor complex from a loosely nuclear binding form to a form which is tightly bound in the cell nucleus (1,2). The molecular mechanism by which the receptor molecule changes upon ligand binding into a DNA-binding protein which regulates gene transcription is not completely clear.

Phosphorylation of the steroid receptor molecule might play an important role in the mechanism of action of steroid hormones. Ample evidence has been provided that steroid hormone receptors can exist in intact cells as phosphoproteins (3-6). It has been postulated that an initial phosphorylation step is necessary for the acquisition of the hormone binding capacity (7-9). A second, hormone dependent, phosphorylation step might be involved in the transformation process and in the regulation of gene transcription (4,10,11). Steroid receptor phosphorylation may result in receptor heterogeneity (4,12). An increased incorporation of [^{32}P]orthophosphate and an upshift in apparent

Abbreviations: LNCaP, Lymph Node Carcinoma of the Prostate; SDS-PAGE, sodium dodecyl sulphate poly acrylamide gel electrophoresis.

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molecular mass on SDS-gels have been observed after hormone treatment of target cells for the progesterone, glucocorticoid, oestradiol and vitamin D receptors (4,11-16).

It is not known whether phosphorylation of the androgen receptor may also occur and might be involved in androgen action. With the recent development of specific polyclonal (17) and monoclonal antibodies against a region within the N-terminal domain of the human androgen receptor, tools were provided to investigate phosphorylation of this receptor molecule. In the present study it is shown that the androgen receptor in human LNCaP cells is a heterogeneous protein that migrates as a doublet on SDS-PAGE. In addition, the results described herein provide evidence that the androgen receptor in these human cells is phosphorylated.

MATERIALS AND METHODS

Materials

17β-hydroxy-17α-[^{3}H]methyl-4,9,11-estratrien-3-one ([^{3}H]R1881) (87 Ci/mmol) and radioinert R1881 were purchased from NEN-Dupont (Dreieich, F.R.G.). Dihydrotestosterone was obtained from Steraloids (Wilton, NH, USA).

Cell culture

The LNCaP cell line was cultured as described previously (18). Two to four days before use the cells were kept on medium containing 5% heat-inactivated, charcoal stripped fetal calf serum.

Preparation of nuclear salt extracts

The preparation of nuclear salt extracts has been described previously (17). 50 mM sodium fluoride and 10 mM sodium molybdate were added as phosphatase inhibitors to all buffers.

In situ photolabeling, preparation of cell lysates and gel slicing have been described elsewhere (18).

SDS-PAGE

SDS-PAGE was carried out according to Laemmli (19) using 6% linear polyacrylamide gels. High molecular weight markers (29,000-205,000 D, Sigma,St. Louis, MO, USA) were used as references for molecular weight estimation.

Fluorography

After SDS-PAGE, the slabgel was fixed in 50% methanol/10% acetic acid for 45 min and soaked in Amplify (Amersham, Buckinghamshire, U.K.) for 20 min, dried under vacuum, and exposed to Hyperfilm-MP (Amersham) for 10 weeks at -80°C.

Western blotting and autoradiography

Western blot analysis, using Sp061 antiserum in a 1:1000 dilution, has been described previously (17). If receptor preparations were labeled with [^{32}P]orthophosphate, the filter was air-dried after colour development and exposed to hyperfilm-MP (Amersham) with two intensifying screens for 8 days at -80°C.

Metabolic labeling with [^{32}P]orthophosphate

For phosphorylation studies LNCaP cells were preincubated for 1 hour at 37°C with a solution of 118.3 mM NaCl, 4.75 mM KCl, 25 mM $NaHCO_3$, 1.2 mM $NaSO_4$, 2.5 mM $CaCl_2$, 0.2% glucose, essential and non-essential amino acids (GIBCO, Grand

Island, NY, USA) and glutamine (GIBCO) in appropriate dilutions at pH 7.3 and subsequently incubated for 4 h with 0.2 mCi/ml [^{32}P]orthophosphate (carrier-free, Amersham). 30 min before the end of the incubation, R1881 (10 nM; final concentration) or vehicle were added. After the incubation period nuclear salt extracts were prepared and androgen receptors were isolated by immunopurification as described below.

Preparation of monoclonal antibodies

A synthetic peptide, corresponding to the amino acids 301-320 of the human androgen receptor (20) was coupled to keyhole limpet hemocyanin and used for immunization. The same peptide has been successfully used for the generation of polyclonal antibodies (17). A Balb/c mouse (12-weeks old) was immunized subcutaneously with the peptide conjugate; 3 times at 5 weeks intervals with 25 µg of the peptide conjugate in Specol (21). Fusion of approximately 10^8 spleen cells with 2.10^7 mouse myeloma cells (SP2/0) was achieved with 40% polyethylene glycol 4000 (Merck) and 5% dimethyl sulfoxide. Fused cells were cultured in RPMI-1640 medium in the presence of azaserine (1 µg/ml) and hypoxanthine (0.1 mM). After 10 days hybridoma's were cultured in RPMI-1640 medium containing hypoxanthine (0.1 mM). Hybridoma supernatants were screened for reactivity with the peptide in an ELISA. Some positive cultures were cloned by limiting dilution and then plated out at one cell per well density.

Immunoprecipitation

200 µl hybridoma supernatant, containing monoclonal mouse anti-androgen receptor antibodies, was mixed with 50 µl anti-mouse IgG-agarose (packed gel) (Sigma) and 150 µl phosphate-buffered saline and incubated for 2 h at 4°C under constant rotation. Following centrifugation (10 sec, 2000 x g) the supernatant was removed and the pellet was washed three times with TEDGF buffer (40 mM Tris, 1 mM EDTA, 10% glycerol, 10 mM dithiothreitol, 50 mM sodium fluoride, 10 mM sodium molybdate, pH 7.4). A portion of 400 µl of the nuclear extract from [^{32}P]orthophosphate labeled LNCaP cells was added together with 2 ml TEDGF buffer, containing 1% Triton X-100, 0.5% desoxycholate and 0.08% SDS. The mixture was incubated for 2 h at 4°C under constant rotation and washed 3 times with the incubation buffer, 3 times with TEDGF buffer containing 0.4 M NaCl and 0.2% Triton X-100, and 3 times with TEDGF buffer without further additions. The pellet was mixed with 100 µl sample buffer (40 mM Tris, pH 6.8, 5% glycerol, 2% SDS, 10 mM DTT), boiled for 2 min and centrifuged (2000 x g, 5 min). The supernatants were subjected to SDS-PAGE.

RESULTS

LNCaP cells were photolabeled with the synthetic androgen [^{3}H]R1881 in the presence or absence of a 100-fold molar excess of unlabeled dihydrotestosterone. After photolabeling cell lysates were prepared and analyzed by SDS-PAGE. Gel slicing revealed that two proteins of 110 kD and 43 kD were labeled covalently with [^{3}H]R1881 (Fig. 1A and ref.18). The radiolabeled 110 kD protein represents the human androgen receptor (18). Fluorography after SDS-PAGE of lysates prepared from photolabeled LNCaP cells revealed that the androgen receptor migrated as a closely spaced doublet around 110 kD (Fig. 1B). Further evidence for heterogeneity of the androgen receptor was obtained after Western blotting of a similar receptor preparation (Fig. 1C). Most of the androgen receptor protein was present in the higher molecular weight form according to the difference in intensity between the two bands. (Figs. 1B and 1C).

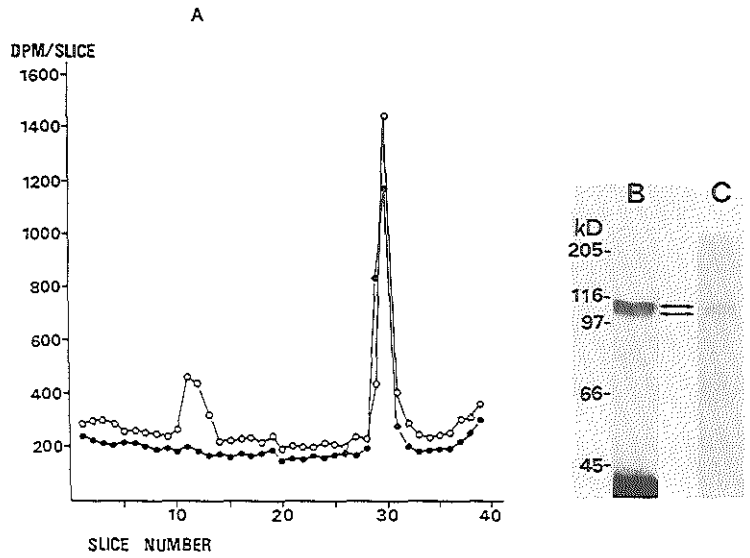

Figure 1
SDS-PAGE of the androgen receptor from LNCaP cell lysates.
A: The cells were incubated for 1 h with 10 nM [^{3}H]R1881 in the presence (●) or absence (○) of a 100-fold molar excess of dihydrotestosterone and were photolabeled. Cell lysates were analyzed on SDS-PAGE. Gel slices were counted for radioactivity.
B: Fluorography of androgen receptor in lysates, prepared after incubation of the cells with 10 nM [^{3}H]R1881 for 1 h and subsequent photolabeling in situ.
C: Immunoblot of the androgen receptor in cell lysates prepared after incubation of the cells with 10 nM R1881 for 1 h.

Whether the relative amounts of the isoforms change during hormone treatment was investigated by analyzing the receptor pattern at different times following the administration of 10 nM R1881 to LNCaP cells. Fig. 2 shows a

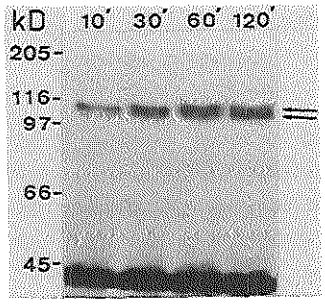

Figure 2
Fluorography after SDS-PAGE of androgen receptor in lysates prepared from photolabeled LNCaP cells. The cells were incubated for 10-120 min with 10 nM [^{3}H]R1881 before photolabeling.

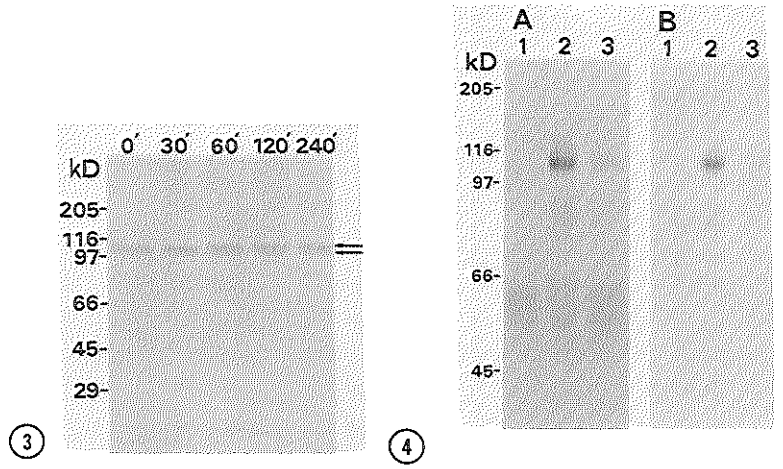

Figure 3
Western blot of androgen receptor in LNCaP cell lysates after exposure of the cells for 0-240 min to 10 nM R1881.

Figure 4
Western blot (4A) and autoradiogram (4B) of androgen receptor immunopurified from nuclear extracts of R1881-treated (lanes 1 and 2) or untreated (lane 3) LNCaP cells. The cells were incubated with 0.2 mCi/ml [^{32}P]orthophosphate for 4 h either in the presence or absence of R1881. Androgen receptors were immunopurified using a specific monoclonal antibody (lanes 2 and 3) or nonspecific mouse IgG (lane 1) and analyzed after SDS-PAGE and Western blotting. Equal amounts of protein from nuclear extracts were used for immunopurification in all cases. The immunoblot was autoradiographed.

fluorogram of lysates of LNCaP cells that were photolabeled with [^{3}H]R1881 at 10-120 min after hormone administration. A pronounced time-dependent change in the ratio of the receptor isoforms in the presence of R1881 could not be detected. The relatively low intensity of photolabeling at 10 min after [^{3}H]R1881 administration might be due to incomplete labeling of the androgen receptor. Based on additional experiments, labeling of the androgen receptor was optimal 30 min after administration of 10 nM [^{3}H]R1881 to LNCaP cells. Western blot analysis of lysates, prepared from LNCaP cells after incubation with 10 nM R1881 for different time periods up to 240 min, also revealed no time dependent changes of the ratio between the androgen receptor isoforms (Fig. 3).

Since isoforms of other steroid hormone receptors appear to be due to differences in their phosphorylation states, it was investigated whether the androgen receptor is also a phosphoprotein. To this end LNCaP cells were incubated with [^{32}P]orthophosphate in the presence or absence of R1881. Androgen receptors were isolated from nuclear extracts with a specific monoclonal antibody and subsequently analyzed by SDS-PAGE, Western blotting and autoradiography (Fig. 4). Fig. 4A shows that from nuclear extracts of cells incubated in the absence of R1881, only a very small amount of androgen

receptor could be immunopurified (lane 3), whereas much more androgen receptor could be isolated from nuclear extracts of R1881-treated cells (lane 2). Using a nonspecific mouse IgG no androgen receptor was isolated (lane 1). As shown by autoradiography (Fig. 4B) the specific monoclonal antibody precipitated a [^{32}P]labeled 110 kD protein from nuclear extracts of R1881-treated cells (lane 2), which was not isolated using a nonspecific mouse IgG (lane 1). From nuclear extracts prepared from cells not treated with R1881, a very faint phosphorylated protein band of 110 kD was precipitated with the specific monoclonal antibody (lane 3).

DISCUSSION

The results presented herein show that the human androgen receptor migrates as a doublet around 110 kD on SDS-PAGE. The doublet was detected at the protein level (using Western blot analysis) and at the steroid binding level (using fluorography), which indicates that both isoforms bind ligand. In addition strong evidence is provided that the human androgen receptor is a phosphoprotein. First, the phosphorylated protein was precipitated specifically using a monoclonal antibody raised against the human androgen receptor. Second, the immunopurified protein co-migrated with the 110 kD androgen receptor on SDS-PAGE. Third, the presence of the phosphorylated protein in nuclear extracts of LNCaP cells was hormone dependent. Isoforms of other steroid hormone receptors reflect differences in their phosphorylation states. The heterogeneous properties of the androgen receptor found in the present investigation might also be due to differentially phosphorylated androgen receptor forms. This variability may reflect differences in the extent as well as in the sites of phosphorylation (22,23).

A time dependent upshift in apparent molecular weight, as shown for the progesterone, estradiol and vitamin D receptors after ligand binding (12,15,16) was not observed for the androgen receptor upon R1881 treatment of LNCaP cells. This might indicate that phosphorylation of the androgen receptor in LNCaP cells does not change markedly after ligand binding. Possibly, ligand-induced phosphorylation of steroid hormone receptors is cell specific, as may be suggested on basis of results on the glucocorticoid receptor (11,14,24).

Within 30 min after R1881 administration to the LNCaP cells the androgen receptor was transformed to the tight nuclear binding form and could be recovered completely from nuclear extracts. Prolonged exposure to R1881 did not result in the extraction of more androgen receptor (not shown). In the absence of ligand estradiol and progesterone receptors are loosely bound to the nucleus, while untransformed glucocorticoid receptor is present both in

the nucleus and in the cytoplasm (25-28). These receptor proteins are recovered from the cytosol after cell fractionation in the absence of ligand. The subcellular localization of the androgen receptor in the absence of hormone is still not known. In the present investigation a small amount of androgen receptor was extracted from nuclear salt extracts of cells that were not treated with R1881, as shown using Western blot analysis. Presently it is not clear whether this small amount of androgen receptor reflects a more tightly bound nuclear receptor population or represents a residual fraction associated to the nucleus due to incomplete washing of the nuclei.

The present experiments with [^{32}P]orthophosphate indicate that the transformed androgen receptor recovered from nuclear extracts of R1881-treated LNCaP cells is a phosphoprotein. It remains to be investigated whether the untransformed androgen receptor is also phosphorylated. The function of androgen receptor phosphorylation remains to be elucidated, but might be essential for ligand binding (7-9) or transcriptional regulation (4,10,11).

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CHAPTER 6

HORMONE-DEPENDENT ANDROGEN RECEPTOR PHOSPHORYLATION IS ACCOMPANIED BY RECEPTOR TRANSFORMATION IN HUMAN LYMPH NODE CARCINOMA OF THE PROSTATE CELLS

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Hormone-dependent Androgen Receptor Phosphorylation Is Accompanied by Receptor Transformation in Human Lymph Node Carcinoma of the Prostate Cells*

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Phosphorylation of the androgen receptor was investigated in the absence of hormone as well as during and after transformation of the receptor to the tight nuclear binding form. Human prostate tumor cells (LNCaP) were labeled for 4 h with [^{32}P]orthophosphate in the presence or absence of steroid. Subsequently, androgen receptors were immunoprecipitated either from total cell lysates or from nuclear extracts using a specific monoclonal antibody. The immunoprecipitated receptor preparations were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, Western blotting, using a polyclonal antiserum, and autoradiography. It was observed that the androgen receptor is already phosphorylated in the absence of hormone, but undergoes a hormone-induced additional phosphorylation. After administration of 10 nM R1881, a 1.8-fold increase in phosphorylation over nonstimulated control cells was reached. Moreover, the amount of nuclear extractable androgen receptor was increased; the acquisition of tight nuclear binding capacity was accompanied by hormone-induced receptor phosphorylation.

Phosphorylation of steroid hormone receptors is thought to be involved in the regulation of steroid hormone action. Ample evidence has been provided that the progesterone, glucocorticoid, and estradiol receptors exist as phosphoproteins in intact cells (1–4). It has been shown that phosphorylation of the estradiol receptor from calf uterus is indispensable for ligand binding (5). In addition, indications for a role of phosphorylation in the acquisition of ligand binding capacity were provided for estrogen, progesterone, glucocorticoid, and androgen receptors (6–11).

Steroid hormone receptors are thought to be mainly nuclear proteins (12–14). In the absence of hormone, the receptor proteins are loosely bound in the nucleus and fractionate into the cytosol. Hormone binding initiates the process of receptor transformation. This results eventually in specific binding of the steroid-receptor complex to hormone-responsive elements of steroid-regulated genes and in transcriptional regulation (15, 16). *In vitro*, the transformation process can be monitored by an increased affinity for nuclei and a concomitant decrease of receptor protein recovered in the cytosol fraction (10).

Additional phosphorylation of estradiol, progesterone, glucocorticoid, and vitamin D receptors has been observed upon hormone binding (2, 17–21). The hormone-dependent phosphorylation might be involved in the process of receptor transformation or in transcriptional regulation (2, 19, 20). The hormone-induced phosphorylation step has no influence on down-regulation of progesterone and glucocorticoid receptors (2, 19).

The androgen receptor is structurally related to other steroid hormone receptors (22–24). However, little is known about phosphorylation of this receptor protein. It has been shown that the hormone-binding capacity of the androgen receptor from rat ventral prostate was lost in ATP-depleted cells, and that androgen-binding activity could be regained when ATP levels were restored (10). In addition, in cell-free systems, inhibition of endogenous phosphatase activity was correlated with increased hormone-binding activity of the androgen receptor (11). These data suggest that androgen receptor phosphorylation is required for hormone binding.

In a previous study (25) we have shown that the androgen receptor-hormone complex, when immunoprecipitated from nuclei of human prostate carcinoma cells (LNCaP),[1] was phosphorylated. In the present report, the kinetics of hormone-dependent androgen receptor phosphorylation is described. The results indicate that the androgen receptor exists as a phosphoprotein in hormone-depleted LNCaP cells. A 1.8-fold increase in androgen receptor phosphorylation was observed after administration of androgens to LNCaP cells. This hormone-dependent phosphorylation is accompanied by transformation of the receptor protein to the tight nuclear binding form.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Androgen Receptor Turnover Time—The incubation period with [^{32}P]orthophosphate which is required to achieve steady state labeling of the androgen receptor in LNCaP cells was investigated. First, pulse-chase experiments were performed to determine the half-life of the androgen receptor. LNCaP cells were cultured for 2 h in the presence of 10 nM [^{3}H]R1881

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[1] The abbreviations used are: LNCaP, lymph node carcinoma of the prostate; SDS, sodium dodecyl sulfate; R1881, 17β-hydroxy-17α-methyl-estra-4,9,11-trien-3-one.

[2] "Experimental Procedures" are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

and subsequently UV irradiated to link the androgen receptor covalently to the radiolabeled ligand. Cells were exposed to a chase of 10 nM unlabeled R1881 for 0.5, 1, 2, 3, or 4 h and lysed subsequently. The amount of covalently labeled androgen receptor was quantified after SDS-polyacrylamide gel electrophoresis by gel slicing and counting of radioactivity in the individual gel slices. Fig. 1 summarizes three separate pulse-chase experiments. It was observed that 50% of covalently labeled androgen receptor disappeared during the chase period within 2–2.5 h. The total amount of androgen receptor did not change during the 4-h incubation period with [^{3}H] R1881 after UV irradiation of the cells (Fig. 1), which indicates synthesis of new receptor protein under the experimental conditions. Based on the observed receptor half-life of 2–2.5 h, the phosphorylation studies were performed using a 4-h metabolic labeling period with [^{32}P]orthophosphate. This 4-h period allows turnover of a large fraction of the androgen receptor.

Hormone-independent Androgen Receptor Phosphorylation—It has been shown previously that the androgen receptor is phosphorylated in hormone-treated LNCaP cells (25). In the present study phosphorylation of the androgen receptor in hormone-depleted LNCaP cells was also investigated. Cells were incubated for 4 h with [^{32}P]orthophosphate, followed by immunoprecipitation of the androgen receptor from the cytosol fraction using an androgen receptor-specific monoclonal antibody (F39.4). As a control, nonspecific mouse IgG was used. Immunoprecipitated proteins were separated by SDS-polyacrylamide gel electrophoresis and subjected to combined immunoblotting and autoradiography. In Fig. 2, it is shown that the 110-kDa androgen receptor was phosphorylated in the absence of hormone.

Hormone-dependent Androgen Receptor Phosphorylation—In Fig. 3*A*, the effect of R1881 treatment on the phosphorylation status of the androgen receptor is shown. In this experiment, the LNCaP cells were incubated for 4 h with [^{32}P] orthophosphate; increasing concentrations of R1881 (0.1, 1, and 10 nM) were added 30 min before the end of the incubation with the radiolabeled phosphate. The androgen receptor was isolated from total cell lysates using the F39.4 monoclonal antibody. It was observed that R1881 treatment resulted in additional receptor phosphorylation; 0.1 nM R1881 already increased phosphorylation and the phosphate content was further increased with 1 and 10 nM R1881. The R1881-induced increase in phosphate content of the androgen receptor is specific for this protein, because nonspecifically precipitated proteins showed no increase in phosphorylation level (Fig. 3*A*). Treatment of LNCaP cells with R1881 did not affect the androgen receptor protein level, as is shown on the corresponding Western blot (Fig. 3*B*).

In the next experiment, phosphorylation of the androgen receptor was studied as a function of time after addition of 10 nM R1881 to LNCaP cells. The cells were incubated for 4 h with [^{32}P]orthophosphate; 10 nM R1881 was added at different times during the incubation with the radiolabeled phosphate. As shown in the autoradiogram (Fig. 4*A*), a small increase in phosphorylation of androgen receptor was observed after incubation of the cells with R1881 for 5 min. Maximal phosphorylation was reached 15 min after R1881 administration. On the corresponding Western blot (Fig. 4*B*) it is shown that the level of receptor protein remained unchanged during the

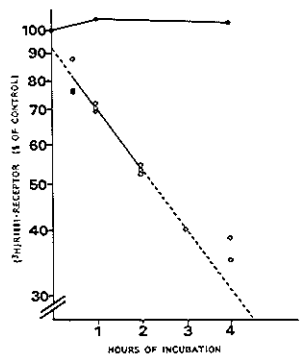

FIG. 1. **Turnover of androgen receptor, covalently labeled with [^{3}H]R1881, in LNCaP cells.** LNCaP cells were incubated with 10 nM [^{3}H]R1881 for 2 h at 37 °C and subsequently UV irradiated. Medium was removed and then cells were either exposed to 10 nM unlabeled R1881 for 0.5, 1, 2, 3, and 4 h (○) or to 10 nM [^{3}H] R1881 for 0, 0.5, or 4 h (●). Cells which were incubated with radiolabeled R1881 were UV irradiated again. Lysates were prepared, and after electrophoresis the 110-kDa receptor bands were excised and subjected to liquid scintillation spectrophotometry as described under "Experimental Procedures." The *solid line* is based on the mean of the data at 0.5, 1, and 2 h.

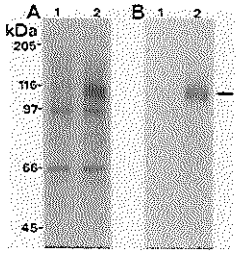

FIG. 2. **Hormone-independent androgen receptor phosphorylation.** LNCaP cells were incubated for 4 h with [^{32}P]orthophosphate (0.15 mCi/ml). Androgen receptor was immunoprecipitated from cytosol with the F39.4 monoclonal antibody (*lane 2*) or nonspecific mouse IgG was used (*lane 1*). Isolated proteins were separated on a 7% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with Sp061 polyclonal rabbit antiserum and an alkaline phosphatase-conjugated second antibody, respectively. The Western blot (*Panel B*) was used for autoradiography (*Panel A*).

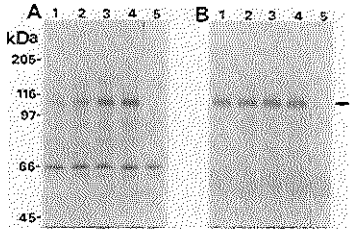

FIG. 3. **Hormone-dependent androgen receptor phosphorylation.** LNCaP cells, cultured for 4 h with [^{32}P]orthophosphate (0.15 mCi/ml), were incubated in the absence (*lane 1*) or presence of 0.1 (*lane 2*), 1 (*lane 3*), or 10 nM (*lanes 4* and *5*) R1881. R1881 was added 30 min before the end of the incubation with radiolabeled phosphate. Receptors were immunoprecipitated from total cell lysates with the F39.4 monoclonal antibody (*lanes 1–4*) or nonspecific mouse IgG was used (*lane 5*). Isolated proteins were separated on a 7% SDS-polyacrylamide gel, transferred to nitrocellulose, and probed with Sp061 antiserum and an alkaline phosphatase-conjugated second antibody, respectively. The Western blot (*Panel B*) was used for autoradiography (*Panel A*).

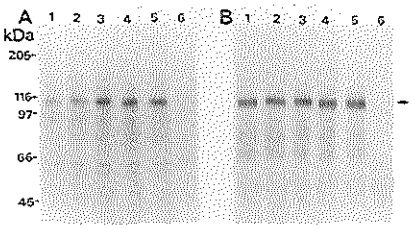

FIG. 4. **Time course of R1881-dependent androgen receptor phosphorylation.** LNCaP cells were incubated for 4 h with [^{32}P] orthophosphate (0.15 mCi/ml) in the absence (*lane 1*) or presence of 10 nM R1881. The ligand was added 5 (*lane 2*), 15 (*lane 3*), 30 (*lanes 4* and *6*), or 240 (*lane 5*) min before the end of the 4-h labeling period. Androgen receptor was immunoprecipitated from total cell lysates using F39.4 monoclonal antibody (*lanes 1–5*) or nonspecific mouse IgG (*lane 6*). Isolated proteins were separated by 7% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose. The nitrocellulose sheet was incubated with the Sp061 antiserum and with an alkaline phosphatase conjugate of goat anti-rabbit antibody, respectively. The Western blot (*Panel B*) was subjected to autoradiography (*Panel A*).

TABLE I

Ligand-dependent phosphorylation of the androgen receptor

LNCaP cells were incubated for 4 h at 37 °C with [^{32}P]orthophosphate (0.15 mCi/ml). 30 min before the end of the labeling, steroids were added to a final concentration of 10 nM. Androgen receptors were immunoprecipitated from total cell lysates using the F39.4 monoclonal antibody. Isolated proteins were subjected to SDS-polyacrylamide gel electrophoresis and Western blot analysis. The nitrocellulose sheet was incubated first with the Sp061 rabbit antiserum and then with ^{125}I-protein A. After color development, the 110-kDa receptor bands were excised and subjected to liquid scintillation counting with a double label setting for ^{125}I and ^{32}P. ^{32}P incorporation was normalized to ^{125}I and within each experiment compared to the normalized incorporation of the receptor protein from cells not incubated with steroid (control) to obtain the relative phosphate content. The mean ± standard deviation from three independent determinations are given.

Ligand	Stimulation of phosphorylation (n = 3)
	mean ± *s.d.*
R1881	1.81 ± 0.05
Dihydrotestosterone	1.81 ± 0.17
Progesterone	1.62 ± 0.06
Estradiol	1.28 ± 0.08
Triamcinolone acetonide	1.02 ± 0.07

incubation with R1881, indicating that the increased amount of phosphorylation is not due to an increased level of receptor protein.

Steroid Specificity of Hormone-dependent Androgen Receptor Phosphorylation—Recently it was shown that the androgen receptor in LNCaP cells also has binding affinity for progesterone and estradiol (29). Therefore, it was investigated whether there is a correlation between binding of different steroids and steroid-induced receptor phosphorylation. To this end, the extent of phosphorylation of androgen receptor from cells incubated in the presence or absence of different steroids (10 nM) was determined with a quantitative Western blot assay, in which both the amount of receptor protein and receptor-specific ^{32}P were assayed. The ratio of the relative phosphate content of androgen receptor isolated from steroid-treated cells to that from control cells is shown in Table I. Compared to the phosphate content of androgen receptors in cells not incubated with steroid, androgen receptor phosphorylation increased 1.8-fold after administration of R1881 or dihydrotestosterone. Progesterone increased phosphorylation with a factor of 1.6, while estradiol induced a minor increase in the extent of receptor phosphorylation. Triamcinolone acetonide, which does not bind to the androgen receptor, and had no effect on androgen receptor phosphorylation (Table I).

Receptor Transformation—It was investigated whether hormone-induced phosphorylation and transformation coincide, or whether these processes can be separated in time. Transformation, characterized by an accumulation of nuclear extractable androgen receptor, and phosphorylation of the transformed (nuclear extractable) androgen receptor were investigated as a function of time after the addition of R1881 to LNCaP cells. It is shown on the Western blot (Fig. 5*B*) that incubation of the cells with R1881 for 5 min increased the amount of nuclear extractable androgen receptor significantly. Incubation of the cells for 15 and 30 min with R1881 resulted in higher phosphate (Fig. 5*A*) and protein levels (Fig. 5*B*) of nuclear extractable androgen receptor. Using the quantitative Western blot assay as described under "Experimental Procedures," it was determined that the ^{32}P to ^{125}I ratio of nuclear extractable androgen receptor was identical after incubation of the cells for 5, 15, and 30 min with R1881 (Table II). The low level of nuclear receptor protein in the absence

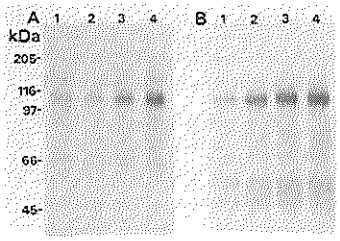

FIG. 5. **R1881-dependent phosphorylation and transformation of androgen receptor in LNCaP cells.** LNCaP cells were cultured at 37 °C with [^{32}P]orthophosphate (0.15 mCi/ml) in the absence (*lane 1*) or presence of 10 nM R1881, which was added 5 (*lane 2*), 15 (*lane 3*), or 30 (*lane 4*) min before the end of the incubation with radiolabeled phosphate. Androgen receptor was immunoprecipitated from nuclear extracts and analyzed by SDS-polyacrylamide gel electrophoresis and Western blotting. The Western blot was used for autoradiography (*Panel A*). After autoradiography, the nitrocellulose paper was incubated first with Sp061 polyclonal antiserum, then with ^{125}I-labeled protein A, and finally with an alkaline phosphatase conjugate of goat anti-rabbit IgG (*Panel B*).

TABLE II

R1881-dependent phosphorylation and transformation of the androgen receptor in LNCaP cells

The experiment was performed as described in the legend of Fig. 5. After color development, 110-kDa receptor bands were excised and subjected to liquid scintillation counting with a double label setting for ^{125}I and ^{32}P. The ^{32}P to ^{125}I ratios of two independent experiments are presented.

Incubation time with R1881	^{32}P/^{125}I ratio	
	Experiment 1	Experiment 2
min		
0	—[a]	—[a]
5	0.0057	0.0056
15	0.0065	0.0053
30	0.0060	0.0062

[a] Not measurable because of low amounts of androgen receptor protein.

of hormone did not allow an accurate determination of the ^{32}P to ^{125}I ratio at 0 min.

DISCUSSION

In a previous investigation it was established that the transformed human androgen receptor in nuclear extracts of hormone-treated LNCaP cells is phosphorylated (25). In the present study it is shown that the androgen receptor is also phosphorylated in cells not treated with hormone. However, the androgen receptor in LNCaP cells undergoes additional phosphorylation after hormone administration. Hormone-dependent phosphorylation has been demonstrated for progesterone, glucocorticoid, and estradiol receptors as well (2, 17–20). The relatively high rate of androgen receptor phosphorylation after administration of R1881 to the cells suggests that pre-existing, rather than newly synthesized receptors, undergo the hormone-dependent phosphorylation step.

Recently a point mutation was detected within the steroid-binding domain of the androgen receptor in LNCaP cells.[3] This mutation might be responsible for the unusually broad binding specificity towards different steroid hormones (29). Not only androgens, but also progesterone and estradiol can bind to this receptor protein. In the present study, it is shown that these steroids are able to induce additional androgen receptor phosphorylation as well. Hormone-induced phosphorylation is restricted to steroids which are bound to the androgen receptor. The phosphorylation status of the androgen receptor remained unchanged when [^{32}P]orthophosphate-labeled LNCaP cells were incubated with triamcinolone acetonide, a synthetic glucocorticoid which does not bind to the androgen receptor. Incubation of the cells with 10 nM estradiol increased androgen receptor phosphorylation to a lesser extent than progesterone or androgens, which were added in the same concentration. This might be due to the relatively lower affinity of the LNCaP androgen receptor for estradiol (K_d approximately 16 nM) (29).

With pulse-chase experiments using covalently labeled androgen receptor, it was determined that the androgen receptor in LNCaP cells is a rapidly turning over protein with a half-life of 2–2.5 h. A similar strategy has been followed to determine estradiol receptor half-life (30). It was shown that covalent labeling of estradiol receptor did not affect the half-life of this receptor protein. The half-life of the androgen receptor in LNCaP cells is in good agreement with estradiol receptor half-life, which ranges from 2 to 4 h in different studies (30–32), but half-lives of 12 and 20 h were observed for progesterone and glucocorticoid receptors, respectively (33, 34).

For phosphorylation studies, LNCaP cells were cultured for 4 h in the presence of [^{32}P]orthophosphate, which allowed almost complete turnover of the androgen receptor during the incubation period of the cells with radiolabeled phosphate. Steady state labeling of the androgen receptor excludes the possibility that the observed increase in [^{32}P] content of the androgen receptor after hormone treatment is due to an increased receptor turnover or to an increased turnover of incorporated phosphate, rather than to additional phosphorylation.

Administration of R1881 to LNCaP cells resulted in an increase of nuclear extractable androgen receptor. The phosphate to protein ratio of androgen receptor, that could be extracted from nuclei 5, 15, and 30 min after R1881 administration to LNCaP cells, stayed constant. This indicates that the hormone-dependent phosphorylation step occurred before or during transformation to the tight nuclear binding form, but not after transformation. Recently it has been reported that the androgen receptor is a nuclear protein (14). In the absence of hormone, the receptor protein is loosely bound in the nucleus and fractionates with the cytosol upon homogenization of the cells (10). It was observed that some androgen receptor protein fractionated with nuclei of cells which were not incubated with hormone. These androgen receptors might possibly represent untransformed androgen receptor or a processed androgen receptor form. Since the nature of this receptor fraction is not known, the phosphate level of this androgen receptor protein, although isolated from cells not incubated with hormone, cannot be considered as the basal phosphorylation level of the nuclear androgen receptor.

The protein kinase(s) which are involved in phosphorylation of the androgen receptor have not yet been identified. It cannot be excluded that hormone-dependent phosphorylation of the androgen receptor requires a different protein kinase from that involved in hormone-independent phosphorylation. The fact that hormone-dependent phosphorylation occurs rapidly following hormone administration indicates that the kinase involved in this process is already present in the cell. Hormone binding may cause a conformational change in the receptor molecule making it a substrate for the specific kinase. It is also possible that the kinase itself is present in an inactive form in the absence of steroid. Binding of the steroid-receptor complex to the protein kinase might be a mechanism for activation of protein kinase activity, as has been suggested for the specific kinase involved in basal phosphorylation of the estradiol receptor in calf uterus (17).

It has been shown that progesterone and glucocorticoid receptors are phosphorylated exclusively on serine (2, 35) while the estradiol receptor from calf and rat uterus is phosphorylated on tyrosine (4). Phosphorylation sites of progesterone and glucocorticoid receptors are predominantly localized on the *N*-terminal half of the receptor molecules (18, 35–37) but the exact location of phosphorylated sites is still not known. Studies will be carried out to identify which amino acid residues are phosphorylated on the androgen receptor. In addition, the location of the phosphorylated amino acid residues with respect to the functional domains of the androgen receptor and their role in the mechanism of action of the androgen receptor remain to be elucidated and will be the subject of further studies.

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SUPPLEMENTAL MATERIAL TO HORMONE-DEPENDENT ANDROGEN RECEPTOR PHOSPHORYLATION IS ACCOMPANIED BY RECEPTOR TRANSFORMATION IN HUMAN LNCaP CELLS

Materials

17β-hydroxy-17α-methyl-4,9,11-estratrien-3-one ([^{3}H]R1881) (87 Ci/mmol) and radioinert R1881 were purchased from NEN-Dupont (Dreieich, F.R.G.). Other steroids were obtained from Steraloids (Wilton, NH, USA).

Cell culture

The LNCaP cell line (derived from a fast growing colony of a Lymph Node Carcinoma of the Prostate) was a gift from Dr. Horoszewicz (26). The cells were cultured in plastic Nunc flasks at 37 °C in RPMI 1640 culture medium, with added glutamine, streptomycin, penicillin and 7.5% (v/v) heat-inactivated fetal calf serum in a humidified atmosphere of 5% CO_2 in air. Two to four days before use of the cells, medium with 7.5% (v/v) heat-inactivated fetal calf serum was replaced by the same medium, but containing 5% heat-inactivated charcoal-stripped fetal calf serum. LNCaP cells between the 67th and 72th passage *in vitro* were used for the present studies.

Metabolic labeling with [^{32}P]orthophosphate

For phosphorylation studies, LNCaP cells were preincubated for 1 h at 37 °C with a phosphate-free Krebs-Ringer buffer at pH 7.3 (118.3 mM NaCl, 4.75 mM KCl, 25 mM $NaHCO_3$, 1.2 mM Na_2SO_4, 2.5 mM $CaCl_2$), containing 0.2% (w/v) glucose. Subsequently, cells were cultured in phosphate-free Krebs-Ringer buffer in the presence of 0.2% (w/v) glucose, essential and non-essential amino acids, according to Eagles Minimum Essential

Medium formulation (GIBCO, Grand Island, NY, U.S.A.), and 0.15 mCi/ml [^{32}P]orthophosphate (carrier-free; Amersham, Cardiff, U.K.). During the labeling with [^{32}P]orthophosphate, cells were incubated either in the absence or presence of steroid.

Preparation of nuclear extracts
Approximately 60 x 10^6 cells were collected in 5 ml of buffer A (pH 7.4) (40 mM Tris-HCl, 1 mM EDTA, 10% glycerol (v/v), 10 mM dithiothreitol, 10 mM Na_2MoO_4, 50 mM NaF, 0.6 mM phenylmethylsulfonyl fluoride, 0.5 mM bacitracin) at 4 °C, and centrifuged (5 min, 800 x g). The pellet was resuspended in 2 ml buffer A and homogenized with a glass/Teflon homogenizer on ice (6 strokes at 1100 rpm). The homogenate was centrifuged at 800 x g for 10 min at 4 °C. The pellet was washed with buffer A (pH 7.4) containing 0.2% (v/v) Triton X-100, and then with buffer A without additions. The nuclear pellet was resuspended in buffer A (pH 8.5) in the presence of 0.4 M NaCl, and extracted for 1 h at 0 °C. The sample was centrifuged at 105,000 x g for 30 min. The androgen receptor was precipitated from the supernatant.

Preparation of cytosol
Approximately 60 x 10^6 cells were collected in 5 ml PBS at room temperature. Cells were centrifuged at 100 x g for 5 min and homogenized with a glass/Teflon homogenizer on ice (6 strokes at 1100 rpm). The cytosol was prepared by centrifugation of the homogenate at 105,000 x g for 30 min at 4 °C.

Preparation of cell lysates
After labeling with [^{32}P]orthophosphate, cells were washed with PBS and lysed subsequently in buffer A, containing 1 % (v/v) Triton X-100, 0.5 % (w/v) sodium deoxycholate and 0.08 % (w/v) SDS, at 0 °C under constant stirring during 10 min. Per 10^7 cells 800 µl of the lysis buffer was used. The lysate was centrifuged (10 min, 1700 x g) and androgen receptor was immunoprecipitated from the supernatant.

Immunoprecipitation
80 µl hybridoma supernatant, containing either monoclonal mouse anti-androgen receptor antibodies (F39.4) (25) or nonspecific mouse IgG, was mixed with 20 µl anti-mouse IgG-agarose (packed gel) (Sigma, St Louis, MO, U.S.A.) and 200 µl PBS. The mixture was incubated for 2 h at 4 °C using end-over-end rotation. Following centrifugation (10 sec, 2000 x g), the supernatant was removed and the agarose beads were washed three times with buffer A. Portions of 400 µl cell lysate or nuclear salt extract from ^{32}P-labeled cells were added and the mixture was incubated using end-over-end rotation for 2 h at 4 °C. Under these conditions, the addition of more F39.4 monoclonal antibody-bound agarose beads did not result in binding of more androgen receptor. After centrifugation for 10 sec at 2000 x g the agarose-antibody-receptor 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 without further additions. The pellet was mixed with 70 µl sample buffer (40 mM Tris-HCl, pH 6.8, 5% (v/v) glycerol, 2% (w/v) SDS, 10 mM dithiothreitol, 0.2% (w/v) bromophenol blue), boiled for 2 min and centrifuged (2000 x g, 2 min). The supernatants were subjected to SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide gel electrophoresis, Western blotting and autoradiography
Immunopurified [^{32}P]orthophosphate-labeled receptor preparations were separated on 7% SDS-polyacrylamide gels according to Laemmli (27). High molecular weight markers (29 - 205 kDa, Sigma) were used as references for molecular weight estimation. After electrophoresis, the slab gels were positioned on nitrocellulose paper (Schleicher and Schuell, 0.45 μm) and placed in a Bio-Rad Trans-Blot cell (Richmond, CA, U.S.A.), filled with 16.5 mM Tris/150 mM glycine/20% (v/v) methanol (pH 8.3). The transfer was performed at 9 °C using 70 V for 17 h. The paper was incubated with an androgen receptor-specific polyclonal rabbit antiserum (Sp061) (28), diluted 1:1000 in PBS/0.05% (v/v) Tween 20 (PBS-Tween) for 1 h at room temperature, washed 4 times for 10 min each with PBS-Tween, and incubated subsequently with alkaline phosphatase-conjugated goat-anti-rabbit IgG (Sigma), diluted 1:1000 in PBS-Tween. After washing of the nitrocellulose paper, the antibody complexes were stained with a solution of 0.3% (w/v) 4-aminodiphenylamine diazonium sulphate (Sigma) and 0.1% (w/v) naphtol phosphate (disodium salt) (Sigma) in 0.2 M Tris-HCl, pH 9.1, containing 10 mM $MgCl_2$. Subsequently, the filter was air-dried and exposed to Hyperfilm-MP (Amersham) with two intensifying screens for 18 h at -80 °C.

Iodination of Protein A
5 μg protein A was incubated for 15 min on ice with 3 μl Na^{125}I (300 μCi) and 20 mg Protag (Baker, Phillipsburg, NJ, U.S.A.) in 1 ml 10 mM H_3BO_3-NaOH, pH 8.2, containing 0.9% NaCl. The mixture was subsequently applied to a PD-10 desalting column (Pharmacia, Uppsala, Sweden), which was pre-equilibrated with PBS in the presence of 1% KI. The column was eluted with the equilibration buffer and the protein fraction eluted from the column was pooled.

Measurement of receptor-specific phosphate levels
To determine the ratio of ^{32}P-label to receptor protein levels, immunoprecipitated [^{32}P]androgen receptors were subjected to SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose as described herein. Incubation of the nitrocellulose sheet for 1 h at room temperature with the polyclonal antiserum was followed by incubation with ^{125}I-Protein A (6 x 10^6 dpm in 20 ml PBS-Tween) for 1 h at room temperature. The nitrocellulose sheet was washed 3 times 10 min with PBS-Tween. The antibody-receptor complex was incubated for 1 h with alkaline phosphatase conjugated goat-anti-rabbit IgG. After colour development, each lane was cut into 2 mm slices. The slices were dissolved in 1 ml of a mixture of 8% (v/v) Triton X-100 and 8% (v/v) 4-azaheptamethylenediamine in water for 18 h at room temperature, and mixed with 10 ml Ultima Gold (Packard, Downers Grove, IL, U.S.A.). ^{32}P and ^{125}I radioactivities in the individual slices were measured in a Packard scintillation spectrometer (model 2500 TR) with a double label setting for ^{32}P and ^{125}I at 98% efficiency for ^{32}P and 60% efficiency for ^{125}I. ^{32}P and ^{125}I were counted in the same sample with no noticeable crossing over using preset channals for ^{32}P and ^{125}I. The amount of each isotope associated with the androgen receptor was determined after subtraction of the background from the area under the peak. This technique yields a linear standard curve for receptor protein levels.

Determination of receptor half life
LNCaP cells were incubated for 2 h at 37 °C in serum-free RPMI 1640 culture medium with 10 nM [^{3}H]R1881. The cells were washed twice with PBS. The flasks were put on the surface of a 300 nm UV transilluminator (UVP, San Gabriel, U.S.A.) and the cells

were irradiated for 2 min. After irradiation, the medium was replaced and the incubation was continued in serum-free RPMI 1640 culture medium in the presence of 10 nM of either unlabeled R1881 or [^{3}H]R1881. Incubation with [^{3}H]R1881 was followed by a second UV-irradiation after different incubation times. Subsequently, cells were lysed in SDS-sample buffer and analyzed by SDS-polyacrylamide gel electrophoresis. The slab gel was cut into 2 mm slices, and proteins were eluted from the individual slices in a mixture of 8% (v/v) Triton X-100 and 8% (v/v) 4-azaheptamethylenediamine in water for 18 h at room temperature. The extracts were mixed with 10 ml Ultima Gold (Packard) and radioactivity was counted. The amount of ^{3}H associated with the androgen receptor was determined after substraction of background from the area under the peak.

CHAPTER 7

GENERAL DISCUSSION

7.1 Cellular systems for analysis of androgen receptor structure and function

The LNCaP cell line, established from a metastatic lesion of a human prostatic adenocarcinoma (Horoszewicz et al., 1983), was used for all studies described herein. This cell line contains a considerable amount of androgen receptors and is the only human cell line, characterized so far, that shows androgen-dependent growth *in vitro* (Horoszewicz et al., 1983; Schulz et al., 1985). In addition, androgens regulate the production and secretion of prostate-specific acid phosphatase and the release of a 40 kDa protein by the cultured cells (Horoszewicz et al., 1983; Berns et al., 1986), suggesting that the cell line is a suitable model to study androgen receptor function. As described in Paragraph 1.4, however, the androgen receptor from this cell line contains a point mutation within the steroid-binding domain (Veldscholte et al., 1990). The point mutation, which was shown to be responsible for an unusually broad spectrum of specificity of steroid hormone binding (Veldscholte et al., 1990), explains the binding activity of the receptor protein for the synthetic progestagen R5020, described in Chapter 3.

At present, it is not known whether the mutation of the androgen receptor in LNCaP cells affects properties, other than ligand-binding characteristics, of this receptor protein. In addition to androgens, it was found that progestagens and estradiol not only can bind to this androgen receptor (Chapter 3; Veldscholte et al., 1990), but also induce additional receptor phosphorylation (Chapter 6), and indeed can stimulate the growth of LNCaP cells (Horoszewicz et al., 1983; Schulz et al., 1985; Berns et al., 1986; Schuurmans et al., 1988). This suggests that these different steroids, when bound to the LNCaP androgen receptor, all induce its transformation and effectuate the subsequent steps towards gene activation. It is not certain, however, to what extent progestagen- and estrogen-induced receptor functions really resemble the effects of androgens.

To study whether basal and hormone-dependent phosphorylation, as observed for the LNCaP androgen receptor (Chapters 5 and 6), are general characteristics of the androgen receptor, future studies on androgen receptor phosphorylation should involve normal target cells for androgens as well. However, the androgen receptor concentration in normal cells is very low, implicating that it will be difficult to study the biochemistry of the receptor protein in such cells. A possibility to obtain a higher cellular concentration of the normal androgen receptor involves the transient expression of androgen receptor

cDNA in COS cells (Brinkmann et al., 1989) or other cell lines, such as the human-derived HeLa cells. However, in this case the normal androgen receptor function is studied in cells which are not androgen target cells. Hence, a major disadvantage of this latter approach is, that it is not known whether the cells contain the particular kinase(s) and phosphatase(s) that could be involved in androgen receptor phosphorylation/dephosphorylation. Transfection of an androgen receptor cDNA expression vector in androgen target cells, such as genital skin fibroblasts, will circumvent this problem since these cells contain all factors necessary for normal receptor function.

7.2 Molecular mass of the androgen receptor

Various proteins, e.g. the cardiac membrane protein phospholamban, glycogen synthase kinase, type II cAMP-dependent protein kinase, and avian ß-adrenergic receptor, show a decrease in their mobility on SDS-gels upon phosphorylation (Hofmann et al., 1975; Ahmad, et al., 1982; Stadel et al., 1983; Wegener and Jones, 1984). The reason for the mobility shift is not clear, since the actual increase in molecular mass of the protein due to phosphorylation is very small. The decreased electrophoretic mobility of proteins upon phosphorylation is not a general characteristic of phosphoproteins and seems to depend on a combination of factors such as protein structure, the extent of phosphorylation and the sites of phosphorylation. It has been suggested that phosphorylation can cause these proteins to undergo a conformational change that is partly preserved in SDS-gels (Wegener and Jones, 1984). The lower mobility of phosphoproteins in SDS-gels could also be due to a decrease in the ability to bind SDS (Wegener and Jones, 1984).

Different methods (*in situ* photoaffinity labeling, Western blot analysis, and metabolic labeling) have been used for detection of the androgen receptor in SDS-polyacrylamide gels, as described in Chapter 2. However, with none of these methods the apparent molecular mass of the androgen receptor in SDS-gels (110 kDa; Chapters 3-6) reflected the predicted molecular mass of 99 kDa of the receptor protein, calculated from the amino acid sequence (Chang et al., 1988; Lubahn et al., 1988; Faber et al., 1989). This has been observed not only for the LNCaP androgen receptor, but also for recombinant androgen receptor cDNA when transiently expressed in COS cells (Brinkmann et al., 1989).

Since the androgen receptor has been shown to exist as a phosphoprotein in LNCaP cells (Chapters 5 and 6), the phosphate groups of the androgen receptor might be responsible for the discrepancy between the calculated molecular mass of the androgen receptor and its actual electrophoretic mobility. However, this discrepancy might also be caused by other properties of the androgen receptor protein. It cannot be excluded that the homopolymeric amino acid stretches of the androgen receptor influence the migration rate in SDS-gels through an effect on SDS-binding. In addition, it is possible that the androgen receptor does undergo additional covalent modifications, such as glycosylation, that not only contributes to the decreased mobility of the receptor, but in fact also may serve a functional role. However, to date no evidence has been provided for glycosylation of any of the steroid receptors.

Steroid receptors, including the androgen receptor (Chapter 5), are heterogenous proteins that exist in multiple isoforms, migrating as doublets or triplets in SDS-gels. Upon ligand binding, the isoforms of progesterone, estradiol, and vitamin D receptors undergo an upshift in apparent molecular mass, simultaneously with additional phosphorylation (Pike and Sleator, 1985; Golding and Korach, 1988; Sheridan et al., 1989; Brown and DeLuca, 1990). Dephosphorylation of the progesterone receptor by means of alkaline phosphatase treatment led to a decrease in the amount of the higher molecular weight isoforms in SDS-gels, and a simultaneous increase in the amount of the lowest molecular weight form. In addition, the ligand-induced upshift of the three isoforms in SDS-gels could be reversed by alkaline phosphatase treatment (Sheridan et al., 1989). This experiment provided evidence that the isoforms of the human progesterone receptor represent differentially phosporylated receptor populations. The structural and functional similarities between the progesterone receptor and the other steroid receptors suggest that all steroid receptor isoforms, including those of the androgen receptor, may represent receptors that differ in their phosphorylation states. However, an upshift in apparent molecular mass upon phosphorylation is not a general characteristic of steroid receptors and cannot be used as a parameter for receptor phosphorylation. This is illustrated by the following. A change in apparent molecular mass of the two androgen receptor isoforms was not observed upon R1881 treatment of LNCaP cells (Chapter 5), whereas the androgen receptor undergoes additional phosphorylation upon hormone treatment of the cells (Chapter 6). Similarly, the 79 kDa and the 110 kDa forms of the avian progesterone receptor, which were shown to be

encoded by the same gene (Conneely et al., 1989), undergo a hormone-dependent phosphorylation, but only the 79 kDa form undergoes a simultaneous upshift in apparent molecular mass on SDS-polyacrylamide gels (Sullivan et al., 1988).

7.3 Future studies on androgen receptor phosphorylation

In the studies described in this thesis, it is shown that the androgen receptor exists as a phosphoprotein in LNCaP cells and undergoes a hormone-induced, additional phosphorylation (Chapters 5 and 6). In this respect, the androgen receptor resembles the progesterone, estrogen, and glucocorticoid receptor. However, many aspects of androgen receptor phosphorylation are still not clear. One major point is whether the androgen receptor is phosphorylated on serine/threonine and/or on tyrosine residues, and also the actual sites of phosphorylation have not yet been identified.

It has been shown that progesterone and glucocorticoid receptors are phosphorylated on serine residues in intact cells (Sheridan, et al., 1988; Smith et al., 1989; Denner et al., 1990), while phosphorylation on tyrosine has been reported for the estrogen receptor in calf uterus (Migliaccio et al., 1986). In the rat glucocorticoid receptor, the phosphorylated serine residues were shown to be located exclusively in the N-terminal domain (Dalman et al., 1988); in the mouse glucocorticoid receptor, phosphorylated serines were localized both in the N-terminal, and in the steroid-binding domain (Smith et al., 1989). Recently, three hormonally regulated phosphorylation sites were identified in the chicken progesterone receptor (Denner et al., 1990). Two sites are present in the N-terminal domain, while the other site, that is very conserved among steroid receptors, is located in the hinge region between the DNA- and hormone-binding domains. Phosphorylation of this site is stimulated 20-fold upon hormone binding. Since the hinge region of the chicken progesterone receptor also contains a transcription activation region (Dobson et al., 1989), it was suggested that this phosphate is involved in the activation of transcription.

The hormone-dependent phosphorylation might occur at new sites or could be due to increased phosphorylation at preexisting sites. Both mechanisms have been observed for the human progesterone receptor (Denner et al., 1990). The overall increase in androgen receptor phosphorylation with a factor 1.8 upon hormone administration to

LNCaP cells (Chapter 6) might thus represent the average of a differential increase in phosphorylation at different sites. It is even possible that certain sites are dephosphorylated in response to hormone. It is clear that the dynamics of the phoshorylation/dephosphorylation process needs further investigation.

The kinases, involved in steroid receptor phosphorylation, have not yet been identified. An endogenous kinase, purified from the cytosolic fraction of calf uterus homogenates, was reported to phosphorylate the calf uterus estradiol receptor *in vivo* and to activate hormone binding. This kinase has been characterized as a calmodulin-stimulated tyrosine kinase (Migliaccio et al., 1984), and its activity is stimulated by the hormone-receptor complex (Auricchio et al., 1987). A phosphatase, thought to be involved in dephosphorylation of estradiol receptor tyrosine phosphates, was partially purified from the nuclear fraction of calf uterus homogenates (Auricchio et al., 1984).

The purified avian progesterone receptor is an *in vitro* substrate, in cell-free systems, for both the serine-specific, cAMP-dependent protein kinase (Weigel et al., 1981), and the tyrosine-specific EGF and insulin receptor kinases (Ghosh-Dastidar et al., 1984; Woo et al., 1986). The rat glucocorticoid receptor can also be phosphorylated by the cAMP-dependent protein kinase *in vitro* (Singh and Moudgil, 1985a). Whether these enzymes are involved in receptor phosphorylation in intact cells remains to be investigated.

Several reports have described the copurification of a Mg^{2+}-dependent protein kinase with the glucocorticoid, and progesterone receptors (Kurl and Jacob, 1984; Singh and Moudgil 1985b; Sanchez and Pratt, 1986; Garcia et al., 1987). This endogenous kinase is able to phosphorylate the receptor proteins *in vitro*. The physiological role of the enzyme is not known, but it cannot be excluded that the kinase is an unrelated abundant enzyme that becomes associated aspecifically with the receptor during the purification procedure.

The amino acid sequence of the human androgen receptor includes several consensus sequences for the serine/threonine-specific proline-directed kinase (Vulliet al., 1989), postulated to be important for functional regulation of transcription factors (Suzuki, 1989). It has been suggested that this kinase is involved in hormone-dependent phosphorylation of the human progesterone receptor (Denner et al., 1990). The most typical substrate motif for the cAMP-dependent kinase ((Arg)-Arg-X-Ser-X; Kemp and Pearson, 1990) is not present in the androgen receptor.

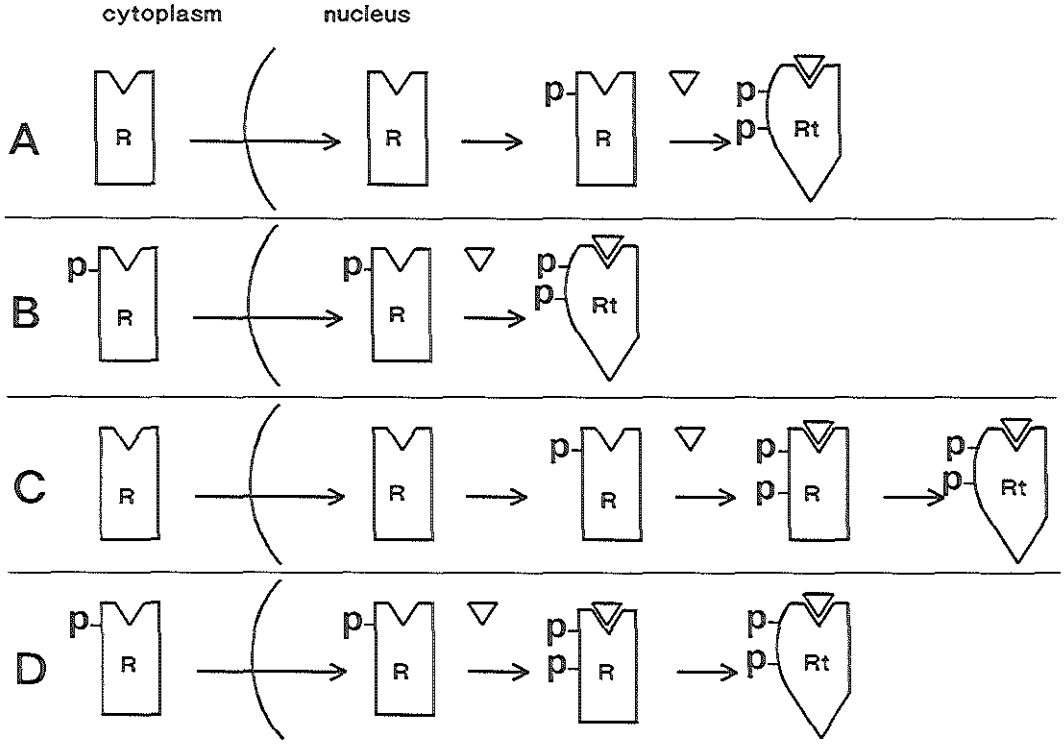

Figure 7.1. Possible sequences of events of basal and hormone-induced androgen receptor phosphorylation, with respect to nuclear translocation and receptor transformation.
A. The androgen receptor is translocated to the nucleus and phosphorylated by a nuclear protein kinase. Hormone-induced receptor phosphorylation and transformation occur simultaneously.
B. The androgen receptor is phosphorylated by a cytoplasmic protein kinase and translocated to the nucleus. Hormone-induced receptor phosphorylation and transformation occur simultaneously.
C. The androgen receptor is translocated to the nucleus and phosphorylated by a nuclear protein kinase. Receptor transformation occurs after hormone-induced phosphorylation.
D. The androgen receptor is phosphorylated by a cytoplasmic protein kinase and translocated to the nucleus. Receptor transformation occurs after receptor hormone-induced phosphorylation.
▽, hormone; R, receptor; Rt, transformed receptor; P, phosphate

Basal, and hormone-induced phosphorylation of the androgen receptor may be catalyzed by the same kinase, but it is also possible that different kinases are involved. In addition, it is not known whether the newly synthesized receptor proteins are phosphorylated by a cytoplasmic kinase before nuclear translocation (Fig 7.1 B,D), or

whether they are translocated to the nucleus first and then phosphorylated by a nuclear kinase (Fig.7.1 A,C). In Figure 7.1, the possible sequences of events are depicted of hormone-induced receptor phosphorylation and transformation to a tight nuclear binding form, that interacts specifically with the hormone responsive elements. As described in Chapter 6, the hormone-dependent phosphorylation of the androgen receptor either occurs simultaneously with receptor transformation (Fig.7.1 A,B), or precedes the transformation process (Fig.7.1 C,D). Not presented in this figure is the possibility that the basal phosphorylation is necessary to acquire hormone-binding activity (receptor activation).

Evidence has been provided for an important role of phosphorylation in the regulation of the activity of many transcription factors, other than steroid hormone receptors. For example, phosphorylation modulates the DNA-binding activity of topoisomerase II (Ackerman et al., 1985), Simian virus 40 large tumor antigen (Mohr et al., 1987; Klausing et al., 1988), and serum response factor (Prywes et al., 1988). It has been suggested that the interaction of components of the transcription machinery with a DNA-bound heat shock factor (Sorger and Pelham, 1988) and with a component of the RNA polymerase transcription complex of vesicular stomatitus virus (Chattopadhyay and Banerjee, 1987) can be modulated by phosphorylation. In addition, there is evidence that dimerization of a cAMP response element binding protein can be regulated by phosphorylation (Yamamoto et al., 1988).

Different functional roles for ligand-induced steroid receptor phosphorylation have been suggested, including dissociation of non-receptor proteins (such as the 90 kDa heat shock protein) from the steroid receptors, receptor dimerization, interaction of the receptors with other transcription factors, and receptor binding to the hormone responsive elements (Sheridan et al., 1988; Hoeck et al., 1989; Orti et al., 1989; Denner et al., 1990). Recently, it has been shown by site-directed mutagenesis that phosphorylation of identified serine residues of the v-*erb*A gene product, which is related to the c-*erb*Aα encoded thyroid hormone receptor, is required for full biological activity (Glineur et al., 1990). The mechanism by which phosphorylation affects the function of this protein is still not known. It was suggested that phosphorylation might contribute to the interaction of the protein either with other factors to stabilize its binding to hormone responsive elements, or with specific transcription factors. In an androgen-insensitive rat, a point mutation, changing arginine 734 to glutamine within the steroid-binding domain,

has been identified (Yarbrough et al., 1990). It was suggested that arginine 734 belongs to a phosphorylation consensus sequence, and that its loss may result in inefficient phosphorylation on the highly conserved serine 735 or threonine 737. Since phosphorylation might be involved in the activation of steroid receptors to a hormone-binding form (Paragraph 1.5), loss of this phosphorylation site may result in impaired hormone binding (Yarbrough et al., 1990). However, the role of basal androgen receptor phosphorylation in ligand binding has not been validated yet, and the role of the hormone-induced receptor phosphorylation remains to be investigated as well. Evidence for the function of androgen receptor phosphorylation can possibly be obtained by means of identification of the phosphorylated amino acid residues and the subsequent site-directed mutagenesis of these sites.

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SUMMARY

Ligand binding is a prerequisite for the acquisition of transcription regulating activity by steroid hormone receptors. The ligand induces a cascade of events, including receptor transformation, specific DNA-binding and modulation of gene transcription by the hormone-receptor complex. These events are still not well understood at the molecular level.

It has been proposed that phosphorylation of steroid receptors has an important function in the regulation of several aspects of their activity. In the study described in this thesis it was investigated whether the androgen receptor is a phosphoprotein in the absence of ligand and also undergoes a subsequent, hormone-induced, phosphorylation; this has been observed for progesterone, estrogen, and glucocorticoid receptors. Since specific androgen receptor antibodies are valuable tools in studies on receptor phosphorylation, polyclonal and monoclonal antibodies have been prepared and used in the studies described herein.

Chapter 1 summarizes the recent literature on the structure, function and phosphorylation of steroid hormone receptors in general and of the androgen receptor in particular.

In **Chapter 2** the development and applications of several new tools for biochemical analysis of the androgen receptor are discussed. These tools, particularly *in situ* photoaffinity labeling, androgen receptor-specific antibodies, and a double-labeling procedure for the androgen receptor, appeared to be very useful during the course of the study described in this thesis.

For all studies, the human LNCaP cell line has been used. In **Chapter 3** the androgen receptor from these cells is characterized with respect to its ligand-binding properties and its apparent molecular mass on SDS-polyacrylamide gels. It is shown that the LNCaP androgen receptor migrates as a 110 kDa protein.

Chapter 4 presents the characterization of three androgen receptor-specific polyclonal antisera. Two of the antisera were raised against synthetic peptides, corresponding to putative immunogenic regions within the N-terminal domain of the human androgen receptor. Another polyclonal antiserum was generated against a fusion protein, that contained a part of the N-terminal domain of the androgen receptor. The polyclonal antisera contained high titers of androgen receptor-specific antibodies and

were shown to be proper tools for immunoprecipitation, immunoblotting, and immunohistochemistry.

In **Chapter 5** evidence is provided that the transformed, human androgen receptor in LNCaP cells is phosphorylated. It is also shown that the androgen receptor is a heterogeneous protein, consisting of at least two isoforms. The isoforms migrate as a closely spaced doublet on SDS-polyacrylamide gels, and might reflect differentially phosphorylated androgen receptor forms. These results could be obtained by using an androgen receptor-specific monoclonal antibody. The preparation of this antibody has been described briefly.

Chapter 6 describes that the androgen receptor in LNCaP cells is phosphorylated already in the absence of hormone, but undergoes an additional phosphorylation step upon hormone-binding. In this regard, the androgen receptor behaves like other steroid receptors. Evidence has been provided that the hormone-induced phosphorylation either precedes receptor transformation or occurs simultaneously with the transformation process. Consequently, hormone-induced androgen receptor phosphorylation might have a role in receptor transformation and/or in processes involved in transcription regulation.

Finally, in **Chapter 7**, some aspects of the results obtained in the previous chapters, particularly the suitability of the LNCaP cell line for studies on androgen receptor phosphorylation and heterogeneity, are discussed. In addition, several possibilities for future research on androgen receptor phosphorylation are proposed.

SAMENVATTING

Androgenen behoren tot de groep van steroïd hormonen. Ze zijn o.a. betrokken bij de ontwikkeling en het instandhouden van de mannelijke geslachtskenmerken, met inbegrip van de spermatogenese. De androgenen testosteron en dihydrotestosteron binden aan specifieke receptor eiwitten, androgeen receptoren, die aanwezig zijn in de kern van doelwitcellen voor androgenen. De binding van het androgeen aan het receptor eiwit heeft een aantal strukturele veranderingen in het receptor molekuul tot gevolg, receptor transformatie genoemd, die leiden tot een specifieke interaktie van het hormoon-receptor complex met bepaalde DNA-sequenties, de hormoon responsieve elementen. Dit resulteert in de regulatie van transcriptie van bepaalde genen.

Op moleculair niveau zijn de mechanismen van het transformatieproces van de androgeen receptor, en de daarop volgende transcriptieregulatie, nog onvoldoende bekend. Voor receptoren van andere steroïd hormonen (progestagenen, oestrogenen, en glucocorticoïden) is gevonden dat deze eiwitten in intakte cellen gefosforyleerd zijn. Na binding van het hormoon worden de steroïd receptoren extra gefosforyleerd, hetgeen suggereert dat fosforylering van de receptor eiwitten een rol speelt in het werkingsmechanisme van steroïd hormonen. De studie die in dit proefschrift wordt beschreven had tot doel te onderzoeken of de androgeen receptor, zoals andere steroïd receptoren, een gefosforyleerd eiwit is, en eveneens een extra, hormoon-afhankelijke, fosforylering ondergaat. Polyclonale en monoclonale antilichamen, gericht tegen de androgeen receptor, waren een belangrijk hulpmiddel bij dit onderzoek. De ontwikkeling en karakterisering van deze antilichamen worden in dit proefschrift eveneens beschreven.

In **Hoofdstuk 1** wordt een overzicht gegeven van de recente literatuur met betrekking tot de struktuur, funktie en fosforylering van steroïd hormoon receptoren in het algemeen, en van de androgeen receptor in het bijzonder.

In **Hoofdstuk 2** worden de ontwikkeling en toepassing van een drietal nieuwe technieken geschikt voor de biochemische analyse van de androgen receptor besproken. Deze technieken, met name het *in situ* covalent koppelen van radioaktief ligand aan het receptor eiwit door middel van fotoactivering (fotoaffiniteitslabeling), het gebruik van androgeen receptor-specifieke antilichamen, en een methode om de relatieve hoeveelheid geïncorporeeerd fosfaat te bepalen, werden gebruikt voor het in dit proefschrift beschreven onderzoek.

Voor alle studies werd een humane prostaat tumor cellijn, LNCaP, gebruikt. In **Hoofdstuk 3** wordt de androgeen receptor van deze cellen gekarakteriseerd met betrekking tot de ligand-bindende eigenschappen en de schijnbare molekuulmassa, bepaald met behulp van SDS-polyacrylamide gel electroforese (SDS-PAGE). Er wordt aangetoond dat de androgeen receptor een schijnbare molekuulmassa van 110 kDa heeft.

In **Hoofdstuk 4** wordt de karakterisering van drie androgeen receptor-specifieke polyclonale antisera beschreven. Twee van deze antisera zijn gericht tegen synthetische peptiden die corresponderen met gebieden in het N-terminale domein van de androgeen receptor. Een ander antiserum was gericht tegen een fusie eiwit dat een deel van het N-terminale domein van de androgeen receptor bevatte. Alle polyclonale antisera hebben hoge titers van androgeen receptor-specifieke antilichamen en bleken zeer geschikt te zijn voor immuunprecipitaties, immunoblotting, en immunohistochemie van de androgeen receptor.

In **Hoofdstuk 5** wordt aangetoond dat de androgeen receptor gefosforyleerd is in intakte LNCaP cellen. Eveneens wordt beschreven dat de androgeen receptor een heterogeen eiwit is en uit minstens twee isovormen bestaat. De isovormen migreren als een doublet op SDS-PAGE. Er wordt gesuggereerd dat de isovormen ontstaan doordat de fosforylering van de androgeen receptor op verschillende manieren kan plaatsvinden. Bij het verkrijgen van deze resultaten werd gebruik gemaakt van monoclonale antilichamen, de bereiding van deze antilichamen wordt kort beschreven.

Hoofdstuk 6 beschrijft dat de androgeen receptor in LNCaP cellen in afwezigheid van hormoon al gefosforyleerd is, maar na binding van ligand een extra fosforylering ondergaat. In dit opzicht gedraagt de androgeen receptor zich als andere steroïd hormoon receptoren. Er werden tevens aanwijzingen verkregen dat de hormoon-afhankelijke androgeen receptor fosforylering ofwel aan het transformatieproces vooraf gaat, ofwel gelijktijdig optreedt met de transformatie tot de DNA-bindende vorm. Hieruit volgt dat de hormoon-afhankelijke fosforylering zowel bij het transformatie-proces, als bij transcriptieregulatie kan zijn betrokken.

Tot slot wordt in **Hoofdstuk 7** een aantal aspecten van de resultaten uit de vorige hoofdstukken bediscusseerd, zoals de voor- en nadelen van het gebruik van de LNCaP androgeen receptor voor het onderzoek naar androgeen receptor fosforylering en heterogeniteit. Mogelijkheden voor toekomstig onderzoek met betrekking tot androgeen receptor fosforylering komen eveneens aan de orde.

LIST OF ABBREVIATIONS

cAMP	adenosine cyclic-3',5'-monophosphate
(c)DNA	(complementary) deoxyribonucleic acid
EGF	epidermal growth factor
IGF-1	insulin-like growth factor-1
kDa	kilo Dalton
LNCaP	lymph node carcinoma of the prostate
RNA	ribonucleic acid
SDS	sodium dodecylsulphate
PAGE	polyacrylamide gel electrophoresis
PDGF	platelet-derived growth factor
vitamin D	1,25-dihydroxycholecalciferol

PAPERS RELATED TO THIS THESIS

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CURRICULUM VITAE

De schrijfster van dit proefschrift werd geboren op 29 juli 1958 te Norg. Na het behalen van het HAVO diploma aan het Westfries Lyceum in Hoorn in 1975 volgde ze de HBO-A opleiding medische microbiologie aan het Ir. van den Broek Instituut in Amsterdam en behaalde daar in 1978 het diploma. Van 1976 tot 1980 was ze werkzaam als analiste op het bacteriologisch laboratorium van de GG&GD in Haarlem. In 1980 begon ze met de studie scheikunde aan de Universiteit van Amsterdam. Het doctoraal examen, met biochemie als hoofdvak en experimentele oncologie als bijvak, werd afgelegd in september 1986.

Het in dit proefschrift beschreven onderzoek werd uitgevoerd binnen het Instituut Biochemie II, thans opgenomen in de Vakgroep Endocrinologie & Voortplanting, van de Faculteit der Geneeskunde en Gezondheidswetenschappen van de Erasmus Universiteit Rotterdam. De schrijfster van dit proefschrift was hier werkzaam als wetenschappelijk assistente van augustus 1986 tot mei 1990. Sinds april 1991 werkt ze als post-doctoraal onderzoekster voor INSERM bij de afdeling "Biochimie des régulations cellulaires endocrines (INSERM Unité 244)" op het "Centre d'Etudes Nucléaires de Grenoble" in Frankrijk.